Tesis Doctoral - Ana P Berbegall – Integración de la genómica en la modelización de los tumores neuroblásticos - 2015

INTEGRACIÓN DE LA GENÓMICA en la MODELIZACIÓN de los TUMORES NEUROBLÁSTICOS

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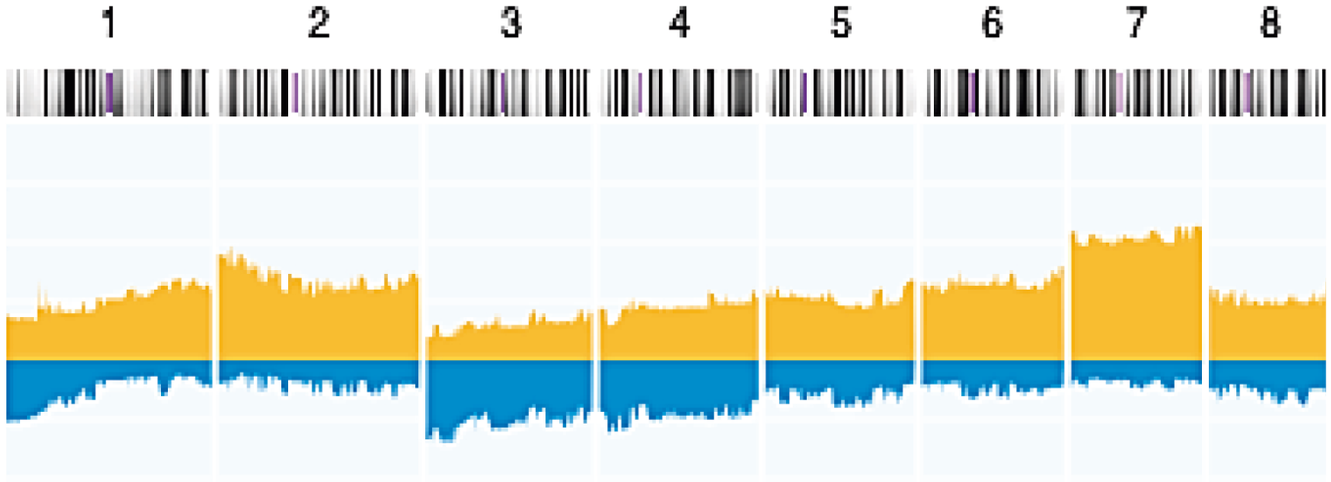
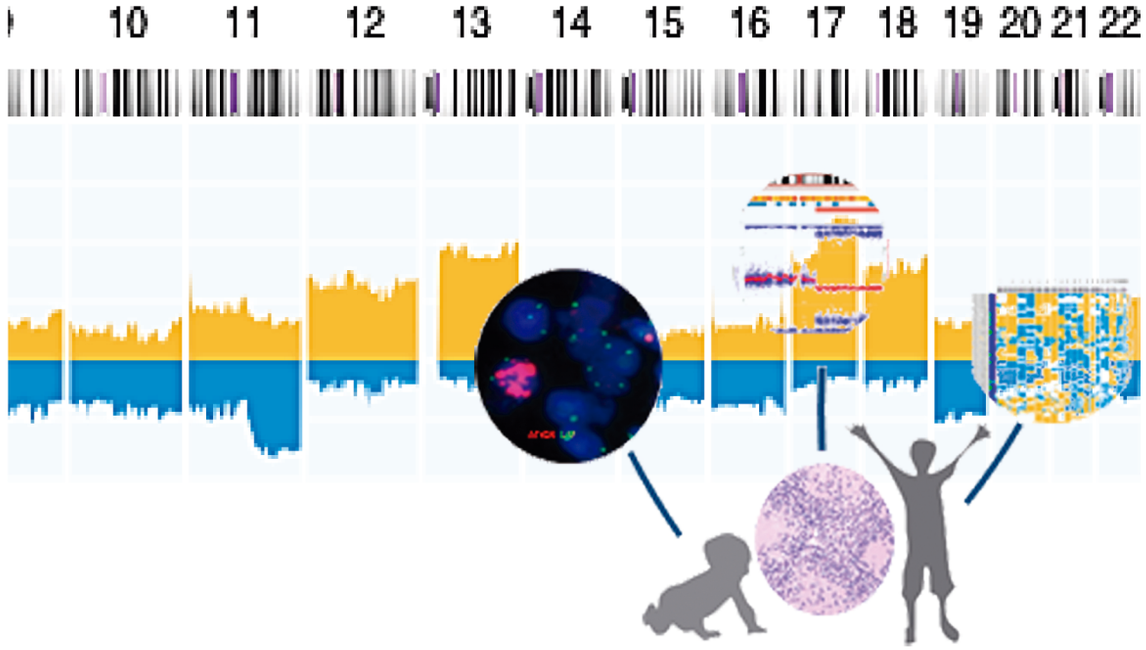
INTEGRATION of GENOMICS in NEUROBLASTIC TUMORS MODELING





Doctoral thesis
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PhD Thesis

INTEGRACIÓN DE LA GENÓMICA EN LA MODELIZACIÓN DE LOS TUMORES NEUROBLÁSTICOS

—

INTEGRATION OF GENOMICS IN NEUROBLASTIC TUMORS MODELING

Doctoral thesis presented by:

Ana Pilar Berbegall Beltrán

in order to obtain the international Doctorate mention, Doctorate Program 285B Pathology of Human Tumors, Department of Pathology, Faculty of Medicine and Dentistry, University of Valencia

Directed by:

Dra. Prof. Rosa Noguera Salvá

Dr. Prof. Samuel Navarro Fos

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Valencia - April 2015

Doña Rosa Noguera Salvá y Don Samuel Navarro Fos, Doctores en Medicina y catedráticos de la Facultad de Medicina y Odontología de la Universidad de Valencia,

CERTIFICAN:

Que Ana Pilar Berbegall Beltrán, licenciada en Biología por la Universidad de Valencia, ha realizado, bajo nuestra dirección, el trabajo que lleva por título:

"*Integración de la genómica en la modelización de los tumores neuroblásticos"*

el cual consideramos satisfactorio y apto para ser presentado y defendido como Tesis Doctoral en el Departamento de Patología de la Universidad de Valencia.

Y para que así conste, expedimos este certificado en Valencia, a 10 de Abril de 2015.

Fdo.: Profª. Rosa Noguera Salvá
Profª. Catedrática de Histología

Fdo.: Prof. Samuel Navarro Fos
Prof. Catedrático de Anatomía Patológica

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RESUMEN

INTEGRACIÓN DE LA GENÓMICA EN LA MODELIZACIÓN DE LOS TUMORES NEUROBLÁSTICOS

Ana Pilar Berbegall Beltrán

Mención Internacional de Doctorado, Programa de Doctorado: Patología de los tumores humanos, Departamento de Patología, Universidad de Valencia

Introducción:

El neuroblastoma es un tumor maligno embrionario del sistema nervioso simpático con una gran heterogeneidad en la presentación clínica, morfológica y genética y en otras características biológicas. La estratificación de grupos de riesgo pretratamiento se basa en los siguientes factores: edad, estadio, histopatológica del tumor, estado del oncogén *MYCN*, integridad del brazo cromosómico 11q y ploidía. Por otra parte, los cambios numéricos y estructurales detectados en el perfil genético se han incluido recientemente en la estratificación terapéutica de los pacientes con bajo riesgo a recaer o morir. El amplio espectro clínico de la enfermedad va desde pacientes con neuroblastoma en estadio 1, 2, 3 y 4S, generalmente caracterizados por su pronóstico favorable, a pacientes con tumores en estadio 4 con una mala evolución de la enfermedad, clasificados como pacientes de alto riesgo. Por otra parte se han descrito cohortes de neuroblastoma con unas características clínicas y biológicas inusuales y con baja prevalencia. Típicamente el neuroblastoma se diagnostica en la época neonatal y en bebés menores de un año y generalmente el oncogén *MYCN* se encuentra amplificado en aproximadamente el 25% de todos los casos. El diagnóstico en pacientes mayores de 10 años, la coexistencia de la amplificación de *MYCN* y deleción del brazo 11q o la amplificación heterogénea de *MYCN* son fenómenos infrecuentes. En los neuroblastomas de alto riesgo y en los raros grupos mencionados se necesitan de forma urgente investigaciones dirigidas a la identificación de nuevos enfoques terapéuticos para lograr una mejora de la evolución de la enfermedad. Una mejor caracterización de los diferentes grupos pronóstico y la identificación de nuevas dianas terapéuticas en el tumor y células del estroma, y en los elementos de la matriz extracelular dará lugar a terapias más adecuadas.

Hasta la fecha son pocos los estudios que han determinado de forma precisa la frecuencia y detallado las alteraciones genéticas en neuroblastomas de pacientes adolescentes y adultos, o en neuroblastomas con amplificación atípica de *MYCN* y por tanto, están lejos de aportar estrategias tangibles para el cuidado de los pacientes. Sin embargo, ampliar esta información es crucial para identificar nuevos enfoques terapéuticos mediante la minería de datos.

Objetivos generales:

En esta tesis perseguimos describir las características biológicas de tres grupos infrecuentes de tumores neuroblásticos identificando las alteraciones genéticas de cada grupo con el fin último de facilitar grandes estudios multicéntricos que puedan lograr una terapia más precisa de los pacientes.

Objetivos específicos:

- Buscar patrones dependientes de la edad avanzanda en el neuroblastoma, asociados con características biológicas particulares, en una cohorte de pacientes mayores de 10 años al diagnóstico. El estudio está centrado en: 1) Determinar los patrones de alteraciones cromosómicas segmentarias mediante la técnica de micromatrices de polimorfismos de un único nucleótido. 2) Investigar la infiltración de las células del sistema inmune en el tejido tumoral mediante la expresión inmunohistoquímica de marcadores celulares (CD4, CD8, CD20, CD11b, CD11c y CD68). 3) Evaluar la expresión de la proteína ATRX mediante inmunohistoquímica.

- Determinar el perfil genético de tumores neuroblásticos con dos alteraciones genéticas agresivas: la amplificación del oncogén *MYCN* y deleción de la región cromosómica 11q, poniendo énfasis en: 1) Identificar las alteraciones cromosómicas segmentarias y definir sus puntos de ruptura cromosómica. 2) Definir las regiones más pequeñas de solapamiento de las alteraciones segmentarias. 3) Describir la heterogeneidad intratumoral de los cambios cromosómicos presentes en este grupo tumoral.

- Describir las características genéticas de los tumores neuroblásticos con amplificación heterogénea del oncogén *MYCN*. La descripción genética se basa en: 1) Identificación de los patrones de alteraciones cromosómicas segmentarias y de los puntos de ruptura cromosómica. 2) Comparación de los perfiles genómicos y de determinados puntos de ruptura cromosómica entre los tumores con amplificación heterogénea de *MYCN* y los subgrupos genéticos agresivos de neuroblastoma (con y sin amplificación homogénea de *MYCN* y deleción de 11q).

Metodología:

El presente trabajo de tesis doctoral es un compendio de tres artículos científicos, en los que la candidata es la primera autora o primera coautora, que tratan sobre tumores neuroblásticos infrecuentes. En el texto del compendio de tesis hemos usado el término ultra-raro para referirnos al conjunto de tumores neuroblásticos caracterizados. Los artículos que constituyen el compendio son los siguientes:

1. Genetic instability and intratumoral heterogeneity in neuroblastoma with *MYCN* amplification plus 11q deletion. Eva Villamón,*, Ana P. Berbegall,*, Marta Piqueras, Irene Tadeo, Victoria Castel, Anna Djos, Tommy Martinsson, Samuel Navarro, and Rosa Noguera. * Equal contribution. PLoS One, 2013;8(1): e53740.

2. Neuroblastoma after Childhood: Prognostic Relevance of Segmental Chromosome Aberrations, ATRX Protein Status, and Immune Cell Infiltration. Ana P. Berbegall, Eva Villamón, Irene Tadeo, Tommy Martinsson, Adela Cañete, Victoria Castel, Samuel Navarro and Rosa Noguera. Neoplasia, 2014; 16(6): 471-480.

3. Comparative genetic study of intratumoral heterogenous *MYCN* amplified neuroblastoma with aggressive genetic profile neuroblastic tumors. Ana P. Berbegall, Eva Villamón, Marta Piqueras, Irene Tadeo, Anna Djos, Peter Ambros, Tommy Martinsson, Inge Ambros, Adela Cañete, Victoria Castel, Samuel Navarro and Rosa Noguera. Oncogene, 2015; Aceptado para su publicación, Abril 2015.

Como se razona a continuación, los artículos reúnen tres desafíos en la investigación del neuroblastoma:

1. Centrarse en una cohorte minoritaria de pacientes afectos de neuroblastoma. Dentro de la enfermedad neuroblástica, considerada como una entidad rara (ORPHA635, http://www.orpha.net/), existen grupos clínica y biológicamente ultra-raros, en los que es necesaria una mayor investigación. La clínica de los pacientes con tumores ultra-raros es problemática, el tratamiento en estos pacientes con mucha frecuencia supone un dilema y todo el proceso de la enfermedad se vuelve más complejo. El escaso avance experimentado en el conocimiento de estos tumores no ha contribuido a la mejora de la supervivencia ni al desarrollo de tratamientos más adecuados. Por lo tanto, a pesar de la infrecuencia de estos tumores su investigación posee un gran valor.

2. Proponer una gestión más adecuada de las muestras. Dado que los grupos ultra-raros de neuroblastoma han sido a menudo excluidos o considerados como parte de otros grandes grupos de neuroblastomas con características clínicas y biológicas distintas a estos, la determinación de sus características biológicas se ha visto comprometida. Además, la escasez de la muestra tumoral disponible para realizar los estudios de investigación dificulta consensuar el papel de los análisis genéticos en la estratificación de riesgo terapéutica. Es crucial resaltar la importancia de la recogida metódica de la mayor cantidad de tejido tumoral posible para ser analizado secuencialmente por hibridación in situ fluorescente y por técnicas pangenómicas para determinar la presencia de heterogeneidad genética intratumoral. Considerar a estos tumores como entidades especiales con protocolos específicos para el manejo de las muestras beneficiaría su diagnóstico e investigación.

3. Considerar la utilidad potencial de los datos. La pretensión final del trabajo de tesis es contribuir a la recogida de datos y modelización del neuroblastoma. La descripción genética de estas cohortes facilitará la consecución de grandes estudios multicéntricos que son necesarios para coordinar proyectos clínicos. La ausencia de consenso diagnóstico respecto a los grupos de edad avanzada, una relativa falta de atención a los casos de neuroblastoma con amplificación atípica de *MYCN*, la escasez de la muestra tumoral y la heterogeneidad genética intratumoral son limitaciones que se pueden superar en el marco de los estudios multicéntricos almacenando adecuadamente los datos. Los tumores neuroblásticos ultra-raros debido a sus características biológicas singulares también pueden servir como modelos que proporcionarían conocimiento preciso sobre la patogénesis del neuroblastoma.

Conclusiones:

Los estudios presentados son de relevancia para nuevos enfoques terapéuticos específicos y para generar estrategias de colaboración que permitan desarrollar propuestas de ensayos clínicos adecuadas.

SUMMARY

INTEGRATION OF GENOMICS IN NEUROBLASTIC TUMORS MODELING

Ana Pilar Berbegall Beltrán

International mention, Doctorate Program: Pathology of Human Tumors, Department of Pathology, Faculty of Medicine, University of Valencia

Background:

Neuroblastoma is an embryonic malignancy of the sympathetic nervous system with a remarkable heterogeneity in clinical, histological, genetic and in other biological features. The pre-treatment risk group stratification is based on the following factors: age, stage, tumor histology, *MYCN* gene status, chromosome 11q integrity and ploidy. Recently, numerical and segmental chromosome aberrations detected in the genetic profile have been included in the treatment stratification of patients with a low-risk to relapse or die. The wide clinical spectrum of the disease ranges from neuroblastoma patients with stage 1, 2, 3 and 4S usually characterized by favorable prognosis, to advanced stage 4 with an adverse outcome and classified as high-risk neuroblastoma. Besides these groups, neuroblastoma cohorts characterized by unusual clinical and biological features have been described with low prevalence. Typically, neuroblastoma is diagnosed during the neonatal period and infancy, and finding that the *MYCN* oncogene is usually amplified in approximately 25% of all cases. Diagnosis in ages over 10 years, coexistence of *MYCN* amplification plus 11q chromosome deletion or heterogeneous *MYCN* amplification are very infrequent events. In high-risk neuroblastoma and in the above-mentioned rare groups, research efforts focusing on the identification of new therapeutic approaches are urgently needed to improve outcome. A better characterization of the different prognostic groups and the identification of therapeutic targets in the tumor and stroma cells, as well as in the extracellular matrix elements would lead to the identification of more specific treatments.

To date, few studies have determined the precise frequency or detailed the genetic alterations of samples derived from adolescent and adult life neuroblastomas, or from tumors with atypical presence of *MYCN* amplification, and therefore almost do not constitute actionable goals for patient care. However, their accurate characterization is crucial for the identification of new therapeutic approaches through data mining studies.

General objectives:

In this thesis research, we pursue to describe the biological features of three ultra-rare cohorts of neuroblastic tumors, searching for any genetic features specific to each group, with the ultimate goal of facilitating large multicenter studies that could lead to the implementation of a more accurate management and care of the patients.

Specifics objectives:

- To search for older-age-dependent patterns in neuroblastoma associated with particular biological characteristics in a cohort of patients older than 10 years at diagnosis. Our actions concentrated on: 1). Exploring the patterns of segmental chromosome aberrations by performing the Single Nucleotide Polymorphisms array technique. 2). Investigating the

presence of immune cells tumor infiltration by immunohistochemistry expression of cell markers (CD4, CD8, CD20, CD11b, CD11c and CD68). 3). Evaluating the expression of the ATRX protein by immunohistochemistry.

- To characterize the genetic profile of uncommon neuroblastic tumors harboring two chromosomal aggressive features, *MYCN* amplification and 11q deletion. Emphasis was placed on: 1). Identification of the segmental chromosome aberrations present and mapping their chromosomal breakpoints. 2). Defining the smallest region of the segmental chromosomal alterations. 3). Describing the intratumoral heterogeneity of the structural chromosome changes present in this tumor cohort.

- To demarcate the genetic characteristics of unusual heterogeneous MYCN amplified neuroblastomas. Our approach consisted of: 1). Identification of the patterns of segmental chromosome aberrations and mapping of the chromosomal breakpoints. 2). Comparison between their genomic profiles and certain breakpoints with those from aggressive genetic subgroups of neuroblastoma (homogeneous *MYCN* amplified tumors and non-*MYCN* amplified with 11q deletion).

Methodology:

The present doctoral thesis research is presented as a compendium of three publications, of which the candidate is the first or co-first author, dealing with ultra-rare neuroblastic tumors; henceforth in this text of the compendium we use the term ultra-rare neuroblastoma to refer to all these characterized tumors. The articles of the compendium are the following:

1. Genetic instability and intratumoral heterogeneity in neuroblastoma with *MYCN* amplification plus 11q deletion. Eva Villamón,*, Ana P. Berbegall,*, Marta Piqueras, Irene Tadeo, Victoria Castel, Anna Djos, Tommy Martinsson, Samuel Navarro, and Rosa Noguera. * Equal contribution. PLoS One, 2013; 8(1): e53740.

2. Neuroblastoma after Childhood: Prognostic Relevance of Segmental Chromosome Aberrations, ATRX Protein Status, and Immune Cell Infiltration. Ana P. Berbegall, Eva Villamón, Irene Tadeo, Tommy Martinsson, Adela Cañete, Victoria Castel, Samuel Navarro and Rosa Noguera. Neoplasia, 2014; 16(6): 471-480.

3. Comparative genetic study of intratumoral heterogenous *MYCN* amplified neuroblastoma with aggressive genetic profile neuroblastic tumors. Ana P. Berbegall, Eva Villamón, Marta Piqueras, Irene Tadeo, Anna Djos, Peter Ambros, Tommy Martinsson, Inge Ambros, Adela Cañete, Victoria Castel, Samuel Navarro and Rosa Noguera. Oncogene, 2015; Accepted for publication, April_2015.

As outlined below, the articles unify three research challenges in neuroblastoma:

1. Focus on minor cohorts of neuroblastoma patients. Within neuroblastoma, considered as a rare disease (ORPHA635, http://www.orpha.net/), there are clinical and biological ultra-rare neuroblastoma groups in which further research is necessary. Ultra-rare neuroblastoma patients' care is problematic, therapeutic decision-making very frequently poses a dilemma and dealing with the disease process becomes more complicated. The scant knowledge of these tumors has led to a failure in patient survival improvement or in the development of better treatments. Therefore, although uncommon tumors their research is worthy and worthwhile.

2. Propose an accurate management of the samples. Since ultra-rare groups of neuroblastoma have often been excluded or considered as part of larger groups of neuroblastoma with dissimilar clinical and biological characteristics, delineation of their biology has been compromised. The scarcity of tumor material available for research studies makes it difficult to achieve a consensus on the role of genetic analyses for establishing a therapeutic risk stratification. It is crucial to stress the importance of a methodical collection of as much tumor tissue as possible to be sequentially analyzed by Fluorescence In Situ Hybridization and pangenomic techniques to ascertain the presence of the genetic intratumoral heterogeneity. Considering these tumors as special entities with specific standard protocols for sample management would benefit their diagnosis and research.

3. Consider potential utility of the data. The final challenge of the thesis work is to contribute to the data collection and modeling of neuroblastic tumors. The genetic description of these cohorts will facilitate the consecution of large multi-center studies needed to coordinate clinical projects. The lack of a diagnostic consensus referent to older age neuroblastoma, the relative lack of attention to ultra-rare neuroblastoma cases with *MYCN* amplification, the sample scarcity and genetic intratumoral heterogeneity are limitations that can be overcome in the frame of multicenter studies with adequate databases. Ultra-rare neuroblastomas, because of their singular biological features, can also serve as models that could provide accurate knowledge concerning neuroblastoma pathogenesis.

Conclusions:

The studies presented are of relevance for new targeted treatment approaches and to generate collaborative strategies to develop proposals for appropriate clinical trials proposals.

ABBREVIATIONS

AYA	Adolescent and young adults
CDs	Cluster of differentiation
CGH	Chromosomal-comparative Genomic Hybridization
CISH	Chromogenic in situ Hybridization
COG	Children Oncology Group
ddmin	Double minutes
EFS	Event free survival
FISH	Fluorescence In Situ Hybridization
GN	Ganglioneuroma
GNB	Ganglioneuroblastoma
hetMA	Heterogeneous MNA
hetSCA	Heterogenous SCA
homNA	Homogeneous MNA
HSRs	Homogeneously stained region
IDRF	Image defined risk factors
INPC	International Neuroblastoma Pathology Classification
INRG	International Neuroblastoma Risk Group
INRGSS	International Neuroblastoma Risk Group Staging System
MLPA	Multiplex Ligation Probe Amplification
MNA	*MYCN* amplification
MNG	*MYCN* gain
NB	Neuroblastoma
NCA	Numerical chromosome aberrations
NGS	Next Generation Sequencing
nonMA	non *MYCN* amplified
OS	Overall survival
PCR	Polymerase Cycle Reaction
SCA	Segmental chromosome aberrations
SIOPEN	Society of Pediatric Oncology, Europe Neuroblastoma
SNPa	Single Nucleotide Polymorphisms array
SRO	Small region of overlap
UCSC	University of California, Santa Cruz
UPD	Uniparental dysomy

COMPENDIUM OF ARTICLES THAT SUPPORT THIS THESIS

This thesis research is based on three publications as a compendium in which the candidate is the first co-author. Full text of the publications is in Annex I and the publications are the following:

ARTICLE I

Genetic instability and intratumoral heterogeneity in neuroblastoma with *MYCN* amplification plus 11q deletion.
Eva Villamón,*, **Ana P. Berbegall**,*, Marta Piqueras, Irene Tadeo, Victoria Castel, Anna Djos, Tommy Martinsson, Samuel Navarro, and Rosa Noguera.
PLoS One, 2013;8(1): e53740.
* Equal contribution.

ARTICLE II

Neuroblastoma after Childhood: Prognostic Relevance of Segmental Chromosome Aberrations, ATRX Protein Status, and Immune Cell Infiltration.
Ana P. Berbegall, Eva Villamón, Irene Tadeo, Tommy Martinsson, Adela Cañete, Victoria Castel, Samuel Navarro and Rosa Noguera.
Neoplasia, 2014; 16(6): 471-480.

ARTICLE III

Comparative genetic study of intratumoral heterogeneous *MYCN* amplified neuroblastoma with aggressive genetic profile neuroblastic tumors.
Ana P. Berbegall, Eva Villamón, Marta Piqueras, Irene Tadeo, Anna Djos, Peter Ambros, Tommy Martinsson, Inge Ambros, Adela Cañete, Victoria Castel, Samuel Navarro and Rosa Noguera.
Oncogene, 2015; Accepted for publication, April_2015.

Full text ARTICLES I to III

ARTICLE I

Genetic instability and intratumoral heterogeneity in neuroblastoma with *MYCN* amplification plus 11q deletion.

Eva Villamón,*, **Ana P. Berbegall**,*, Marta Piqueras, Irene Tadeo, Victoria Castel, Anna Djos, Tommy Martinsson, Samuel Navarro, and Rosa Noguera.

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PLOS ONE

Genetic Instability and Intratumoral Heterogeneity in Neuroblastoma with *MYCN* Amplification Plus 11q Deletion

Eva Villamón[1☯], Ana P. Berbegall[1☯], Marta Piqueras[1], Irene Tadeo[2], Victoria Castel[3], Anna Djos[4], Tommy Martinsson[4], Samuel Navarro[1], Rosa Noguera[1]*

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Abstract

Background/Aim: Genetic analysis in neuroblastoma has identified the profound influence of *MYCN* amplification and 11q deletion in patients' prognosis. These two features of high risk neuroblastoma usually occur as mutually exclusive genetic markers, although in rare cases both are present in the same tumor. The purpose of this study was to characterize the genetic profile of these uncommon neuroblastomas harboring both these high risk features.

Methods: We selected 18 neuroblastomas with MNA plus 11q loss detected by FISH. Chromosomal aberrations were analyzed using Multiplex Ligation dependent Probe Amplification and Single Nucleotide Polymorphism array techniques.

Results and Conclusion: This group of tumors has approximately the same high frequency of aberrations as found earlier for 11q deleted tumors. In some cases, DNA instability generates genetic heterogeneity, and must be taken into account in routine genetic diagnosis.

Citation: Villamón E, Berbegall AP, Piqueras M, Tadeo I, Castel V, et al. (2013) Genetic Instability and Intratumoral Heterogeneity in Neuroblastoma with *MYCN* Amplification Plus 11q Deletion. PLoS ONE 8(1): e53740. doi:10.1371/journal.pone.0053740

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Competing Interests: The authors have declared that no competing interest exist.

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☯ These authors contributed equally to this work.

Introduction

Genetic instability and the presence of genetically heterogeneous cell populations are well-known features in neuroblastoma (NB), the most common extra-cranial solid neoplasm in childhood [1 5]. This disease is characterized by a diverse behavior, and both prognosis and response to therapy can vary widely [6]. Genetic markers of NB include *MYCN* amplification (MNA) and allelic loss of 11q. In addition, presence of gains and losses of heterozygosity (LOH) have been reported for other chromosomal regions [1,7]. The use of Multiplex Ligation dependent Probe Amplification (MLPA), Comparative Genomic Hybridization (CGH) and Single Nucleotide Polymorphisms arrays (aSNP) has provided accurate and rapid identification of genome abnormalities at high resolution [8 10]. In fact, numerical chromosome aberrations (NCAs), have been associated with excellent survival, while tumors with any type of segmental chromosome aberrations (SCAs) are related to a high-risk of relapse [1,11].

MNA is present in about 17 20% of all NB cases, being associated with unfavorable cases with advanced stage disease, aggressive behavior and high-risk of relapse [6]. Intratumoral coexistence of amplified neuroblastic cells alongside non-amplified neuroblastic cells in the same tumor is regarded as heterogeneous MNA (hetMNA) [12 16] and presence of MNA in all neuroblastic cells is considered homogeneous MNA (homMNA) [13]. The study of heterogeneity according to the *MYCN* status requires single cell approach techniques such as fluorescence in situ hybridization (FISH). Several lines of investigation indicate that the q arm of chromosome 11 contains NB suppressor gene(s) [17 19]. Rearrangements in chromosome 11q occur also in approximately 30% of primary NB; they are associated with poor outcome and have recently been included as an independent risk factor in the International Neuroblastoma Risk Group (INRG) pretreatment risk classification [20]. An inverse relationship between MNA and 11q loss has been described, indicating distinct genetic subtypes of aggressive NB [19,21]. In fact, infrequent cases with MNA plus 11q loss have been described with an unexpected complexity and dramatic decline of survival rates [22].

We carried out MLPA/aSNP studies of eighteen NB cases with MNA plus 11q loss detected by FISH, to characterize chromosomal aberrations and breakpoints and to describe the genetic cell heterogeneity in these unusual cases.

Materials and Methods

Tumor Material

Between 1999 and 2007, 905 tumor samples were referred to the Spanish Reference Centre for NB Biological and Pathological studies at the time of diagnosis. Nineteen samples were selected according to FISH status results for MNA and 11q deletion. Paraffin slides were stained with hematoxylin-eosin and examined by a pathologist (SN) to evaluate the amount of neuroblastic cells, and histopathologically categorized according to the International *Neuroblastoma* Pathology Classification (INPC) [23].

This study was approved by the Experimental Research Ethics Committee of the Spanish Society of Pediatric Hematology and Oncology (SHEOP) (File number: 59C18ABR2002; EC number: 2010-021396-81). Participants or their family members/informants signed written informed consent forms.

FISH

MYCN copy number and integrity observed in chromosomal regions such as 1p36, 11q24 and 17q 22 were investigated in touch preparations and/or paraffin slides with commercial cocktail probes: *MYCN*(2p24)/*LAF*(2q11); *MLL*(11q23)/SE11; *MPO*(17-q22)ISO17q/*p53*(17p13), (Kreatech, Biotechnology) and 1p36(*D1Z2*)/centromere Chromosome 1 (Qbiogene). Assessment and interpretation of FISH results were performed according to previously published procedures [24,25].

Ploidy

DNA content was analyzed by image cytometry following the protocol as previously described with minor changes, and the INRG recommendations [13,26].

Multilocus/Pangenomic Techniques

DNA from frozen tumors was extracted using phenol/chloroform/isoamyl alcohol extraction after proteinase K treatment. More than one tumor piece was analyzed in some hetMNA cases in order to extend the description of the tumor genotype. MLPA was used as a cost-effective first approach method in the detection of frequent SCAs in NB.

The technique was performed using the SALSA MLPA Kit P251/P252/P253 developed by MRC-Holland in co-operation with International Society of Paediatric Oncology European Neuroblastoma (SIOPEN). The SALSA MLPA P251 probemix contains probes for chromosomes 1, 3 and 11; P252 probemix for chromosomes 2 and 17; and P253 probemix for chromosomes 4, 7, 9, 12 and 14. Each panel includes control probes located in chromosome regions rarely altered numerically in NB. The technique and the interpretation guidelines are described elsewhere [8,13].

For the array experiments GeneChip Human Mapping 250K arrays were used following the protocol provide by the supplier (Affymetrix, Inc., Santa Clara CA). The primary data analysis was made using GDAS software (Affymetrix), while genomic profiles were generated using CNAG (Copy Number Analyzer for Affymetrix GeneChip Mapping arrays) Version 3.0 with the AsCNAR (allele-specific copy-number analysis using anonymous references) function [27]. The UCSC genome browser, assembly February 2009 was used to visualize gene regions.

Statistical Analysis

Overall survival (OS) was defined as the time to disease-related death or last follow-up. The clinical data were compared of 28 patients suffering from tumors with homogeneous MNA without 11q deletion (homMNA w/o 11q-del) tumors [28]. Survival curves were analyzed using the Kaplan-Meier method and compared using the log-rank test.

Results

Patients and Tumor Characteristics

Sample inclusion in this study was based solely on the co-existence of the MNA and 11q deletion aberrations detected by FISH in each sample. A total of nineteen primary tumors without previous treatment were selected for study using MLPA/aSNP. Clinical features of the patient cohort are summarized in Table 1. Patient age at time of diagnosis ranged from 9 to 108 months (median 24). Most of the patients (13/19) were older than 18 months. A slight predominance of males was present (63% versus 37%). The primary tumor was abdominal in all cases. All but two cases presented advanced stages, and thirteen patients had metastases at diagnosis. Bone and bone marrow metastases were the most frequent. Patients were treated according to Spanish Society of Paediatric Oncology (SEOP)/SIOPEN protocols depending on the date of diagnosis. Time to first relapse ranged from 4 to 28 months (mean 11.4 months). Mean follow-up was 32 months. Five patients remained alive 13 to 132 months after the initial diagnosis, 3/7 patients with hetMNA and 2/12 with homMNA (Table 2). Fourteen patients died, eleven due to disease progression and three because of sepsis or complications with chemotherapy and transplant. In terms of OS (Supplementary Figure S2), in the 3-year OS rate no statistically significant differences were found between patients with either hom and hetMNA plus 11q-deleted tumors and patients with homMNA w/o 11q-del tumors (49.2% $\pm$13 versus 53% $\pm$9.5, $p = 0.335$), although the slope was somewhat delayed in the latter group. When considering the patients with homMNA plus 11q-deleted tumors and the patients suffering homMNA w/o 11q-del tumors the OS were 33.3$\pm$13 and 53$\pm$9.5 respectively ($p = 0.138$).

Regarding histopathology, according to INPC guidelines eight tumors were undifferentiated NB, ten poorly-differentiated NB and one a nodular ganglioneuroblastoma [23] (Tables 1 and 2). Tumor cell content in all samples was 60% or more.

Overview of Genetic Markers

In our series of NB analyzed by FISH, MNA was found in 18% of the samples (163 out of 905 tumors). FISH was used to detect aberrations at 1p, 11q and 17q. In this series, 1p deletion was observed in 25% of the tumors (117 out of 477 samples), 11q deletion was detected in 19% of the tumors (92 cases out of 486 samples) and 38% of the samples showed 17q gain (160 out of 420 tumors). Of the 486 cases, 19 primary tumors (4%) presented MNA and 11q loss simultaneously. Table 2 summarizes the FISH and ploidy results for the nineteen primary tumors. Of the nineteen primary tumors, seven showed hetMNA, these cases are described in further detail below. Fifteen cases showed deletion in the 1p36 region. 17q gain was observed in all but one of the fourteen cases studied by FISH. Ploidy was analyzed in sixteen samples. Nine cases were triploid, one of which presented a pentaploid population.

We describe below the limits of the *MYCN* amplicon, the shortest regions of overlap (SROs) in the most recurrent deleted and gained regions, as well as other SCA sizes detected in the original tumors. In addition we provide the genetic intratumoral heterogeneity data.

Characterization of genetic markers. A good concordance was observed between MLPA and aSNP procedures in eighteen out of the nineteen original NB, taking into account the different resolutions of the methods (Figure 1; Table 3 and Table

Table 1. Clinical characteristics and outcome.

ID	Sex	Age at diagnosis (months)	Stage	Metastases	Pathology	Protocol Treatment	Treatment Response	Relapse	Time to first relapse (months)	Outcome	Follow-up time (months)
1	M	12	1	N	pdNB	INES	CR	Y	14	DOD	15
14	M	41	2	N	nGNB	LNESG1	SurPR	Y	4	DOD	53
5	F	18	3	N	pdNB	N-II-92		N		DOS	1
8	M	33	3	N	uNB	HR-NBL1	PR	Y	28	DOD	58
15	F	9	3	N	pdNB	INES	PR	N		AWD	76
18	M	24	3	N	pdNB	HR-NBL1	PR	N		DTC	7
2	F	41	4	Y(B+BM)	pdNB	HR-NBL1		N		DOD	8
3	M	10	4	Y (B+BM)	uNB	INES	DP	Y	5	DOD	7
4	M	20	4	Y (B+BM)	uNB	NAR-99	DP	Y	12	DOD	12
6	M	19	4	Y (LN)	uNB	NAR-99	VGPR	N		ADF	132
7	F	108	4	Y(B+BM)	pdNB	HR-NBL1	DP	N		DOD	10
9	M	20	4	Y (B+ST)	uNB	HR-NBL1	VGPR	Y	9	DOD	12
10	F	52	4	Y(B+BM)	pdNB	HR-NBL1		N		DTC	3
11	M	14	4	Y (BM)	uNB	NAR-99	CR	Y	9	DOD	10
12	F	40	4	Y (B+BM)	pdNB	HR-NBL1	PR	Y	18	DOD	42
13	M	96	4	Y (B+BM)	pdNB	HR-NBL1	PR	N		AWD	13
16	M	50	4	Y (B+BM)	uNB	HR-NBL1	CR	Y	4	DOD	27
17	F	15	4	Y(B+BM+LN+ST)	pdNB	HR-NBL1	VGPR	N		AWD	48
19	M	41	4	Y (B+BM)	uNB	N-II-92	CR	N		ADF	77

M, male; F, female; N, no; Y, yes; B, bone; BM, bone marrow; LN, lymph nodes; ST, soft tissue; uNB, undifferentiated neuroblastoma; pdNB, poorly differentiated NB; nGNB, nodular ganglioneuroblastoma HR-NBL1, High-Risk Neuroblastoma Study 1;INES,Infants Neuroblastoma European Study, SIOPEN protocols; CR, complete response; VGPR, very good partial response; PR, partial response; DP, disease progression; SurPR, surgical partial resection; DOD, died of disease; DOS, died of sepsis; DTC, died of treatment complication, AWD, alive with disease; ADF, alive disease-free.
The patients have been listed according to the stage of disease. ID was assigned according to genetic aberrations of chromosome 2.
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Table 2. Histopathological and genetic characteristics of the tumors.

		FISH results				
ID	**Pathology**	**% MNA**	**% 11q loss (% 11q imbalance)**	**%1p loss (% 1p imbalance)**	**% 17q gain**	**Ploidy**
1	pdNB	95	60	25 (10)	60	T
14	nGNB	<5 (het)	60	0	ND	ND
5	pdNB	98	80	90	80	D
8	uNB	95	65	10 (10)	65	T
15	pdNB	5 (het)	50	20	80	T
18	pdNB	90	20	0 (10)	50	D
2	pdNB	95	20 (50)	ND	ND	TE
3	uNB	95	65	95	65	D
4	uNB	90	35	15 (80)	50	BD -TE
6	uNB	95*	20	31 (64)	50	T
7	pdNB	98	20	98	0	T
9	uNB	95	50	90 (10)	60	T
10	pdNB	90	20	80	50	D
11	uNB	15 (het)	35 (35)	60 (20)	90	T
12	pdNB	30 (het)	50	10 (5)	60	T
13	pdNB	10 (het)	70	ND	ND	D
16	uNB	35 (het)	45	40	ND	T-P
17	pdNB	15 (het)	25	20 (70)	ND	ND
19	uNB	98	50	50	60	ND

uNB, undifferentiated neuroblastoma; pdNB, poorly differentiated NB; nGNB, nodular ganglioneuroblastoma; %, percentage; het, heterogeneous; ND, not done; D, diploid; BD, borderline diploid, T, triploid; TE, tetraploid; P, pentaploid.
All cases presented dmin except case number 5 (* dmin plus HSR).
The patients have been listed according to the stage of disease. ID was assigned according to genetic aberrations of chromosome 2.
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S1). Using aSNP results a high number of SCAs were found in the majority of the cases. The mean SCA of all the cases was 10.7 (range 4 23) (Figure 1 and Table S1). Figure S1 shows a graphic representation of MLPA/aSNP results in case 3 as an example.

Fifteen cases showed MNA by multilocus/pangenomic techniques. A great variability in the total region size of the *MYCN* amplicon (from 600 kb to 6.3 Mb) and amplicon gene composition between the tumors was found in both homMNA and hetMNA (Figure 1 and Table S1). Two different MNA patterns were found: simple amplification with one continuous region amplified (ten cases; three of which were hetMNA) and complex rearrangements with several discontinuous amplification regions (five cases; see Figure 1 cases indicated with *, and Figure 2A, 2B). Five out of fifteen cases showed both MNA and 2p gain. Three groups can be formed according to the genes included in the amplified *MYCN* region: only *MYCN* gene (case 5), *DDX1 MYCN*

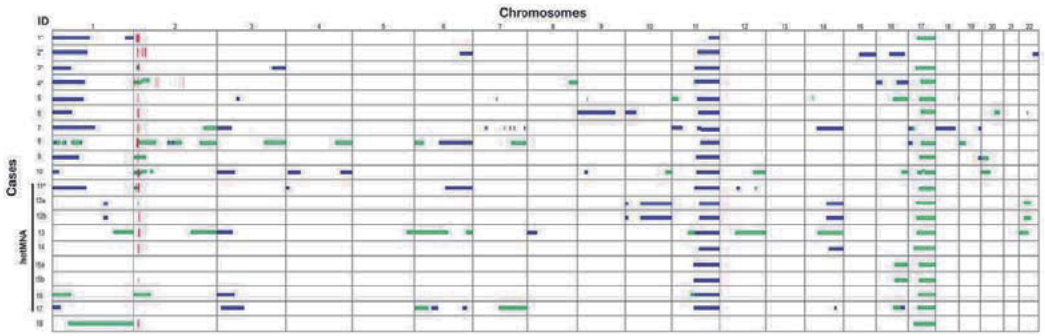

Figure 1. Summary of aSNP data of segmental chromosome alterations. Horizontal lines show segmental loss (blue) and gain (green). Red-vertical lines show amplification. Cases with hetMNA are indicated to the left. Complex MNA are marked by*. The cases have been listed according to chromosome 2.aberrations.
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Table 3. MLPA data.

ID	MLPA results
1	1p ;1q ; MNA; 11q ; +17q
2	1p ; MNA; 11q
3	1p ; MNA; +3q; 11q ; +17q
4	1p ; MNA; +2p; 11q ; +17q
5	1p ; MNA; 3p ; 9p ; +11p; 11q ; +17q
6	1p ; MNA; 9pq ; 11q ; +17q
7	1p ; MNA;+2q; 3p ; 11q ; 14q ;17p ; +17p
8	1p ;+1p; MNA; +2p; +2q; +3q; +4q; +7q; 11q ; 17p ;+17q
9	1p ; MNA; +2p; 11q ; +17q
10	1p ; MNA; +2p; 3p ; 4p ; 4q ; 9p ; 11q ;+17q
11	1p ; MNA; +2p; 6q ; 11q ; +12q; +17q
12a	*MYCN* gain; 11q ; 14q ; +17q;
12b	MNA; +11p, 11q ; 14q ; +17q
13	+1q; MNA; +2q; 3p-; +11p; 11q ; +12q; +14q; +17q
14	MNA; 11q ; 14q-; +17pq
15a	11q ; +17q
15b	*MYCN* gain; 11q ; +17q
16	+1p; +2p; 3p ; 11q ; +17q
17	1p ; 3p ; +7q; 11q ; 14q +17q
18	+1pq; MNA; +17q

INRG Biology Committee definitions [11]. *MYCN* amplification (MNA), up to 4-fold excess of signal numbers of the chromosomal region of interest compared with the reference signals; Gain (+), unbalanced ratio (high signal excess) between the signals of a gene and all other probes located on the same chromosome; Loss ( ), unbalanced ratio (low signal excess) between the signals of the chromosomal region of interest (at least two adjacent probes) and the reference signals (at least two) of the chromosomal region of interest. The cases have been listed according to chromosome 2 aberrations using pangenomic techniques.

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gene (cases 4 and 6), and *DDX1-MYCN* with *NBAS* (entirety or in part) genes (twelve samples). In the first two groups, most telomeric breakpoints of the *MYCN* amplicon were located slightly proximal to the fragile site *FRA2C*tel at 2p24.3. In the third group the distal breakpoints were mapped within *FRA2C*tel, a fragile site located at 2p24.2 (eight cases) or located more distally to this critical site (four cases) (Figure 2A). Case 2 presented an amplified peak at p23.2-23.1 containing the whole gene *ALK* within. Case number 4 showed one small amplification (2q 31.3 32.1) concomitantly with four amplification peaks in 2p region (Figure 2B). MLPA/aSNP verified deletion of the long arm of chromosome 11 in seventeen cases. The consensus region of 11q loss was from 111.7 to 134.5 Mb (q23-qter), SRO being 22.8 Mb. The mean size of 11q loss was 53.7 (range 22.8 64.9 Mb). The deleted region was uninterrupted in all cases except in case 7 (two breakpoints were displayed) (Figure 3A).

Twelve cases showed 1p loss using MLPA/aSNP techniques confirming the FISH results (Figure 3B). The consensus region of 1p loss ranged from 3.5 to 8.6 Mb, with 5.1 Mb of SRO. The mean size of 1p deletion was 65.3 Mb. Continuous deletion along 1p36 region was present in eleven cases with a larger deleted region in ten cases (deletion size >20 Mb) (Figure 3B). Case 8 presented complex chromosome 1 rearrangements with up to 7 SCAs in 1p-arm. Gain of 1q was observed in case 13. Loss of 1q was detected as terminal (case 1) and interstitial (case 12) (Figure 1 and Table S1). Case 18 presented a large gain region covering both the p and the q arms.

Sixteen cases presented 17q gain. In addition, case 8 showed 17p loss. In case 7 17p loss was observed (pter-14.4 Mb) by MLPA/aSNP without associated 17q gain (Figure 1 and Table S1). The mean 17q gain was 45.3 Mb. The consensus gained segment was rather large, extending from 47.1 Mb to qter (37.1 Mb) (Figure 3C).

Deletion in 3p was observed in 33% of cases (6/18). In cases 5 and 17, interstitial deletions were observed, while the rest of the cases showed common terminal 3p deletions. In case 10, deletions in both arms of chromosome 4 were seen. A small 4p deletion was observed in case 11 (size 10 Mb). Cases 5 and 10 showed an interstitial 9p deletion of 0.4 and 12.2 Mb respectively. A hemizygotic deletion of *CDKN2A/B* and *MTAP* genes, located in 9p21.3, was observed in case 5. Other chromosome rearrangements were found in several cases (Figure 1 and Table S1).

An aSNP allele analysis revealed several large copy neutral loss of heterozygosity (CN-LOH) in different chromosomes. CN-LOH was detected in four cases (1, 5, 10 and 12), although without satisfying the requirement for matched constitutional DNA. In case 1, eight chromosome regions presented CN-LOH (1q, 2p, 5q, 7p, 9p, 9q, 10p and 17p). Cases 5, 10 and 12 presented CN-LOH in chromosome 9p, 11p and 18q respectively.

Intratumoral heterogeneity. SCAs diagnosed by presence or absence of specific DNA sequences using fluorescent probes can be complex, with a miscellany of clones, some of which can remain hidden when using MLPA/aSNP approaches, resulting in different genetic states associated with the *MYCN* gene, and 11q, 1p and/or 17q chromosome regions within a single tumor. Intratumoral heterogeneity was present in cases 11 to 18 (42%) and considered as heterogeneous SCA (hetSCA) cases. Total percentages of neuroblasts with SCAs detected by FISH in the tumor cohort are presented in Table 2. Details of the different frequencies of clusters with SCA, NCA as well as disomic cells seen in the hetSCA tumors are presented in Table 4. Co-existence of cells with SCA along with NCA cells in the hetSCA tumors was often present for all analyzed markers. Co-existence of hetMNA and het11q-del was present in the 31.5% of the cases (six out of nineteen samples) analyzed by FISH. However, cases 14 and 15 showed co-existence of MNA neuroblastic cells and disomic tumor cells confirmed by histopathological study (data not shown). Multilocus/pangenomic data are shown in Figure 1, Table 3 and Table S1. MNA was detected in fourteen out of eighteen (77.8%) of the cases with these approaches. Regarding detection of hetMNA status (cases 11 to 17), multilocus/pangenomic techniques were accurate in three cases. Four out of seven hetMNA cases showed MNA in some of the pieces analyzed by MLPA/aSNP (cases 11 to 14). Case number 15 presented *MYCN* gain in one of the two pieces studied. A 2p gain of a large region (56 Mb) was detected in case 16. No SCA involving 2p was found in case 17. Three hetMNA cases (samples 12, 15, 16) were diagnosed as having heterogeneous 1p deletion. The percentage of tumor cells with this alteration by FISH ranged from 10 to 40%. Heterogeneity of 11q deletion (het11q-del) was found in case 18 (5.5%), where this aberration was revealed by FISH in only 20% of tumor cells, but was not shown by multigenomic analysis.

Discussion

NB tumors show a diverse behavior, and an algorithm of clinical, histopathological, and genetic factors has stratified the risk and delineated therapeutic decisions [6,13,20]. Nevertheless, NB demands improved characterization and a better understanding of

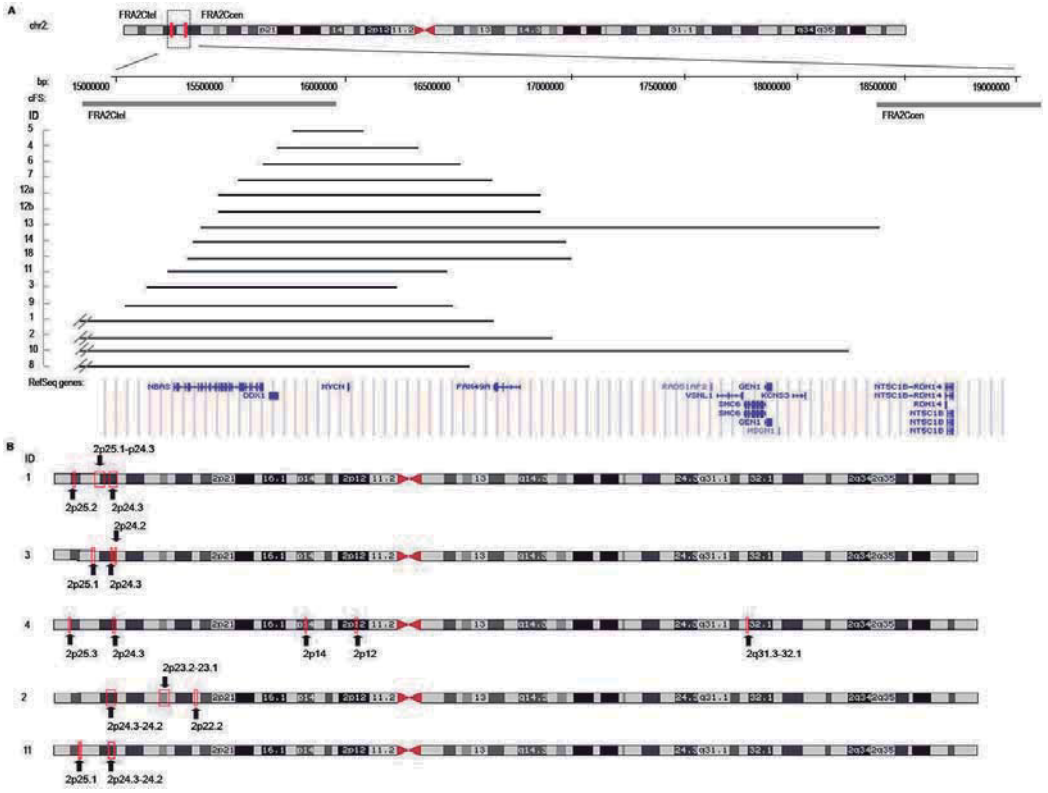

Figure 2. Schematic representation of the amplicons in chromosome 2. All data are illustrated according to the NCBI build Hg19. (A) Genes included in the *MYCN* amplicon. The crossed lines in cases 1, 7, 9 and 10 indicate that the breakpoint mapped more distal in chromosome 2 than the area represented. (B) Boxes and arrows indicate position of the different amplicons in the cases with complex amplification. The cases have been listed according to chromosome 2 aberrations.
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how tumor biology drives specific clinical behavior, especially for those patients identified as having a high-risk phenotype [6]. The present study analyzes, for the first time, the genetic characteristics in the largest cohort to date of unusual neuroblastic tumors carrying MNA plus 11q deletion, using the aSNP technique in addition to FISH and MLPA techniques to obtain high resolution detection and mapping of numerical and structural genomic changes in these tumors.

MNA and allelic loss of 11q with single copy of *MYCN* are associated with advanced stage NB, and both are independent prognostic indicators for clinically high-risk patients [19,22,29]. Despite this, hetMNA status is not prognostically interpretable worldwide, and the effect of 11q loss on NB biology is not sufficiently clear [15,21]. Several hypotheses have already been published related to the aggressiveness of hetMNA clone(s), varying from a 'premalignant' status lacking malignant properties to an invasiveness and metastatic potential comparable to homMNA tumors [12,15,16,30]. Of the surviving patients described in this study, 60% correspond to cases with hetMNA; however, no significant prognostic differences in survival were found between patients with homMNA plus 11q-deleted tumors and the patients with hetMNA plus 11q deletion tumors. The homMNA w/o 11q-del and hom and hetMNA plus 11q-deleted groups were similar in terms of overall survival in the analyzed cohort. Previous studies showed a worse outcome in the group of MNA tumors plus 11q loss than in patients with MNA alone [22]. These differences could be due both to the presence of hetMNA tumors in our cohort and the low number of cases with homMNA. In fact, the survival analysis excluding hetMNA tumors showed a trend toward statistical differences in OS rates, suggesting a less aggressive nature for tumors with hetMNA than tumors with homMNA. The SIOPEN is now studying the dilemma created by these unusual cases. Studies of a large number of samples have shown differences in age at diagnosis between the MNA group and 11q-deleted group [22,29]. In a recent study, the median age at diagnosis in MNA cases was lower than in the 11q loss group [19]. In the cohort of infrequent tumors herein presented, the median age at diagnosis was comparable to that of the group with MNA. A significantly higher frequency of chromosomal breaks has been reported in NB with 11q deletion than in MNA NB cases (12 versus 4) [19]. The cases analyzed here shared a high number of SCAs (mean 10.7; median 10) with the 11q-deleted group.

Both simple and complex types of MNA amplicon organization have been described using the aSNP technique in NB [31 33].

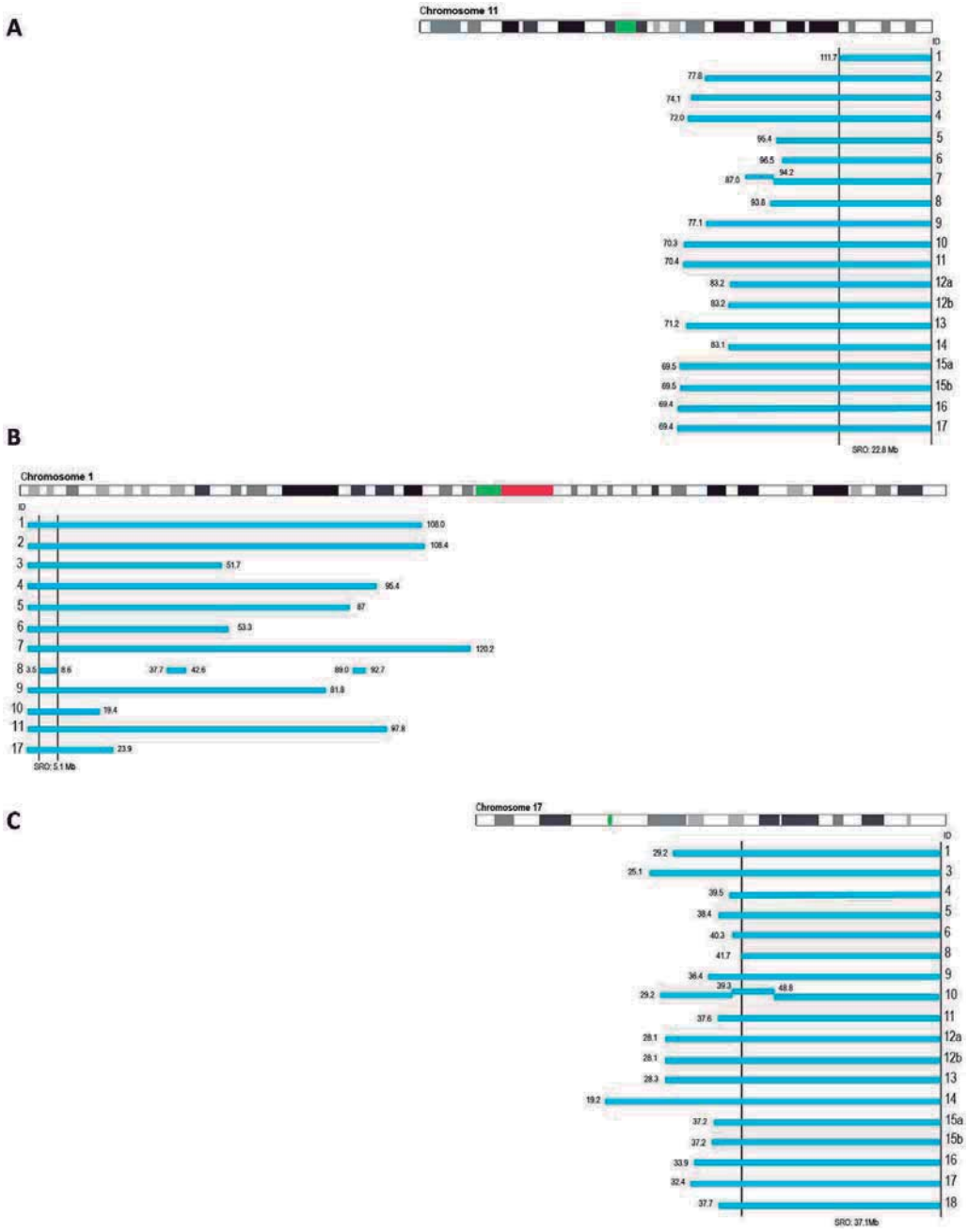

Figure 3. Graphic representation of chromosomes 11, 1 and 17. Bars illustrate the deleted/gain region. The positions of the breakpoints are indicated in megabases. (A) Deletions of chromosome 11q. SRO: 1117.7 to qter (22.8 Mb). (B) Deletions of chromosome 1p. SRO: 3.5 to 8.6 Mb (5.1 Mb). (C) Gain of chromosome 17q. SRO: 41.7 to qter (37.1 Mb). The cases have been listed according to chromosome 2 aberrations.
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Table 4. FISH results in the heterogeneous cases (hetMNA and het11q del).

ID	MYCN	11q	1p	17q
11	**5:35 15%;** 5:5 80%; 2:2 5%	**2:1 35%; 3:2 35%;** 3:3 15%; 2:2 15%	**(4:1, 5:1, 6:1) 60%;** (4:2,5:2,6:2,4:3,5:3,6:3) 20%; 2:2 20%	**2:4 30%; 3:5 30%; 3:4 30%;** 2:2 10%
12	**2:50–100 dmins 10%, 4:50–100 dmins 20%;** 4:4 40%; 2:2 30%	**2:1 25%; 3:1 25%;** 3:3 10%; 2:2 40%	**2:1 10%; 4:2 5%;** 4:4 35%; 2:2 50%	**2:3 50%; 2:4 15%; 2:5 15%;** 2:2 20%
13	**2:>100 dmins 10%;** 2:3 40%; 4:4 10%; 2:2 40%	**3:1 70%;** 3:3 10%; 4:4 5%; 2:2 15%	ND	ND
14	**2:20 dmins <5%;** 2:2 95%	**2:1 60%;** 2:2 40%	3:3 10%; 4:4 15%; 2:2 75%	ND
15	**2:>100 dmins 5%;** 2:2 95%	**2:1 25%; 3:1 25%;** 3:3 10%; 2:2 40%,	**2:1 20%;** 2:2 80%	**2:3 50%; 2:4 15%; 2:5 15%;** 2:2 20%
16	**(2:10,2:12) 35%;** (4:6,2:3,2:6) 30%; 2:2 35%	**2:1 15%; 3:1 30%;** 3:3 15%; 2:2 30%	**2:1 40%;** 2:2 60%	ND
17	**4:>50 dmins 10%; 2:>50 dmins 5%;** 4:4 60%; 8:8 5%; 2:2 10%	**3:1 25%;** 3:3 15%; 2:2 60%	**4:1 10%; 2:1 10%;** 4:2 70%; 2:2 10%	ND
18	**3:>100 dmins 20%, 2:>100 dmins 20%, 4:>100 dmins 50%;** 2:2 10%	**2:1 20%;** 2:2 80%	**2:3 10%;** 4:4 10%; 2:2 80%	**2:3 50%;** 2:2 50%

Disomic cells (ratio 2:2) and numeric alterations (ratios 3:3; 4:4…), balanced ratio between the signal numbers of chromosomal region of interest and the reference signals on the opposite arm of the chromosome; Cells with gain (ie ratios 2:4; 3:4…), signal numbers of the chromosomal region of interest exceed up to 4-fold the number of reference signals; Cells with imbalance (ie ratios 3:2; 4:3…), imbalance ratio between the signal numbers of chromosomal region of interest and the reference signals with more than 1 signals of chromosomal region of interest; Cells with deletion (ie. ratios 2:1; 3:1 4:1…), unbalanced ratio between the signal numbers of the chromosomal region of interest and the reference signals with only 1 signal of the chromosomal region of interest; hetMNA, occurrence of clusters or as single cells with amplification (at least five cells per slide) surrounded by non-amplified tumor cells.
ND, not done.
The cases have been listed according to chromosome 2 aberrations using pangenomic techniques.
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The copy number of the *DDX1* gene, located in the close vicinity of *MYCN*, has controversial prognostic implication and was amplified in all but one of the cases analysed [32,34 38]. *NABS*, overexpressed via *MYCN* co-amplification, may perturb the quantitative balance of complexes involved in cell-cycle-related events and membrane trafficking [36]. This gene was also frequently co-amplified with *MYCN* in our series. Co-amplification of the entire *ALK* gene is reported to occur rarely, it is observed only in patients with poor outcome, and is not a statistically significant independent marker for survival [39]. In this series, only one case presented *ALK* amplification. Recently, Blumrich et al. [40] suggested that *MYCN* amplicons arise from extra replication rounds of secondary DNA structures accumulated at *FRA2C*tel and/or *FRA2C*cen. Genomic location of the *FRA2C* subregion has been described, being able to accurately define the borders of the region using BAC clones. The complex genomic rearrangements in the boundaries of the common fragile sites (cFS) has led to the proposal of a dual role for the cFS in the generation of gross chromosomal rearrangements, either after DNA breakage or by inducing extra replication rounds [40]. Our results are consistent with this study and revealed that most amplicon borders were clustered within the *FRAC2*tel, and that most of the proximal amplicon borders were located in the region spanning 15.5 and 16.6 Mb on 2p. An association between MNA and 2p gain has been described previously [31,32,41]. In our study, 33% of the tumors with MNA presented 2p gain using MLPA/aSNP but, and in agreement with other studies, no relevant clinical differences in overall survival were observed between these samples and the samples without 2p gain (data not shown) [41].

In 11q-deleted cases, different candidate suppressor genes have been proposed [17 19]. The loss of one copy of the *H2AFX* gene (11q23.2 q23.3) has been described as the factor responsible for the genetic instability in the 11q-deleted NB due to its role in double-strand break repair and cancer susceptibility [19]. This hypothesis implies accepting that the 11q deletion event must occur early in tumorigenesis, despite the relatively old age of patients seen in some studies. However, studies of gene expression profiles suggest that 11q loss is acquired in unfavorable NB as a result of selective pressure and confers specific properties to the tumor, while MNA would be an earlier event [21,42]. All the patients of the infrequent group described in this study presented tumors with neuroblasts with losses of gene *H2AFX* and with homMNA or hetMNA along with genetic instability and young age at diagnosis. We can hypothesize that a younger age can be related to loss of gene *H2AFX* and hetMNA as early events in tumorigenesis.

Simultaneous genomic imbalances can strengthen one another or act in combination, which may affect other perfectly-balanced genomic regions [43]. Deletions of chromosome arm 1p have been well characterized, and different SROs have previously been reported in NB, occurring in approximately 30 35% of primary NB and being highly correlated with MNA [6,19]. There is evidence that one or more tumor suppressor genes involved in NB initiation and/or progression are localized to this region [44]. Tumors with MNA have 1p deletions extending proximal to 1p36, while *MYCN* non-amplified tumors, including cases with 11q loss, more often have small terminal deletions of 1p36 [19,31]. Caren et al. [31] located an interstitial SRO between 17.2 Mb and 37 Mb for the MNA tumors and an SRO from 0 to 10.4 Mb for the second group. A larger study established the SRO at 5.3 Mb and 6.1 Mb [19]. Our SRO apparently matches with the SRO previously reported in tumors without MNA. Recently, a recurrent 17.9 Mb terminal deletion on distal chromosome 1q has been reported in association with high-risk NB with 11q deletion without MNA [45]. In our study, only one case presented a similar terminal deletion (19 Mb, q42.13-qter). Two possible candidate tumor suppressor genes located within the deleted segment are *FH* (fumarate hydratase) and *EGLN1* (EGL nine homolog 1), implicated in other types of tumors [45]. Furthermore, different experiments suggested that chromosome 1q gain, seen in one case of this cohort, is associated with poor outcome in NB patients [1].

It is not surprising that the majority of the cases presented gain of 17q. Gain of genetic material on the long arm of chromosome 17 is very common in aggressive NB, irrespective of presence or absence of MNA. 17q gain and the breakpoint position on 17q may influence tumor behavior [46]. In our study, the MLPA/aSNP profile was confirmatory of a single 17q breakpoint (leading to a single region of gain) in all cases but one. The resulting copy number gain for 17q invariably implicated a large segment encompassing at least the distal part from approximately 41.7 Mb to qter. These results are in keeping with the results obtained in other high resolution studies where various oncogenes have been proposed [46]. Critical genes sensitive to a gene dosage effect contributing to NB pathogenesis could be located in the SRO (37.1 Mb) of our study. In addition, three of our cases presented 17p loss, an alteration that has been related with chemoresistant NB [47].

Loss of chromosome 3p is a frequent event in NB, occurring preferentially in tumors that exhibit loss of 11q [22]. In this study, samples showing loss of 3p had patterns equivalent to those previously described. Critical regions of minimal 3p loss contain multiple candidate tumor suppressor genes, which may contribute to NB pathogenesis [31,48]. Other typical SCA with unclear prognostic significance in NB, such as deletion of 4p or 9p, were detected in the tumors analyzed [1]. Although, CN-LOH and homozygous deletions are rare events in NB, some cases have been reported and were present in the tumor cohort analyzed. Their clinical impact is unknown [31,49]. As no matched constitutional DNA was investigated for any of our samples, no conclusion about acquired or inherited CN-LOH can be drawn.

High intra-sample genomic heterogeneity, characteristic of some NB, as well as stromal DNA contamination may explain discordant results between the techniques [8,50]. When using multilocus/pangenomic techniques to analyse tumor samples, intratumoral genetic hetereogeneity, complex patterns of CNA, LOH events and polyploidy as well as normal DNA contamination effects must be considered. Current algorithms, although robust computational methods, try to correct for the presence of normal cells allowing at the same time the detection of CNA occurring in only a subset of cells. Up to now it has often been proved to be sub-optimal [27,51]. Especially in NB, a single statistical framework able by itself to overcome the complex genetic heterogeneity should be used to improve the analysis of tumor pangenomic data. In this regard, FISH has a higher sensitivity because it detects the gene or region copy number at the single cell level and allows the correlation of morphologic details, while at the same time it is not affected by stromal contamination. As we present here, hetSCAs were not observed easily by MLPA/aSNP. Thus far, hetSCA status in NB can remain masked and is therefore difficult to diagnose, creating the need for additional information for diagnosis, prognostication and therapeutic decision making [12,15,30].

In summary, we have made a detailed study of a subgroup of neuroblastoma with both MNA and 11q deletion. We found that this group of tumors has approximately the same high frequency of aberrations as earlier found for 11q deleted tumors, albeit not higher. These tumors represent a valuable model of genetic instability, revealing intratumoral heterogeneity in NB. An in-depth investigation of the genome of these same cases should be made in future studies, using new generation sequencing techniques to identify candidate genes associated with tumor progression and potential target genes for drug therapy in this subgroup of high-risk patients.

Supporting Information

Figure S1 Graphic representation of MLPA/aSNP results in case number 3. Chromosomes with segmental aberrations are indicated by an arrowhead. (A) Graphic results obtained using NB probemixes P251, P252 and P253. The thresholds for loss and gain detection were set at 0.75 and 1.25, respectively. Normal values are showed in yellow bars, gains and amplification in green bars and losses in red bars. (B) Whole genomic profile of the aSNP and single view of chromosome 2p arm. The figure displays the sequential amplicons detected.
(TIF)

Figure S2 Kaplan-Meier overall survival curves. (A) Patients with hom and hetMNA plus deleted tumors (n = 16) and homMNA w/o 11q-del tumors (n = 28); 3-year overall survival: 49.2% ±13 versus 53% ±9.5, p = 0.335. (B) Patients with homMNA plus 11q-deleted tumors (n = 9) and homMNA w/o 11q-del tumors (n = 28); 3-year overall survival 33.3±13 versus 53±9.5, p = 0.138.
(TIF)

Table S1 Summary of aSNP data of segmental chromosome alterations. The table shows the sizes of different segments with amplification (red), gain (green) or loss (blue). Complex MNA are marked with an asterisk * in the first row 'ID
(TIF)

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Author Contributions

Conceived and designed the experiments: TM SN RN. Performed the experiments: EV APB MP IT AD. Analyzed the data: EV APB MP IT VC AD TM SN RN. Contributed reagents/materials/analysis tools: TM SN RN. Wrote the paper: EV APB TM RN.

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Fig. S1

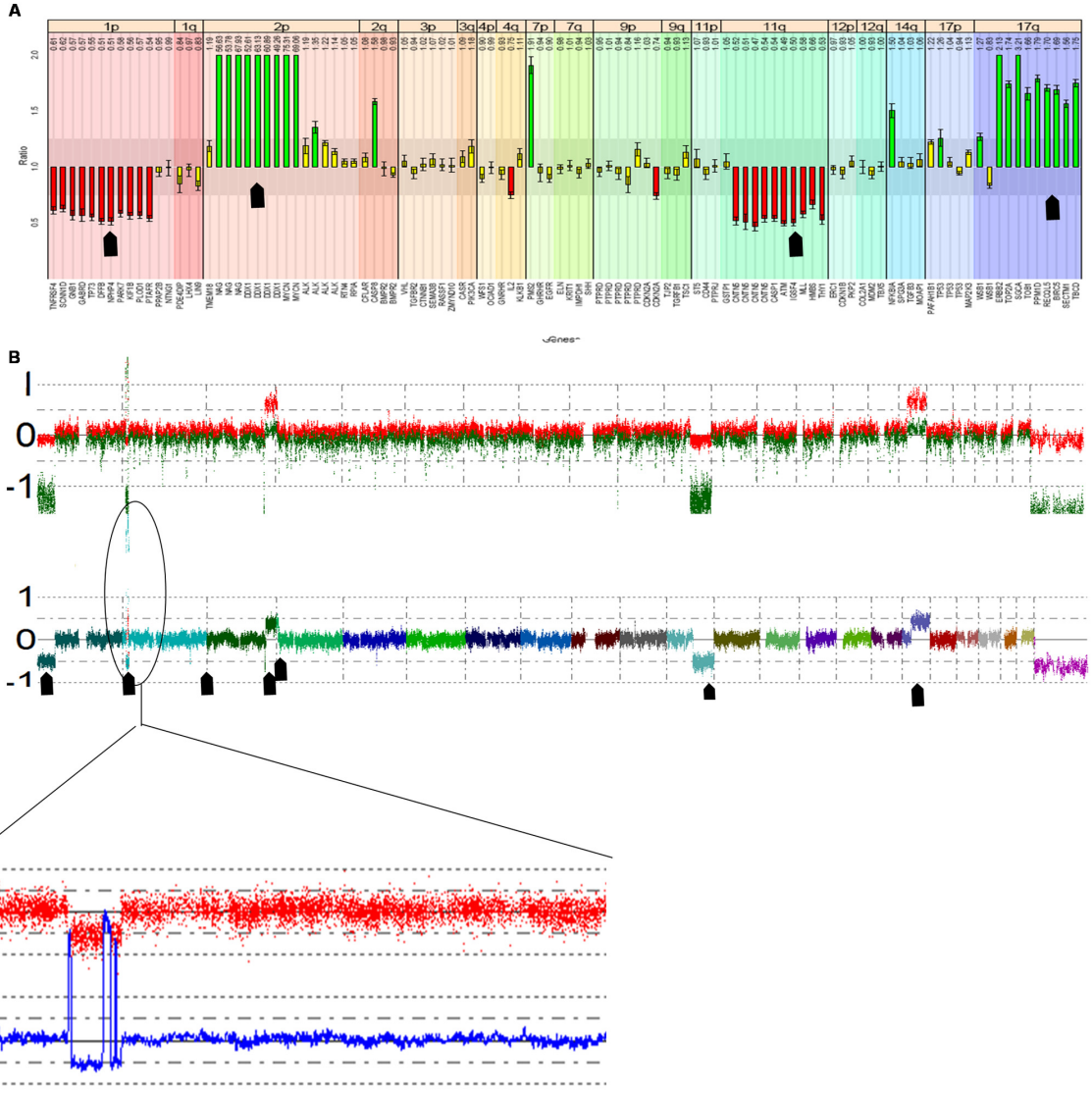
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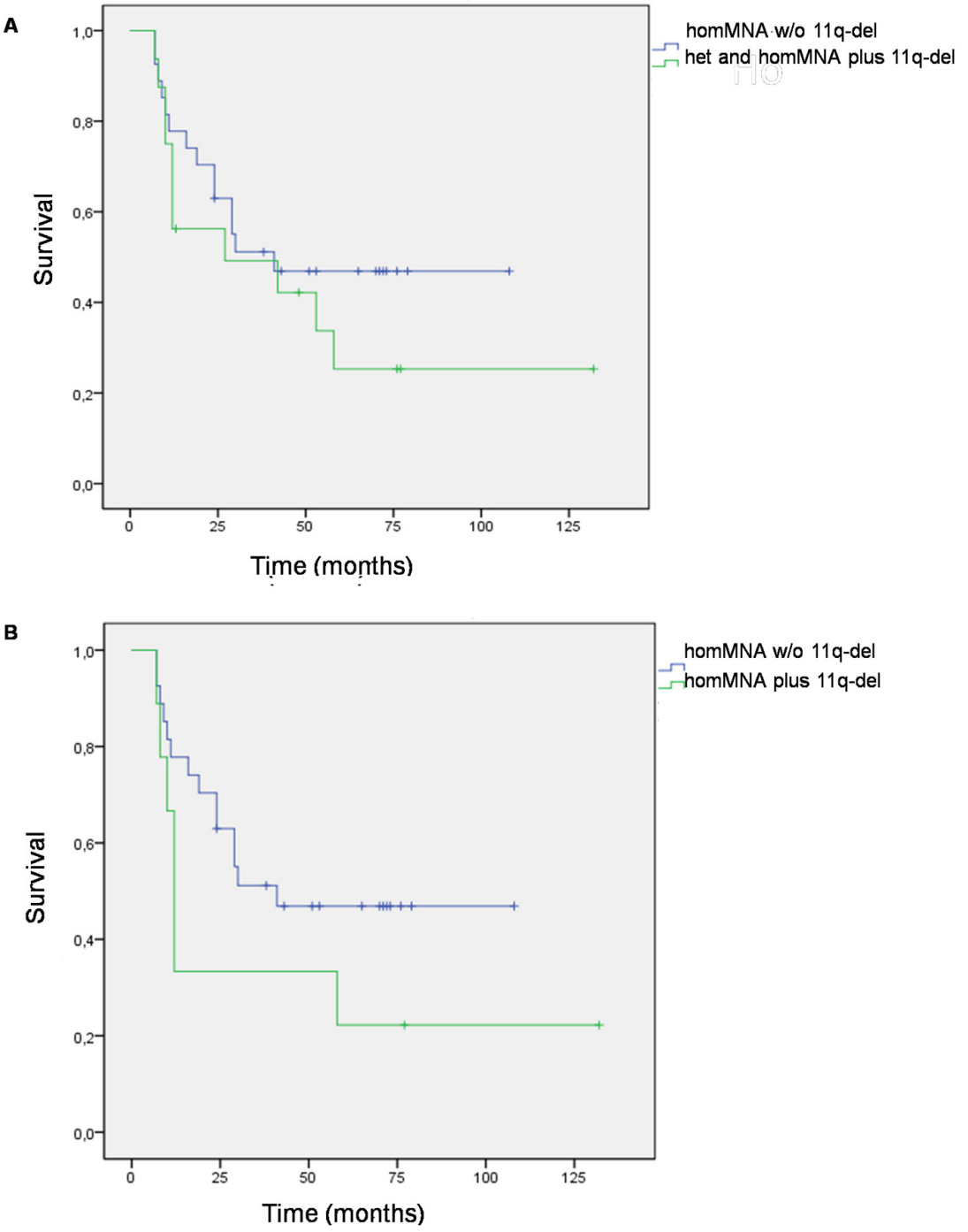
Fig. S2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Time (months)
homMNA w/o 11q-del
het and homMNA plus 11q-del
B
Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
25
50
75
100
125
Time (months)
homMNA w/o 11q-del
homMNA plus 11q-del

-XXX-

Table S1
Summary of SCA aSNP data

hetMNA

ID		1*	2*	3*	4	5	6	7	8	9	10*	11*	12a	12b	13	14	15a	15b	16	17	18
Chromosomes	p	pter-108.0	pter-51.7	pter-95.4	pter-87.0	pter-53.3	pter-120.2	pter-3.5 3.5-8.6 8.6-22.9 27.4-37.7 37.7-42.6 43.7-89 89-92.7	pter-81.8	pter-19.4	pter-108.4	pter-97.8							pter-52.8	pter-23.9	pter-48.6
1	q	226.7-qter											154.4-161.3	154.4-161.3	189.2-qter						
	p	5.4-5.7 10.7-13.7 14.5-16.8	10.4-11.0 11.0-15.3 15.3-16.5 16.5-16.8 16.8-17.3 17.3-17.8	pter-3.9 3.9-4.4 4.4-15.6 15.6-16.6 16.6-21.2 21.2-50.0 67-67.3 79.71-80.5	15.6-16.2	15.6-16.6	15.4-16.8	10.4-16.7 16.7-69.7	pter-15.0 15-16.4 16.4-41.4	pter-14.0 14-18.3 18.3-25.1 25.1-43.4 53.8-58.9	14.4-17.0 28.34-30.66 37.4-38.3	pter-7.3 7.3-8.0 8.0-15.2 15.2-17.0	15.3-16.9	15.3-16.9	15.4-18.8	15.4-17		15.5-16.5	pter-56.0		14.4-15.4 15.4-16.5
2	q			182.5-183.5			234.0-qter	114.3-121.7 121.7-128.7 128.7-134.5 134.5-161.4 222.6-240.0							189.72-243.0						
3	p				60.0-66.0		pter-45.0			pter-54.2					pter-50.0				pter-67.0	10.3-79.5	
	q		165.4-166.8 168.6-qter					142.1-qter													
4	p		pter-5.21							6.0-44.8		pter-10.1									
	q							145.0-qter		162.0-qter											
5	p																				
	q														154.7-180.6						
6	p							pter-26.8							pter-113.6 155.6-qter					pter-44.0 54.0-71.2 145.0-157.0	
	q							73.1-qter			136.4-163.5	108.9-qter									
7	p						34.8-37.5 40.4-41.3														
	q				68.5-69.7		93.2-97.6 108.0-116.9 119.7-137.2 151-153.6	123.4-qter												75.9-qter	
8	p														pter-29.3						
	q			120.4-qter																	
9	p				21.8-22.2					20.0-32.2											
	q					pter-110															
10	p					pter-30.6							pter-8.1	pter-8.1							
	q									121.7-qter			44.6-qter	44.6-qter							
11	p				pter-27		pter-30														
	q	111.7-qter	74.1-qter	65.9-72.0 72.0-qter	95.4-qter	96.5-qter	87.0-94.2 94.2-qter	93.8-qter	77.1-qter	70.3-qter	77.8-qter	68.2-70.4 70.4-qter	83.2-qter	83.2-qter	51.4-71.2 71.2-qter	83.1-qter	69.5-qter	69.5-qter	60.0-69.0 69.4-qter	69.4-qter	
12	p											51.0-62.0									
	q									112.5-qter		108-113.1			45.3-qter						
13	q																				
14	q				21.4-22.2		35.5-qter						61.0-qter	61.0-qter	61.0-qter	67.2-qter				96.0-106.0	
15	q										54.1-qter										
16	p			pter-24																	
	q			72.0-qter	55.0-qter					79.3-qter	45.3-88.7						66.0-qter	66.0-qter		54.3-76.9 77.1-88.6	
17	p						pter-14.4 15.2-22.6	pter-14.5								19.2-qter					
	q	29.2-qter	25.1-qter	39.5-qter	38.4-qter	40.3-qter		41.7-qter	36.4-qter	29.2-39.3 39.3-48.8 48.8-qter		37.6-qter	28.1-qter	28.1-qter	28.3-qter		37.2-qter	37.2-qter-	33.9-qter	32.4-qter	pter-37.7
18	p																				
	q				74.8-qter		pter-61.0														
19	p							pter-16.9													
	q						46.6-qter		52.0-qter												
20	p								pter-20.4	pter-26.2											
	q					52.4-qter															
21	q																				
22	q					23.7-24.2					39.8-qter		15.5-34.3	15.5-34.3	pter-25.0						
N° breaks		7	12	16	11	8	18	23	8	18	9	12	8	8	14	4	3	4	6	11	4

Red = amplification; Green = gain; Blue = loss. Complex MNA are marked with an asterisk * in the first row 'ID'.

ARTICLE II

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

Ana P. Berbegall, Eva Villamón, Irene Tadeo, Tommy Martinsson, Adela Cañete, Victoria Castel, Samuel Navarro and Rosa Noguera.

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Neuroblastoma after Childhood: Prognostic Relevance of Segmental Chromosome Aberrations, ATRX Protein Status, and Immune Cell Infiltration[1]

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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-thalassemia/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.tcaq.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 CDs and ATRX and the dilutions used are listed in Table 2. Formalin-fixed paraffin-embedded 4-μm 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 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).

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

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	Y
5			SCA	5	N	2	Y	Y	N	N
6			SCA	12	N	5	Y	Y	N	N
7			SCA	8	N	2	Y	Y	N	N
8			NCA	0	N	12	N	N	N	N
12			SCA	17	Y	0	N	Y	Y	Y
13			SCA	5	N	1	Y	Y	Y	N
15		Adolescents	SCA	11	Y	1	Y	Y	Y	N
16			SCA[a]	3[a]	N	1[a]	Y	Y	ND	ND
20			SCA	5	N	10	N	N	Y	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
29		Middle aged adults	SCA	8	N	2	N	N	N	N
30			SCA	3	N	0	N	Y	Y	N
31			hetMNA	2	N	0	Y	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			SCA	4q, 11q, 17q	3p, 11q		7		12q amp	
15		Adolescents	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
20			SCA	1q, 5q, 12q	5p, 10q		6, 7, 8, 9, 13, 17, 18, 20, 21	11	4q	
25	Group 2	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			SCA	4p, 17q, 18q					+20p	
31			hetMNA		3p, 11q					6p

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

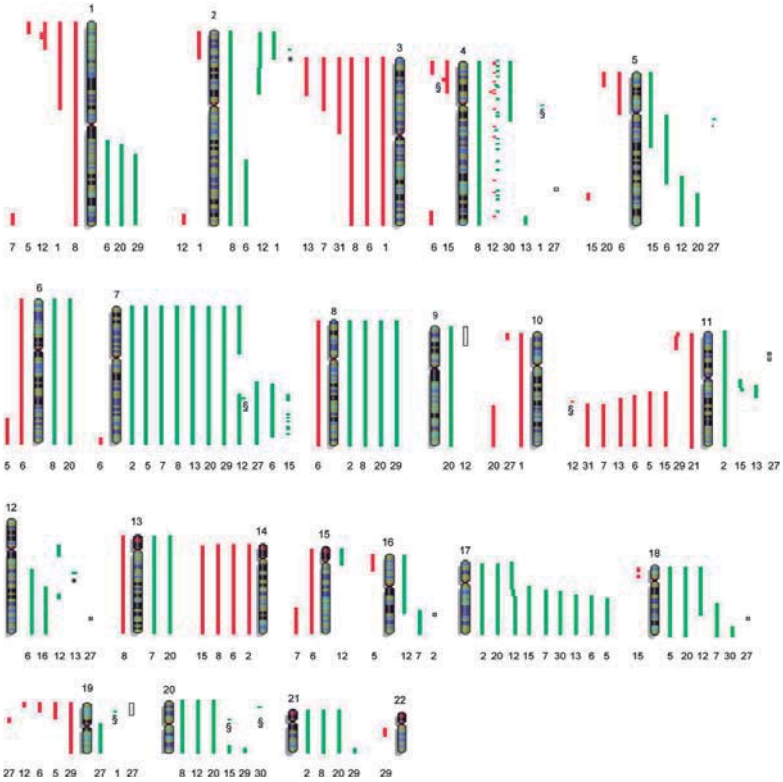

Figure 1. Representation of the genomic profiles by aSNP. Losses are indicated by a plain bar on the left, gains by a plain bar, and cnLOH by an empty bar on the right of each chromosome ideogram. Chromosome number and ID of the patients are indicated above and below respectively. Chromothripsis is indicated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

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

Patient ID	Main Age Group	Age Subgroup	Sex	ATRX 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	ADF
26			f	Mosaic	DOD
28		Middle aged adults	m	Mosaic	DOD
30			f	Mosaic	AWT
31			f	Negative	DOD

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

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,

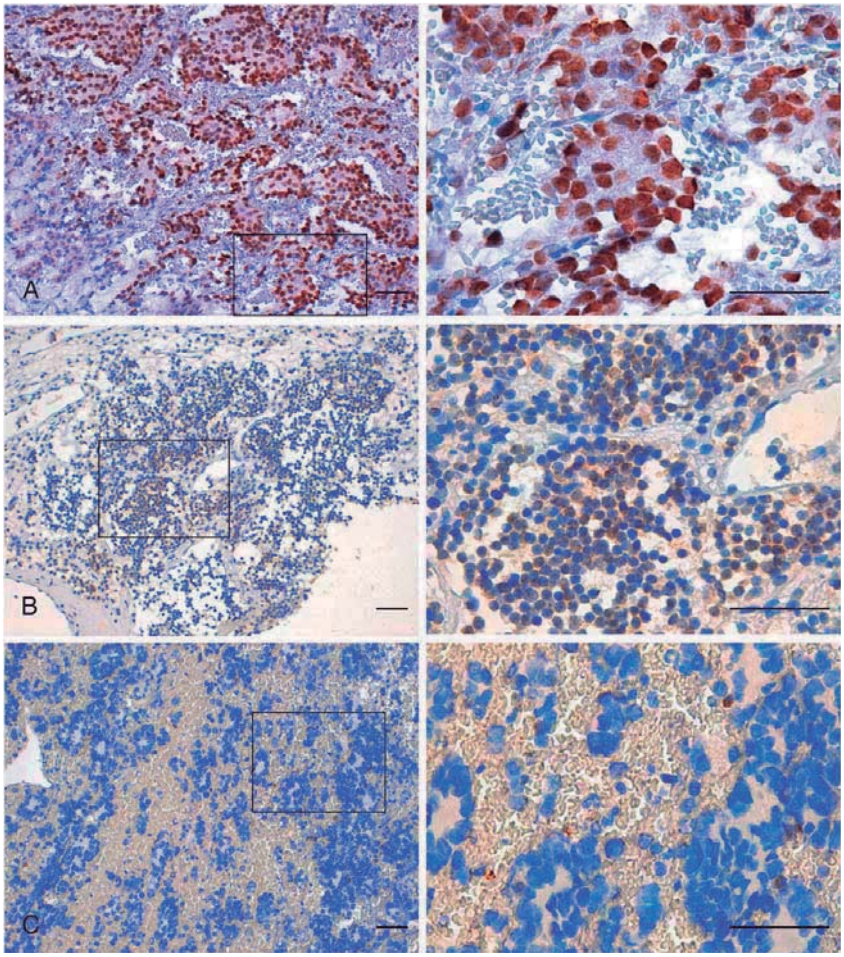

Figure 2. ATRX protein expression by IHC in three NB tumors. (A) Strong positive nuclear expression of neuroblast cells. (B) Mosaic expression of neuroblast cells, less than half of the neuroblasts cells retained the expression. (C) Negative nuclear expression. Scale bar, 50 μm (image at $\times$40 and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

Comparative genetic study of intratumoral heterogenous *a* ¸ */b* amplified neuroblastoma with aggressive genetic profile neuroblastic tumors.

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Final Decision made for ONC-2015-00018RR

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Oncogene@us.nature.com <Oncogene@us.nature.com> 11 de mayo de 2015, 18:25
Responder a: Oncogene@us.nature.com
Para: ana.berbegall@uv.es

Dear Miss Berbegall:

Here is a copy of the decision letter for manuscript "Comparative genetic study of intratumoral heterogenous mycn amplified neuroblastoma versus aggressive genetic profile neuroblastic tumors" by Rosa Noguera, Ana Berbegall, Eva Villamón, Marta Piqueras, Irene Tadeo, Anna Djos, Peter Ambros, Tommy Martinsson, Inge Ambros, Adela Cañete, Victoria Castel, and Samuel Navarro [Paper #ONC-2015-00018RR], which you were a Contributing Author.

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Sincerely,

Editor-in-Chief
Dr Douglas Green
Oncogene
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Subject: ONC-2015-00018RR Decision Letter

10th May 15

Ms Rosa Noguera
Medical School of Valencia
Department of Pathology
Avda. Blasco Ibañez, 15
Valencia, Valencia 46010
Spain

Final Decision made for ONC-2015-00018RR.

RE: MS#ONC-2015-00018RR

Dear Ms Noguera,

I am very pleased to inform you that your manuscript entitled "**Comparative genetic study of intratumoral heterogenous mycn amplified neuroblastoma versus aggressive genetic profile neuroblastic tumors" is acceptable for publication in Oncogene, a**nd will be forwarded to the publisher once your manuscript has all of the required materials and forms.

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Receiving Editor (Comments to the Author):

Manuscript # ONC-2015-00018RR
Title Comparative genetic study of intratumoral heterogenous mycn amplified neuroblastoma versus aggressive genetic profile neuroblastic tumors
Corresponding Author Rosa Noguera

Dear Dr. Noguera

I am delighted to inform your hat your revised manuscript is acceptable to Oncogene.

Sincerely

Reviewer #1 (Comments to the Author):

The authors have addressed all of my concerns.

Reviewer #2 (Comments to the Author):

The authors have satisfactory addressed all comments.

Ana Berbegall <ana.berbegall@uv.es>

Comparative genetic study of intratumoral heterogenous *MYCN* amplified neuroblastoma versus aggressive genetic profile neuroblastic tumors

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Running title

HetMNA neuroblastoma

Keywords

heterogeneous *MYCN* amplification; homogeneous *MYCN* amplification; genomic profiles; 11q loss; neuroblastoma; intratumoral heterogeneity

Abstract

Intratumoral heterogeneous *MYCN* amplification (hetMNA) is an unusual event in neuroblastoma with unascertained biological and clinical implications. Diagnosis is based on the detection of *MYCN* amplification surrounded by non-amplified tumor cells by FISH. To better define the genetic features of hetMNA tumors, we studied the Spanish cohort of neuroblastic tumors by FISH and Single Nucleotide Polymorphism arrays. We compared hetMNA tumors with homogeneous MNA (homMNA) and nonMNA tumors with 11q deletion (nonMNA w11q-). Of 1,091 primary tumors, 28 were hetMNA by FISH. Intratumoral heterogeneity of 1p, 2p, 11q and 17q was closely associated with hetMNA tumors when analysing different pieces for each case. For chromosome 2, sixteen cases showed 2p intact, 4 focal gain at 2p24.3 and eight MNA. The lengths of the smallest regions of overlap (SROs) for 2p gains and 1p deletions were between the SRO lengths observed in homMNA and nonMNA w11q- tumors. Co-occurrence of 11q- and +17q was frequently found with the largest SROs for both aberrations. The evidence for and frequency of different genetic subpopulations representing a hallmark of the hetMNA subgroup of NB; indicates, on the one hand, the presence of a considerable genetic instability with different SRO of either gains and losses compared with those of the other NB groups and highlights, on the other hand, the need for multiple sampling from distant and macroscopically and microscopically distinct tumor areas. Narrowing down the different SRO for both deletions and gains in NB groups would be crucial to pinpointing the candidate gene(s) and the critical gene dosage with prognostic and therapeutic significance. This complexity of segmental chromosomal aberration patterns reinforces the necessity for a larger cohort study using FISH and pangenomic techniques to develop a suitable therapeutic strategy for these patients.

INTRODUCTION

The *MYCN* proto-oncogene, on chromosome 2p24, is found to be amplified in different human malignancies.[1] *MYCN* amplification (MNA) is par excellence a marker of poor outcome, having clinical and treatment implications in neuroblastoma (NB).[2, 3] In addition, defining new therapeutic strategies in NB targeting *MYCN* is a demanding task.[4, 5] Fluorescence *in situ* hybridization (FISH) for *MYCN* gene analysis is critical when describing the *MYCN* status because of its high sensitivity and rapid morphological correlation.[6, 7] Homogeneous MNA (homMNA) is defined by the International NB Risk Group (INRG) Biology committee as the presence of a more than 4-fold increase in the *MYCN* signal number compared to the reference probe located on chromosome 2 in all tumor cells. Although rare, a proportion of tumor cells may also show *MYCN* gain (MNG).

Intratumoral heterogeneity for MNA (hetMNA), refers to the coexistence of amplified (frequently including tumor cells with MNG) as well as non-amplified tumor cells in the tumor as cluster (focal) or as single (scattered) cells.[8] In addition, temporospatial differences in MNA leading to a hetMNA status have also been described, as well as marked variations in the relation of *MYCN* amplified versus non-amplified tumor cells.[9, 10] Since the MNA clones in hetMNA tumors are frequently small, their diagnosis requires a thorough examination and confirmation, and is therefore difficult or impossible in small or inadequate biopsies. In addition, validation of hetMNA, ideally in paraffin sections by FISH, can be crucial to exclude false-positive results by 'contamination' of MNA cells from another tumor.[11] If these points are not considered, then hetMNA cases can be either missed entirely or included in studies as non-amplified *MYCN* (nonMNA) or as MNA tumors.[6, 12, 13] The few studies of hetMNA existing in the literature revealed that only 1-2% of all NB are hetMNA tumors, while MNA is detected in 20-25%, especially in stage 4 patients (~40%).[3, 7, 9, 10, 13-17] HetMNA is more frequently described in advanced stages 3, 4 and in stage 4S than in localised tumors. [discussed in [18]] Theissen et al, in a review of German cooperative trials, concluded that a small amount of MNA cells is not correlated with adverse outcome.[9] However, due to the limited clinical information on the hetMNA phenomenon, clinicians are faced with a significant dilemma when deciding on treatment strategy.[12,18]

Pangenomic analyses are essential in current diagnosis and treatment allocation for NB patients; since, in addition to the *MYCN* status, the overall genetic profile is essential for outcome prediction.[7, 19, 20] Seven recurrent segmental chromosome aberrations (SCA, i.e. losses at 1p, 3p, 4p, 11q and gains at 1q, 2p and 17q), which can also show intratumoral heterogeneity, were recently used for therapy stratification in a European SIOPEN study (LINES, Low and Intermediate Neuroblastoma European Study).[21] Since MNA tumors display an SCA pattern distinct from those of nonMNA tumors, it might be useful to further investigate SCAs and possible intratumoral heterogeneities of hetMNA tumors to ascertain their pattern and to detect indications of intratumoral genetic instability which could influence outcome.[22, 23] Furthermore, as genetic characterization by means of single nucleotide polymorphisms array (SNPa) is able to reveal gains and losses of chromosomal fragments of any size and subpopulations, we decided to study the hetMNA cases using this high-throughput technique in several pieces of the same tumor in order to improve the identification of a heterogeneous SCA (hetSCA) status.[7, 20, 24-26] To better demarcate the genetic characteristics of hetMNA tumors, we also studied the following aggressive genetic subgroups of NB: homMNA tumors with and without deletion of 11q (w11q-; w/o11q-, respectively) and nonMNA tumors with and without these specific aberrations.[26] Because many SCA in NB are large and encompass numerous genes, narrowing down the different SRO for both deletions and gains in NB groups would be crucial to pinpointing the candidate gene(s) and the critical gene dosage with prognostic and therapeutic significance. We also discuss the biological and prognostic implications in the cohort of NBs with a heterogeneous *MYCN* status.

RESULTS

Overview of clinical features

The main clinical features of the 28 patients with hetMNA are provided in Table 1. For patients with hetMNA tumors, age at diagnosis ranged from 5 to 86 months (mean 24.5, median 22). Advanced disease stages 3 and 4 were the most prevalent (70.3%). The mean overall survival for the hetMNA cohort was 92 months (CI 67-116) with an estimated 5-year overall survival (OS) and event free survival (EFS) rate of 51% (SE 0.13) and 58% (SE 0.11) respectively. For the homMNA cohort, the 5-year OS and EFS were 35% (SE 0.09) and 30% (SE 0.10)

respectively; and for the nonMNA w11q- cohort, 30% (SE 0.08) and 39% (SE 0.09) respectively .Eight patients (30.7%) experienced relapse (mean 24.5 months). To date, eight (30.7%) patients have died of disease after a mean of 26.6 months (median 32) within the hetMNA cohort.

Overview of the genetic profiles of the entire cohort

The final diagnosis of *MYCN* status of the 1,091 tumors studied by FISH, revealed that 28 cases presented hetMNA (2.5%), 197 homMNA (18.1%) and 866 nonMNA (79.4%). Of the 273 tumors analysed by SNPa, 28 showed hetMNA (10.3%) while of the remainder, 37 were homMNA (13.5%) and 208 nonMNA cases (76.1%). Different genetic profiles were revealed by SNPa analyses in each *MYCN* status subgroup. In hetMNA tumors, 26 cases had an SCA profile (92.9%), 4 of which had only one SCA, and 2 cases had a numeric chromosome aberrations (NCA) profile (7.1%). All homMNA tumors had an SCA profile. Of the nonMNA tumors, 117 cases had an SCA profile (56.3%), 55 cases w11q- (47%) and 62 cases w/o11q- (53%), 87 cases had an NCA profile (41.8%) and 4 a flat genetic profile (neither SCA nor NCA, 1.9%).

Out of 180 cases with an SCA profile, the highly recurrent SCA, including hetSCA, affected: 1p-, +1q, +2p, -3p, 11q- and +17q. Co-occurrence of 1p- plus +17q (15 cases, 53.5%), 11q- plus +17q (12 cases, 42.8%) or +2p plus +17q (10 cases, 35.7%) were frequently found in hetMNA tumors; while in homMNA tumors, co-occurrence of 1p-, +2p and +17q occurred in 43%. For the remaining cases (nonMNA w11q- and w/o11q-) the highly recurrent typical SCA was +17q (90.9% and 54.8% respectively). In the hetMNA cohort, uniparental isodisomies (UPDs) were found in 39.3%, UPDs for chromosome 11 were more frequent in older than in younger patients (12% versus 9%), and 11q- with large terminal SRO was observed in 42.8% by SNPa. In homMNA, UPD at chromosome 11, and 11q aberrations, were rare; present in 0% and 24.3% of the cases respectively, despite the bias introduced by the inclusion of our previously published unusual homMNA plus 11q cohort. [19] No association patterns for the remaining typical and atypical SCA or NCA were found for hetMNA, homMNA or nonMNA cases. Regarding the number of chromosomal breakpoints for each subgroup, hetMNA cases had an intermediate number of breakpoints, 7.3 (median 7), while homMNA and nonMNA w and

w/o 11q- had an average of 6.7 (median 6), 10 (median 10) and 3.96 (median 3) breakpoints, respectively (p=0.04 for hetMNA and nonMNA w11q-; and p=0.000 for hetMNA and nonMNA w/o 11q- groups). Considering homMNA w11q- tumors, the average number of breakpoints was 10.5 (median 7), versus 5.2 (median 5) for homMNA w/o11q-. Interestingly, this tendency for a higher number of breakpoints for 11q- tumors was also seen within hetMNA cases, with 8.9 breakpoints for the 11q- tumors and 5.2 for those w/o11q- (median 7.5 and 6.5 respectively). When grouping the patients above and below 18 months of age, little difference in the number of SCA was found (6.6 vs 7.8), although most of the hetMNA w11q- tumors belonged to patients older than 18 months (8 out of 12 cases).

Intratumoral heterogeneity for hetMNA

The genomic findings for hetMNA tumors are detailed in Table 2. Alterations of the 1p, 2p, 11q and 17q chromosome regions are represented in Figures 1 and 3. HetMNA occurred either as small foci with 5-10 cells (n=11), large foci with 50-55 cells (n=2) or scattered cells (n=15). Intratumoral hetSCA, occurred more often within hetMNA tumors (50%, 14 cases) than within homMNA (18.9%, 7 cases) or nonMNA tumors (20.9%, 13 cases). A diversity in the *MYCN* status was found when analysing more than one piece by FISH (4 cases) or by SNPa (6 cases) and/or by divergent results obtained by the two techniques (Table 2, Figure 1 and 2). For cases with different *MYCN* status analysed by FISH, hetMNA and nonMNA were detected in three tumors (cases 10, 11 and 20) and hetMNA and homMNA in one tumor (case 17). In case 11 hetMNA, MNG and nonMNA were found. In two other tumors (cases 18 and 19) the hetMNA findings were unequal, showing an evident disparity in the number of amplified cells (10-50%) and variation in the number of double minutes (from 15 to 100) between the two tumor pieces in each case. In these six cases, complex *MYCN* heterogeneity was present, since different results were also found by SNPa. HetMNA was confirmed in paraffin-embedded whole tissue sections from all cases by FISH. Regarding the SNPa results (4 cases), MNG (found as micro gain on 2p24.3) was detected in three cases, and in the fourth case, MNA was detected only in one of the pieces analysed, while the second piece showed no trace of MNA cells (cases 9, 19, 27 and 20 respectively). Furthermore, in two more cases, MNG was detected in the first piece and MNA in the second piece (cases 17 and 28). There was also a clear difference in the level

of amplification between the two pieces of case 22. Among all the hetMNA cases, SNPa showed nonMNA in 16 cases, MNA in 8, and MNG in 4 cases. Presence of hetMNA plus MNG was found in 12 cases by FISH, which resulted as MNA or MNG in 6 cases, and nonMNA in the remainder, by SNPa. Specifically, MNA plus gain at 2p was found in cases 12 and 20 by SNPa, while in case 22 only MNA was found. In cases 24 and 28, MNG plus gain at chromosome arm 2p was found, while in case 19 only MNG was detected by SNPa (Figure 1). Six out of 8 cases that showed MNA and 3 out of 4 cases that showed MNG by SNPa had ≤10 MNA cells per slide by FISH. The cases with 35-90 MNA cells per slide (n=5) presented nonMNA status by SNPa. The amplicon or the gained region extended from 15.2 to 18.7 Mb, depending on the sample; the smaller region ranged from 15.8 to 16.2, affecting only the *MYCN* gene (case 12). The *NBAS* gene was excluded in two cases, while the *DXX*1 and *MYCN* genes were usually involved. One or two extra amplifications distal to *MYCN* amplicon were observed in 2 hetMNA and 9 homMNA cases and in 1 nonMNA tumor (Figure 3b).

Intratumoral hetSCA was found when analysing several pieces with SNPa and when comparing FISH and SNPa results. With regard to the discrepant results in the detection of 1p deletion by FISH and SNPa, we hypothesise that 1p deletion in hetMNA tumors occurred in a similar fashion to that of MNA (Figure 2). Clones with 1p deletion are admixed with clones without aberration at 1p in a heterogeneous manner, and thus the aberration may remain hidden. In view of the discrepant results in the detection of 1p deletion by SNPa we hypothesize that 1p deletion in hetMNA tumors occurs similar to the detection of hetMNA by FISH (Figure 2). Heterogeneity for large segmental UPDs (11p, 12q, 14q, 17q, 18p and 22q) was found in 4 cases, in half of these, loss of the same chromosome regions was seen instead of the UPD in the second piece; interestingly, for the case with 17q UPD, a gain of 17q was seen by FISH. The case with the highest number of SCA, in addition to hetUPD (14q and 22q), also presented heterogeneity for up to three SCA, and for one micro SCA (+5p) and different *MYCN* status (MNA/MNG), indicating a high intratumoral genetic instability. In two cases, heterogeneity in the SNPa was present in relation to 2p alterations other than *MYCN*: w and w/o amplification at 2p.25.1 and w and w/o gain in 2p24.1. Altogether, the average number of chromosomal breakpoints was 7.3 (median 7). The most frequent NCA in hetMNA tumors were +7 and +17, alone or together with other NCAs (13 cases, 47%). Micro SCAs were present and randomly

distributed along the genome in 9 cases (32.1%), two of which were heterogenous cases, except for the *MYCN* gain region. Large UPD events (11 cases, 39.3%) were randomly distributed along the genome and more frequent for the cases with an increased number of breakpoints (72.7%, 8/11 cases). UPD and micro SCA were also present in NCA cases. Chromothripsis-like pattern was present in four chromosomes (5, 6, and 11) of three cases, all with ≥7 breakpoints.

Patterns of 1p-, +2p, 11q- and +17q in all subgroups analysed

We focused on frequency (FISH and SNPa data), length and type (intrachromosomal, terminal, micro SCA and SROs) of the typical SCAs 1p-, +2p, 11q- and +17q. A representation of SNPa results for each region of the aggressive subgroups is shown in Figure 3. The differential distribution of other typical and atypical SCAs is shown in Table 3.

1p deletion: of the total of 180 SCA tumors, this deletion was present in 44.4% (80 cases). HetMNA tumors showed less 1p deletions than homMNA (67.8 vs 86.4%). For nonMNA w and w/o11q- the difference was less marked (23.6 versus 25.8%). The SRO extended from pter in all cases (except for one homMNA tumor), to p36.21 for the hetMNA tumors (13.8 Mb), to p36.12 for homMNA (27.3 Mb) and to p36.22 for nonMNA w11q- cases (9.5 Mb) (Figure 3a). The differences between groups in the size of the 1p- region were statistically significant (p-value=0.007). An interstitial deletion, from p36.32 to p36.23 (3.5 to 8.6 Mb), was detected in one homMNA case, creating an interstitial SRO. The median length in the hetMNA cases was 66 Mb, 53.3 Mb for the homMNA and 28 Mb for the nonMNA w11q-.

2p gain: +2p (62 cases) accounted for 34.4 % of all the SCA cases. For the hetMNA tumors, +2p was less common than for homMNA and nonMNA w11q- tumors (35.7, 43.2 and 45.4%, respectively). However, in SCA tumors within the subgroup of nonMNA w/o11q-, proximal +2p accounted for 19.3%. In the strictest sense, the +2p SRO for the homMNA cases was from p24.3 to p24.1 (3.3 Mb) due to loss of genetic material between the two amplified regions observed in one case. This fact was considered as a false effect of the amplification peaks, and when discounted the SRO ranged from p25.1 to p24.1 (9.6 Mb). The hetMNA cases had an intermediate SRO, from p25.3 to p24.3 (14.3 Mb), and the nonMNA with 11q deletion

had a shorter SRO, involving only the p24.3 region (0.7 Mb) (Figure 3b). The large 2p gain in hetMNA tumors was associated with 11q- in 41.6%, in contrast to 33% in homMNA tumors.

11q deletion*:* out of all SCA tumors, 11q- was present in 76 cases (42.2%); 55 were nonMNA (72.4%) and 21 het or homMNA (27.6%), being more frequent in hetMNA than in homMNA cases (42.8 vs 24.3%). Furthermore, hetMNA tumors had larger deletions than the homMNA and nonMNA subgroup of tumors (median 62 Mb, 44 Mb and 59 Mb, respectively) (p=0.038) (Figure 3c). When considering only the SRO, the differences between the deleted regions became more apparent, with a large terminal SRO for the hetMNA, from 11q14.1 (84.7 Mb) to qter, a short terminal SRO for the homMNA cases, from 11q23.1 (111 Mb) to qter, and an interstitial SRO for nonMNA cases, from 11q21 to 11q23.3 (96.8-118 Mb). In the latter subgroup, only one tumor out of 55 showed no terminal deletion; this meant that the most common 11q- type for nonMNA was also a terminal deletion with an SRO of a size between that from hetMNA and homMNA. One homMNA case showed two additional breakpoints located at 94.1-99.8 Mb. None of the hetMNA tumors presented a breakpoint between 112 and 117 Mb, where the FRAC11B and FRAC11G fragile sites are located, contrary to some of the homMNA and nonMNA tumors.

17q gain: as expected, +17q was the most frequent SCA, present in 134 cases (75.7%). The occurrence of +17q was evenly spread across the 3 genetic subgroups, although more frequent in nonMNA w11q- tumors (90.9%) (Figure 3d). The gain always included the telomeric region; having more than one breakpoint in 14 cases (14.5%) and interrupted by a UPD in one case. Although the average size of the 17q gain was similar in all the subgroups (45 Mb), the SRO was larger for the hetMNA (breakpoint at 45.3 Mb, q21.31-qter), intermediate for the nonMNA (breakpoint at 52.7 Mb, q23.2-qter) and smaller for the homMNA tumors (60.9 Mb, q23.3-qter). Grouping all the cases according to the 17q gain breakpoint positions and associating with better survival, as described by Theissen et al., the frequencies were almost identical to those described: 11.5%, 77% and 11.5% for <26.6 Mb, 26.6-42.5 Mb and ≥42.5 Mb breakpoint positions respectively.[27] The majority of nonMNA11q- cases mapped within the range 26.6-42.5 Mb, being more dispersed in both hetMNA and homMNA tumors.

DISCUSSION

To date, hetMNA studies in NB have comprised small cohorts providing limited genetic data, there have been no pangenomic studies, and the related clinical features remain unclear. [reviewed in [18]] We present a large cohort of hetMNA NBs studied using a pangenomic approach intending to identify the genetic profile, distinguish the underlying genetic pattern of intratumoral hetMNA, describe the presence of intratumoral heterogeneity of SCA and compare the SRO for both deletions and gains. The incidence of hetMNA in primary tumors in the largest cohort reported so far, is 1.1% (15 out of 1341), with similar small frequencies reported by others, and similar to the results of the present study.[7, 9, 15, 16] The low frequency of hetMNA described might be explained by the complex and not always feas ble work-up required for its diagnosis; and also by the presence of focal genetic alterations, methodologically difficult to identify, which lead to an underestimation of the frequency. In hetMNA tumors, either focal or scattered MNA neuroblasts are admixed with non-MNA neuroblasts, producing a speckled or mottled appearance. HetMNA should therefore be considered as a single mass sprinkled with diverse tumor cell clones, including MNA cells, tumor nonMNA cells and stromal cells. While a hetMNA tumor evaluated by FISH shows a combination of MNA cells (usually in low percentages and with less *MYCN* copies per cell than in homMNA), with nonMNA and occasionally MNG neuroblastic cells, SNPa results do not always show conspicuities in the *MYCN* region. HetMNA cases, ideally when more than one tumor piece is analyzed, can show a more than 4-fold peak (as compared to the chromosome 2 baseline) at 2p24.3 (usually at lower levels as compared to homMNA tumors), or a gain (up to 4-fold) restricted to the *MYCN* gene locus (MNG, equal to a micro 2p24.3 gain, *DDX1* and *NAG* genes can be included), not found in either MNA (only in case of low tumor cell contents) or in nonMNA tumors. However, hetMNA may also be present without any visible alteration in the *MYCN* gene region by SNPa, even when several fragments are studied. Our results support those previously reported by others which use methods that rely on pooled DNA samples in hetMNA NB, and reflect the admixture with tumor DNA not showing MNA or normal cell DNA that can hinder the detection of the genetic alteration.[10] Such findings strengthen the use of FISH for *MYCN* status diagnosis, with an internal control of somy of chromosome 2, in conjunction with pangenomic techniques. The International Society of Pediatric Oncology, Europe Neuroblastoma Group (SIOPEN) is involved

in the assembly of clinical and FISH data of hetMNA tumors from eight participating countries (Ambros IM and Noguera R, manuscript in preparation).

Apart from the intertumoral genetic heterogeneity characteristic of NB, intratumoral diversity, although not very frequently reported, is also known to be present in primary NB.[28] Our group and others have previously described intratumoral hetSCA, of 1p and 17q by FISH and genome-wide techniques without ascertaining biological and clinical significance.[7, 19, 29-31] HetMNA presented genetic profiles ranging from pure NCA to SCA with multiple rearrangements. The data on hetMNA tumors reveal a spectrum of genetic heterogeneity, here referred to as intratumoral hetSCA, for typical SCA, but which also appeared in non-typical SCA. This heterogeneity among hetMNA NB was higher compared to homMNA and nonMNA w11q- tumors. A close association between homMNA and 1p deletion is well-known; a similar close coexistence of hetMNA and het1p deletion was revealed. Therefore, analyzing several pieces of hetMNA tumors would not only help in clarifying *MYCN* status, but also in obtaining an accurate and complete genetic profile of each tumor. This is especially important in treatment approaches for low-risk patients, i.e. localised stages in children aged ≤18, and Ms stage.[32] In the present cohort, four patients presented with localized disease and two with Ms stage. Whether this intratumoral heterogeneity might be a result of a clonal evolution process and would lead to clinical progression and resistance to chemotherapy is difficult to assess. It could be reasoned that in NBs with NCAs, the presence of hetMNA (and hetSCA) represents an earlier stage in NB evolution on its way to reaching the threshold of malignant transformation.[23, 33] Unfortunately, no differences in age could be seen in patients between any of the genetic subtypes studied.

Remarkably, the predominance of SCA tumors was noticeable, with large regions of 17q gain, with large regions of 11q loss, or intermediate regions of 2p gain. 17q gain is almost ubiquitous in SCA profiles of NBs, assuming an oncogenic gene dosage effect for this aberration.[8, 34, 35] Recently, a better prognosis has been ascertained for patients with a more distal 17q gain breakpoint (26.6 Mb).[9] However, the more proximal breakpoint identified herein was not associated with poorer survival for most of the hetMNA patients. In addition to +2p and +17q, 1p- is also known to be strongly correlated with homMNA tumors. Interestingly, several tumor suppressor genes located in this region have been explored.[36, 37] Nevertheless, our results and

those of previous studies did not detect a strong correlation between hetMNA tumors and 1p deletion. Half of the hetMNA tumors show mainly het1p-, denoting that the overall proportion of deleted cells is low.[7, 9] Compared to 1p-, 11q- was more frequently represented in hetMNA, than in homMNA tumors, especially in older patients with advanced stage tumors and was associated with a higher number of SCA.[26, 38, 39] As 11q- rarely co-occurs in homMNA tumors, it is recognized as an alternative mechanism by which full oncogenic potential is attained through haploinsufficiency of the genes mapping to the deleted region.[19, 26, 40, 41] 11q- tumors are distinguishable also at the mRNA and miRNA level, but the genetic factors that mediate the poorer outcome on 11q- tumors are not clear.[42, 43] HomMNA is known to occur at an earlier stage than 11q deletion and with less associated SCA. On the other hand, unfavorable nonMNA w11q- tumors have a later onset and frequently an increased number of associated SCA, suggesting that nonMNA w11q- tumors achieve their strong oncogenic potential by acquisition of genetic instability. Interestingly, hetMNA tumors seem to have a very 'unstable' genetic profile, different to homMNA tumors, and frequently with 11q- associated. Identification of hetSCA and genomic amplification other than *MYCN*, if present, can help in narrowing the region to search for target genes with impact on outcome, even genes implicated in the *MYCN* pathway.[44, 45] In this regard, 2p25, 1p31 and 1p34.2, among other amplifications, have been detected in homMNA and are associated with a tendency towards poorer prognosis than homMNA only.[46, 47] We found these amplifications in two low SCA tumors (stage 1 and 3) both in patients aged >18 months. The implications of other chromosomal alterations, such as 1p imbalance have been discussed in a short report, questioning whether nonfavorable neuroblastic clones are indeed evolving from favorable clones, and could be investigated in hetMNA tumors. [48] The high presence of chromosome 11 UPDs associated with younger age, localized disease stage and aneuploidy founded in the Austrian cohort (data not shown) will be discussed elsewhere (Ambros IM, manuscript in preparation). In addition, epidemiologic differences may also be involved in this issue. Defining the SROs of both gains and deletions in each NB group will certainly aid in identifying the distinct gene dosages responsible for tumor progression. The finding that the SCA profile of hetMNA differed from that of the homMNA and nonMNA tumors has importance for identifying whether a distinct gene dosage or different

tumor suppressor gene effects are related to the presence of multiple tumor cell subpopulations and their relationship with the microenvironment.

In conclusion, hetMNA tumors harbour an 'unstable' genetic profile, with genetically diverse subpopulations of tumor cells that diminish the utility of defining the cut-off for the number of MNA cells by FISH, and demands, when possible, performing multiple sampling from macroscopically and microscopically distinct tumor fragments. Further efforts using FISH together with pangenomic techniques are essential to developing a targeted therapeutic strategy for this subgroup of patients with hetMNA tumors, especially for those with NCA or low SCA profile.

MATERIALS AND METHODS

Clinical data and tumor material

Between January 1997 and January 2014, 1,091 tumor samples were referred to the Department of Pathology (Medical School of Valencia) at the time of diagnosis. Clinical data are available in the Spanish NB database. In summary all patients were studied and staged according to the INSS and from those diagnosed after 2009 according to the INRG. Except for one patient with a nodular ganglioneuroblastoma, all hetMNA cases were histopathologically classified as poorly-differentiated NB, according to the International NB Pathology Classification (INPC).[49] Informed consent from parents or guardians was obtained for all patients. Histologic and genetic studies were approved by Spanish Society of Pediatric Hematology and Oncology (file number: 59C18ABR2002) and as well as by the Ethical Committee of the University of Valencia and University Clinic Hospital of Valencia.

Fluorescence in situ hybridization (FISH)

Tumor touch imprints and paraffin sections were hybridized for FISH analyses in two to four fragments of each tumor, using commercial cocktail probes: 1p36(D1Z2)/centromere chromosome 1 (Qbiogene), *MYCN*(2p24)/LAF(2q11), *MLL*(11q23)/SE11 and *MPO*(17q22) ISO17q/p53(17p53) (Kreatech, Biotechnology). Assessment and interpretation of FISH results was performed in accordance with previously published procedures and all hetMNA cases were centrally reviewed by the International Society of Pediatric Oncology Europe Neuroblastoma

Biology Group [50]. In this manuscript, the term intratumoral hetMNA has only been used as defined by the INRG Biology Committee, a definition also used by Theissen et al.[8, 9] For the final diagnosis of the *MYCN* gene status, a FISH diagnosis of hetMNA prevailed of a normal *MYCN* status found by SNPa.

Genetic study by SNPa

DNA was extracted from fresh (n=237) and formalin-fixed paraffin-embedded (FFPE) tissue (n=36) as previously reported.[19] All samples had tumor cell content >50%, except for five samples with only 50% of tumor cell content (cases 10,15,17,18 and 24). Two SNPa platforms were used: Genechip Human Mapping Nsp Array (262,256 markers) in 90 tumors and HumanCytoSNP-12 DNA Analysis BeadChip (299,140 markers) in 183 tumors from Affymetrix (Affymetrix, Inc., Santa Clara, CA) and Illumina (Illumina Inc., San Diego, CA), respectively. Previously described experimental procedures were used for all array platforms.[19, 26] The complex genetic intratumoral composition demanded the analysis, when possible, of different pieces of the same tumor to achieve a precise and comprehensive diagnosis (at least two fragments in 14 cases, with a third fragment in cases 11, 21 and 22). The most aggressive genetic profile was taken as final diagnosis of *MYCN* gene status by SNPa (i.e. MNA prevails over MNG or nonMNA status, and MNG prevails over nonMNA status). For each hetMNA tumor we determined: a) NCAs, b) hetSCA (which would lead to different diagnoses for SCA status in distinct tumor fragments within the same tumor), c) amplifications, d) focal or micro SCA, smaller than 2 Mb and excluding copy number variations (CNVs), and e) copy neutral loss of heterozygosity considered as UPD. MNA and MNG were detected by SNPa using AsCNAR (allele-specific copy-number analysis) and cnvPartition functions from CNAG and KaryoStudio software respectively. MNA regions are tracked in dark red, and MNG regions in pale red for Affymetrix arrays. For Ilumina arrays, a value of 4 was assigned to the MNA region, and a value of 3 to the MNG region. In addition, MNA and MNG status was confirmed by visual inspection of the difference between the increased signals in the copy number plot. Micro SCAs were not taken into account when counting the number of SCA breakpoints, and hetSCAs by FISH were considered as a single event. For final diagnosis of SCA in cases with hetSCA, the most aggressive profile (i.e. the highest number of SCA) was used. SCAs in hetMNA cases were

classified as: NCA/low SCA (0-1), intermediate (3-7) and high SCA (>7) profiles. Comparison of SRO size and number of breakpoints between groups (hetMNA, homMNA and nonMNA) were done using Mann-Whitney *U* test (2 groups) and Kruskal-Wallis (3 groups), all p-values were two-sided. Some data for hetMNA w11q- cases (n=7), homMNA w11q- cases (n=11), and nonMNA w11q- cases (n=53) have already been published.[19] (Javanmardi and Berbegall et al. 2015, manuscript in preparation).

CONFLICT OF INTEREST

The authors disclose no potential conflict of interest.

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TABLES LEGEND

Table 1.

Abbreviations. EFS, event free survival; OS, overall survival; ADF, alive disease-free; AWD, alive with disease; AWT, alive with treatment; DOD, died of disease; DOC, died of other cause; ND, no data.

Table 2.

No numerical chromosome aberrations are detailed; [a], micro segmental chromosomal aberrations (< 2 Mb); UPD, uniparental disomy; (a), amplification; cth, chromothripsis-like pattern; [b], additional aberrations found by FISH (shown in Table 2).

Table 1. Main clinical features of hetMNA tumors

Patient No	Age (months)	Stage	Relapse	Outcome	EFS (months)	OS (months)
1	22	3	no	AWT	0	1
2	11	2	no	ADF	0	70
3	5	4S	no	ADF	0	34
4	12	1	no	ADF	32	32
5	14	3	yes	AWT	14	111
6	19	1	no	ADF	5	5
7	8	4	no	ADF	80	80
8	41	2	yes	DOD	4	40
9	8	3	no	AWT	52	52
10	42	4	yes	DOD	11	40
11	38	4	no	ADF	0	116
12	20	ND	ND	ND	ND	ND
13	5	2	no	ADF	92	92
14	38	4	yes	DOD	9	16
15	20	3	no	DOC	0	0
16	49	4	yes	DOD	32	36
17	26	4	no	ADF	0	12
18	61	4	yes	AWT	83	138
19	28	3	no	ADF	4	4
20	25	4	no	DOD	1	1
21	39	4	yes	DOD	18	42
22	5	4S	yes	DOD	25	28
23	17	2	no	ADF	71	71
24	51	4	ND	ND	ND	ND
25	72	4	no	ADF	0	131
26	13	4	no	DOD	0	10
27	14	4	no	AWD	49	49
28	86	4	no	AWT	12	12

Table 2. SNPa findings of hetMNA tumors from Spanish NB Registry

Patient No.	Segmental chromosomal aberrations	Other chromosomal alterations	Heterogenous chromosomal alterations	Breaks No.
1		UPD(12p, 20pq)		0
2	+2p21[a]	UPD(6p)		0
3				1[b]
4	+16q22.1[a]			1[b]
5		UPD(3p)	+2p24.1[a], UPD(7p, 13q), +17q/UPD(17q)	1[b]
6	+17pq	1p34.2(a), 1p31.3(a)		1
7	+1q, +19q			3[b]
8	11q-, +17pq	2p24.3(a)		3[b]
9	11q-, +16q, +17q		+2p24.3[a]	4[b]
10	+9p, 11q-, +17q(2)	UPD(11q)		4
11	11p-, 11q-, +15q, +20pq			5[b]
12	1p-, +2p, 15q-, +17q	2p24.3(a)	2p25 3(a)	5[b]
13	+2p, 11p-, 11q-, -14q			6[b]
14	1p-, +2p(2), -7q, +12q, +17q			6
15	+2p, 3p-, +7q, 11q-, +12q, +17q			7[b]
16	1p-(2)-, +1q, +7p(2), +7q, +8pq	UPD(2p,10q,16p,16q), cth(5,11)		7
17	+4q25[a], 7q(2)-, 9p(4)-	UPD(7q, 8 ,9, 11q)	2p24.3(a)/+2p24.3[a]	8[b]
18	1p-, +2p, 4q(2)-, +4q13.3[a], 9p(2)-, 16q-, +17q			8
19	+1q, 3p-, 4p(2)-,11q12.3-[a], +11q, 11q-, +17q , 21q-		+2p24.3[a]	8
20	+2p, 2q-, 4q32.1-[a], +7q, +12p, 14q-, +17q		2p24.3(a), 19p-, +19q	8
21	+1q, +2p, 2q-[a], 3p-, 4p-, 11q-, +12q, -14q, +17q			9[b]
22	1q(2)-, 10p-, 10q-, 11q-, 14q-, 16q23.1-[a], +17q, +22q	2p24.3(a)	UPD(11p)	9[b]
23	1p-, +1q, +2p, 3p-, +3q, -9q, +12q(2), 14q-, +17q	2p24.3(a), UPD(8p)		10
24	1p-, +2p24.3[a], -8p, +12pq, +12q(3), -14q, +17q(2), 19p-, -22q	UPD(12q)		11
25	1p-, +2p, 4p-, 6q-, 11q-, 12q(2)-, +13q31.1[a], +17q, +18p, +22q	2p24 3(a)	12q-/+12q, UPD(12q), 18p-/UPD(18p)	12
26	1p-, +12q, +13q(5), 14q(2)-, +17q, 20q(3)-	cth(5)		13
27	1p-, 3p(2)-, 5p(2)-, +6q, 6q(2)-, +7q, 9p-, 9q-, 11q-, 14q-,15q-,16p-, 16q-, +16q, +17q, 22q-	cth(6)	+2p24.3[a]	19
28	+1q, +2p, +2q, 3p-, +2q, +5q, -6q(2), +7q, -8p, +11p, 11q-, +12q, +17q, +18pq, +22q		2p24 3(a)/2p24.3[a], +4q(2), +7q(2), +14q/UPD(14q), +5p13.33[a], UPD(22q)	21

Table 3. Frequency of SCA on hetMNA, homMNA, nonMNA w11q- and nonMNA w/o 11q- tumors

Group	1p-	+1q	1q-	+2p	3p-	4p-	+7p	+7q	9p-	10q-	11q-	+12q	14q-	16p-	+17q	19p-
hetMNA	67 9%	21.4%	3.5%	35.7%	21.4%	10.7%	3 5%	17.9%	10.7%	3.5%	42.8%	28.6%	28 6%	3.5%	78.5%	7.1%
homMNA	86.4%	5.4%	2.7%	43 2%	21.6%	5.4%	2.7%	13.5%	5.4%	9. 0%	24.3%	18.9%	8.0%	0%	75.7%	2.7%
nonMNA w11q-	23 6%	30.9%	10.9%	45.4%	45.5%	20.0%	9%	25.4%	7.2%	24 3%	100%	25.4%	14 5%	3.6%	90.9%	9.0%
nonMNA w/o 11q-	27.4%	17.7%	0%	9.7%	8%	19.4%	3 2%	12.9%	4.8%	0%	0%	4.8%	6.5%	0%	54.8%	22 6%

FIGURE LEGENDS

Figure 1. Correlation between FISH (touch imprints) and SNPa results in hetMNA tumors. HetMNA status, ranging from a low to high number of amplified cells, was confirmed by FISH in paraffin-embedded tissue sections in all cases. For *MYCN* gene results, the correlation between techniques was independent of the number of MNA and/or the percentage of MNG cells. In some cases where 1p deletion was missed by SNPa, non-deleted tumor cells in conjunction with 1p imbalance and deleted cells were observed by FISH. Intratumoral homogeneity and heterogeneity was found for 11q and 17q chromosome regions respectively. [a/b], indicates equal results between fragments (when different results both results are indicated); MNA, *MYCN* amplification; f, focal (1-2 MNA foci); s, scattered MNA cells; nonMNA, no *MYCN* amplification; homMNA, homogeneous *MYCN* amplification; MNG, *MYCN* gain; G, Gain; het, intratumoral heterogeneity of *MYCN* /chromosome aberration; D, deleted; ND, Non-deleted; Im, 1p imbalance; *, >50% difference in copy number from cell to cell of double minutes and different percentages of 1p-, 11q- or +17q cells; ↑or ↓, higher or lower level *MYCN* amplification. FISH colour box definition: no colour, intratumoral heterogeneity of *MYCN* status/chromosome aberration; purple, no SCA detected; red, SCA only detected for one fragment; blue, SCA detected. SNPa colour box definition: no color, concordant *MYCN* gene status with FISH results orange, discordant with FISH results (described when nonMNA or SCA); green, concordant with FISH results. Case 11c, additional piece analyzed with different results.

Figure 2. Schemas presenting the various possible spatial distributions of tumor cells, chromosome 2p SNPa profiles and FISH images of nonMNA, homMNA and hetMNA cases to illustrate the effects of MNA DNA dilution within DNA of nonMNA. Schemas showing nonMNA neuroblasts (green circles), MNA cells (red circles) and stromal cells (blue ovals) in a whole tumor (large cylinder) and pieces of the tumor (small cylinders demarcated by dashed lines). Tumor touch imprints of the surface of each fragment were used for FISH and the whole the fragment for SNPa analyses. FISH images show *MYCN* gene in red, LAF region in green and DAPI nuclear counterstaining in blue. (**a**) In nonMNA tumors, the two techniques detect only nonMNA cells. (**b**) In homMNA tumors, homogeneous distribution and large amount of MNA cells without nonMNA neuroblasts facilitates the detection of the MNA by either SNPa or FISH.

(**c**) In hetMNA tumors, the heterogeneous distribution and differing amount of MNA cells, nonMNA neuroblasts and stromal cells generates discrepant FISH and SNPa results. MNA and tumor nonMNA cells are detected in all FISH touch preparations. (**d**) The low ratio between MNA and nonMNA cells does not allow the detection of 2p24.3 alteration by SNPa. (**e**) The higher presence of MNA cells enables their detection as a 2p24.3 high peak by SNPa. (**f**) The amount of intermingled MNA cells is reflected as *MYCN* gain by SNPa due to the dilution of the MNA DNA content. nonMNA, no MYCN amplification; homMNA, homogeneous MYCN amplification; MNG, MYCN gain; hetMNA, heterogeneous MYCN amplification; f, focus of MNA cells; s, scattered MNA cells.

Figure 3. Representation of chromosome aberrations detected by SNPa for hetMNA (the reference number for each patient is indicated at the top of each bar), homMNA and nonMNA w11q- subgroups of tumors. Length of small regions of overlap (SROs) of each aberration is indicated below the tittle of each subgroup, SROs are indicated by dashed horizontal lines. Ideograms on the left of **a**-**d** represent 1p, 2p, 11q and 17q chromosome arms, respectively. Vertical bars represent the length of 1p and 11q deletions in a and c, and the length of 2p and 17q gains in b and d. Horizontal bars represent amplifications, micro SCA are marked by §, and UPDs by a grey bar. Two oblique lines at the end of the vertical bars indicate where the alteration extends to the other chromosome arm. When other breakpoints are present in the altered regions, the bars are out of alignment.

Patient No	FISH					SNPa				
	hetMNA[a/b] (number cells)	MNG[a/b] (% cells)	1p	11q	17q	hetMNA	2p	1p	11q	17q
1	5 f									
2	10 f									
3	5 f		hetD					a/b		
4	10 f		hetD			a/b	a/b	a/b	a/b	a/b
5	10 f				hetG	a/b	a/b	a/b	a/b	G/NG
6	20 s	G 15			G					
7	10 f		D							
8	>10 f/<5 f*		hetD[a/b]*	hetD[a/b]*	hetG[a/b]*	MNA				
9	5 s		D	hetD	G	G/nonMNA	a/b	a/b	a/b	a/b
10	5 s/nonMNA		a/b	D	G					
11[c]	90 s/nonMNA			D	G	nonMNA[a/b]	a/b		a/b	a/b
12	5 s	G 10	Im		G	MNA[a/b]	a/b	D[a/b]	a/b	a/b
13	55 f	G 40	hetD		hetG	a/b	a/b	D/ND	D[a/b]	a/b
14	20 s	G 60	hetD		G					
15	35 s	G 20	D	hetD	G					
16	20 s		D							
17	5 s/homMNA		hetD		G	G/MNA	a/b	a/b	a/b	a/b
18	5 s	G 70	D+Im		G					
19	<15 s/>50 s*	G 5		Im	G	G	a/b	a/b	D[a/b]	a/b
20	10 s/nonMNA	G 75			G	MNA/nonMNA	a/b	a/b	a/b	a/b
21	80 s		hetD+Im	D	G	a/b	G[a/b]	a/b	a/b	a/b
22	30 s	G 5	hetD+Im	D	G	MNA↑/MNA↓	a/b	a/b	a/b	a/b
23	50 f		D		G	MNA	G			
24	5 f	G 50	D		G	G				
25	5 f		D+Im	D+Im	G	MNA[a/b]	G[a/b]	a/b	a/b	a/b
26	15 s	G 35	D+Im		G					
27	5 f		D+Im	D	G	G/nonMNA		a/b	a/b	a/b
28	10 s	G 40		D	G	G/MNA		a/b	a/b	a/b

Figure 1

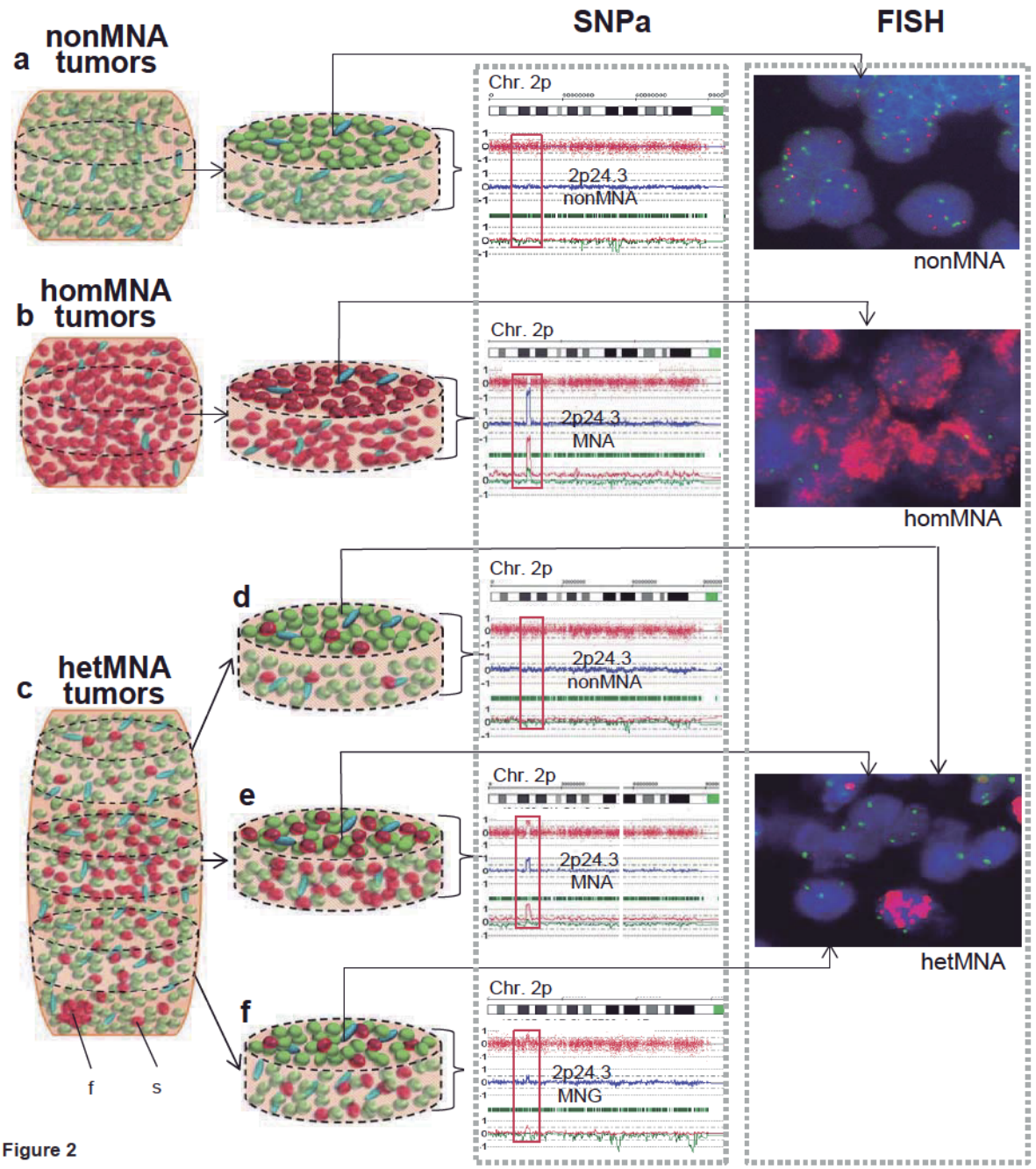
SNPa
FISH
a nonMNA tumors
Chr. 2p
2p24.3
nonMNA
nonMNA
b homMNA tumors
Chr. 2p
2p24.3
MNA
homMNA
c hetMNA tumors
d
Chr. 2p
2p24.3
nonMNA
e
Chr. 2p
2p24.3
MNA
hetMNA
f
Chr. 2p
2p24.3
MNG
f
s

Figure 2

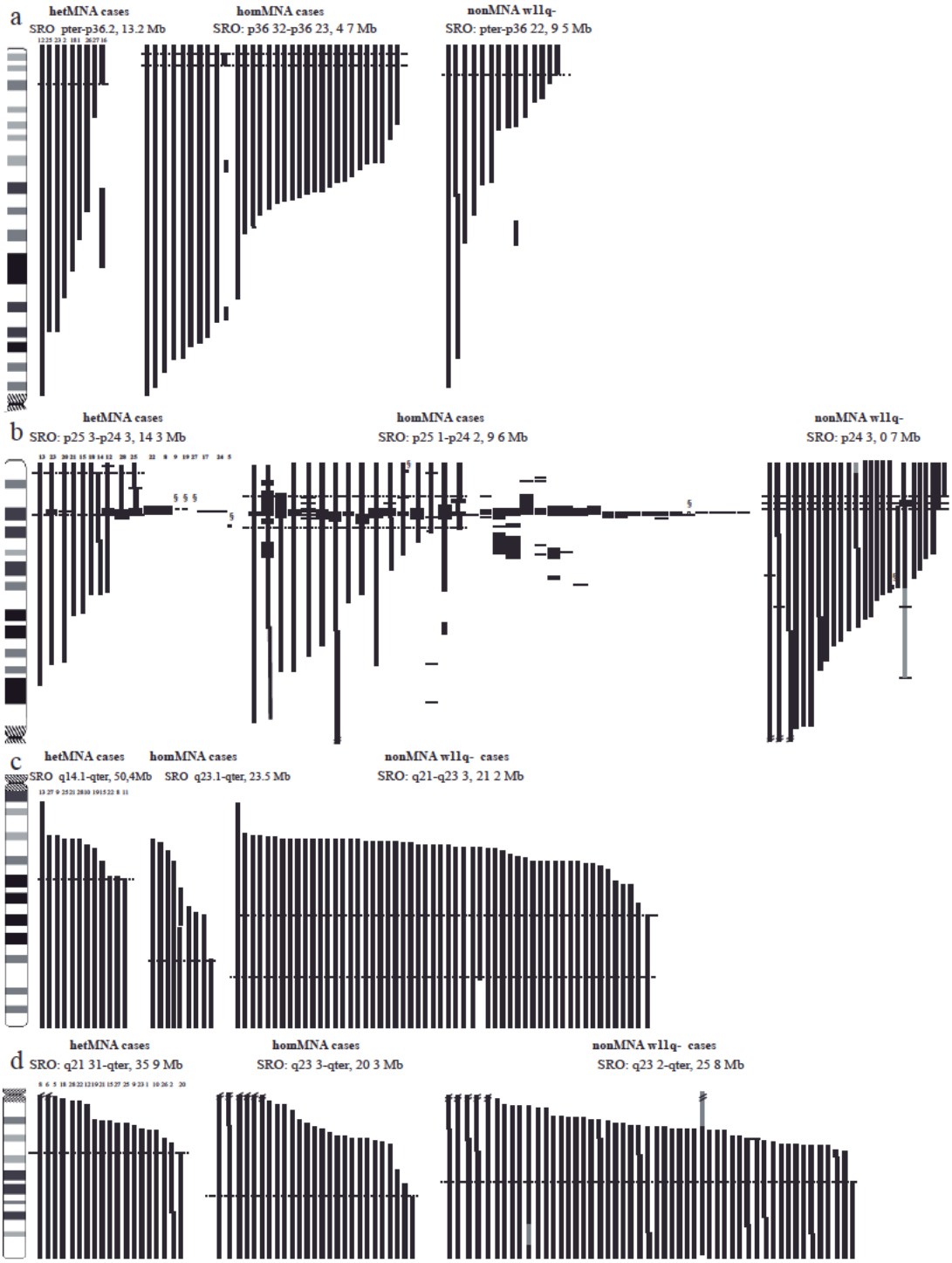
a
hetMNA cases
SRO pter-p36.2, 13.2 Mb
homMNA cases
SRO: p36 32-p36 23, 4 7 Mb
nonMNA w11q-
SRO: pter-p36 22, 9 5 Mb
b
hetMNA cases
SRO: p25 3-p24 3, 14 3 Mb
homMNA cases
SRO: p25 1-p24 2, 9 6 Mb
nonMNA w11q-
SRO: p24 3, 0 7 Mb
c
hetMNA cases
SRO q14.1-qter, 50,4Mb
homMNA cases
SRO q23.1-qter, 23.5 Mb
nonMNA w11q- cases
SRO: q21-q23 3, 21 2 Mb
d
hetMNA cases
SRO: q21 31-qter, 35 9 Mb
homMNA cases
SRO: q23 3-qter, 20 3 Mb
nonMNA w11q- cases
SRO: q23 2-qter, 25 8 Mb

Figure 3.

SCRIPT OF THE THESIS

1. INTRODUCTION

1.1. BASIC ASPECTS OF NEUROBLASTIC TUMORS

1.1.1. Epidemiology

Peripheral neuroblastic tumors are embryonic malignancies. They represent the most frequent extracranial solid tumor in childhood, accounting for 7.6% of all worldwide childhood malignancies, under the age of 15, and being 8% of these malignancies in Spain [1, 2]. In the text we will use the well-known term neuroblastoma (NB) instead of the more precise but less common term: peripheral neuroblastic tumor. The median age at diagnosis is 17 months [1, 3, 4], with a peak in diagnosis at the first year of age. The survival rate is high up to the first year of diagnosis and then drops steeply. In fact, while for NB of all ages 5-year survival is of 70.6% (95% CI, 68.4-72.6) for the children aged less than one year the 5-year survival is of 91% (95% CI, 89.6-92.5) [5] . Over the age of 10 years, in adolescent and adult lifehood, NB is very rarely diagnosed [6]. As a result of the low number of cases, adolescent and young adult (AYA) NBs are usually included as older patients, whereas NB in middle-aged and elderly adults are often ignored in the NB studies [7-11]. 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 known biological markers [9, 12, 13]. Moreover, studies of older high-risk NB patients show an age-dependent pattern in overall response; the patients older than 18 years appeared to have a higher response rate than the adolescent population [14]. Nonetheless, clinical and biological features of older NB remain obscure. We carried out a characterization of genetic features, among others, in a cohort of NB patients older than 10 years; these results have been published as part of the **article II** of the present thesis work.
The male-to-female ratio is 1.2 for all NB ages [1]. The precise aetiology of NB is poorly understood, with genetic factors among others involved [15-18]. The majority of the cases are sporadic and only 1-2% are familial, being the latter more frequently diagnosed at an early age [19].

1.1.2. Embryological origin and pathology classification

NB belongs to the small, blue, round cell tumors of childhood. It originates from precursor cells of the Sympathetic Nervous System which develop from the trunk neural crest cells and possess the characteristics of self-renewal and multipotency [20]. In fact, NB tumors express genes cells cells express stem cell and pluripotency related genes, such as *SOX2,* that have been correlated to aggressive disease or *LIN28B, KLF2 and KLF4 that are upregulated by*

MYCN [21]. Location of primary neuroblastic tumors is dependent on the embryonic migration of trunk neural crest cells, arising anywhere along the sympathetic ganglia or paraganglia as well as in the adrenal medulla. Around 65% of the tumors are located in the adrenal medulla and lumbar sympathetic ganglia while the rest arise along the paravertebral axis (i.e. neck, chest or pelvis) [4].

The current International Neuroblastoma Pathology Classification (INPC) based upon modifications of the histoprognostic classification proposed by *Shimada et al* [22, 23], has proved a role in predicting outcome in NB [24-26]. INPC distinguishes favorable and unfavorable tumors depending on the age at diagnosis, the amount of Schwannian stromal content, grade of neuroblastic differentiation and the mitosis-karyorrhexis index (MKI). Peripheral neuroblastic tumors are classified into four basic morphologic categories and their grade of neuroblastic differentiation: 1) neuroblastoma (Schwannian stroma-poor), undifferentiated, poorly differentiated, and differentiating; 2) ganglioneuroblastoma, intermixed (Schwannian stroma-rich); 3) ganglioneuroblastoma, nodular (composite Schwannian stroma-rich/stroma dominant and stroma-poor); and 4) ganglioneuroma (Schwannian stroma-dominant), maturing and mature [23].

1.1.3. Clinical presentation and current prognostic risk stratification

Clinical spectrum of NB ranges from tumors that spontaneously regress or mature into benign ganglioneuromas, to tumors with a very aggressive onset and fatal outcome [4, 27-29]. In fact, NB shows the highest rate of spontaneous regression among malignant tumors [30]. Nonetheless, a relevant fact is that survivors have an increased risk of developing complications later in life; especially for patients who undergo on multimodal treatments rather than surgery alone and efforts are being made to reduce therapy-related toxicities [31, 32].

Prognostic factors classify NB patients according to risk of relapse or death and are used for treatment stratification. Stage is one of the most important prognosis factors in NB. A task effort was conducted by the International NB Risk Group (INRG) in order to facilitate the world-wide comparison of risk-based groups with the development of a new staging system, called the INRG Staging System (INRGSS) [33, 34]. The patients are now stratified, previous to any treatment, based on tumor imaging rather than the extent of surgical resection of the previous staging system (INSS) [33, 35]. The extent of locoregional disease is now determined by the absence or presence of image-defined risk factors (L1 and L2, respectively). Stage M is used for widely disseminated disease, and MS describes metastatic NB limited to skin, liver,

and bone marrow without cortical bone involvement in children aged 0 to 18 months. This new staging system is not intended to be an immediate substitute for the INSS, and currently it is recommended that both systems should be used in parallel during a transitional period. The patients included in the articles of this thesis studies are staged according to the INSS system. INRG has also developed a new risk stratification system in order to establish a consensus pretreatment risk stratification approach using seven factors that were considered clinically relevant and also highly statistically significant (Table 1). In brief, this comprehensive risk stratification is based on clinical (patient age, tumor stage), pathological (histological category and grade of tumor differentiation) and specific biological (*MYCN* oncogene, and presence/absence of 11q aberrations, and tumor cell ploidy) prognosis factors.

Table 1. International Neuroblastoma Risk Group (INRG) Consensus Pretreatment Classification [33]

INRG Stage	Age (months)	Histologic Category	Grade of Tumor Differentiation	*MYCN*	11q Aberration	Ploidy	Pretreatment Risk Group
L1/L2		GN maturing; GNB intermixed					A Very low
L1		Any, except GN maturing or GNB intermixed		NA			B Very low
				Amp			K High
L2	< 18	Any, except GN maturing or GNB intermixed		NA	No		D Low
					Yes		G Intermediate
	≥ 18	GNB nodular; neuroblastoma	Differentiating	NA	No		E Low
					Yes		H Intermediate
			Poorly differentiated or undifferentiated	NA			
				Amp			N High
M	< 18			NA		Hyperdiploid	F Low
	< 12			NA		Diploid	I Intermediate
	12 to < 18			NA		Diploid	J Intermediate
	< 18			Amp			O High
	≥ 18						P High
MS	< 18			NA	No		C Very low
					Yes		Q High
				Amp			R High

GN, ganglioneuroma; GNB, ganglioneuroblastoma; Amp, amplified; NA, not amplified; L1, localized tumor confined to one body compartment and with absence of image-defined risk factors (IDRFs); L2, locoregional tumor with presence of one or more IDRFs; MS, metastatic disease confined to skin, liver and/or bone marrow in children < 18 months of age; M, distant metastatic disease (except stage MS).

Using this system, four broad categories with different 5-year free survival rates (EFS) are identified: very low risk (more than 85%), low risk (more than 75% to ≤ 85%), intermediate risk (≥ 50% to ≤ 75%) and high risk (less than 50%).

Clinical and pathological factors included in the INRG are surrogate markers of the underlying tumor biology. Histology, tumor grade of differentiation and MKI are separated from age, as the latter has been included as an independent variable in the risk stratification schema. Although outcome gradually worsens with increasing age, an age cut-off of 18 months was chosen as suggested by London *et al* because of practical reasons for the analyses [3, 6].

However, for stage M patients with diploid and nonMN*A* tumors a more conservative age cutoff of 12 months was maintained [36].

1.1.4. Therapy

NB patients are treated according to the INRG patient´s stratification risk. The biological heterogeneity demands diversity in the treatment approaches. Patients with low and intermediate risk disease have an excellent prognosis [37]and for these patients the trend over the last decade has been a reduction in the therapeutic intensity and a tumor resection as complete as possible [31]. Low risk patients are treated with surgery alone, intermediate risk patients with surgery plus moderate-intensity chemotherapy and patients with disease stage 4S/MS will receive supportive care (the "wait and see" approach) [28, 38]. On the contrary, in high-risk patients, that have shown only a low to moderate improvement in survival, the management consisted in intensified chemoradiotherapy. In general, the classic treatment approaches for these patients is performed in three phases: 1) intensive chemotherapeutic induction of remission followed by surgery, 2) consolidation of the remission to eradicate remaining tumor cells by myeloablative chemotherapeutic doses followed rapidly by rescue with autologous hematopoietic progenitor cells, and 3) a maintenance phase with isotretinoin administration [31, 39, 40]. Additionally, in high-risk patients the immunotherapy with anti-GD2 plus cytokines has demonstrated an effect in short survival and is used to eradicate residual NB cells [41, 42]. Other novel targeted therapies include: ALK inhibition (Crizotinib®), effective in other malignancies with *ALK*-rearrangements and already used in NB early-phase clinical trials, clinical inhibitors of PI3K, mTOR or AKT, aurora kinase inhibitors, HDAC8-selective inhibitors or antiangiogenic therapy [43-50].

However, the stratification system although very useful misclassifies the risk of some groups of patients that are therefore over- or under-treated. Therefore, poor prognosis and impact on quality of life demands to better distinguish non high-risk from high-risk patients, in which conventional risk stratification fails [51, 52]. This is especially important for the high-risk cohorts, which constitute half of all patients and where less than 50% of these patients will be cured [4, 53]. Identifying the biomarkers predictive for response to treatment is a great request for ongoing research projects [51, 54].

1.2. MOLECULAR ASPECTS OF NEUROBLASTIC TUMORS

This epigraph summarizes the molecular aspects, referred to DNA modifications and protein expression, focusing on the most relevant aspects for the present thesis research.

1.2.1. *MYCN* gene amplification and 11q chromosomal aberration

NB is one of the first tumors for which genomic alterations, *MYCN* amplification (MNA) and more recently 11q aberration have been included in therapeutic management [55]. Generally, high-risk NB either show MNA or 11q deletion. Genomic amplification of the *MYCN* oncogene has been used to predict outcome in NB for over 30 years, being Fluorescence in situ hybridization the gold-standard technique used for its status determination [55-59]. The gene amplification can be detected as double minutes (dmins) or as homogeneously stained regions (HSRs). It is defined by the INRG Biology committee as the presence of a more than 4-fold increase in the *MYCN* signals number compared to the reference probe located on chromosome 2 in all tumor cells [55]. In addition to MNA cells, MYCN gained cells (MNG), with 1 to 4-fold increase in *MYCN* number of signals, can be found in MNA tumors. Homogeneous MNA is present in about 20% of all NB cases, being associated with unfavorable cases, advanced stage of disease, aggressive behavior and high-risk of relapse. It is found in 30% to 40% of patients with advanced disease and 5% to 10% of patients with low stages of disease and stage 4S [4]. *MYCN* amplification results in an increase of MYCN oncoprotein, which is an oncogenic transcription factor with effects on cell proliferation, angiogenesis, differentiation and invasive potential by mechanisms still not clear [60]. Therefore, both the protein and the gene are very attractive therapeutic targets [61, 62]. A relevant number of genes are regulated by *MYCN* and the ongoing research suggests that the aggressive phenotype of *MYCN* might not only be associated with MNA, but with other signaling pathways that regulate MYCN [62, 63]. MNA tumors often display other unfavorable genetic markers such as 1p deletion and 17q gain but very few other segmental chromosome aberrations (SCA) and compared with other high-risk tumors, a less complex genome-wide pattern of SCAs [64]. MNA in a few cases is found as the only alteration of the tumor and even so the prognosis is very poor [65, 66]. In general the term MNA refers to the presence of homogeneous MYCN amplification (homMNA) but MNA can also occur as heterogeneous status (hetMNA). HetMNA NB is an infrequent event (1-2%), in which *MYCN* amplified cells frequently including tumor cells with *MYCN* gain coexist with *MYCN* non-amplified not gained tumor cells [67, 68]. Mainly because it is a rare event and difficult to be diagnosed, hetMNA clinical and biological implications remain unclear. In our **article III** we present the studies performed on hetMNA tumors diagnosed by our research

group. In these report, we compare the genetic background of hetMNA tumors to that of homMNA and nonMNA tumors with 11q deletion.

11q loss is the second molecular marker with more relevance in current prognostic risk stratification [69-71]. Rearrangements in chromosome 11q occur in approximately 30% of primary NB; they are associated with poor outcome. 11q deletion is more frequently present in older-aged patients with advanced disease stage and in tumors with many more SCA [72, 73]. There is an inverse relationship between MNA and 11q loss, indicating distinct genetic subtypes of aggressive NB [65, 72, 74-76]. In fact, infrequent cases with MNA plus 11q loss have been described with an unexpected complexity and dramatic decline of survival rates [77]. 11q deletion very often co-occurs (33%-43%) with 3p deletion and older age [66, 78-80]. Different studies have described several putative tumor suppressor genes located on 11q deleted region but due to the variation on the size of the deleted region, a gene dosage effect seems more plausible explanation for the involvement of the 11q region in the development of NB rather than a single gene [81-83]. Furthermore, the lack of homozygous deletions on 11q might mean that a tumorigenic effect is being rendered through haploinsufficiency of one or more genes on chromosome 11q. Several genes, such as *AT*M and *H2FAX,* involved with genomic instability have been proposed [72, 84-87]. We described in the **article I** of this thesis the genetic characterization of a group of NB in which alterations, MNA (either homMNA or hetMNA) and 11q deletion, were simultaneously present.

1.2.2. Other typical chromosomal aberrations

The most frequent chromosomal alteration in NB is gain of 17q, typically involving the 17q21-qter chromosome region. It occurs in around 70% of all NB tumors; in 45% of tumors classified as low- and intermediate-risk and in almost 100% of the high-risk tumors, either MNA or nonMNA [64, 79, 87]. This rearrangement is very often caused by unbalanced translocations with the distal part of chromosomes 1p or 11q. Therefore, it is found associated with 1p and 11q deletions and also MNA, but so far its independent prognostic significance is controversial [88-91].

Other frequently described chromosomal aberrations are 1p-, 3p- ,4p-, 9p-, 12p-, 14q- +1q, +2p [64, 66, 78, 87, 92-96]. Collaborative studies using a relatively large number of samples with pangenomic techniques (either MLPA or CGH) analysed the typical or recurrent chromosome regions (1p, 1q, 2p, 3p, 4p, 11q and 17q) and concluded that the presence of these aberrations clearly have a prognostic significance [55, 97, 98]. A beneficial advance from bench to bedside has been the inclusion of the genetic profiles into treatment stratification

schema such as into the European trial for localized NB (EudraCT 2010-021396-81). Therefore, the INRG Biology Committee recommends the analysis of the status of all these afore mentioned markers and others and preferentially the analyses of the whole genomic profile as performed in the **articles I, II** and **III** of this thesis and revised bellow**.** Furthermore, in this genetic context high resolution Single Nucleotide Arrays (SNPa) results are important to refine the regions were genetic biomarkers could be identified, as small regions of overlap (SROs) (**article I and II)** [72, 99, 100].

1.2.3. Genetic profiles

Advances from the classical cytogenetic techniques to metaphasic or conventional chromosomal-comparative genomic hybridization (CGH), Multiplex Ligation Probe Amplification (MLPA), different CGH arrays (cDNA microarray-CGH in-house bacterial artificial chromosome array-CGH) and more recently the commercial oligonucleotide cDNA arrays and SNPa and Next Generation Sequencing (NGS) techniques, have demonstrated as powerful tools in generating a more precise genetic characterization of NB [[64, 97, 99, 101]. As a result of these technical advances and research efforts, a large number of complete or partial gains and losses of chromosome material, numerical chromosome aberrations (NCA) and SCA respectively, have been described in NB; most of the latter are the result of unbalanced translocations [55]. The huge genetic variety shown by high throughput methods, although it complicates the identification of clinically significant markers, leads to the belief on the potential identification of distinct well-defined risk biomarkers, associated with each NB disease type.

In brief and based on the genome-wide analyses, NB are classified into two broad genetic subtypes: tumors with only NCA and tumors with SCA with or without MNA and/or NCA. The SCA genomic type in general have a higher hazard of progression or relapse than the NCA tumors [92]. Low-risk tumors are mainly NCA tumors with very few or no SCA. NCA tumors are currently classified by INRG and also by the Children Oncology Group (COG) as low- or intermediate risk [64, 101]. NCA tumors show better progression, free survival without disease and without relapses, and when present always salvaged regardless of age and stage. NCA profiles correlate with pseudotriploidy, with age ≤ 18m and non-stage 4 [102-105]. Whole chromosomes losses (3, 4 and 14) and gains (7 and 17) are the most frequently NCAs found [64, 65, 79, 104]. NCA tumors also corresponded frequently to differentiating NB and localised disease compared with SCA tumors. Disease stage 4S are frequently purely NCA tumors [92] [65, 101]. The high-risk group included mostly near diploid tumors, with SCA, including *MNA* or

11q deletion as well as few or many other genetic aberrations [92]. Interestingly, the presence of SCA identified patients within the low- and intermediate-risk groups with poorer progression free survival [92]. Also a high number of SCA has been associated with a poor prognostic impact. Patient's older than 18 months had a higher number of SCAs [72, 104, 106-108]. Deletions of the chromosome regions 3p, 4p and 11q are frequently present in nonMNA tumors. On the contrary, 1p deletions can be found in nonMNA and MNA tumors but are strongly associated with MNA and 17q gain [65, 66]. Furthermore, 30% of the tumors with a SCA profile co-occur with complete chromosome gains and losses and have a pseudotriploid DNA index similar to that found in the NCA only tumors, but are diagnosed in older patients [92, 104]. A similar pattern of NCA aberrations is shared. Amplifications in genes other than *MYCN* such as *ALK*, *MDM2* and *CDK4* genes are recurrently present in NB, but their role is not fully understood and needs to be further investigated [109-111]. Also chromothripsis has been described in high-stage tumors and it frequently affected genes involved in NB pathogenesis and was associated with amplification of *MYCN* or *CDK4* and loss of heterozygosity of 1p [112, 113]. In low-stage NB tumors no chromothripsis has been found and these tumors lacked of recurrent gene alterations (neither mutations nor structural variations).

There is an additional group of tumors with almost none or few SCA or NCA, termed as silent or flat profile, that represents 5-8.4% [65, 66, 92] of the NB and very frequently with di/tetraploid DNA index [65]. It has been suggested, after having excluded that the amount of neuroblastic cells was not sufficient (at least 50%), that these tumors might represent NB at an early stage of carcinogenesis with an early oncogenic hit. Additional studies at a higher resolution should determine whether hidden genetic aberrations have been undetected [92].

Alongside large chromosome SCAs, focal gains or deletions (<2 Mb) are also detected in around 38% of cases with unknown role in NB pathogenesis [87]. Some studies suggested that these small changes in copy number could contribute to the establishment of metastatic potential in NB could be a mere consequence of global genomic instability. More in depth studies have linked some of these focal changes to miRNAs that mediate in *MYCN* signaling [87, 100].

Summing-up, the global view of NB genetic instability illustrated by genome-wide studies has demonstrated that the overall genomic pattern and the associations of SCA and/or NCAs can more precisely predict the clinical behavior, rather than considering a sole individual chromosomal alteration and is able to subclassify tumors into different genomic subtypes with different risk criteria [66, 92].

1.2.4. Most relevant findings on mutation

In addition to all the somatic changes above mentioned, germ-line and acquired mutations have been described in familial and sporadic NB, respectively [114, 115]. Familial NB is very rare (1-2%) and in 90% of the cases the genes *ALK* or *PHOX2B* are mutated [45, 116-119])ALK is a tyrosine kinase receptor important in neural differentiation, proliferation, and survival. Functional studies performed in NB cell lines with mutated *ALK* showed a gain of function effect that results in increased proliferation. *ALK* is mutated in 80% of familial NB but also *ALK* mutations are identified in sporadic NB tumors both at diagnosis (8-10%) and at relapse (17%) [114, 120, 121]. The recurrent activating mutations but also copy number changes, overexpression and translocation found in NB, together with its normal expression confined to developing neural tissues, made *ALK* a promising therapeutic target in high-risk NB as well as an emergent molecular marker for treatment stratification [43, 50, 122-125]. Moreover, other variations in SNP locus have been identified as predisposing for sporadic NB (i.e. 6p22 locus; *BARD1*, 2q35 and *LMO1*, 11p15) and also specific copy number variants for the genes *NBPF23* (deletion) and *LMO1* (duplication) loci [114, 126-128]. Some of these variations, 6p22 loci among others, are associated also with familial NB and with more aggressive behavior [92].

The recent whole-genome and exome sequence studies in around 400 tumors aimed to uncover somatic tumor-driving mutations found very few genes with early recurrent somatic mutations, with frequencies for mutations of the genes ranging from 0.83 to 11%. The genes identified and the frequencies were the following: *ARID1A* and *ARID1B* (11%), *ALK* (7-9.2%), *TIAM1* (3%), *PTPN11* (2.9%), *ATRX* (2.5%, an additionally 7.1% had focal deletions), *MYCN* (1.7%, a recurrent p.Pro44Leu alteration), and *NRAS* (0.83%) [112, 113]. Nevertheless, a higher mutation frequency strongly correlated to high-stage tumors, worst survival and older age at diagnosis (>18 m) but was independent of *MYCN* status. Most of the recurrent alterations in high-stage tumors were found on genes involved in neuronal growth cones (i.e. *PTPRD, ODZ3, ODZ2, ATRX).* [129, 130].

Within the high-risk cohort there are ongoing attempts to substratify the patients with worse survival that do not adequately respond to high-risk NB standard therapy, the so-called 'ultra-high-risk' NB [131-135]. Finding the criteria to discriminate outcome becomes difficult given the extensive heterogeneity in biological and clinical features. In addition, it requires gathering a high number of patients with known clinical data, as well a long follow-up and available good tumor material. Nevertheless, some recent genomic classification models combine gene and epigenetic expression to generate data to revise the risk estimation and treatment

stratification of the patients and to get signatures that seem to overcome these difficulties and more accurately predict outcome and intensify or de-escalate treatment regimens.

1.2 5. Expression profiles

Expression profiling studies intend to identify relevant NB genes. *MYCN* expression is important in the early developmental process and a decrease in its expression is required later on for a complete embryonic development differentiation. *MYCN* expression is controlled at multiple levels (upstream gene transcription regulators, mRNA turnover, protein activation or decay upon phosphorylation) and in addition, by microRNAs with oncogenic and tumor suppression functions [135]. Therefore, the understanding of MNA association with aggressive behavior is not completely understood. Usually MNA results in N-myc protein overexpression but this is not always achievable [136]. Several N-myc targets have been identified, such as genes involved in cell cycle regulation, apoptosis, drug resistance and *MYCN* stability (ie.*MDM2, ODC, TP53, MRP*1 and *Aurora kinase A*). Early research studies demonstrated that less aggressive tumors expressed differentially the tyrosine kinase receptor genes that mediate in differentiation, apoptosis and neural survival. Usually, expression in tumors of markers that are expressed in later stages of differentiation, such as TRKA are indicative of a better prognosis. TRKA and TRKC expression correlate with more favorable prognosis while TRKB expression correlates with MNA and more aggressive tumors, in which it may enhance proliferation of the tumor [137-140]. General mRNA expression has been expansively studied and different signatures of several or multiple genes have been linked to prognosis [132-134, 141-144]. Other important genes, such as *ATRX,* have been recently identified with a putative role in NB tumor growth. *ATRX* is a chromatin-modifier with a suggested role in telomere maintenance [145]. In fact, defects in *ATRX* gene, deletions or mutations, and loss of the protein expression have been associated with older NB [113, 130, 146, 147]. In **article II** *ATRX* protein expression in AYA NB was evaluated.

The increasing amount of data generated by genomic analysis requires the use of mathematical tools to more easily perform the gene copy number, gene aberrations, mutations and expression analyses, visualization and evaluation. In this sense, different tools have been created. Some examples of databases are the following: ACTuDB (http://bioinfo.curie.fr/actudb), arrayCGHbase (http://medgen.ugent.be/arrayCGHbase), arrayMap (http://arraymap.org/) and R2 (http://r2.amc.nl). These tools enable analysis of copy number, gene aberrations and expression analyses and for meta-analyses in NB and comparison with other cancers. To facilitate the progress of research and the comprehension

of the results integration of all the amount of heterogeneous genetic data gathered, other biological data, such as those of stromal cells and extracellular matrix elements, are a major need.

1.3. STROMAL CELLS AND EXTRACELLULAR MATRIX OF NEUROBLASTIC TUMORS

The tumor microenvironment is comprised of a variety of cell types, lying among a dense network of various extracellular matrix fibers merged with the interstitial fluid and gradients of several chemical species, which constantly interplay with cancer and host cells [148]. These components are important in tumor progression and metastasis, for example tumor angiogenesis implies degradation of the extracellular matrix, and inflammatory infiltration can acquire a tumor-promoting phenotype cells when molded by cancer cells [149].

1.3.1. Immune system cells

Because NB can spontaneously regress, de novo anti-tumour immunity in patients is warranted [150, 151]. Moreover, multiple ongoing immunotherapeutic approaches have been successfully applied in patients that suffered from relapsed or refractory stage 4 NB [41, 151-154]. Lymphocytic infiltration of NB has generally been observed in low-stage cases with the associated opsoclonus-myoclonus syndrome [155]. Recent studies have recognized the potential importance of the background inflammatory cells in the pathophysiology and prognosis of NB [156, 157]. Early expression studies on anti-tumor response in NB tissues showed that T-cells, CD4+ and CD8+, infiltrated the peritumoral stroma but less frequently the tumor nests. Results of some of the studies are related with current prognostic risk stratification factor. A highly enriched immunity signature has been found, including much higher expression of human leukocyte antigen molecules, in a subset of high-risk NBs nonMNA, all stage 3 and 4, and most of them with 11q- and/or +17q, but no 1p- [158]. Expression of tumor-associated macrophages and inflammation related genes are higher in metastatic NB and showed an age-dependent expression pattern (>18m) [157]. Accordingly in **article II,** we have studied the immune cell expression patterns of macrophages and other immune system cells and its association with age in a group of AYA NB.

1.3.2. Other stromal cells and extracellular matrix

Schwann cells, fibroblasts, stem cells and vascular endothelial cells among other cells are also found in the context of NB stroma. Although there are some controversial results, it seems that origin and genetic characteristics of Schwann and neuroblastic cells are clearly different [159-162]. It has been demonstrated that cross-talks between Schwan cells and neuroblasts triggers tumor differentiation and inhibits angiogenesis [163]. Furthermore, this cross-talk between NB cells and the extracellular matrix influences NB differentiation and recent studies have identified key genes that regulate these interactions [2]. Moreover, studies on NB extracellular matrix suggest that its rigidity may also play an important role in this cross-talk with tumor cells affecting their differentiation. Understanding this process would be critical to achieve an optimal differentiation [164-167]. In this respect, our group performed a preliminary study where some differences regarding ECM elements between favorable and unfavorable neuroblastic tumors were described [167]. In a further study, we observed that tumors associated with poor prognostic factors according to INRG presented a decreased amount of glycosaminoglycans, scarce collagen type I bundles and elastic fibers, found as intercellular scaffolding, reticulin fibers networks forming a stiff and poorly porous extracellular matrix and larger, more abundant and more irregularly-shaped blood vessels, where capillaries were less abundant and sinusoid-like blood vessels more abundant (Irene Tadeo doctorate thesis and manuscript in preparation). The usefulness of the extracellular matrix elements characterization to define an Ultra-high Risk group of Neuroblastic Tumors within the High Risk Cohort has been also described (manuscript send to be considered to publication).

The integration of the data from basic research and clinical practice through data modeling techniques will hopefully improve our understanding and treatment of NB.

1.4. MODELING OF NEUROBLASTIC TUMORS

Diagnostic risk classification and treatment stratification for cancer patients are restricted by our incomplete picture of the complex and unknown interactions between the patient's organism and tumor tissues (transformed cells supported by tumor stroma). Moreover, all clinical factors and laboratory studies used to indicate treatment effectiveness and outcomes are by their nature a simplification of the biological system of cancer, and cannot yet incorporate all possible prognostic indicators. A close integration of data generation with mathematical algorithms is the process of creating models for systems biology. The fields

of theoretical and mathematical biology have pioneered the development of mathematical and computational models of biological systems. Systems biology has contributed workflows for data-driven modeling and modeling-driven experimentation to the life sciences. Systems medicine can be seen as a further development of systems biology and bioinformatics toward applications of clinical relevance [168]. The key to dealing with personalized therapy lies in the mathematical modeling. The use of bioinformatics in patient-tumor-microenvironment data management allows a predictive model in neuroblastoma [167].

2. OBJETIVOS, JUSTIFICACIÓN Y CONTEXTO DE LOS ARTÍCULOS INCLUIDOS EN ESTA TESIS

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2. OBJECTIVES, JUSTIFICATION AND SCOPE OF THE THESIS AS A COMPENDIUM

2.1. OBJETIVOS

2. 1.1. Objetivo general

En esta tesis perseguimos describir las características biológicas de tres grupos infrecuentes de tumores neuroblásticos raros identificando las característica genéticas de cada grupo con el fin último de facilitar grandes estudios multicéntricos que puedan lograr una terapia más precisa de los pacientes.

2.1.2. Objetivos específicos

Artículo I: El propósito de este estudio fue caracterizar el perfil genético de tumores neuroblásticos con dos características cromosómicas agresivas, amplificación de MYCN y deleción de 11q. Con este fin nos centramos en:

1. Identificar las alteraciones cromosómicas y definir sus puntos de ruptura cromosómica.
2. Definir las regiones más pequeñas de solapamiento de las alteraciones segmentarias.
3. Describir la heterogeneidad intratumoral de los cambios cromosómicos presentes en este grupo tumoral.

Artículo II: Buscar patrones dependientes de la edad avanzada en el neuroblastoma, asociados con características biológicas particulares, en una cohorte de pacientes mayores de 10 años al diagnóstico. Nuestras investigaciones se concentraron en:

1. Explorar los patrones de alteraciones cromosómicas segmentarias mediante la técnica de micromatrices de polimorfismos de un único nucleótido.
2. Investigar la presencia de la infiltración células inmunes en el tejido tumoral mediante la expresión inmunohistoquímica de marcadores celulares (CD4, CD8, CD20, CD11b, CD11c y CD68).
3. Evaluar la expresión de la proteína ATRX por inmunohistoquímica.

Artículo III: El objetivo del estudio fue describir las características genéticas de los tumores neuroblásticos con amplificación heterogénea de *MYCN.* La investigación se centró en:

1. Identificar los patrones de las alteraciones cromosómicas presentes y definir los puntos de ruptura cromosómica.
2. Comparación de los perfiles genómicos y de determinados puntos de ruptura cromosómica entre los tumores con amplificación heterogénea de *MYCN* y los subgrupos genéticos agresivos de neuroblastoma (con y sin amplificación homogénea de *MYCN* y deleción de 11q).

2.2 JUSTIFICACIÓN DE LA TESIS COMO COMPENDIO DE PUBLICACIONES Y ALCANCE DE LOS RESULTADOS

El presente trabajo de tesis doctoral es un compendio de tres publicaciones que caracterizan neuroblastomas poco frecuentes; utilizaremos el término 'neuroblastomas ultra-raros' para referirnos a todos ellos como grupo. Como se razona a continuación, los artículos recogen tres desafíos de la investigación del neuroblastoma:

2.2.1. Centrarse en una cohorte minoritaria de pacientes afectos de neuroblastoma

Dentro de la enfermedad neuroblástica, considerada como una entidad rara (ORPHA635, http://www.orpha.net/), existen grupos clínica y biológicamente ultra-raros, en los que es necesario investigar más. La clínica de los pacientes con tumores ultra-raros es problemática, el tratamiento en estos pacientes con mucha frecuencia supone un dilema y todo el proceso de la enfermedad se vuelve más complejo. El escaso avance experimentado en el conocimiento de estos tumores no ha contribuido a la mejora de la supervivencia ni all desarrollo de tratamientos más adecuados. Por lo tanto, a pesar de la infrecuencia de estos tumores su investigación posee un gran valor.

2.2.2. Proponer una gestión más adecuada de las muestras

Dado que los grupos ultra-raros de neuroblastoma han sido a menudo excluidos o considerados como parte de otros grandes grupos de neuroblastomas con características clínicas y biológicas distintas a estos, la determinación de sus características biológicas se ha visto comprometida. Además, la escasez de la muestra tumoral disponible para realizar los estudios de investigación dificulta consensuar el papel de los análisis genéticos en la estratificación de riesgo terapéutica. Es crucial resaltar la importancia de la recogida metódica de la mayor cantidad de tejido tumoral posible para ser analizado secuencialmente por hibridación in situ fluorescente y por técnicas pangénomicas para determinar la presencia de heterogeneidad genética intratumoral. Considerar a estos tumores como entidades especiales con protocolos específicos para el manejo de las muestras beneficiaría su diagnóstico e investigación.

2.2.3. Considerar la utilidad potencial de los datos

La pretensión final del trabajo de tesis es contribuir a la recogida de datos y modelización del neuroblastoma. La descripción genética de estas cohortes facilitará la consecución de estudios multicéntricos grandes que son necesarios para coordinar proyectos clínicos. La ausencia de consenso diagnóstico respecto a los grupos de edad avanzada, una relativa falta de atención a los casos de neuroblastoma con amplificación atípica de *MYCN*, la escasez de la muestra tumoral y la heterogeneidad genética intratumoral son limitaciones que se pueden superar en el marco de los estudios multicéntricos almacenando adecuadamente los datos.

La modelización del tumor forma parte de un extenso proyecto de investigación de nuestro grupo. Algunos datos clínicos, histológicos (factores de la matriz celular y extracelular) y los datos genéticos están integrados en una base de datos de diseño propio y de uso interno previamente publicada (NeuPat) [169]. Los datos obtenidos del análisis de las micromatrices de polimorfismos de un único nucleótido se han cargado recientemente en una base de datos (arrayMap) con la intención de conseguir una más visualización fácil que ayude en el análisis e

investigación de las alteraciones genéticas pudiendo combinar los diferentes datos y seleccionar los diferentes valores genéticos y otras variables. Los tumores neuroblásticos ultra-raros debido a sus características biológicas singulares también pueden servir como modelos que proporcionarían conocimiento preciso sobre la patogénesis del neuroblastoma.

2.1. OBJECTIVES

2. 2.1. General

In this thesis research we sought to understand the biological features of three ultra-rare cohorts of neuroblastic tumors by identifying the genetic features specific of each group, with the ultimate goal of facilitating large multicenter studies that could lead to the achievement of a more accurate management of the patients.

2.1.2. Specifics

Article I: The purpose of this study was to characterize the genetic profile of uncommon neuroblastomas harboring two chromosomal aggressive features, *MYCN* amplification plus 11q deletion. Attention was focused on:

1. Identifying the chromosome aberrations present and mapping their breakpoints.
2. Charactering the breakpoints and defining the smallest region of the segmental chromosomal alterations.
3. Describing the intratumoral heterogeneity present in this specific cohort.

Article II: To search for older-age-dependent patterns associated with certain biological characteristics in a cohort of patients older than 10 years at diagnosis. Our actions were concentrated on:

2. Exploring the patterns of segmental chromosome aberrations by performing Single Nucleotide Polymorphism array.
3. Investigating the presence of the immune cell tissue infiltration by immunohistochemistry expression of immune cell markers (CD4, CD8, CD20, CD11b, CD11c and CD68).
4. Evaluating the expression of the ATRX protein by immunohistochemistry.

Article III: The aim of the study was to demarcate the genetic characteristics of unusual heterogenous *MYCN* amplified tumors. Our approach consisted of:

1. Identifying the patterns of segmental chromosome aberrations chromosome aberrations present and mapping of the breakpoints.
2. Comparison between their genomic profiles and breakpoints with those present in the aggressive genetic subgroups of neuroblastoma (homogeneous *MYCN* amplified tumors and segmental chromosome aberrations non-*MYCN* amplified tumors with and without 11q deletion).

2.2 JUSTIFICATION AND SCOPE OF THE THESIS AS PUBLICATION COMPENDIUM

The present doctoral thesis research is presented as a compendium of three publications dealing with uncommon neuroblastoma tumors; henceforth we will use in this compilation the term ultra-rare neuroblastoma to refer to all of them. As outlined below, the papers unify three research challenges:

2.2.1. Focus on minor cohort of neuroblastoma patients

Within this rare neuroblastoma disease (ORPHA635, http://www.orpha.net/) there are clinical and biological ultra-rare neuroblastoma groups for which further research is necessary. Ultra-rare neuroblastoma patients' care is problematic, therapeutic decision-making very frequently poses a dilemma and dealing with the disease process becomes more complicated. The scant knowledge of these tumors means the failure to develop better treatments and consequent lack of improvement in survival. Therefore, although uncommon, research into these tumors is worthy and worthwhile.

2.2.2. Propose an accurate management of the samples

Since ultra-rare groups of neuroblastoma have often been excluded or considered as part of larger groups of neuroblastoma with dissimilar clinical and biological characteristics, delineation of their biology has been compromised. Consensus on the role of genetic analyses for establishing the therapeutic risk stratification is in addition impeded because of the scarce tumor sample material obtained for research studies. Methodical collection of as much tumor tissue as possible to be analyzed by FISH and pangenomic techniques becomes crucial in these groups of tumors to ascertain the presence of biological heterogeneity. Considering these tumors as a separate entity with specific standard protocols for sample management would benefit their diagnosis and research.

2.2.3. Consider potential utility of the data

The final challenge of the thesis work would be to contribute to the data collection and modeling of the tumor. The genetic description of these cohorts would facilitate large multicenter studies needed to coordinate clinical projects. The failure to achieve a diagnostic consensus, the relative lack of attention to ultra-rare neuroblastoma cases, the sample scarcity and genetic heterogeneity are limitations that can be overcome in the frame of multicenter studies. To set out the biological features, mainly chromosomal alterations, genetic consensual findings from other neuroblastoma cohorts were applied.

Generating the model is a huge project at our group of research. Some clinical, histological (cellular and extracellular matrix variables) and genetic data are integrated in an in-house database (NeuPat) [169]. SNPa data have recently been uploaded in a genetic repository (arrayMap) with the intention of allowing an easy visualization, aiding further investigation of the genetic aberrations and to combine all the data with the possibility of sorting or selecting the different genetic values or other factors. Ultra-rare neuroblastoma because of their singular biological features can also serve as models that could supply an accurate knowledge about the pathogenesis of neuroblastoma.

3. RESULTS AND DISCUSSION

The NB ultra-rare groups compiled in this thesis (**article II**: AYA NB; **article I**: MNA plus 11q- tumors; and **article III**: hetMNA tumors) were diagnosed between 1994 and 2014 at the Pathology Department of the Medical School, University of Valencia (DP-UV). During this period a total of 1.134 samples from patients have been received for genetic diagnosis and histopathological review. The average number of primary tumors per year is of 54 for the whole cohort and 3.8 for all ultra-rare tumors (Fig.1). We defined ultra-rare NB groups based on the low incidence (<5%) in our cohort or an unusual clinical and/or genetic feature of the tumors.

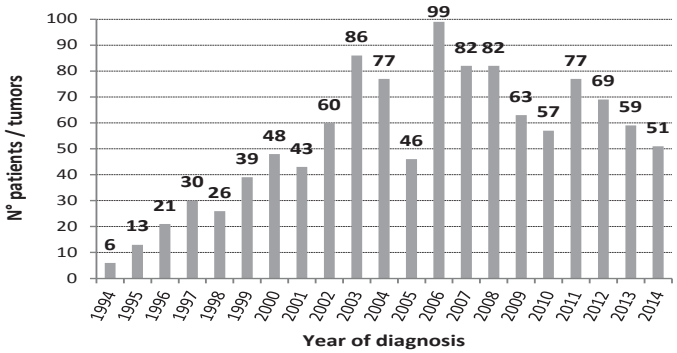

Figure 1. Bar graph showing the number of tumors diagnosed by year.

Because of the lack of pangenomic data for some tumors or because they have been diagnosed after the publication of the articles of this thesis work only 71 out of the 80 ultra-rare are included in the article I, II and III.

3.1. GENOMICS RELATED TO OTHER PROGNOSIS FACTORS

NB is typically diagnosed during the first year of life. Since then its incidence drops after being rarely diagnosed in patients over the age of 10 [170]. The natural history, biology and prognostic factors in older NB patient populations is unknown. Age definition has been always a challenge and finding an older age cut-off with prognostic implication remains complicated. Older age may not be an independent poor prognosis factor but may represent a proxy for a biological genotype [11].

3.1.1. Older age

3.1.1.1. Update of the thesis cohort

Age at diagnosis was known for 911 (80.3%) patients and ranged from 0 to 731.7 months (mean: 28.4 months, median: 15 months). For descriptive and comparative purposes, this cohort of patients can be divided into the age groups shown in Table 1.

Table 1. Age group distribution of NB patients at DP-UV

Age groups (months, years)	Thesis cohort n=911 (%)	MNA plus 11q- article I N= 19 (%)	AYA article II N=31 (%)	HetMNA article III N=28 (%)
0 - <18 months	487 (53.4)	5 (26.3)	-	11 (39.2)
≥18 months - <5 years	312 (34 2)	12 (63.1)	-	14 (50)
≥5 - <10 years	77 (8.4)	2 (10.5)	-	13 (46.4)
≥10 - <18 years	28 (3.1)	-	24 (77.4)	-
≥18 - 21 years	2 (0.2)	-	2 (6 5)	-
>21 years	5 (0.6)	-	5 (16.2)	

Updating the number of AYA patients of **article II**, four new patients older than 10 years and younger than 18 years have been included giving a percentage of AYA patients of 3.8% (35/911) similar to the 4.1% (31/750) of article II.

3.1.1.2. National population: data from the RETI-SEHOP

The cohort here studied although it does not include all the tumors diagnosed in Spain is somehow representative of the entire NB population. Number of patients registered at our laboratory and the total patients registered at the Spanish National Registry of Childhood Tumours (estimation from RETI-SEHOP, http://www.uv.es/rnti/), are presented in Table 2. RETI-SEHOP uses a slightly different age subgroups and shorter range of age, from 0 to 19 years, following the International Agency for Research on Cancer (IARC) and the International Association of Cancer Registries (IACR) guidelines.

Table 2. Patients registered at RETI-SEHOP/DP-UV distributed following IARC/ IACR guidelines age groups

Age groups (months/years)	Patients diagnosed in Spain[a]	Thesis cohort n=906 (%)
<1 year	729.8	409 (48)
1-4 years	690.4	390 (43)
5-9 years	183.7	77 (8.5)
10-14 years	42	26 (3)
15-19 years	45.7	4 (2.8)

[a], data transferred by Prof. Rafael Peris-Bonet (RETI-SEHOP, University of Valencia).

For estimation of the representativeness of our cohort of NB tumors, the expected number of primary tumors in Spain as well as the coverage of our laboratory (proportion of tumors registered at our laboratory) was calculated from age 0 to 19, during 1994-2014 (Table 3, estimation from RETI-SEHOP). The age range 10-19 years partly corresponds with the AYA cohort studied in article II.

Table 3. Number of cases observed and expected in Spain and percentage of coverage at DP-UV

	10-14 years (120-179 months)			15-19 years (179-239 months)			Total cohort (n=906)		
	No. observed	No. expected	coverage %	No. observed	No. expected	coverage %	No. observed	No. expected	coverage %
1994	0	2.4	0.0	0	2.8	0.0	2	75.1	2.7
1995	0	2.3	0.0	0	2.8	0.0	4	73.2	5.5
1996	1	2.2	45.2	0	2.7	0.0	19	72.1	26.4
1997	2	2.1	94.4	0	2.6	0.0	30	71.5	42.0
1998	0	2.0	0.0	0	2.5	0.0	23	70.6	32.6
1999	2	2.0	100.8	0	2.4	0.0	27	70.5	38.3
2000	0	1.9	0.0	1	2.3	44.3	32	71.7	44.6
2001	1	1.9	52.6	0	2.2	0.0	31	73.1	42.4
2002	2	1.9	105.4	0	2.1	0.0	51	75.1	67.9
2003	4	1.9	210.0	0	2.1	0.0	79	78.1	101.2
2004	1	1.9	52.8	0	2.0	0.0	67	80.4	83.3
2005	1	1.9	53.1	1	2.0	50.1	44	82.9	53.1
2006	0	1.9	0.0	2	2.0	100.9	88	85.5	102.9
2007	2	1.9	106.5	0	2.0	0.0	76	87.9	86.5
2008	2	1.9	105.9	0	2.0	0.0	62	91.0	68.2
2009	1	1.9	52.5	0	2.0	0.0	44	92.1	47.8
2010	1	1.9	51.7	0	1.9	0.0	48	90.9	52.8
2011	2	2.0	101.7	0	1.9	0.0	37	90.6	40.8
2012	0	2.0	0.0	0	1.9	0.0	39	89.4	43.6
2013	1	2.0	49.5	0	1.9	0.0	54	86.2	62.7
2014	3	2.1	145.8	0	1.8	0.0	49	84.0	58.3

For the first years (1994-2001, colored in grey at Table3) the number of tumors registered at our laboratory are far from the expected. Our group was not designated as Reference Center for Biology Studies until 1998. Since then, more than 50% of tumors expected to be diagnosed were registered at our laboratory, except for three years (2009, 2011, 2012) where the coverage dropped to 40%. 25 patients aged 10 to 14 were expected to be diagnosed from 2002 to 2014 and 20 patients were registered at our laboratory (80% of coverage). Patients over 15 years old are under registered (coverage 12%).

These data revealed that AYA NB population follows with more difficulty the standard genetic NB management guidelines. Probably because once the patients exceed the pediatric age there is a less exigent genetic routine of diagnosis or they are not included in laboratory registry. As a consequence, a delay or a lack of an accurate genetic diagnosis is very plausible, further meaning that these patients would have difficult access to the clinical relevant trials.

Currently, there is paucity of survival and also of treatment and outcome data for the older patients owing to the rarity of NB in this population [11]. In NB as in other paediatric cancers, AYA patient care is complicated – it is "Less a speciality incorporating a set of diseases that affect a defined age group but, rather, a recognition of the need to address the way in which the service is provided" [171-172]. The lower probability of cure suggests the importance of novel therapies [11]. In this context, the determination of the clinically relevant biologic factors

as well as setting standard treatment guidelines or chemotherapy protocols are crucial for improving older NB management [173].

3.1.1.3. Review of the literature: AYA incidence and main features

For comparison of our data and confirmation of the rare occurrence of older NB, most of the cases published during the last 20 years are presented in Table 4 with the clinical and genetic findings summarised.

Table 4. Literature review of AYA NB

First author, reference	No. patients (%)	Age, years (%)	Stage (%)	*MYCN* status (No. tumors analysed)	Main differential findings	
					Unfavorable	Favorable and others
Blatt J, 1995 [174]	17/136 (12)	6-15	4 (77)	NonMNA (17)	Bone met. 3-y survival	Favorable histology
Franks LM, 1997 [13]	16	13-17 (37) 19-36 (63)	2 (13) 4 (56)	NonMNA (6)	Multiple recurrences Low treat. tolerance Fatal outcome	Pelvic and thoracic location Met. at unusual sites Chronic disease
Kushner BH, 2003 [173]	30	12-41	4 (90)	NonMNA (22)	Unfavorable histology	—
Gaspar N, 2003 [175]	28/943 (3)	>10-<18	1,2,3 (54) 4 (46)	13% MNA (15)	St. 4 poorer survival than st. 3 Poor response to treatment	Met. unusual sites
Conte M, 2006 [9]	53/1663 (3)	10-18	3 (25) 4 (58)	11% MNA (35)	Poor outcome (especially local.) 1p- in 40% tumors	Indolent disease Met. unusual sites
Esiashvili N, 2007 [10]	234/2054 (11)	>10-19 (38) >20 (62)	3 (18) 4 (64)	—	Adolescents had worst survival than adults	Lower frequency of advanced st. for adults
Podda MG, 2010 [8]	27	12-17 (55) 18-69 (45)	3 (22) 4 (59)	10% MNA (10)	Poor outcome Met. at lymph node	Indolent disease
Castel V, 2010 [12]	22/576 (4)	10-17 (86) 18-24 (14)	3 (27) 4 (54)	NonMNA (22)	St. 4 poor outcome 11q- and +17q	—
Mosse YP, 2013 [11]	200/8800 (2.3)	>10-21	3 (16) 4 (59)	9% MNA (148)	Poor survival	Low/Intermediate MKI Indolent disease 1p LOH in 20%

—, data not available; y, year; treat. treatment; st, stage; met, metastases; LOH, loss of heterocigosity; local, localised stages; MKI, Mitosis-Karyorrhexis Index.

As shown in Table 4, age cut-off to consider older NB patients varies in the literature difficulting the comparisons. For the purposes of the study in article II, the patients were divided into two main groups: 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). Nonetheless the accepted age cut-off of previous studies, were maintained and are shown in Table 1. Regarding stage and outcome, our results were in agreement with earlier reports: high frequency of advanced disease stages, especially stage 4 (80.6%) and poor outcome (overall survival at 5-years: 44.8%). Patients with more favorable features (localized stage and NCA tumor) remained without recurrence or progression in accordance with results described in childhood NB [176].

Genetically we have described MNA and nonMNA tumors, with the NCA profile and with the SCA profile (11q deleted and non-deleted), as described in childhood NB [55, 101, 177, 178]. The rarity of MNA in the cohort studied was not unexpected. Its low frequency is shown in

Table 4. Little is known about genetic background of AYA NB, with an absence of pangenomic studies (Table 4). The predominant profile of SCAs found in the previously defined group 1 is in accordance with the last INRG report on AYA [11]. The drift towards a mixed profile (with recurrent SCA and NCA) correlated with the fact that this profile is less frequently found in younger patients within the childhood NB group [104]. However, we found a higher frequency of cases with >3 SCA than that reported for general childhood NB (88% versus 53%). No differences in either EFS or in overall survival (OS) in relation to the number of SCA were ascertained [104].

Finally, *ATRX* mutations and the subsequent loss of the nuclear protein expression are more frequently identified in older patients with disease stage 4 [113, 146]. In our cohort, negative and mosaicism expression (cells with positive expression and cells with loss of the expression) predominated in group 1 and 2, respectively.

3.1.2. MNA plus 11q deletion

MNA and 11q deletion are considered the most powerful genetic markers of aggressiveness in NB. Although both alterations do not mutually exclude each other, their co-occurrence is unusual and consequently there is little data about the genetic background of these tumors and the prognosis of the patients with tumors harbouring the double genetic hit.

3.1.2.1. Update of the thesis cohort

Frequency of tumors with MNA (homMNA and hetMNA) plus 11q deletion was specifically determined in the **article I**, where FISH was used for screening. Also data from the tumors presenting one or both alteration by FISH and SNPa were described and evaluated in **article III**; only cases presenting 11q deletion by SNPa were finally considered**.** Table 5 shows frequency of MNA plus 11q deletion in each article in relation to the global number of cases analysed

Table 5. MNA plus 11q deletion in NB at DP-UV

Study, year intervals (technique)	N° cases	MNA (%)	11q- (%)	homMNA plus 11q- (%)	hetMNA plus 11q- (%)	homMNA no 11q- (%)	hetMNA no 11q- (%)
Article I, 1994-2011 (FISH)	905	163/905 (18.01)	92/486 (18.9)	12/486 (2.4) [a]	7 (0.7)	144/486 (29.6)	—
Article III, 1994-2014 (SNPa)	1091	167/1091 (14.8)	76/273 (27.8)	9/273 (3.2) [a]	12/273 (4.4)	28/273 (10.3)	16/273 (5.9)
Whole cohort, 1994-2014 (FISH and/or SNPa)	1134	196/1134 (17.3)	179/934 (19.1)	14/934 (1.5) [b]	12/934 (1.3)	124/934 (13.3)	17/934 (1.9)

MNA, *MYCN* amplification; homMNA; homogeneous MNA; hetMNA, heterogeneous MNA. Note: Number of cases slightly differs between articles I and III, mainly because of the detection approaches used and the cohort included in each article. [a] Three cases of article I (11q- defined by FISH) were not included in article III (11q- defined by SNPa) for different reasons: i) 11q deletion by FISH and not by SNPa (case 18); ii) no SNPa performed (case 19); iii) clinical update reveal to be a treated tumor (case 9). [b,] Two new cases included in retrospective analyses of 11q- were deleted by FISH or MLPA, no SNPa could be performed.

Updating of MNA (homMNA and hetMNA) plus 11q deletion incidence showed two newly diagnosed cases were diagnosed, both homMNA, giving a frequency of 2.8% (26/934); the infrequency of its co-occurrence was confirmed.

In total, heterogeneity of 11q deletion (het11q-) together with MNA, was found for three cases (3 out of 26). In one of the cases (case 18**,** article I), this aberration was revealed by FISH in only 20% of tumor cells, but it was not shown by multigenomic analysis (neither MLPA nor SNPa). For the two other cases, a new homMNA tumor from the whole cohort and a hetMNA tumor from article III (case 13), the inverse situation occurred.

3.1.2.2. *Review of the literature*

For comparison of the data and confirmation of the rare co-occurrence of MNA plus 11q deletion, most of the cases published are summarised in Table 6 together with the clinical findings reported.

In some studies, mainly focused on *MYCN* gene and/or 11q alterations, the occurrence of MNA plus 11q- was inexistent [69, 78, 179, 180], even within patients with disease stage 4 [181], or reported as very rare without providing any frequency [177]. All these studies showed a frequency around 1-3% confirmed in our results, even if only homMNA plus 11q deletion cases were considered (1.3%, article I and 1.5%, whole cohort).

Advanced stages in the studied cohort (especially metastatic, 68.5%) were predominant in accordance to literature (Table 6). A trend for worst OS was found in the survival analysis for homMNA plus 11q deletion compared to homMNA without 11q deletion NB patients (**article I**, 33.3± 13 and 53± 9.5, respectively, p=0.138) as described [65, 71]. Similar to the results of *Buckley et al*, median age at diagnosis (24 months) was analogous to that of patients with MNA tumors and not to that of 11q deleted tumors; the latter being usually associated with older age and short-survival patients [72, 74, 77]. Within high-risk patients, a more complex pattern of SCA have been associated with nonMNA tumors, including 11q deleted tumors, and with short OS; while MNA tumors are associated with a less complex pattern of SCA [177]. Interestingly, the MNA plus 11q deletion cases analysed in article I showed a high number of

SCAs (mean 10.7; median 10) comparable to the number of SCAs reported for 11q-deleted groups [177].

Table 6. Literature review of MNA plus 11q deletion NB

First author, reference	Country	No. cases (%)	Technique	Age (months)	Stage[a] (No. cases)	Outcome (No. cases)
Spitz R, 2003 [80, 182]	Germany	3/142 (2.1) 3/179 (1.6)	FISH	136, 62, 53	1 (1) 4 (2)	DOD(2)
Spitz R, 2006] [77]		13/611 (2.1)	FISH [b]	—	—	Dramatic decline of survival rate
Attiyeh EF, 2005 [71]	USA	12/905 (1.3)	Southern blot, FISH or qPCR	—	—	—
Stallings RL, 2006 [87]	USA, Ireland, Australia	2/49 (2)	Southern blot, FISH or PCR	—	—	—
Michels E, 2007 [66]	Belgium	1/75 (1.3)	FISH and aCGH	> 12	4	DOD
Tomioka N, 2008 [65, 92]	Japan	4/236 (1.7)	aCGH	> 12	3-4	Worse outcome
Janoueix-Lerosey I, 2009 [92]	France	3/473 (0.6)	aCGH	—	—	—
Jeison M, 2010 [183]	Israel	6/177 (3.3)	FISH and MLPA	—	3 and 4	—
Carén H, 2010 [72]	Sweden	1/165 (0.6)	SNPa	48	L2	DOD
Cetinkaya C, 2013 [106]		2/34 (5.8)	FISH and/or SNPa and aCGH	41 and 48	L2 and M	
Buckley PG, 2010 [74]	Germany and Ireland	4/160 (2.5)	aCGH	17, 18, 19 and 35	3 (2) and 4(2)	3 alive 1 DOD

—, data not available; [a], INSS or INRGSS; [b] no distinction between 11q deletion and 11q imbalance is given (LOH analyses); DOD, death of disease. Note: Some cases are simultaneously included in several studies of the same country or research groups.

3.1.3. HetMNA

In this thesis, the term hetMNA has been used as defined by the INRG Biology Committee [55]. Intratumoral hetMNA, refers to the coexistence of *MYCN* amplified (frequently including tumor cells with MNG) as well as *MYCN* non-amplified tumor cells in the tumor found as a cluster (focal) or as single (scattered) cells [55]. A hetMNA tumor evaluated by FISH shows a combination of MNA cells (usually in low percentages and with less *MYCN* copies per cell than in homMNA), with nonMNA and occasionally MNG neuroblastic cells. Validation of hetMNA, ideally in paraffin sections by FISH, is crucial to exclude false-positive results by 'contamination' of MNA cells from another tumor [58]. If all these points are not considered, then hetMNA cases can be missed and included in studies as non-amplified *MYCN* (nonMNA) or as MNA tumors [184-186].

3.1.3.1. Update of thesis cohort

Frequency of tumors with hetMNA was specifically determined by FISH in **article III** and an update of the number of hetMNA cases is shown in Table 7. Again, some cases were

already presented **in article I**. Similar to the study performed in article I, in article III the hetMNA cases where divided according to the presence or absence of 11q deletion.

Table 7. Genetic profile of HetMNA NB

hetMNA NB (%)				
Article III SNPa (N=273)			Whole cohort (FISH and/or SNPa) (N=1134)	
28 (2.6)			30 (2.6)	
NCA	**SCA**		**11q data**	**11q no data**
2 (7.1)	26 (92.9)		29	1
	11q-	**no 11q-**	**11q-**	**no 11q-**
	12 (42.8)	16 (57.2)	12 (41.38)	17 (58.62)

Two new hetMNA cases were found in the whole cohort but SNPa was not performed in any of the two tumors, because of the low tumor cells content. One case showed 1p imbalance and neither 11q nor 17q alteration. The other tumor did not present 1p aberrations and 11q and 17q regions were not analysed. A balanced proportion of cases in which hetMNA was focal (14 cases) and scattered MNA cells (16 cases) was found in the whole cohort, dissimilar to the findings of others studies [67]. 11q deletion occurred more frequently in hetMNA than in homMNA tumors, especially in older patients with advanced stage of disease [72, 106, 107]. No further statement on the frequency of 11q deletion in hetMNA NB can be given as none of the studies provides 11q status for hetMNA cases.

3.1.3.2. Review of the literature

The Table 8 summarizes the NB reported as hetMNA and their clinical and genetic findings. In none of the previous studies referenced in Table 6, hetMNA event is reported.

Definitions of hetMNA NB can be diverse over the literature. A careful distinction from the frequently observed genetic phenomena intrinsic to NB was clearly stated by Ambros PF *et al* in 2001 and years later, the INRG Biology Committee defined the hetMNA term and provided the guidelines for its diagnosis [55, 187]. In addition, temporospatial differences in MNA leading to a hetMNA status have also been described, as well as marked variations in the relation of *MYCN* amplified versus non-amplified tumor cells [67, 188]. Typically studies pose attention to the presence or absence of amplification and no information is given about the presence of other nonMNA tumoral cells within the amplified regions.

The few studies of hetMNA existing in the literature revealed a frequency of only 1-2% of all NB as hetMNA tumor [67, 185-189], while MNA is detected in 20-25%, especially in stage 4 patients (~40%) [57, 67, 115, 186-191]. The study of Theissen *et al*, is somehow similar to our article III in terms of frequency and total number cases analysed (1.1%, 15 out of 1341), and represents the largest cohort reported so far. The incidence of hetMNA in article III (2.6%, 28/1091) in primary tumors is similar to the small frequencies reported by others [67, 187, 189, 191]. The higher frequencies presented in Table 8 might be biased as only selected samples or patients enrolled for a particular trial study are considered [186, 188].

Table 8. Literature review of hetMNA NB

First author, reference	Country	No. cases (%)	Technique	HetMNA definition / cells population	MNA and other findings	1p status (No.)	Age (months)	Stage (No.)	Outcome (No.)
Ambros PF, 2001 [187]	Austria, France and Italy	3/300 (1)	FISH	MNA foci and areas with nonMNA cells	Moderate MNA[a]	1p D (1)	—	Local (2) 4S (1)	—
Noguera R, 2002 [192]	Spain	1	FISH	MNA cells, MNG cells and nonMNA cells	Moderate MNA[a]	1p D	5	4S	DOD
Kerbl R, 2002 [185]	Austria	1	FISH	regions with MNA and regions with nonMNA cells	MNA cells at the resection margin	1p Im. nonMA cells	10	4S	ADF
Spitz R, [191]	Germany	2/659 (<1)	FISH	MNA diffuse cells	Universal MNA at relapsed tumor	—	—	—	—
Valent A, 2004 [189]	France	4/200 (2)	FISH	MNA cells, MNG cells and nonMNA cells	Low[b] and high MNA	—	3-72	4S (2) 3 (1) 4 (1	ADF (1) AWT (2) DOT (1)
Thorner PS, 2006 [188]	Canada	5/41 (12.2)	CISH and FISH and/or SB and/or PCR	MNA and nonMNA cells	≥50% difference in *MYCN* copies in neighbour cells	—	—	—	—
Cañete A, 2009 [186]	SIOPEN	5/46 (10.9)	FISH	—	—	—	<12	2, 3, 4, 4S	—
Theissen J, Res 2009 [67]	Germany	15/134 1 (1.1)	FISH	MNA (single cells or cluster) and nonMNA cells	MNA as clusters predominance	D (3) Im (2)	2-72	1 (2) 4S (2) 3 (5) 4 (6)	ADF (7) Prog. (3) DOD (5)
Bishop MW, 2014[193]	USA	1	FISH		MNA (focal) and nonMNA cells	—	3	4S	ADF

[a], moderate MNA: a 4 amplification fold increase to 17 copies of *MYCN* compared with the reference or 10-50 signals for *MYCN;* [b], low level MNA: a *MYCN* amplification fold increase compared with the reference; —, no data; MNA, *MYCN* amplified; MNG, *MYCN* gain; Local.; localised stage; BM, bone marrow; ADF, alive disease free; AWT, alive with treatment; DOT, death of toxicity; Prog., local progression of tumor; CISH, Chromogenic in situ Hybridization; SB, Southern blot; D, deletion; Im, imbalance, SIOPEN, International Society of Pediatric Oncology, Europe Neuroblastoma Group. Note: Only tumors at diagnosis time where considered.

Although we do not know the prognostic impact of hetMNA, no differences were found in terms of OS/EFS between homMNA and hetMNA tumors in our cohort, although data showed a tendency to a lesser aggressiveness (**article I**).

3.1.3.3. Preliminary result of SIOPEN hetMNA project

International Society of Pediatric Oncology, Europe NB Group (SIOPEN) launched an ongoing collaborative study on hetMNA tumors with 87 patients from 8 participating countries, being Austria and Spain the countries with the largest included cohorts. One of the aims of this study is to elucidate the genomic background of hetMNA tumors in comparison to homMNA by means of pan-genomic techniques (preferentially aCGH and SNPa, but also MLPA). As the study is in process the data are very preliminary [68]. Information from SIOPEN hetMNA tumors, is given in the following Table 9 according to groups of age (>/< 18 months). Data from Austrian and Spanish tumors are given separately. We present SNPa results from

Austrian (21/286 cases) and Spanish (28/273 cases, **article III**) hetMNA tumors from in which genetic analyses although preliminary were more completed than those of the rest of the participating countries of SIOPEN hetMNA project.

Table 9. HetMNA SIOPEN cohort features

Cohort	No. cases	% of patients </> 18 months		Stage		Ploidy		% Relapse	% SCA profile	% UPDs
				3	4	% diploid	% aneuploid			
SIOPEN	33	**<18**	63	40	20	18	82	50	75	—
		>18	37	20	70	37.5	62.5	40	100	—
Austria	26	**<18**	59.1	40	10	0	100	0	61.5	76.9[a]
		>18	40.9	30	80	43	57	60	87.5	50[a]
Spain	28	**<18**	40.7	20	30	0	100	52	81.8	90[b]/1[a]
		>18	59.3	20	60	43	57	18	94	6[b]/1[a]

—, no data available. SCA, segmental chromosome aberrations; UPD, uniparental dysomies; [a], whole UPDs; [b], partial UPDs.

Considering the entire SIOPEN cohort, a predominance of younger patients was observed (56.4%). HetMNA tumors were found in all disease stages. Older patients had more frequently disease stage 4 and poorer outcome, while younger patients had either stage 3 or stage 2 (30% each) and less frequently stage 4 (20%). Non-diploid tumors were always predominant, independent of age, and also tumors with a SCA profile, being the later more frequent in tumors from older patients (93.1% versus 76.9%).

Austrian and Spanish cohorts are the largest cohorts of the SIOPEN with a similar number of cases. We observed a predominance of metastatic stages and older patients in the Spanish cohort. SCA profile was always predominant, notably in the spanish cases. When studying the genomic profiles according to age, the distribution of SCA tumors was predominant in patients >18 months. In both cohorts the number of SCA varied from none to a very high number (>18) with a similar mean of breakpoints (>7). In our hetMNA cohort, uniparental dysomies (UPDs) were randomly distributed along the genome, not exclusively in chromosome 11 (11 cases, 39.3%), and more frequent for the cases with an increased number of breakpoints (72.7%, 8/11 cases). Also of note, in the spanish cohort, UPDs where mainly present in diploid versus aneuploid tumors (100%, 6 cases vs 25%, 4 cases). A high effort must be done to know the prognostic impact of hetMNA in the SIOPEN hetMNA project.

3.2. VALUE OF INTRATUMORAL GENETIC HETEROGENEITY INVESTIGATION

Intertumor genetic heterogeneity is a frequent finding in NB, as well as in other group of tumors, and becomes easily apparent with a wide range of genetic profiles and alterations exhibited in the tumors belonging to the same group. Intratumoral genetic heterogeneity is known to exist in NB and can be detected by FISH. However, several pieces of the same tumor are infrequently explored to establish a precise definition of the multiple consequences derived from different number and spatial distribution of the tumor clones. The results

obtained through analyzing several pieces of the same tumor specimen in some of the ultra-rare NB tumors compiled in this thesis clearly evidence the existence of this hallmark in this group of NBs.

3.2.1. Intratumoral hetSCA detected by FISH

The use of FISH for *MYCN* status analyses is currently required for clinical use. When hetMNA is detected in touch imprints, confirmation of the hetMNA diagnosis in paraffin-embedded whole tissue sections by FISH is mandatory [55]. Intratumoral hetSCA detected in touch imprints by FISH and defined as SCA cells (diagnosed as unbalanced ratio between chromosomal specific DNA sequence signals and the number of the reference signal probes) intermixed with NCA tumor cells (diagnosed as balanced ratio between signal probes, different than disomic), can be complex to analyze in paraffin embedded tissue sections as sometimes it appears as a miscellany of multiple clones that need to be differentiated from cut nucleus effect.

In **article I,** the genetic cell heterogeneity, by FISH technique, is described for *MYCN* gene, 1p, 11q and 17q chromosome regions for the hetMNA tumors but not for the homMNA plus 11q- tumors. Heterogeneity was notably found for 1p chromosome region. 1p chromosome region status was previously reported for the hetMNA tumors (reviewed in Table 8) as deletion (20-33% of cases, mainly restricted to MNA cells) and although more rare, as imbalances (13% of cases) [67, 185, 187]. In Table 10 the results of heterogeneity are summarised for the whole thesis cohort.

Table 10. Heterogeneity detected by FISH

	1p-(%)	11q- (%)	+17q (%)
nonMNA (N=835)	21/393 (5.3)	22/362 (6)	7/78 (9)
homMNA no 11q-(N=126)	5/69 (7.2)	0	5/90 (5.5)
homMNA 11q-(N=14)	0/14 (0)	0/14 (0)	0/13 (0)
hetMNA (N=30)	8/30 (26.6)	3/30 (10)	3/30(10)

hetMNA, heterogeneous MNA; homMNA, homogeneous MNA. In some cases heterogeneity was present for more than one marker.

As shown in Table 10 in hetMNA tumors higher presence of hetSCA was detected. The FISH high sensitivity, specificity and speed by which the FISH assays can be performed have made FISH a pivotal cytogenetic technique that has provided significant advances in both the research and diagnosis in NB.

3.2.2. Evidence of intratumoral hetSCA dilution effect

To investigate the different status of the *MYCN* gene and other SCA in several pieces of the same tumor, when possible, analyses by FISH and SNPa have to be performed **(article III).**

3.2.2.1. MYCN status

For hetMNA tumors, diversity in the *MYCN* status was found when analysing more than one piece by FISH (at least two touch imprints of different pieces per case) or by SNPa (at least two pieces in 16 out of 28 cases) and/or by comparing different results between the techniques (Tables 11-12).

Table 11. *MYCN* status by FISH in hetMNA tumors

1 hetMNA	hetMNA nonMNA	hetMNA + MNG + +nonMNA	hetMNA + homMNA
24	2	1	1

MNA, *MYCN* amplification; hetMNA, heterogeneous MN homMNA, homogeneous MNA; MNG, *MYCN* gain.

Table 12. *MYCN* status by SNPa in hetMNA tumors

nonMNA	MNG + nonMNA	MNA + nonMNA	MNA + MNG	MNG	MNA
16	3	1	2	1	5

MNA, *MYCN* amplification; hetMNA, heterogeneous MNA; homMNA, homogeneous MNA; MNG, *MYCN* gain.

A disparity in the number of MNA status, number of MNA cells (10 50%) and variation in the number of double minutes (from 15 to 100) between tumor pieces was observed by FISH.

Final diagnosis of *MYCN* gene status by SNPa was: MNA in 28.6%, MNG in 14.3% and nonMNA in 57.1% of the hetMNA cases. HetMNA tumors tended to present a less complex *MYCN* amplicon, when detected, than homMNA by SNPa. In some cases *MYCN* amplicons were not so high or seen as a micro gain, reflecting the fact that only a sub population of the cells carries the amplification. Furthermore, in some cases with a considerable number of MNA cells per slide (35 90) no trace of MNA cells could be detected by SNPa.

3.2.2.2. Other genetic markers

The detection of the alteration by SNPa in the hetSCA tumors from Table 10 is shown at Table 13. Although the number of cases studied was small detection of 1p deletion was more difficult compared to the detection of 11q deletion and 17q gain.

Table 13. SCA status by SNPa in hetSCA tumors

hetSCA tumors (FISH)	1p- (SNPa), (%)	11q- (%)	+17q (%)
nonMNA	5/15 (33)	13/13 (100)	2/2 (100)
homMNA no 11q-	2/3 (66.6)	0	4/4 (100)
hetMNA	2/8 (25)	3/3 (100)	2/3 (66.6)

Note: SNPa data was not always available for all hetSCA tumors from Table 10.

Furthermore, heterogeneity detected for 1p, 11q and 17 chromosome regions status when several pieces were analysed and/or comparison of results obtained by FISH and SNPa techniques are described in Table 14 and also in article I and III.

Table 14. Frequencies of heterogeneous tumors between FISH and SNPa results

	Total (%)	1p- (%)	11q-(%)	+17q (%)
nonMNA NCA[a]	20/84 (23.8)	7/84 (8.3)	3/84 (3.6)	10/84 (11.9)
nonMNA SCA	19/130 (14.6)	14/130 (10.7)	5/130 (3.8)	3/130 (2.3)
homMNA no 11q-	6/38 (15.8)	0/38 (0)	3/38 (7.9)	3/38 (7.9)
hom MNA 11q-	0/14 (0)	0/14 (0)	0/14 (0)	0/14 (0)
hetMNA	12/28 (42.8)	9/28 (32.1)	1/28 (3.6)	3/28 (10.7)

Note:[a] NCA profile according to SNPa, the SCA were found by FISH. In some cases heterogeneity was present for more than one marker.

Coexistence of intratumoral hetSCA, is rarely reported either by FISH or by pangenomic techniques mainly due to methodological difficulties of FISH interpretation as well as the recent use of high throughput pangenomic techniques for NB profile determination. We found higher amount of heterogeneity for 1p (32%) than the reported (10% of all NB specimens and in 15% of hetMNA NB tumors [67, 187]) probably due to the examination of more than one fragment by FISH.

Heterogeneity for 1p chromosome was inexistent for homMNA tumors and scarce for homMNA plus 11q deletion in contrast to hetMNA tumors where heterogeneity for 1p region was mainly found. As previously described, a strong correlation between hetMNA tumors and 1p deletion was not detected [67, 187]. Surprisingly, 11q heterogeneity was rarely found in the cases analysed, suggesting a distinct mechanism of clonal acquisition of this alteration compared to the1p and 17q alterations.

In addition, in hetMNA tumors heterogeneity for segmental UPDs (11p, 12q, 14q, 17q, 18p and 22q) was found in 14% of cases and in half of these, loss of the same chromosome regions was seen instead of the UPD in the second piece (using SNPa). Heterogeneity was also present for focal SCA and amplifications (other than MNA) when studying more than one tumor fragment. These heterogeneous events were found altogether within the same tumor indicating a high intratumoral genetic instability.

The clinical implications of hetSCA remain elusive. NB cases in which a progress to universal MNA at relapse from focal MNA cells at diagnosis have been described, indicating changes in the cytogenetic aberration pattern [191]. It is possible that further studies could benefit mainly the patients with lower risk disease profiles who may have *MYCN* amplification that is heterogeneous in nature. It is necessary to analyze the hetSCA status, specifically in hetMNA tumors, to establish a more accurate genetic diagnosis. How to effectively treat heterogeneous tumors is a major challenge in cancer therapy.

3.3. USEFULNESS OF DATABASES FOR MODELING

Genetic data form part of more extensive studies that aims to generate models in NB tumors by means of integrating clinical, histopathology, genetic and ECM markers data. Diverse studies have proposed or delineated different models or algorithms in NB, such as models based on the hypoxia signature, or models for age dependence in unfavorable NB or algorithm in the process leading up to aneuploidy, but further efforts are needed [106, 194, 195].

Our group designed and created, in cooperation with the Institute of Instrumentation for Molecular Imaging from the Technical University of Valencia (Spain), a database that contains some clinical data and results of the histopathology and genetic evaluations performed on the NB registered at our laboratory [169]. This database makes more feasible the working-up and further selection of the cohorts of tumors with specific features of interest for both diagnosis and research. The database is a flexible tool in which new data from retrospective and/or prospective studies can be filled in different fields maximizing its future efficiency. Hence by the time of writing the articles and the thesis research, the number of samples vary because

cases newly diagnosed were registered or new data from previous samples were incorporated into the database.

More recently, in an effort to easily identify genetic patterns, research collaboration with Dr. Michael Baudis (Institute of Molecular Life Sciences, University of Zurich) was established with the support on array map repository. Raw SNPa data from ultra-rare and from the rest of tumors diagnosed at our laboratory have been uploaded in the array dataset repository, arrayMap [196, 197]. So far, 331 samples from 295 patients have been uploaded (281 tumors at diagnosis and 14 tumors at relapse).

Raw probe values from Affymetrix were processed and pre-processed and log2 intensity and B allele frequency (BAF) values from Illumina platforms were used. Probes were segmented to derive regions of uniform copy number and gain/loss or balanced status was assigned to the regions. An automatic parameter for input quality data can be used to select samples for a more reliable whole genome copy number profiling, eliminating samples with low quality. For "optimal" copy number chromosome aberration interpretation, critical and empirical review of the results is recommended and needed in some cases. So far, for our cohort of cases, this review is ongoing for the cases with more difficult interpretation and cases with lower quality results. The interface allows to query and sort the sample groups, for example in accordance to different parameters such as clinical data (age, stage and survival data) or the genetic features. Generation of the patterns is ongoing with the linked annotation clinical data matching each sample included. This tool generates several visualizations of the genetic profile of the samples as shown in Figure 2.

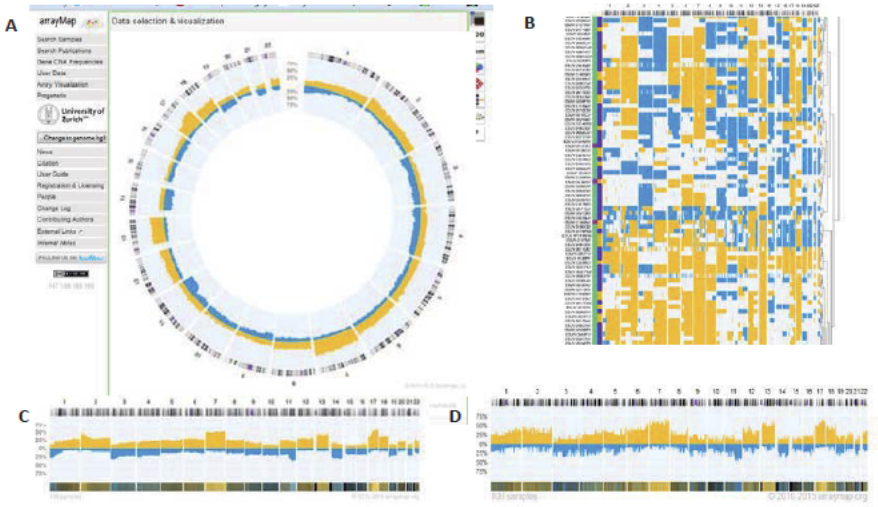

Figure 2. Copy number profiling on arrayMap repository. **A)** Circle plot with chromosomes histograms showing 331 samples. **B)** Matrix plot of a set of NB cases represents at the X axis the genetic profile and at the Y axis the samples. **C)** and **D)** Chromosomal histograms and heatmaps of copy number alterations frequencies (on the bottom) for 198 samples where critical review has been already performed and for 135 samples were review is ongoing, respectively. Every altered genetic material detected is colored, in yellow for gain and in blue for loss (http://arraymap.org/).

As example the genomic profile of 15 homMNA plus 11q deletion and 11 hetMNA tumors are given in Figure 3, A and B respectively. All adjustments needed for copy number profiling are finished.

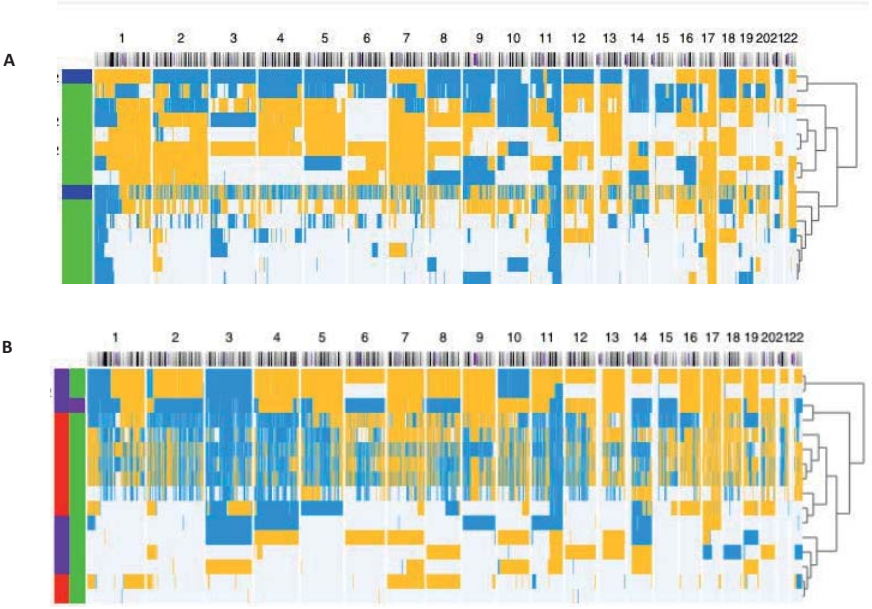

Figure 2. Genomic profiling of homMNA plus 11q deletion and hetMNA 11q deleted tumors by arraMap.

ArrayMap can prove especially useful in identifying the genetic aberration patterns of the ultra-rare tumor samples when the outcoming collaborative projects are carried-out. We intend to recognize more easily patterns of non-random genetic aberrations related to the different NB types, and to pinpoint involvement of specific genes. In this sense, arrayMap tool makes feasible the large-scale data mining, such as identification of more precise genomic patterns to find molecularly definable types of NB.

To our view, the ultra-rare tumors here studied represent *sui generis* situations in NB: AYA NB, NB with an unique and specific psychosocial needs; MNA plus 11q- tumors, with a double unfavorable hit within the same specimen; hetMNA tumors, harbouring a high genetic heterogeneity and that consequently could aid to understanding of the pathogenesis of the disease by means of mathematical tools.

It is crucial to compilate of all biological data, including genomic profiles using high throughput techniques, *ALK* and *ATRX* genes data (copy number and mutation status) and biotensegral information (from stromal cells and extracellular matrix elements), together with the clinical features, to mathematically infer new prognostic markers and to set a strong base for new treatments in these incomparable ultra-rare NB. Similar approaches should be adopted in the ongoing and future European cooperative studies.

4. CONCLUSIONES / CONCLUSIONS

La existencia de centros Nacionales de Referencia para el estudio de neuroblastoma es esencial para definir, detectar y analizar los grupos infrecuentes del neuroblastoma. Como Centro de referencia Nacional de estudios Histopatológicos y Biológicos de neuroblastoma y con una cobertura de más del 50% de los tumores neuroblásticos españoles, hemos definido, tres grupos ultra-raros de neuroblastoma basándonos en la baja incidencia (menor del 5%) de la cohorte de pacientes con edad avanzada y de tumores con características genéticas atípicas. Los tres artículos que componen esta tesis persiguen la identificación de los perfiles genéticos y de algunas de las características particulares que subyacen en la biología de los tumores que aparecen en pacientes adolescentes y adultos jóvenes, de neuroblastomas con amplificación del gen *MYCN* asociada a la deleción de 11q y de los que presentan amplificación heterogénea de *MYCN* para impulsar los estudios multicéntricos enfocados a mejorar la toma de decisiones terapéuticas. Para lograr el objetivo final de la investigación traslacional que es mejorar el diagnóstico, el tratamiento, la supervivencia y el bienestar general de los pacientes, con tumores ultra-raros se requiere información adicional.

En lo que se refiere al estudio de tumores neuroblásticos diagnosticados tras la infancia y la revisión bibliográfica del tema, concluimos:

1. Existe en estos tumores una alta incidencia de neuroblastomas sin amplificación de *MYCN* y con alteraciones cromosómicas segmentarias así como con expresión completamente negativa o en mosaico para la proteína ATRX.

2. Las características genéticas de los tumores neuroblásticos de pacientes preadolescentes y adolescentes son diferentes a las de los tumores presentes en adultos jóvenes y adultos de mediana edad. Existe un mayor número de alteraciones cromosómicas segmentarias en los tumores neuroblásticos de preadolescentes y adolescentes (6.5 versus 3.5), la deleción de 11q se encontró en el 75% versus el 17% y la ganancia de 17q en el 88% versus el 16.6% de los tumores sin amplificación de *MYCN*. Los neuroblastomas de pacientes adultos jóvenes y de mediana edad, por su parte tienen una infiltración de células del sistema inmune más evidente y mayor diversidad poblacional.

3. Corroboramos el predominio de estadios avanzados en nuestra cohorte y la mala evolución de los pacientes adolescentes y jóvenes adultos con neuroblastoma. La tasa de supervivencia global a los 5 años fue del 44,8%. Son necesarios más estudios con un mayor número de pacientes para establecer mejor el patrón genético y de infiltración celular inmune relacionada con los tumores de aparición en edad avanzada.

En lo que se refiere al estudio de tumores neuroblásticos con amplificación de *MYCN* y deleción de 11q y con la revisión bibliográfica del tema, concluimos:

1. Consideramos como un modelo excepcional de inestabilidad genética la presencia combinada de los dos marcadores genéticos asociados a pronóstico desfavorable junto con el elevado número de alteraciones cromosómicas segmentarias.

2. El mayor número de alteraciones cromosómicas segmentarias detectadas en los tumores con amplificación de *MYCN* y deleción de 11q en comparación con el número de alteraciones cromosómicas detectado en los tumores con amplificación de *MYCN* y sin deleción de 11q,

podría considerarse como una implicación de la región cromosómica 11q como un segundo "driver" genético en neuroblastoma o como efecto secundario de la inestabilidad genética previa del tumor.

3. Es necesaria una investigación pangenómica de un mayor número de casos en estudios futuros integrando otros datos biológicos relacionados con la inestabilidad específica descrita.

En referencia al estudio de neuroblastomas con amplificación heterogénea de *MYCN*, y la revisión de la literatura, concluimos:

1. La compleja composición genética intratumoral encontrada pone de manifiesto la extraordinaria importancia de un manejo meticuloso y concienzudo del material tumoral. Todas las siguientes determinaciones deben realizarse cuando se disponga de material tumoral suficiente realizando el diagnóstico definitivo con la combinación de los resultados de todas las técnicas y de todos los fragmentos analizados: a) Realizar varios análisis en improntas y / o cortes de secciones completas del mismo fragmento con la técnica de hibridación in situ fluorescente, b) Analizar los diferentes fragmentos del mismo tumor con la técnica de hibridación in situ fluorescente, c) Determinar el perfil genómico completo en más de una pieza tumoral con técnicas pangenómicas.

2. Los clones con amplificación de *MYCN* y los clones con alteraciones cromosómicas segmentarias en ocasiones han permanecido ocultos cuando se han utilizado diferentes técnicas pangenomicas y / o cuando se han estudiado varias piezas sin existir una correlación con la cantidad de clones tumorales encontrados al utilizar la técnica de hibridación in situ fluorescente.

3. La confirmación de las diferencias halladas respecto a la región más pequeña de solapamiento para la deleción de 11q y la ganancia de 17q en tumores con amplificación heterogénea de *MYCN*, con amplificación homogénea de *MYCN* y con deleción de 11q sin amplificación de *MYCN* debe realizarse en un grupo tumora más ampliol.

Finalmente reuniendo todos los estudios concluimos que,

La integración de la genómica en la modelización de tumores neuroblásticos requiere el uso de herramientas como las bases de datos. El archivo de los datos biológicos (en Neupat y arrayMap) de las muestras de pacientes incluidos en estos estudios de neuroblastomas ultra-raros será clave para la futura clasificación de riesgo terapéutica que ha de constituir uno de los pilares en las investigaciones actuales. Además, los ensayos de medicina personalizada basados en la futura modelización proporcionarán información esencial para priorizar las direcciones a tomar en investigaciones futuras.

The existence of neuroblastoma National Reference Centers is essential to define, detect and analyse the ultra-rare neuroblastoma groups. As the Spanish National Reference Center of Pathology and Biology studies, covering more than 50% of Spanish neuroblastic tumors, we have detected three ultra-rare neuroblastoma groups based on the low frequency (< 5%) of the older age patient cohort and of tumors with atypical genetic features. The ultimate goal of translational neuroblastoma research is to improve diagnosis, treatment, survival and general welfare of the patients, and to accomplish this ultimate goal in ultra-rare neuroblastoma cohorts additional information is necessary. The three articles that comprise this thesis pursue the identification of the genetic profiles and particular features pertaining to the biological background of adolescent and young adult patients, *MYCN* amplified plus 11q deletion, and heterogeneous *MYCN* amplified neuroblastomas, to encourage multicentric studies focused on the improvement of prognostication and therapeutic decision-making.

In relation to the neuroblastoma study of tumors diagnosed after childhood and to the review of the relevant literature, we conclude:

1. There is a high prevalence of non *MYCN* amplified neuroblastomas with segmental chromosome aberrations and neuroblastomas with completely negative or showing mosaic expression of ATRX protein.

2. The genetic and immune cell infiltration characteristics of preadolescent and adolescent neuroblastic tumors are different from those of young adult and middle-aged adult neuroblastoma patients. We have shown a higher number of segmental chromosome aberrations in tumors from preadolescent and adolescent than in tumors from young adult and middle-aged adult neuroblastoma patients (6.5 vs 3.5), 11q deletion is detected in 75% versus 17% and 17q gain in 88% versus 17% of the non *MYCN* amplified tumors. Young adult and middle-aged adult neuroblastoma present a more evident infiltration and a more diverse composition in comparision with to preadolescent and adolescent neuroblastoma.

3. We corroborate the predominance of advanced stages in our cohort and the poor outcome of the adolescent and young adult neuroblastoma patients. Overall survival rate at 5 years is 44.8%. More studies on a larger number of patients are needed to a better establish the older age-dependent genetic pattern and the immune cell infiltration of these tumors. Confirmation of the differences relating to both number and type of segmental chromosomal aberration, profile, as well as the composition and distribution of other tumor elements, as the immune cells, should lead to a better understanding of disease outcome in neuroblastoma after childhood.

With regard to the study of *MYCN* amplified plus 11q deletion neuroblastoma and the review of the relevant literature, we conclude:

1. We consider as an exceptional model of genetic instability to be the combined presence of the two strong negative prognostic markers along with a high number of chromosomal aberrations.

2. The higher number of segmental chromosomal aberrations in *MYCN* amplified plus 11q deletion tumors compared to the number found in *MYCN* amplified non-11q deleted tumors must indicate an involvement of 11q deletion as a second genetic driver and/or as a secondary effect of the previous genetic instability of the tumor.

3. An analysis of these cases with pangenomic techniques in a larger number of tumors should be performed in future studies integrating other biological data.

With regard to the study of heterogeneous *MYCN* amplified neuroblastoma, and the review of the relevant literature, we conclude:

1. The complex genetic intratumoral composition emphasizes the great importance of a conscientious and meticulous management of the tumor material. All the following determinations should be performed when sufficient tumor material is available, establishing the final diagnosis by combining the results of all techniques and fragments studied: a) perform repeated analyses with fluorescence in situ hybridization in touch preparations and/or whole paraffin sections of the same tumor fragment, b) analyze different fragments of the same tumor with the fluorescence in situ hybridization technique, c) determine the whole genomic profile in more than one piece of tumor using pangenomic techniques.

2. The clones with *MYCN* amplification or with segmental chromosomal aberrations in occasions have remained when we used different pangenomic techniques and/or when we studied several tumor fragments without there existing a correlation with the amount of tumor clones found by using fluorescence in situ hybridization technique.

3. The confirmation of the differences found in relation to the smallest region of overlap for 11q deletion and 17q gain in heterogeneous *MYCN* amplified, homogeneous *MYCN* amplified and non-*MYCN* amplified plus 11q deletion tumors should be performed in a larger cohort.

Putting it all together we conclude that,

Integration of genomics in neuroblastic tumor modeling requires the use of databasing solutions. Archiving of the biologic data (Neupat and arrayMap) of patients included in ultra-rare neuroblastoma will prove to have an implication in the future treatment classification of the patients, and is therefore one of the pillars of the current research. In addition, genomic-based personalized medicine trials will provide essential information for prioritizing future research directions.

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ANNEX

OTHER COAUTHOR PUBLICATIONS RELATED BUT NOT INCLUDED IN THE THESIS

A. Genomics related to other prognostic factors

1. Clinical:

Influence of segmental chromosome abnormalities on survival in children over the age of 12 months with unresectable localised peripheral neuroblastic tumours without MYCN amplification. Defferrari R, Mazzocco K, Ambros IM, Ambros PF, Bedwell C, Beiske K, Benard J, Berbegall AP, Bown N, Combaret V, Couturier J, Erminio G, Gambini C, Garaventa A, Gross N, Haupt R, Kohler J, Jeison M, Lunec J, Marques B, Martinsson T, Noguera R, Parodi S, Schleiermacher G, Tweddle DA, Valent A, Van Roy N, Vicha A, Villamon E, Tonini GP. *British journal of cancer* 2015, **112**(2):290-295.

2. Protein expression:

Anaplastic lymphoma kinase expression in neuroblastomas and its relationship with genetic, prognostic, and predictive factors. Berthier A, Piqueras M, Villamon E, Berbegall A, Tadeo I, Castel V, Navarro S, Noguera R. *Human pathology* 2011, **42**(2):301-302.

Intragenic anaplastic lymphoma kinase (ALK) rearrangements. translocations as a novel mechanism of ALK activation in neuroblastoma tumors. Fransson S, Hansson M, Ruuth K, Djos A, Berbegall A, Javanmardi N, Abrahamsson J, Palmer RH, Noguera R, Hallberg B, Kogner P, Martinsson T. *Genes, chromosomes & cancer* 2015, **54**(2):99-109.

Neuroblastoma patient-derived orthotopic xenografts retain metastatic patterns and geno- and phenotypes of patient tumours. Braekeveldt N, Wigerup C, Gisselsson D, Mohlin S, Merselius M, Beckman S, Jonson T, Borjesson A, Backman T, Tadeo I, Berbegall AP, Ora I, Navarro S, Noguera R, Pahlman S, Bexell D. *International journal of cancer Journal international du cancer* 2015, **136**(5):E252-261.

3. Extracellular matrix:

Biotensegrity of the extracellular matrix. physiology, dynamic mechanical balance, and implications in oncology and mechanotherapy. Tadeo I, Berbegall AP, Escudero LM, Alvaro T, Noguera R. *Frontiers in oncology* 2014, **4**:39.

B. Usefulness of databases

Comparative study of MLPA-FISH to determine DNA copy number alterations in neuroblastic tumors. Villamon E, Piqueras M, Berbegall AP, Tadeo I, Castel V, Navarro S, Noguera R. *Histology and histopathology* 2011, **26**(3):343-350.

NeuPAT. an intranet database supporting translational research in neuroblastic tumors. Villamon E, Piqueras M, Meseguer J, Blanquer I, Berbegall AP, Tadeo I, Hernandez V, Navarro S, Noguera R. *Computers in biology and medicine* 2013, **43**(3):219-228.

Quantitative modeling of clinical, cellular, and extracellular matrix variables suggest prognostic indicators in cancer. a model in neuroblastoma. Tadeo I, Piqueras M, Montaner D, Villamon E, Berbegall AP, Canete A, Navarro S, Noguera R. *Pediatric research* 2014, **75**(2):302-314.