FEBS Letters 360 (1995) 26–28 FEBS 15143

A critical mutation in both *WT1* alleles is not sufficient to cause Wilms' tumor

Haruhito Kikuchi[a,b], Yoshikiyo Akasaka[c], Yoshihiro Kurosawa[d], Hiroshi Yoneyama[d], Shingo Kato[e], Jun-ichi Hata[a,*]

[a]*Department of Pathology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 130, Japan*
[b]*Laboratory of Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 130, Japan*
[c]*2nd Department of Pathology, Toho University School of Medicine, 5-21-16 Ohmorinishi, Ohta-ku 143, Tokyo, Japan*
[d]*Department of Pediatrics, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 130, Japan*
[e]*Department of Microbiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 130, Japan*

Received 3 January 1995

Abstract **The *WT1* gene is a tumor suppresser gene for Wilms' tumor (WT). Inactivation of both alleles has been proposed as the cause of WT. We encountered a patient with Denys–Drash syndrome associated with WT whose *WT1* gene had a homozygous point mutation not only in WT but also in renal tissue adjacent to the WT and in the germline. These findings indicate that factor(s) other than the loss of *WT1* are required for WT to develop.**

Key words: Wilms' tumor; *WT1* gene

1. Introduction

Wilms' tumor (WT) or nephroblastoma is a childhood kidney tumor which is thought to arise from cells of the metanephric blastema, a fetal kidney structure. Based on cytogenetic and molecular genetic studies, there are at least three responsible loci for this tumor [1]. Only one of these genes, *WT1*, has been identified in the distal 11p13 region [2,3]. The product of this gene is a transcriptional factor with four zinc fingers, and it binds to a specific DNA sequence [2,4]. Because expression of *WT1* is limited to the developing glomeruli of the kidney, it is thought to have a functional role in renal organogenesis [5]. On the other hand, a mutation occurs in the *WT1* gene, loss of normal regulation of proliferation, leading to tumor formation. The existence of intragenic mutations of the gene, including point mutations found in the DNA from WT, supports this hypothesis [6–12]. A case of Denys–Drash syndrome associated with WT is inconsistent with this concept, however, because we found the same homozygous mutation in the germline, the kidney and in tumor tissue. This finding forces us to reconsider the role of the *WT1* gene in tumorigenesis.

2. Materials and methods

2.1. Genomic DNA isolation

Genomic DNAs were prepared from WT and renal tissue adjacent to the WT (the tumor-free state confirmed by microscopic examination) using the SDS-proteinase K method with slight modification, as described in our previous report [9]. To extract DNA from peripheral leukocytes, 10 ml of whole blood was lysed and centrifuged. The sediment was then subjected to the same procedure as the tissues.

*Corresponding author. Fax: (81) (3) 3353 3290.

2.2. DNA blot analysis and PCR-SSCP analysis [12]

DNA blot analysis [13] and PCR-SSCP analysis [14] were performed as previously described. To identify mutations in *WT1* exon 8, we amplified genomic DNA from white blood cells (WBCs), non-tumor renal tissue, and WT with the following oligonucleotides: 8-S (forward): 5′-AGATCCCCTTTTCCAGTATC-3′ and 8-A (reverse): 5′-GCCAGCAATGAGAAGTGAAC-3′.

2.3. DNA sequences

PCR was performed with 1 μg of genomic DNA in a total volume of 100 μl using the unlabeled primers described above. The amplified mixture was extracted with phenol/chloroform, passed through a Micro Spin column S-400 (Pharmacia) and precipitated with ethanol. For direct sequencing, half of the reaction mixture was used for dideoxy sequencing using an AutoCycle kit (Pharmacia) and FITC-labeled primer 8-S or 8-A. The products were analyzed on 6% polyacrylamide gels containing 7 M urea using an A.L.F. DNA sequencer (Pharmacia). PCR products were also sequenced after cloning using the p-GEM-T vector system (Stratagene) with the same kit.

3. Results

3.1. Patient description and pathological findings

The patient was a girl who was 1 year and 1 month old at the time of admission. Her chief complaint was abdominal distention. Intravenous pyelography, computerized tomography and ultrasound examination revealed a tumor occupying the lower half of her left kidney. She also had proteinuria. Left nephrectomy was performed and was followed by chemotherapy. As a result of histological examination, the tumor was classified as a WT, triphasic type. Most of the glomeruli showed focal, segmental mesangial sclerosis (Fig. 1). Global sclerosis of the mesangium was also detected in some glomeruli. These histological findings were consistent with Denys–Drash syndrome.

3.2. PCR-SSCP analysis of WT1 of the patient

The PCR-SSCP method was used to screen for mutations in *WT1* exons 7, 8, 9, 10 (zinc finger domain I–IV). We could not find any mobility shift in exons 7, 9 or 10 (data not shown). We analyzed the exon 8 PCR products from the genomic DNA of WT, renal tissue adjacent to tumor tissue and WBC. The results clearly demonstrated an altered migrating fragment (Fig. 2). No fragments comigrated with the unaffected human placental DNA.

3.3. Sequence analysis of WT1

Direct sequencing of the PCR product on exon 8 from the WT and renal tissue revealed a G to A transition at nucleotide

SSDI 0014-5793(95)00071-2

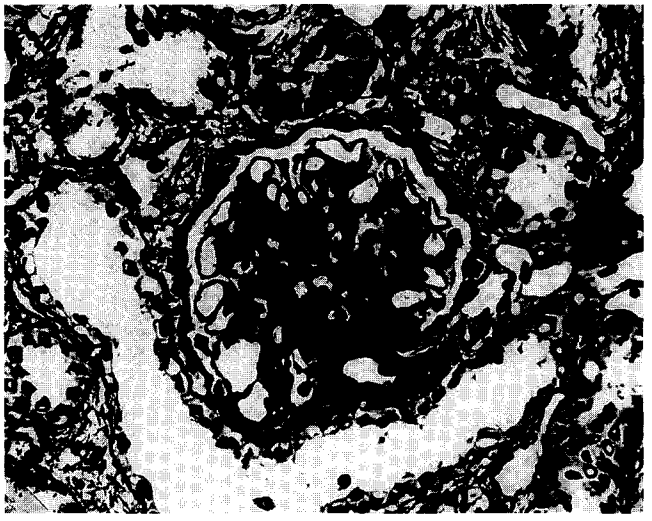

Fig. 1. Histology of the renal tissue adjacent to the Wilms' tumor of the case (PAM stain) showing features of diffuse mesangial sclerosis.

position 1064 (Fig. 3). This mutation results in the substitution of Tyr^{355} for Cys. The same sequence was obtained with both the forward and with the reverse primer. To determine whether this mutation was present in the germline, we sequenced the PCR product of a DNA sample from the patient's WBCs. All of the nucleotide sequences obtained coincided with those from the kidney and WT.

3.4. DNA blot analysis

We performed DNA blot analysis to confirm that one of the *WT1* genes had not been deleted. No gross deletion or duplication of *WT1* was found (Fig. 4). The 3.1 kb fragments, which include exon 8, of the DNA from the patient tissues had the same density as the normal control. Because our DNA blot analysis could detect loss of one allele [13], there are two alleles containing exon 8. Judging from this finding and the results of PCR-SSCP and sequence analysis, the DNA samples from this patient exhibited the same point mutation on exon 8 of both *WT1* alleles.

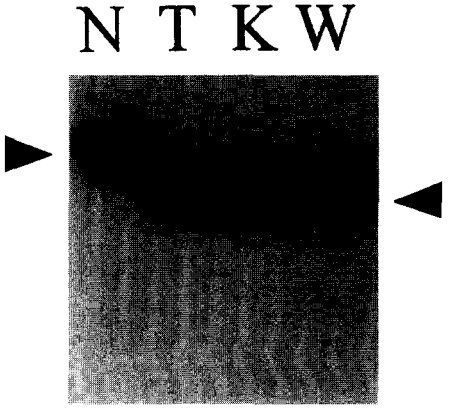

Fig. 2. PCR-SSCP analysis of *WT1* exon 8. The forward primer was radiolabeled with 32P. The amplified sense fragments (indicated by arrowheads) of genomic DNA from Wilms' tumor of the case (T), kidney (K) and WBCs (W) moved to downward positions from that of the control unaffected human placental DNA (N).

4. Discussion

According to the results of PCR-SSCP and sequence analysis, the *WT1* gene of the patient carried with a point mutation of exon 8. No abnormalities was shown by DNA blot analysis which can distinguish one copy and two copies of DNA sequence per single cell [13]; the density of the fragments containing exon 8 were not different from that of normal control. From these results, the Wilms' tumor, kidney and WBCs of the patient have two *WT1* alleles with the same point mutation. Namely, the mutation in exon 8is homozygous. As the same abnormality was found in different tissues, the mutation is likely to be present in the germline. Sakai et al. examined the *WT1* gene in the DNA isolated from WBCs of the patient and her mother, though they did not analyzed the DNA from patient's kidney tissue or Wilms' tumor. They found the same point mutation as ours in the patient's WBCs although her mother had no mutation in *WT1* including exon 8 [15]. Unfortunately, the patient's father refused any DNA analysis. Since themajority of patients with the syndrome have been shown to carry de novo mutations, perhaps the patient's father did not have the mutation. The most probable explanation is as follows. The first point mutation had occurred during oogenesis or spermatogenesis. Just after fertilization the second episode occurred, which might be gene conversion transferring the point mutation to the opposite allele. Because the case that had the ^{1614}G to A conversion has not been reported except this case, it is not likely that the identical rare mutation accidentally arose separately. We could not deny the possibility, however, that the mutation had been transmitted from the patient's father, for we [14] and Coppes et al. [16] identified the patients having the *WT1* mutation observed in Denys–Drash syndrome without any signs or symptoms of the syndrome.

Conversion of the Cys residue chelating the zinc in zinc finger I was also detected in another WT [10]. Because this Cys residue is thought to be one of the key amino acids that chelate zinc and give rise to the finger-like protrusion binding to target DNAs in the second zinc finger [17,18], the homozygous mutation in the patient should greatly affect the function of the *WT1* products. There is a possibility that there is another mutation in the patient's *WT1* gene because we analyzed only *WT1* exon 7–10, although it will be contribute weaker effects on Wilms' tumorigenesis.

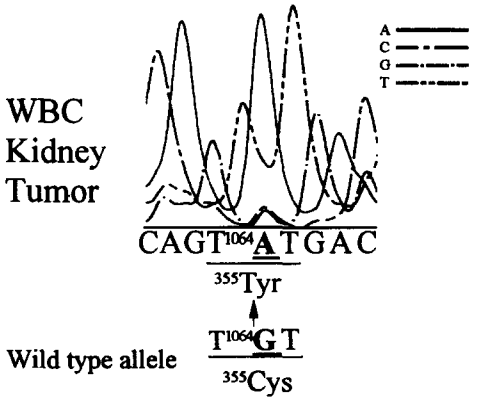

Fig. 3. Direct sequence analysis of *WT1* exon 8 with A.L.F. DNA sequencer (described in section 2). The genomic DNAs from Wilms' tumor, kidney and WBCs of the case had the same point mutation, replacement G to A.

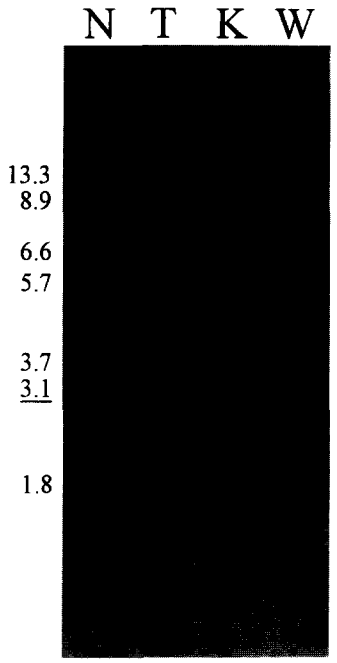

Fig. 4. DNA blot analysis of *WT1* (probe *Eco*RI fragment of WT33 [2]). Numbers indicating sizes (kb) of fragments. All fragment of DNA samples from patient's tumor (T), kidney (K) and WBCs (W) were same intensity as those of the control unaffected human placental DNA (N). The underscored 3.1 kb fragments contain *WT1* exon 8.

DNA from both WTs and normal tissue has been analyzed in several studies, and intragenic *WT1* mutations have been detected [7–12]. In all of these studies, the *WT1* gene was mutated in both alleles in the WTs, but in only one or neither allele in normal tissue. These findings suggested that complete loss of function of WT1 might be sufficient to cause Wilms' tumors [19]. The findings in our patient, however, are incompatible with this hypothesis because there were cells in the kidney(s) of the patient that had no normal *WT1* alleles and did not undergo malignant transformation. Therefore, the case reported here is an example which refutes the hypothesis stated above, and factor(s) in addition to mutation of the *WT1* gene is/are needed for WT to develop.

Recently, Park et al. described two cases in which WT and nephrogenic rests (considered to be precursor lesions of WT) contained the same mutated *WT1* gene [20]. This report and ours both adopt the same viewpoint regarding the necessity of other factor(s) for malignant transformation. They did not, however, find mutations of *WT1* in the germline. They categorized the nephrogenic rests as clonal pre-cancerous lesions whose formation is the rate-limiting step in tumorigenesis in the 'two-hit' model. In contrast, the mutation of *WT1* in our patient is not likely to be a rate-limiting step for malignant transformation because the transversion of the critical amino-acid residue had probably been present in the germline.

A candidate for the factor required for development of WT, is an additional tumor suppresser gene located at 11p15. Interactions between *WT1* and a putative suppresser gene on 11p15 in tumorigenesis have been suggested [6]. Another possibility is co-operation between mutated *WT1* and certain oncogenes. Haber et al. have reported the cooperation of a dominant *WT1* mutation with the viral oncogene *E1A* in the transformation of primary kidney cells [21].

In conclusion, mutation of both *WT1* alleles is not sufficient to cause WT to develop. Additional factor(s) remain to be identified to clarify the mechanisms of the development of Wilms' tumor.

Acknowledgements: We thank Dr. Koichi Shimizu and Takao Kosaka (National Children's Hospital) for providing materials. We also thank Yoshiyuki Hiraishi for his technical assistance. This work is supported by a grant-in-aid for cancer research from the Ministry of Health and Welfare and the Ministry of Education in Japan (03454174), funds provided by the Entrustment of Research Program of the foundation for Promotion of Cancer Research in Japan, a grant from the Vehicle Racing Commemorative Foundation, and Clinical Pathology Research Foundation of Japan.

References

[1] Haber, D.A. and Housman, D.E. (1992) Adv. Cancer Res. 59, 41–68.
[2] Call, K.M., Glaser, T., Ito, C.Y.,Buckler, A.J., Pelletier, J. Haber, D.A., Rose, E.A., Kral, A., Yeger, H., Lewis, W.H., Jones, C. and Housman, D.E. (1990) Cell 60, 509–520.
[3] Gessler, M., Poustka, A., Cavenee, W., Neve, R.L., Orkin, S. and Bruns, G.A.P. (1990) Nature 343, 774–778.
[4] Bickmore, W.A., Oghene, K., Little, M.H., Seawright, A., van Heyningen, V. and Hastie, N.D. (1992) Science 257, 235–237.
[5] Pritchard-Jones, K., Fleming, S., Davidson, D., Bickmore, W., Porteous, D., Gosden, C., Bard, J., Buckler, A., Pelletier, J., Housman,. D., van Heyningen, V. and Hastie, N. (1990) Nature 346, 194–197.
[6] Haber, D.A., Buckler, A.J., Glaser, T., Call, K.M., Pelletier, J., Sohn R.L., Douglass, E.C. and Housman, D.E. (1990) Cell 61, 1257–1269.
[7] Huff, V., Miwa, H., Haber, D.A., Call, K.M., Housman, D., Strong, L.C. and Saunders, G.F. (1991) Am. J. Hum. Genet. 48, 997–1003.
[8] Pelletier, J., Bruening, W., Kashtan, C.E., Mauer, S.M., Manivel, J.C., Striegel, J.E., Houghton, D.C., Junien, C., Habib, R., Fouser, L., Fine, R.N., Silverman, B.L., Haber, D.A. and Housman, D. (1991) Cell 67, 437–447.
[9] Kikuchi, H., Akasaka, Y., Nagai, T., Umezawa, A., Iri, H., Kato, S. and Hata, J. (1992) Am. J. Pathol. 140, 781–786.
[10] Bruening, W., Bardessy, N., Silverman, B.L., Cohn, R.A., Machin, G.A., Aronson, A.J., Housman, D. and Pelletier, J. (1992) Nature Genet. 1, 144–148.
[11] Baird, P.N., Groves, N., Haber, D.A., Housman, D.E. and Cowell, J.K. (1992) Oncogene 7, 2141–2149.
[12] Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K. and Sekiya, T. (1989) Proc. Natl. Acad. Sci. USA 86, 2766–2770.
[13] Hiraishi, Y., Kato, S., Ishihara, T. and Takano, T. (1992) J. Med. Genet. 29, 897–901.
[14] Akasaka, Y., Kikuchi, H., Nagai, T., Hiraoka, N., Kato, S. and Hata, J. (1993) FEBS Lett. 317, 39–43.
[15] Sakai A,, Tadokoro, K., Yanagisawa H., Nagafuchi, S., Hoshikawa, N., Suzuki, T., Kohsaka, T., Hasegawa, T., Nakahori, Y. and Yamada, M. (1993) Hum. Mol. Genet. 2, 1969–1970.
[16] Coppes, M.J., Liefers, G.J., Higuchi, M., Zinn A.B., Balfe, J.W. and Williams, B.R.G. (1992) Cancer Res. 52, 6125–6128.
[17] Miller, J., McLachlan, A.D. and Klug A. (1985) EMBO J. 4, 1609–1614.
[18] Lee, M.S., Gippert, G.P., Soman, K.V., Case, D.A. and Wright, P.E. (1989) Science 245, 635–637.
[19] Coppes, M.J., Liefers, G.J., Paul, P., Yeger, H. and Williams B.R.G. (1993) Proc. Natl. Acad. Sci. USA 90, 1416–1419. 11, 1969–1970.
[20] Park, S., Bernard, A., Bove, K.E., Sens, D.A., Hazen-Martin, D.J., Garvin, A.J. and Haber D.A. (1993) Nature Genet. 5, 363–367.
[21] Haber, D.A., Timmers, H.T.M., Pelletier, J., Sharp, P.A. and Housman D.E. (1992) Proc. Natl. Acad. Sci. USA 89, 6010–6014.