available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Allelic variants of *CAMTA1* and *FLJ10737* within a commonly deleted region at 1p36 in neuroblastoma

Kai-Oliver Henrich[a,*], *Andreas Claas*[a,d], *Christian Praml*[a], *Axel Benner*[b], *Jan Mollenhauer*[c], *Annemarie Poustka*[c], *Manfred Schwab*[a], *Frank Westermann*[a]

[a]*Division of Tumour Genetics B030, German Cancer Research Center DKFZ, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany*
[b]*Central Unit Biostatistics, German Cancer Research Center DKFZ, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany*
[c]*Division of Molecular Genome Analysis B050, German Cancer Research Center DKFZ, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany*

ARTICLE INFO

Article history:
Received 23 June 2006
Received in revised form
12 September 2006
Accepted 19 September 2006

Keywords:
CAMTA1
FLJ10737
1p
Neuroblastoma
Deletion
LOH

ABSTRACT

Deletion of a distal portion of 1p is seen in a wide range of human malignancies, including neuroblastoma. Here, a 1p36.3 commonly deleted region of 216 kb has been defined encompassing two genes, *CAMTA1* and *FLJ10737*. Low expression of CAMTA1 has been recently shown to be an independent predictor of poor outcome in neuroblastoma patients. The present study surveys *CAMTA1* and *FLJ10737* for genetic alterations by fluorescence-based single strand conformation polymorphism (SSCP) using a panel of DNAs from 88 neuroblastomas, their matching blood samples and 97 unaffected individuals. Nucleotide variants encoding amino acid substitutions were found in both genes. One CAMTA1 variant (T1336I) was not detected in 97 unaffected individuals, another (N1177K) resides in a conserved domain of the CAMTA1 protein and was found hemizygous in six neuroblastomas. We found no evidence for somatic mutations in *FLJ10737* or *CAMTA1*. Further investigations are needed to address the functional impact of the identified variants and their possible significance for neuroblastoma.

1. Introduction

Neuroblastoma is an embryonal tumour consisting of neural crest derived undifferentiated neuroectodermal cells that accounts for ~9% of all childhood cancers. The clinical hallmark of this tumour is its extreme heterogeneity, ranging from spontaneous regression to malignant progression. An aggressive subtype of neuroblastomas is characterised by genetic aberrations including amplification of the *MYCN* oncogene[1,2] and deletion of a distal portion of 1p. The incidence of 1p deletion in neuroblastoma is ~30% and it has been shown that 1p36 loss of heterozygosity (LOH) is an independent predictor of disease progression.[3,4] Functional evidence supporting a role of 1p in neuroblastoma derives from experiments in which the introduction of 1p chromosomal material into neuroblastoma cells resulted in reduced tumourigenicity.[5] Thus, it is generally believed that 1p contains a gene (or genes) important in the development of neuroblastoma.

For almost two decades numerous studies have attempted to narrow down the 1p deleted region in order to increase the chances for identifying the gene(s) of interest.[6–14] However, so far, the success in defining candidate genes is limited as most of the identified regions were too large to pinpoint single genes. Recent studies made it possible to considerably narrow down a smallest region of consis-

* *Corresponding author:* Tel.: +49 6221 423220; fax: +49 6221 423277.
E-mail address: k.henrich@dkfz.de (K.-O. Henrich).
[d] Present address: Population Genetics Technologies Ltd., Babraham Research Campus, Babraham, Cambridgeshire CB2 4AT, UK.
0959-8049/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2006.09.023

tent heterozygous deletion that is shared by most neuroblastomas with 1p deletions and spans 261 kb between 1p36.3 markers *D1S2731* and *D1S214*.[15–20] This region encompasses two genes, *FLJ10737* and *CAMTA1*.

By homology, *FLJ10737* is predicted to encode a member of the DnaJ protein family and it is expressed in a wide variety of human tissues.[18] In neuroblastoma, no evidence for an association of *FLJ10737* expression with clinicobiologic variables or outcome was found.[15] DnaJ proteins regulate chaperone functions of Hsp70 proteins and participate in protein folding, protein degradation and assembly/disassembly of protein complexes.[21] The identification of the Drosophila tumour suppressor gene l(2)tid provided first proof for causal involvement of DnaJ proteins in neoplastic transformation,[22] and a human homologue of l(2)tid encodes two opposing modulators of apoptosis.[23] Therefore, one could imagine that FLJ10737 as a member of the DnaJ protein family plays a role not only restricted to the mediation of 'classical' chaperone functions but also in processes related to tumour onset or progression.

CAMTA1 is a member of a recently described protein family designated as calmodulin-binding transcription activators (CAMTAs).[24] The protein's primary structure contains a nuclear localisation signal, two DNA-binding domains (CG-1 and TIG), calmodulin-binding motifs (IQ motifs) and ankyrin repeats. CAMTA1 is a transcription activator potentially involved in cell cycle regulation[25] that may, similar as its homologue AtCAMTA1, interact with Ca2+/calmodulin and be engaged in calcium signalling.[24] Although the expression of *CAMTA1* is seen in various organs, highest levels are found in neuronal tissues.[18,26,27] In neuroblastoma, low *CAMTA1* expression is significantly associated with poor outcome of patients. Intriguingly, in multivariate survival analysis the prognostic information of *CAMTA1* expression is independent of the established risk markers *MYCN* amplification, 1p deletion, age at diagnosis and advanced tumour stage.[15] These data suggest (i) that *CAMTA1* expression represents a powerful prognostic variable with the potential of improving current risk stratification for neuroblastoma, and (ii) that *CAMTA1* is a strong candidate for being involved in the development of neuroblastoma and other tumours with 1p deletion. This is further supported by a recent report that identified *CAMTA1* as the only gene mapping to a 1p36 minimal region of overlapping deletions in glioma tumours.[27]

To address the question, whether mutations or sequence variants of *FLJ10737* or *CAMTA1* exist that are associated with neuroblastoma, we have analysed the coding regions of both genes in a panel of up to 88 neuroblastomas by single strand conformation polymorphism (SSCP) and sequencing. To estimate the population allele frequencies of individual sequence variants, blood samples from 97 unaffected individuals were used as a reference panel. Our results suggest that somatic mutations of *CAMTA1* or *FLJ10737* are not likely to be involved in neuroblastoma development. However, allelic variants, predicted to cause amino acid substitutions, were found in both genes. The significance of these variants with respect to neuroblastoma development needs to be addressed in further investigations.

2. Materials and methods

2.1. Tumours and blood specimens

Genomic DNA was extracted from 88 fresh or snap-frozen neuroblastoma tumours. Of these, 22 were stage 1, 11 were stage 2, 6 were stage 3, 34 were stage 4, 6 were stage 4s and 9 were of unknown stage. Staging was according to the International Neuroblastoma Staging System (INSS). Twenty-seven of the 88 tumours had LOH at 1p36 (markers *D1S468*, *D1S253* and *D1S244*; data not shown). All tumours were obtained from hospitals cooperating within the framework of the German Neuroblastoma Study Group. DNA was also extracted from peripheral blood of the patients and 97 unaffected individuals.

2.2. CE-SSCP and sequencing

The entire coding region of both *CAMTA1* (23 exons) and *FLJ10737* (16 exons) was analysed by capillary electrophoresis single strand conformation polymorphism (CE-SSCP).[28] Initially, *FLJ10737* was surveyed in 25, *CAMTA1* in 30 neuroblastoma tumours (Table 1). Selected sequence variants were

Table 1 – Clinical and genetic parameters of the initially analysed set of tumours

Patient	Tumour stage	1p LOH	MYCN-amplification
N-1	1	–	–
N-2	1	–	–
N-3	4	+	–
N-4	4	+	+
N-5	1	+	–
N-6	4	+	–
N-7	4	+	+
N-8	4	+	–
N-9	4	+	+
N-10	1	–	–
N-11	1	–	–
N-12[a]	4	–	–
N-13[a]	1	–	–
N-14	4	+	–
N-15	1	+	–
N-16	1	+	–
N-17[a]	2	–	–
N-18	4	+	+
N-19	4s	+	–
N-20[a]	2	–	–
N-21	4	+	+
N-22[a]	4	+	+
N-23	1	+	–
N-24	2	+	+
N-25	4s	–	+
N-26	4	+	+
N-27	1	–	–
N-28	4	+	+
N-29	4	+	+
N-30	4	+	+

Column 3: +, 1p LOH; –, no 1p LOH; column 4: +, *MYCN*-amplification; –, no *MYCN*-amplification. Tumours were staged according to the International Neuroblastoma Staging System criteria (INSS).
a Not analysed for *FLJ10737* variants.

then analysed in additional tumours up to the maximum number of 88 available samples. DNA variants causing amino acid residue substitution were also analysed in up to 97 blood DNAs from unaffected individuals to estimate the population allele frequency.

If the size of a PCR target sequence exceeded 350 bp in case of larger exons, the respective exons were PCR-amplified as overlapping fragments. Sequences and annealing temperatures of the primers used are given in Table 2. PCR was carried out in 50 μl reactions with 1.5 U Ampli*Taq* (Applied Biosystems, Foster City, CA), 1× reaction buffer, 200 μM of each dNTP, 1.5 mM $MgCl_2$ and 0.2 μM of each fluorescent labelled primer (HEX, FAM, respectively; Applied Biosystems, Foster City, CA). For GC-rich amplicons, 1.5× PCR× Enhancer Solution (Invitrogen, Carlsbad, CA) or DMSO (3–5%) was included. Cycle conditions have been reported before.[28] SSCP analysis was performed with dilutions of PCR products using an ABI 310 genetic analyser (Applied Biosystems, Foster City, CA). PCR products showing one common specific peak pattern, suggesting allelic identity, were grouped and several candidates

Table 2 – Primers used for SSCP and sequencing analyses of CAMTA1 and FLJ10737

	Forward primer (5′–3′)	Reverse primer (5′–3′)	Annealing temperature (°C)
CAMTA1-EX1	CGGAGAGTAGTGAGACCCCTGGTGC	CCGATGCCCGGGATGTCC	60[a]
CAMTA1-EX2	GGCAGGAATATCACAGAAGAGC	ATAATTACTGCCACTGCCAAGC	57
CAMTA1-EX3	TCACATTGACTAAACTGAGCTGC	GTGAAGCAACCTAAAAAATTTGC	59
CAMTA1-EX4	CTCTCAGGATCCAATGTGAGC	AATTCACTGGCCAAATCAGG	59
CAMTA1-EX5	AATTATGTTCCAGCAAATGTGG	ATGACATTTGTGCACCAAGG	57
CAMTA1-EX6	TGTGGCCCCTTCTTGCTC	CAGACCCTTCCCGAGCAC	57[b]
CAMTA1-EX7	CTCCATGCCACCCTCATG	GTTGATGCCAGCCTGGTTC	61
CAMTA1-EX8	ACCCACGGGCTCTGACAG	CAGCAGGGACAGCCATGAC	64
CAMTA1-EX9-a	CATGTGTGCCTGCGTGTG	AGACCTCGGTGCTGCTCTG	61
CAMTA1-EX9-b	GCTCCAGCCGTGAGAAGAG	GTGGGGGACATGGTGTACAC	58
CAMTA1-EX9-c	CGCCACGGTGTTCATGTC	TACGTCTGGCCCTGCTTTG	58[c]
CAMTA1-EX9-d	GAAACCACCATGAACTTTGACC	GTGGTGCTGGGACTCAGG	56[c]
CAMTA1-EX9-e	AGCTCCCTCACCCTGACC	GTCTCCACGTGACCGCTG	56[c]
CAMTA1-EX9-f	ATGCCCACGGTGAAAACG	GGGTAGAGTCCCTGCACCAC	59
CAMTA1-EX9-g	CGGAGACCAACGGGGTAAT	GGAGGCACATCTCTGCCTG	58[c]
CAMTA1-EX9-h	CACGGCGCTCTCACAGTC	CCTTAGCTGCAGGGTGGC	62
CAMTA1-EX10	TCCCACAGTGGCTCACACTC	GTGCTCCTCAGGCCATCAC	56
CAMTA1-EX11	CCAGGCTGTAGGTACCCACC	CCTCTCCCTTGGTGCCTG	61
CAMTA1-EX12	AACACAACCTCACTCTTCCTCC	GATCCATCCACTGCCTCG	60
CAMTA1-EX13	GGCCTTTAGTCCTGAGGTCG	CAGGAAGAATGTGAGCGAGG	55[c]
CAMTA1-EX14	TTAACGGTGGTGAATAGTGTGG	CAGACTGGAAGAACAGCAAGG	59
CAMTA1-EX15	CTGATTGAGAACGCTTGCTG	TGTCAAGCTCTGAGATGTCATG	58
CAMTA1-EX16-a	CTCCTGTCCTGACTATCCTTTC	TCCTACAGGCTGAGATCCAG	61
CAMTA1-EX16-b	CTTCAGCCGGGAACTCTCC	CAGCCTCTGCGGGCTTAC	63
CAMTA1-EX17	GAGGCAGGGAGGTAGACCTG	CGGGAATCTATGCTGGGTAG	64
CAMTA1-EX18	GTGGTGCAAATCAATCATTAATC	GAATGTCACAAAACGAAGGTTC	56
CAMTA1-EX19	TAAACCTGGGATCTCAACTCTG	CTTTAGCCAAACCAGGATCC	57
CAMTA1-EX20	CCCTGTTGTTACTGTGTCGC	CCCTAGGGAGCTTTACCTCC	56
CAMTA1-EX21	AGTCCTCTGTCACTGCTGACC	GTTGCTTCATCATTCTCCCTG	62
CAMTA1-EX22	TTCTGTTGTGACCCTCATGTG	ATGGAAGAGAAGAGAAACTGGC	60
CAMTA1-EX23	ACCCCTTTCCTTTACTTGGTC	AATGTCACATTGCTAAGGGATG	60
FLJ10737-EX1	GTCCCTGACGCGGATCAC	GTGGGGACGCCTCCATC	57[b]
FLJ10737-EX2	ATTGGGACGTGGCTGATG	CCTCTACAAATCGCACCTCC	57
FLJ10737-EX3	TCTCAGGAAGGACTGAAGGTG	TTGGGGAGATGTTCTGTAACTG	57
FLJ10737-EX4	CTCCTGGGTCCTCAACTGTC	ACCTGGCTTCTAGTTATGAGGC	57
FLJ10737-EX5	TGTTCAATTAAAGCGAGCTGG	CCCTTGCTGTACTCGGAGAG	57
FLJ10737-EX6	CTTTAGGCTGCTGCCATTG	GCACAGACAACACAACTCAGC	57
FLJ10737-EX7	ACATACCTGAGCCTGGGTTG	GACAAGATAGTGCCAACCACTG	57
FLJ10737-EX8	GAGCAGGTGCTATCCAGGAC	TCCTCCCAGAGCTCTGTCTG	57
FLJ10737-EX9	AGAGTCACACGCCCTAGCC	CACCAACTGGTTCCACTGTG	57
FLJ10737-EX10	TCTTCTCAAATGACAGCTGCC	GGTGACAGGCATTCCTTGAC	57
FLJ10737-EX11	CTCTTCCTGTGCCTGACACC	AACTGAGGCACAGGGCTG	57
FLJ10737-EX12	CTGTGTTGGCTGGAAGCTG	CCTGAGACCAACATGTGGG	57
FLJ10737-EX13	GGCCCTGAGCAAGTATGTTG	GAGGAAGGCCTCCTCCAG	57
FLJ10737-EX14	GTCTCTAAGCCACACAAGGAGG	TTATAGGCCTGAGTCACCGC	57
FLJ10737-EX15	GTGCTGGTCTTAGACCGTCTG	ACACTGAGCAGCCGCTTC	57
FLJ10737-EX16	TCTGATCACCTGCGTCTGTC	TGCAGTCTGGTTCCCAGTG	55

a 1.5× PCR× Enhancer Solution (Invitrogen) was included in the PCR.
b 5% DMSO were included in the PCR.
c 3% DMSO were included in the PCR.

from each group were sequenced using BigDye Terminator chemistry and an ABI 310 genetic analyser (Applied Biosystems, Foster City, CA). Nomenclature of sequence variants was as recommended by the Nomenclature Working Group.[29]

2.3. RT-PCR

In case of an intronic sequence variant localised in the vicinity of a splice site, we performed RT-PCRs to identify potential products of aberrant splicing. Since tumour RNA was not available, RNA was isolated from neuroblastoma cell lines (Kelly, IMR-32, CHP-134, SH-EP, NMB, SK-N-SH, Vi-856, GI-ME-N) after their allelic status for the respective variant had been determined. RNA was isolated with TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. First strand cDNA was synthesised from 1 μg of total RNA using a 1st strand cDNA synthesis kit (Roche, Indianapolis). Of this 20 μl cDNA reaction, a volume of 1 μl served as template in a PCR with primers flanking the tested exon. PCR was performed as described for CE-SSCP but with unlabelled primers and 35 cycles of 30 s at 94 °C, 45 s at 59 °C and 45 s at 72 °C.

2.4. Statistics

An exact test was used to test the null hypothesis that the Hardy–Weinberg equilibrium holds.[30] Distributions of allele frequencies were compared using Fisher's exact test. Calculation of allele frequencies for tumours with 1p36 LOH was based on non-deleted alleles only. Chi-squared tests on the distribution of genotypes were performed if observed frequencies were compared with estimated probabilities. An effect was judged statistically significant at a p-value not larger than 5%. All statistical calculations were done using the software package R, version 1.8.[31]

3. Results

3.1. Genetic variants of FLJ10737and CAMTA1

Sequence variants are given as deviations from the cDNA sequences GenBank AK001599.1 (*FLJ10737*) and GenBank AB020640.3 (*CAMTA1*), respectively. Exon numbering refers to genomic structure as described previously[15] (illustrated in Fig. 1).

3.1.1. FLJ10737

For *FLJ10737*, we detected seven nucleotide variants of which two were intronic (Tables 3 and 4). Of the five variants in the coding region, two dictate amino acid substitution.

One of the two respective variants, 799A > G, causes methionine (ATG) to be replaced by valine (GTG). The 799G allele is common (average allele frequency 87.6%), indicating that the 799A allele to be found in the reference sequence AK001599.1 is the rarer variant. Out of 25 tumours, 799A was found heterozygous in one and hemizygous in a second tumour with the matching blood DNAs being 799A/G heterozygous. In unaffected individuals, 799A was observed homozygous in one, heterozygous in 24 of 97.

The second of the two amino acid substituting variants, 869C > G, encodes an amino acid substitution from threonine (ACC) to serine (AGC). Of 25 tumours, 869G was found heterozygous in three, hemizygous in four. All blood DNAs corresponding to tumours displaying this variant were 869C/G heterozygous. Four of 97 unaffected individuals were homozygous for 869G, 33 heterozygous.

Another *FLJ10737* variant chosen for more detailed analysis is an intronic 1-bp deletion in a polythymidine tract (T_{18}) preceding the splice acceptor site of the intron 10–exon 11 junction (IVS10-15delT, Table 4). Among 25 tumours, the T_{17} allele of IVS10-15delT was found homozygous in two, heterozygous in three and hemizygous in nine tumours. In the exemplarily investigated blood DNAs of four patients, the same status as in the corresponding tumours was seen ($2\times T_{18}/T_{18}$, $2\times T_{17}/T_{17}$). Alterations in polythymidine tracts preceding splice acceptor sites have the potential to decrease the recognition of an adjacent exon during the splicing process.[32] To test whether the shortened polythymidine tract might associate with exclusion of exon 11 from the *FLJ10737* transcript, we performed RT-PCR on cDNAs from seven neuroblastoma cell lines exclusively displaying the T_{17} allele (Kelly, IMR-32, CHP-134, SH-EP, NMB, SK-N-SH, Vi-856), and one neuroblastoma cell line exclusively displaying the T_{18} allele (GI-ME-N). The exon 11-flanking primers 5′-TGGGACGGTGGTGGAGTAC-3′ (homologous to exon 10) and 5′-TGGGACGGTGGTGGAGTAC-3′ (homologous to exon 12) were used. The size of the resulting RT-PCR products indicates that exon 11 was not excluded from the *FLJ10737* transcript in any of the examined cell lines (Fig. 2).

3.1.2. CAMTA1

In *CAMTA1*, we detected 15 sequence variants of which eight were intronic and one was located in the 5′UTR (Tables 3 and 4). Of the remaining six variants in the coding region, three dictate amino acid substitutions.

One of these three, 3531C > G, encodes substitution at codon 1177 from AAC (asparagine) to AAG (lysine). This amino acid variant resides in an ankyrin repeat (Fig. 1), a domain known to be involved in protein–protein interactions.[33] The crystal structure of several ankyrin domain containing proteins is available (Protein Data Bank, PDB, http://www.rcsb.org/pdb). A computer aided homology search revealed that the maximum homology of the CAMTA1 ankyrin domain to an ankyrin domain with described 3-D structure is 33% (to an artificial ankyrin repeat protein, PDB ID: 1MJ0). This degree of homology is not sufficient for the construction of a model predicting the influence of the amino acid substitution on the spatial structure of the protein. The localisation of 3531C > G (N1177K) in a conserved domain led us to examine its status in an extended cohort of 88 neuroblastomas (27 of 88 with 1p36 deletion). 3531G was found heterozygous in 15, hemizygous in six (five stage 4, one stage 1) of 88 tumours. One of these six displayed 3531G homozygous in the corresponding blood DNA. All other blood DNAs corresponding to tumours displaying the 3531G-allele were 3531G/C heterozygous. The distribution of genotypes among unaffected individuals did not significantly deviate from that predicted under the conditions of Hardy–Weinberg equilibrium (exact test for Hardy–Weinberg equilibrium, $p = 0.36$), but in these the 3531G-allele was found only in the heterozygous form (25/94). To investigate the possibility of selection for the

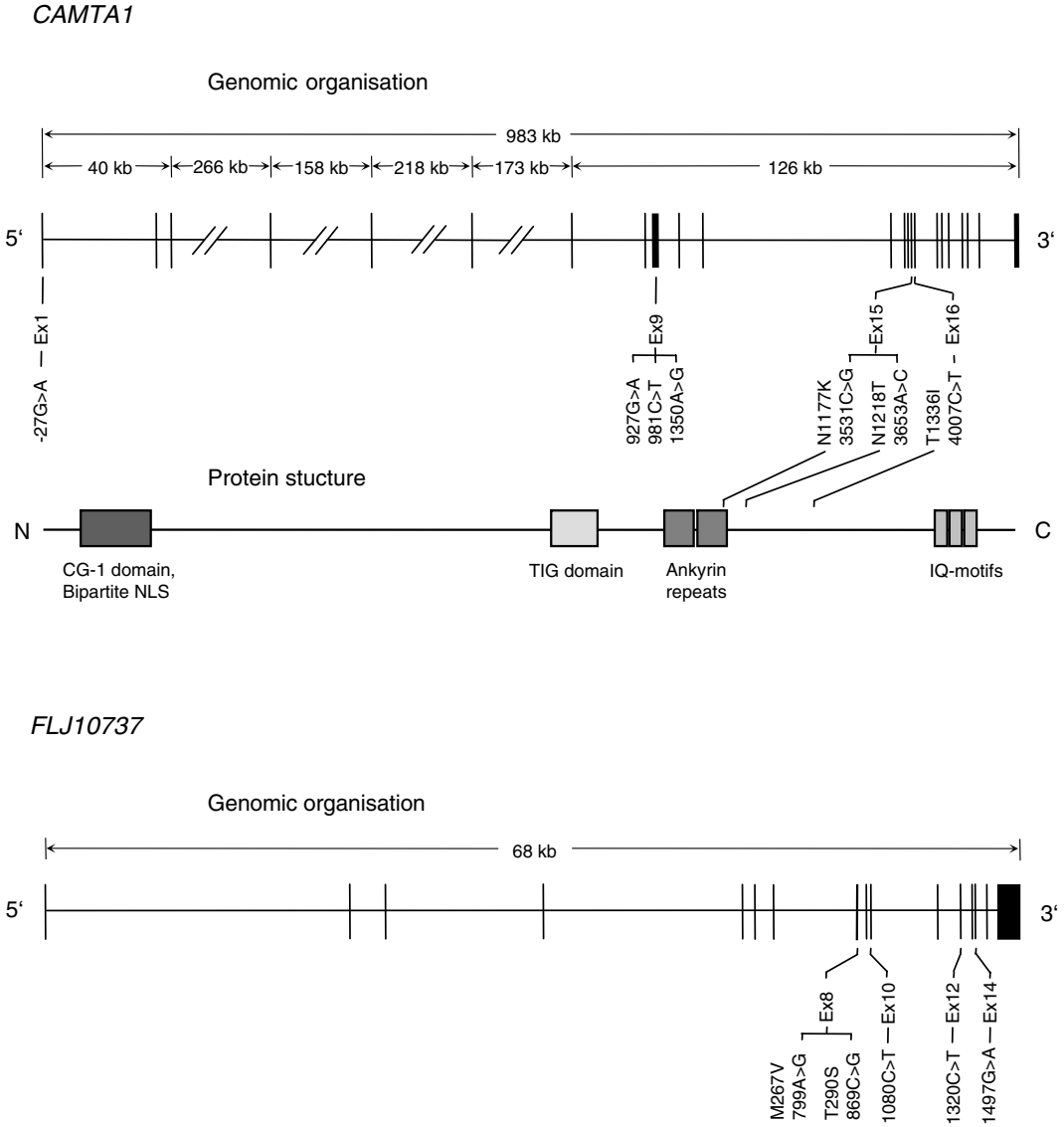

Fig. 1 – Schematic representation of the CAMTA1 protein and genomic organisation of *CAMTA1* and *FLJ10737* with position of the observed sequence variants. Arrows illustrate genomic distances. Exons are represented by vertical black lines or bars. Protein domains are indicated by grey boxes. NLS, nuclear localisation signal. Further indicated are CG-1 and TIG domains, ankyrin repeats and IQ-motifs. Identity and position of the domains are taken from Bouché et al.[24] and were confirmed by a conserved domain search (http://www.ncbi.nlm.nih.gov).

3531G allele, the status of the variant was assessed in blood DNAs from all 27 patients whose tumours exhibited 1p36 LOH. The 3531G frequency in 1p36 LOH patients was 18.5% compared to 13.3% in unaffected individuals and 14.1% in all 88 tumours analysed. One of the 27 blood DNAs displayed 3531G homozygous, eight heterozygous. Of these eight, 1p36 loss affected the 3531C allele in five, the 3531G allele in three cases. Thus, the number of six 3531G/– hemizygous tumours resulted from (a) an initial higher frequency of the variant in patients whose tumours exhibited 1p36 LOH (18.5%) and (b) 3531C allele loss with a rate of 62.5%. However, the deviation of the observed number of 3531G/– hemizygous tumours from the expected genotype distribution, based on (i) the allele frequency found in controls, (ii) the assumption of Hardy–Weinberg equilibrium and (iii) random deletion of one allele in 27 tumours, was not statistically significant (χ^2-test, $p = 0.17$).

Another *CAMTA1* variant, 3653A > C, is predicted to cause an amino acid substitution from asparagine (AAC) to threonine (ACC). Of the 76 tumours investigated, 3653C was found heterozygous in one and hemizygous in a second tumour with both corresponding blood DNAs displaying the heterozygous form. Of 41 unaffected individuals, the allele was observed in one as 3653A/C heterozygous.

A further rare sequence alteration (4007C > T), causing a substitution of codon 1336 from ACT (threonine, polar) to ATT (isoleucine, non-polar), was detected in one of 30 tumours. Both the tumour and matching blood DNA of this patient were 4007C/T heterozygous. 4007T was not present in the DNA of 97 control individuals.

Table 3 – CAMTA1 and FLJ10737 sequence variants leading to amino acid substitution

Gene	Exon	Nucleotide variant	Codon variant	dbSNP ID	Allele frequencies[a]			Genotype frequencies		
					NB tumours	Unaffected individuals	*p*-value[d]	Genotype	NB tumours	Unaffected individuals
CAMTA1	15	3531C > G	N1177K	–	21/149 (14.1%)	25/188 (13.3%)	0.87	C/G	15/88[b]	25/94
								G/G	0/88	0/94
								G/–	6/88[c]	0/94
								C/C	46/88	69/94
								C/–	21/88	0/94
		3653A > C	N1218T	–	2/125 (1.6%)	1/82 (1.2%)	1.00	A/C	1/76[b]	1/41
								C/C	0/76	0/41
								C/–	1/76[b]	0/41
								A/A	48/76	40/41
								A/–	26/76	0/41
	16	4007C > T	T1336I	–	1/40 (2.5%)	0/194 (0%)	0.17	C/T	1/30[b]	0/97
								T/T	0/30	0/97
								T/–	0/30	0/97
								C/C	9/30	97/97
								C/–	20/30	0/97
FLJ10737	8	799A > G	M267V	rs12137794	29/31 (93.5%)	168/194 (86.6%)	0.39	A/G	1/25[b]	24/97
								G/G	5/25	72/97
								G/–	18/25	0/97
								A/A	0/25	1/97
								A/–	1/25[b]	0/97
		869C > G	T290S	rs200454	7/31 (22.6%)	41/194 (21.1%)	0.82	C/G	3/25[b]	33/97
								G/G	0/25	4/97
								G/–	4/25[b]	0/97
								C/C	3/25	60/97
								C/–	15/25	0/97

Nomenclature of sequence variants as recommended by Antonarakis.[29] Variants are given as deviations from the reference sequences AB020640.3 (*CAMTA1*) and AK001599.1 (*FLJ10737*). The A of the initiator Met codon ATG is denoted +1. NB: neuroblastoma. dbSNP: NCBI single nucleotide polymorphism database.

a Calculation of allele frequencies is based on non-deleted alleles.

b The variant was found heterozygous in the corresponding blood DNA sample(s).

c The variant was found heterozygous in five of the corresponding blood DNA samples, homozygous in one.

d Two-sided *p*-value of Fisher's exact test. Allele frequencies in NBs *versus* allele frequencies in unaffected individuals.

Table 4 – CAMTA1 and FLJ10737 sequence variants not leading to amino acid substitution

Gene	Exon	Nucleotide variant	Codon variant	dbSNP ID	Allele frequencies[a]		Genotype frequencies		
					NB tumours	Unaffected individuals	Genotype[b]	NB tumours	Unaffected individuals
CAMTA1	1	−27G>A	–	–	6/39 (15.4%)	n.d.	G/A	2/29	0/1
							A/A	1/29	0/1
							A/–	2/29	0/1
	4	IVS4+48T > C	–	–	2/40 (5%)	n.d.	T/C	0/30	0/1
							C/C	0/30	0/1
							C/–	2/30	0/1
	6	IVS5-66C > T	–	–	1/40 (2.5%)	n.d.	C/T	0/30	0/1
							T/T	0/30	0/1
							T/–	1/30	0/1
		IVS6+35T > C	–	rs1201529	2/40 (5%)	n.d.	T/C	1/30	0/1
							C/C	0/30	0/1
							C/–	1/30	0/1
	8	IVS8+25C > T	–	–	1/40 (2.5%)	n.d.	C/T	1/30	0/1
							T/T	0/30	0/1
							T/–	0/30	0/1
	9	927G > A	–	rs3737907	5/40 (12.5%)	n.d.	G/A	2/30	0/1
							A/A	0/30	0/1
							A/–	3/30	0/1
		981C > T	–	rs3737906	5/40 (12.5%)	n.d.	C/T	1/30	1/1
							T/T	1/30	0/1
							T/–	2/30	0/1
		1350A > G	–	rs12128526	18/39 (46.2%)	n.d.	A/G	3/29	0/1
							G/G	2/29	0/1
							G/–	11/29	0/1
		IVS9+25C > A	–	rs4908671	13/40 (32.5%)	n.d.	C/A	2/30	0/1
							A/A	2/30	1/1
							A/–	7/30	0/1
	11	IVS11+6A > G	–	rs3011926	22/40 (55%)	n.d.	A/G	3/30	0/1
							G/G	3/30	0/1
							G/–	13/30	0/1
	13	IVS13+41A > G	–	rs2071985	25/40 (62.5%)	n.d.	A/G	5/30	0/1
							G/G	3/30	1/1
							G/–	14/30	0/1
	14	IVS14+26C > G	–	–	1/40 (2.5%)	n.d.	C/G	0/30	0/1
							G/G	0/30	0/1
							G/–	1/30	0/1
FLJ10737	1	IVS1+87G > A	–	rs3761925	14/31 (45.2%)	n.d.	G/A	3/25	0/1
							A/A	1/25	1/1
							A/–	9/25	0/1
	10	1080C > T	–	rs200458	3/31 (9.7%)	n.d.	C/T	1/25	0/1
							T/T	0/25	0/1
							T/–	2/25	0/1
	11	IVS10-15delT	–	–	16/31 (51.6%)	n.d.	18×T/17×T	3/25	0/1
							17×T/17×T	2/25[c]	0/1
							17×T/–	9/25	0/1
	12	1320C > T	–	–	1/31 (3.2%)	n.d.	C/T	1/25	0/1
							T/T	0/25	0/1
							T/–	0/25	0/1
	14	1497G > A	–	rs12134083	1/31 (3.2%)	n.d.	G/A	0/25	0/1
							A/A	0/25	0/1
							A/–	1/25	0/1

Nomenclature of sequence variants as recommended by Antonarakis.[29] Variants are given as deviations from the reference sequences AB020640.3 (*CAMTA1*) and AK001599.1 (*FLJ10737*). The A of the initiator Met codon ATG is denoted +1. Intron variants are designated by the Intron (IVS) number, positive numbers starting from the G of the donor site invariant GT, negative numbers starting from the G of the acceptor site invariant AG. NB: neuroblastoma. n.d.: not determined as only one unaffected individual was analysed for variations not leading to amino acid residue substitution. dbSNP: NCBI single nucleotide polymorphism database.

a Calculation of allele frequencies is based on non-deleted alleles.

b Only genotypes containing the variant allele are shown.

c In the constitutional DNA of four patients, the same status as in the corresponding tumours was observed ($2\times T_{17}/T_{17}$, $2\times T_{18}/T_{18}$).

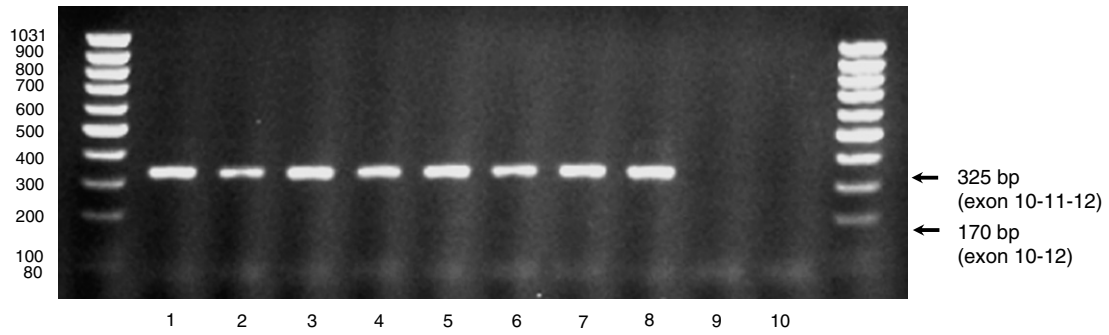

Fig. 2 – RT-PCR analysis specific for *FLJ10737*, exon 11 in neuroblastoma cell lines. Using primers flanking exon 11, PCR was performed on cDNA of the cell lines Kelly (1), IMR-32 (2), CHP-134 (3), SH-EP (4), NMB (5), GI-ME-N (6), SK-N-SH (7), Vi-856 (8), genomic DNA (9) and H_2O (10). The expected size for a PCR product from the transcript including exon 11 is 325 bp. A 170 bp product would result from the transcript lacking exon 11. All amplification products were consistent with the size of the product including exon 11.

Collectively, in *FLJ10737* we found two variants causing amino acid substitutions and one intronic deletion close to a splice acceptor site. In *CAMTA1*, we found three variants causing amino acid substitution of which one was not observed in an unaffected control group (4007T), and a second was found as the only allele in six neuroblastomas but exclusively heterozygous in unaffected controls (3531G). Allele frequencies of all described amino acid variants were statistically not significantly different comparing neuroblastomas and unaffected controls (Table 3). No somatic mutations were detected.

4. Discussion

Deletion of a distal portion of 1p is one of the most frequent chromosomal alterations in human neuroblastomas. The genomic localisation of *FLJ10737* and *CAMTA1* to a recently defined 261 kb 1p36.3 smallest region of consistent deletion[15–20] has led us to examine the coding regions of these genes for sequence alterations in neuroblastoma tumours.

Combined SSCP and sequencing analysis revealed two variants dictating amino acid change in *FLJ10737* and three variants dictating amino acid change in *CAMTA1*. Since all of them were also observed in the matching blood DNA of the patients, we conclude that somatic mutations of these genes are unlikely to be involved at significant incidence in neuroblastoma development. This is in line with a recent study that failed to detect somatic mutations of *CAMTA1* in a set of gliomas, a tumour entity in which *CAMTA1* maps to a 1p36 minimal deleted region of only 150 kb.[27] However, there might be additional sequence alterations that remained undiscovered in our study since CE-SSCP has previously failed to detect about 4% of genetic variants.[28] In addition, alterations may reside in introns or promotor regions that were not addressed in this study. While no somatic mutations were detected in *FLJ10737* and *CAMTA1*, sequence variants exist that might entail different protein activities.

Variant 799A in *FLJ10737* was found in neuroblastomas and unaffected individuals. The ATG codon at position 799 resides in the Kozak context. This codon might be selected as an alternative start site by mechanisms like leaky ribosomal scanning,[34] internal ribosomal entry[35] or ribosomal shunting.[36] Whether a corresponding FLJ10737 isoform exists needs to be clarified in further analyses.

We also found an intronic 1-bp deletion within a polythymidine tract T_{18} affecting a splice acceptor site (*FLJ10737*, IVS10-15delT) in neuroblastoma cell lines and tumours. It has been shown that alterations in polythymidine tracts preceding splice acceptor sites can decrease the recognition of the adjacent exon and finally result in exon skipping.[32] However, RT-PCR on cDNAs from cell lines with IVS10-15delT did not produce evidence for a similar mechanism acting for *FLJ10737*. Independent from the presence of the T_{17} or T_{18} variant, all transcript amplicons were of a size corresponding to exon 11 retention. It is possible that the detected splice acceptor variant might cause retention of introns or usage of cryptic splice acceptors, but, as exon skipping, these splicing aberrations most likely would have resulted in altered length of the RT-PCR product.

In *CAMTA1*, we observed one rare amino acid substituting sequence variant that was not found in unaffected controls (4007C > T, T1336I). Another *CAMTA1* sequence variant predicted to lead to amino acid substitution was found in an ankyrin domain. It was proposed that CAMTAs could interact with other proteins or form heteromeric/homomeric complexes by means of their ankyrin domain.[24] The observed amino acid substitution might modify such protein–protein interactions like it has been shown for cancer-related amino acid substitutions in the ankyrin domains of the cell cycle inhibitor p16.[37] We found the CAMTA1 ankyrin domain variant (3531C > G, N1177K) hemizygous in six neuroblastomas but exclusively in the heterozygous form in unaffected individuals. Thus, solely in neuroblastomas and one matching blood sample the 3531G variant was found as the only allele. Five of the six blood DNAs corresponding to the 3531G hemizygous tumours were heterozygous for this variant, indicating that in these cases LOH resulted in retention of the less common allele.

LOH can result in unmasking of particular polymorphic sequences that are associated with cancer risk.[38,39] Addressing the question, whether there is selection for the 3531G allele, we found a higher 3531G frequency in 1p36 LOH patients compared to controls and deletion of the 3531C allele with a slightly higher rate (5/8 = 62.5% *versus* 3/8 = 37.5% 3531G-dele-

tion). However, the difference between the observed number of 3531G/– hemizygous tumours and the genotype distribution expected assuming absence of selection did not reach statistical significance in the cohort examined here.

Polymorphic sequences in cancer susceptibility genes can contribute to cancer risk[40,41] and it has been discussed that weak susceptibility genes may influence the patterns of LOH involving preferential deletion of allelic variants that confer lower risk.[42] The main limitation of studies associating common variants with cancer risk is their lack of power without large sample sizes. This is especially true for alleles of weak effect. Further analyses in extended cohorts should investigate whether influences on disease through the identified gene variants exist.

Despite the absence of mutations in our study cohort, the high prevalence of *CAMTA1* deletion in both neuroblastoma and glioma tumours[16,18,27] and the independent predictive power of low *CAMTA1* expression for poor neuroblastoma outcome[15] support a role of this gene in tumourigenesis. The expression signature found in neuroblastomas is consistent with the idea that low *CAMTA1* levels mediate a selective advantage for developing neuroblastoma cells. Significantly lower *CAMTA1* expression in 1p deleted tumours[15] is in line with a haploinsufficiency model where a single copy of *CAMTA1* would result in a lower transcript level than the normal two copies.

Conflict of interest statement

None declared.

Acknowledgements

This work was supported by the Deutsche Krebshilfe and the Bundesministerium für Bildung und Forschung (Nationales Genomforschungsnetz-2).

REFERENCES

1. Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. *Science* 1984;**224**(4653):1121–4.
2. Schwab M, Alitalo K, Klempnauer KH, et al. Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature* 1983;**305**(5931):245–8.
3. Maris JM, Weiss MJ, Guo C, et al. Loss of heterozygosity at 1p36 independently predicts for disease progression but not decreased overall survival probability in neuroblastoma patients: a Children's Cancer Group study. *J Clin Oncol* 2000;**18**(9):1888–99.
4. Attiyeh EF, London WB, Mosse YP, et al. Chromosome 1p and 11q deletions and outcome in neuroblastoma. *New Engl J Med* 2005;**353**(21):2243–53.
5. Bader SA, Fasching C, Brodeur GM, Stanbridge EJ. Dissociation of suppression of tumorigenicity and differentiation in vitro effected by transfer of single human chromosomes into human neuroblastoma cells. *Cell Growth Differ* 1991;**2**(5):245–55.
6. Fong CT, Dracopoli NC, White PS, et al. Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification. *Proc Natl Acad Sci USA* 1989;**86**(10):3753–7.
7. Weith A, Martinsson T, Cziepluch C, et al. Neuroblastoma consensus deletion maps to 1p36.1-2. *Genes Chromosomes Cancer* 1989;**1**(2):159–66.
8. Schleiermacher G, Peter M, Michon J, et al. Two distinct deleted regions on the short arm of chromosome 1 in neuroblastoma. *Genes Chromosomes Cancer* 1994;**10**(4):275–81.
9. Takeda O, Homma C, Maseki N, et al. There may be two tumor suppressor genes on chromosome arm 1p closely associated with biologically distinct subtypes of neuroblastoma. *Genes Chromosomes Cancer* 1994;**10**(1):30–9.
10. Martinsson T, Sjoberg RM, Hedborg F, Kogner P. Deletion of chromosome 1p loci and microsatellite instability in neuroblastomas analyzed with short-tandem repeat polymorphisms. *Cancer Res* 1995;**55**(23):5681–6.
11. White PS, Maris JM, Beltinger C, et al. A region of consistent deletion in neuroblastoma maps within human chromosome 1p36.2-36.3. *Proc Natl Acad Sci USA* 1995;**92**(12):5520–4.
12. Schwab M, Praml C, Amler LC. Genomic instability in 1p and human malignancies. *Genes Chromosomes Cancer* 1996;**16**(4):211–29.
13. Caron H, Spieker N, Godfried M, et al. Chromosome bands 1p35-36 contain two distinct neuroblastoma tumor suppressor loci, one of which is imprinted. *Genes Chromosomes Cancer* 2001;**30**(2):168–74.
14. Ohira M, Kageyama H, Mihara M, et al. Identification and characterization of a 500-kb homozygously deleted region at 1p36.2-p36.3 in a neuroblastoma cell line. *Oncogene* 2000;**19**(37):4302–7.
15. Henrich KO, Fischer M, Mertens D, et al. Reduced expression of CAMTA1 correlates with adverse outcome in neuroblastoma patients. *Clin Cancer Res* 2006;**12**(1):131–8.
16. Bauer A, Savelyeva L, Claas A, Praml C, Berthold F, Schwab M. Smallest region of overlapping deletion in 1p36 in human neuroblastoma: a 1 Mbp cosmid and PAC contig. *Genes Chromosomes Cancer* 2001;**31**(3):228–39.
17. Hogarty MD, Liu X, Guo C, et al. Identification of a 1-megabase consensus region of deletion at 1p36.3 in primary neuroblastomas. *Med Pediatr Oncol* 2000;**35**(6):512–5.
18. White PS, Thompson PM, Gotoh T, et al. Definition and characterization of a region of 1p36.3 consistently deleted in neuroblastoma. *Oncogene* 2005;**24**(16):2684–94.
19. White PS, Thompson PM, Seifried BA, et al. Detailed molecular analysis of 1p36 in neuroblastoma. *Med Pediatr Oncol* 2001;**36**(1):37–41.
20. Katoh M, Katoh M. Identification and characterization of FLJ10737 and CAMTA1 genes on the commonly deleted region of neuroblastoma at human chromosome 1p36.31-p36.23. *Int J Oncol* 2003;**23**(4):1219–24.
21. Kelley WL. The J-domain family and the recruitment of chaperone power. *Trends Biochem Sci* 1998;**23**(6):222–7.
22. Kurzik-Dumke U, Gundacker D, Renthrop M, Gateff E. Tumor suppression in Drosophila is causally related to the function of the lethal(2) tumorous imaginal discs gene, a dnaJ homolog. *Dev Genet* 1995;**16**(1):64–76.
23. Syken J, De-Medina T, Munger K. TID1, a human homolog of the Drosophila tumor suppressor l(2)tid, encodes two mitochondrial modulators of apoptosis with opposing functions. *Proc Natl Acad Sci USA* 1999;**96**(15):8499–504.
24. Bouché N, Scharlat A, Snedden W, Bouchez D, Fromm H. A novel family of calmodulin-binding transcription activators in multicellular organisms. *J Biol Chem* 2002;**277**(24):21851–61.

25. Nakatani K, Nishioka J, Itakura T, et al. Cell cycle-dependent transcriptional regulation of calmodulin-binding transcription activator 1 in neuroblastoma cells. *Int J Oncol* 2004;**24**(6):1407–12.
26. Nagase T, Ishikawa K, Suyama M, et al. Prediction of the coding sequences of unidentified human genes. XII. The complete sequences of 100 new cDNA clones from brain which code for large proteins in vitro. *DNA Res* 1998;**5**(6):355–64.
27. Barbashina V, Salazar P, Holland EC, Rosenblum MK, Ladanyi M. Allelic losses at 1p36 and 19q13 in gliomas: correlation with histologic classification, definition of a 150-kb minimal deleted region on 1p36, and valuation of CAMTA1 as a candidate tumor suppressor gene. *Clin Cancer Res* 2005;**11**(3):1119–28.
28. Walz T, Geisel J, Bodis M, Knapp JP, Herrmann W. Fluorescence-based single-strand conformation polymorphism analysis of mutations by capillary electrophoresis. *Electrophoresis* 2000;**21**(2):375–9.
29. Antonarakis SE. Recommendations for a nomenclature system for human gene mutations. Nomenclature Working Group. *Hum Mutat* 1998;**11**(1):1–3.
30. Emigh T. Comparison of tests for Hardy–Weinberg Equilibrium. *Biometrics* 1980;**36**:627–42.
31. R Development Core Team. R: A language and environment for statistical computing. In: R Foundation for Statistical Computing. Vienna; 2003.
32. Chu CS, Trapnell BC, Curristin S, Cutting GR, Crystal RG. Genetic basis of variable exon 9 skipping in cystic fibrosis transmembrane conductance regulator mRNA. *Nat Genet* 1993;**3**(2):151–6.
33. Sedgwick SG, Smerdon SJ. The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem Sci* 1999;**24**(8):311–6.
34. Kozak M. Emerging links between initiation of translation and human diseases. *Mamm Genome* 2002;**13**(8):401–10.
35. Hellen CU, Sarnow P. Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes Dev* 2001;**15**(13):1593–612.
36. Yueh A, Schneider RJ. Translation by ribosome shunting on adenovirus and hsp70 mRNAs facilitated by complementarity to 18S rRNA. *Genes Dev* 2000;**14**(4):414–21.
37. Mosavi LK, Cammett TJ, Desrosiers DC, Peng ZY. The ankyrin repeat as molecular architecture for protein recognition. *Protein Sci* 2004;**13**(6):1435–48.
38. Mollenhauer J, Herbertz S, Holmskov U, et al. DMBT1 encodes a protein involved in the immune defense and in epithelial differentiation and is highly unstable in cancer. *Cancer Res* 2000;**60**(6):1704–10.
39. Hu YJ, Diamond AM. Role of glutathione peroxidase 1 in breast cancer: loss of heterozygosity and allelic differences in the response to selenium. *Cancer Res* 2003;**63**(12):3347–51.
40. Tavtigian SV, Simard J, Teng DH, et al. A candidate prostate cancer susceptibility gene at chromosome 17p. *Nat Genet* 2001;**27**(2):172–80.
41. Ewart-Toland A, Briassouli P, de Koning JP, et al. Identification of Stk6/STK15 as a candidate low-penetrance tumor-susceptibility gene in mouse and human. *Nat Genet* 2003;**34**(4):403–12.
42. Balmain A, Gray J, Ponder B. The genetics and genomics of cancer. *Nat Genet* 2003;**33**(Suppl.):238–44.