WWOX: A candidate tumor suppressor gene involved in multiple tumor types

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We previously reported the construction of a P1-derived artificial chromosome (PAC) contig encompassing a set of homozygous deletions of chromosome 16q23–24.1 found in primary ovarian tumor material and several tumor cell lines. Using these PAC clones in a cDNA selection experiment, we have isolated a *Sau*3A fragment homologous to the *WWOX* transcript (GenBank accession no. AF211943) from normal human ovarian surface epithelial (HOSE) cells. We demonstrate the homozygous deletion of *WWOX* exons from ovarian cancer cells and three different tumor cell lines. We also identify an internally deleted *WWOX* transcript from a further primary ovarian tumor. In three of these samples the deletions result in frameshifts, and in each case the resulting *WWOX* transcripts lack part, or all, of the short chain dehydrogenase domain and the putative mitochondrial localization signal. Sequencing revealed several missense polymorphisms in tumor cell lines and identified a high level of single nucleotide polymorphism (SNP) within the *WWOX* gene. This evidence strengthens the case for *WWOX* as a tumor suppressor gene in ovarian cancer and other tumor types.

A high incidence of loss of heterozygosity (LOH) is demonstrated by 16q23 in breast, prostate, and hepatocellular carcinoma (1–3), with LOH of D16S516 in 67%, 53%, and 52% of cases, respectively. We previously identified homozygous deletions of this region in primary ovarian tumor material and several tumor cell lines (4). An accompanying paper reported a further tumor cell line exhibiting homozygous deletion of 16q23 (5). The lack of cellular heterogeneity, with ubiquity of homozygously deleted cells within these tumors, suggests a selective advantage for cells lacking the 16q23 region, implying the existence of a tumor suppressor gene. Recently, the *WWOX* (*WW* domain containing *ox*idoreductase) gene, encoding a protein with two WW domains and a short-chain dehydrogenase (adh) domain, has been mapped to this region (6). The *WWOX* gene was independently cloned by a second group (7), who named the gene *FOR* (*f*ragile-site FRA16D *o*xido*r*eductase), and reported several alternative splice forms (*FORI-FORIII*) with unique 3′ sequences.

The homologous mouse protein (Wox1; GenBank accession no. AAF31693) has been shown to be an essential mediator of Tnf-α-induced apoptosis (8). Furthermore, mouse Wox1 protein binds directly to p53, and blocking Wox1 by expression of antisense mRNA abolishes p53-mediated apoptosis in NIH 3T3 cells. The high conservation of WWOX protein between *Homo sapiens* and *Mus musculus* (93% identity) supports a similar, important role in apoptosis for human WWOX. We describe here the mutation screening of this candidate tumor suppressor gene in human cancer.

Materials and Methods

Ethical Approval. Institutional ethical approval was granted for this work by the Lothian University National Health Service Trust Medicine/Clinical Oncology Research Ethics subcommittee.

cDNA Selection from Normal Human Ovarian Surface Epithelial (HOSE) Cell mRNA. Primary HOSE cell cultures were established as described (9). RNA extracted from confluent HOSE cell monolayers was used as a cDNA source. Genomic DNA for the experiment was obtained from P1-derived artificial chromosome (PAC) clones from the 700-kb PAC contig we had previously constructed across the 16q23 region (4). The cDNA selection protocol used was a modified version of the method described by Morgan *et al.* (10), and was kindly provided by Ruth Suffolk (Medical Research Council Human Genetics Unit). Full details of the method are available on request. Briefly, double-strand cDNA was prepared from the HOSE cell RNA and was hybridized to the biotinylated PAC DNA. DNA·cDNA hybrids were isolated by using streptavidin coated magnetic beads, and the cDNA was eluted and PCR amplified. Following three cycles of enrichment, the cDNA was cloned. The clones were sequenced and precipitated according to the manufacturer's instructions (ABI PRISM dRhodamine dye-terminator kit, Perkin–Elmer Applied Biosystems) and analyzed on an AB3700 machine (Agnes Gallacher, Medical Research Council Human Genetics Unit).

Tumor Cell Line Material. Ninety-five tumor cell lines of different tumor types (see Table 4, which is published as supporting information on the PNAS web site, www.pnas.org) were obtained from American Type Culture Collection or the Imperial Cancer Research Fund Cell Culture Facility and cultured under standard conditions. DNA was extracted from cell lines by using the Nucleon BACC2 kit (Anachem, Luton, UK). RNA was prepared from cell lines by using TRI reagent (Sigma, Dorset, UK), and was treated with DNaseI (Boehringer Mannheim, Lewes, UK).

Tumor Material and Blood Samples from Cancer Patients. Primary tumor samples were collected from 15 ovarian cancer patients, obtained from the multidisciplinary gynae-oncology clinic, Western General Hospital National Health Service Trust, Edinburgh, and from 34 colorectal cancer patients, obtained from the Sir Alistair Currie Cancer Research Campaign Laboratories (C. Millwater and A. Wylie), Western General Hospital, Edinburgh. Paired normal tissue was available for each of these 49

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Abbreviations: HOSE, normal human ovarian surface epithelial; SNP, single nucleotide polymorphism; RT, reverse transcription.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF325423–AF325432 (PCR products of *WWOX* exons 1–9) and AF325433 (cryptic *WWOX* exon)].

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primary tumors. DNA was extracted from primary tumor and normal tissue by using the Nucleon BACC2 kit (Anachem). RNA from 31 frozen ovarian tumor samples was obtained from the Imperial Cancer Research Fund Medical Oncology Unit (S. Langdon).

DNA Samples from Individuals Without Cancer. DNA from the blood of 54 normal individuals was obtained from the Medical Research Council Human Genetics Unit (S. Farrington).

Mutation Screening of the *WWOX* Gene. Primary PCR of *WWOX* exons was carried out on genomic DNA under standard conditions. Primers and PCR conditions for each exon are detailed in GenBank (accession nos. AF325423–AF325432). Products amplified from tumor cell lines were run on a Transgenomic WAVE machine (Cheshire, U.K.) as described (11), and those showing heteroduplexes were purified by treatment with Exonuclease I and shrimp alkaline phosphatase (Amersham Pharmacia) and then sequenced as described above. Because of the high degree of polymorphism detected, heteroduplex analysis was not useful for reducing the number of samples requiring sequencing, and therefore subsequent PCR products from blood and normal tissues were purified and sequenced directly.

PolyA primed cDNA was prepared by using the 1st Strand cDNA Synthesis Kit (Boehringer Mannheim) and reverse transcription (RT)-PCR was carried out under standard conditions as follows: 25 μl PCR reactions containing 2 μl of reverse transcription reaction, 1× reaction buffer, 2 mM $MgCl_2$, *Taq* polymerase (1 unit, Imperial Cancer Research Fund PIC *Taq*, Promega), and 0.8 μM each PCR primer and dNTPs (200 μM each); initial denaturation at 94°C for 90 s; ten cycles of 94°C for 30 s, 69°C (−1°C per cycle) for 30 s, and 72°C for 1 min; thirty cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 1 min; and a final extension step of 72°C for 3 min. Nested RT-PCR reactions were performed on 1 μl of primary PCR product in 25 μl reactions as above; initial denaturation at 94°C for 90 s; ten cycles of 94°C for 30 s, 67°C (−1°C per cycle) for 30 s, and 72°C for 30 s; thirty cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s; and a final extension step of 72°C for 3 min. Products were either directly sequenced (as previously described) or cloned into pGEM-T Easy (Promega) before sequencing as described above.

PCR primer sequences are WOXfor1 (5′-GAGTTCCTGAGCGAGTGGAC-3′), WOXrev1 (5′-ACTTTCAAACAGGCCACCAC-3′), WOXfor2 (5′-GAATTCAGGTGCCTCCACAGTCAGCC-3′), and WOXrev2 (5′-GAATTCTGTGTGCCCATCCGCTCTG-3′).

Northern Blot of Tumor Cell Lines. Total RNA was isolated from tumor cell lines by using TRI reagent (Sigma). For each RNA sample, 20 μg was separated on a 1.5% agarose-formaldehyde gel and then transferred to a MSI nylon membrane (Micron Separations, Westboro, MA). A 1.29-kb *WWOX* coding region probe was generated by nested RT-PCR using the WOXfor2 and WOXrev2 primers as described above. The membrane was hybridized with radiolabeled probe under standard conditions, and signal was detected by exposure to Kodak X-Omat film for 5 days.

Results

The *WWOX* Gene Is Homozygously Deleted in Several Tumor Types. A 763-bp *Sau*3A fragment of the *WWOX* gene was isolated from HOSE cell cDNA by cDNA selection. BLAST searching the *WWOX* gene revealed homology to several BAC clones from the human genome project. These data were combined with the physical map we previously constructed (4), revealing that the *WWOX* gene spanned more than 1 Mb and encompassed the common fragile site, FRA16D (data not shown). PCR amplification of individual *WWOX* (*FORII*) exons from 95 tumor cell lines of various tumor types (see Table 4) identified homozygous loss of coding exons in four samples. PEO6 [a cell line that is derived from the ovarian cancer patient in whom we identified the original 16q23 homozygous deletion (4), and is 1 of 17 ovarian tumor cell lines screened] was homozygously deleted for *WWOX* exons 4–8. WX330 and NCI-H69 (2 of 12 small cell lung carcinoma cell lines analyzed) showed homozygous deletion of exons 6–8, and PANC1 (1 of 2 pancreatic carcinoma cell lines screened) showed loss of exons 7–8. These homozygous deletions were confirmed by Southern blot hybridization of genomic DNA (data not shown).

***WWOX* Exhibits Internally Deleted Transcripts in Primary Ovarian Tumors and Tumor Cell Lines.** To confirm and extend these findings, RT-PCR was performed on RNA isolated from these deleted cell lines plus a further 34 cell lines, 31 primary ovarian tumor samples, and normal HOSE cells by using the *FORII*-transcript-specific primers WOXfor1 and WOXrev1 (Fig. 1*A*). As predicted from the genomic PCR, the PEO series (PEO1, $PEO1^{CDDP}$, PEO4, and PEO6) and WX330 revealed aberrant transcripts (Fig. 1*A*, transcripts G and D) lacking exons 4–8 and exons 6–8, respectively, as confirmed by sequencing the RT-PCR product. Several samples produced faint RT-PCR products (e.g., PEO16) and so all samples were reamplified in a nested PCR using the primers WOXfor2 and WOXrev2 (Fig. 1*B*). This nested PCR revealed the expected aberrant RT-PCR products in NCI-H69 and PANC1, lacking the homozygously deleted exons (Fig. 1*B*, transcripts C and D), as confirmed by sequencing. Northern Blots of tumor cell line RNA hybridized with a *WWOX* coding region probe confirmed the expected short transcripts, and no full-length *WWOX*, in PEO6, WX330, and NCI-H69 (Fig. 1*C*). No hybridization signal was detected for PANC1.

RT-PCR detected full-length product (Fig. 1, transcript A) in the majority of the cell lines and ovarian tumors, and also in normal HOSE cells. In contrast, two shorter transcripts were detected in the primary ovarian tumor sample HOV25 (Fig. 1 *B* and *D*, transcripts E and F). Sequencing these products demonstrated that both transcripts lack exons 5–8, although transcript E also contained an insertion of 76 bp, with homology to a long terminal repeat element, between exons 4 and 9 (Fig. 1*D*). One explanation of these aberrant transcripts is that HOV25 contains a homozygous deletion. However, because of the stromal contamination in the HOV25 sample, no genomic deletion could be identified and a low level of full-length *WWOX* transcript could be detected (data not shown). It therefore remains possible that the aberrant transcripts in HOV25 result from RNA processing or a hemizygous deletion. However, the predominance of transcripts E and F in the RT-PCR, and also the absence of these products in the other cell lines and primary tumors analyzed, suggest that the HOV25 tumor is homozygously deleted for exons 5–8.

***WWOX* (*FORII* Transcript) Demonstrates Alternative Splicing.** Several cell lines (e.g., 59 M) and primary ovarian tumours (e.g., HOV93) exhibited a full length RT-PCR product (Fig. 1*B*, transcript A) and two additional, smaller products (Fig. 1*B*, transcripts B and D). These transcripts do not correspond to previously reported splice forms for *WWOX*. Sequencing the smaller of these additional bands revealed a *WWOX* transcript lacking exons 6–8. We predict from its size that transcript B lacks exon 7. Some of the tumor cell lines showing these alternative splice forms (e.g., MCF7) were found to be heterozygous for single nucleotide polymorphisms (SNPs) in exons 6, 7, or 8 (see Table 4). This suggests that, in these samples at least, the alternative transcripts were not caused by genomic deletion of exons on a single *WWOX* allele. It is therefore likely that these

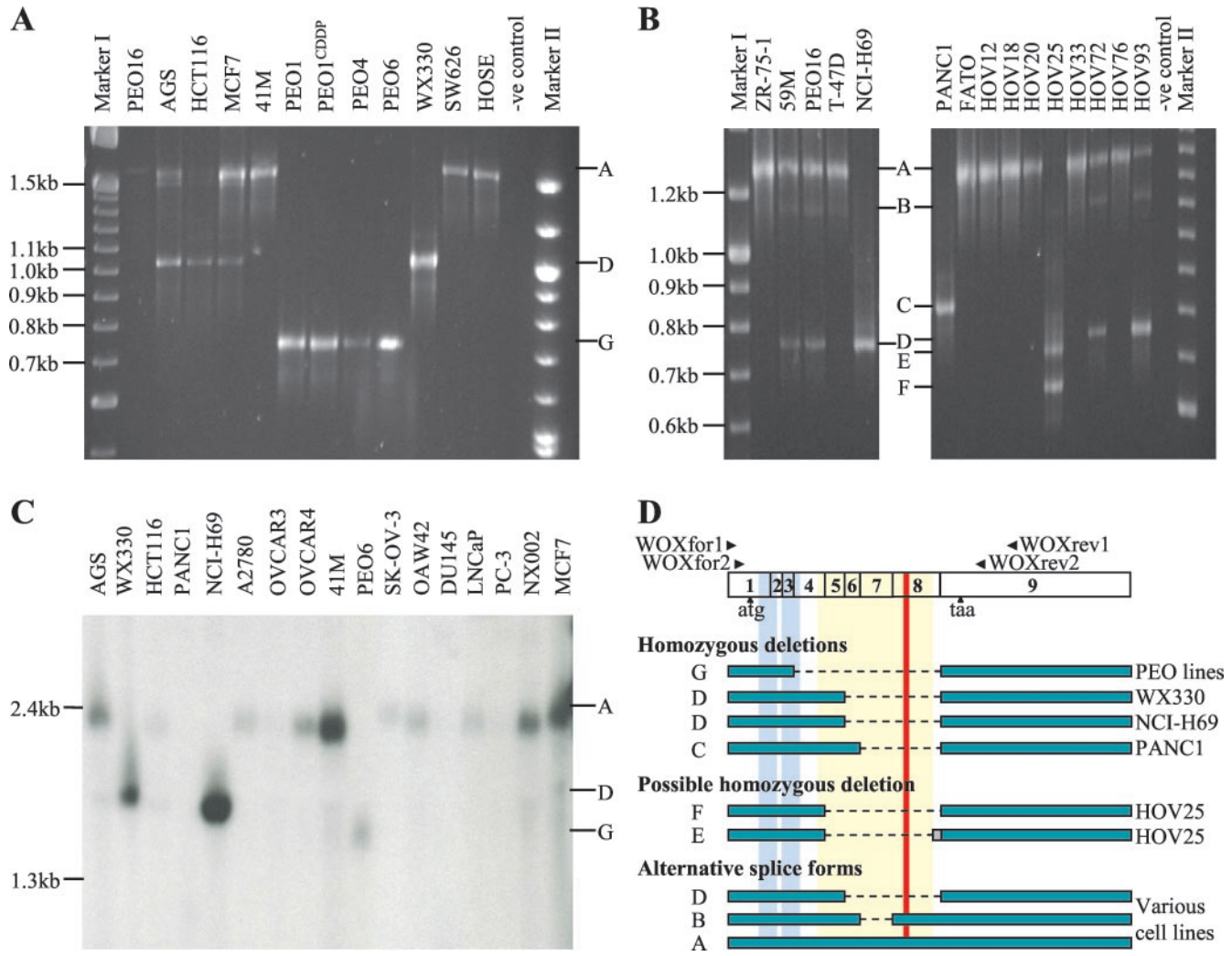

Fig. 1. (*A*) An example RT-PCR from tumor cell line RNA using WOXfor1 and WOXrev1 primers. PCR products were separated on 2% Metaphor agarose (Cambrex, East Rutherford, NJ) in 1× 0.04 M Tris-acetate/0.001 M EDTA (TAE) at 70 V for 4 h. The expected 1.56-kb product representing normal *FORII* (transcript A) can be seen in several cell lines and in HOSE. Aberrant transcripts D and G can be seen in the homozygously deleted WX330 and PEO6 cell lines, respectively. The PEO1^{CDDP}, PEO1, and PEO4 cell lines were derived at different time points from the same ovarian cancer patient as the PEO6 cell line. All four of these lines exhibit homozygous deletion of 16q23 and produce the aberrant transcript G. Several cell lines (e.g., MCF7) show both transcript A and transcript D. (*B*) An example nested RT-PCR from tumor cell line and primary ovarian tumor (HOV) RNA using WOXfor2 and WOXrev2 primers. PCR products were separated on 2% Metaphor agarose in 1× TAE at 70 V for 4 h. The expected 1.29-kb product representing normal *FORII* (transcript A) can be seen in several lanes. Aberrant transcripts C and D can be seen in the homozygously deleted PANC1 and NCI-H69 cell lines, respectively. Two aberrant transcripts (E and F) were detected in the primary ovarian tumor sample HOV25. Several samples exhibited both transcript A and additional products B and D. (*C*) Northern Blot of tumor cell lines hybridized with a *WWOX* coding region probe. Aberrant, short transcripts were detected from WX330 and NCI-H69, and from PEO6, corresponding to the predicted sizes of transcripts D and G, respectively—thus confirming the homozygous loss of *WWOX* exons in these samples. No signal was detected from PANC1. Transcript A was found to be the predominant *WWOX* splice form in the remaining cell lines. (*D*) Schematic representation of the different transcripts identified by RT-PCR. Positions of PCR primers used for primary and nested RT-PCR are shown. White boxes indicate the nine exons of the *WWOX* (*FORII*) transcript, blue boxes show the position of the WW domains, a yellow box indicates the adh domain, and a red line represents the putative substrate-binding site. Each transcript is shown by a green bar with the dashed line representing the deleted portion of the transcript. The gray box in transcript E represents the 76-bp insertion (GenBank accession no. AF325433). Aberrant transcripts C, E, F, and G are out-of-frame deletions, whereas transcript D exhibits an in-frame deletion. However, in every case, the substrate-binding site, putative mitochondrial localization signal, and most of the adh domain are deleted.

transcripts are produced by RNA processing, as reported for the *FHIT* gene (see *Discussion*).

RT-PCR of normal HOSE cDNA (Fig. 1*A*) and a CLONTECH cDNA panel of 16 normal tissues (data not shown) amplified only full-length WWOX (transcript A), suggesting that the alternative transcripts B and D may be cancer-associated. However, screening of a larger panel of normal samples will be necessary to confirm that these splice variants are not physiological.

Missense Polymorphisms Were Detected in Primary Tumors and Tumor Cell Lines. DNA from 95 tumor cell lines, 15 ovarian cancers, and 34 colorectal cancers was screened for point mutations within the *WWOX* gene. We included ovarian and colorectal tumors because we had previously identified homozygous deletions of 16q23 in these tumor types (4). Independent, primary PCR products spanning each exon of the *FORII* and *FORIII* splice forms were analyzed for heteroduplexes by using Transgenomic's WAVE machine and then sequenced, or were sequenced directly (see *Materials and Methods*). Constitutive DNA from 54 individuals without cancer and from normal tissues of the 49 cancer patients was also sequenced to identify common SNPs. In total, thirty-four polymorphic bases (16 exonic SNPs and 18 intronic SNPs) were detected in the tumor cell lines (Tables 1 and 2). The

Table 1. Exonic polymorphisms within the *WWOX* gene

Variant name	Nucleotide position	Exon location	Resulting amino acid change	Frequency of variant allele: Tumour cell lines, %	Frequency of variant allele: Blood from patients, %	Frequency of variant allele: Blood from normals, %
C121T	121	1	none (5′ UTR)	35.6	26.1	33.3
C418T	418	4	Pro-98 → Leu	6.4	1.1	2.9
C457G	**457**	**4**	**Thr-111 → Ser**	**0.9**	**0.0**	**0.0**
C476G	476	4	Leu-117 → Leu	2.7	0.0	0.0
C483T	**483**	**4**	**Arg-120 → Trp**	**1.8**	**2.4**	**0.0**
A660G	660	6	Ala-179 → Thr	57.4	34.8	36.0
A628G	**628***	**6a**	**Lys-182 → Glu†**	**6.6**	**1.1**	**0.0**
C932T	932	8	Asn-269 → Asn	1.3	0.0	1.0
G941T	941	8	Leu-272 → Phe	1.3	0.0	1.0
C969G	969	8	Pro-282 → Ala	18.8	5.7	6.7
T1001G	1001	8	Ala-292 → Ala	1.3	0.0	0.0
G1066A	**1066**	**8**	**Arg-314 → His**	**2.6**	**0.0**	**0.0**
G1403C	1403	9	none (3′ UTR)	3.0	0.0	0.0
G1414T	1414	9	none (3′ UTR)	1.0	0.0	0.0
C1442T	1442	9	none (3′ UTR)	15.0	3.4	0.0
T1497G	1497	9	none (3′ UTR)	54.1	37.2	34.1

Sixteen single nucleotide polymorphisms detected in the exons of the *WWOX* gene. Nucleotide positions are taken from GenBank AF211943 (*FORII* transcript) or GenBank AF187015 (*, *FORIII* transcript). Resulting amino acid changes are predicted from translation of the *FORII* or *FORIII* (†) sequences. The frequencies of the variant allele for each SNP in tumor cell lines, blood from cancer patients, and blood from individuals without cancer are given as a percentage of informative samples. Four missense polymorphisms within the *WWOX* coding region that were not detected in individuals without cancer are shown in bold. For a full list of tumor cell lines and their corresponding SNP data, see Table 4.

frequency with which each of these SNPs was detected in the tumor cell lines was highly variable and ranged from 0.7% (Isnp6) to 64.5% (Isnp17) of informative samples. Fourteen of these SNPs were also identified in normal individuals and 17 in blood from cancer patients (Tables 1 and 2). Eleven polymorphisms (three silent and eight missense), but no truncating point mutations, were identified within the coding region of *WWOX* (Table 1, Fig. 2). Four of these missense polymorphisms (shown in bold in Table 1) were not detected in normal individuals, suggesting that they may be cancer-associated. The Arg-120 → Trp and Arg-314 → His changes alter highly basic residues conserved in mouse and *Drosophila*, to noncharged or weakly basic residues. Both were detected as homozygous changes in tumor cell lines (Table 3), possibly indicating loss of the second allele, or endoreduplication. The Lys-182 → Glu change also alters a basic residue, giving an acidic amino acid in the unique

Table 2. Intronic polymorphisms within the *WWOX* gene

Variant name	Nucleotide change	Nucleotide position	Frequency of variant allele: Tumour cell lines, %	Frequency of variant allele: Blood from patients, %	Frequency of variant allele: Blood from normals, %
Isnp1	C → T	111 bases 5′ of exon 2	20.0	19.7	15.9
Isnp2	G → T	12 bases 5′ of exon 2	38.5	25.5	23.5
Isnp3	C → T	69 bases 5′ of exon 3	51.0	n/d	n/d
Isnp4	A → C	16 bases 3′ of exon 4	37.5	n/d	n/d
Isnp5	T → C	169 bases 5′ of exon 5	31.1	n/d	n/d
Isnp6	G → T	78 bases 5′ of exon 5	0.7	0.0	0.0
Isnp7	A → T	56 bases 5′ of exon 5	1.3	0.0	0.0
Isnp8	C → T	6 bases 3′ of exon 5	19.3	34.5	22.2
Isnp9	G → A	50 bases 3′ of exon 5	23.9	14.8	12.7
Isnp10	C → G	48 bases 5′ of exon 6	3.3	0.0	0.0
Isnp11	C → T	37 bases 3′ of exon 6	11.9	6.7	3.1
Isnp12	G → T	11 bases 5′ of exon 6a	9.4	4.5	1.0
Isnp13	C → A	3 bases 5′ of exon 6a	57.5	37.5	20.8
Isnp14	T → G	18 bases 3′ of exon 6a	0.9	0.0	0.0
Isnp15	G → A	17 bases 5′ of exon 7	23.8	7.8	0.0
Isnp16	C → G	64 bases 3′ of exon 8	4.1	n/d	n/d
Isnp17	C → T	40 bases 5′ of exon 9	64.5	28.6	n/d
Isnp18	T → C	14 bases 5′ of exon 9	13.8	0.0	0.0

Eighteen single nucleotide polymorphisms detected in the introns of the *WWOX* gene. The frequencies of the variant allele for each SNP in tumor cell lines, blood from cancer patients, and blood from individuals without cancer are given as a percentage of informative samples. n/d, not determined. For a full list of tumor cell lines and their corresponding SNP data, see Table 4.

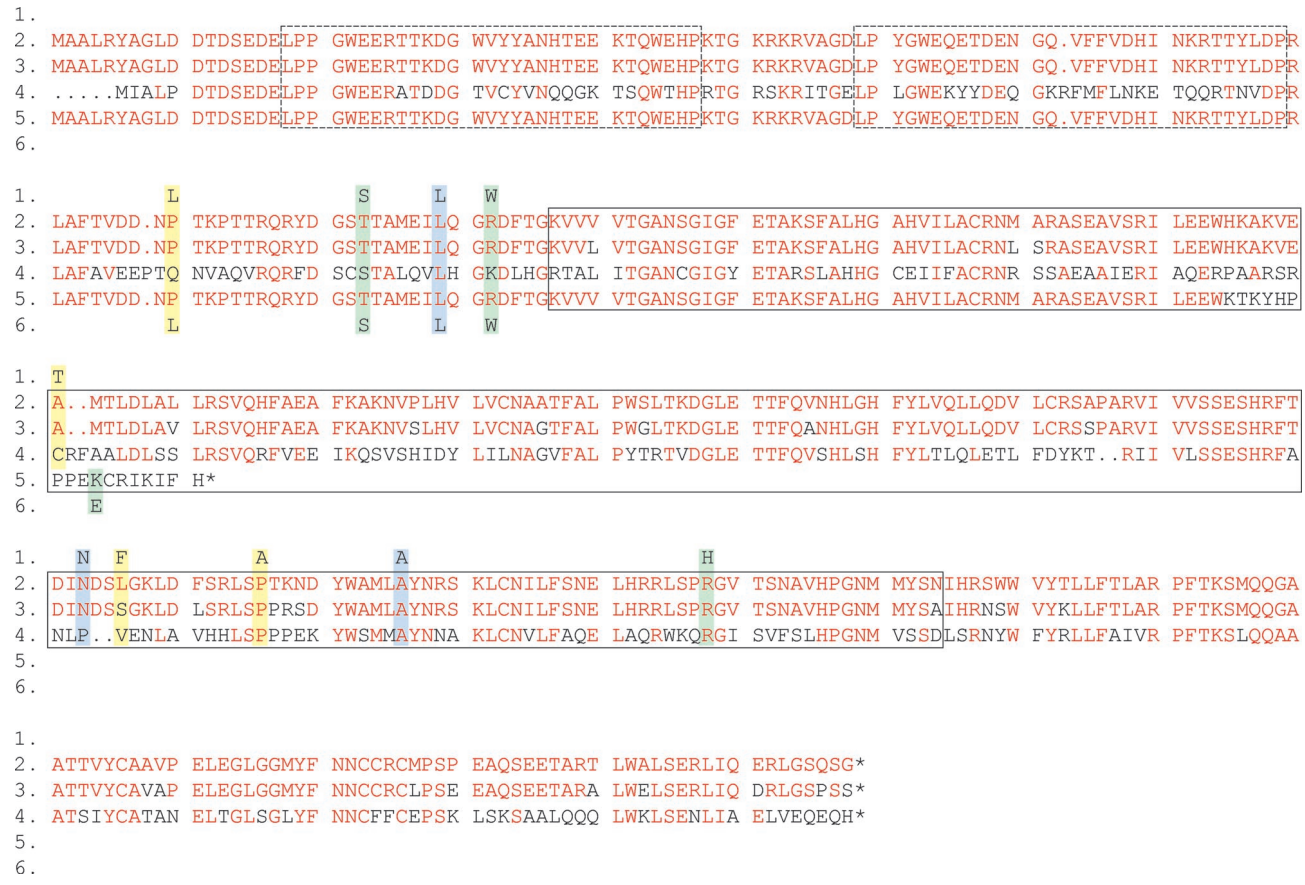

Fig. 2. Polymorphisms within the coding region of the *WWOX* gene. CLUSTALX alignment of WWOX protein sequences showing amino acid residues altered by nucleotide changes identified through mutation screening. Line 1: Polymorphic residues in Human WWOX (FORII isoform) corresponding to polymorphisms listed in Table 1. Line 2: Human WWOX (FORII isoform). Line 3: Mouse Wox1. Line 4: Drosophila Wwox (GenPept accession no. AAF52587). Line 5: Human WWOX (FORIII isoform). Line 6: Polymorphic residues in Human WWOX (FORIII isoform) corresponding to polymorphisms listed in Table 1. Amino acids indicated in red show conservation with the Human WWOX FORII isoform (Line 2). Blue boxes show silent changes where the amino acid is unaltered by the nucleotide change. Yellow boxes indicate polymorphic residues due to SNPs. Green boxes indicate missense polymorphisms detected only in tumor cell lines or normal tissue from cancer patients. The WW domains are shown as dashed boxes and the adh domain as a solid box.

C terminus of the FORIII isoform, whereas the Thr-111 → Ser alteration produces only a conservative change. Tumor cell lines and patient samples exhibiting these cancer-associated polymorphisms are listed in Table 3. Further analysis will determine whether these alterations abrogate WWOX protein function.

Discussion

A number of publications have suggested that 16q23 contains a tumor suppressor gene involved in multiple tumor types. The *WWOX* gene is a candidate for this tumor suppressor gene for the following reasons: (*i*) the *WWOX* gene maps to a region showing loss of heterozygosity (LOH) and homozygous loss in human cancer; (*ii*) a combination of cDNA selection, exon trapping (4), and gene prediction from the human draft genome sequence (data not shown) identified no other genes from this 700-kb region; (*iii*) mouse Wox1 protein binds directly to p53, and enhances TNF-α-mediated cell death, suggesting a role for human WWOX protein in the regulation of apoptosis; and significantly, (*iv*) we now demonstrate homozygous deletion of *WWOX* exons in primary ovarian tumor cells and three tumor cell lines, and aberrant transcripts in a second primary ovarian tumor, possibly also due to homozygous deletion. The deletions in the two ovarian tumors and the pancreatic tumor cell line result in a frameshift, whereas the two small-cell lung cell lines exhibit in-frame deletions. However, in each case the adh domain is disrupted and both the putative mitochondrial localization signal and substrate-binding site are lost, thus abrogating the predicted activity of the protein.

A previous study reported no mutation of *WWOX* in breast carcinoma (6). We have identified homozygous deletion of *WWOX* exons, but sequencing *WWOX* from primary ovarian and colorectal tumors and 95 tumor cell lines (including six breast tumor cell lines) revealed no evidence of truncating point mutations. However, four missense polymorphisms were identified that were not detected in normal individuals (none of which occurred in breast tumor cell lines) and a high degree of polymorphism was found (≈1 polymorphism per 100 bp). A precedent for these unexpected observations exists in the *FHIT* gene, which exhibits many similar features to *WWOX*. It is a large gene (>1Mb) that spans a common fragile site (12) and lies in a region of loss of heterozygosity (LOH) (13). *FHIT* also exhibits a high frequency of alternative splicing because of exon skipping as detected by RT-PCR (12, 14). Additionally, extensive screening of the *FHIT* gene in many cancers has revealed a high incidence of homozygous deletions, but a very low frequency of inactivating point mutations (14), a remarkably similar observation to that seen for *WWOX*. The most likely explanation for this is the intragenic fragile site. Common fragile sites are chromosomal regions that frequently exhibit DNA strand breaks, often following exposure to chemicals that delay DNA replication. It has been proposed that repair of these double-strand breaks involves homologous recombination between

Table 3. Tumor samples exhibiting alterations within the coding region of *WWOX*

Tumor sample	Tumor type	Alteration of *WWOX* gene detected
PEO1	Ovarian adenocarcinoma	Homozygous deletion of exons 4–8
PEO1^{CDDP}	Ovarian adenocarcinoma	Homozygous deletion of exons 4–8
PEO4	Ovarian adenocarcinoma	Homozygous deletion of exons 4–8
PEO6	Ovarian adenocarcinoma	Homozygous deletion of exons 4–8
WX330	SCLC	Homozygous deletion of exons 6–8
NCI-H69	SCLC	Homozygous deletion of exons 6–8
PANC1	Pancreatic ductal adenocarcinoma	Homozygous deletion of exons 7–8
HOV25	Primary ovarian tumor	Aberrant transcript lacking exons 5–8
MOLT-4	Acute lymphoblastic leukaemia (T cell)	Heterozygous Lys-182/Glu-182*
P3HR-1	Burkitt lymphoma	Heterozygous Thr-111/Ser-111
U-937	Histiocytic lymphoma	Homozygous Trp-120/Trp-120
OH5 Hela	Cervical carcinoma (Hela variant)	Homozygous His-314/His-314
SAOS-2	Osteosarcoma	Heterozygous Lys-182/Glu-182*
SK-OV-3	Ovarian adenocarcinoma	Heterozygous Lys-182/Glu-182*
COR-L105	NSCLC	Heterozygous Lys-182/Glu-182*
COR-L311	SCLC	Heterozygous Lys-182/Glu-182*
NCI-H1672	SCLC	Homozygous Glu-182/Glu-182*
colo11	Primary colorectal tumor	Heterozygous Lys-182/Glu-182*
colo53	Primary colorectal tumor	Heterozygous Arg-120/Trp-120
colo95	Primary colorectal tumor	Heterozygous Arg-120/Trp-120

Tumor cell lines and primary ovarian and colorectal tumors exhibiting homozygous deletion of *WWOX* exons, aberrant *WWOX* transcripts, or one of four cancer-associated missense polymorphisms (shown in bold in Table 1). Amino acid alterations are predicted from translation of the *FORII* or *FORIII* (*) sequences.

flanking repeat elements [long interspersed repeat elements (LINEs) or short interspersed repeat elements (SINEs)], thus resulting in an interstitial deletion (15). The frequency of breakage at the fragile sites may explain why deletion rather than point mutation is the major mechanism of inactivation of *WWOX* and *FHIT*.

Although the fragile site may explain the occurrence of homozygous deletions, the clonal selection of these deletions in tumors suggests an associated disruption of a tumor suppressor gene. Further evidence from *in vitro* genetic analysis and a *Fhit* knockout mouse model supports a role for Fhit as a tumor suppressor important in several cancer types (16, 17). The heterozygote *Fhit* knockout mouse demonstrated an increased incidence of neoplasia associated with loss of Fhit expression, on exposure to *N*-nitrosomethylbenzylamine. This suggests that second-hit disruption of the fragile site, and therefore *Fhit*, was a consequence of carcinogen exposure. We would predict a similar carcinogenic mechanism for *Wox1* inactivation in a heterozygous mouse model. Functional analysis of the various missense and deleted *WWOX* transcripts is currently underway to determine the physiological function of WWOX and its role in cancer.

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