

The role of Wilms' tumor genes

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Abstract : The constitutional chromosomal deletion within the short arm of one copy of chromosome 11, at band p13, which often correlated with WAGR syndrome consisting of Wilms' tumor with aniridia, genitourinary malformation, and mental retardation, provided the first clue to the genetic events in the development of Wilms' tumor. *WT1* gene is encoded by 10 exons, resulting in messenger RNA subject to a complex pattern of alternative splicing. *WT1* gene encodes a zinc finger transcription factor, which binds to GC-rich sequences and functions as a transcriptional activator or repressor for many growth factor genes. WT 1 protein is mainly expressed in developing kidney, testis, and ovary, indicating that it is involved in the differentiation of genitourinary tissues, all thought to be the sites of origin of Wilms' tumor. The point mutation of *WT1* results in Denys-Drash syndrome. The other Wilms' tumor gene, *WT2* at 11p15.5, is linked to Beckwith-Wiedemann syndrome. The possibility that *WT1* is involved in the etiology of rhabdoid tumor of the kidney was discussed.

WT1 is expressed in immortalized hematologic cells such as EBV-LCL and hematologic malignancies, but not in PBL or IL-2L. High level *WT1* expression in leukemia cells and a poor prognosis are linked in patients with leukemia, making the gene a novel marker for leukemia cells. A correlated expression between *WT1* and *mdr-1* in vincristine resistant cells indicates a close relation with multi-drug resistance and is a promising diagnostic marker for chemoresistance in hematologic malignancies. *J. Med. Invest.* 46 : 130-140, 1999

Key words : *WT*, tumor suppressor gene, Wilms' tumor, rhabdoid tumor, leukemia, chemoresistance

Etiology of WAGR syndrome

In 1964, Miller *et al.* reported an association of Wilms' tumor with aniridia, hemihypertrophy and other congenital malformations (1). A new disease entity, WAGR syndrome consisting of Wilms' tumor with aniridia, genitourinary malformations, and mental retardation was subsequently proposed (2). Wilms' tumor is the most common childhood intraabdominal solid tumor of the kidney (3). Thereafter,

Riccardi *et al.* found a correlation between WAGR syndrome and karyotypic abnormality within the short arm of one copy of chromosome 11, at band p13 (4). This constitutional chromosomal deletion provided the first clue to the genetic events in the development of Wilms' tumor.

In 1971, Knudson compared cases showing the earlier age of onset and the bilateral presentation of retinoblastomas (RB) in children with a family history of this disease with those showing the uni-

Abbreviations : PBL, peripheral blood lymphocytes ; IL-2L, interleukin 2 activated lymphocytes ; CML, chronic myelogenous leukemia ; EL, erythroleukemia ; MDS, myelodysplastic syndrome ; AML, acute myelogenous leukemia ; APL, acute promyelocytic leukemia ; AMoL, acute monocytic leukemia ; MegL, megakaryocytic leukemia ; EoL, eosinophilic leukemia ; cALL, common type acute lymphocytic leukemia ; T-ALL, T-cell type ALL ; ATL, adult T-cell leukemia/lymphoma ; T-CLL, T-chronic lymphocytic leukemia ; Afn BL, African Burkitt's lymphoma ; B-ALL, B-cell

type ALL ; Jpn BL, Japanese BL ; Imbl, immunoblastic type malignant lymphoma ; MM, multiple myeloma.

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lateral tumors and proposed a model to explain the etiology of this disease, the two-event hypothesis (5). This model predicts that tumor formation depends on two-rate limiting genetic events. Tumorigenesis in children who have a constitutional lesion, either inherited from a parent or resulting from a spontaneous mutation, needed only one new genetic event. Contrary to this, sporadic cases required two independent somatic mutations. A similar model for Wilms' tumor was proposed later (6). Subsequent genetic studies of a number of tumors confirmed that the two postulated genetic events are caused by the inactivation of both alleles of a tumor suppressor gene (7). Now, that Wilms' tumor is caused by the Wilms' tumor suppressor gene *WT1* is demonstrated. The Wilms' tumor gene related diseases are summarized in Table 1.

Structure and regulatory mechanisms of WT1

The *WT1* gene that is mapped to chromosome locus 11p13 is encoded by 10 exons, resulting in messenger RNA subject to a complex pattern of alternative splicing (8, 9). The *WT1* promoter has three transcription initiation sites and the gene is a member of the GC-rich, TATA-less and CCAAT-less class of RNA polymerase II genes (10, 11). The *WT1* transcript is ~ 3.5 kb long and encodes a zinc-finger protein, WT1, with a predicted molecular weight *M_r* of 47kd to 49kd, depending on the presence or absence of two alternatively spliced exons. The first alternative splicing introduces exon 5, encoding 17 amino acids (+17aa), just proximal to the first of four zinc fingers. The second results in an insertion of three amino acids (+KTS) between the third and fourth zinc fingers. The most prevalent *WT1* mRNA variant has both of the insertions present (+KTS and +17aa), and the least common form is missing both insertions (-KTS and -17aa) (9). The insertion of three amino

acids (+KTS) disrupts the distance between zinc fingers 3 and 4 and thus alters its DNA-binding specificity (10). The four patterns of alternative splicing are shown in Figure 1.

The elements responsible for regulating the tissue specific expression of *WT1* are not known, although Sp1 and GATA-1 have been shown to positively modulate *WT1* expression (11-13). In addition, a cell type-specific enhancer has been identified within the 3' end of the human *WT1* gene (14). Recently, Pax 2 and Pax 8 were shown to contribute to the regulation of *WT1*. When Pax 2 and Pax 8 expression becomes maximal, WT1 levels begin increasing (15). Recently, it was reported that the ectopic expression of p50 and p65 subunits of NF-κB stimulated the murine *WT1* promoter activity by 10-30-fold in a transient transfection assay (16). Subsequently, the regulators or signal cascades that could modulate the function of WT1 were studied. Evidence was obtained that WT1 protein expressed exogenously in fibroblasts was phosphorylated *in vivo* and that treatment with forskolin, which ac-

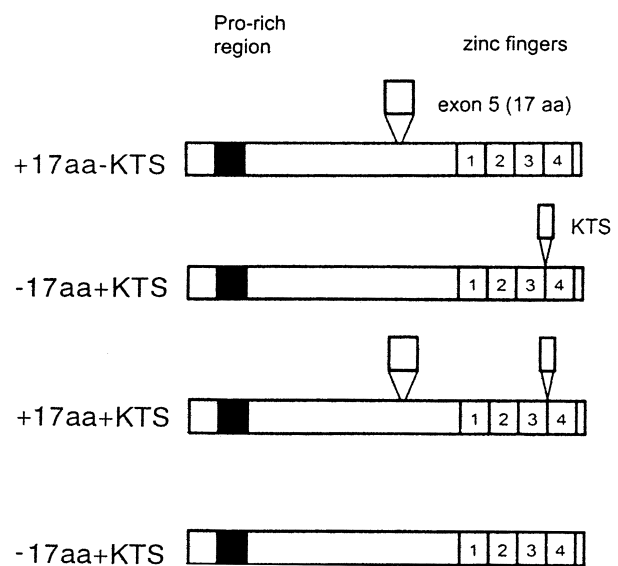


Figure 1. The four patterns of alternative splicing of the *WT1* gene. The details are explained in the text.

Table 1. Wilms' tumor genes and related diseases

Disease name	Clinical manifestations	Genetic abnormality	Incidence of Wilms' tumor
WAGR synd.	Aniridia, genitourinary malformations, mental retardation, Wilms' tumor	Deletion at 11p13 (<i>WT1</i>)	> 30 %
Denys-Drash synd.	Intersexual disorders, nephropathy, Wilms' tumor	<i>WT1</i> point mutation	> 90%
Beckwith-Wiedemann synd.	Macroglossia, organomegaly, hemihypertrophy, embryonal tumors	Duplication of paternal allele of 11p15 (<i>WT2</i>)	< 5 %

tivates the cAMP-dependent protein kinase (PKA) *in vivo*, induced phosphorylation of additional sites at Ser-365 and Ser-393 in WT1, resulting in abolishment of the DNA binding capacity of WT1 *in vitro* (17). In addition, Maheswaran and coworkers presented evidence that p53 physically interacts with WT1 in transfected cells and that WT1 transcriptional activity might be modulated by p53 (18).

Whereas in NIH-3T3 cells wild type WT1 repressed expression, its transfection into Saos-2 cells, which lack endogenous p53, resulted in increased transcription from a promoter into which epidermal growth factor receptor 1 (EGR-1) consensus binding sites had been added (18). However, the WT1 transcriptional activity was independent of *p53* genotype in the induction of P3 promoter activity of mouse insulin-like growth factor II gene (IGF-2) in primary cultures derived from p53 wild type (p53+/+) and knock-out (p53+/-) mouse embryo (19). There is a report that upregulation of *WT1* expression is associated with an expression marker for the differentiation into monocytes/macrophages of *c-fms* and *Mac-1* in murine myeloblastic cell line, M1, cells cultured in leukemia inhibitory factor (LIF), resulting in apoptotic cell death (20).

Functions of WT1

The *WT1* gene encodes a zinc finger transcription factor, which binds to GC-rich sequences (5'-GCGGGGCG-3') and functions as a transcriptional activator or repressor (21). It represses transcription of growth factor [platelet derived growth factor-A (PDGF-A) chain, colony stimulating factor-1 (CSF-1), and IGF-2] and growth factor receptor (IGF-1R) genes, and the other genes [retinoic acid receptor-alpha (RAR- α), *c-myc*, and *bcl-2*](22-27). In addition, it has been known that *WT1* mediates the expression of genes such as EGR-1, EGF-R, inhibin- α , Pax2, transforming growth factor-beta (TGF- β) and *WT1* itself (28-33). The transient transfection of *WT1* constructs into NIH-3T3 or 293 cells results in the transcriptional repression of a number of co-transfected promoters containing the EGR-1 consensus sequence (34). The EGR-1 consensus is found upstream of many transcriptional start sites, leading to the identification of a number of promoters that bind *in vitro*-translated *WT1* and are repressed in transient transfection assay. Haber and coworkers transfected the four wild type *WT1*

isoforms into RM1 human anaplasia of the kidney cell line, a variant form of Wilms' tumor, inoculated into nude mice and observed that each isoform independently suppressed the emergence of colony formation (35). Subsequently, they found that stable transfection of *WT1* into RM1 cells results in induction of endogenous IGF-2 but not of other previously postulated WT1 target genes (34).

WT1 is expressed only in specific types of cells, which is a major difference from *RB* or *p53* (36). The pattern of normal *WT1* expression has provided important clues to the function of *WT1* during differentiation. The WT1 protein is mainly expressed in developing kidney, testis, and ovary, indicating that it is involved in the differentiation of genitourinary tissues. Other organs expressing the protein are spleen and the mesothelial cells of heart, lung and abdomen (36-38). In the kidney, *WT1* is expressed only in condensing blastemic cells, renal vesicles, and glomerular epithelium, all thought to be sites of origin of Wilms' tumor. Renal *WT1* expression peaks around the time of birth and then rapidly declines as the organ matures (37, 38). In contrast to its transient expression in the developing kidney, *WT1* is expressed continuously in mesothelial cells, Sertoli cells of testis, and granulosa cells of the ovary (39). The critical developmental role of *WT1* is evident in the severe genitourinary and mesothelial abnormalities of mice whose *WT1* alleles have been deleted. *WT1* knock-out mice die before birth with failure of kidney and gonadal development in addition to hypoplasia of the heart and lungs (40).

The WT1 protein is present in normal breast tissue and appears to be developmentally regulated in that a high percentage of breast tumor cells express little or no WT1 protein. It is known that breast tumor growth is controlled by the genes encoding components of the IGF and TGF- β signaling system and, recently, altered expression of *WT1* in breast cancer was also reported (41).

WT2 at 11p15

The second Wilms' tumor gene was identified at chromosome locus 11p15 and designated *WT2* (42, 43). This locus is linked to the Beckwith-Wiedemann syndrome (BWS), showing manifestations such as visceromegaly, macroglossia, or hemihypertrophy in some patients (43, 44). *p57* was recently identified as a cyclin-dependent kinase (CDK) inhibitor,

which is found to have the strongest tumor suppressor activity among the genes of the *p21* family (45, 46). As shown in Figure 2, like related family members such as *p21* and *p27*, *p57* binds to several G₁-cyclin/CDK complexes and arrests the cell cycle at the G₁ phase (47). Human *p57* is found at chromosome locus 11p15.5, a region implicated in

the etiology of embryonal tumors including Wilms' tumor and BWS. Frequent loss of 11p15.5 in Wilms' tumor indicates that *WT2* is located in this region (45, 48).

Role in rhabdoid tumor of the kidney

The author established two cell lines of rhabdoid tumor of the kidney (RTK), SWT-1 and SWT-2, as shown in Figure 3 (49). RTK was once proposed to be a variant of Wilms' tumor and subsequently segregated from an unfavorable type of sarcomatous renal tumor that was grouped with clear cell sarcoma (CCSK) and anaplasia of the kidney (50-53). It is well known that RTK has a much poorer prognosis than Wilms' tumor (50, 52, 53). In an attempt to identify the etiology of RTK, the mRNA expression of genes including *WT1*, *p57*, *IGF-2*, *p53*, *N-myc*, and *c-myc* was tested using reverse transcriptase-polymerase chain reaction (RT-PCR) in the two cell lines. The expressions were compared with those in a biopsied kidney tissue and a normal mesangial cell line, MCP-3. Coinciding with the karyotype of SWT-2 cell lines, del 11p13 as shown in Figure 4, no mRNA expression of

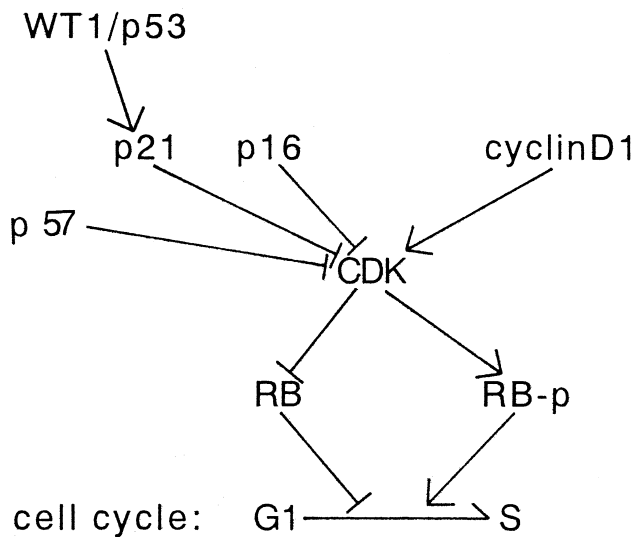


Figure 2. The relation between the proteins that regulate cell cycle progression.

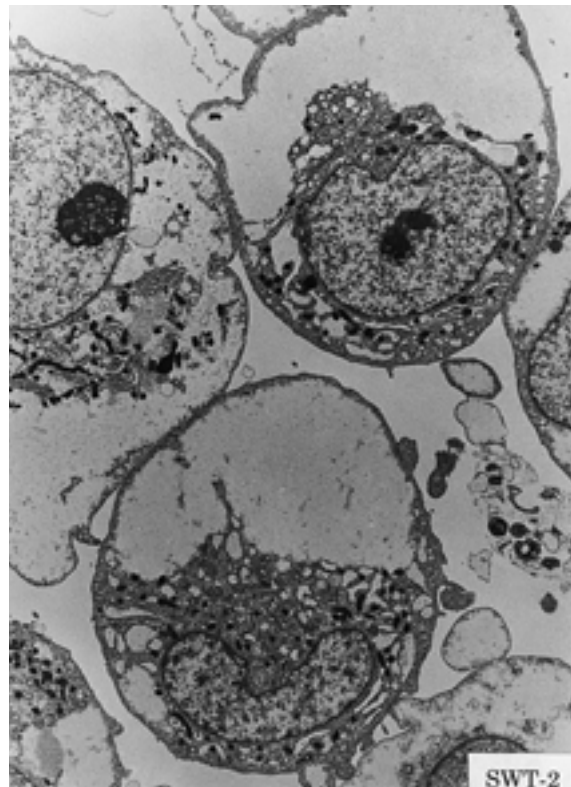
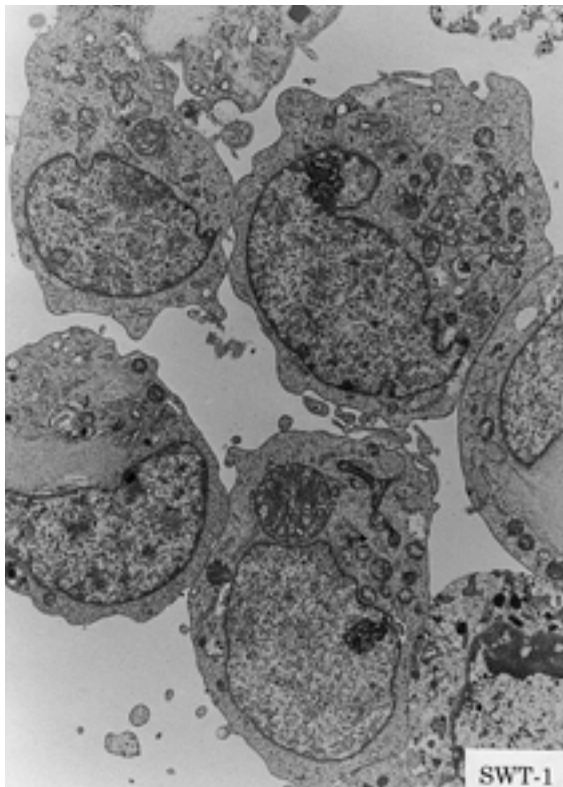


Figure 3. Ultrastructure of SWT-1 and SWT-2. Intermediate filaments characteristic for the rhabdoid tumor can be seen around the perinuclear field.

WT1 was found. Interestingly, SWT-1, which showed a normal karyotype at least on the G-banding analysis, also lacked the *WT1* expression as shown in Figure 5. In addition, SWT-2 lacked the expression of two genes, *p57* and *IGF-2*, while SWT-1 expressed them. Both *N-myc* and *c-myc* expression was detected in the two cell lines (data not shown). These results strongly suggest that *WT1* is involved in the etiology of RTK. However, transient transfection of *WT1* did not reduce colony formation in the other RTK cell line, SM 2 (35). The etiology of RTK may need other genetic abnormalities in addition to *WT1*. The presence of *p57* mRNA in SWT-1 implies that *WT2* is not involved in the etiology of this disease.

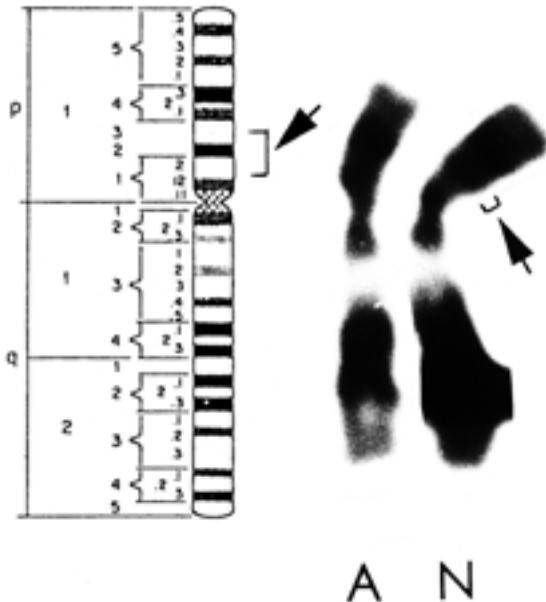


Figure 4. Structure of the short arm of chromosome 11 and the karyotype of SWT-2. SWT-2 shows del 11p13. The *WT1* and aniridia gene of *Pax6* are located at band 11p13, and *p57* and *IGF-2* are located at band 11p15.5.

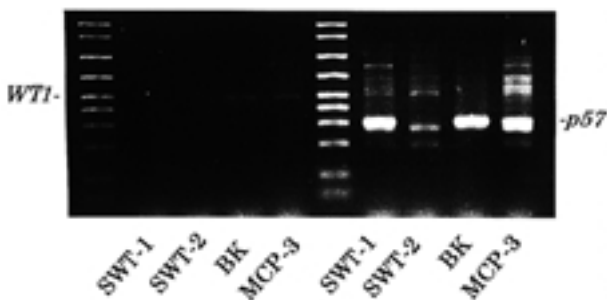


Figure 5. No *WT1* mRNA expression in two RTK cell lines. Neither RTK cell line expresses *WT1* whereas the biopsied kidney sample (BK) and normal mesangial cell line (MCP-3) show weakly positive expression.

Other functions of WT1

cDNA constructs encoding the four human *WT1* splice variants caused programmed cell death, which was associated with a reduced synthesis of EGF-R in human osteosarcoma cell lines, U2OS and Saos-2 (54). The effect of this assay was independent of the presence of the *p53* gene. However, the nature of the recipient cells themselves is an important factor in these transient transfection studies. WT1 and p53 proteins are associated *in vivo* and transfection of *WT1* into cells deleted of the *p53* gene results in transcriptional activation, an effect that is suppressed by the reinduction of wild-type p53 (18). *WT1* was transiently transfected into the p53-negative Hep3B and the p53-positive Hep2G hepatoma cell lines, resulting in the induction of apoptosis in both cell lines by the wild type splice variant, whereas the *WT1* (+KTS) isoform did not induce apoptosis (55). This result is independent of the effect of p53, which seems to contradict a previous publication in which the expression of the *WT1* (-/-) isoform was reported to suppress p53-induced apoptosis in both U2OS and E1A-transformed baby rat kidney (18). Thus, the observation that the effect of the *WT1* isoforms is cell type dependent may explain these discrepancies. Subsequently, it was demonstrated that inducible expression of *WT1* in osteosarcoma cells triggers programmed cell death through an effect that is associated with transcriptional repression of endogenous EGFR. This *WT1* mediated apoptosis was preceded by induction of the CDK inhibitor p21, associated with G1 phase arrest (56).

Microinjection of the *WT1* cDNA into quiescent cells or cells in early to mid G1 phase blocked serum-induced cell cycle progression into S phase, from which inhibition of the activity of cyclin/CDK complexes may be involved in mediating the *WT1*-induced cell cycle block (57).

Mutation of WT1

Mutations in the *WT1* gene underlie 5 % to 10 % of cases of sporadic Wilms' tumor (58). Although the majority follow Knudson's two-hit hypothesis for tumor suppressor genes, it is now clear that a substantial minority (~30 %) of Wilms' tumors retain one normal *WT1* allele, suggesting that in some cases, heterozygous mutation is sufficient for tumorigenesis. Five different types of mutations

are commonly found in Wilms' tumors: large deletions of part of the gene, nonsense or frameshift mutations affecting amino acids in the zinc fingers critical for DNA binding, missense mutations affecting the putative activation or repression domains, and mutations preventing correct splicing. Approximately 75% of the *WT1* mutations found in sporadic Wilms' tumor produce a truncated protein, whereas missense mutations in the zinc finger region predominate in Denys-Drash syndrome (DDS) (58). Wilms' tumor patients have an increased frequency of leukemias as second primary tumors, some of which may be due to *WT1* mutation (59). The biological significance of DNA binding and transcriptional regulation by WT1 is underscored by the finding of small deletions and point mutations in the *WT1* zinc-fingers that abolish DNA binding in a number of Wilms' tumors, especially in tumors associated with the DDS (60). Consequently, the ability of the mutant *WT1* allele, containing an in-frame deletion within the DNA-binding domain, to transform baby rat kidney was tested. The mutant *WT1* gene was found to cooperate with the adenoviral *E1A* gene in transforming baby rat kidney cells, whereas the wild-type *WT1* gene in all of its alternatively spliced forms neither suppressed E1A-induced focus formation in soft agar nor cooperated with E1A (61).

Expression in hemopoetic cells

The *WT1* gene is strongly over-expressed in leukemic blasts compared with immature hematopoietic progenitors with an increase in its expression levels at relapse and an inverse correlation between its expression levels and prognosis, making it a novel marker for leukemia cells. The poor prognosis and the higher level of *WT1* gene expression are linked in patients with leukemia. Thus, it was supposed that the aberrant over-expression of *WT1* contributed to the pathogenesis of AML (62, 63). In addition, *WT1* was shown to be useful in the monitoring of minimal residual disease in patients with hematologic malignancy. However, a consequential result that normal CD34 positive stem cells as well as acute myeloid leukemia cells equally express *WT1* mRNA was obtained (64).

It was reported that K562 cells down regulate *WT1* mRNA during induced erythroid and megakaryocytic differentiation (65). The same phenomenon was observed in HL60 cells, suggesting that sustained

high levels of *WT1* are incompatible with differentiation (66). Likewise, the expression of *WT1* inversely correlated with the differentiation level of acute leukemias (67). *WT1* antisense oligomers inhibit the cell growth of both leukemic cell lines and fresh leukemic blasts from patients with acute leukemia or chronic myeloid leukemia, indicating that the WT1 protein may be important for the sustained proliferation of leukemia cells (68). Contrary to these findings, the monoblastic cell line, U937, constitutively expressing either of the isoforms, (-KTS) or (+KTS), did not respond to differentiation induction by retinoic acid or vitamin D3, whereas the cell line unable to express this gene responded to these substances (69). A recent result indicated that *WT1* expression competes with the differentiation-inducing signal mediated by G-CSF receptor and constitutively activated Stat3, resulting in the blocking of differentiation and subsequent proliferation (70). *WT1* mutation is associated with a failure to achieve complete remission and a lower survival rate in AML, confirming that *WT1* mutations underlie a similar proportion of cases of AML to that seen in Wilms' tumors and its normal role in hemopoiesis is at a very early progenitor stage (71).

Relation between WT1 and multidrug resistance (MDR)

The drug resistance that is a major obstacle to cancer treatment develops at the initial stage of the therapy or during chemotherapy after relapse (72). p170-kd permeability-related glycoprotein (P-GP) encoded by the *mdr-1* gene has ATPase activity which reduces the intracellular drug accumulation by increasing the efflux of anti-cancer drugs from cells (73, 74). p190-kd multidrug resistance-associated protein (MRP) encoded by the *mrp* gene is a member of the ATP-binding cassette super family of membrane transporter proteins that displays minor homology to P-GP (75). It is known that both P-GP and MRP are expressed in normal peripheral blood lymphocytes (PBL), monocytes, and granulocytes as well as cells of hematologic disease. However, the spectrum of cells expressing P-GP is narrow as compared with those expressing MRP, which exists in a wider spectrum of cells or tissues than P-GP (76-79). In recent years, a 110-kd lung resistance-related protein (LRP) has been found

(80), which is reported to be expressed in monocytes among the bone marrow cells (81).

In an attempt to understand the cause of the poor prognosis of leukemia patients whose leukemia cells expressed a high level of *WT1*, the author investigated the relation between *WT1* and multidrug resistance (MDR). The expression level of the *WT1* gene in human hemopoetic cells such as peripheral blood lymphocytes (PBL), interleukin-2 activated lymphocytes (IL-2L), the lymphoblastoid cell lines immortalized by EBV (EBV-LCL), and the cultured cell lines derived from human hematologic malignancies was compared with that of *mdr-1*, *mrp*, and *lrp* in Table 2. Positive expressions of MDR-related genes as above were detected in all PBL, IL-2L, and EBV-LCL, while the *WT-1* expression was found in immortalized cell lines such as EBV-LCL and hematologic malignancies. However, the results using normal cells or wild type cell lines did not demonstrate any positive correlation between MDR-related genes and *WT1*. Subsequently, the author tested the expression of *WT1* in vincristine (VCR) resistant cells. As shown in Figure 6, in three VCR-resistant cells that induced *mdr-1*, *WT1* correlated with the progression of drug resistance and the increase of *mdr-1* expression. The disappearance of *p53* may suggest a linkage with *mdr-1* and *WT1*. Thus, it became obvious that the high level of *WT1* expression is a promising diagnostic marker for drug resistance (82). Another study reported the relation between *WT1* mutations and chemoresistance (81). Although it is apparent that the *WT1* gene is linked to the poor prognosis that is caused by the chemoresistance in hematologic malignancies, little is known of the

role of *WT1*. The role of *WT1* in the hematologic malignancies merits further investigation.

Table 2 . Expression of MDR-related genes and WT1

Lineage	Cell lines	Origin	<i>mdr-1</i>	<i>mrp</i>	<i>lrp</i>	WT-1			
PBL	PBL	PBL	2+	2+	1+	-			
			2+	2+	2+	-			
			2+	2+	2+	-			
			2+	2+	2+	-			
T-cell	IL-2L	PBL	2+	2+	2+	-			
			2+	2+	2+	-			
			2+	2+	2+	-			
			+	2+	2+	-			
B-cell	EBV-LCL	PBL	w	2+	2+	+			
			+	2+	1+	+			
			+	w	1+	+			
			+	2+	2+	w			
Myeloid	K-562 KOPM-28 HEL P39/tsu ML-1 PL-21 HL-60 THP-1 P31/fuj CMK EoLE5	CML CML EL MDS AML APL APL AMoL AMoL MegL EoL	+	+	w	+			
			-	2+	-	-			
			+	2+	1+	w			
			-	+	2+	2+			
			-	w	2+	2+			
			-	2+	2+	+			
			-	+	2+	+			
			-	+	2+	2+			
			-	2+	2+	2+			
			+	+	+	+			
			-	w	1+	2+			
			nT, nB	P30/ohk Reh KOPN-1 KM-3 Nalm-6	cALL cALL cALL cALL cALL	-	+	2+	+
						-	2+	2+	-
						-	2+	2+	+
-	2+	2+				+			
-	2+	2+				2+			
-	2+	2+				2+			
T-cell	P12/ich CCRF-CEM RPMI-8402 MOLT-3 MOLT-4F HPB-ALL ATL-1K MT-2 SKW-3	T-ALL T-ALL T-ALL T-ALL T-ALL T-ALL ATL ATL T-CLL	-	2+	1+	2+			
			-	2+	2+	+			
			-	+	w	2+			
			-	2+	2+	2+			
			-	+	1+	+			
			-	2+	w	2+			
			-	2+	1+	-			
			+	w	2+	-			
			-	2+	2+	2+			
			B-cell	Raji P3HR-1 Daudi BALL-1 BL-TH P32/ish A3/kaw A4/fuk U-266	Afn, BL Afn, BL Afn, BL B-ALL Jpn, BL Jpn, BL Imbl Imbl MM	-	w	-	w
-	w	-				w			
-	+	-				2+			
-	2+	-				-			
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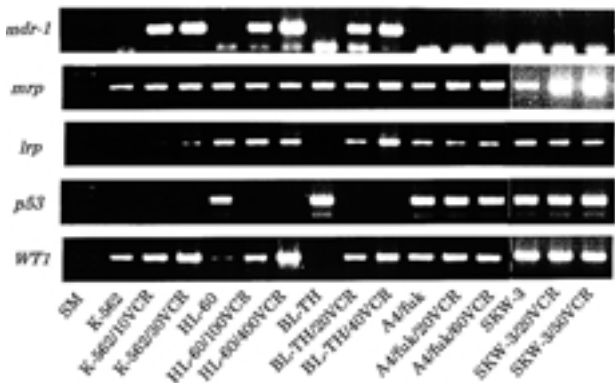


Figure 6. The mRNA expression of *WT1* and MDR-related genes in vincristine resistant hematologic cell lines. The PCR was initiated with 50 ng of cDNA and every PCR went through 28 cycles because the products increased linearly until 30 PCR cycles. Products were analyzed using NIH image.

The level of expression was analyzed using NIH image and the positive expression was divided into three levels by a proper criterion : -, negative expression ; positive expression was described in the order of w<1+<2+.

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