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Lack of evidence for mutations or deletions in the CDKN2A/*p16* and CDKN2B/*p15* genes of Brazilian neuroblastoma patients

C.L. Bassi[1], L. Martelli[1], R. Cipolotti[2], C.A. Scrideli[2], R. Defávery[2] and L.G. Tone[2]

Departamentos de [1]Genética, and [2]Pediatria e Puericultura, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brasil

Correspondence
L.G. Tone
Departamento de Pediatria e Puericultura
HC, FMRP, USP
Av. Bandeirantes, 3900, 7º andar
14048-900 Ribeirão Preto, SP
Brasil
E-mail: lgtone@fmrp.usp.br

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Abstract

Neuroblastoma, the most common extracranial tumor in childhood, has a wide spectrum of clinical and biological features. The loss of heterozygosity within the 9p21 region has been reported as a prognostic factor. Two tumor suppressor genes located in this region, the CDKN2B/*p15* and CDKN2A/*p16* (cyclin-dependent kinase inhibitors 2B and 2A, respectively) genes, play a critical role in cell cycle progression and are considered to be targets for tumor inactivation. We analyzed CDKN2B/*p15* and CDKN2A/*p16* gene alterations in 11 patients, who ranged in age from 4 months to 13 years (male/female ratio was 1.2:1). The most frequent stage of the tumor was stage IV (50%), followed by stages II and III (20%) and stage I (10%). The samples were submitted to the multiplex PCR technique for homozygous deletion analysis and to single-strand conformation polymorphism and nucleotide sequencing for mutation analysis. All exons of both genes were analyzed, but no deletion was detected. One sample exhibited shift mobility specific for exon 2 in the CDKN2B/*p15* gene, not confirmed by DNA sequencing. Homozygous deletions and mutations are not involved in the inactivation mechanism of the CDKN2B/*p15* and CDKN2A/*p16* genes in neuroblastoma; however, these two abnormalities do not exclude other inactivation pathways. Recent evidence has shown that the expression of these genes is altered in this disease. Therefore, other mechanisms of inactivation, such as methylation of promoter region and unproperly function of proteins, may be considered in order to estimate the real contribution of these genes to neuroblastoma genesis or disease progression.

Key words
• *p15* gene
• *p16* gene
• Deletion
• Mutation
• Neuroblastoma

Neuroblastoma originates in pluripotential cells derived from the neural crest and is the most common childhood solid extracranial neoplasm with a world incidence of approximately 8.0/million per year in children under 15 years of age and of 7.3/million in Brazil (1,2).

MYCN/N-myc amplification is one of the most important prognostic factors among the genetic events which occur in neuroblas-

toma. However, some cases of advanced disease were described without MYCN/N-myc amplification, indicating that other genetic events are also responsible for neuroblastoma evolution. Takita et al. (3) reported that the loss of heterozygosity of the short arm of chromosome 9 (9p) is associated with the advanced stage and a poor prognosis, regardless of MYCN/N-myc amplification. Two tumor suppressor genes, CDKN2B/*p15* and CDKN2A/*p16* (cyclin-dependent kinase inhibitors 2B and 2A, respectively), are located in the 9p21 region and can be targets of inactivation in cases with heterozygosity loss in this region (4,5). Both are cyclin-dependent kinase inhibitors and, once these genes are inactivated, the cells become free of the restrictions imposed by them and uncontrolled cell reproduction can occur.

In the present investigation we have searched for deletion and/or mutation of the CDKN2B/*p15* and CDKN2A/*p16* genes in neuroblastomas in an attempt to correlate gene alterations with the origin and progression of the tumor.

We analyzed tumor samples from 11 patients attended at the Pediatric Oncology Unit of the University Hospital, School of Medicine of Ribeirão Preto, University of São Paulo, from 1988 to 1997. Neuroblastoma diagnosis was confirmed by histopathology. The study was approved by the Ethics in Research Committee, process HCRP No. 2865/99, and the persons responsible for the patients gave written informed consent to participate. Clinical and pathological data and patient outcome are listed in Table 1. The patients ranged in age from 4 months to 13 years (median = 5 years) and the male/female ratio was 1.2:1. Ten patients were classified according to the International Neuroblastoma Staging System (INSS; Ref. 6): the tumor was defined as stage I in 1 patient (10%), as stage II in 2 patients (20%), as stage III in 2 patients (20%), and as stage IV in 5 patients (50%). Four patients are still alive, 5 patients died because of failure to achieve complete remission or relapse and the outcome is unknown for 2 patients who were lost to follow-up.

Table 1. Clinical pathological data and outcome of patients with neuroblastomas.

Patient	Sex	Age	Diagnosis	Primary site of tumor	Stage	Outcome
1	M	2 years	NB	Retroperitoneal	III	Alive
2	M	7 years	NB	Retroperitoneal	IV	Dead
3	F	4 months	NB	Paravertebral	I	Alive
4	M	1.5 years	NB	Abdominal	IV	Dead
5	M	2.5 years	NB	Suprarenal	III	Dead
6	F	11 years	NB	Extramedullary	II	Dead
7	M	13 years	GNB	Abdominal	II	Dead
8	F	2.5 years	NB	Retroperitoneal	IV	WF
9	M	1 year	NB	Posterior mediastinum	ND	WF
10	F	6 years	NB	Adrenal	IV	Alive
11	F	8 months	NB	ND	IV	Alive

The tumors were staged according to the International Neuroblastoma Staging System (INSS; Ref. 6). M = male; F = female; NB = neuroblastoma; GNB = ganglioneuroblastoma; WF = without follow-up; ND = not determined.

The multiplex PCR technique was used to detect deletions in the exons of the genes (CDKN2B/*p15* and CDKN2A/*p16* consist of 2 and 3 exons, respectively, all of which were studied in the present investigation). We used specific primers for each exon, which were simultaneously amplified with the ß-globin gene as an internal control. The PCR product was then applied to 2% agarose gel and the electrophoretic run was performed with Tris/borate EDTA (TBE, 0.089 M Tris, 0.089 M borate, 2 mM EDTA) buffer 1X at 80 V, for approximately 30 min. The samples were prepared using the PCR product, 0.001% SDS/EDTA and staining solution. The samples were then denatured at 94ºC for 10 min, placed on ice and applied to 6% acrylamide gel. Electrophoresis was performed with TBE buffer 1X at 200 V, 40 mA and 8 W for 4.5 to 6.0 h. The gel was stained with silver, developed with a sodium carbonate solution, fixed in 10% acetic acid and dried on transparent paper. The PCR products exhibiting abnormally migrating bands were submitted to manual sequencing reactions with the T7 sequencing kit™ (Amersham Pharmacia Biotech, Uppsala, Sweden)

according to manufacturer recommendations and labeling was performed with the [α^{35}S]-dATP radioisotope. The samples were submitted to electrophoresis on 6% polyacrylamide denaturing gel, transferred to Whatman filter paper, vacuum dried, and exposed in a cassette to X-ray film for 5 to 7 days.

The frequency of genetic alterations of the CDKN2B/*p15* and CDKN2A/*p16* genes is extremely variable in acute lymphoblastic leukemia, the most common cancer in children, according to the origin of the patients studied (7-9). Few studies have investigated the mutation involving deletion in these genes in neuroblastomas (3,10-13).

In the present study, no deletion was detected in any exon of the CDKN2B/*p15* and CDKN2A/*p16* genes. Single-strand conformation polymorphism analysis allowed the screening of the samples to be sequenced. Only one showed a different migration for exon 2 of gene CDKN2B/*p15*, but no alteration was detected in the base sequence of the exon by nucleotide sequencing. Thus, no mutation or deletion was detected in any of the analyzed exons.

According to studies carried out on neoplasms and cell lines, deletion of the CDKN2A/*p16* gene is a rarely detected event in neuroblastomas, regardless of the technique used (10-13). There is only one report of deletion in a cell line described by Easton et al. (14). Our results suggest that deletion is not an important mechanism of CDKN2A/*p16* gene inactivation in neuroblastomas. Few mutations in the CDKN2A/*p16* gene have been described in neuroblastomas, either in neoplasms or in cell lines, and most represent polymorphisms or substitutions that do not affect the protein structure (10-12,15). The only significant alteration was a nonsense mutation in codon 52 described by Takita et al. (3).

The CDKN2B/*p15* gene has not been extensively investigated up to now for deletion and mutation in neuroblastomas, and no alteration was detected in the available studies (12). Our results corroborate the findings of Iolascon et al. (12). It has been suggested that deletion and mutation are not the mechanisms of CDKN2B/*p15* inactivation in neuroblastomas. However, the number of studies carried out does not permit any conclusions.

Although no changes were detected in these genes, it should be kept in mind that deletion and mutation are not the only gene inactivation mechanisms. Transcription inactivation of the CDKN2B/*p15* and CDKN2A/*p16* genes associated with hypermethylation of the CpG islands (areas of increased density of the dinucleotide sequence cytosine-phosphate diester-guanine) have been described in several neoplasms (16,17). Inactivation of the CDKN2A/*p16* gene has even been found in cancers in which homozygotic deletion is rare (18) indicating that this gene may be inactivated much more frequently in human neoplasms than detected in deletion studies. Methylation of the promoter region of the CDKN2A/*p16* gene has been detected in neuroblastoma cell lines and tumors, associated or not with the absence of gene expression (3,12,17). The possible role of methylation in the inactivation of the CDKN2B/*p15* gene in neuroblastomas has not yet been investigated.

Another inactivation mechanism which should be considered is the interaction of proteins coded by these genes with a regulator protein, which could block the action of cyclin-dependent kinase inhibitors. Some studies have reported progression of the cell cycle in neuroblastomas even in the presence of high Cdkn2b/p15 and Cdkn2a/p16 expression levels, suggesting a mechanism that does not interfere with gene expression, but interferes with protein activity (12,14,16). Easton et al. (14) suggested that a complex of the Cdkn2a/p16 protein with another regulator protein may explain these data, since there is evidence that the Cdkn2a/p16 protein product, and perhaps also the Cdkn2b/p15 protein product, can be inactivated by interaction with the oncoprotein Tax of the

leukemia human type 1 virus. Interestingly, Omura-Minamisawa et al. (19) reported that neuroblastomas in unfavorable stages exhibit expression of Cdkn2a/p16 mRNA and protein more frequently than those in the favorable stages, and were significantly associated with a lower overall survival. Cdkn2a/p16 protein expression did not correlate with the phosphorylation status of pRb, and these investigators suggested that Cdkn2a/p16 protein may not be functioning properly to regulate the Cdk4-6/cyclin D/pRb pathway. Therefore, the absence of gene alterations in our samples may indicate that the CDKN2A/*p16* gene is really intact and expressing itself, and its action could be blocked by some pathway, leading to a poor prognosis in the great majority of patients. The CDKN2B/*p15* and CDKN2A/*p16* genes are important for cell cycle control and consequently are considered to be the preferred target for inactivation in tumors that, like neuroblastomas, commonly present loss of heterozygosity in 9p21. However, it has been demonstrated that the loss of heterozygosity can involve neighboring regions of the CDKN2B/*p15* and CDKN2A/*p16* genes, suggesting the presence of another tumor suppressor gene in this region. Giordani et al. (20) have identified two regions of frequent allelic loss in neuroblastomas, which appear to be distant from the CDKN2B/*p15* and CDKN2A/*p16* genes, suggesting that other genes may be involved in 9p deletion. It has been recently reported that, besides abnormal expression of the CDKN2A/*p16* gene, alterations in the expression of p14/ARF (but not of the CDKN2B/*p15* gene) are associated with a poor prognosis in neuroblastomas, suggesting that p14/ARF, identified in the same region of the CDKN2B/*p15* and CDKN2A/*p16* genes in 9p21, can contribute to tumorigenesis in this disease (21).

To conclude, our results is in agreement with previous reports, which demonstrated that homozygous deletion and mutation are not preferential mechanisms of inactivation of CDKN2B/*p15* and CDKN2A/*p16* genes in neuroblastoma.

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