Expression of the Wilms' Tumor Gene *WT1* in Human Malignant Mesothelioma Cell Lines and Relationship to Platelet-Derived Growth Factor A and Insulin-Like Growth Factor 2 Expression

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Mutations in the *WT1* tumor suppressor gene are known to contribute to the development of Wilms' tumor (WT) and associated gonadal abnormalities. *WT1* is expressed principally in the fetal kidney, developing gonads, and spleen and also in the mesothelium, which lines the coelomic cavities. These tissues develop from mesenchymal components that have subsequently become epithelialized, and it has therefore been proposed that *WT1* may play a role in this transition of cell types. To test the possible involvement of this gene in malignant mesothelioma, we have first studied its expression in a panel of human normal and malignant mesothelial cell lines. *WT1* mRNA expression levels varied greatly between the cell lines and no specific chromosomal aberration on 11p, which could be related to the variation in *WT1* expression in these cell lines, was observed. Furthermore, no gross deletions, rearrangements, or functionally inactivating point mutations in the *WT1* coding region were identified. All four *WT1* splice variants were observed at similar levels in these cell lines. The *WT1* gene encodes a zinc-finger transcription factor and the four protein isoforms are each believed to act as transcriptional repressors of certain growth factor genes. Lack of *WT1* expression is thus predicted to result in growth stimulation of tumor cells. Binding of one particular WT1 isoform construct to the insulin-like growth factor 2 (*IGF2*) and platelet-derived growth factor A (*PDGFA*) gene promoters has been demonstrated to result in repression of these genes in transient transfection studies. Analysis of *IGF2* and *PDGFA* mRNA expression levels compared with *WT1* mRNA expression levels failed to demonstrate an inverse correlation in the mesothelial cell lines, which endogenously express these genes. Finally, the putative role of WT1 in the transition of cell types was investigated. No obvious correlation between *WT1* expression levels and cell morphology of the malignant mesothelial cell lines was evident from this study. Moreover, no change in *WT1* expression was observed in normal mesothelial cells which were, by alteration of culture conditions, manipulated to switch from the mesenchymal to epithelial morphology. *Genes Chromosom Cancer 12:87–96 (1995).* © 1995 Wiley-Liss, Inc.

INTRODUCTION

Human malignant mesothelioma is a mesodermally derived tumor, which is most often found in the pleura. Its incidence is strongly associated with exposure to asbestos fibers (Wagner et al., 1960). Malignant mesothelioma is thought to develop from mesothelial cells, which form a specialized epithelium lining the coelomic cavities. Malignant mesothelioma was mentioned as one of the so-called second risk tumors in a few patients who had recovered from the pediatric kidney malignancy Wilms' tumor (WT) (Austin et al., 1986). This suggests the possible involvement of a common underlying genetic event in both malignancies.

The *WT1* locus on 11p13 has been identified as one of the chromosomal loci contributing to WT development (Call et al., 1990; Gessler et al., 1990). *WT1* encodes a protein with DNA-binding capacity, elicited by four zinc-fingers at the C-terminal part, which bind to DNA sequences with the core consensus element 5'-GCGGGGGCG-3' (Rauscher et al., 1990). The WT1 protein additionally possesses transcriptional regulatory activity exerted through the N-terminal glutamine- and proline-rich regions of the protein (Madden et al., 1991). Alternative splicing of the *WT1* gene at two independent splice sites has been shown to result in the formation of four *WT1* splice variants. In tumor tissue and fetal kidney there appears to be little variation in the ratios of these four isoforms (Haber et al., 1991; Brenner et al., 1992).

In transient transfection studies insulin-like growth factor 2 (*IGF2*), platelet-derived growth factor A (*PDGFA*), and *IGF1R* have recently been identified as potential target genes for transcriptional repression by WT1 (Drummond et al., 1992; Gashler et al., 1992; Wang et al., 1992; Werner et

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al., 1993). These and other results have led to the hypothesis that the WT1 protein functions as a tumor suppressor gene product and that reduction or absence of *WT1* expression that is seen in a proportion of WTs results in an increased expression of certain growth factor genes. However, transactivation by WT1 was also seen, depending on the presence of wild-type TP53 protein, the number of WT1 binding sites in the promoter of the target gene, or the presence of specific missense mutations within the transregulatory domain (Maheswaran et al., 1993; Park et al., 1993a; Wang et al., 1993).

Despite these results on repression or activation of certain target genes by WT1 in transient assays, not much is known about the physiological role of WT1. It has been suggested that WT1 may play a role in mediating the shift from a mesenchymal to an epithelial phenotype, as it is expressed in the nephrogenic epithelia, in epithelial cells of the gonads, and in the mesothelium (Pritchard-Jones et al., 1990; Pelletier et al., 1991; Armstrong et al., 1992). These are all cells of mesodermal origin that have undergone the transition to the epithelial morphology. However, other cell types undergoing this transition do not express the *WT1* gene, whereas in embryonic mice distinct regions in the spinal cord and brain that are of ectodermal origin display *WT1* expression, arguing that there should be other tissue-specific roles for WT1 in development (Armstrong et al., 1992). Recently, a crucial role for *WT1* in early urogenital development was established in a model system by gene targeting in murine embryonic stem cells (Kreidberg et al., 1993). In these *WT1* knockout mice a failure in kidney development was observed. This was suggested to be caused by inhibition of inductive events leading to the formation of the metanephric kidney. In addition, abnormal development of the gonads, the heart, and the mesothelium was seen as well. Abnormalities in the phenotypes of these tissues support the idea of a role for WT1 in the mesenchymal to epithelial cell transition.

As *WT1* expression was observed in the human and mouse developing and mature mesothelium (Pritchard-Jones et al., 1990; Armstrong et al., 1992; Park et al., 1993b), we were interested to determine whether *WT1* was also expressed in its malignant counterpart as this might indicate a possible involvement of *WT1* in the pathogenesis of malignant mesothelioma. In this respect, it is worth noting that cytogenetic analysis of 40 confirmed mesothelioma patients revealed karyotypic abnormalities in several chromosomes, but only in a few cases were rearrangements in 11p seen (Hagemeijer et al., 1990). We have studied expression of the *WT1* gene in a panel of human normal and malignant mesothelial cell lines. Expression levels were related to the morphology (epithelial or mesenchymal) of the mesothelioma cells in vitro in order to clarify putative WT1 involvement in cell type transition. We also investigated whether gross alterations or point mutations could be detected at the *WT1* locus, whether the four different alternatively spliced mRNAs were present in the various normal and malignant mesothelial cell lines, and whether the *WT1* mRNA expression level in these cell lines could be correlated to their *PDGFA* and *IGF2* mRNA levels.

MATERIALS AND METHODS

Cell Lines, Growth Conditions, Characterization, and Cytogenetics

Experiments were performed using the human pleural malignant mesothelioma cell lines Mero-14, -25, -41, -48b, -48c, -72, -82, -83, -84, -95, -96, and -123 (Versnel et al., 1988, 1989) and the normal mesothelial cell lines NM-1, -4, -5, -9, and -12 (Versnel et al., 1991, 1993; Langerak et al., in preparation). All mesothelioma cell lines were derived from mesothelioma patients whose diagnosis was based on routine cytology, which was histologically or ultrastructurally confirmed. Cell lines were routinely cultured as described earlier (Versnel et al., 1988, 1989). Cytogenetic analysis was performed as described earlier (Versnel et al., 1988).

Northern and Southern Blot Analyses and Probes

Northern blotting and Southern blotting were performed as described in Langerak et al. (1992). For hybridization of Northern and Southern blots a 1.8 kb *Eco*RI *WT1* fragment derived from the WT33 cDNA was used (Call et al., 1990). Northern blots were rehybridized with the 1.3 kb *Eco*RI *PDGFA* fragment (Betsholtz et al., 1986), the 1.4 kb *Xba*I-*Eco*RI *IGF2* fragment from pIGF-II (Jansen et al., 1985), and the 0.7 kb *Eco*RI-*Pst*I glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) fragment (Benham et al., 1984). Rehybridization of Southern blots was performed with a 1.3 kb *Pst*I *IGF2* fragment from pKT218 (Jansen et al., 1985).

Reverse Transcription (RT) Polymerase Chain Reaction (PCR) Analysis

RT of RNA, isolated from the normal and malignant mesothelial cell lines, and subsequent PCR

analysis were performed as described previously (Langerak et al., 1992). For amplification of the alternative splice variants the sense primers B297 (5′-TTG GTC GAC ATG ACC TGG AAT CAG/C ATG-3′; located in *WT1* exon 4) or B439 (5′-CTT GTA CGG TCG GCA TCT-3′; located in *WT1* exon 7) were used in combination with antisense primer B298 (5′-TGC AAG CTT CAG CTG AAG GGT/C TTC/T TC-3′; located in *WT1* exon 10) (Little et al., 1992). Thirty-five cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C were performed. PCR products were analyzed on 10% polyacrylamide gels using *Pst*I digested lambda DNA as a marker.

Point Mutation Analysis

Chemical cleavage mismatch analysis using hydroxylamine and osmium tetroxide (HOT analysis) was performed as described (Cotton et al., 1988; Prosser et al., 1990). Templates were generated by RT-nested PCR (Hanson et al., 1993), a strategy required due to low yields of available RNA. The *WT1* region encoding the transregulatory domain was amplified with outer primers D609 sense (5′-CAA ACA GGA GCC GAG CTG G-3′) and D610 antisense (5′-GCA CAT CCT GAA TGC CTC TG-3′) followed by inner primers 1 and 3 (Brown et al., 1992). The DNA-binding domain was similarly amplified with primers C582 sense (5′-AAA TGG ACA GAA GGG CAG AGC-3′) and C583 antisense (5′-TTG GAA GTT GGA TGA AGA AGA TC-3′) followed by primers 2 and 4 (Brown et al., 1992). PCR conditions for D609/D610 are step 1, 1 min at 94°C; step 2, 30 cycles of 30 sec at 94°C, 1 min at 54°C, and 1–2 min at 72°C; step 3, 10 min at 72°C. Touchdown PCR conditions for C582/C583 are step 1, 1 min at 94°C; step 2, 30 cycles of 30 sec at 92°C, 1 min at 62–57°C and 2 min at 72°C; step 3, 10 min at 72°C. Samples generating cleaved fragments after HOT analysis were subjected to a second round of RT-nested PCR. Then the products were purified by β-Agarase I (New England Biolabs, Beverly, MA) and subsequently sequenced directly as described (Winship, 1989).

RESULTS

WT1 mRNA Expression in Mesothelial Cell Lines

Expression of the *WT1* gene was studied on Northern blots, containing total RNA from normal and malignant mesothelial cell lines of human origin. *WT1* mRNA was found to be consistently expressed in the normal mesothelial cell lines, although some variation in the levels was observed (Fig. 1). The *WT1* expression level was highly variable in the investigated panel of malignant mesothelioma cell lines, ranging from very high (Mero-25) to nearly or totally undetectable (Mero-41, -72, -82, -83, -95) (Fig. 1). *WT1* mRNA expression levels were normalized over *GAPDH* mRNA levels after quantification by densitometric analysis (see Table 1).

Based on their histology, malignant mesotheliomas can be classified as epithelial, mesenchymal, or biphasic. The morphology of the cultured mesothelioma cells in monolayer can differ from the primary tumor-tissue morphology (see Table 1). In an attempt to clarify the possible role of the *WT1* gene product in the transition of certain cell types from a mesenchymal toward an epithelial morphology, *WT1* mRNA expression levels in the cultured mesothelioma cells were therefore related to the epithelial or fibrous/mesenchymal morphology of these cell lines. Although the highest *WT1* mRNA level was observed in Mero-25, which has the clearest epithelial phenotype, *WT1* expression was not completely confined to cell lines showing an epithelial or biphasic morphology. *WT1* mRNA was undetectable in several cell lines with a fibrous morphology like Mero-41 and -72, but other fibrous cell lines did express *WT1* (Mero-83, -96, and -123). In cell lines that were predominantly either fibrous or epithelial, expression was observed occasionally (Mero-48b, -48c, and -84), and in others not at all (Mero-82 and -95).

The putative involvement of *WT1* in the mesenchymal to epithelial shift was further studied using a second approach. It has been described that cultured normal mesothelial cells can adopt a mesenchymal (fibrous) or epithelial cell shape depending on the presence or absence of epidermal growth factor (EGF) in the culture medium, respectively (Connell and Rheinwald, 1983). Therefore, the *WT1* mRNA level was determined in two normal mesothelial cell lines under these conditions. The two cell lines showed a more epithelial morphology upon removal of EGF [and hydrocortisone (HC)] for 3 days from the standard culture medium. However, analysis of RNA isolated from the mesothelial cells cultured for 72 hr in the absence of EGF and HC did not result in a significantly altered level of *WT1* mRNA (data not shown).

Alternative Splicing Pattern of *WT1*

The *WT1* gene is capable of producing four different mRNAs by alternative splicing, which can lead to the insertion of an extra 51 bp (exon 5) upstream of the zinc-fingers and/or 9 bp (giving rise

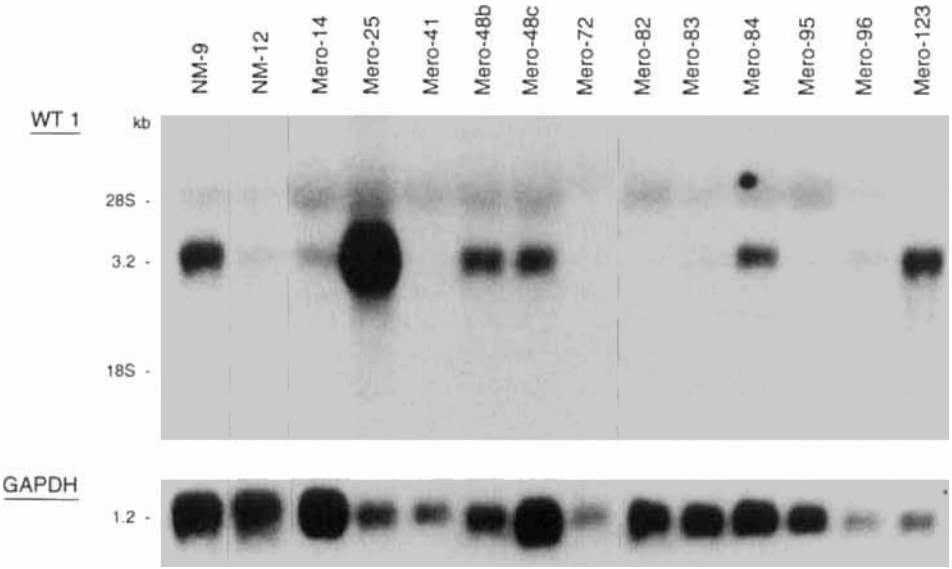

Figure 1. Northern blot analysis with 25 μg total RNA from normal (NM) and malignant (Mero) mesothelial cell lines. RNA was hybridized to *WT1* and *GAPDH* probes.

TABLE 1. Summary of Cytogenetics, Tumor Cell Line Morphology, and *WT1* Expression in Normal (NM) and Malignant (Mero) Mesothelial Cell Lines

Cell line	Tumor cell line morphology	Modal chromosome No.	No. of copies of chromosome (arm)			*WT1* mRNA level[a]	*WT1* gene structure (Southern blot)	*WT1* gene (mutations)	*WT1* mRNA (alternative splicing)
			Normal No. 11	Rearranged No. 11p	Total No. 11p				
NM-1	—	46	2	—	2	0.81	ND[b]	ND	4 isoforms
NM-4	—	46	2	—	2	0.28	ND	ND	ND
NM-5	—	46	2	—	2	0.12	ND	ND	ND
NM-9	—	46	2	—	2	0.26	Normal	ND	ND
NM-12	—	46	2	—	2	0.08	ND	ND	ND
Mero-14	Fibrous	75	3 (4)	—	3 (4)	0.04	Normal	Not found	4 isoforms
Mero-25	Epithelial	67	3 (4)	1×inv (11)(p11q14)	4 (5)	9.59	Normal	Not found	4 isoforms
Mero-41	Fibrous	72	4	—	4	0	Normal	ND	NA[c]
Mero-48a	Epithelial (fibrous)[d]	71–75	3	1×mar (t(9p;11p))	4	ND	Normal	Not found	ND
Mero-48b	Fibrous (epithelial)	71–75	3	1×mar (t(9p;11p))	4	0.43	Normal	Neutral transition	ND
Mero-48c	Fibrous (epithelial)	71–75	3	1×mar (t(9p;11p))	4	0.12	Normal	Neutral transition	4 isoforms
Mero-72	Fibrous	42	2	—	2	0	Normal	ND	NA
Mero-82	Fibrous (epithelial)	49	2	—	2	0	Normal	ND	NA
Mero-83	Fibrous	75–85	2	2×add 11p15	4	0.04	Normal	Not found	4 isoforms
Mero-84	Fibrous (epithelial)	38	0	t(6p + ;11p−), +der(11)t(11;22)	2	0.14	Normal	Not found	4 isoforms
Mero-95	Biphasic	54–58	3	—	3	0	Normal	ND	NA
Mero-96	Fibrous	72–78	2 (3)	2×6q−(6p::11p)	4 (5)	0.26	Normal	Not found	4 isoforms
Mero-123	Fibrous	55	3	—	3	0.91	ND	Neutral transition	4 isoforms

[a]Shown over *GAPDH* expression levels as determined by densitometry.
[b]ND, not determined.
[c]NA, not applicable.
[d]In parentheses: minority of the cells.

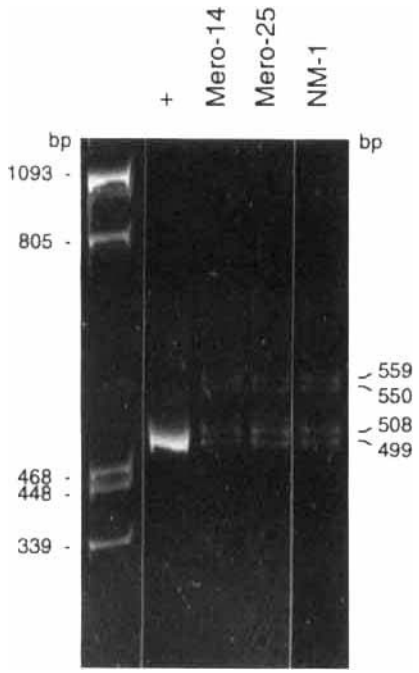

Figure 2. RT PCR analysis with RNA from malignant mesothelioma cell lines Mero-14 and -25 and normal mesothelial cell line NM-1. WT33 cDNA was used as a positive control (+). In the left lane *Pst*I digested lambda DNA was loaded as a size marker.

to the KTS amino acid sequence) between zinc-fingers 3 and 4 (Haber et al., 1991; Brenner et al., 1992). In this study the occurrence of these distinct *WT1* mRNA forms was examined in clonal cell lines. For this purpose cDNA derived from total RNA of several of the *WT1* expressing malignant mesothelioma cell lines (see Table 1) was used in a PCR reaction with primers B297 and B298. Four distinct DNA fragments of 499 bp (−51 bp, −KTS), 508 bp (−51 bp, +KTS), 550 bp (+51 bp, −KTS), and 559 bp (+51 bp, +KTS), resulting from two independent alternative splicing events, were observed (see Table 1). All four splice variants were detected in normal mesothelial cell line NM-1 as well. In Figure 2 these results are shown for the cell lines NM-1 and Mero-14 and -25. Using primers B439 and B298 in all cell lines studied, two distinct fragments of 320 and 329 bp were seen, which result from the 9 bp alternative splicing event (data not shown). In our *WT1* mRNA expressing mesothelial cell lines, little variation was observed in the ratios of the four isoforms.

Cytogenetic and Genomic Data Concerning *WT1*

The variation in *WT1* mRNA expression that we observed in the malignant mesothelioma cell lines could be due to differences in the whole or partial copy number of chromosome arm 11p. Furthermore, rearrangements of the *WT1* gene or gene amplification may also be involved. To see if any of these possibilities may explain the variation in *WT1* mRNA level in the mesothelioma cell lines, cytogenetic analysis and Southern blotting were employed.

Cytogenetic data from the mesothelioma cell lines were obtained by studying metaphase cells. Analysis of chromosome arm 11p in the mesothelioma cell lines did not point toward any specific chromosomal aberration that could be correlated to their *WT1* mRNA expression level (see Table 1). The aneuploidy of chromosome arm 11p and the *WT1* mRNA level in the various cell lines did not correlate either (Table 1).

To see if gene rearrangements had occurred in those cell lines showing very low or undetectable amounts of *WT1* mRNA, the *WT1* gene was studied by Southern blot analysis. No differences were found in the *Eco*RI and *Hin*dIII digestion pattern of DNA from any of the malignant mesothelioma cell lines studied compared to the pattern of normal mesothelial cell line NM-9 (Fig. 3; also data not shown). This indicated that gross rearrangements in the *WT1* gene had not occurred in the malignant mesothelial cell lines and thus were not likely to be the cause of undetectable *WT1* mRNA expression in Mero-41, -72, -82, and -95.

By Southern blot analysis we did detect small differences in the intensity of the bands for the various cell lines, which could in principle be caused by variation in the number of *WT1* gene copies. However, rehybridization of the filter containing *Hin*dIII digested DNA with a probe for the *IGF2* gene, which is also located on the short arm of chromosome 11 at 11p15, showed similar differences in intensity (data not shown). This meant that the *WT1* gene was not differentially amplified in any of the cell lines. Variation in the number of copies of 11p may exist but in general the number of chromosome arm 11p largely balanced the total chromosome number, which suggests that the small differences in intensity are most probably due to small variations in loading of the gels.

WT1 Point Mutation Analysis

The HOT technique of chemical cleavage mismatch analysis was used to scan virtually the entire coding sequence of *WT1* for point mutations. Only the first 256 bp in exon 1 were omitted from this analysis, as the high GC content of this region renders it refractory to PCR amplification. The malig-

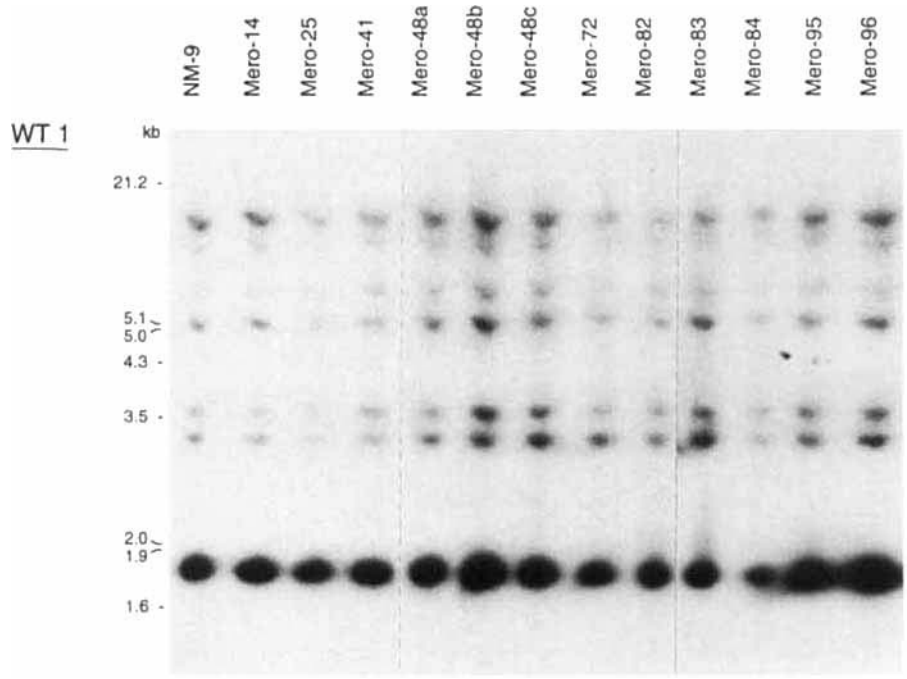

Figure 3. Southern blot analysis of the *WT1* gene. *Eco*RI-digested genomic DNA from a normal (NM-9) and several malignant (Mero) mesothelial cell lines was hybridized to a ^{32}P-labeled *WT1* probe.

nant mesothelioma cell lines Mero-14, -48b, -48c, -83, -84, and -123 were scanned for point mutations in exons 1–6, encoding the transregulatory domain, and Mero-14, -25, -48b, -48c, -83, -84, -96, and -123 were scanned for point mutations in exons 6–10, encoding the DNA-binding domain. The only base change detected was a novel C to T transition at nucleotide 768 (numbered according to Gessler et al., 1990). This exon 1 transition is a third base change and does not cause amino acid substitution (AAC/T encodes an Asn residue). It is present in the homo/hemizygous state in Mero-48b, -48c, and -123 (data not shown). No functionally inactivating nonsense or missense mutations were detected (see Table 1).

WT1 mRNA Expression in Relation to *PDGFA* and *IGF2* mRNA Expression

In transient transfection assays WT1 was reported to repress the expression of *PDGFA* and *IGF2* promoter constructs. In order to see if the described variation in *WT1* mRNA expression in our mesothelial cell lines could be related to different levels of *PDGFA* and *IGF2* mRNA, blots were rehybridized with probes for these two genes and analyzed by densitometry. The results from a representative experiment are presented as arbitrary units over *GAPDH* signals in Table 2.

TABLE 2. mRNA Expression Levels of *WT1*, *PDGFA*, and *IGF2* (6.0 and 4.8 kb) in Normal (NM) and Malignant (Mero) Mesothelial Cell Lines, Expressed as Relative Densitometric Units Over *GAPDH* Expression Levels

			IGF2	
Cell line[a]	*WT1*	*PDGFA*[b]	6.0 kb	4.8 kb
NM-1	0.81	0.65	0	0.53
NM-4	0.28	0.78	0.19	0.66
NM-9	0.26	ND[c]	0	0.23
NM-5	0.12	0.62	0.12	0.48
NM-12	0.08	ND	0.23	0.48
Mero-25	9.59	1.42	0	0.73
Mero-123	0.91	2.22	0	0.68
Mero-48b	0.43	1.41	0.28	1.02
Mero-96	0.26	3.74	1.72	6.55
Mero-84	0.14	1.55	0.02	0.23
Mero-48c	0.12	0.50	0.29	0.43
Mero-14	0.04	0.04	0	0.16
Mero-83	0.04	1.18	0.01	0.18
Mero-82	0	1.02	0	0.21
Mero-72	0	2.59	2.37	2.62
Mero-95	0	2.62	0.07	0.44
Mero-41	0	7.54	0.24	1.27

[a]Cell lines are arranged in descending order of *WT1* expression levels.
[b]Totaled levels of 2.8, 2.3, and 1.9 kb *PDGFA* transcripts.
[c]ND, not determined.

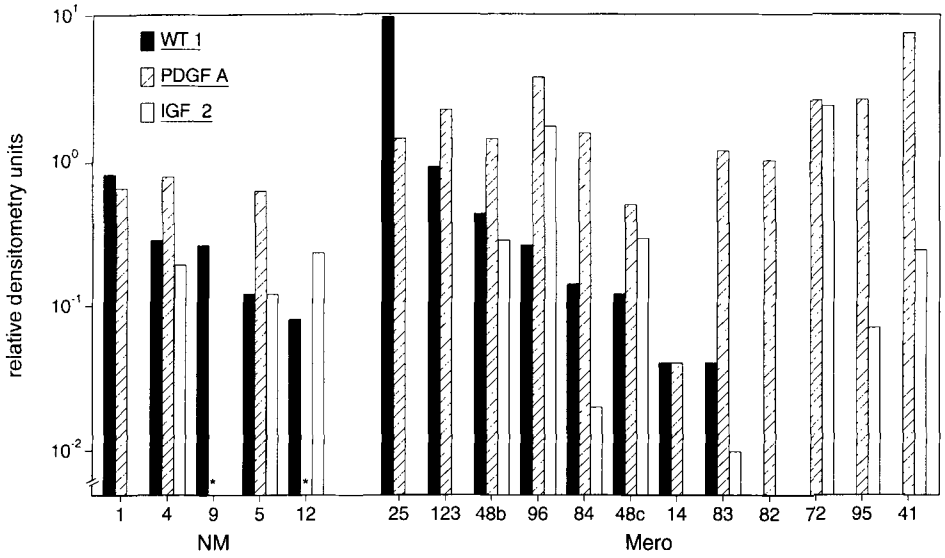

Figure 4. Relative densitometric units of the mRNA expression levels of *WT1*, *PDGFA* (2.8, 2.3, and 1.9 kb totaled) and *IGF2* (6.0 kb) in normal (NM) and malignant (Mero) mesothelial cell lines. The data are presented on a log scale. See Table 2 for the exact densitometric units. Cell lines are arranged in descending order of *WT1* expression levels. Expression levels that were not determined are indicated by an asterisk.

The densitometric data of the three *PDGFA* transcripts (2.8, 2.3, and 1.9 kb) were totaled, as expression of these three messengers is under control of a single promoter region that contains consensus WT1 binding sequences. Our mesothelioma cell lines demonstrated variation in *PDGFA* mRNA expression, but in general the expression level was higher than in normal mesothelial cell lines. In a few cell lines lacking *WT1* mRNA, a relatively high *PDGFA* expression was seen (Mero-41, -72, and -95), but other cell lines which did show *WT1* mRNA expression, i.e., Mero-96 and -123, displayed a similar *PDGFA* mRNA level. Cell line Mero-25, in which a very high *WT1* mRNA level was seen, showed an intermediate level of *PDGFA* expression. This intermediate *PDGFA* level, however, was also seen in cell lines with a lower *WT1* mRNA content than Mero-25. So, as is also illustrated in Figure 4, no clear correlation was observed between *WT1* and *PDGFA* expression in our mesothelial cell lines.

The 6.0 kb *IGF2* transcript is the product of the fetal P3 promoter, which contains WT1 consensus binding sequences, whereas the 4.8 kb transcript is expressed from a different fetal promoter (P4), also containing consensus WT1 binding sequences. Relatively high *WT1* mRNA levels were found in cell lines which do not express the 6.0 kb *IGF2* transcript (NM-1 and Mero-25 and -123), but other cell lines which demonstrated very low or no 6.0 kb *IGF2* mRNA expression, like NM-9 and Mero-14, -82, -83, -84, and -95, showed a low or intermediate *WT1* mRNA level. In the panel of normal and mesothelial cell lines no clear relationship, reciprocal or otherwise, was observed between the 6.0 kb *IGF2* and *WT1* mRNA level (see also Fig. 4). The same holds true for the expression level of the 4.8 kb *IGF2* transcript.

DISCUSSION

The WT gene *WT1* on 11p13 acquired its name, as it was originally mapped by deletion analysis of individuals with the WAGR (WT, aniridia, genitourinary abnormalities, and mental retardation) syndrome (Riccardi et al., 1978; van Heyningen et al., 1985). The demonstration of constitutional and somatic intragenic deletions in the *WT1* gene in a proportion of WT patients has confirmed that it is a WT predisposition gene (Haber et al., 1990; Cowell et al., 1991; Huff et al., 1991; Brown et al., 1992; Tadokoro et al., 1992). In expression studies in human and mouse embryos the *WT1* gene was reported to be involved in normal genitourinary development (Pritchard-Jones et al., 1990; Armstrong et al., 1992). Because of this limited spatial expression, *WT1* was suggested to be important in tissues which are of mesodermal origin and which undergo a mesenchymal to epithelial transition, although expression was also observed in the spinal cord and developing brain. In situ hybridization studies showed high *WT1* expression in the developing Sertoli cells of the testis and granulosa cells of the ovary (Pelletier et al., 1991; Armstrong et al., 1992).

As the studies by Pritchard-Jones et al. (1990) and Armstrong et al. (1992) had revealed the expression of *WT1* in the mesothelium, a specialized epithelium lining the coelomic cavities, an obvious question was if expression of the *WT1* gene could be detected in mesothelioma as well. This prompted us to study *WT1* expression in a panel of normal and malignant human mesothelial cell lines. Northern blot analysis revealed the consistent presence of *WT1* mRNA in cultured normal mesothelial cells, whereas in cultured malignant mesothelioma cells a variation in the expression level, ranging from very high to undetectable, was seen. We also found that the apparent lack of *WT1* mRNA expression in several of these cell lines probably was not due to deletions or rearrangements in the *WT1* gene. Furthermore, differences in the *WT1* mRNA expression level between different cell lines could not be accounted for by gene amplification or a specific chromosomal aberration on 11p. Differences in transcription initiation or RNA degradation thus most probably account for the variation in *WT1* mRNA expression between the malignant mesothelioma cell lines.

When the *WT1*-expressing malignant mesothelioma cell lines were analyzed for more subtle alterations within the coding sequence, no nonsense or missense mutations were found. Three lines contained an identical C to T transition in the sequence encoding the transregulatory domain. However, this mutation is predicted to be silent at the protein level and therefore most likely pathologically insignificant. Recently a homozygous *WT1* missense mutation that alters a Ser residue in the transregulatory domain has been reported for a single case of human peritoneal mesothelioma (Park et al., 1993b). This case is unusual in that the mesothelioma was not asbestos-related and was not actually a malignant tumor but rather a developmental abnormality. In addition, Park et al. (1993b) found no *WT1* mutations in 32 specimens of asbestos-related mesothelioma. For our samples it is possible that there are undetected *WT1* mutations in the 5′-most coding region of exon 1 or in the untranslated or intronic sequences of the gene. The cell lines that fail to show *WT1* mRNA expression may additionally have mutations in the promoter/control regions of the gene. Differences in the occurrence of the four alternative splicing products, which may result in altered specificity for DNA binding sites (Bickmore et al., 1992), were not observed in our panel of *WT1* expressing mesothelial cell lines. All four variants were identified earlier in WT tissue and in fetal kidney (Haber et al., 1991; Brenner et al., 1992). In these tissues the transcripts with the 9 bp alternative splice were suggested to be slightly predominant, whereas in our mesothelial cell lines we did not observe this, but a more quantitative assay has to be performed to unravel this putative discrepancy.

Taken together, the results obtained in our panel of malignant mesothelioma cell lines thus suggest that the *WT1* gene may play a role as a tumor suppressor gene in a minority of human mesotheliomas. To test this in vivo, we started to study primary tumor material from mesothelioma patients. *WT1* mRNA expression could be observed in cells from pleural effusions of four malignant mesothelioma patients (data not shown). These pleural fluids, however, contain tumor cells in combination with several other cell types, which means that this expression cannot be simply attributed to tumor cells, even though pleural fluids with a high percentage of mesothelioma cells were analyzed. Immunofluorescence staining with WT1 antibodies and/or RNA in situ hybridization would be more informative in this respect.

As the ovarian surface epithelium is considered to be a specialized mesothelium (Papadaki and Beilby, 1971) and several ovarian carcinoma cell lines demonstrated a comparable *PDGFA* and *PDGFB* mRNA expression to malignant mesothelioma cell lines (Versnel et al., 1994), we also analyzed several ovarian carcinoma cell lines for *WT1* mRNA expression. Comparable to the expression in malignant mesothelioma cell lines, in three of six serous ovarian carcinoma cell lines studied, *WT1* expression was observed on Northern blots, while in the other three no *WT1* transcripts were detected (data not shown). Furthermore, we recently observed a very high *WT1* mRNA level in cell line COV-434, which is derived from a granulosa tumor of the ovary and thus is not of mesothelial origin (data not shown). This expression is in agreement with the observed *WT1* expression in ovarian granulosa cells (Armstrong et al., 1992).

Malignant mesotheliomas are classified as epithelial, fibrous/mesenchymal, or biphasic. We therefore tried to correlate the morphology of the various malignant mesothelioma cell lines with their *WT1* mRNA expression level. Although the highest expression was found in cell line Mero-25, which has the most obvious epithelial morphology, no clear correlation could be observed between *WT1* mRNA expression and morphology. Moreover, normal mesothelial cells which can switch in morphology depending on the addition or removal of EGF from the culture medium, showed similar

WT1 expression levels independent of the phenotype of the cells. So in these mesothelial cell lines no evidence could be found for a WT1 role in the mesenchymal to epithelial transition of cells. However, this may be different in vivo. Therefore, mesothelioma tissue from patients with a biphasic (i.e., with mesenchymal and epithelial elements) malignant mesothelioma should be studied with WT1 antibodies or by RNA in situ hybridization to investigate the putative correlation between *WT1* expression and morphology in vivo.

It has been shown in transient transfection assays that WT1 represses *PDGFA* (Gashler et al., 1992; Wang et al., 1992) and *IGF2* mRNA expression (Drummond et al., 1992). Our panel of normal and malignant mesothelial cell lines showing variation in *WT1* mRNA expression was analyzed for *PDGFA* and *IGF2* mRNA levels as well. No clear relationship, reciprocal or otherwise, between *WT1* and *PDGFA* or *IGF2* expression was found in our cell lines. The fact that no clear correlation could be found in cells endogenously expressing these genes is in contrast with the repression seen in the forementioned studies. However, these data were obtained in cells upon transfection of *WT1* expression constructs together with *PDGFA* and *IGF2* reporter constructs, whereas we looked at endogenous expression levels in a panel of cell lines. It may also be that in our mesothelial cell lines other factors are also involved in regulating *PDGFA* and *IGF2* mRNA expression, thereby masking regulation by WT1. Alternatively, mutations in the WT1 binding sequences in the promoters of these genes may prohibit WT1 regulation. It remains to be determined, whether WT1 can regulate expression of these genes in a physiological context. Evidence for this may come from stable transfection of the *WT1* gene in Mero cell lines lacking *WT1* or knocking out the endogenous *WT1* gene expression in *WT1* expressing cell lines.

In summary, we have shown that *WT1* mRNA is consistently expressed in normal mesothelial cell lines and that there is no expression in a minority of malignant mesothelioma cell lines. No indications were found for chromosomal aberrations, deletions, rearrangements, functionally inactivating missense or nonsense mutations, or an aberrant alternative splicing pattern in these cell lines. The *WT1* expression level does not seem to correlate with the mesenchymal or epithelial morphology of the various cell lines in vitro. No inverse correlation between *WT1* and *PDGFA* or *IGF2* mRNA expression was seen in our panel of mesothelial cell lines, which endogenously express these genes.

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