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Genomic Organization of the Human WT1 Gene

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We have analyzed the genomic structure of the human WT1 gene, one of the recessive oncogenes for Wilms' tumor at chromosome 11p13. By analyses of three cosmids covering the WT1 gene as well as products generated by polymerase chain reaction, cleavage sites for 10 restriction enzymes were mapped in a region of about 80 kb, and the positions of 10 exons were defined. We also mapped two polymorphic sites for *TaqI*. Our genomic map will be useful to analyze DNA abnormalities sometimes found in the tumors, as well as loss of heterozygosity.

Key words: Wilms' tumor — Recessive oncogene — Restriction map — Polymorphic sites — Loss of heterozygosity

The WT1 gene is one of the recessive oncogenes responsible for genesis of Wilms' tumor, a childhood nephroblastoma. Based on cytogenetic observation of interstitial deletion at 11p13 especially found in Wilms' tumor-aniridia association, the WT1 gene was cloned in 1990 by two groups.[1,2] Although the gene is now believed to be a transcriptional factor involved in differentiation processes of the urogenital organs,[3-10] DNA abnormalities of the gene have only been detected in a small fraction of the tumors to date.[11] This is quite different from evidence of a high frequency of inactivation of the RB gene in retinoblastomas.[12] This may be explained by involvement of other genes in the genesis of Wilms' tumor. Chromosome 11p15 is the second genetic locus implicated by chromosomal abnormalities found in Beckwith-Wiedemann syndrome and by loss of heterozygosity.[13-15] Familial linkage studies have excluded the involvement of both the 11p13 and 11p15 regions.[16-18] Therefore, at least three genes have been suggested to be involved. Several cases of intragenic homozygous deletion of the WT1 gene have confirmed the idea that the WT1 gene is one of the recessive oncogenes for Wilms' tumor.[19-21] However, we still do not know whether inactivation of one WT gene is enough to generate tumors, or whether there is some interaction among the three possible WT genes.

We have been analyzing Wilms' tumor DNA isolated from Japanese patients to see what fraction of the tumors could be explained by inactivation of the WT1 gene. Since the incidence of the tumor in Japan as well as several South-East Asian countries is lower than that in Caucasians,[22] we are interested to see whether the WT gene inactivation pattern is dependent upon genetic background. After screening of 42 tumor samples by Southern hybridization probed with a cDNA clone of the WT1 gene, we have detected several cases of DNA rearrangement, and precisely analyzed one case of intragenic homozygous deletion.[19] To do this, we have constructed a genomic map for the 3′ half of the gene. Since then, we have extended the map to cover the 5′ half of the gene. In this report, we describe a genomic map of the WT1 gene with restriction sites and positions of exons.

MATERIALS AND METHODS

DNA probes and PCR primers A cDNA clone of the WT1 gene (WT33) and three cosmids (L109, L156 and L159)[1] were kindly provided by D. E. Housman and D. A. Haber. Polymerase chain reaction (PCR) primers used for intron cloning were as follows: 2EF, 5′-TCCCCAACCACTCATTCAAGC and 3ER, 5′-CGTCCTCAGCAGCAAAGCCT. They were synthesized by using a DNA/RNA synthesizer model 392 (Applied Biosystems) and used after evaporation.

PCR reaction Our standard conditions were as follows unless otherwise specified. Reaction mixtures consisted of 10–100 ng of human DNA, 0.5 μM of each primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris pH 8.3 and 1 unit of *Taq* polymerase (AmpliTaq, Takara) in a volume of 20 μl. Amplification was carried out using a programed temperature control system (PC-700; Astec) for 30 cycles consisting of 1 min at 92°C for strand separation, 1 min at an annealing temperature, and 2 min at 72°C for extension, except for 5 min for strand separation in the first cycle and 10 min for extension in the last cycle. The PCR products were analyzed by electrophoresis through either a 0.7% agarose gel, a 1–2% NuSieve 3:1 gel (SeaKem), or a 5–10% polyacrylamide gel in TBE buffer depending on the size of products, and detected by staining with ethidium bro-

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mide or silver staining kit (Bio-Rad). To produce longer DNA fragments or fragments of unknown size, for example, to clone introns using primers on exons, the extension time and concentration of substrates were increased to 10 min and 400 μM, respectively.

Other methods related to DNA manipulation Conditions used for DNA isolation, Southern hybridization, plasmid isolation, subcloning, and DNA sequencing were standard methods and described previously.[19, 23] The restriction map was constructed primarily by an end assignment method in triple digests.[24] For cloning of PCR products containing introns, PCR products were purified by a glass milk method (Geneclean, Bio 101), incubated with polynucleotide kinase and DNA polymerase, and then ligated into an *Sma*I site of pUC18. Several PCR fragments were cloned onto pCR1000 plasmid (Invitrogen) without any purification. Nucleotide sequences were determined by a Sequenase ver. 2 kit (United States Biochemicals) using plasmid derivatives and universal primers or synthetic oligonucleotides.

RESULTS AND DISCUSSION

Genomic map of the WT1 gene DNA abnormalities of the WT1 gene in Wilms' tumors have been screened in most laboratories by Southern hybridization using a cDNA as a probe after digestion with *Eco*RI, since the sum of the size of detected fragments is relatively large. An example of such hybridization after digestion with various enzymes is shown in Fig. 1. Such analyses could

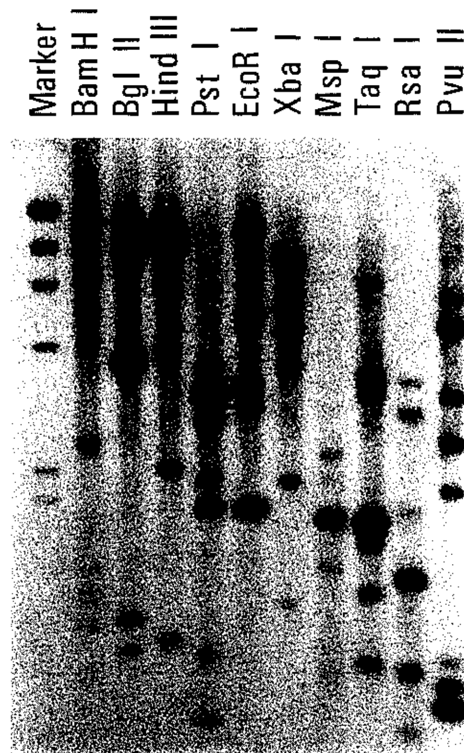

Fig. 1. Autoradiogram of Southern hybridization probed with a cDNA clone of the WT1 gene after digestion of genomic DNA with various restriction enzymes. A DNA sample isolated from leukocytes of a normal individual was digested with indicated enzymes, and hybridized with WT33.

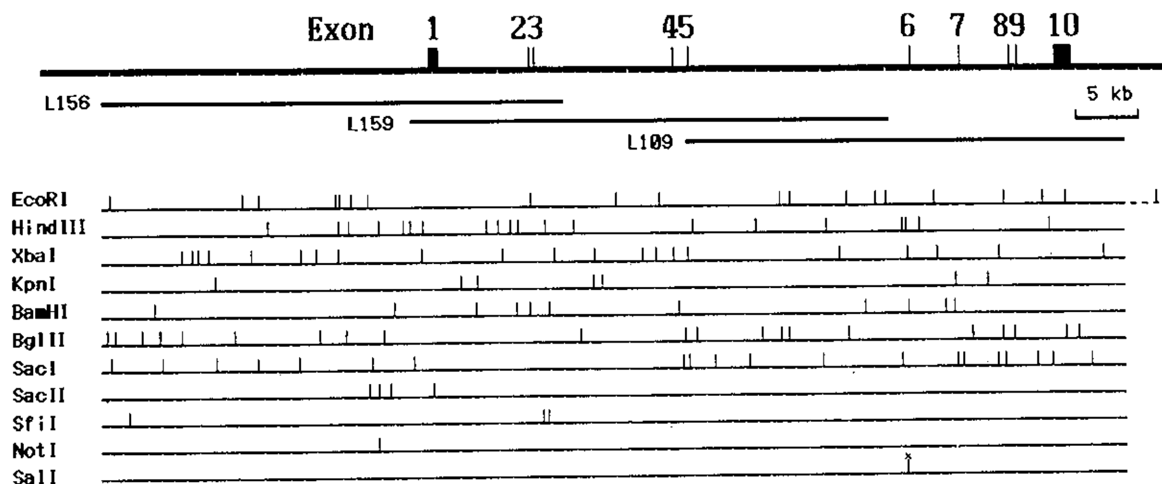

Fig. 2. A genomic structure of the human WT1 gene. The map was constructed by analyses of three cosmids and PCR products generated from normal DNA. The boundary of each cosmid was defined by *Not*I sites in the vector. A polymorphic *Sal*I site is indicated with an X. Although we tried to draw this map to scale as far as possible with the aid of a personal computer, there is some technical limitation to the resolution. Several tiny homo-end fragments are not included. Exons from 2 to 9 are too small to draw to scale.

Table I. Representative Plasmids Used to Analyze Genomic Organization of the WT1 Gene

Name	Insert	Origin	Site	Vector
pMY1062	10.0 kb *Eco*RI-*Eco*RI[a]	L156	*Eco*RI	pUC18
pMY1068	1.3 kb *Eco*RI-*Eco*RI	L156	*Eco*RI	pUC18
pMY1064	5.9 kb *Eco*RI-*Eco*RI	L156	*Eco*RI	pUC18
pMY1073	5.5 kb *Sac*I-*Sac*I[b]	L156	*Sac*I	pUC18
pMY1061	13.0 kb *Eco*RI-*Eco*RI	L156	*Eco*RI	pUC18
pMY1079	6.6 kb *Eco*RI-*Eco*RI	L159	*Eco*RI	pUC18
pMY1095	3.3 kb *Eco*RI-*Eco*RI	L159	*Eco*RI	pUC18
pMY1085	9.4 kb *Eco*RI-*Eco*RI	L159	*Eco*RI	pUC18
pMY1051	0.8 kb *Eco*RI-*Eco*RI[c]	L109	*Eco*RI	pUC18
pMY1028	4.5 kb *Eco*RI-*Eco*RI	L109	*Eco*RI	pUC18
pMY1030	2.2 kb *Eco*RI-*Eco*RI	L109	*Eco*RI	pUC18
pMY1043	0.8 kb *Eco*RI-*Eco*RI[d]	L109	*Eco*RI	pUC18
pMY1032	3.7 kb *Eco*RI-*Eco*RI	L109	*Eco*RI	pUC18
pMY1025	5.5 kb *Eco*RI-*Eco*RI	L109	*Eco*RI	pUC18
pMY1027	3.1 kb *Eco*RI-*Eco*RI	L109	*Eco*RI	pUC18
pMY1031	1.8 kb *Eco*RI-*Eco*RI	L109	*Eco*RI	pUC18
pMY1026	4.7 kb *Eco*RI-*Eco*RI[e]	L109	*Eco*RI	pUC18
pMY1090	ex2-ex3	PCR	*Hph*I	pCR1000
pMY1048	ex4-ex5	PCR	*Sma*I	pUC18
pMY1049	ex6-ex7	PCR	*Sma*I	pUC18
pMY1050	ex7-ex8	PCR	*Sma*I	pUC18
pMY1023	ex8-ex9	PCR	*Sma*I	pUC18
pMY1021	ex9-ex10	PCR	*Sma*I	pUC18

a) *Eco*RI or *Sac*I fragments are ordered in the direction from left to right in Fig. 2.
b) Containing 3 small *Eco*RI-*Eco*RI fragments.
c) A 0.8 kb *Eco*RI fragment at the left and sensitive to *Bgl*II.
d) A 0.8 kb *Eco*RI fragment at the right and sensitive to *Nco*I.
e) One of the *Eco*RI sites was derived from the vector.

reveal homozygous deletion or some rearrangements creating a different size of detected fragments. However, it seems to be difficult to detect other types of rearrangement, especially heterozygous deletion, because only a portion of the genomic sequences is hybridized with a cDNA clone and the intensity of detected fragments varies extensively. In order to facilitate DNA analyses, we made a genomic map of the WT1 gene. Three approaches were followed. First, we arranged the detected fragments in the same order as in the genome using various parts of the cDNA fragment as probes in Southern hybridization. Second, we tried to clone introns by PCR[25] on the basis of a reported nucleotide sequence for the cDNA.[1,26] Third, we obtained three cosmids (L109, L156 and L159) covering the entire region of the gene.[1] Several genomic fragments were subcloned from the cosmid, and then restriction sites and nucleotide sequences were determined in part. Again, PCR using new primers was applied to normal DNA to clone additional segments of the genomic sequence. Putting these results together, a genomic map was constructed as shown in Fig. 2. Relevant plasmids are listed in Table I. Although the results for L109 was previously reported,[19] they are included in this map to form a complete map of the gene.

Inconsistency among three cosmids The three cosmids partly overlapped with each other to form a continuous stretch of about 80 kb. Restriction sites in the overlapping region were in good accord with each other. However, L159 had two additional *Bam*HI sites located at each end of the insert, very close to *Not*I and *Eco*RI sites in the vector. We determined nucleotide sequences using the L109 cosmid in the region corresponding to the right end of the L159 cosmid. Moreover, several PCR products generated from normal individuals were sequenced, since the region was near the deletion end point in a 1389 Wilms' tumor sample and also near a polymorphic *Taq*I site.[19] Since no *Bam*HI sites were found in the corresponding region by such analyses, we did not include those *Bam*HI sites in our map. However, we could not exclude the possibility that those *Bam*HI sites in L159 were caused by polymorphism.

Intronic *Eco*RI fragments In a previous report,[19] we described two intronic 0.8 kb *Eco*RI fragments between the 9.4 and 4.5 kb *Eco*RI fragments by analysis of L109. Again, cosmid L159 contained two 0.8 kb *Eco*RI frag-

ments, of which one had *Bgl*II sites and the other had an *Nco*I site. The order of *Eco*RI fragments around this region was reconfirmed by analyses of L159.

Position of exon 4 We previously defined the position of exons from 5 to 10, and discussed inconsistencies in several reports from other laboratories.[19] The position of exon 4 was only suggested on the basis of the size of PCR products between exons 4 and 5, since L109 did not contain exon 4. Now, the cosmid L159 contained exon 4. A restriction map of pMY1048 carrying a PCR product between exons 4 and 5 completely coincided with a portion of L159, especially in the order and distance between *Bam*HI-*Sac*I-*Bgl*II-*Xba*I-*Sac*I. Thus, the 1.2 kb distance between exons 4 and 5 as reported previously is confirmed.

Position of exons 1, 2 and 3 According to a cDNA sequence,[2] exon 1 had one *Sac*II site and one *Xho*I site, and those sites were separated by 442 bp. There were only 4 *Sac*II sites in the entire region, all of which were located in the 13.0 kb *Eco*RI fragment. The *Eco*RI fragment contained several *Xho*I sites, but all of them but two were located in the middle of the fragment. The distance between the 3rd *Sac*II site and an *Xho*I site was about 900 bp, while the distance between the 4th *Sac*II site and another *Xho*I site was about 450 bp. Therefore, we positioned exon 1 on the region containing the 4th *Sac*II site. Partial sequencing around the 4th *Sac*II site confirmed that the region actually contained the cDNA sequence. pMY1090 carrying a PCR product between exons 2 and 3 contained an *Eco*RI site. According to a cDNA sequence,[1] exon 2 had one *Bam*HI site and one *Bst*EII site. Among the *Eco*RI sites in the entire region, only a distal *Eco*RI site of the 13.0 kb *Eco*RI fragment was located close to a *Bam*HI site. We found that pMY1061 contained two *Bst*EII sites, one of which was mapped near the right end of the insert and close to *Bam*HI. Therefore, we positioned exons 2 and 3 close to the *Eco*RI site between 13.0 and 6.6 kb *Eco*RI fragments.

Mapping polymorphic *Taq*I sites We previously reported two *Taq*I polymorphic systems in the WT1 gene.[27] One of the polymorphic *Taq*I sites (corresponding to system B) was deduced to be in intron 7, and one *Taq*I site found in a previously reported sequence[19] was polymorphic (EMBL, Genbank and DDBJ accession number D01249, data not shown). Another polymorphic *Taq*I site (system A) was deduced to be in intron 5 (data not shown). In the effort to design PCR conditions to detect it, we found that the polymorphic *Taq*I site was exactly the same as the polymorphic *Sal*I site, since the sequence around it was GCCGAC or GTCGAC. *Sal*I is one of the rare cutters for a mammalian genome and is sometimes used for long-range mapping. Therefore, the polymorphic *Sal*I site was indicated in our map, although L109 did not contain it. PCR conditions to detect such *Taq*I polymorphisms together with other intragenic polymorphisms will be reported elsewhere.

Comparison of our map with maps generated by other laboratories Bonetta *et al.* reported a long-range map of *Eco*RI and *Hin*dIII which covered about 130 kb including the WT1 and WIT-1 genes.[28, 29] The map seemed to have been generated by partial digestion with an enzyme(s) after labeling one end of a cosmid. It is astonishing to find that their *Eco*RI and *Hin*dIII sites are fundamentally in agreement with ours, although they missed several small *Eco*RI fragments. This may be accounted for by limitation in the resolution of the techniques that they used. In contrast, they map several restriction sites for rare cutters, including *Not*I and *Sfi*I. They found only one *Sfi*I site in the middle of the WT1 gene, but we found two *Sfi*I sites separated by 0.5 kb in the corresponding region. Although the distance between the sites for these rare cutters (*Sfi*I-*Not*I-*Sfi*I) agreed with our map, they were incorrectly positioned in relation to *Eco*RI or *Hin*dIII sites. It seemed likely that they mapped *Sfi*I sites independently of the experiments with *Eco*RI or *Hin*dIII.

During preparation of this manuscript, two groups reported on exon-intron boundaries of the WT1 gene.[30, 31] Our sequencing results were in accordance with those reports. One of the reports presented a genomic organization including *Eco*RI sites and position of exons.[31] When compared to our map, several intronic *Eco*RI fragments were missed and incorrectly ordered in their map. Moreover, several exons were also incorrectly positioned. It seemed that their genomic organization was not deduced based on experimental data, but should be regarded as a schematic presentation. Therefore, only our map truly reflects the genomic organization of the human WT1 gene. Our map will be useful in analyses of DNA abnormalities in Wilms' tumors.

Although we drew the map shown in Fig. 2 to scale as far as possible with an aid of a personal computer, it is difficult to show precise relationships. Further information is available upon request.

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