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The two human homologues of yeast *UFD2* ubiquitination factor, *UBE4A* and *UBE4B*, are located in common neuroblastoma deletion regions and are subject to mutations in tumours

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ABSTRACT

Chromosomes 11q and 1p are commonly deleted in advanced-stage neuroblastomas and are therefore assumed to contain tumour suppressor genes involved in the development of this cancer. The two *UFD2* yeast gene human homologues, *UBE4A* and *UBE4B*, involved in the ubiquitin/proteasome pathway, are located in 11q and 1p, respectively. *UBE4B* has previously been analysed for mutations and one mutation in the splice donor site of exon 9, c.1439 + 1G > C, was found in a neuroblastoma tumour with fatal outcome. We speculated that the homologue *UBE4A* might be involved in an alternative tumourigenesis pathway. The coding exons of *UBE4A* were therefore sequenced. One putative missense mutation (1028T > C, leading to I343T, residing in exon 8) was found in neuroblastoma tumour 20R8; this finding was confirmed by sequencing in both directions. The change, isoleucine (non-polar) to threonine (polar), was situated in a highly conserved amino acid region. In addition, two novel variants were also found in intronic sequences of *UBE4A*. It might be speculated that the proteins generated from *UBE4B* and *UBE4A* are involved in protecting the cell from environmental stress and that inactivation of either of them could contribute to malignancy.

1. Introduction

Neuroblastoma (NB) is a tumour of the sympathetic nervous system (SNS), mostly affecting small children. It is the most common solid tumour of childhood; 40% of the cases are diagnosed by age one and 75% before the age of four. Most NB tumours are composed of neuroblasts, undifferentiated sympathetic nerve cells arising from the neural crest. Primary tumours are located in areas of the SNS; about half of all NBs originate from the adrenal medulla and the rest arise in thoracic or abdominal paraspinal sympathetic ganglia or in pelvic ganglia. Metastases often spread to regional lymph nodes, bone and bone marrow. NB displays a high degree of heterogeneity, including a milder or a benign tumour, lethal tumour progression despite intensive therapy and the unusual ability to regress spontaneously, the latter particularly occuring in infants. Favourable tumours are likely to have near-triploid karyotypes with few structural rearrangements. Aggressive stage 4 tumours often have near-diploid karyotypes and structural rearrangements, including deletions of 1p or 11q, unbalanced gain of 17q and amplification of the *MYCN* protooncogene [1].

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The genetic heterogeneity of NB has evoked the idea that tumourigenesis involves the differential inactivation of several tumour suppressor genes. Maris and colleagues observed loss of heterozygosity (LOH) on chromosome 11 in 44% of primary NB and defined the commonly deleted region as 11q14-23. A strong inverse correlation was also found between 11q LOH and the important prognostic marker *MYCN* amplification [2]. In a different study, a distinct genetic subtype of 11q loss without *MYCN* amplification or loss of 1p was detected. This group was mainly found in stage 4 tumours and was significantly associated with loss of 3p, 4p and 14q [3]. Guo and colleagues have performed another investigation, consisting of LOH studies at 24 microsatellite loci spanning 11q, in which a single region of 2.1 cM within 11q23.3 was found to be deleted in all samples with 11q LOH, flanked by markers D11S1340 and D11S1299. In patients with a single copy of *MYCN*, 11q LOH was associated with advanced-stage disease and poor prognosis [4]. This indicated that a tumour suppressor gene might reside in 11q23.3.

The distal part of 1p shows LOH in 20–40% of NB tumours, indicating that one or several tumour suppressor genes (TSG) reside in this region [1]. The consensus region often involved in 1p deletion is located within 1p36.2-3. We have previously identified the shortest region of overlap (SRO) of deletions in our tumour material to a 25 cM region [5,6]. This was narrowed down to a 5 cM SRO by including a germ cell tumour with a 1p deletion [7]. A homozygously deleted region of 500 kb at 1p36.2-3 was found in a NB cell line by Ohira and colleagues, which further supported the accuracy of the consensus region [8]. We have focused on the region containing the following genes: *UBE4B-KIF1B-PGD-CORT-DFFA-PEX14*. The genes have been screened for mutations; some have indeed been found [9,10]. The gene transcripts have also been demonstrated to be down-regulated in high-stage, compared to low-stage tumours [11]. We have previously identified a novel gene, *APITD1*, located at 1p36.22 [12]. This gene has a low level of expression in NB tumours and is implicated in apoptosis through the p53 pathway, suggesting TSG properties.

According to Knudson's two-hit hypothesis, inactivation of a TSG requires two successive mutations, turning a normal cell into a malignant one [13]. We have previously

Table 1 – PCR primers for amplification of the UBE4A coding exons

Exon	Primer	Annealing temperature (T_A) (°C)	Product size (base pairs)	Primer sequence
2	FP[a]			5′-AGGAATTGGAAAATCTTAACC-3′
	RP	55	493	5′-GGGGAGGGACTCACAAC-3′
3	FP			5′-GTGGTAGGATTTATAGCCAG-3′
	RP	55	486	5′-CAGGGAGTGCAGTGAGTAGGT-3′
4	FP			5′-ATGCTGTCAAGATCAGTTGTC-3′
	RP	50	259	5′-GAACAGTGAGCAGAATTCAG-3′
5	FP			5′-ATTCAAATCTGTGATTCTTGC-3′
	RP	55	485	5′-TACGGAAAGGAGACTAAAG-3′
6	FP			5′-AATTAATTTTCAAGCATCCAC-3′
	RP	50	397	5′-CCTCCTCCTTAATTTATACCA-3′
7	FP			5′-AAAGAGCTAGTGTGTAAAGGC-3′
	RP	55	349	5′-TAGTGTCTTCAGGATAGGGAA-3′
8	FP			5′-CCACCCCATCACCTTATCT-3′
	RP	55	418	5′-TACAGGCTTGAGCTACCATGC-3′
9	FP			5′-ACTGCCAGTTGCTAAGGTTTG-3′
	RP	50	490	5′-AAAGGATAAGGGAGTGAATGC-3′
10	FP			5′-ACTGTATTTCTGTAGACGGTG-3′
	RP	55	438	5′-GCCTCAAGCAATCCTT-3′
11	FP			5′-CTGCAACTCCCTACTATCCTT-3′
	RP	55	488	5′-TAAACTTCCAAAGTGACCCA-3′
12	FP			5′-CATTTATTGACTTAACGACCA-3′
	RP	55	397	5′-TAACTTCTGAAGGCAACC-3′
13	FP			5′-GGGTGGATTTTGATTCTTATG-3′
	RP	50	458	5′-ATGGTGACAATGGGATAATGT-3′
14 + 15	FP			5′-ACTCTTCCTCTTATGGCACCG-3′
	RP	55	477	5′-GTACTAGGGGCATAAGGACT-3′
16	FP			5′-TTTCCCAGCTTAGTTGACTG-3′
	RP	55	378	5′-ACCAGGGTCTGAATTCTTAGT-3′
17	FP			5′-CAGTAGTTCCCAGAGGTCTC-3′
	RP	50	411	5′-AAGATTCCTTCATCAAGCAAC-3′
18	FP			5′-TTGTTCTGCAGCCTACCTAT-3′
	RP	55	278	5′-TGACAACGTGGTTCTTAATGA-3′
19	FP			5′-CACTGTCAAGGGTTATTAATG-3′
	RP	50	373	5′-GGTGCAAAGTACTGTCTTAGG-3′
20	FP			5′-GGCATTTGACTCAAAGTTATA-3′
	RP	55	332	5′-CCAGAAAGAGAGAAACCACA-3′

a Column 2: FP, forward primer; RP, reverse primer.

explored epigenetics as an alternative route to inactivation. Methylation of CpG islands in the promoter regions does not seem to be the explanation for the down-regulation seen in gene transcripts [11]. Ubiquitination factor *E4B* (*UBE4B*) is a strong candidate TSG implicated in the ubiquitin/proteasome pathway [10,14]. We have previously identified one novel *UBE4B* mutation in a NB patient. As in the case of other genes in the region, gene expression is down-regulated in high-stage NB. A strong homologue to *UBE4B* has been found in *UBE4A* (*Ub*iquitination factor *E4A*), one among a few genes in the 2–5 cM consensus 11q-deleted region. *UBE4A* has been mapped to the 11q23.3 critical region, playing a part not only in NB but also in other specific cancers [15]. The gene is composed of 20 exons, 19 of which are coding, covering a length of more than 40 kb (URL: http://www.ensembl.org). The only known motif of *UBE4A* is a U-box domain spanning approximately 70 amino acids (URL: http://genome.ucsc.edu). We have previously speculated that a skewed expression of either *UBE4B* (located in 1p36.3) or *UBE4A* (in 11q23.3) is an important step in advanced-stage NB development and/or progression [10] and we therefore wanted to investigate the *UBE4A* gene in relation to NB tumours.

2. Patients and methods

2.1. *Patient and control material*

DNA was extracted from frozen samples of 71 tumours, one healthy tissue sample from a NB patient and 90 samples from healthy control individuals. A total of 69 NB patients contributed to the tumour material, meaning that three samples originated from the same patient, with biopsies taken at different times.

2.2. *DNA amplification*

Primers were designed for the coding exons and flanking intronic sequences of transcript NM_004788.2 from UCSC Genome Browser May 2004. Oligo Primer Analysis Software version 6.0 (Molecular Biology Insights, Inc., Cascade, CO) was used and primers were ordered from Life Technologies, Inc., Gaitherburg, MD (Table 1). Standard reactions of 20 μl were used, containing 25–100 ng DNA, 1.5 mM $MgCl_2$, 2 mM dNTP, 0.6–0.75 μM primers and 1 U Taq polymerase. Reactions were denatured at 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 s, annealing for 30 s, 72 °C for 1 min and ending with a 7-min extension step.

2.3. *Sequencing*

PCR products were purified with Exo-SAP-IT™ (USB Corporation, Cleveland, Ohio). Sequencing was performed with forward or reverse primer using the ABI Prism BigDye™ cycle sequencing Ready Reaction Kit (Applied Biosystem, Foster City, CA). The products were precipitated and resuspended in 10 μl Hi-Di formamide (Applied Biosystem) and analysed in an ABI 3100 Genetic Analyzer (Applied Biosystem). Analyses of the runs were performed with SeqScape version 2.1.1 (Applied Biosystem).

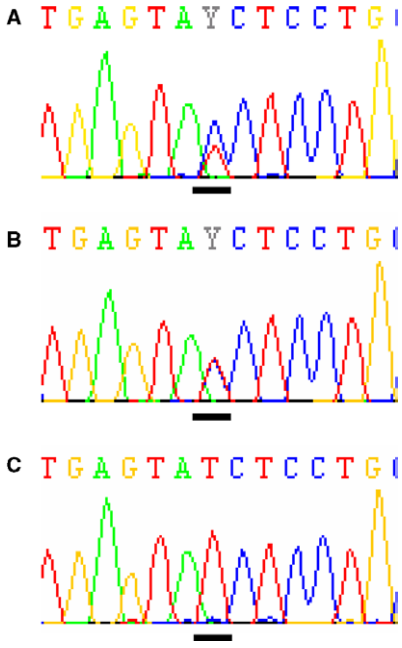

Fig. 1 – *UBE4A* missense mutation in a NB primary tumour. Bars under each chromatogram indicate the mutation position. (A) The 1028T > C mutation in patient 20R8 gives rise to an amino acid change from Ile to Thr. Note the lower dose of the normal T allele, compared to B. (B) Normal tissue from patient 20R8, heterozygous for C/T. (C) Healthy control individual.

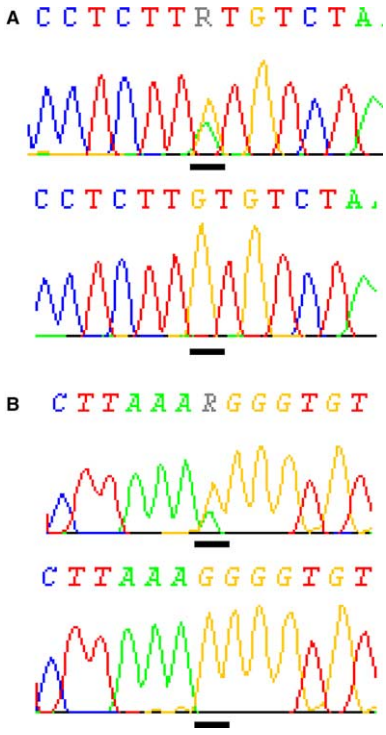

Fig. 2 – Variations detected in *UBE4A*. Bars under the chromatograms indicate the position of the variation. (A) Variation IVS1-26A/G. Upper panel: Base change from G/G to A/G in patient 27R1. Lower panel: Healthy control. (B) Variation IVS2-36A/G. Upper panel: Patient 26R0 displays a heterozygous A/G. Lower panel: Healthy control.

3. Results

The coding exons of *UBE4A* and some flanking intronic sequences were searched for mutations. A putative missense mutation was found in one patient as well as a novel polymorphism, both located within a coding exon. Two different variations, residing in flanking introns, were also found. The mutation 1028T > C leading to a putative missense mutation, p.I343T, residing in exon 8, was found in patient 20R8 and was confirmed by sequencing in both directions (Fig. 1). The variation was heterozygous (C/T) yielding alternative codons, ATC and ACC, thus resulting in a potential amino acid change from isoleucine (Ile) to threonine (Thr). Normal tissue from 20R8 also contained the variation C/T. In addition, two more samples derived from the same patient but at different points in time, one with the same origin as 20R8 and the other harvested later, were investigated, resulting in a variable degree of heterozygosity. Ninety healthy control individuals were also screened for the variation. Out of the 180 alleles, none had the alteration, strongly suggesting that this was not a polymorphism.

A novel polymorphism in exon 16, 2583C/T p.I861I, was detected and confirmed. Three NB patients, 15R3, 24R3 and 18E9A, carried the variation C/T, as did three of 150 alleles from healthy controls. No amino acid change (Ile-Ile) would result from modification of codon ATC to ATT.

A third variation, IVS1-26A/G, was found in patient 27R1. The alteration from a homozygous G/G state to a heterozygous A/G state resided 26 bases upstream of exon 2 in the 5′ flanking intron (Fig. 2). A fourth sequence variation, IVS2-36A/G, was detected in patient 26R0, residing 36 bases upstream of exon 3 in the flanking intron (Fig. 2). None of these variations were detected in 90 healthy control individuals (180 alleles).

4. Discussion

Guo and colleagues had previously presented data on the 11q LOH in NB primary tumours [4,16]. They showed that the consensus deletion region was present in a 2 cM region in chromosome band 11q23.3 and that it was flanked by markers *D*11S1340 and *D*11S1249. Using the most recent version of the human gene map (data from UCSC data base, May 2004 assembly), we can conclude that the *UBE4A* gene is one of a few genes located in this region; *D*11S1340 (base pars from 11pter:116,092,04-8-116,092,409) – *UBE4A* (117,735,511–117,775,134) – *D*11S1299 (119,139,500–119,140,047) (Fig. 4B).

We have previously studied several genes in the 1p36.2 LOH consensus region in NB tumours, including the gene *UBE4B* [7,9–12,17,18]. In an earlier study we detected one true mutation in the *UBE4B* gene in a fatal-outcome NB tumour, i.e., a splice donor site mutation of exon 9, c.1439 + 1G > C [10]. This variant was not present in the constitutional DNA of the patient and still represents, to our knowledge, the only true mutation found so far in a 1p-SRO-located gene in primary NB tumours.

The fact that the two human homologues of the yeast gene *UFD2*, i.e., *UBE4A* and *UBE4B*, had been shown to reside in two small LOH regions on 1p (500 kb; Fig. 4A) and on 11q (3 Mb, Fig. 4B) prompted us to investigate the *UBE4A* gene, located in the 11q23.3 consensus deletion region, in NB primary tumour material as well. One very interesting variation was detected, i.e., a 1028T > C in exon 8, that changes a homozygous T/T into a heterozygous C/T (Figs. 1 and 4C). The codon thus alternates between ATC and ACC with an alteration from the former to the latter giving rise to an amino acid change from isoleucine (Ile) to threonine (Thr), Ile343Thr. The variant was detected in a stage 3 NB tumour. No alteration at this site was found among the 90 healthy control individuals, i.e., 180 alleles. This strongly suggests that the allele variant is a

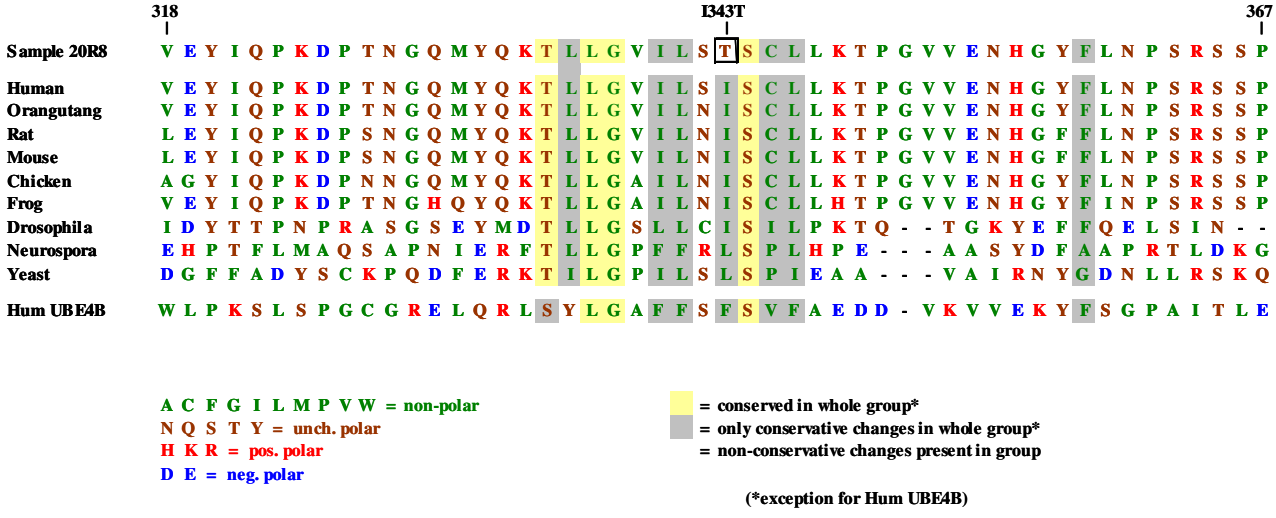

Fig. 3 – Alignment of amino acid sequences with homology to *UBE4A*. The putative missense mutation, Ile343Thr, observed in sample 20R8, is viewed on top of the alignment. The region has been highly conserved throughout evolution and it is likely that the isoleucine to threonine change leads to a non-functional protein. Sequences were derived from GenBank and aligned with the multiple alignment function through ExPaSy (URL: http://www.expasy.org).

mutation that is significant for the progression to advanced-stage NB. The non-polar amino acid isoleucine in the above-mentioned position has been highly conserved throughout evolution among most investigated species (Fig. 3) and it is likely that the change to the polar amino acid threonine in this position is deleterious for the proper function of the protein. This also indicates that the variation is a mutation rather than a neutral polymorphism. Furthermore, the alleles are not equal in dose in the tumour, compared to the constitutional state of the patient (Fig. 1A and B). This is indicative of loss of the normal allele in the tumour tissue, leaving only the mutated C allele copy. There were varying proportions of normal cells in our tumour material and the normal cell content of each tumour sample thus varied, depending on the dissection site. A large amount of normal cells in the samples might be an explanation for the variation in allelic loss detected in the tumour samples from 20R8. In order to investigate this hypothesis, two additional tumour samples from the patient deriving from biopsies taken at different times, the first having the same origin as 20R8 and the second harvested later, were extracted in the hope of finding an unequivocal allelic loss. However, no definite conclusions could be drawn, since the first sample was similar to the putative mutation and the second resembled normal tissue. In summary, one interpretation might be that the variation seen as C/T is a hereditary mutation, explaining its detection in normal tissue. A subsequent mutational event could subsequently have shifted the heterozygous C/T state to a hemizygous C allele by LOH, although that loss of the T allele is diffuse because the sample was contaminated by normal heterozygous cells. It should be noted that none of the 90 healthy controls (180 alleles) screened for the variation carried the C allele, again indicating that the C allele is indeed a mutation.

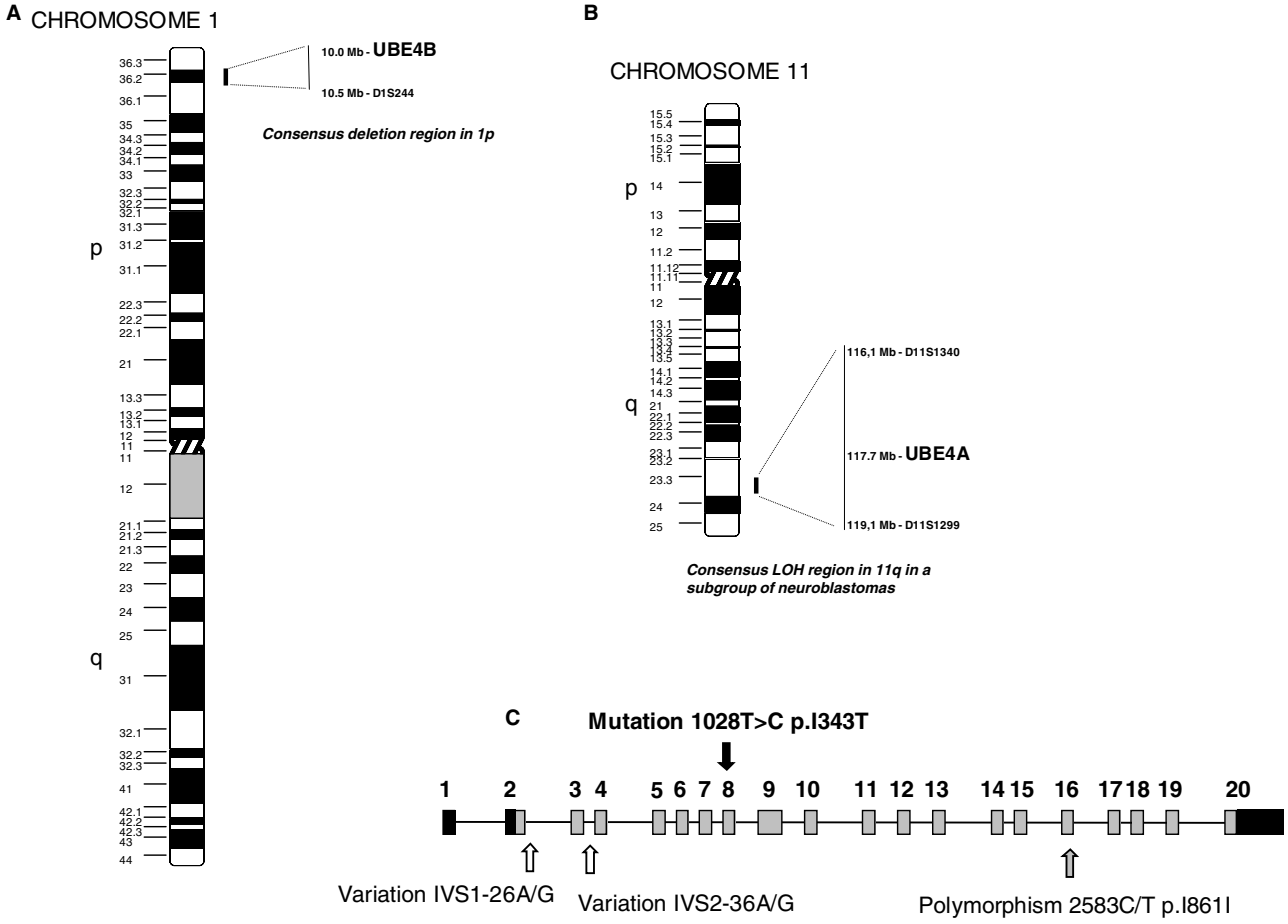

Fig. 4 – Consensus deletion regions in 1p and 11q in NBs, the relative localisation of the *UBE4A* and *UBE4B* genes and location of detected mutations and variations in *UBE4A*. (A) The *UBE4B* gene is located in the 500 kb consensus deletion region, represented by data from Ohira and colleagues [8] and Krona and colleagues [10]. (B) The *UBE4A* gene is located in the 3Mb consensus deletion region presented by Guo and colleagues [4]. (C) A schematic representation of *UBE4A* and a summary of all sequence variations detected. Shaded boxes represent the coding regions of exons 2–20 and dark boxes represent non-coding regions. The putative missense mutation, p.I343T, found in exon 8 is shown as a black arrow and the novel polymorphism, p.I861I, resides in exon 16, shown as a grey arrow. Variation IVS1-26A/G is localised 26 bases upstream of exon 2 and variation IVS2-36A/G resides 36 bases upstream of exon 3, both indicated as white arrows. Data for position of markers in A and B are derived from the UCSC genome browser May 2004 assembly.

An alteration, 2583C/T I861I, was also found in exon 16. The variation is not annotated as a single nucleotide polymorphism (SNP) in the UCSC genome browser, indicating that a rare polymorphism has been found. The normal C allele has been substituted to a heterozygous C/T and the alternate codon ATT (from ATC) does not replace the amino acid isoleucine. Interestingly, all three NB samples carrying the variation were from stage 4 patients. However, three of 75 controls were also found to carry the variation in a heterozygous state, indicating that the variation is a non-pathogenic alteration.

The third variation found, IVS1-26A/G, resides in the first intron of *UBE4A*, 26 bases upstream of exon 2, where a homozygous G/G has been changed into a heterozygous A/G state (Fig. 2A). Interestingly, the A allele, together with the succeeding bases T and G, might yield a base triplet of ATG, which gives the translation start codon AUG in RNA. Since the DNA coding for proteins begins within exon 2, it could be argued that the variation found flanking the 5′ end of exon 2 yields an alternative translation initiation site. This would result in an entirely different protein with other properties. In the case of NB, it is probable that replacement of the normal protein with another protein would lead to a loss of ubiquitination function, giving rise to malignancy. However, it is not easy to influence the translation initiation site since efficient initiation codon recognition requires additional factors. This, and the fact that the sample carrying the variation came from a favourable-prognosis stage 2 case, strongly suggest that the variation is a neutral base change that is not harmful. The alteration was not found in any of the healthy controls (180 alleles) screened.

The fourth alteration, IVS2-36A/G, detected in the intron 36 bases upstream of exon 3, was carried by a stage 4 patient (Fig. 2B). The variation was not detected in any of the 90 healthy control individuals (180 alleles). Nothing indicated that the variation caused disease. For a schematic view of the variations found, see Fig. 4C.

In conclusion, we have detected a putative missense mutation in *UBE4A*. The *UBE4A* gene resides in a SRO of deletions in NB, localised in the long arm of chromosome 11 (11q23.3). In an earlier study, a true mutation in the splice donor site of exon 9: c.1439 + 1G > C, was found in the homologue *UBE4B* in a stage 3 NB patient with fatal outcome. The splice site mutation resided in the 1p SRO of deletions in the short arm of chromosome 1 (1p36.2-3) [10]. Both the *UBE4A* (in 11q) and the *UBE4B* (in 1p) genes are implicated in the ubiquitin proteasome pathway by encoding proteins that are homologues of the yeast protein Ufd2 [19]. It might be speculated that the proteins generated from *UBE4B* and *UBE4A* work together to protect the cell from environmental stress and that inactivation of either of them contributes to malignancy.

Conflict of interest statement

None declared.

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