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The human homologue of *unc-93* maps to chromosome 6q27 – characterisation and analysis in sporadic epithelial ovarian cancer

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Abstract

Background: In sporadic ovarian cancer, we have previously reported allele loss at *D6S193* (62%) on chromosome 6q27, which suggested the presence of a putative tumour suppressor gene. Based on our data and that from another group, the minimal region of allele loss was between *D6S264* and *D6S149* (7.4 cM). To identify the putative tumour suppressor gene, we established a physical map initially with YACs and subsequently with PACs/BACs from *D6S264* to *D6S149*. To accelerate the identification of genes, we sequenced the entire contig of approximately 1.1 Mb. Seven genes were identified within the region of allele loss between *D6S264* and *D6S149*.

Results: The human homologue of *unc-93* (UNC93A) in *C. elegans* was identified to be within the interval of allele loss centromeric to *D6S149*. This gene is 24.5 kb and comprises of 8 exons. There are two transcripts with the shorter one due to splicing out of exon 4. It is expressed in testis, small intestine, spleen, prostate, and ovary. In a panel of 8 ovarian cancer cell lines, UNC93A expression was detected by RT-PCR which identified the two transcripts in 2/8 cell lines. The entire coding sequence was examined for mutations in a panel of ovarian tumours and ovarian cancer cell lines. Mutations were identified in exons 1, 3, 4, 5, 6 and 8. Only 3 mutations were identified specifically in the tumour. These included a c.452G>A (W151X) mutation in exon 3, c.676C>T (R226X) in exon 5 and c.1225G>A(V409I) mutation in exon 8. However, the mutations in exon 3 and 5 were also present in 6% and 2% of the normal population respectively. The UNC93A cDNA was shown to express at the cell membrane and encodes for a protein of 60 kDa.

Conclusions: These results suggest that no evidence for UNC93A as a tumour suppressor gene in sporadic ovarian cancer has been identified and further research is required to evaluate its normal function and role in the pathogenesis of ovarian cancer.

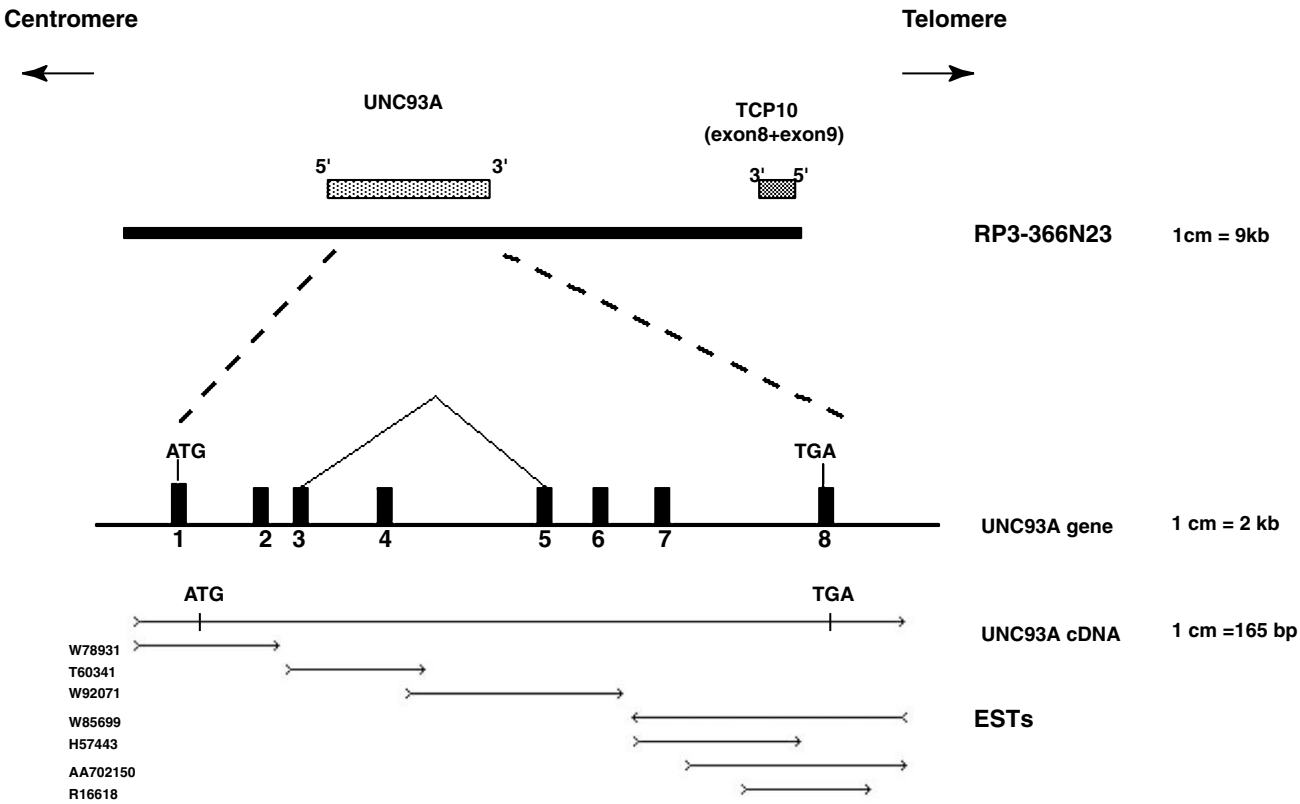


Figure 1
Genomic structure and ESTs corresponding to UNC93A. PAC 366N23 contains the entire gene for UNC93A and exons 8 and 9 of TCP10. The individual exons of UNC93A are shown diagrammatically. The alternative splice variant is without exon 4. Individual ESTs (accession numbers) are shown based on their sequence homology to UNC93A cDNA.

Background

Ovarian cancer is the most frequent cause of death from gynaecological malignancies in the Western World [1]. Epithelial ovarian cancer constitutes 70–80% of ovarian cancer and encompasses a broad spectrum of lesions, ranging from localised benign tumours and neoplasms of borderline malignant potential to invasive adenocarcinomas. The p53 gene has been found to be frequently mutated (50%) in sporadic malignant ovarian tumours [2]. The BRCA1 and BRCA2 genes are mutated in a proportion of patients with familial breast/ovarian cancer [3]. However, familial ovarian cancer accounts only for 5–10% of all ovarian tumours [4]. In tumours from patients with sporadic ovarian cancer, only five mutations in the BRCA1 gene and four in the BRCA2 gene have been reported suggesting that they are rare in sporadic ovarian cancer [5,6]. However, epigenetic inactivation due to methylation has been reported for BRCA1 and 2 in a proportion of sporadic tumours [7]. Mutations in the mismatch repair genes have been reported at a frequency of 10% in sporadic ovarian cancer [8]. More recently, the PTEN gene has been reported to be mutated in 20% of endometrioid subtype of sporadic ovarian tumours [9]. However, detailed evaluation by mutation analysis and immunohistochemistry has shown inactivation of PTEN in other subtypes of ovarian cancer [10]. Thus tumour suppressor genes that may be more critical in tumour progression in sporadic ovarian cancer have not yet been fully characterised [11].

Chromosome 6 has been implicated to contain a putative tumour suppressor gene important in the pathogenesis of ovarian cancer, both by karyotypic analysis and allele loss studies. The analysis of 70 malignant ovarian tumours using cosmids mapping to chromosomal arm 6q initially defined the minimal region of allele loss between *D6S149* and *D6S193* (1.9 cM) in one tumour [12,13]. Subsequent studies have shown increased frequency of allele loss on 6q around the same region, although a minimal region was not defined [14,15]. Based on the analysis of 56 malignant ovarian tumours we showed previously that the minimal region of allele loss on 6q27 is between *D6S297* and *D6S264* (3 cM) (Figure 1). The maximal frequency of

Table 1: Intron-exon structure of UNC93A.

EXON	EXON SIZE (bp)	INTRON SIZE (bp)	5' SPLICE SITE *	3' SPLICE SITE *
1	164	2941	gattgttttt/CCCATGCCTC	GAGCCTGCAG/gtatgtgtt
2	181	1334	gttcccacag/AGCAGCCTGT	TCGCCAGCTG/gtacgcagcc
3	229	1684	ttacccacag/GTACACTTTG	CCCAGCCAAG/gtaaaaggaa
4	125	5613	gacttcatag/AGACCCTTCC	ATCTACACTG/gtacgagctc
5	214	1768	gtcctttcag/GGAGTGGTGT	ATACACAAGG/gtatgaacga
6	135	1729	ctctccccag/TCCTATGTCA	TACGTGCTGG/gtaggtatca
7	131	7277	gcacccccag/GCGCGGTGAC	CAAAACAATG/gtgagtcccc
8	827		tcctctgcag/CTCTCTACGG	AAAAATGTGA/gagcagtgag

* Letters in lower case correspond to intron sequence and the upper case to exon sequence.

allele loss occurred at *D6S193* (62%) and *D6S297* (52%) which are approximately 55 Kb apart. Three tumours showed loss of *D6S193* only, while retaining flanking markers thus suggesting that the putative tumour suppressor gene was close to *D6S193*. The allele loss was observed in all types of epithelial ovarian cancer [16–18]. Further evidence that there might be a putative tumour suppressor gene around *D6S193* is provided by FISH studies using YACs on direct metaphase spreads from fresh ovarian tumours which suggested that this change might be important even in early ovarian tumours [19,20]. More recently, a homozygous deletion has been mapped centromeric to *D6S193* in one ovarian cancer cell line [21]. Further, it is possible that the same region is implicated in a subset of lymphomas and breast cancer [22–26].

To identify the potential tumour suppressor gene on chromosomal band 6q27 implicated in the pathogenesis of ovarian cancer, we undertook a positional cloning approach. We have constructed an extended bacterial clone contig in PACs/BACs from *D6S264* until *D6S149* which encompasses the maximal possible region of allele loss from our data and that previously reported [12,16]. Subsequently, we undertook sequencing of BACs/PACs which mapped to the key polymorphic markers *D6S193* and *D6S297* and we now have almost complete sequence of the extended contig [27]. Seven genes were identified within the interval between *D6S264* and *D6S149*. This paper reports the identification of the human homologue of *unc-93* in c. *elegans* [28] in the minimal region of allele loss and its analysis in ovarian tumours.

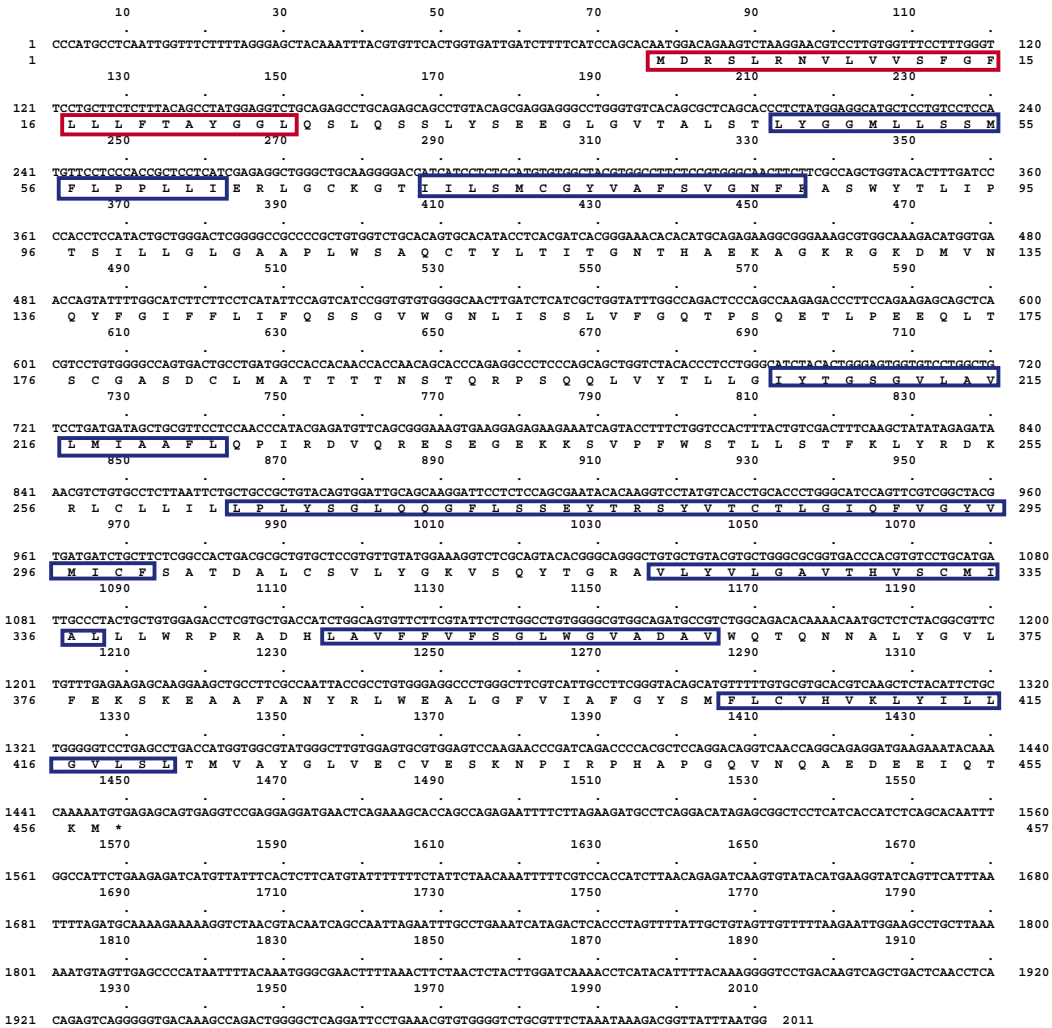
Results

Physical mapping, genomic structure and cDNA sequence of UNC93A

Using the previously defined integrated genetic map of chromosome 6q24-27 [17], we constructed an extended bacterial clone contig (PACs/BACs) between D6S264 and D6S149 [27]. To accelerate the identification of genes in this region, we chose to sequence the entire region. We now have contiguous sequence of approximately 1.1 Mb between D6S264 and PAC RP11-178P20. The sequence is incomplete between RP11-178P20 and RP3-431P23 that contains D6S149. We identified seven genes in this region by a variety of strategies. UNC93A and the last two exons of TCP10 were identified by analysis of the sequence from RP3-366N23 with a suite of exon prediction programmes. Several ESTs were also mapped to the same genomic region (RP3-366N23) that was identified to contain the predicted gene (Fig. 1.) The gene for UNC93A is 24.5 kb in length and comprises 8 exons. All the intron exon boundaries of UNC93A follow the GT/AG rule (Table 1).

The predicted cDNA of UNC93A was 1451 bp. Sequence analysis of the individual ESTs confirmed that the full length cDNA was slightly longer due to 3' untranslated sequence (2011 bp) (Fig. 2). The putative initiation methionine was preceded by an inframe stop codon and had the best Kozak's consensus sequence. The primary structure of the protein predicts a leader peptide (1–25 aa) followed by seven transmembrane domains. The size of the predicted protein is 50 kDa. The amino acid sequence of UNC93A is 30% homologous to the *unc-93* sequence in *C.elegans* (Fig. 3). There is another predicted homologous protein in *C.elegans* (acc.no.Q93380) and two homologous predicted proteins in *Drosophila* (acc.no. Q9Y115 and Q9V4S6). The overall similarity in primary sequence, particularly over the predicted transmembrane regions, is highlighted.

As UNC93A mapped within the minimal region of allele loss, it was potentially an attractive candidate as a tumour suppressor gene. We analysed this gene in detail to try to understand its expression in normal cells and its possible involvement in the tumourigenesis of sporadic ovarian cancer.



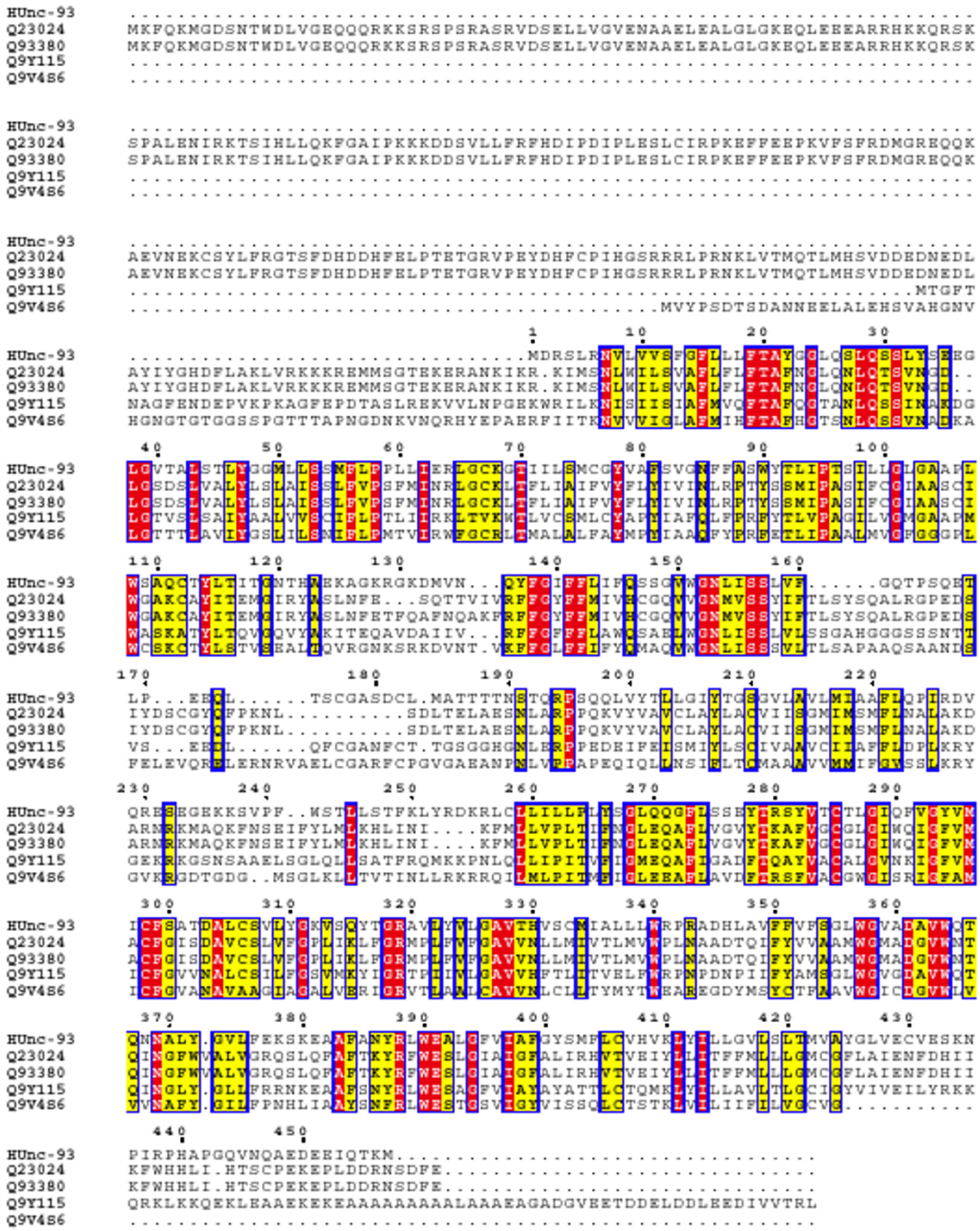
A

Figure 2
cDNA and amino acid sequence of UNC93A. The entire cDNA of UNC93A is shown with the predicted peptide sequence. Primary structure analysis of the protein has indicated that there is a leader peptide (boxed red) and seven transmembrane domains (boxed blue). The above prediction was obtained by the analysis of the protein by several structure prediction programmes including SignalIP prediction, TMPred, TMHMM, TopPred, PredictProtein, and HMMTOP.

Expression

None of the EST clones contained the full length UNC93A cDNA upon sequencing. To isolate full length cDNA clones and also to confirm the sequence of the predicted cDNA, primers were designed to amplify the coding sequences of UNC93A. RT-PCR was performed using total RNAs from normal ovary (epithelial cells) and the panel of 8 ovarian cancer cell lines as templates. Two products of 1.4 and 1.3 kb were observed in cell lines 41M and OAW28 (Fig. 4A). Direct sequencing of these two products have shown that the 1.4 kb amplicon represented the entire coding sequence of UNC93A and the 1.3 kb amplicon did not have exon 4. Another set of RT-PCR was carried out to confirm the above result by using the same panel of RNAs as templates with primers designed to amplify exon 3–5 (Fig. 4B). Again, two major amplicons were observed in cell lines 41M and OAW28. Sequencing of both PCR products showed that the smaller product did not contain exon 4. Therefore, there are two splice variants of UNC93A, with one containing all of the 8 exons and one missing exon 4.

The UNC93A cDNA (1.4 kb) containing the entire open reading frame was used as a probe in Northern analysis. Hybridisation of UNC93A against a Northern blot of multiple human tissues, revealed a major transcript of about



B

Figure 3
Alignment of UNC93A across species. Q23024 is the original *unc-93* sequence in *C. elegans*. Q93380 is a predicted second *unc-93* like protein in *C.elegans*. Q9Y115 and Q9Y4S6 are two predicted proteins in *Drosophila*. Residues shaded red are identical and in yellow are similar. The alignment was performed using Pileup (Blosum 62) and displayed using ESPRipt.

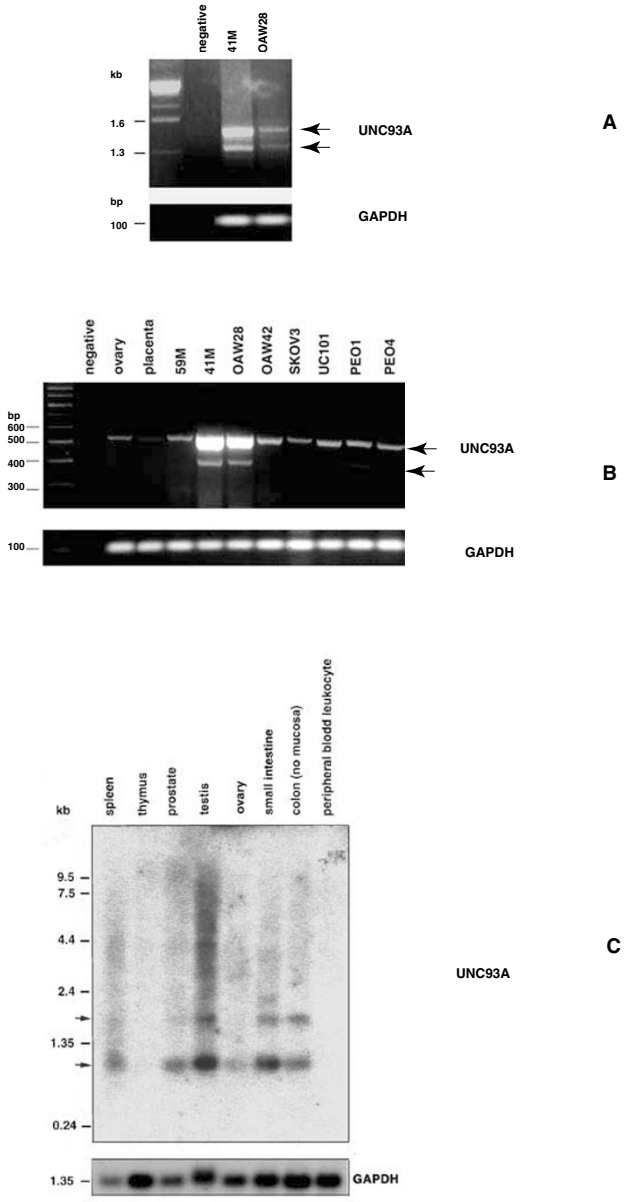


Figure 4

A) The expression of UNC93A in ovarian cancer cell lines by RT-PCR. Top panel: Ethidium-bromide stained agarose gel of amplification products from the RT-PCR using primers located in exon 1 and exon 8 of the cDNA of UNC93A to amplify the entire coding sequence. The template for the RT-PCR were the total RNAs from indicated ovarian cancer cell lines (2 μg each). Two products of 1.4 kb and 1.3 kb were observed in 41M and OAW28. Bottom panel: GAPDH was amplified by RT-PCR using the same panel of RNAs as those used in the RT-PCR of the top panel. **B) Amplification of exon 3–5 by RT-PCR**. Top panel: Ethidium-bromide stained agarose gel of amplification products from the RT-PCR using primers located in exon 3 and exon 5 of the cDNA of UNC93A. The template of the RT-PCRs were the total RNAs from normal ovary tissue, normal placenta tissue and 8 ovarian cancer cell lines (2 μg each). A major RT-PCR amplicon about 550 bp was observed in all the samples except in the negative control. Another amplicon about 400 bp was observed in cell lines 41M, OAW28. Bottom panel: GAPDH was amplified by RT-PCR using the same panel of RNAs as those used in the RT-PCR of the top panel. **C) Northern blot analysis of the UNC93A expression in adult tissues.** A Northern blot of poly $(A)^+$ mRNA (2 μg each lane) from 8 adult tissues (Clontech) was hybridised to full length cDNA of UNC93A. The membrane was exposed to Fuji film at -70°C for 5 days. Two major transcripts of 1.1 and 2.2 kb were observed in spleen, prostate, testis, ovary, small intestine, and colon.

2.1 kb in testis, small intestine and colon; a smaller transcript of 1.1 kb was also observed in spleen, prostate, ovary, small intestine, and colon (Fig. 4C). Using the same probe to hybridise the Northern blot of 8 ovarian cancer cell lines, no expression of this gene could be detected (data not shown).

The entire coding sequence of UNC93A (nucleotide 78–1448, Fig. 2A) was cloned in frame into pEGFP-N3 with the GFP protein at the C-terminal end of UNC93A. Transient expression of this construct in 293-T cells, showed clear membrane localisation of the fusion protein (Fig. 5A,5B,5C). In addition, immunoprecipitation of the GFP-UNC93A fusion protein from the transfected cells, identified a specific band of 90 kDa (Fig. 5D). Thus, in vivo, the UNC93A protein is approximately 60 kDa in size which is slightly larger than that predicted from the amino-acid sequence. The increase from the predicted size could be due to post-translational modifications.

Mutation analysis

We analysed the entire coding sequence of UNC93A for mutations using initially SSCP (^{32}P), then SSCP with fluorescent labelled oligonucleotide primers using an ABI 377 machine (F-SSCP) and then DHPLC in a panel of 36 malignant ovarian tumours. We have also sequenced the entire cDNA in 8 ovarian cancer cell lines to detect mutations.

Mutation detection using ^{32}P-SSCP and F-SSCP

Primers were designed to amplify each exon of the UNC93A gene (Table 2). Two sets of primers were designed to amplify exon 8 with the PCR products (smaller than 200 bp) overlapping each other (Table 2). Ten malignant ovarian tumours with allele loss of the key marker D6S193 [16] were selected initially for ^{32}P SSCP analysis of all of the 8 exons of UNC93A. Variant bands were detected in T68 in exon 3, T39 in exon 4 (Fig. 6A). Interestingly, the variant bands detected in the tumours were also present in the matched normal control for each tumour compared to the placental DNA.

Subsequently, 36 malignant tumours (including the previous 10 samples) were analysed by F-SSCP in all 8 exons of UNC93A. PCR samples were run on an ABI 377 Sequencer with the corresponding SSCP settings. Abnormal peaks, which represented the variant bands in this study, were observed in 5 tumours (T32, T60, T50, T30, T68) in exon 3, in 4 tumours (T28, T34, T25, T39) in exon 4 and in 5 tumours in exon 8 (T19, T34, T44, T49, T54) (Fig. 6B) compared to normal control.

Mutation detection using DHPLC

To employ a mutation detection technique which would not be affected by PCR product size, we used the DHPLC (Denaturing High-Performance Liquid Chromatography) method on a Transgenomic – WAVE machine to further screen for mutations on the same panel of tumour samples. We also used DHPLC to evaluate exons in tumours which were normal by the previous methods.

A new set of primers was designed to amplify the whole of exon 8 to confirm the SSCP results for exon 8. In total, exons 1, 2, 5, 8 from the 36 tumour samples were amplified by primers flanking the exons using the "touchdown" programme [29] and subjected to the analysis on the WAVE machine. The results from this study showed that there were additional abnormal peaks in 6 tumours (T26, T29, T32, T36, T37, and T59) in exon 1, and in one tumour (T43) in exon 5 (Fig. 6C).

Mutations in UNC93A

Ovarian Tumours

In exon 3, 5 tumours were identified with abnormal SSCP patterns (Table 3, Fig. 6A, 6B). Direct sequencing of the exons showed that all of them had an identical single base change c.452G>A that would alter amino-acid *Trp* to a stop codon thereby truncating the protein. For 4 of these tumours (T32, T68, T60 and T50), an identical alteration was found in matched normal control DNA, suggesting that this was not tumour specific. In tumour sample T30, however, there was a heterozygous alteration c.452G>A at this base which was not observed in the matched normal DNA (Fig, 7A).

In exon 4, there were two tumours (T39 and T28) which showed an abnormal SSCP pattern (Table 3, Fig. 6A,6B). In both tumours, the abnormal variant in exon 4 was also present in the matched normal DNA (Fig. 6A). Direct sequencing of this exon in both tumours revealed that there was a splice site mutation at the first base in the intron at the 3' end of the exon (c.625+1G>C). This mutation was also present in the normal matched DNA (Fig. 7B). Direct sequencing of the variant band in T39 (Fig. 6A) confirmed this alteration.

In exon 5, one tumour (T43) had an abnormal pattern on DHPLC (Fig. 7C). Direct sequencing of this exon revealed that this was due to a mutation c.676C>T which would alter amino acid *Arg* to a stop codon, thereby truncating the protein. This mutation was tumour specific as it was not present in the matched normal DNA (Fig. 7C).

In exon 8, there were abnormal patterns detected by SSCP or DHPLC in 5 tumours (Fig. 6B, Table 4). All sequence variants were present in the matched normal control DNA (Fig. 8A,8B,8D) except one mutation in T19 where a single base change c.1225G>A altered *Val* to *Ile* which is a conservative alteration (Fig. 8C).

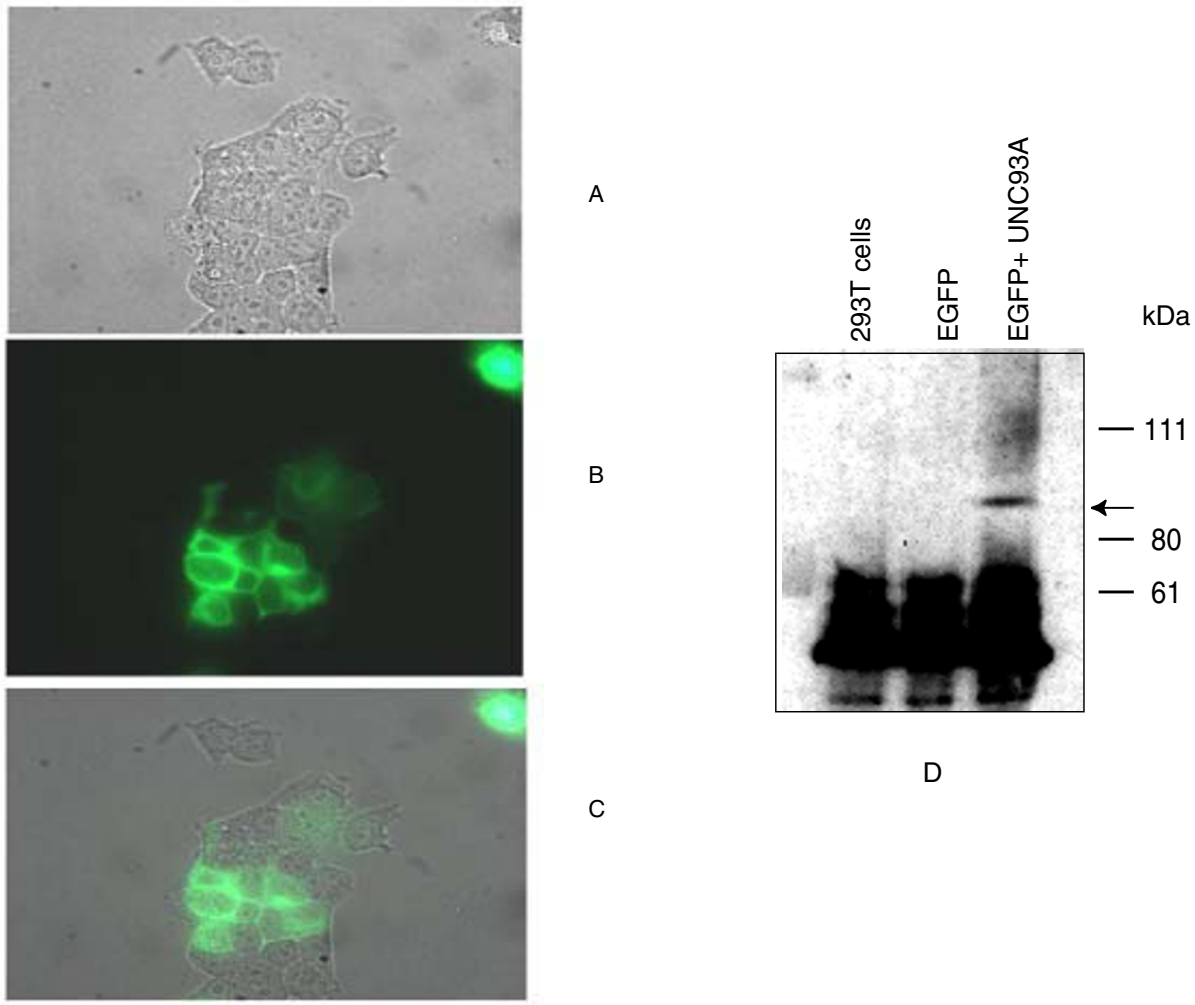


Figure 5
***In vivo* expression of UNC93A. Cellular expression and localisation of GFP-UNC93A.** The ORF of UNC93A was subcloned inframe into a fluorescent GFP tagged mammalian expression vector (EGFP-N3) and was transiently transfected into 293-T cells. 24 hours after the transfection, the cells were transferred to cover slips for a further 24-hour culture, followed by fixation and observed under fluorescent microscope. The fusion protein was localised on the membrane of the transfected cells and was abundant from 48 to 72 hours after the transfection. **A**) cells observed under phase contrast, *B*) cells observed under UV light, **C**) merged image. **D) Immunoprecipitation and immunoblotting of the EGFP-UNC93A fusion protein.** Cell lysate from untransfected 293-T cells, 293-T cells transfected with EGFP vector, and 293-T cells transfected with EGFP-UNC93A (the EGFP + UNC93A lane) were immunoprecipitaed with GFP monoclonal antibody, resolved on an 10% SDS-PAGE gel, and Western blotted. A specific fusion protein of about 90 kDa was detected with GFP monoclonal antibody and anti-Mouse HRP in the cells transfected with EGFP-UNC93A, but not in the untransfected cells nor cells transfected with EGFP vector (indicated by arrow).

In exon 1, there were 6 tumours (T26, T29, T32, T36, T37 and T59) which showed an abnormal pattern on DHPLC (Fig. 6C). Direct sequencing of this exon in all of the 6 tumours identified a single base change 32 bp upstream of the ATG in the 5' untranslated sequence of UNC93A (c.1-32T>G). Sequencing of exon 1 from matched control normal DNA for all of these tumours showed an identical alteration (data not shown).

Ovarian cell lines

Direct sequencing of all of the exons of UNC93A was performed in eight ovarian cancer cell lines (Table 5). In exon 1, 6/8 cell lines had the same mutation observed in tu-

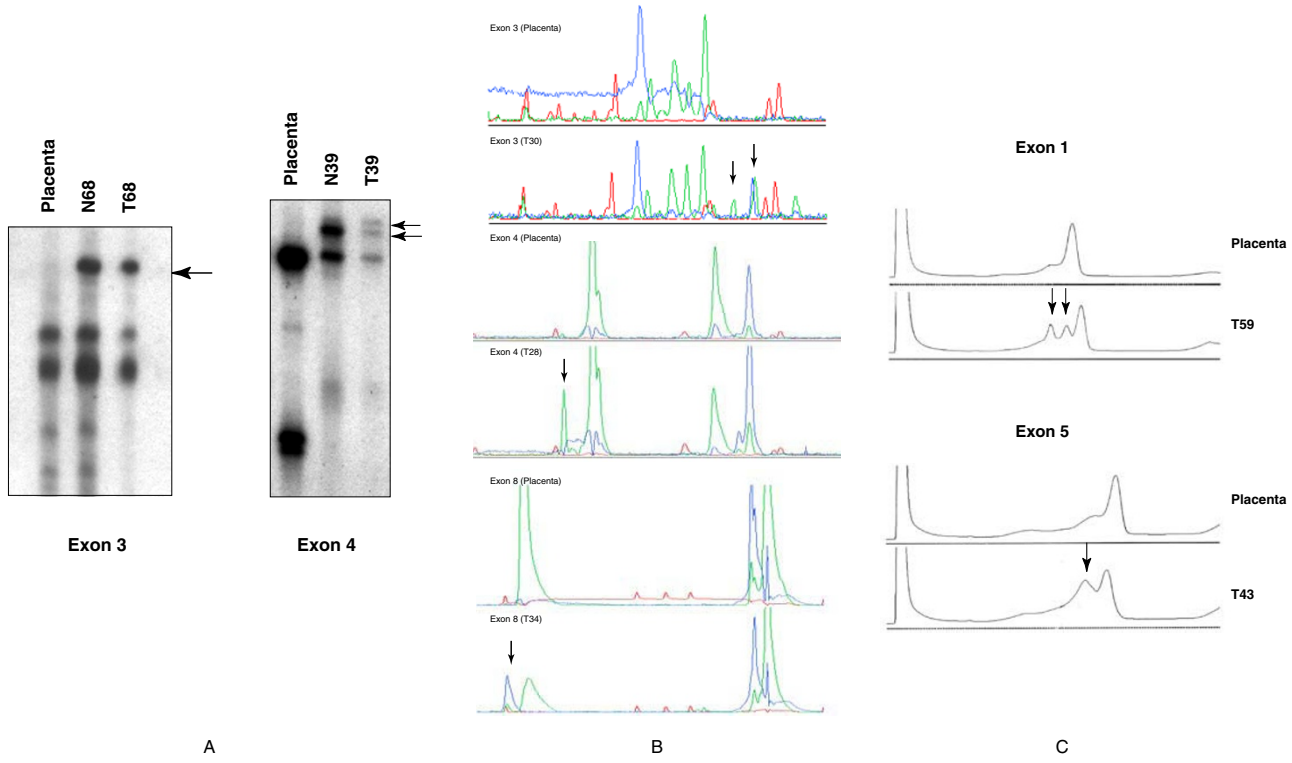


Figure 6

Mutation detection of UNC93A in ovarian tumors. A) Mutation detection using P^{32}-SSCP method. Each exon was amplified with DNAs from tumour sample, its matched control and normal placenta tissue. The variant bands in exon 3 and 4 in the tumour samples and their matched normals compared with normal placenta tissue are indicated by arrows. **B) Mutation detection using F-SSCP method.** Representative examples are shown for exons 3, 4, and 8. The primers were labelled with fluorescent dye (the forward primers were labelled with HEX, the reverse primers were labelled with FAM). The electrograms are a graphical display of the fluorescent intensity on the y-axis and the mobility along the x-axis. The red peaks are the GeneScan 500 size standard (Perkin Elmer) labelled with TAMRA, which functions as an internal control for each lane. Placenta DNA was used as a normal control. The abnormal peaks in the tumours are indicated by arrows. **C) Mutation detection using DHPLC method.** PCR was performed using primers flanking exons by 'touchdown' PCR and subjected to DHPLC analysis. The electrograms are a graphical display of the amount of DNA run through the column (intensity peak) on the y-axis and the retention time of the DNA in the column (minute) along the x-axis. The abnormal peaks in exon1 (T59) and exon 5 (T43) are indicated by arrows. PCR products from placenta DNA was used as control.

mours in the 5' untranslated sequence, c.1-32T>G. An identical stop codon mutation in exon 3, c.452G>A, as observed in tumours was detected in cell lines PEO1, PEO4, OAW42, and SKOV3. The alteration was heterozygous in two cell lines (OAW42, SKOV3) and homozygous in two (PEO1 and PEO4). A missense mutation in exon 5, c.659C>T was observed in UCI101 which would confer a change of *Ala* to *Val*. A missense mutation in exon 6, c.874G>A was observed in 6/8 cell lines. This mutation would alter amino acid residue *Val* to *Ile*, which is a conservative alteration. The mutations observed in exons 5 and 6 in these cell lines were not observed in tumours. In exon 8, identical in them alterations as that observed in tumours were also identified (Table 5).

Normal population

As the mutation in exon 3 (c.452G>A), which creates a stop codon, was present in one of the two alleles in several matched normal samples, it was possible that this variant was a polymorphism. We analysed exon 3 by F-SSCP in a panel of 50 normal DNA samples (Caucasian). 3 samples showed a different pattern on SSCP compared to placenta (Fig. 9A). Direct sequencing of exon 3 from these 3 normal DNA samples, revealed that two were heterozygous for the mutation c.452G>A and one was homozygous (Fig 9B). Similarly, the frequency of the mutation in exon 5, c.676C>T, which creates a stop codon, in the normal population was also evaluated. This mutation creates a new restriction site for the enzyme Nde1 (CA↓TATG). There-

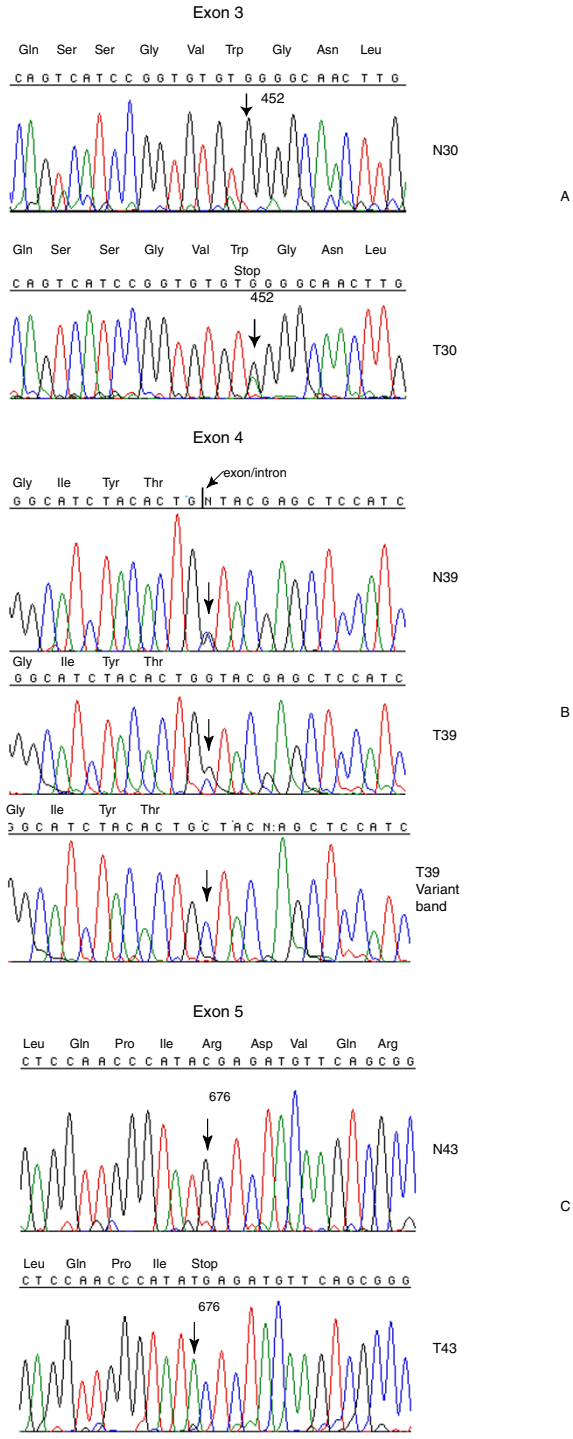


Figure 7
DNA sequence traces of the mutations detected in exon 3, 4, and 5 of UNC93A. A) Sequence trace of the nucleotide variant c.452G>A in exon3, which confers a stop codon, in tumor T30 compared with its matched normal DNA. **B)** Sequence trace of the nucleotide variant c.625+1G>C at the 3' splice site of exon 4 in tumor T39, its matched normal and the variant band from the P^{32}-SSCP gel. **C)** Sequence trace of the nucleotide variant c.676C>T in exon 5, which confers a stop codon, in tumor T43 and its matched normal. Corresponding amino acids are shown at the top of each sequence trace.

Table 2: Primers for amplification of UNC93A genomic DNA.

EXON	PRIMER NAME	PRIMER SEQUENCE (5'-3')	PCR PRODUCT SIZE (BP)	ANNEALING TEMPERATURE AND MG^{++}	IN DHPLC DENATURING TEMPERATURE (°C)	IN DHPLC GRADIENT START (INITIAL Buffer B%)
1	1f	GAATGGGACTTCTTGGTACTGATTG	225	61° C, 1 mM	59	52
	1r	TTCAGGTAATTTGATGACCGGACACA				
2	2f	CTGTGCTCTGACAGGGCTGGCTTTGT	244	62° C, 1.5 mM	65	52
	2r	GGGTGGGCAGGGGGTGGTGGCTGCT				
3	3f	CTATGGGTCTGCATTTTACCC	282	61° C, 1.5 mM	61	54
	3r	ACAATTGCTTGCCCCTTTTCCTTT				
4	4f	ATTTGCCGTCATCTCATGTCT	195	61° C, 2 mM	-	-
	4r	CTGCCCTGGGCCGATGGAGCTCGT				
5	5f	TGAAAGCTGAAGCCTTTGCTATGT	276	61° C, 1.5 mM	60	55
	5r	GAGCCACCGGCCCTCACTTTCGTT				
6	6f	GCCTGGGAGCGTCCATGACGTGGC	204	61° C, 1.5 mM	-	-
	6r	GGATGGGACCCACGCTGATACCTA				
7	7f	TCCCCTCTGCACCCCCAGGCGCGG	167	61° C, 2 mM	-	-
	7r	CTGGGCTGGGGACTCACCATTGTT				
8 a	8f	TCACTCCGCTCTCTCCTCTGCAGC	166	58° C, 1.5 mM	-	-
	8r2	GACCCCCAGCAGAATGTAGAGCTT				
8b	8f2	AAGCTCTACATTCTGCTGGGGGTC	173	58° C, 1.5 mM	-	-
	8r	TTCATCCTCCTCGGACCTCACTGCTCT				
8	8f	TCACTCCGCTCTCTCCTCTGCAGC	315	62° C, 1 mM	63	55
	8r	TTCATCCTCCTCGGACCTCACTGCTCT				

Table 3: Mutations in UNC93A in tumours

					ALLELE LOSS						
TUMOUR	AGE	STAGE	HISTOLOGY	DIFF.	149	297	193	264	EXON	MUTATION	NORMAL
32	62	III	endometrioid	poorly	NI	NI	NI	NI	3	c.452G>A, W151X*	Identical to the tumour
68	54	II	mucious	well	-	NI	-	-	3	c.452G>A, W151X *	Identical to the tumour
60	75	III	undifferentiated	poorly	NI	+	+	-	3	c.452G>A, W151X *	Identical to the tumour
50	47	I	serious	moderately	NI	-	-	-	3	c.452G>A, W151X *	Identical to the tumour
30	52	I	clear cell	moderately	-	NI	NI	+	3	c.452G>A, W151X *	Normal
28	49	III	clear cell	moderately	-	-	NI	-	4	c.625+1G>C *	Identical to the tumour
39	53	II	endometrioid	poorly	-	+	+	-	4	c.625+1G>C *	Identical to the tumour
43	72	III	serious	moderately	-	-	+	-	5	c.676C>T, R226X	Normal

$, indicates the first base downstream of the splice donor site of exon 4. Diff.= differentiation; NI, not informative or unable to amplify; -, both alleles retained; +, LOH (loss of heterozygosity). Data as previously published [16]. All mutations are homozygous except when indicated by *. Normal refers to matched DNA from peripheral blood. The location of each mutation in the cDNA sequence is based on numbering from the inititiator codon ATG.

fore, this exon was amplified by PCR from 50 normal DNA samples and the PCR products were digested with Nde1. One sample (ORK29) had an altered restriction pattern suggestive of a heterozygous mutation (Fig. 10). This was confirmed by direct sequencing of exon 5 in this sample.

Discussion

The region between *D6S264* and *D6S149* is now well mapped except for a gap between RP11-178P20 and RP3-431P23. The latter PAC contains the telomeric marker *D6S149*. The entire region has been sequenced (1.1 Mb) and we have identified 7 genes [27]. One of the candidate genes identified within this interval was the human homologue of *unc-93* in *C.elegans*. We chose to analyse this gene in detail as it was expressed in the ovary and

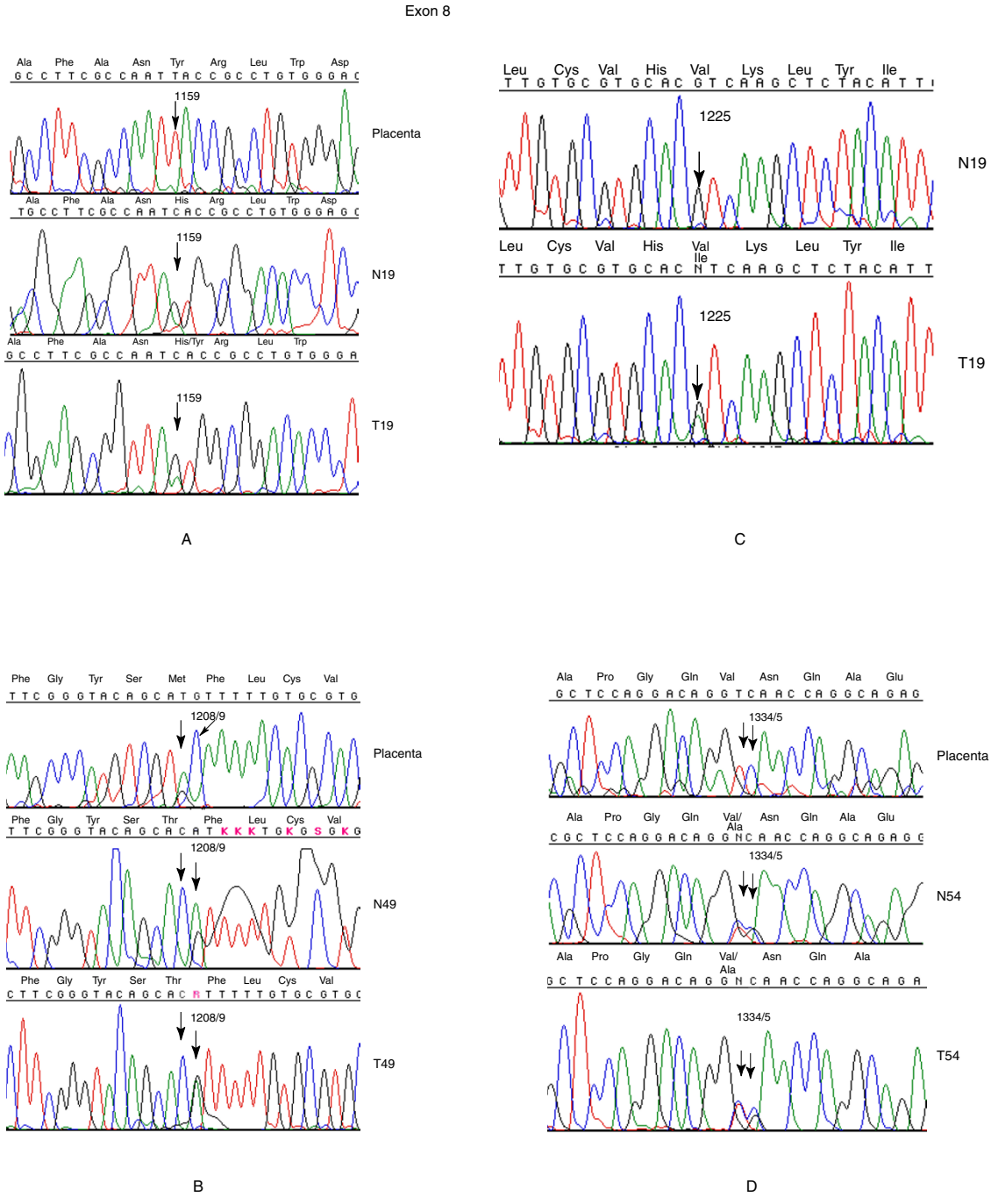


Figure 8
Sequence traces of variants in exon 8 of UNC93A. **A**) Sequence trace of the nucleotide variant c.1159T>C in tumour sample T19, matched normal DNA N19 and placental DNA. **B**) Sequence trace of the nucleotide variant c.1208T>C+1209G>A in tumour sample T49, matched normal DNA N49 and placental DNA. **C)** Sequence traces of the nucleotide variant c.1225G>A, in tumour sample T19, matched normal DNA N19 and placental DNA. **D)** Sequence variant c.1334T>C+1335C>G in tumor sample T54, matched normal N54 and placenta. Corresponding amino acids are shown at the top of each sequence trace.

Table 4: Mutations in exon 8 in UNC93A in tumours

					ALLELE LOSS					
TUMOR	**AGE**	**STAGE**	**HISTOLOGY**	**DIFF**	**149**	**297**	**193**	**264**	**MUTATION**	**NORMAL**
19	59	III	Serous	poorly	NI	NI	+	MI	c.1159T>C, Y387H* c.1208T>C, M403T c.1225G>A, V409I* c.1334T>C+1335C>G, V445A*	c.1159T>C, Y387H* c.1208T>C, M403T c.1334T>C+1335C>G, V445A*
34	74	III	Serous	mod-erately	+	+	NI	NI	c.1159T>C, Y387H * c.1208T>C, M403T c.1334T>C+1335C>G, V445A*	Identical to the tumour
44	58	III	Serous	poorly	NI	NI	-	-	c.1159T>C, Y387H * c.1208T>C, M403T c.1225G>A, V409I * c.1334T>C+1335C>G, V445A*	Identical to the tumour
49	44	Ic	Serous	mod-erately	-	NI	-	-	c.1159T>C, Y387H * c.1208T>C, M403T c.1208T>C+1209G>A*, M403T, c.1334T>C+1335C>G, V445A*	Identical to the tumour
54	28	Ia	Mucinous	well	NI	NI	NI	-	c.1159T>C, Y387H * c.1208T>C, M403T c.1334T>C+1335C>G, V445A*	Identical to the tumour

NI, not informative or unable to amplify; -, both alleles retained; +, LOH (loss of heterozygosity); MI, microsatellite instability. Data as previously published [16]. All mutations are homozygous except when indicated by *. Normal refers to matched DNA from peripheral blood.The location of each mutation in the cDNA sequence is based on numbering from the inititiator codon ATG.

mapped within the interval of allele loss between *D6S149* and *D6S264*. We have published the results of analysis of other candidate genes within this interval separately [27].

In *C. elegans*, *unc-93* is one of a set of five interacting genes (*unc-93*, *sup-9*, *sup-10*, *sup-11* and *sup-18*) involved in the regulation or co-ordination of muscle contraction [30–32]. Mutation of this gene gives a characteristic "rubber-band" phenotype in the worm [28]. When a rubber band mutant is touched on its head, the worm contracts and then quickly relaxes without moving backwards, whereas a wild-type worm simply moves backwards. Thus, the regulation or co-ordination is defective in the rubber band mutants, although they can contract their body wall muscles. These mutants are sluggish, flaccid, and defective in egg laying. Mutations are quite diverse but the major ones are *e1500* which alters G388R in a membrane spanning domain, the *n200* animals have two mutations, A49V and G562V which are in the hinge domains and in the *n234* animals the *Trp* at position 534 is altered to a stop codon [28]. It has been proposed that these mutations produce aberrant proteins which are toxic and cause defects in the regulation of muscle contraction. Interestingly, loss of function, or null, mutations in *unc-93* confers no visibly abnormal phenotype. It has been concluded that this is because the set of five genes involved in muscle contraction are functionally redundant of each other [28]. The putative *unc-93* proteins have two distinct regions: the N-terminal is extremely hydrophilic, while the rest of the protein has multiple potential membrane-spanning domains. The expression of *unc-93* in *C.elegans* is quite low compared to other unc genes. It is suggested that *unc-93* is likely to encode a membrane-associated muscle protein in *C. elegans* [28].

As none of the available ESTs for UNC93A encoded full length cDNA, the entire coding sequence was amplified by RT-PCR and sequenced completely. The amino acid sequence suggests that following a short leader peptide there are at least seven membrane spanning domains similar to that of *unc-93*. Comparison of the cDNA sequence with that of the genomic sequence showed that the gene encodes 8 exons as predicted by the exon prediction programmes. The data form RT-PCR, confirmed by sequencing suggests that there are two splice variants expressed naturally. The second splice variant does not contain exon 4. UNC93A was expressed as two transcripts, 2.1 kb and 1.1 kb on Northern analysis. There is relatively less expression of the smaller transcript in the ovary compared to testis and small intestine. Importantly, none of the ovarian cancer cell lines expressed UNC93A on northern analysis although by more sensitive RT-PCR we did find expression in cell lines. Upon transfection of the GFP tagged UNC93A, there is clear fluorescence at the membrane,

Table 5: Mutations in UNC93A in ovarian cancer cell lines

CELL LINE	EXON 1	EXON 3	EXON 4	EXON 5	EXON 6	EXON 8
41M	-	-	-	-	c.874G>A, V292I *	c.1159T>C, Y387H * c.1208T>C, M403T c.1208T>C+1209G>A*, M403T, c.1334T>C+1335C>G, V445A*
59M	c.1-32T>G[a]	-	-	-	c.874G>A, V292I *	c.1159T>C, Y387H * c.1208T>C, M403T * c.1334T>C+1335C>G, V445A*
OAW28	-	-	-	-	c.874G>A, V292I *	c.1159T>C, Y387H * c.1208T>C, M403T c.1334T>C+1335C>G, V445A*
OAW42	c.1-32T>G	c.452G>A, W151X *	c.500-15T>G[b]	-	c.874G>A, V292I *	c.1159T>C, Y387H * c.1208T>C, M403T c.1208T>C+1209G>A*, M403T, c.1334T>C+1335C>G, V445A*
PEO1	c.1-32T>G	c.452G>A, W151X	-	-	-	c.1159T>C, Y387H * c.1208T>C, M403T c.1208T>C+1209G>A*, M403T, c.1334T>C+1335C>G, V445A*
PEO4	c.1-32T>G	c c.452G>A, W151X	-	-	-	c.1159T>C, Y387H * c.1208T>C, M403T c.1208T>C+1209G>A*, M403T, c.1334T>C+1335C>G, V445A*
SKOV3	c.1-32T>G	c.452G>A, W151X*	-	-	c.874G>A, V292I	c.1159T>C, Y387H * c.1334T>C+1335C>G, V445A*
UCI-101	c.1-32T>G	-	-	c.659C>T A220V*	c.874G>A, V292I	c.1159T>C, Y387H * c.1208T>C+1209G>A*, M403T, c.1334T>C+1335C>G, V445A*

[a]: 32 bases upstream of the 'ATG' site. [b]: 15 bases upstream of start of exon4. All mutations are homozygous except when indicated by an *. The location of each mutation in the cDNA sequence is based on numbering from the inititiator codon ATG.

confirming that it is a membrane associated protein. It is now apparent there are homologous *unc-93* genes in *Drosophila* and a second *unc-93* gene in *C.elegans*. There is quite significant similarity among the species, in particular in the membrane spanning domains.

To evaluate if this gene is involved in the pathogenesis of sporadic ovarian cancer, mutation detection analysis was carried out on a panel of 36 malignant tumours using methods such as SSCP (both ^{32}P-labelled and fluorescent dye-labelled primers) and DHPLC. In general, there was good concordance between conventional SSCP and F-SSCP. However, DHPLC did detect the mutations in exons 5 and 1 which were not identified by SSCP. The use of both methods significantly increased the success of identifying mutations. Overall, 18/36 tumours were identified to have mutations that might have functional significance (Fig. 11). Both missense and nonsense mutations were identified. However, surprisingly the majority of these mutations were present in the matched normal DNA for each individual tumour. Only in three tumours (8%) were specific mutations identified. Firstly, in exon 3 in T30, c.452G>A which alters residue *Trp* to a stop codon. Secondly, in exon 5 in T43, c.676C>T which alters *Arg* to a stop codon. Both the above mutations should alter the reading frame and truncate the protein. Thirdly, one missense mutation in T19 in exon 8, c.1225G>A which alters *Val* to *Ile*. Direct sequencing revealed that many of the mutations identified in the tumours were also present in the cell lines. However, there was one missense mutation c.874G>A in exon 5 identified (*Ala-Val*) only in UCI101 and a frequent mutation in exon 6, c.874G>A (*Val-Ile*) which were observed in 6/8 cell lines. The mutations identified in UNC93A were more numerous than that identified in *unc-93* mutants in *C.elegans* and their location was not identical. It is possible that the mutations in UNC93A may be due to the presence of a pseudogene. Analysis of UNC93A sequence in the database has not identified a pseudogene or any other related genes. Secondly, Southern analysis of normal genomic DNA confirmed that UNC93A is a single copy gene (data not shown).

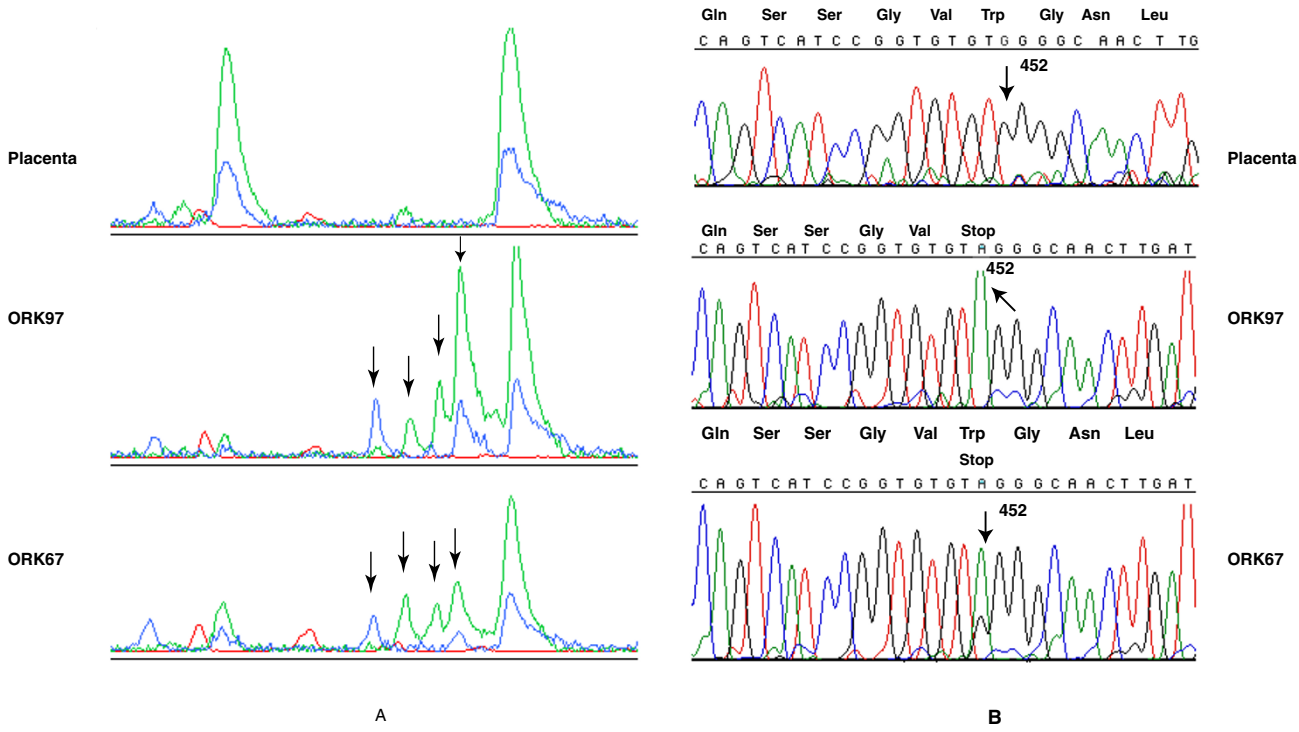


Figure 9
c.452G>A mutation in exon 3 in normal DNA. A) Electrogram of F-SSCP of exon 3 from DNA from placenta and two normal controls ORK67 and ORK97 are shown. Abnormal peaks are indicated by arrows. **B)** Sequence trace showing the mutation c.452G>A in exon 3 with homozygous (ORK97) and heterozygous (ORK 67) alteration compared to placenta.

The identification of the mutation W151X in exon 3 in 4 matched normal DNA samples as well as in tumours (11%), suggested that this might be present in the normal population. The frequency of this polymorphism in the normal population was 3/50 (6%). This included at least one sample having a homozygous mutation. In exon 5, the mutation c.676C>T, which also creates a stop codon, was identified in one tumour (<3%). Screening of 50 normal DNA samples identified this sequence change in one sample (2%). The identification of these two mutations in the normal population suggest that these represent low frequency polymorphisms. Such polymorphisms are rare and an extensive search of the literature identified one report involving BRCA2 where a stop codon polymorphism was found in one patient [33]. However, recently genome wide scanning for SNPs in coding sequences have identified a low frequency of such mutations in less than 1% [34,35] of the genes analysed. The functional impact of these rare mutations are at present unknown. It is estimated that on average the number of polymorphic sites per kilobase of DNA was 3.4 in the coding region and 50% of them coded for an aminoacid change [35]. In the coding sequence of UNC93A, the frequency is much higher (6.4/kb). Only genes of the HLA complex and the P450 cytochrome enzymes have higher rates of polymorphism than this.

Conclusions

The frequency and nature of the mutations in UNC93A in ovarian tumours and cell lines suggests the following. Firstly, UNC93A is an unusual gene which is highly polymorphic and the truncating mutations may not result in any deleterious function in the normal population. This might be due to compensation by other related genes yet to be identified. Secondly, because of identification of significant mutations in the germline of these patients, it is probable that these mutations predispose to ovarian cancer. Polymorphic variants of genes in individuals may increase the risk of a particular cancer. Extensive research is being performed to identify such genes which might ex-

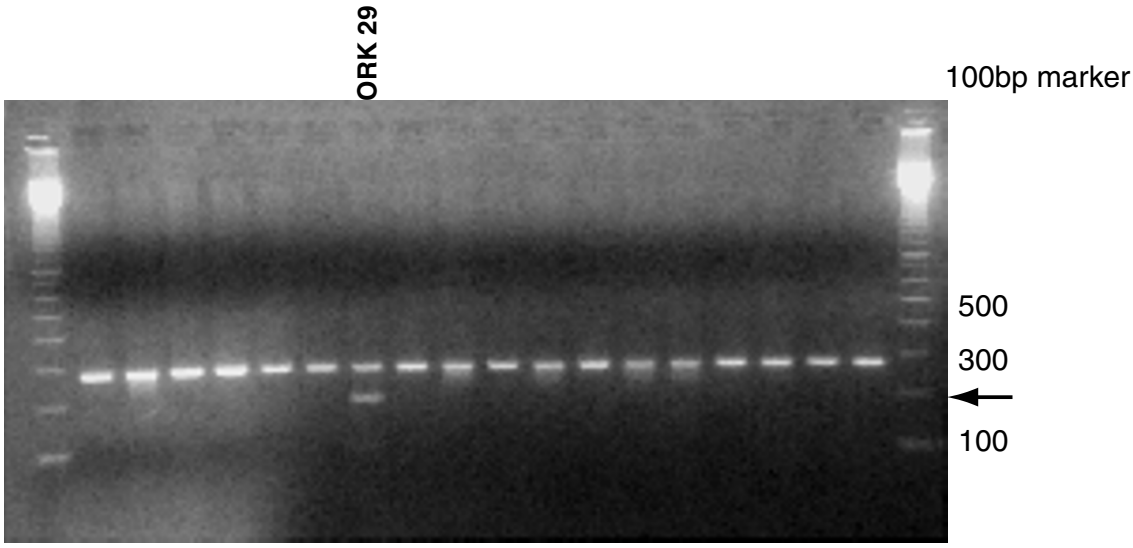


Figure 10
c.676C>T mutation in normal DNA. Agarose gel showing restriction digest by Nde1 of exon 5 amplified by PCR from normal DNA samples. The PCR product from ORK 29 shows an additional band of appropriate size suggesting heterozygous alteration, c.676C>T.

plain polygenic diseases. Although several candidate genes have been evaluated for such association in ovarian cancer, none have been identified that could significantly increase the risk of ovarian cancer. Further research is required to discover the allele frequency of the sequence variants in the coding sequence of UNC93A in the normal population and their possible association with ovarian cancer. However, the results presented in this paper do not suggest that UNC93A is the tumour suppressor gene to explain the allele loss observed between D6S264 and D6S149 in ovarian tumours.

Methods

Tumour tissues and blood samples

Fresh tissues (tumours, normal ovary and placenta) and peripheral blood samples were collected from patients in John Radcliffe and Churchill Hospitals, Oxford. Tissue samples were collected at surgery and stored in liquid nitrogen. Blood samples were collected in sodium chloride-EDTA tubes and processed for DNA extraction. Formalin fixed paraffin embedded tumour samples and matched normal tissues were obtained from pathology archives.

Primers

All the primers, including the fluorescent labelled (HEX, FAM) primers were synthesised by the Oligonucleotide synthesis laboratory, Clare Hall, ICRF.

Cell lines

Eight ovarian cancer cell lines [36,37] and the 293-T cell line were supplied byCell Services, Clare Hall, ICRF. The cells were cultured in media and 10% fetal calf serum and maintained in 5% CO_2 at 37°C.

DNA extraction

DNA was extracted from tumours and cell lines following standard protocols. Sections from paraffin blocks were mixed with 100% Xylene (1 ml/4–5 paraffin sections). The mixture was incubated at room temperature for 30 min to 1 hr and spun at 10, 000 g for 3 to 5 min. The supernatant was discarded and the precipitate was washed twice, air dried and digested in buffer (50 mM Tris-HCl, pH 8.0; 25 mM EDTA, 1% Tween 20, with freshly added 200 μg/ml proteinase K) at 37°C overnight followed by 95°C for 10 min to inactivate the proteinase K.The samples were then spun for 1 min at 10,000 g and the supernatant was used for PCR. DNA from dried polyacrylamide gel was obtained by soaking the band cut from the gel in 50–100 μl of dH_2O overnight at 4°C and the supernatant

was used as template in PCR. DNA from blood samples were extracted as previously described [16].

Northern analysis

The membrane was prehybridised in ExpressHyb™ Hybridisation Solution (Clontech) at 68°C for 1 hour. UNC93A full length cDNA was labelled with ^{32}P-dCTP using Megaprime™ labelling systems (Amersham) and purified through G-50 Sephadex column. The membrane was hybridised in ExpressionHyb™ Hybridisation Solution (Clontech) at 68°C with recommended amount of denatured probe overnight. Membranes were washed according to manufacturer's instructions.

RT-PCR

Total cellular RNA was isolated from 5–10 × 10^7 cells of each cell line with RNeasy Midi/Maxi kit (QIAGEN) according to manufacturer's instructions. Total RNA from normal ovary and placenta were extracted using RNAZol B (Biogenesis) according to manufacturer's instructions. 2 μg of RNA from each cell line was used in a RT-PCR reaction using QIAGEN One Step RT-PCR Kit (QIAGEN) according to manufacturer's instructions (forward primer sequence in exon 3: TTTGATCCCCACCTCCATACTG; reverse primer sequence in exon 5: GGAGAGGAATCCTTGCTGCAATC). The reaction was performed on a PTC-200 (GRI Lab Care Service) for synthesis of first strand (50°C, 40 min), initial PCR activation step (95°C, 15 min), 40 cycles of denaturation (94°C, 40 sec), annealing (64°C, 1 min), extension (72°C, 1 min), and 1 cycle of final extension (72°C, 10 min).

Mutation detection analysis

P^{32}-SSCP

Individual exons were amplified from genomic DNA from a panel of 10 malignant tumours, labelled with α^{32}P-dCTP, and resolved using Mutation Detection Enhancement (MDE) gel (FMC Corporation). The PCR reactions were paused at the 25th cycle, 10 μl of each reaction was taken out for evaluating the PCR product on an conventional agarose gel, and 0.5 μl α-^{32}P-dCTP together with 0.2 μl Taq polymerase were added to each sample. The PCR reaction was continued for a further 5 cycles. 1 μl of each α-^{32}P labelled PCR sample was mixed with 10 μl of sample loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue and 0.25% xylene cyanol) on a 96-well plate (a standard SSCP gel can run 32 samples). Samples were denatured at 95°C for 3 min and immediately transferred to ice before loading 4 μl of each sample on to a 0.5 × MDE gel with 10% glycerol. The MDE gel was prepared according to manufacturer's instruction. The samples were run at a constant power of 8–12 Watts for 12 to 16 hours at room temperature. The gel was dried for 2 hours at 68°C and then exposed to X-ray film.

F-SSCP

Individual exons were amplified by PCR from genomic DNA from a panel of 36 malignant tumours using primers labelled with HEX (green, forward primers), and FAM (blue, reverse primers) and applied to MDE gel on a ABI 377 system (PE Applied Biosystems). Standard PCR reactions were carried out to amplify the individual exon in a 50 μl reaction using these primers. One μl of each fluorescence-dye labelled PCR sample was diluted (1:5 to 1:10). One μl of the diluted sample was combined with 3.0 μl of loading buffer (Deionized formamid, 0.5 μl of GeneScan size standard (TAMRA), 0.5 μl of 100 mM NaOH, and 0.5 μl of Blue dextran EDTA (50 mM EDTA, 50 mg/ml blue dextran), denatured at 90°C for three minutes and loaded to the ABI 377 Sequencer. The gel (0.5% MDE and 10% glycerol) was run at the settings for wattage-limiting module (GS Run 60W C CHILLER) for 10–12 hours using filter set C. The temperature of the gel was controlled at 19°C by a cooling system (Betta Tech). The data was collected by ABI 377 software and analysed using the GeneScan 672 software (Version 3.1).

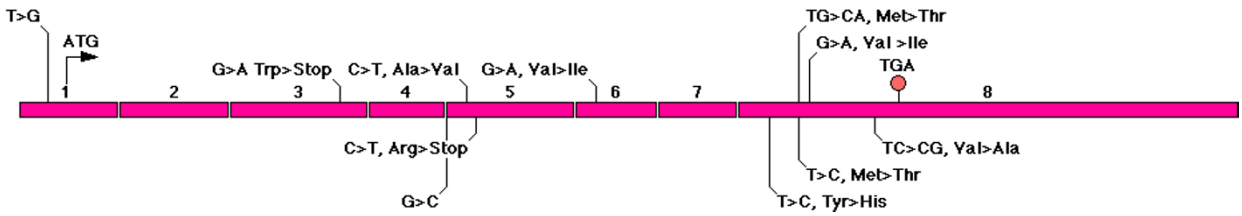


Figure 11
Mutations in UNC93A All the mutations observed in tumours or cell lines in individual exons are as indicated.

DHPLC analysis

'Touchdown' PCR [29] was performed to amplify individual exons. Briefly, a 50 μl PCR reaction contained 30 ng genomic DNA, 10 pmol of each primer, 0.5 mM of dNTPs, 0.3 μl of a combination of Amplitaq Gold (Perkin Elmer) and Pfu Turbo (Stratagene) at 9:1 ratio, 5 μl 10 × Ampli-Taq Gold buffer (Perkin Elmer), and 3 to 5 μl of $MgCl_2$ (25 mM) optimised for each primer set (Table 2). Amplification was carried out on a PTC-200 (GRI Lab Care Service) without oil. PCR conditions were initial denaturation step for one cycle (10 min at 95°C); 14 cycles of denaturation (30 sec at 95°C), annealing (30 sec, at temperature 7.5°C higher than the optimised annealing temperature for each primer pair at the first cycle and subsequently decreased by 0.5°C per cycle), and extension (30 sec at 72°C); a further 20 cycles of denaturation (30 sec at 95°C), annealing(30 sec at the optimal temperature shown in Table 2), and extension (30 sec at 72°C), and a final extension of 1 cycle at 72°C for 7 min. Touch-down PCR products were subjected to DHPLC analysis according to conditions (Table 2) on a WAVE Machine (Transgenomic, Omaha, USA). Standard buffer mixtures were used, as recommended by Transgenomic: buffer A, 0.1 M triethylammonium acetate (TEAA), pH 7.0; buffer B, 0.1 M TEAA, pH 7.0 + 25% acetonitrile. Gradients were designed to increase the percentage of buffer B at a rate of 2%/minute for four minutes. Since DHPLC detects mismatches between DNA sequences, not actual mutations, we consequently analysed PCR products from tumour DNA alone as well as tumour DNA mixed 1:1 with normal DNA from an individual of known sequence. This was peformed by denaturation at 95°C for 4 minutes, followed by 42 cycles of decreasing temperature at 1.6°C per cycle for 1 minute until 4°C.

Automated DNA sequencing

PCR products were purified with QIAquick PCR Purification kit (QIAGEN) and sequenced with BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) on an Applied Biosystems model 377 DNA Sequencer (PE Applied Biosystems) according to manufacturer's instruction. Sequencing to confirm any variation from normal sequence was performed twice.

Construction of pEGFP-UNC93A and fluorescence

The entire coding region of UNC93A was amplified by RT-PCR with 1 μg of total RNA from cell line OAW28 using QIAGEN One Step RT-PCR Kit (QIAGEN) according to manufacturer's instructions (forward primer sequence: CTTTTCATCCAGCACAATGG; reverse primer sequence: TTCATCCTCTGCCTGGTTG; the annealing temperature was 60°C) and was subcloned into pGEM-T-Easy Vector (Promega). The cDNA of UNC93A was then released by digestion with HindIII and EcoRI, and subcloned into pEGFP-N3 mammalian expression vector in frame with the fluorescent EGFP coding region at its C-terminal end.

To visualise the expression and location of the EGFP-UNC93A fusion protein in the 293-T cells transfected by this construct, transfected cells were transferred to sterile cover slips 24 hours after the transfection. After a further culture of 24 hours, the cells were fixed with PBS/4% paraformaldehyde and visualised under a fluorescent microscope according to manufacturer's instructions.

Immunoprecipitation and immunoblotting

293-T cells were transfected with the EGFP-UNC93A construct, and EGFP vector respectively with Effectene (QIAGEN). After incubation for 48 hours at 37°C, transfected cells were lysed by adding 1.0 ml lysis buffer (1% Nonindet P 40, 50 mM Tris (PH 7.5), 150 mM NaCl, 10 mM EDTA.) containing freshly added protease inhibitors (1 μg each of Leupeptin and Pepstin per ml, 10 μg each of Aprotinin and Trypsin per ml, and 0.5 mM of Phenylmethylsulfonyl Fluoride per ml) at 0°C for 30 minutes. The lysate was clarified by centrifugation for 30 min at 16,000 g at 4°C. The supernatant was incubated with monoclonal GFP antibody (1:500 dilution) at 4°C for 3 hours and then with 25 μl of protein A-agarose beads at 4°C for two hours. The immune complexes were washed twice in ice-cold cell lysis buffer supplemented with 0.5 M NaCl, and twice in ice-cold cell lysis buffer and denatured by boiling for 5 min in 2 × SDS sample sample buffer (50 mM Tris-HCl, PH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.25% bromophenol blue). The immune complexes were resolved by 10% SDS polyacrylamide gel electrophoresis (PAGE) and then transferred to Hypond-P nitrocellulose membrane (Amersham). The membrane was blocked overnight in PBSA (5% non-fat milk, 0.1% Tween 20) at 4°C, rinsed with PBSA (0.1% Tween 20), and then incubated with monoclonal GFP antibody (1:3000 dilution) for 1 hour in PBSA (0.5% non-fat milk, 0.1% Tween 20), rinsed with PBSA (0.1% Tween 20), and incubate with anti-Mouse Horse-Radish-Peroxidase (1:5000 dilution, Clontech) for 1 hour in PBSA (0.5% non-fat milk, 0.1% Tween 20). The membrane was then washed with PBSA (0.5% non-fat milk, 0.1% Tween 20) for 50 min at room temperature with several changes of the wash solution. Bound proteins were detected by chemiluminescence (Amersham).

Accession numbers

The accession number of sequence for RP3-366N23: AL021331, RP3-431P23:AL009178. RP11-178P20: AL592444. The approved symbol for the human homologue of *unc-93* is UNC93A and the accession number is AJ508812.

Authors Contributions

Authors Y.L., P.D., G.E., and R.S.W. carried out the molecular genetics studies. Authors A.M., I.D., and S.B. are part of the chromosome 6 sequencing group atSanger Centre, Cambridge. Author F.M.C. provided the clinical samples. Author T.S.G. designed and co-ordinated the study and wrote the manuscript together with authors Y.L. and P.D..

All authors read and approved the final manuscript.

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