

Neuroblastoma after Childhood: Prognostic Relevance of Segmental Chromosome Aberrations, ATRX Protein Status, and Immune Cell Infiltration¹

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Abstract

Neuroblastoma (NB) is a common malignancy in children but rarely occurs during adolescence or adulthood. This subgroup is characterized by an indolent disease course, almost uniformly fatal, yet little is known about the biologic characteristics. The aim of this study was to identify differential features regarding DNA copy number alterations, α -thalassemia/mental retardation syndrome X-linked (ATRX) protein expression, and the presence of tumor-associated inflammatory cells. Thirty-one NB patients older than 10 years who were included in the Spanish NB Registry were considered for the current study; seven young and middle-aged adult patients (range 18-60 years) formed part of the cohort. We performed single nucleotide polymorphism arrays, immunohistochemistry for immune markers (CD4, CD8, CD20, CD11b, CD11c, and CD68), and ATRX protein expression. Assorted genetic profiles were found with a predominant presence of a segmental chromosome aberration (SCA) profile. Preadolescent and adolescent NB tumors showed a higher number of SCA, including 17q gain and 11q deletion. There was also a marked infiltration of immune cells, mainly high and heterogeneous, in young and middle-aged adult tumors. ATRX negative expression was present in the tumors. The characteristics of preadolescent, adolescent, young adult, and middle-aged adult NB tumors are different, not only from childhood NB tumors but also from each other. Similar examinations of a larger number of such tumor tissues from cooperative groups should lead to a better older age-dependent tumor pattern and to innovative, individual risk-adapted therapeutic approaches for these patients.

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Introduction

Neuroblastoma (NB), a common solid tumor in childhood, is infrequent in patients over 10 years of age. Adolescent and young adult (AYA) NBs are usually included as older patients, whereas NB in middle-aged and elderly adults is often ignored in these studies [1–5]. Several studies have demonstrated that tumors of older NB patients present a worse prognosis than their childhood NB counterparts, despite the presence of very few unfavorable biologic markers [3,6–8]. Recent results from studies of older high-risk NB patients showed an age-dependent pattern in overall response, in that patients older than 18 years appeared to have a higher response rate than adolescent patients [9]. Franks et al. reported that the course of the disease was highly dependent on stage; the majority of adolescents presented with stage 4, in comparison with localized disease observed among adults [7].

Abbreviations: aSNP, single nucleotide polymorphism array; AYA, adolescent and young adults; cnLOH, copy-neutral loss of heterozygosity; FSCA, focal segmental chromosome aberration; Het, heterogeneous; Hom, homogeneous; IHC, immunohistochemistry; MLPA, multiplex ligation probe amplification; MNA, *MYCN* amplified; MNNA, *MYCN* not amplified; NB, neuroblastoma; NCA, numerical chromosome aberration; SCA, segmental chromosome aberration

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Nevertheless, resistance to cytotoxic therapy, a phenomenon that is probably multifactorial in etiology, has been shown to be a major indicator of poor survival in older NB patients [2,3,10].

In childhood NB, a genomic profile is a key requirement for the accurate identification of molecular prognostic markers, especially indicated when the *MYCN* oncogene is not amplified (MNNA) [11–13]. At present, segmental chromosome aberrations (SCAs) at 1p, 11q, and 17q are commonly seen in AYA and middle-aged adult NB tumors, while *MYCN* amplifications (MNA) are a very rare event [1,3,6]. In addition, Cheung et al. in 2012, using whole-genome sequencing, identified that mutations on the α -thalassaemia/mental retardation syndrome X-linked (*ATRX*) gene are recurrently present in older NB and less frequent in childhood NB patients [14]. *ATRX* is a tumor suppressor gene involved in chromatin remodeling, and its recurrent somatic mutations have also been associated with stage 4 NB patients [14,15]. Moreover, *ATRX* mutations are mutually exclusive of MNA tumors and have been associated with complete or mosaic loss of protein expression [14–16].

The lack of efficient therapies and the limited knowledge on genomic prognostic markers are challenging exigencies to improve the suboptimal survival of AYA and middle-aged adult NB patients. Recent studies have recognized the potential importance of the background inflammatory cells in the pathophysiology and prognosis of NB [17,18]. Depending on the type of stimuli, immune cells of the tumor microenvironment can adopt different activation states that are associated with tumor-permissive, tumor-promoting, and/or tumor-inhibitory phenotypes [19]. Immune cell infiltration at the intratumoral level could play a role in the slow tumor growth rate observed in AYA and middle-aged adult NB [1,10,14]. Moreover, multiple ongoing immunotherapeutic approaches have been successfully applied in childhood and older relapsed or refractory stage 4 NB [20–24].

Our group has previously studied clinical and histopathologic features and multiplex ligation probe amplification (MLPA) profiles of 22 NB cases [6]. In the present study, the group carried out a more comprehensive study, substantially extending the characterization of older NB by updating the clinical data, adding nine cases, incorporating single nucleotide polymorphism array (aSNP) results, *ATRX* expression data, and a description of immunomarker-based stromal cell heterogeneity. The purpose of the study was to search for hypothetical older age-dependent patterns in NB, reflected as SCA, along with a polarization of immune cell infiltration and/or *ATRX* protein aberrations as the most salient features to search for innovative therapeutic approaches.

Materials and Methods

Clinical and Histopathologic Characteristics

The database of the Spanish Society of Hematology and Pediatric Oncology includes 31 of the 750 (3.4%) NB patients ≥ 10 years of age diagnosed between January 1997 and December 2012. Updated and enlarged clinical and histopathology data of the 31 cases are provided in Table 1. Age at diagnosis ranged from 10 to 60 years (mean 14.5 years, median 12.8 years). For the purposes of the study, the patients were divided into two main groups as follows: group 1, preadolescents and adolescents (range 10–17 years, $n = 24$) and group 2, young adults and middle-aged adults (range 18–60 years, $n = 7$). Median follow-up time was 50.7 months (range 2–179). To aid in-depth analysis by age, subdivisions of groups according to accepted classifications are shown in Tables 3–6. Updating previously published cases (2010) increased

follow-up time to an average of 49.9 months for 10 cases [6]. Tumors were histopathologically classified according to the International NB Pathology Classification system [25]. Primary tumors for 30 patients and the lymphatic ganglia metastasis for one patient (patient 18) were studied. The mitosis-karyorrhexis index (MKI) was scored as low, intermediate, or high in 27 cases [25].

Genomic Profile Determination

For determination of the final genomic profiles, aSNP, MLPA, and fluorescence *in situ* hybridization (FISH) analyses were used in tumors with adequate DNA quality (17 cases). New and published data obtained by these techniques are shown in Table 1. DNA was extracted from fresh ($n = 14$) and formalin-fixed paraffin-embedded tissue ($n = 3$) in samples with at least 50% of tumor cell content, as previously reported [26,27]. The following two aSNP platforms were used: Genechip Human Mapping Nsp Array (262,256 markers) and HumanCytoSNP-12 DNA Analysis BeadChip (299,140 markers) from Affymetrix, Inc (Santa Clara, CA) and Illumina Inc (San Diego, CA), respectively. For the Affymetrix arrays, the protocol provided by the supplier was used in eight cases as previously described (<http://www.affymetrix.com>) [26]. The primary data analysis was made using the GDAS software (Affymetrix), while genomic profiles were generated using CNAG v3.0 (Copy Number Analyzer for Affymetrix GeneChip Mapping arrays) with the AsCNAR (allele-specific copy-number analysis) function [28]. DNA amplification, tagging, and hybridization to Illumina chips were performed in six cases according to the manufacturer's protocol (<http://www.illumina.com>). Data were analyzed using GenomeStudio Genotyping and KaryoStudio software (Illumina) with standard settings. For exclusion of constitutional copy number polymorphisms, the Database of Genomic Variants was used (<http://projects.tcag.ca/variation>). Genomic position annotations were based on the hg19 build of the human genome sequence (<http://genome.ucsc.edu/>). To describe the number of numerical chromosome aberrations (NCAs) and SCA per case, only aSNP results were considered. The MLPA technique was performed using the SALSA Kit P251/P252/P253 (MRC-Holland, Amsterdam, Netherlands), and only data from three cases were used for the genomic profile determination (marked in Table 1 as “c”). The technique and the interpretation guidelines are described elsewhere [29,30]. *MYCN* status was classified by FISH results in the entire cohort as MNNA, homogeneous MNA (homMNA, all tumor cells were amplified), and heterogeneous MNA (hetMNA, coexistence of amplified and non-amplified tumor cells) [29].

Immunohistochemical Analysis

Immune cell infiltration and/or *ATRX* expression were evaluated by immunohistochemical (IHC) analysis in 20 cases. In 13 cases, adequate material for both cluster of differentiation (CD) and nuclear *ATRX* protein analysis was available. Commercially available antibodies for CD4 and *ATRX* and the dilutions used are listed in Table 2. Formalin-fixed paraffin-embedded 4- μ m sections were automatically IHC stained (Autostainer Link 48; Dako, Glostrup, Denmark). For the description of immune cell location within the tumor, two regions were differentiated: the stroma-rich region, region A, and the neuroblast-rich region, region B. Positivity for the immune cell infiltrate in each region was semiquantitatively graded according to the following criteria: 1) minimal, less than 10% of positive cells; 2) moderate, 10% to 25% with positive expression; 3) high, between 25% and 50%; and (4) very high, when positive expression was

Table 1. Summary of Published^a and New Clinical and Biologic Features of the Cohort.

Patient ID	Age (years)	Sex	Location	Metastases	Stage	Event	Time to First Relapse/Progression (Months)	Outcome	Histopathology (MKI)	MLPA ^a Profile	Final Genetic Profiles ^b
1	10.1	f	AbNA	LG, O	4	R	22	DOD	pdNB (low)	–	homMNA, SCA
2	10.18	f	A	–	1	–	–	ADF	pdNB (intermediate)	–	MNNA, NCA
3	10.25	f	AbA	–	3	–	–	AWD	nGNB (low)	–	MNNA, low tumor content
4	10.88	m	AbNA + Tx	LG, O	4	–	–	ADF	uNB (low)	–	MNNA, low tumor content
5	10.93	m	A	B, BM	4	R	18	DOD	NOS (low)	SCA	MNNA, SCA
6	11.14	m	A	BM	4	R	4	DOD	pdNB (intermediate)	SCA	MNNA, SCA
7	11.15	f	A	BM, LG	4	R	13	DOD	pdNB (intermediate)	SCA	MNNA, SCA
8	11.27	f	A	–	1	ND	ND	ADF	pdNB (low)	NCA	MNNA, NCA
9	11.33	f	A	–	3	R	5	DOD	NOS (low)	–	MNNA, low tumor content
10	11.37	f	AbA	–	3	R	13	ADF	NOS (low)	–	MNNA, low tumor content
11	11.48	m	AbA	BM	4	R	15	DOD	ND	–	ND, low tumor content
12	11.53	f	AbNA	B, LG	4	–	–	AWR	pdNB (intermediate)	–	MNNA, SCA
13	11.91	m	AbA	LG	4	P	14	DOD	pdNB (low)	SCA	MNNA, SCA
14	12.73	f	AbA	ND	4	R	14	DOD	NOS	–	MNNA, low tumor content
15	12.89	m	AbA	–	3	–	–	AWD	pdNB (intermediate)	SCA	MNNA, SCA
16	13.33	f	A	B, BM	4	R	106	AWT	uNB (low)	SCA	MNNA, (SCA) ^c , no DNA available
17	13.55	m	Tx	–	1	–	–	ADF	iGNB	–	MNNA, low tumor content
18	13.73	f	AbA	B, BM,	4	R	23	DOD	NOS (low)	–	MNNA, low tumor content
19	13.86	m	AbNA	B, BM	4	R	32	DOD	pdNB (low)	–	MNNA, low tumor content
20	14.37	f	AbA	B, LG	4	R	20	DOD	dNB (low)	NCA	MNNA, SCA
21	14.65	m	P	B	4	R	88	AWT	ND (low)	–	MNNA, low tumor content
22	14.94	m	P	B, BM, O	4	R	24	DOD	ND	–	ND, low tumor content
23	16.55	m	A	B, LG, ST	4	R	38	AWT	uNB (low)	–	MNNA, low tumor content
24	16.98	m	A + coeliac	–	3	R	9	DOD	pdNB (high)	–	MNNA, low tumor content
25	18.59	m	P	–	2B	R	1	ADF	pdNB (low)	–	MNNA, (SCA) ^c , no DNA available
26	19.21	f	A	–	3	ND	ND	ADF	NOS	–	MNNA, low tumor content
27	21.71	m	P	B, BM	4	R	11	DOD	uNB (low)	–	MNNA, SCA
28	24.69	m	A	CNS	4	–	–	DOD	NOS (low)	–	MNNA, no DNA available
29	36.43	f	A	B, BM	4	–	–	AWT	GNB (low)	–	MNNA, SCA
30	39.74	f	A	–	2	–	–	ADF	pdNB (low)	–	MNNA, SCA
31	60.98	f	ND	–	2	ND	ND	DOD	pdNB (high)	–	hetMNA, SCA

^a Data published in Castel et al. [6].^b Data from aSNP, MLPA, and FISH.^c Cases included in Table 2 with only MLPA profile; f, female; m, male; AbA, abdominal adrenal; AbNA, abdominal non-adrenal; A, adrenal; B, bone; BM, bone marrow; CNS, central nervous system; LG, lymphatic ganglia; O, other; P, pelvic; ST, soft tissue; Tx, thoracic; R, relapse; P, progression; DOD, died of disease; ADF, alive disease-free; AWD, alive with disease; AWT, alive with treatment; ND, no data; pdNB, poorly differentiated NB; uNB, undifferentiated NB; GNB, ganglioneuroblastoma; iGNB, intermixed GNB.

>50%. Diverse ATRX expression patterns were described as follows: 1) negative expression or complete loss of expression, none of the neuroblast cells were positive; 2) mosaic expression, <50% of the neuroblast cells were positive; and 3) positive expression, >50% of the neuroblasts present in the sample were positive to a moderate or high intensity. For negative ATRX expression, an ATRX gene-deleted tumor from our paraffin tumor bank was used as a control.

Results

Clinical and Histopathologic Characteristics

The primary tumor location was predominantly abdominal (25 cases, 83.3%). Advanced disease stages were highly prevalent (80.6%). Bone and bone marrow were the most frequent sites of metastases; an unusual metastasis at the central nervous system was documented for only one case. The most predominant histology shown

Table 2. List of Antibodies, Source, and Dilution.

Antibodies	Source	Dilution
Anti-CD4	Dako	Not diluted
Anti-CD8	Dako	Not diluted
Anti-CD20	Dako	1/100
Anti-CD11b	Novus Biologicals (Cambridge, UK)	1/100
Anti-CD11c	Novus Biologicals (Cambridge, UK)	1/100
Anti-CD68	Dako	1/5000
Anti-ATRX	Sigma-Aldrich (St. Louis, MO, USA)	1/500

was poorly differentiated NB (13/29, 45%). The results of the MKI evaluation were given as follows: low in 20/27 tumors (74.1%), intermediate in 5/27 (18.5%), and high in 2/27 (7.4%). The median overall survival for the analyzed cohort was 43 months (CI 23.1–62.58) with an estimated overall survival rate at 5 years of 44.8% (SE 0.09). Mean time to first relapse was 24.7 months (median 15 months). To date, 16 patients have died of disease after a mean of 28.6 months (median 25.5 months).

Genomic Findings

A summary and detailed description of the genetic findings are presented in Tables 3 and 4, respectively; a summary representation of the genomic profiles by aSNP is shown in Figure 1. Two of 29 cases (0.7%) were MNA (Table 1). By aSNP and/or MLPA, 2 of 17 cases had the NCA profile (12%), 2 other cases showed an SCA profile with MNA (12%), and the remaining 13 cases had an SCA profile without MNA (76%). The two NCA cases exhibited several chromosome losses and gains. In one of these, two copy-neutral losses of heterozygosity (cnLOH) were also found. These were the only stage 1 cases in the cohort. As expected, fewer SCAs were seen in the MNA cases. In the homMNA tumor, NBAS and DDX1 genes co-amplified with MYCN, presenting 1p and 2p losses and a gain of 2q as SCAs. Gains of small chromosomal regions (0.2–2 Mb), known as focal SCAs (FSCAs), were also found. The hetMNA tumor showed an amplification of MYCN and 3p and 11q losses. The MNNA cases with only MLPA genomic data ($n = 3$) had an SCA profile with between one and three SCAs per

Table 3. Summary of the Genetic Findings by aSNP/MLPA.

Patient ID	Main Age Group	Age Subgroup	Genetic Group	Number of SCAs	ct	Number of NCAs	11q-	+17q	FSCA	cnLOH		
1	Group 1	Preadolescents	homMNA	3	N	2	N	N	Y	N		
2			NCA	0	N	7	N	N	N	N	Y	
5			SCA	5	N	2	Y	Y	N	N	N	
6			SCA	12	N	5	Y	Y	N	N	N	
7			SCA	8	N	2	Y	Y	N	N	N	
8			NCA	0	N	12	N	N	N	N	N	
12			SCA	17	Y	0	N	Y	Y	Y	Y	
13			SCA	5	N	1	Y	Y	Y	Y	N	
15			Adolescents	SCA	11	Y	1	Y	Y	Y	N	N
16				SCA ^a	3 ^a	N	1 ^a	Y	Y	ND	ND	ND
20				SCA	5	N	10	N	N	Y	N	N
25			Group 2	Young adults	SCA ^a	2 ^a	N	1 ^a	N	N	ND	ND
26					SCA ^a	1 ^a	N	1 ^a	N	N	ND	ND
27	SCA	4			N	0	N	N	Y	Y	Y	
29	Middle-aged adults	SCA			8	N	2	N	N	N	N	N
30		SCA			3	N	0	N	Y	Y	N	N
31		hetMNA			2	N	0	Y	N	N	N	Y

^a Genomic profile from MLPA data; Y, yes; N, no; ND, no data; ct, chromothripsis.

case. The distribution of the SCAs, detected by aSNP, in the 10 MNNA cases was rather heterogeneous with an average of 7.8 SCAs (range 3-17, median 5). FSCAs affected six cases and cnLOH affected two cases. Most of the SCA cases were also affected by NCA (10/13, 77%). Considering older age-dependent genomic patterns, tumors in group 1 patients had a higher median of SCAs when compared with group 2 (6.5 vs 3.5). The recurrent chromosome regions ordered by frequency were given as follows: +17q, 11q-, +1q, +2p, 1p-, 3p-, and 4p-. For group 1, 11q- was present in all MNNA tumors except two, while it was absent in all MNNA tumors from group 2. Moreover, a higher frequency of 17q gain occurred in group 1 tumors compared with group 2 tumors (87.5% vs 16.6%). A shattered pattern of chromothripsis at chromosome 7q, and chromosome 4 were observed in two cases. For the 7q arm chromothripsis, 10 breakpoints leading to gained SCAs were annotated. For chromothripsis at the whole chromosome 4, >30 SCAs (losses and gains) were present. Finally, gain of chromosome 7 (nine cases) was the most frequent NCA in the entire cohort.

Immune Cell Infiltration Study

The IHC results of the immune cell marker expression are shown in Table 5. In general, the number of positive cells was higher in the stroma-rich region (A) than in the neuroblast-rich region (B). A tendency toward higher infiltration of immune cells in tumors from group 2 was also seen. In addition, group 2 tumors had a heterogeneous pattern related to infiltration percentages of CD expression in cells. In summary, related to the age-dependent immune cell infiltration pattern, tumor region A from group 2 patients presented a clearly higher percentage of immune cells positive for CD4, CD8, CD20, and CD68 markers and a slightly higher percentage for CD11b+ and CD11c+ cells, compared with both their region B counterpart and both regions in tumors from group 1. In relation to the genetic pattern-dependent immune cell infiltration, the NCA tumors tended to present small percentages of positive immune cell markers. Both MNA tumors showed the highest quantity of CD11b+ cells and a high amount of CD68+ cells in region A; only the hetMNA case presented a high

Table 4. Detail of the Genetic Findings by aSNP/MLPA.

Patient ID	Main Age Group	Age Subgroup	Genetic Group	Partial Chromosome Gains	Partial Chromosome Losses	Regions with ct	Complete Chromosome Gains	Complete Chromosome Losses	FSCA	cnLOH		
1	Group 1	Preadolescents	homMNA	2q	1p, 2p	-	-	3, 10	+4q, +19q	-		
2			NCA	-	-	-	7, 8, 11, 17, 20, 21	14	-	7q, 16q	-	
5			SCA	17q	1q, 6q, 11q, 16p	-	7, 18	-	-	-	-	
6			SCA	1q, 2p, 5q(i), 7q(i), 12q, 17q	4p, 4q(i), 5p, 7q, 11q, 19p	-	-	3, 6, 8, 14, 15	-	-	-	
7			SCA	16q, 17q, 18q	1q, 3p, 11q, 15q, 19p	-	7, 13	-	-	-	-	
8			NCA	-	-	-	1, 2, 4, 6, 7, 8, 20, 21	3, 13, 14, 19	-	-	-	
12			SCA	2p(2), 5q, 7p, 7q, 12pq(i), 12q(i), 15q, 16pq, 17pq, 17q(i), 17q, 18pq	1p, 1p(i), 2q, 19p	4	-	-	+7q, 11q-	9p	-	
13			Adolescents	SCA	4q, 11q, 17q	3p, 11q	-	7	-	12q amp	-	-
15				SCA	5p, 11q(2), 17q, 20p, 20q	4p, 5q(i), 11q, 18p(i), 18p	7q	-	14	4p-, +20p	-	-
16				SCA ^a	12q, 17q	11q	-	7	-	ND	ND	ND
20				SCA	1q, 5q, 12q	5p, 10q	-	6, 7, 8, 9, 13, 17, 18, 20, 21	11	4q-	-	-
25			Group 1	Young adults	SCA ^a	1q	4p	-	17	-	ND	ND
26					SCA ^a	-	4p	-	7	-	ND	ND
27	SCA	7q, 19pq			10p, 19p	-	-	-	-	+5p	4q, 11p, 11p, 12q, 19p	
29	Middle-aged adults	SCA	1q, 2p, 20q, 21q	11p(2), 19p, 22q	-	7, 8	-	-	-	-		
30			4p, 17q, 18q	-	-	-	+20p	-	-	-	-	
31			hetMNA	-	3p, 11q	-	-	-	-	-	6p	

^a Genomic profile from MLPA data; (i), intrachromosomal (two breakpoints); ct, chromothripsis; amp amplification.

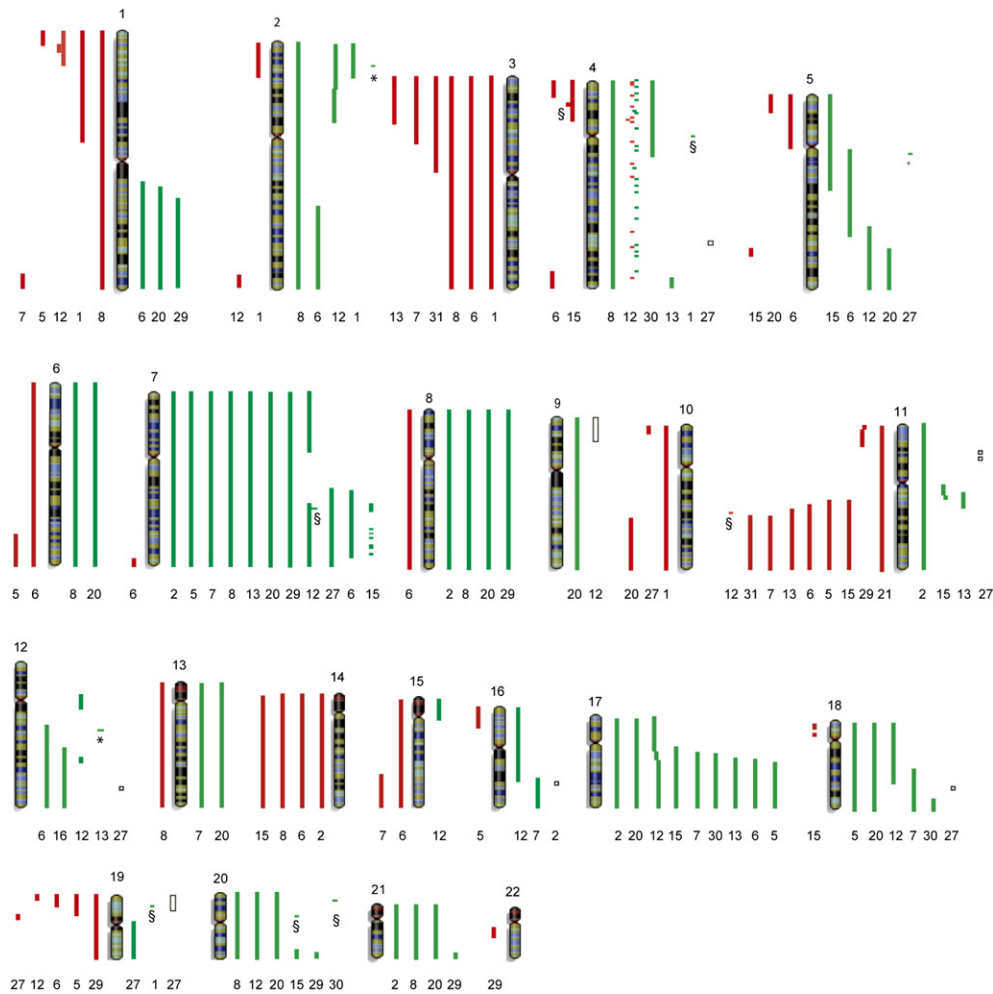


Figure 1. Representation of the genomic profiles by aSNP. Losses are indicated by a plain bar on the left, gains by a plain bar, and cnLOH by an empty bar on the right of each chromosome ideogram. Chromosome number and ID of the patients are indicated above and below respectively. Chromothripsis is indicated to the right of the chromosome ideograms. FSCAs and MNA are marked by * and §, respectively.

amount of CD68+ cells in region B. For the SCA cases, a wide variation in percentages was seen.

ATRX Expression

A summary of clinical data and nuclear ATRX protein expression is presented in Table 6 and Figure 2. Thirteen of 17 cases (76.5%) were negative. Seven cases had completely negative tumor cell staining, and in six cases, some tumor cells retained ATRX and some lacked ATRX nuclear staining. Four positive cases (23.5%) without *ATRX* gene deletion were found. The homMNA case showed the highest immunopositivity for ATRX. A mosaicism immunophenotype was found in the *ATRX* gene deleted control case.

Discussion

Applying high-density aSNP and IHC techniques, the present study has identified similarities and differences between tumors from preadolescent and adolescent patients (group 1) and young adult and middle-aged adult patients (group 2). Genetically, as is described in childhood NB, we have identified cases with MNA (hom and het), with MNNA, with the NCA profile, and with the SCA profile (11q deleted and non-deleted) [29,31,32]. Patients with a localized stage and NCA tumor remained without recurrence or progression in

accordance with results previously described in childhood NB [11]. In childhood NB, homMNA is frequent at a median age of 28 months, and tumors with homMNA appear to have rapid growth [31,33–36]. These facts indicate that homMNA tends to be an early phenomenon in oncogenesis, implying a different route of tumor evolution [11,34,37]. According to the literature, hetMNA in childhood NB tends to be more frequent in advanced stages; nevertheless, in our study hetMNA was present in a low stage [38]. Neither median age nor impact of hetMNA on outcome has been reported in large cohorts to date [38,39]. The rarity of homMNA in NB tumors after childhood was not unexpected. Its low frequency has been consistently described throughout the literature; however, hetMNA has not previously been described in older NB [1,3,4,7,8,40,41]. In our study, the hetMNA case was found in the oldest patient, which may indicate that the acquisition of hetMNA may be a late event associated with early death after diagnosis. Furthermore, in contrast to childhood tumors, a drift toward a mixed profile with recurrent SCA and NCA has also been found [32]. The predominant SCA profile found in group 1 is in accordance with some reports, including the latest AYA International NB Risk Group [1,6]. Schleiermacher et al. suggested a hypothesis in childhood NB for progression of NCA tumors, whereby they evade clinical examination and then acquire

Table 5. Expression of Immune Cell System Markers by IHC.

Patient ID	Main Age Group	Age Subgroup	Genetic Group	CD4		CD8		CD20		CD11b		CD11c		CD 68		
				A	B	A	B	A	B	A	B	A	B	A	B	
1	Group 1	Preadolescents	homMNA	1	1	1	1	1	1	4	1	1	1	4	0	
2			NCA	0	0	2	1	2	1	1	0	1	1	0	0	
5			SCA	0	0	2	2	1	1	2	2	0	0	2	1	
7			SCA	0	0	2	2	3	3	1	1	0	0	2	1	
8			NCA	0	0	2	1	1	1	1	1	1	1	1	1	
13			SCA	0	0	2	1	1	1	1	1	1	1	2	1	
15		Adolescents	SCA	1	1	2	1	1	1	1	1	1	1	2	0	
16			SCA ^a	0	0	1	1	0	0	1	1	1	1	2	2	
23			NE	0	0	4	1	1	0	1	1	1	0	1	0	
24			NE	0	0	2	1	2	1	1	1	1	2	2	2	
25		Group 2	Young adults	SCA ^a	1	1	2	0	4	1	2	1	2	1	3	1
26				SCA ^a	2	1	4	2	4	3	2	2	2	1	4	3
28			Middle-aged adults	NE	1	0	2	1	1	0	2	1	1	1	4	2
29	SCA			1	0	3	1	3	0	2	1	4	1	3	1	
30	SCA			3	1	4	2	4	1	1	1	2	1	4	4	
31	hetMNA			0	0	1	1	1	0	4	1	4	0	3	4	

^a Genomic profile from MLPA data; A, stroma-rich region; B, neuroblast-rich region; 0, negative; 1, minimal; 2, moderate; 3, high; 4, very high; NE, not evaluated.

SCAs. In agreement with this hypothesis, the present cohort of older age patients at diagnosis correlates with the high frequency of this mixed profile. Most of the patients with SCA tumors with NCAs are short-term survivors [42]. A complete gain of chromosome 7, the most frequently gained NCA in childhood NB, was also present in our cases [31,43]. The negative impact of the recurrent SCAs in NB is well reported [32,44,45]. Nevertheless, the role of the amount of SCAs in the oncogenic pathophysiology is not clear. Several studies have explored the SCA cutoff number to discriminate the impact in event-free survival (EFS) and/or overall survival. Among all NB tumors with an SCA profile, a threshold of three SCAs could distinguish between long- and short-term survivors in high-risk children [46]. Moreover, it has been proposed that a higher number (more than seven SCAs) has prognostic impact [42]. The present study found a higher frequency of cases with more than three SCAs than that reported for general childhood NB (88% vs 53%) [42]. Indeed, the average number of SCA per sample, despite the dispersion, falls within the range described in a recent study for stage 4 NB in patients older than 18 months [43,46]. The present study found no differences in either EFS or in OS in relation to the number of SCA (data not shown). An interesting finding is that most of the tumors with a lower number of SCA were from group 2; this may indicate a distinct evolutionary

mechanism that requires investigation. When considering the SCA in NB of all ages, 11q deletion is linked to a higher age at diagnosis (41-48 months) and to a higher instability [34,37]. Inconsistent data have been reported in relation to 11q- frequency and older age NB: a decreased presence of 11q- when considering patients over 7 years of age at diagnosis and a relatively stable proportion of 11q- tumors in patients from 18 months to >10 years of age [1,35]. Our study included a slightly higher proportion of 11q- tumors than previously reported (41% vs 32-33%) [1,35]. The most outstanding and recent complex genetic finding is chromothripsis; this has been found with high prevalence in neuroepithelial tumors (NB, medulloblastoma, and glioblastoma) [15,47-50]. In NB of all ages, it affects chromosomes 2, 5, 6, 7, and 8 and is associated with 1p deletion and amplification of *CDK4* or *MNA* [48]. Until now, chromothripsis at chromosome 4 has been described in only one large-scale study, although structural variants in genes located at chromosome 4q (i.e., *ODZ3*, 4q35.1) have been found in aggressive NB tumors [15,51]. Restructuring of the *ODZ* gene family, implicated in the neuronal growth cone, has been found in NB lacking *MNA* as a frequent alteration associated with chromothripsis [15]. Strikingly, the chromothripsis of chromosome 4 coexisted with a focal loss of the *ODZ4* gene and with a restructuring at 5q affecting the *ODZ2* gene. Although this catastrophic event has been associated with poor prognosis, it is not clear if it contributes to tumor development as a driver mutation or if it represents a secondary event as a consequence of genomic instability with different implications for tumor progression [48]. The fact that both patients are still alive without relapse leads to the hypothesis that chromothripsis in these tumors might be a marker of an underlying genomic instability contributing to tumor inhibition.

The identification of genetic mutations in older NB has attracted much interest as its protracted course is not completely understood. *ATRX* mutations and the subsequent loss of the nuclear protein expression are more frequently identified in patients older than 12 years with stage 4 disease. Exome sequencing studies point to an inactivation of both *ATRX* copies through mutation and chromosome X inactivation [14,15]. Immunolabeling loss of *ATRX* is also associated with mutation in other cancers [52,53]. Cheung et al. found *ATRX* gene deletions in 43% of older NB (>12 years old) and in 11% of childhood NB (5-12 years) patients [14]. Some of the *ATRX* deletions described are too small (16 kb) to be detected with the aSNP platforms,

Table 6. *ATRX* Expression and Main Clinical and Genetic Features of the Tumors.

Patient ID	Main Age Group	Age Subgroup	Sex	<i>ATRX</i> Protein	Outcome
1	Group 1	Preadolescents	f	Positive	AWT
2			f	Positive	ADF
3			f	Negative	ADF
5			m	Negative	ADF
8			f	Negative	ADF
9			f	Negative	DOD
13		m	Mosaic	DOD	
15		Adolescents	m	Negative	ADF
16			f	Negative	ADF
18			f	Mosaic	DOD
20			f	Positive	DOD
24		m	Mosaic	DOD	
25		Group 2	Young adults	m	Positive
26	f			Mosaic	DOD
28	Middle-aged adults		m	Mosaic	DOD
30			m	Mosaic	AWT
31			f	Negative	DOD

f, female; m, male; AWT, alive with treatment; ADF, alive disease-free; DOD, died of disease.

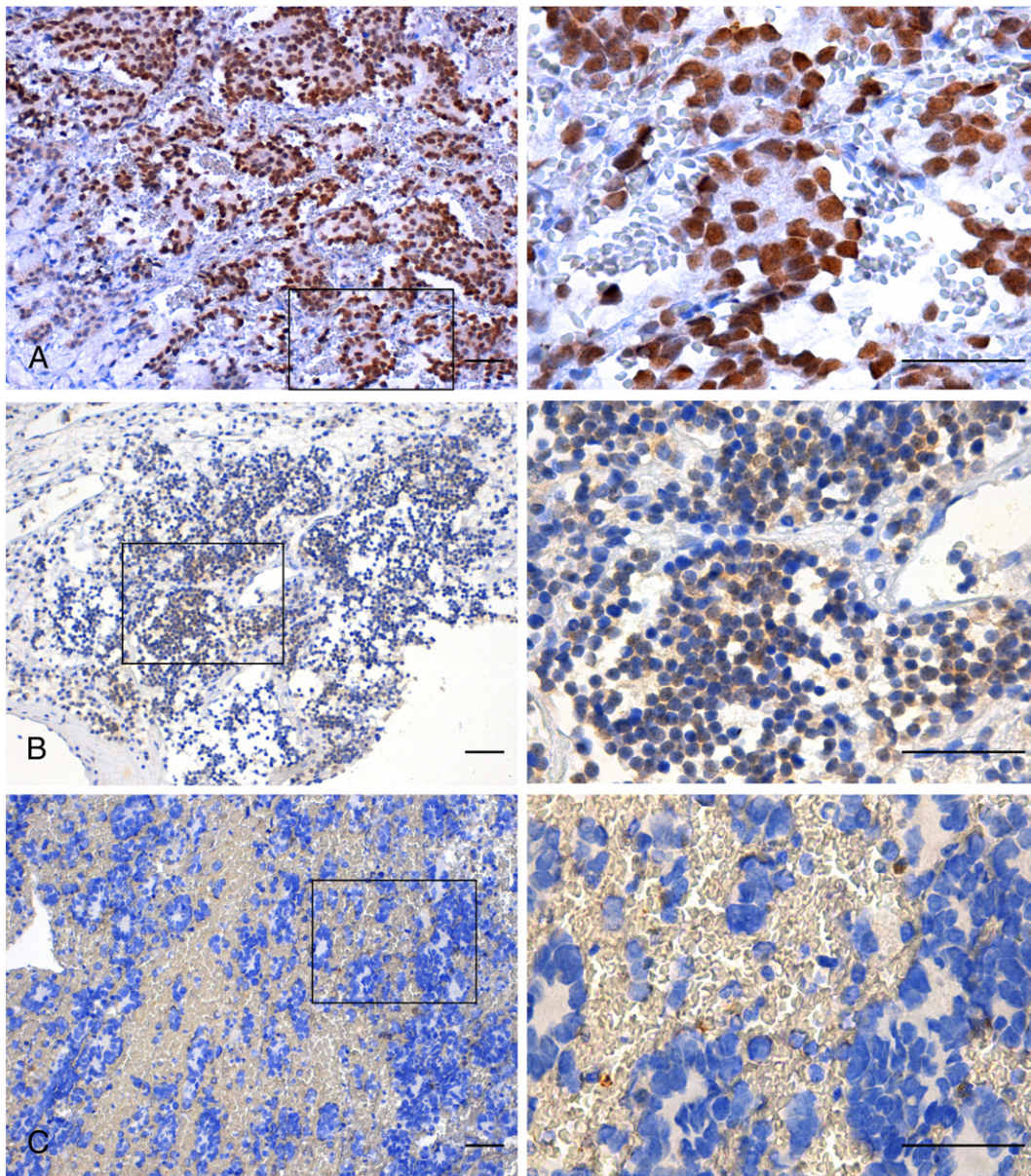


Figure 2. ATRX protein expression by IHC in three NB tumors. (A) Strong positive nuclear expression of neuroblast cells. (B) Mosaic expression of neuroblast cells, less than half of the neuroblasts cells retained the expression. (C) Negative nuclear expression. Scale bar, 50 μm (image at $\times 40$ and $\times 100$).

which could explain why none of the cases of the present cohort displayed the *ATRX* deletion [51]. Loss of ATRX protein expression, as in mosaicism, is less often represented than the complete negative expression in older NB [11]. In our cohort, mosaicism is more closely linked to the tumors of group 2.

Additional prognostic factors such as the NB microenvironment, a complex structure relating to the interaction between neuroblasts and Schwann cells, normal host cells, and extracellular matrix elements are now being considered as a potential focus for research [54,55]. In childhood NB, data describing immune cell infiltration are limited except in NB associated with Opsoclonus-Myoclonus syndrome [17,56,57]. It is assumed that the host immune response to a human solid tumor could be reflected in immune cell infiltration, and chronic local inflammation has been described as being involved in the initiation of many adult neoplasias [58–62]. It has been

postulated that analyzing the composition, distribution, and architecture of the immune infiltrate for each tumor type could offer new prognostic or predictive biomarkers [63,64]. Assuming that the innate and adaptive immune system must be well developed beyond the age of 10, an interesting comparative study of pediatric and adult tumors was carried out by Vakkila et al. [65]. This comparative study revealed that pediatric tumors are characterized by a less diverse leukocyte composition, consisting almost exclusively of macrophages, with fewer infiltrating leukocytes, a lack of dendritic cells, and a more scattered distribution of infiltrating cells. A diverse composition and organized distribution of the tumor-associated leukocytes in adult tumors was ascertained. Nevertheless, no significant differences in the total number of tumor-associated immune cells were detected between adult and pediatric tumors. The age-related infiltration pattern existing between groups 1 and 2

resembled that of Vakkila et al. [65]. A pattern with a low diversity of immune cells, almost exclusively macrophages in composition, was observed in group 1. In our cohort, it is remarkable that infiltration and diverse composition was more evident in group 2, both in the stroma and the neuroblast-rich regions. Neuroblastic cells present a low expression of major histocompatibility complex class I and II molecules and may be much better targets for natural killer (NK) cells than for cytotoxic T lymphocytes [24]. The presence of the CD8+ T cells alone does not necessarily imply an anti-tumor response but, in combination with high percentages of CD11b+ cells, prompts us to hypothesize that the CD8 cells become activated and display effector actions like tumor-growth and progression or tumor permissiveness [66]. Based on the literature, it is plausible to assign the extended positivity found for the CD11b marker in our samples to a subset of naïve CD8+ T cells. Consequently, it should be ascertained whether or not this subset of cells completely overlaps the CD8+ T cells, especially for the cells within the neuroblast-rich region [67–69]. In a study describing the differentiated distribution of immune cells in childhood NB tissue, few CD4+ and CD8+ cells were able to infiltrate the peritumoral stroma but were unable to infiltrate the tumor nests [70]. The present study detected different percentages of immune cell infiltration between stroma-rich and neuroblast-rich regions. Although the percentages were higher in the stroma-rich region, CD8 and CD20 positive cells were also present in the neuroblast-rich region. Regarding the hetMNA case, despite being in group 2, the infiltration pattern was similar to that of group 1; the stroma-rich region was highly infiltrated with CD11b+ and CD68+ cells in comparison to the neuroblast-rich region. It could be useful, using quantitative analysis, to study the balance of the immune cell infiltration in both regions [71,72].

In summary, we corroborate the high prevalence of SCA, low MNNA, and negative expression of ATRX in NB after childhood. Confirmation of the differences relating to both number and type of SCA as well as the composition and distribution of the immune cells should lead to a better understanding of disease outcome. It is imperative that biologic studies by cooperative groups continue on a larger number of such valuable tumor tissues to better establish differences among the patterns found in this cohort.

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