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The potential tumour suppressor role for caspase-9 (*CASP9*) in the childhood malignancy, neuroblastoma

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Abstract

The clinical aggressiveness of neuroblastoma, a childhood embryonal tumour of neuroectodermal cells derived from the neural crest, is considered to be dictated by the competitive interactions between cell proliferation, differentiation and apoptosis. Caspase-9 is a central effector enzyme in the apoptotic mechanism. Recent studies with caspase-9 (*CASP9*) knockout mice indicate a primary defect in the brain caused by decreased apoptosis during the early stages of nervous system development. It is our hypothesis that silencing of *CASP9* through genetic mutations may promote neuroblastoma tumorigenesis. Here, we report the outcome of screening neuroblastoma tumours for silencing mutations in *CASP9*. cDNA prepared from RNA isolated from 22 neuroblastoma tumours representing the full range of neuroblastoma clinicopathological disease stages was sequenced. Single nucleotide changes were detected in all neuroblastoma tumours, but were found not to represent silencing mutations, but rather sequence polymorphisms. These polymorphisms did not associate with the clinicopathological stages of disease or the predicted clinical outcomes of the patients. Silencing mutations of *CASP9* are therefore unlikely to be causal to neuroblastoma tumorigenesis.

Keywords: Neuroblastoma; Caspase-9 (*CASP9*); Apoptosis; Mutation; Polymorphism; Tumour suppressor gene; Tumorigenesis

1. Introduction

Neuroblastoma, an embryonal tumour of neuroectodermal cells derived from the neural crest, manifests in the sympathetic nervous tissue and is one of the most common solid tumours of infancy. Clinically, neuroblastoma is characterised by a staging system involving five distinct clinicopathological stages. Four of these stages (stages 1–4) identify distinct groups which are defined by increasing levels of tumour dissemination and the fifth (stage 4S) as having a localised primary tumour as well as remote disease which is confined to the liver, skin or bone marrow [1]. Such clinicopathological staging is one of the more important prognostic factors for neuroblastoma, with patients presenting with the localised (stage 1, 2) and 'special' (stage 4S) tumours surviving longer than those with bilateral and metastatic (stage 3, 4) tumours. At the molecular level, the amplification of the *N-MYC* oncogene has also proven to be a powerful indicator of outcome in neuroblastoma patients [2].

Neuroblastoma is characterised by a high rate of spontaneous regression, which has been proposed to be due to delayed apoptosis [3]. Both primary and metastatic neuroblastomas have been reported to spontaneously regress, a scenario that usually takes place over 6–12 months, leaving most children clinically free of the disease. Spontaneous regression is especially prevalent in patients presenting with stage 4S tumours [3,4]. It is now widely accepted that the competitive outcome of interactions between cellular proliferation, differentiation and apoptosis dictate the clinical aggressiveness of neuroblastoma.

Essential mediators of apoptosis include members of the caspase family of cysteine proteases. Caspases cleave target proteins at specific aspartate residues [5]. A key initiator of the apoptotic caspase activity is the formation

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of a ternary complex between the apoptotic protease-activating factor-1 (Apaf-1) and caspase-9 with cytochrome *c* following its release from mitochondria [6]. Activated caspase-9 in turn cleaves and activates other caspases resulting in a cascade of caspase activity and cell destruction [5,6]. Gene deletion studies have shown that caspase-9 and Apaf-1 play a central role during the development of the mouse brain, and is essential for apoptosis of neural progenitor cells [7]. Specifically, deletion of *CASP9* caused perinatal lethality, increased brain size due to neuronal hyperplasia, grossly abnormal brain development with protrusions of the cranial tissues and defective mitochondrial-mediated apoptosis of the proliferative neuroepithelium [7]. Mice homozygous for a null allele of *APAF1* demonstrated similar marked enlargement of the brain due to reduced apoptotic response in the periventricular proliferative zone [8]. Furthermore, Soengas and colleagues [9] demonstrated that inactivation of either Apaf-1 or caspase-9 mimicked the loss of p53 and promoted oncogenic transformation of *Myc*-expressing mouse embryo fibroblasts, leading to the prevention of apoptosis, enhancement of cell survival and promotion of tumorigenic potential. Neuroblastoma is one of the few tumours where *TP53* mutations do not appear to influence tumorigenesis [10]. However, amplification of *N-MYC* is common in this malignancy and related to patient outcome [2]. In humans, the gene for *CASP9* is located at chromosome 1p34-1p36.1 [12], a region subject to loss of heterozygosity (LOH). A range of studies, summarised by Mora and colleagues [11], estimate LOH of this region is found in at least 40% of neuroblastoma tumours. On this basis, we formed the hypothesis that inactivation of caspase-9 may be causally linked to neuroblastoma tumorigenesis. As such, the *CASP9* gene could act as a tumour suppressor gene in neuroblastoma. To investigate this hypothesis, we determined whether mutations of the *CASP9* gene existed in neuroblastoma tumours.

2. Patients and methods

2.1. Tumour samples

The neuroblastoma tumour samples were generously supplied by Professor Michelle Haber and Associate Professors Murray Norris and Glenn Marshall of the Children's Cancer Institute Australia. The RNA from 22 neuroblastomas, with at least two tumour samples representing each clinicopathological stage of the disease (stage 1, 2, 3, 4 and 4S), were included in this study. For all samples, the number of copies of the *N-MYC* oncogene per haploid genome had previously been independently determined. Tumour RNA was extracted and cDNA prepared as previously described in Ref. [13].

2.2. Caspase 9 sequencing

The entire *CASP9* coding region was amplified in three overlapping fragments using the following primer pairs: F1 5′ GCC TGG AGT CTT AGT TGG CTA CTC 3′ and R1 5′ CAG ACC CAA TGT CCA CTG GTC TGG 3′, F2 5′ CCA GAG GTT CTC AGA CCG GAA ACA 3′ and R2 5′ CAT GGT CTT TCT GCT CCC CAC CAC 3′, F3 5′ CCT GGG AGG AAA GCC CAA GCT CTT 3′ and R3 5′ TTG GGG TGC AAG ATA AGG CAG GGT 3′. Polymerase chain reaction (PCR) reaction mixtures (25 μl) containing 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.8), 50 mM KCl, 0.01% gelatin, 200 μM deoxynucleotide triphosphate (dNTP), 5 μg/ml each primers and 1 unit *Taq* polymerase (Perkin-Elmer) were amplified with a 5-min initial incubation at 94 °C followed by 30 cycles of 45 s at 94 °C, 45 s at 55 °C and 90 s at 72 °C. A final elongation step of 10 min at 72 °C was included. PCR products were purified by precipitation with polyethylene glycol (PEG) solution (26.7% polyethylene glycol 8000, 0.6 M NaOAc pH 5.2, 6.5 mM $MgCl_2$) prior to sequencing. The precipitate was washed in 95% (u/v) cold ethanol and dried by heating to 42 °C on a heating block and subsequently resuspended in 10 μl sterile water. A 2 μl aliquot of each sample was verified for quantity and specificity by electrophoresis through a 1% 40 mM tris acetate 2mM EATA (TAE) agarose gel. The three fragments underwent PCR sequencing according to the ABI PRISM protocol using the respective amplifying primers so as to cover each fragment and, consequently, the entire coding region, in both the forward and reverse directions. The sequence data was obtained and analysed using the ABI software.

3. Results

Following PCR amplification of each sample in our cohort, we observed that a single product of the expected size was amplified for each fragment, indicating that no major deletions or insertions were present in *CASP9*. Sequence analysis indicated seven codons which contained single nucleotide differences when compared with the previously published *CASP9* sequence Mch6 (Genbank Acc No. U60521). Three of the nucleotide changes corresponding to codons 32 (A→C, S32R), 197 (C→T, P197L) and 277 (A→G, G277G) were found in all 22 neuroblastoma samples (see Fig. 1 at ◆, for example). However, these nucleotide changes were also identified in another previously published sequence for *CASP9*, ICE-LAP6 (Genbank Acc No. 56390) and as such, represent differences from the published Mch6 sequence.

The remaining four single nucleotide variations occurred within codon 28 (C→T, A28V), 31 (C→T, S31S), 136 (T→C, F136F) and 221 (A→G, Q221R).

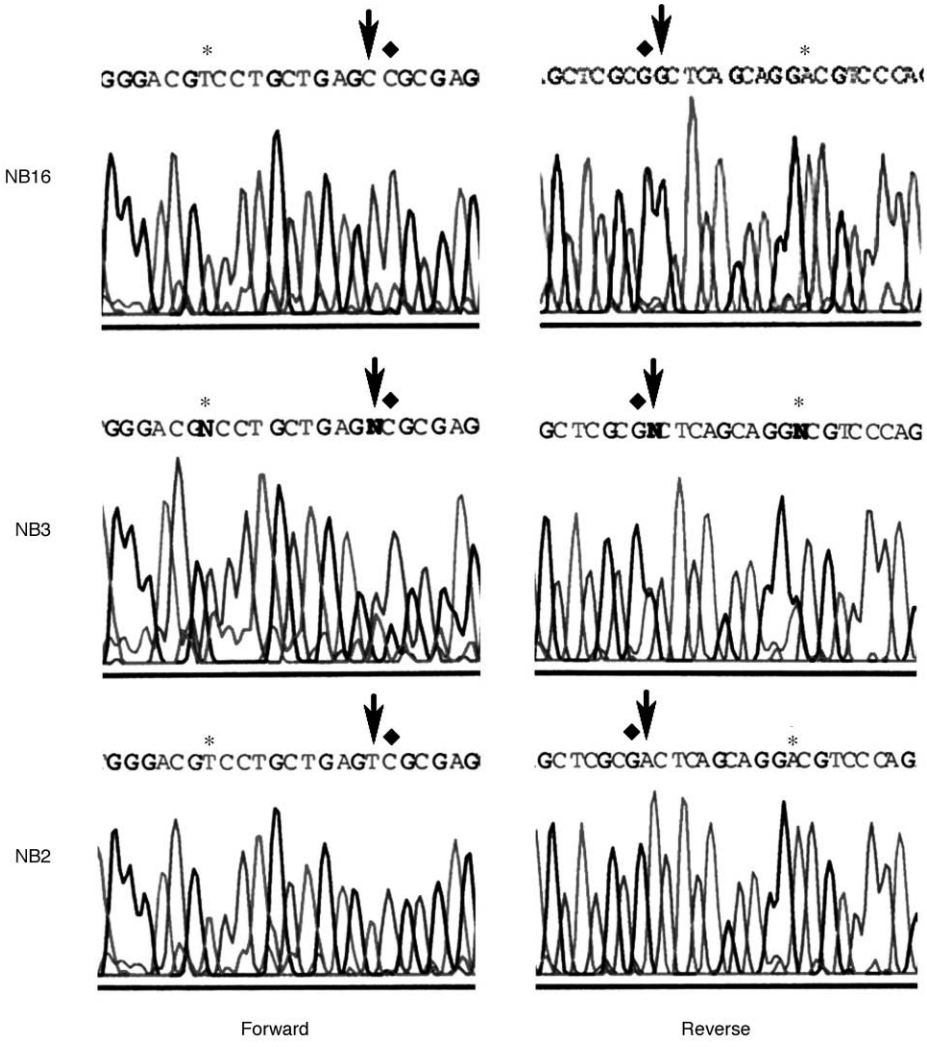

Fig. 1. Sequence profiles of the newly identified C→T polymorphism in caspase-9 at codon 31 (↓). Sequence from both the forward and reverse directions are shown. Three neuroblastoma samples are displayed. NB16 and NB2 represent neuroblastomas that are homozygous for the C or the T polymorphic site, respectively. NB3 represents a heterozygous example of this polymorphism. The asterisk (∗) highlights the position of the polymorphism at codon position 28 (cf. Table 1) identified through comparison with the previously published ICE-LAP6 and Mch6 sequences with both homozygous (NB2, NB16) and heterozygous (NB3) examples depicted. The nucleotide indicated with the diamond (◆) represents the sequence change at codon position 32. As indicated in these and all other samples, only the cytosine (C) is present, corresponding to the ICE-LAP6 sequence.

Screening of the neuroblastoma tumour samples indicate that each nucleotide change was represented throughout our cohort (Table 1). Comparison with the Mch6 and ICE-LAP6, however, indicated the presence of each nucleotide possibility for codon 28, 136 and 221 in either of the two published sequences. 12 of the 22 patients were found to have single nucleotide changes in at least one of these three codons compared with ICE-LAP6, with seven samples having changes in all three codons (Table 1). By contrast, the single nucleotide change detected within codon 31 was not found in either the Mch6 or ICE LAP6 published sequences (Fig. 1). These *CASP9* polymorphisms did not appear on the NCBI Single Nucleotide Polymorphism database [14].

4. Discussion

It is only just emerging how aberrant caspase activity, which results from altered gene expression or genetic mutations, may be associated with the aetiology of human diseases, in particular cancer. Frameshift mutations in the mononucleotide repeat within the coding region of *CASP5* have been described in tumours associated with the hereditary nonpolyposis colorectal cancer syndrome [15], while deletions and inactivating point mutations in the *CASP8* gene have been reported in human head and neck carcinoma [16]. Patients with the inherited Autoimmune Lymphoproliferative Syndrome have been identified as carrying point mutations which affect the large subunit of caspase-10 subsequently

Table 1
Summary of single nucleotide polymorphisms in the *CASP9* coding region detected in 22 staged neuroblastoma tumours[a]

Tumour sample	Stage	N-*myc*	Polymorphism			
			Codon 28 GCC→GTC A→V	Codon 31 AGC→AGT S→S	Codon 136 TTT→TTC F→F	Codon 221 CAG→CGG Q→R
	Good prognosis					
NB1	1	–	T	T	C	A/G
NB2	1	–	T	T	C	G
NB3	1	–	C/T	T/C	T	A/G
NB4	1	–	T	T	C	G
NB5	2	–	C	C	C	A/G
NB6	2	–	T	C	C	G
NB20	4S	–	C	C	T/C	A
NB21	4S	–	C	C	T/C	A
	Poor prognosis					
NB7	3	S	T	T/C	C	G
NB8	3	S	T	T	C	G
NB9	3	S	T	C	–	G
NB10	3	S	T	C	T	A/G
NB22	3	S	C/T	C	T	A
NB11	3	A	C	C	T	A
NB12	3	A	C	C	T/C	A
NB13	3	A	T	C	C	G
NB14	4	S	C/T	C	–	G
NB15	4	S	T	C	C	G
NB16	4	A	T	C	C	G
NB17	4	A	T	C	C	A/G
NB18	4	A	T	T	C	G
NB19	4	A	C	C	T	A
	Genbank Accession Number					
Mch6	U60521		C	C	T	A
ICE-LAP6	U56390		T	C	C	G

S, single copy N-*myc*; A, amplification of N-*myc* (>10 copies).

[a] Each polymorphism is described by the codon number, the nucleotide sequence for that codon and the amino acid change predicted. Dark shaded boxes represent codon sequences identified in the previously published Mch6 sequence. The lightly shaded boxes illustrate those tumours having the newly identified polymorphism in codon 31.

leading to altered levels of apoptosis in lymphocytes [17]. In studies specifically involving neuroblastoma, *CASP1* has been found to be preferentially expressed in tumours with a known favourable outcome, but is conspicuously absent in advanced staged neuroblastoma (stage 3 and 4) with amplified *N-MYC* [18]. Similarly, an increase in expression of *CASP3* has been reported to significantly correlate with neuroblastoma tumours with a high level of TrkA expression, single copy *N-MYC*, younger age of disease onset, lower clinicopathological stages (stage 1, 2 and 4S) which are features of favourable prognosis [19]. This study further demonstrates that active caspase-3 can be located in the fragmented nuclei of apoptotic cells from stage 4S tumour which strongly suggests that activation of this caspase is involved in the regression of neuroblastoma [19].

Sequence analysis of the caspase-9 coding region within our cohort of neuroblastoma tumours revealed seven single nucleotide changes. With each single nucleotide change either a conserved or identical amino acid change would be predicted. However, as these sequence variations were found in the previously published Mch6 and ICE-LAP6 sequences, we considered them to be simple polymorphisms and not gene mutations. These polymorphisms were dispersed randomly among the samples and did not appear to be associated with tumour clinicopathological stage, *N-MYC* status or predicted patient outcome.

The single nucleotide change detected within codon 31 was, however, not found on comparison to either Mch6 or ICE LAP6 published sequences. As this single nucleotide change would not cause an amino acid

alteration we conclude it to be a new polymorphism (Fig. 1). This polymorphism did not associate with those identified at codons 28, 136 and 221 and appeared predominantly in neuroblastoma samples having a good prognosis (50% versus 21%) (Table 1). It would be necessary to screen a greater number of neuroblastoma samples to further confirm a preferential distribution of the codon 31 polymorphism in the good prognosis neuroblastomas. Apart from these single nucleotide polymorphisms, no other genetic changes were detected in the *CASP9* coding region of the neuroblastoma tumours.

As a consequence, we conclude that the *CASP9* gene is not subject to single somatic mutations and does not behave as a classical tumour suppressor gene in neuroblastoma tumours. However, other modes of caspase 9 inactivation need to be considered before its involvement in neuroblastoma tumorigenesis can be excluded. Epigenetic silencing of caspases has been proposed to regulate the tumour suppressor potential of caspases. For example, DNA methylation inactivates *CASP8* in neuroblastoma tumours with *N-MYC* overexpression [20]. However, following reverse transcriptase PCR, the detection of an abundance of each amplified gene fragment suggests that it is unlikely that *CASP9* silencing could occur through altered epigenetic regulation. Likewise, the overexpression of the truncated dominant-negative variant, caspase-9S, blocks caspase-9 activity through a competitive interaction with Apaf-1 [21]. Similarly, increased activity of the phosphoinositide 3-kinase/Akt kinase signal tranduction pathway may play a causal role in the development and progression of neuroblastoma via regulation of caspase-9 activity through serine phosphorylation [22]. Therefore, caspase-9 inactivation specifically through altered post-translational mechanisms, should also be investigated before defective caspase-9 function, and hence, a tumour suppressor role, can be excluded during the aetiology of neuroblastoma.

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