UNIVERSITY OF NAPOLI "FEDERICO II"

Doctorate School in Molecular Medicine

Doctorate Program in Genetics and Molecular Medicine
Coordinator: Prof. Lucio Nitsch
XXV Cycle

Searching for Common Hereditable Genetic Variants Associated with Risk of Neuroblastoma Development and Progression.

Dr. Francesca Totaro

Napoli 2013

“…e ti senti una favola,

e ti sembra che tutta la vita

non è solamente retorica,

ma sostanza purissima,

che ti nutre le cellule,

e ti fa venir voglia di

vivere fino all'ultimo attimo…”

(L. Jovanotti “Terra degli Uomini”)

Table of contents.

List of publications.

Capasso M, Diskin S, **Totaro F**, Longo L, De Mariano M, Russo R, Cimmino F, Hakonarson H, Tonini GP, Devoto M, Maris JM, Iolascon A. **"Replication of GWAS-identified neuroblastoma risk loci strengthens the role of BARD1 and affirms the cumulative effect of genetic variations on disease susceptibility."** Carcinogenesis 2013.

Totaro F, Cimmino F, Pignataro P, Acierno G, De Mariano M, Longo L, Tonini GP, Iolascon A, Capasso M. "**Impact of interleukin-6 promoter polymorphism and gene expression interleukin-6 level on childhood cancer neuroblastoma."** Cancer Epidemiology, Biomarkers & Prevention 2013. [submitted]

Abstract.

Abstract.

Several neuroblastoma (NBL) susceptibility loci located in diverse genes (LINC00340, BARD1, LMO1, DUSP12, HSD17B12, DDX4, IL31RA, HACE1 and LIN28B) have been identified by genome-wide association studies (GWAS). The replication of association studies are mandatory to validate and comprehensively evaluate the impact of the identified NBL variants on disease risk and phenotype. To this purpose, during my PhD program I have genotyped in an Italian population 14 single nucleotide polymorphisms (SNPs), that have already been associated with NBL in European Americans. All NBL susceptibility genes replicated in the Italian dataset except for two of them, and the most significant SNP was rs6435862 in BARD1. Interestingly, BARD1 showed an additional and independent SNP association (rs7585356). This variant also influenced BARD1 mRNA expression in LCLs and NBL cell lines. Moreover, a cumulative effect of risk variants on NBL risk and development of high-risk phenotype was observed in a dose-dependent manner. These results provide further evidence that the risk loci identified in GWAS contribute to NBL susceptibility in distinct populations and strengthen the role of BARD1 as major genetic contributor to NBL risk.

Recent studies have demonstrated a role for interleukin(IL)-6, a pro-inflammatory cytokine, in progression and development of diverse cancers. Moreover, it has been demonstrated that the SNP rs1800795 in IL-6 promoter is associated with inferior clinical outcomes in patients with high-risk NBL. In particular, the major allele is reported as associated with lower survival. Thus, it has been analyzed an Italian population to validate these data. The results showed that the SNP was not implicated in susceptibility to NBL development while the minor allele is significantly associated with a reduction of the overall survival, advanced stage, and high-risk phenotype. Moreover, the analysis of expression indicate the minor allele as correlated with increased level of IL-6 expression. Kaplan-Meier analysis demonstrated that high levels of IL-6 were associated with poor outcome. These findings indicate that the biological effect of this SNP in relation to promotion of cancer progression is consistent with the observed decreased survival time.

Background.

Background.

Clinical of neuroblastoma.

NBL is a neuroendocrine tumor, arising from any neural crest element of the sympathetic nervous system. About half of NBLs arise in the adrenal medulla, while the other half arise in paraspinal sympathetic ganglia in the chest or abdomen or in pelvic ganglia. NBL is an important clinical problem, accounting for more than 7% of malignancies in children and around 15% of pediatric oncologic deaths (Gurney JG et al. 1997).

NBL is diagnosed at a median age of about 17 months and it is the most common cancer diagnosed during the first year of life. The incidence of NBL is 10.2 cases per million children under 15 years of age (London WB et al. 2005; Linet MS et al. 1999).

Primary tumors in the neck or upper chest can cause Horner's syndrome (ptosis, miosis, and anhidrosis). Tumors along the spinal column can expand through the intraforaminal spaces and can cause cord compression, with resulting paralysis. Although many lower-stage NBLs are encapsulated and can be surgically excised with little chance of complications, higher-stage tumors often infiltrate local organ structures, surround critical nerves and vessels such as the celiac axis, and are largely unresectable at the time of diagnosis. NBLs typically metastasize to regional lymph nodes and to the bone marrow by means of the hematopoietic system. Tumor cells metastatic to marrow can infiltrate cortical bone. NBLs can metastasize also to the liver, most notably in patients with stage 4S tumors, in whom involvement can be extensive; however, transient and complete regression often occurs with no intervention other than supportive care.

Indeed, NBL is an heterogenous tumor with the highest rate of spontaneous regression of all human cancers. However, most children of more than 1 year of age have extensive or metastatic disease with poor prognosis at diagnosis and around 50% of the patients show resistance to chemotherapy. This clinical heterogeneity is due to the complex genomic abnormalities acquired in tumor cells.

The International Neuroblastoma Staging System (INSS) stratifies NBL according to the anatomical presence at diagnosis (Brodeur GM et al. 1988 and 1993)(Figure 1):

- Stage 1: localized tumor confined to the area of origin;
- Stage 2A: unilateral tumor with incomplete gross resection;
- Stage 2B: unilateral tumor with complete or incomplete gross resection;
- Stage 3: tumor infiltrating across midline with or without regional lymphnode involvement;
- Stage 4: dissemination of tumor to distant lymphnodes, bone marrow, bone, liver or other organs except as defined by Stage 4S;
- Stage 4S: localized primary tumor in infants younger than 1 year, with dissemination limited to liver, skin, or bone marrow.

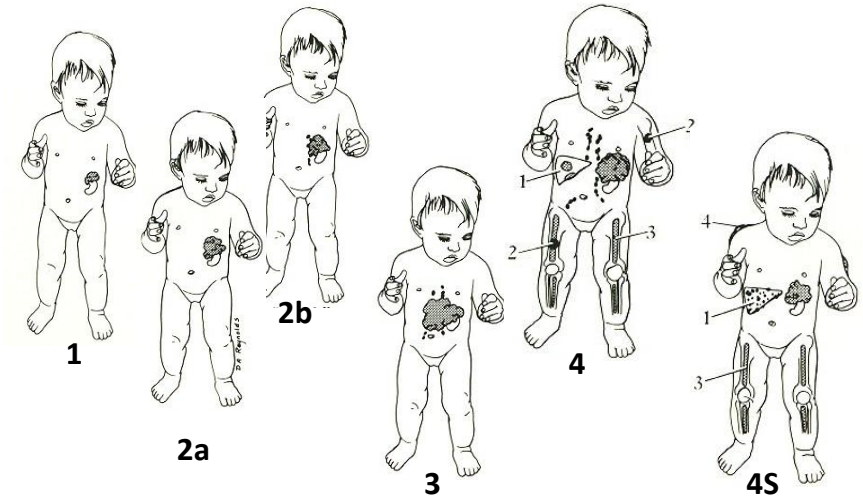

Figure 1. Stratification of NBL patients according to INSS.

According to Brodeur GM et al. (1993), the stage of disease, as formulated in the INSS, can also be considered a surrogate marker of the tumor burden and underlying tumor biology. Moreover, because of the complexity of the disease, age at diagnosis, clinical stage based on the INSS and tumor histology are important factors in predicting the outcome of NBL and these information can be useful in the selection of therapy (Maris JM et al. 2007).

At the extreme ends of the spectrum with respect to age and stage of disease, there is little controversy concerning risk classification. Older children with stage 4 (metastatic) disease are at high risk for death from refractory disease. In contrast, infants with localized tumors are almost always cured, often without cytotoxic therapy. For patients who fall between these extremes, however, it has been difficult to reach a consensus, owing to both the relative rarity of the condition and the evolving nature of molecular diagnostics. To address this issue of classification, Cohn and Pearson (2009) conducted a study that led to the development of a new tumor staging system that divides localized tumors into two groups on the basis of the presence or absence of

rigorously defined surgical risk factors (Cecchetto G et al. 2005; Monclair T et al. 2009) and an age cutoff point of 18 months was proposed. An International Neuroblastoma Risk Group (INRG) classification system with a total of 16 statistically distinct risk groups was proposed. This classification is based on the assessment of 13 potential prognostic factors. Four broad categories - very low risk, low risk, intermediate risk, and high risk - were proposed in terms of 5-year event-free survival rates of >85%, >75 to ≤85%, ≥50 to ≤75%, and <50%, respectively, on the basis of the analysis of age at diagnosis, INRG tumor stage, histologic category, grade of tumor differentiation, DNA ploidy, and copy-number status at the MYCN oncogene locus and at chromosome 11q.

Tumor-derived genomic information has been used since the 1980s to predict the course of newly diagnosed NBLs, with the discovery that the MYCN oncogene is the target of the extremely high-level amplifications at chromosome band 2p24 observed in about 20% of NBL cases. Because MYCN amplification has a profound effect on the clinical outcome, it is routinely used as a biomarker for treatment stratification (Schwab M et al. 1983; Brodeur GM et al. 1984; Seeger RC et al. 1985). Since the initial discovery of MYCN, many prognostic biomarkers have been proposed for NBL, the most intensely studied of which include histopathological classification, the tumor-cell DNA index (ploidy), and specific recurrent segmental chromosomal aberrations. More recently, microarray-based technologies have permitted the detailed dissection of the NBL genome and transcriptome, and several outstanding studies indicate that patterns of DNA-based or RNA-based aberrations have substantial predictive power (Oberthuer A et al. 2006; Asgharzadeh S et al. 2006; Ohira M et

al. 2005; Wei JS et al. 2004; Spitz R et al. 2006; Janoueix-Lerosey I et al. 2009).

Taken together, the available data suggest that DNA copy-number aberrations fall into two broad prognostic categories: whole-chromosome gains that result in hyperdiploidy and are associated with a favorable prognosis and segmental chromosomal aberrations, such as amplification of MYCN and regional loss or gain of chromosomal material, that tend to be associated with a worse outcome (Janoueix-Lerosey I et al. 2009) (Figure 2).

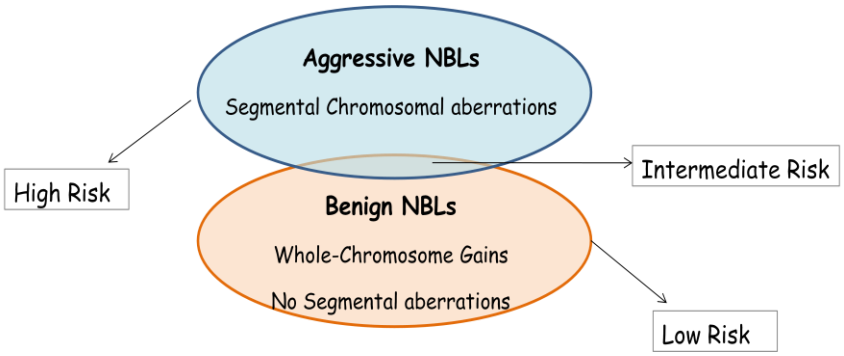

Figure 2. Classification of NBLs according to INRG.

Genetics of neuroblastoma.

The aetiology of NBL is unknown. A subset of patients inherits a genetic predisposition to NBL and NBLs can be classified into subtypes that are predictive of clinical behavior based on the patterns of genetic change.

The genetic aberration most consistently associated with poor outcome in NBL is the genomic amplification of MYCN (Brodeur GM et al. 1986 and 1987). MYCN amplification occurs in roughly 20% of primary tumors and is strongly correlated with advanced stage disease

and treatment failure (Brodeur GM et al. 1984; Seeger RC et al. 1985) (Figure 3).

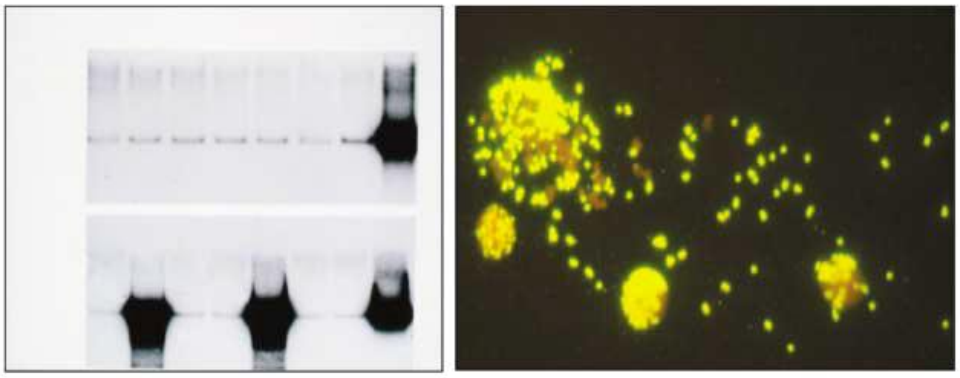

Figure 3. MYCN amplification in NBL.

Deletions of the short arm (p) of chromosome(chr) 1 can be identified in 25–35% of NBLs. These deletions correlate not only with MYCN amplification, but also with advanced stage of the disease (White PS et al. 1995; Gehring M et al. 1995; Martinsson T et al. 1995; White PS et al. 2001). However, the gene or genes within chr1p involved in the pathogenesis of NBL have not been identified yet. Whether the loss of heterozygosity due to deletion of alleles from chr1p is an independent indicator of prognosis remains controversial. However, evidence suggests that allelic loss at chr1p36 predicts an increased risk of relapse in patients with localized tumors (Gehring M et al. 1995; Caron H et al. 1996; Maris JM et al. 2000; Spitz R et al. 2002).

Allelic loss of the long arm (q) of chr11 is present in 35–45% of primary tumors (Guo C et al. 1999; Plantaz D et al. 2001; Spitz R et al. 2003). This genomic aberration is rarely seen in tumors with MYCN amplification, yet remains highly associated with other high-risk features.

A gain of additional chr17q copies, often through unbalanced translocation with chr1 or chr11, can also correlate with a more aggressive phenotype suggesting that a dosage effect of one or more genes provides a selective advantage (Bown N et al. 1999; Schleiermacher G et al. 2004).

Other frequently detected structural genetic alterations in NBL were reported, including allelic loss of genetic material located on chr3p, 4p, 9p, 14q, 16p and 19q, as well as segmental gains of 1q, 5q and 18q (Schwab M et al. 2003) and most of these markers have been associated with unfavorable features of the disease. Furthermore, copy number changes of whole chromosomes are also commonly observed in NBL, and are closely associated with the ploidy of the tumors (Brodeur GM 2003). In most cases, karyotypes of NBL cells are either in the diploid ('near-diploid') or hyperdiploid ('near-triploid') range. A near-triploid DNA content has been shown to be associated with favorable features such as stages 1-3 and 4S, a younger age at diagnosis, a lack of structural chromosomal aberrations and a more favorable outcome of the patient (Spitz R et al. 2006; Look AT et al. 1991; Kaneko Y et al. 1990).

Recently, genome-wide association studies (GWAS) focusing on single nucleotide polymorphisms (SNPs) provided the basis for the identification of activating mutations in the anaplastic lymphoma kinase (ALK) oncogene. These mutations appear to be responsible for most of the rare cases of hereditary NBL, and might also be relevant for a smaller fraction of the 6-9% of sporadic tumors (Chen Y et al. 2008; Janoueix-Lerosey I et al. 2008; Mossè YP et al. 2008). Interestingly, an even higher percentage of primary NBL (20-25%) present copy number increases at the ALK locus on chr2p23. Together with reports on elevated ALK gene-expression levels in aggressively growing NBL,

these findings strongly support an important role of this oncogene for NBL tumorigenesis (Wang Q et al. 2006; Oberthuer A et al. 2008; Osajima-Hakomori Y et al. 2005).

Prior to this revelation, the PHOX2B transcription factor, a homeobox gene functioning as an important regulator of normal autonomic nervous system development, was shown to be mutated in a small subset (~2%) of neuroblastic tumors (Mossè YP et al. 2004; Trochet D et al. 2004; van Limpt V et al. 2004).

The figure 4 illustrates the most common genomic aberrations in NBL.

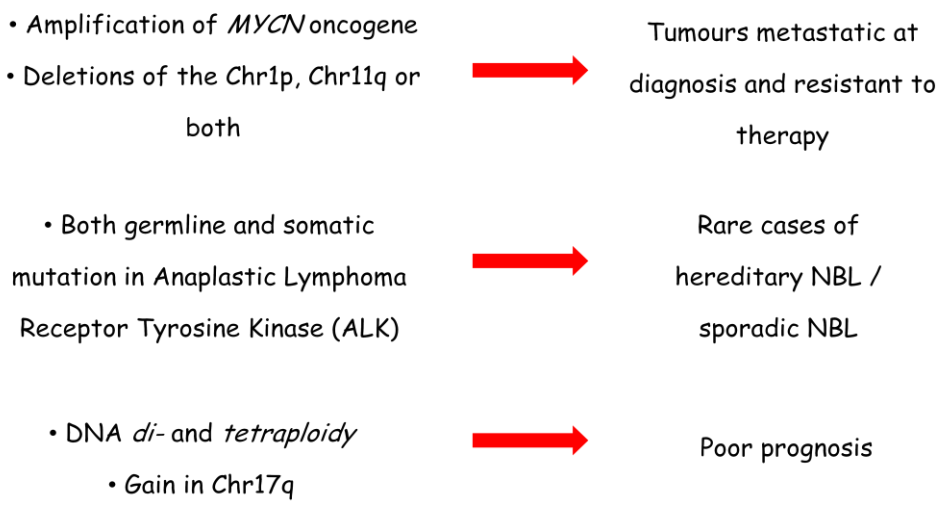

Figure 4. Genomic aberrations in NBL.

Genome Wide Association Studies (GWAS) of neuroblastoma.

Very little is known about elements predisposing to NBL as no specific environmental factors have been identified (Olshan et al. 1999_1 and 1999_2). The predisposition seems to be genetically heterogeneous. So, the tumor onset could be due to the presence of more alterations. This suggests that NBL is a genetic complex disease and its tumorigenesis could be the result of different genetic alterations.

A classical human genetic association study is based on a biological hypothesis. Indeed, this kind of study is used to test for a correlation between disease status and genetic variation to identify candidate genes or genome regions that contribute to a specific disease (Figure 5).

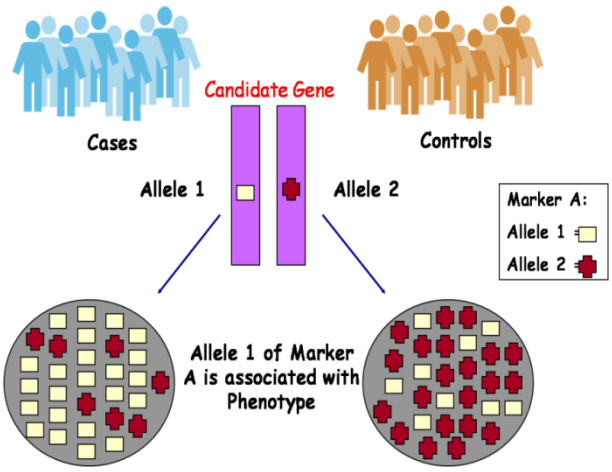

Figure 5. Human Genetic Association Study.

In contrast to this method, GWAS is a method free-of-hypothesis that investigates the whole genome. GWAS is an examination of many common genetic variants in different individuals to see if any variant is

associated with a trait. Generally, GWAS focus on associations between SNPs and disease. The approach of GWAS is the case-control setup which compares a healthy control group and a case group affected by a disease. The SNPs genotyped typically are 550K or a million (Figure 6).

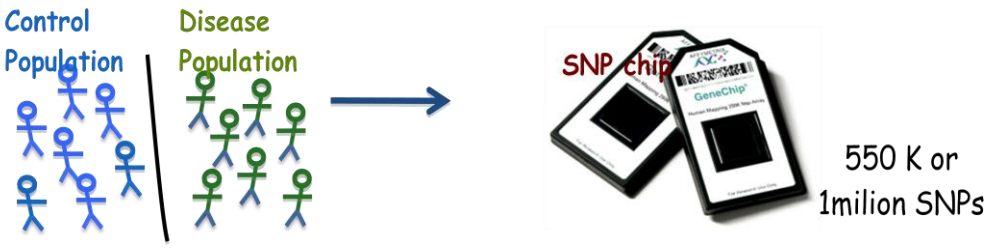

Figure 6. GWAS approach.

For every SNPs on the chip is evaluated if the allele frequency is significantly altered between the two populations studied.

In such setups, the fundamental parameters are the ORs and the p-value for the significance of the ORs (generally calculated by chi-squared test). Subsequently, it is created a "Manhattan plot" that shows the negative logarithm of the p-value as a function of genomic location. In this way, the most significant associated SNPs will be out on the plot (Figure 7). The p-value threshold for significance is corrected for multiple testing issues and the exact threshold is typically very low (about 10^{-7} -10^{-8}) because of the millions of tested SNPs.

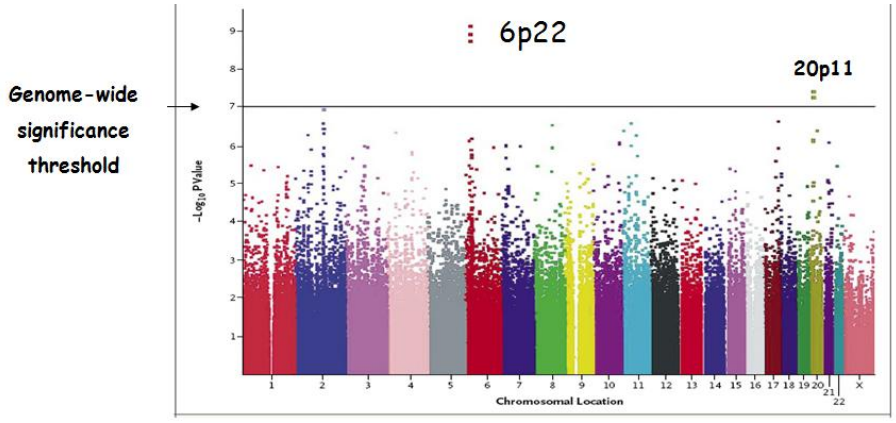

Figure 7. Manhattan plot (adapted from Maris et al. 2008)

In the last years GWAS have been performed to study NBLs and it has been demonstrated that common variants in DNA predispose to NBL onset. Indeed, three intronic SNPs in the predicted gene FLJ22536 (chr6p22) have been associated to NBL development and poor prognosis (Maris et al. 2008).

Moreover, a case/control study has demonstrated that BARD1 gene is a new gene of susceptibility to NBL high risk (Capasso et al. 2009). BARD1 is a tumor-suppressor gene that herodimerizes with BRCA1 (Baer et al. 2002; Simons et al. 2006) playing an active role in tumor suppression. In literature, BARD1 is reported as necessary for progression in the phase S of the cell cycle and as necessary for contact inhibition in mammal cells. Moreover, BARD1 is involved in the apoptotic response mediated by p53 in genotoxic stress (Figure 8) (Irminger-Finger et al. 2001; Jefford et al. 2004).

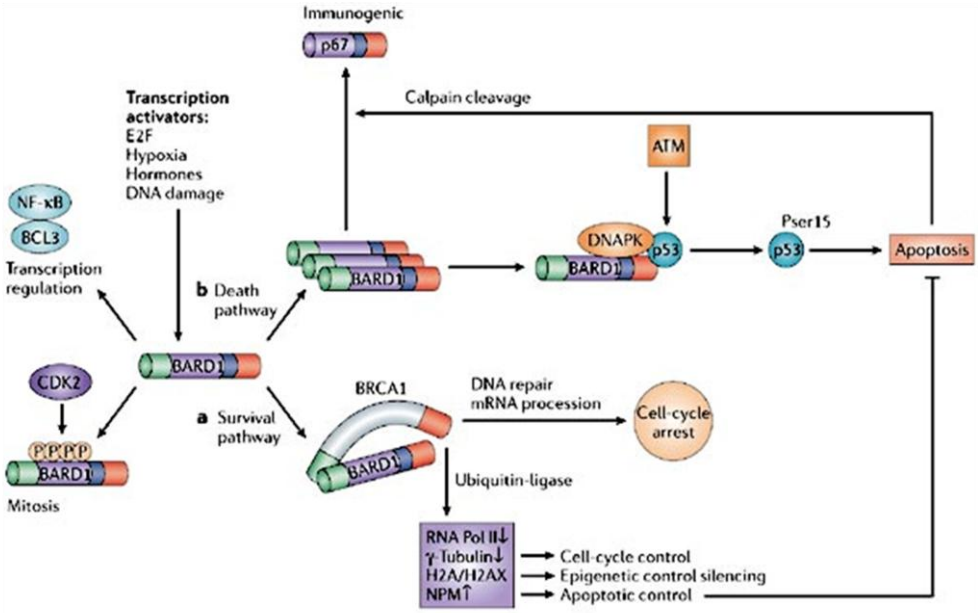

Figure 8. BARD1 role in death and survival pathways.

BARD1 gene is polymorphic and some of its SNPs, in particular six intronic SNPs, three non-synonymous SNPs and one SNP localized in the regulatory region of the gene, have been strongly associated with high risk NBL in the European American population (Capasso et al. 2009).

Recently, another study has identified LMO1 as a NBL oncogene (Wang et al. 2011). LMO1 encodes a cysteine-rich transcriptional regulator. The SNP risk alleles and somatic copy number gains were associated with increased LMO1 expression in NBL cell lines and primary tumors, consistent with a gain-of-function role in tumorigenesis.

The SNP associations reported above were particularly enriched in the high-risk group, whereas, SNPs within dual specifity phosphatase 12 (DUSP12), hydroxysteroid (17-beta) dehydrogenase 12 (HSD17B12), DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (DDX4) and interleukin 31 receptor A (IL31RA) were enriched in the low-risk

group. Additional SNPs within HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1 (HACE1) and lin28 homolog B (C. Elegans) (LIN28B) have been found to be associated with NBL but not with disease phenotype (Diskin et al. 2012).

Other recent studies have demonstrated a role for IL-6, a pro-inflammatory cytokine, in progression and development of multiple myeloma, colon cancer, melanoma, renal cancer, Hodgkin's disease, non-Hodgkin's lymphoma, prostate cancer, breast cancer (Heikkilä K et al. 2008).

In particular, the increase of IL-6 has been associated to the up-regulation of the expression of adhesion receptors on endothelial cells and to the production of growth factors. These events promoted the formation of favorable microenvironment for metastasis and suggested a role for IL-6 as a stimulator of metastasis (Hirano T 1997; Hutchins D et al. 1994). Moreover, it has been demonstrated that the peripheral blood levels of IL-6 correlated with NBL progression and development (Egler RA et al. 2008) and that IL-6 promoted growth and survival of NBL cells in the bone marrow (Ara T et al. 2009). Interestingly, the studies by Lagmay et al. (2009) reported the SNP rs1800795 (-174 G>C) in IL-6 promoter as associated with inferior clinical outcomes in patients affected by high-risk NBL.

All these findings suggest a "common variant-common disease" model for NBL: the interaction of multiple genetic variations can influence the development of the disease.

Model of neuroblastoma tumorigenesis.

The figure 9 shows a hypothetical model of NBL tumorigenesis based on germline mutations and common genetic variations associated to the disease.

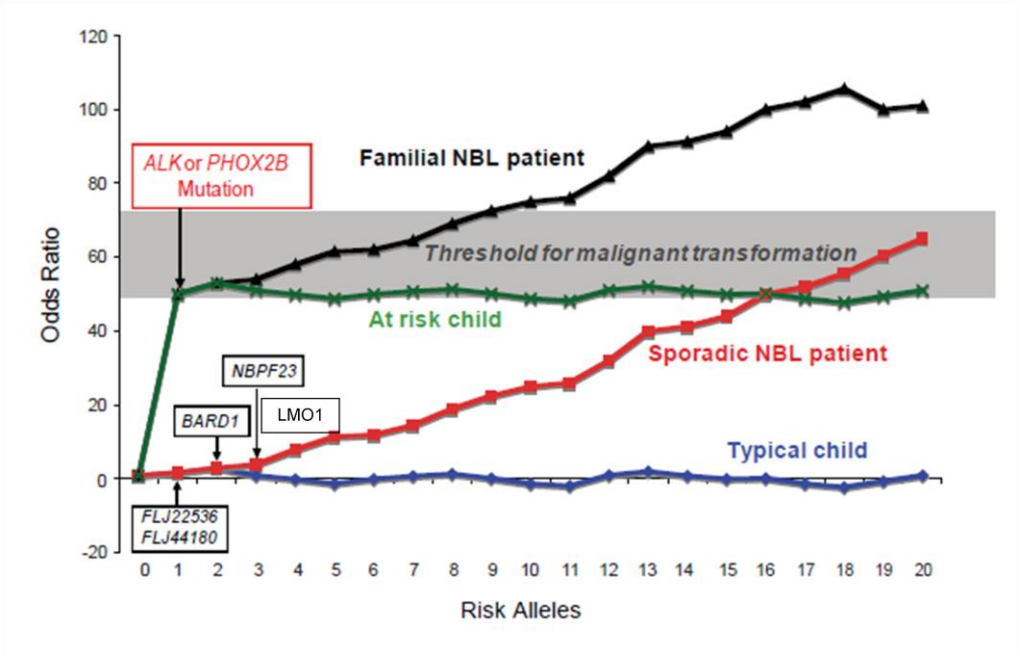

Figure 9. Graphical model of the genetics of NBL tumorigenesis.

The model reports the number of co-occurring risk alleles in a child's germline DNA along with the corresponding odds ratio (ORs) assuming an additive effect. The threshold for malignant transformation is set at an OR of 50. The blue line represents the typical child: the germline DNA presents only a small subset of risk alleles and thus the child is not at risk for developing the disease.

The green line shows a child with mutations within ALK or PHOX2B that is considered at risk for developing NBL. In presence of additional risk alleles, this child may become a familial NBL patients (black line), while, in absence of additional risk alleles, the child will be considered at risk but not develop the disease.

The red line shows a sporadic NBL patient that lacks mutations within ALK or PHOX2B likely requires the presence of 20 or more risk alleles before reaching the threshold for the malignant transformation.

Aims of the Study.

Aims of the Study.

The main objective of our study is to validate the identified GWAS NBL risk variants. To this purpose, I performed a case-control study to demonstrate that SNPs associated to NBL in European Americans are also associated to NBL in Italians. In particular, it has been assessed the association of NBL with 14 SNPs (rs6939340, rs4712653 located in LINC00340, rs6435862, rs3768716, rs7585356, rs2070094, rs2229571, rs1048108 located in BARD1, rs110419 and rs4758051 in LMO1, rs1027702 in DUSP12, rs11037575 in HSD17B12, rs2619046 in DDX4 and rs10055201 in IL31RA) (Capasso et al. 2013). The results of the Italian case-control study were compared to those of the European American GWAS composed of 1627 cases and 2575 controls (Nguyen le et al. 2011). I also replicated the SNP rs1800795 in IL-6 promoter found associated with negative prognosis in high-risk NBL patients (Lagmay et al. 2009).

The expected outcome for this study is to deliver to the research community a validated catalog of NBL susceptibility variants across the spectrum of allele frequencies and effect sizes, which could have implications in providing fundamental new insights into NBL tumorigenesis. All these tasks may help to identify the events responsible of NBL progression and development.

Materials and Methods.

Materials and Methods.

DNA samples used for the replication of GWAS-identified neuroblastoma risk loci.

This study consisted of 370 NBL patients collected through the Italian Neuroblastoma Group and 809 cancer-free controls of Italian origin. The population has been described in detail in our previous paper (Capasso et al. 2013).

Clinical and biological characteristics of the patients are shown in table 1. Samples were assigned into two risk groups (not high-risk and high-risk) based on the COG risk assignment.

DNA samples used for the replication study on IL-6 promoter polymorphism.

The study consisted of 326 DNAs extracted from peripheral blood of NBL patients collected through the Italian Neuroblastoma Group and 511 DNAs from cancer-free controls of Italian origin (mean age 9.67 ± 5.41 year). Clinical and biologic characteristics of the patients are shown in table 2. Samples were assigned into two risk groups (not high-risk and high-risk) based on the COG risk assignment. DNA samples have been described in full detail in our paper appended to the thesis (Totaro F et al. 2013_submitted).

Table 1. Clinical characteristic of study samples for replication of GWAS.

Variable	Italians (N=370) Number (%)
Age	
≥18 months	170 (46.8)
< 18 months	193 (53.2)
Unknown	7
INSS Stage	
4	145 (40.2)
1, 2, 3, 4S	216 (59.8)
Unknown	9
MYCN	
Amplified	80 (24.0)
Not Amplified	254 (76.0)
Unknown	36
Risk	
High Risk	212 (57.3)
Not High Risk	158 (42.7)
Unknown	-

Table 2. Clinical characteristic of study samples for IL-6 SNP association.

	n
Cases	326
Controls	511
Age	
≥ 18 months	152
< 18 months	168
Unknown	6
Sex	
Males	178
Females	141
Unknown	7
INSS stage	
Stage 1 + 2	103
Stage 3 + 4	192
Stage 4S	23
Unknown	8
Risk	
High-risk	144
Not high-risk	182
MYCN	
Amplified	73
Not Amplified	221
Unknown	32

SNPs selection and genotyping.

The SNPs selection and the genotyping methods are described precisely in our previous papers (Capasso et al. 2013; Totaro F et al. 2013_submitted).

Briefly, for the replication of GWAS-identified NBL risk loci, we selected 14 genetic variants that have previously been associated with NBL in European American children. We included the two most significant SNPs identified in LINC00340 (intronic rs6939340, rs4712653) (Maris et al. 2008), in BARD1 (intronic rs6435862, rs3768716) (Capasso et al. 2009), and in LMO1 (intronic rs110419 and intergenic rs4758051) (Wang et al. 2011) as previously reported. In addition, we genotyped the putative functional variation rs7585356 located 3' downstream of BARD1, and its coding variants rs2070094, rs2229571, and rs1048108 (Capasso et al. 2009). We also analyzed four SNPs (intergenic rs1027702 near DUSP12, intronic rs11037575 in HSD17B12, rs2619046 in DDX4, and rs10055201 in IL31RA) recently found to be associated with low-risk NBL (Nguyen le et al. 2011). The Italian DNA samples were genotyped by TaqMan assay or by Restriction Fragment Length Polymorphism (RFLP).

To test NBL risk variants cumulative effect, we also used genotype data of rs4336470 (HACE1) and rs17065417 (LIN28B) already demonstrated to be associated with NBL in this Italian case-control sample (Diskin et al. 2012).

For the study of the polymorphism in IL-6 promoter, DNA samples were screened for the SNP rs1800795 using RFLP mapping to identify each patient's genotype. The RFLP strategy is the same described by Lagmay et al. (2009).

***In silico* gene expression correlation analysis.**

As described in our paper (Capasso et al. 2013), the influence of SNPs on gene expression was evaluated using two web tools, SNPexp v1.2 (http://app3.titan.uio.no/biotools/tool.php?app=snpexp) (Holm et al. 2010) and Genevar v3.1.0 (http://www.sanger.ac.uk/resources/software/genevar/) (Yang et al. 2010).

SNPexp calculates correlation between HapMap genotypes and gene expression levels in LCLs using linear regression under an additive model. For this analysis, 198 unrelated HapMap3 subjects were chosen.

In Genevar, a linear regression (under an additive model) was used to calculate the SNP-gene expression correlations performing a Matched Co-Twin Analysis. The expression and genotype data were obtained from LCLs of 170 individuals separate in two sets, Twin 1 (81 subjects) and Twin 2 (89 subjects). This analysis permits immediate replication and validation of the identified SNP-gene expression associations.

Also for IL-6 the SNP-gene expression correlation analysis, the influence of the SNP has been evaluated using SNPexp v1.2 (http://app3.titan.uio.no/biotools/tool.php?app=snpexp). For this analysis, 198 unrelated HapMap3 subjects were chosen. In addition, *in silico* analysis of IL-6 gene expression-outcome correlation has been performed. Two independent sets of normalized gene expression data and clinical annotations were downloaded from the website R2, microarray analysis and visualization platform (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi):

1) Versteeg dataset composed of 88 NBL samples;

2) Seeger dataset composed of 102 NBL samples.

Genotyping of neuroblastoma cell lines.

For routine maintenance, cells were grown in RPMI 1640 complete media or DMEM complete media.

NBL cell lines were genotyped by direct sequencing for the HSD17B12-SNP rs11037575 and for the BARD1-SNP rs7585356.

The isolation of genomic DNA and PCR strategy, including primers sequences, are reported in our previous paper (Capasso et al. 2013).

Gene expression of neuroblastoma cell lines.

As reported in detail in our published paper (Capasso et al. 2013) RNA was isolated from NBL cell lines using the TRIzol reagent (Invitrogen Life Technologies). Two hundred ng of total RNA was reverse transcribed into cDNA using iScript cDNA Synthesis Kit (BIORAD). Quantitative RT-PCR (qRT-PCR) using Power SYBR Green PCR Master Mix (Applied Biosystems) was performed to evaluate the gene expression of HSD17B12 and BARD1.

Samples were amplified on an Applied Biosystems 7900HT Sequence Detection System and data collected and analyzed by 2^-Δct method. β-actin gene was used as housekeeping gene. Primers overlapping the exon-exon junction were used and they were specifically designed to detect the cDNA full lenght isoforms.

IL-6 gene expression analysis in primary tumors.

RNA from 31 NBL tissues has been isolated using the TRIzol reagent (Invitrogen Life Technologies). Two thousand ng of total RNA was reverse transcribed into cDNA using iScript cDNA Synthesis Kit (BIORAD). To evaluate the gene expression of IL-6, quantitative real time PCR (qRT-PCR) was performed using Power SYBR Green Master Mix (Applied Biosystems). Samples were amplified on an Applied Biosystems 7900HT

Sequence Detection System and data were collected and analyzed by 2^-Δct method. HPRT was used as housekeeping gene. Primers overlapping the exon-exon junction were used and the primers for IL-6 were specific for the full length isoform (Totaro F et al. 2013_submitted).

Statistical analysis.

The statistical analysis are reported in full detail in our previous paper (Capasso et al. 2013; Totaro F et al. 2013_submitted).

Briefly, for the replication of GWAS-identified NBL risk loci, Hardy-Weinberg equilibrium (HWE) was evaluated. Two-sided chi-square tests were used to evaluate differences in the distributions of allele frequencies between all patients and controls, and only high-risk patients and controls.

ORs and 95% confidence intervals (CIs) were calculated to assess the relative disease risk conferred by a specific allele. We defined replication as a p-value of ≤0.05 with a consistent direction of association. We did not correct for multiple tests because the analyzed genes were previously reported associated to NBL in multiple independent datasets (Capasso et al. 2009; Latorre et al. 2012; Maris et al. 2008; Nguyen le et al. 2011; Wang et al. 2011).

To compare the differences in the mRNA expression levels between NBL cell lines stratified according to genotype Student's t test was used.

Linkage disequilibrium (LD) and haplotype analysis were performed using the website tool SNAP v2.2 (http://www.broadinstitute.org/mpg/snap/index.php) (Johnson et al. 2008) and Haploview v4.2 software (Barrett et al. 2005).

The cumulative effects of the independent significant risk loci on NBL initiation and phenotype have been assessed counting the number of risk alleles in each subject and modeling the summary variable categorically in

logistic regression analysis. This analysis was performed using IBM SPSS 19.0 software.

Gene-gene interaction was tested using logistic regression additive model by adding an interaction term between the genotypes from all pair of SNPs investigated. This analysis was performed by PLINK software v1.06 (Purcell et al. 2007).

Also for the replication study on IL-6 promoter, HWE was evaluated using the goodness-of-fit chi-square test in control subjects. Two-sided chi-square tests were used to test for associations of the existence of polymorphism versus each other clinical factor, all patients, and only high-risk patients. ORs and 95% CIs were calculated to assess the disease risk conferred by a specific allele or genotype.

Overall Survival (OS) was calculated by Kaplan-Meier method to generate survival curves which were compared using a log-rank test.

The Cox regression model was used to test for the independent predictive ability of the SNP after the adjustment for other significant factors: MYCN amplification, age, INSS stages. P-values of <0.05 were considered statistically significant.

Results and Discussion.

Results.

Replication of GWAS-identified neuroblastoma risk loci.

The allele frequencies of all selected genetic variants among cases and controls and their association with NBL and high-risk phenotype are shown in table 3.

The observed genotype frequency among the control subjects was in agreement with HWE, except for rs2070094 ($p<0.001$). So this SNP was excluded from further analyses.

In the population studied significant differences of allele distributions were observed for all SNPs except rs4758051 (LMO1), rs2619046 (DDX4) and rs10055201 (IL31RA). SNP rs6435862 within BARD1 was the most significant (table 3, $p=8.4x10^{-15}$).

In Italian children, all SNPs showed a pattern of association to NBL that is similar to that reported in European Americans (Capasso et al. 2013). Moreover, all SNPs of LINC00340 and BARD1, and one of LMO1 were significantly associated with high-risk NBL both in Italians and in Americans (Capasso et al. 2013) (table 3).

Table 3. Association of sixteen previously identified SNPs with NBL in Italian children.

				Italians						SNP-gene expression associations					
SNP ID	Chromosome band	Function	[a]Allele	Case genotypes	High risk genotypes	Control genotypes	Case MAF (No.)	High risk MAF (No.)	Control MAF	[c]P	[c]OR (95% CI)	[d]P	[d]OR (95% CI)	[e]P HapMap	[f]P Twin1	[f]P Twin 2
LINC00340 rs6939340	6p22	-	A/G	74, 162,103	29, 71, 48	196, 390, 175	0.54	0.56	0.49	0.01	1.25 (1.04-1.50)	0.01	1.37 (1.06-1.76)	-	-	-
LINC00340 rs4712653	6p22	-	T/C	78, 155, 108	31, 62, 57	199, 376, 197	0.54	0.59	0.50	0.05	1.20 (1.00-1.44)	5.3×10^{-3}	1.43 (1.11-1.83)	-	-	-
BARD1 rs6435862	2q35	Intronic	T/G	120, 155, 69	49, 67, 33	423, 312, 49	0.43	0.45	0.26	8.4×10^{-15}	2.10 (1.73-2.53)	1.2×10^{-10}	2.28 (1.76-2.94)	0.50	4.8×10^{-6}	3.0×10^{-4}
BARD1 rs3768716	2q35	Intronic	A/G	155, 153, 50	60, 74, 20	473, 289, 45	0.35	0.37	0.23	2.9×10^{-9}	1.78 (1.50-2.16)	6.3×10^{-7}	1.91 (1.48-2.48)	0.16	5.0×10^{-4}	4.0×10^{-4}
BARD1 rs7585356	2q35	3' downstream	G/A	204, 120, 19	94, 51, 7	370, 305, 94	0.23	0.21	0.32	2.0×10^{-5}	0.63 (0.51-0.78)	2.2×10^{-4}	0.57 (0.43-0.77)	3.0×10^{-4}	1.0×10^{-4}	7.7×10^{-8}
BARD1 rs2070094	2q35	exon 6 (Val507Met)	G/A	219, 83, 24	103, 33, 9	419, 247, 109	0.20	0.18	0.30	1.8×10^{-6}	0.59 (0.47-0.73)	2.0×10^{-5}	0.50 (0.36-0.68)	-	-	-
BARD1 rs2229571	2q35	exon 4 (Arg378Ser)	G/C	165, 124, 61	74, 53, 24	238, 363, 208	0.35	0.33	0.48	7.1×10^{-9}	0.58 (0.49 - 0.70)	2.5×10^{-6}	0.54 (0.42-0.70)	-	1.0×10^{-3}	6.8×10^{-6}
BARD1 rs1048108	2q35	exon 1 (Pro24Ser)	C/T	156, 98, 28	69, 39, 10	296, 355, 99	0.27	0.25	0.37	4.5×10^{-5}	0.64 (0.52-0.79)	3.9×10^{-4}	0.57 (0.42-0.78)	-	-	-
LMO1 rs110419	11p15	Intronic	G/A	84, 152, 87	36, 62, 39	271, 370, 133	0.50	0.51	0.41	5.0×10^{-5}	1.46 (1.21-	2.0×10^{-3}	1.50 (1.16-	0.24	0.03	0.60

LMO1 rs4758051	11p15	Intergenic	A/G	114, 156, 70	47, 72, 23	246, 405, 141	0.44	0.42	0.43	0.94	1.76) 1.00 (0.84-1.21)	0.57	1.94) 0.93 (0.72-1.20)	0.34	0.20	0.22
DUSP12 rs1027702	1q23.3	Intergenic	C/T	141, 156, 52	61, 67, 22	258, 385, 128	0.37	0.37	0.42	0.05	0.83 (0.69-1.00)	0.14	0.83 (0.64-1.06)	4.0×10^{-7}	0.42	0.27
HSD17B12 rs11037575	11p11.2	Intronic	T/C	123, 168, 52	52, 74, 21	322, 379, 84	0.40	0.39	0.35	0.03	1.23 (1.02-1.47)	0.13	1.22 (0.94-1.57)	1.1×10^{-8}	7.2×10^{-6}	2.0×10^{-4}
DDX4 rs2619046	5p15.2-p13.1	Intronic	G/A	180, 130, 25	78, 60, 8	456, 300, 44	0.27	0.26	0.24	0.19	1.15 (0.93-1.41)	0.52	1.10 (0.83-1.46)	0.30	0.24	0.69
IL31RA rs10055201	5p15.2-p13.1	Intronic	A/G	199, 123, 20	90, 54, 7	475, 264, 36	0.24	0.23	0.22	0.26	1.13 (0.91-1.40)	0.75	1.05 (0.78-1.41)	0.68	0.26	0.41
HACE1 rs4336470	6q16.3	Intronic	C/T	176, 136, 38	63, 68, 18	348, 329, 103	[b]0.30	0.35	[b]0.34	[b]0.06	[b]0.71 (0.52-0.97)	0.84	1.03 (0.79-1.33)	0.02	0.23	0.60
LIN28B rs17065417	6q21	Intronic	A/C	298, 48, 5	126, 22, 2	600, 155, 8	[b]0.08	0.09	[b]0.11	[b]0.03	[b]0.83 (0.68-1.00)	0.75	0.75 (0.49-1.16)	-	0.92	0.14

[a] Major/minor Alleles; Italians: 370 cases and 809 controls; [b] Data as reported in Diskin et al. 2012; [c] P-values and ORs from comparison of Case versus Control; [d] P-values and ORs from comparison of High risk versus Control; [e] P-values from SNP-gene expression correlation using 198 unrelated HapMap subjects (SNPexp web tool); [f] P-values from SNP-gene expression correlation using 170 subjects (Genevar web tool).

Subsequently it has been tested for SNP-gene expression associations at all 14 SNPs and at 2 previously reported SNPs (Diskin et al. 2012). The analysis of gene expression variation using genome-wide expression arrays from LCLs of 198 unrelated HapMap individuals demonstrated that SNPs rs7585356 (BARD1), rs1027702 (DUSP12), rs4336470 (HACE1) and rs11037575 (HSD17B12) affected expression of the respective genes (table 3). These results were confirmed in 170 additional LCLs only for rs7585356 (BARD1) and rs11037575 (HSD17B12) when we performed a Matched Co-Twin Analysis, which permits immediate replication and validation of expression quantitative trait loci discoveries.

The qRT-PCR gene expression analysis showed that mRNA expression of full length BARD1 isoform was significantly higher in NBL cell lines heterozygous at rs7585356 (AG) (Figure 9a). A trend toward association between high mRNA levels of full length HSD17B12 isoform and presence of the risk allele C was observed for rs11037575 without reaching the threshold for statistical significance (Figure 9b).

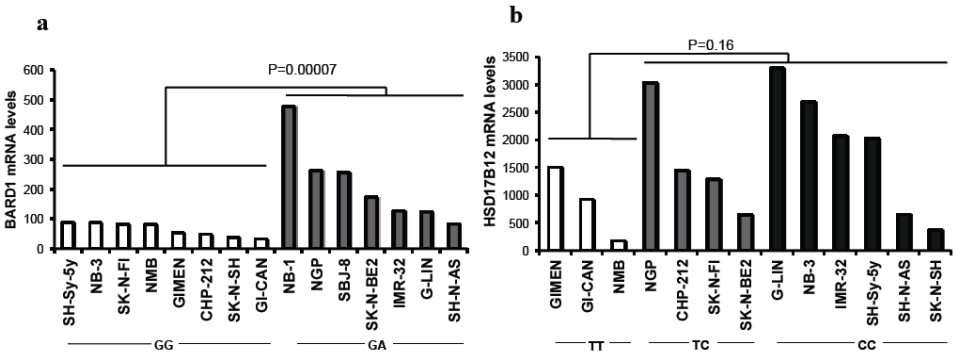

Figure 9. Gene expression analysis of BARD1 and HSD17B12 in NBL cell lines.

To select those SNPs independently associated with NBL among the 13 replicated ones, we performed a LD analysis using data from 1000 Genomes Project (http://www.broadinstitute.org/mpg/snap/index.php). SNPs with a r^2 >0.10 with the most significant SNP within each gene were removed: eight SNPs (rs6939340 (LINC00340), rs6435862 and rs7585356 (BARD1), rs110419 (LMO1), rs1027702 (DUSP12), rs11037575 (HSD17B12), rs4336470 (HACE1) and rs17065417 (LIN28B) were selected for further analyses. Interestingly, the LD and haplotype analyses demonstrated that SNPs rs6435862 and rs7585356 of BARD1 were located in two independent genetic loci.

To demonstrate the independence of their association with NBL risk, we conducted a logistic regression analysis by adjusting for either of the two SNPs. The results showed that both SNPs still remained significant in Italians (rs6435862: $p=1.7x10^{-4}$, OR=1.97, 95% CI: 1.60-2.43; rs7585356: p=0.04, OR=0.80, 95% CI: 0.64-0.99).

The association of these eight independent risk variants and NBL stratified by clinical stage (stage 4 versus not stage 4), MYCN status (amplified versus not amplified), risk assessment (high-risk versus not high-risk), and age at diagnosis (age ≥18 months and <18 months) was further evaluated among cases. As shown in table 4, the genetic variants of LINC00340 (Maris et al. 2008), BARD1 (Capasso et al. 2009) and LMO1 (Wang et al. 2011) confirmed the association with more clinically aggressive phenotypes, but only rs7585356 resulted to be significantly associated with stage 4 and age≥18 months (P=0.04, OR= 0.67, 95% CI=0.46-0.98 and P=0.02, OR=0.64, 95% CI=0.44-0.92).

Table 4. Association of the eight genetic variants with pathologic characteristic of NBL.

SNP ID	Stage 4 (%)	Not Stage 4 (%)	P	OR (95% CI)	MYCN Amp (%)	Not MYCN Amp (%)	P	OR (95% CI)	High Risk (%)	Not High Risk (%)	P	OR (95% CI)	Age≥ 18 (%)	Age< 18 (%)	P	OR (95% CI)
LINC00340 rs6939340	155 (0.56)	206 (0.54)	0.52	1.11 (0.81-1.51)	89 (0.57)	245 (0.54)	0.44	1.15 (0.80-1.67)	176 (0.55)	192 (0.53)	0.60	1.08 (0.80-1.47)	160 (0.55)	203 (0.55)	0.91	1.02 (0.75-1.39)
BARD1 *rs6435862*	119 (0.43)	169 (0.43)	0.95	1.01 (0.74-1.38)	75 (0.47)	187 (0.41)	0.17	1.29 (0.90-1.85)	142 (0.44)	151 (0.41)	0.45	1.12 (0.83-1.52)	134 (0.45)	155 (0.41)	0.24	1.20 (0.88-1.64)
BARD1 *rs7585356*	52 (0.19)	98 (0.25)	0.04	0.67 (0.46-0.98)	35 (0.23)	106 (0.23)	0.92	1.02 (0.66-1.58)	65 (0.21)	93 (0.24)	0.36	0.84 (0.59-1.21)	59 (0.19)	93 (0.26)	0.02	0.64 (0.44-0.92)
LMO1 rs110419	131 (0.51)	187 (0.50)	0.98	1.01 (0.73-1.38)	71 (0.49)	230 (0.52)	0.60	0.91 (0.62-1.32)	150 (0.51)	176 (0.50)	0.92	1.02 (0.74-1.38)	140 (0.52)	179 (0.49)	0.25	1.20 (0.88-1.65)
DUSP12 *rs1027702*	104 (0.38)	154 (0.38)	0.99	0.99 (0.73-1.37)	59 (0.37)	179 (0.38)	0.81	0.96 (0.66-1.39)	123 (0.38)	137 (0.37)	0.72	1.06 (0.78-1.44)	116 (0.38)	142 (0.34)	0.87	1.03 (0.75-1.40)
HSD17B12 *rs11037575*	106 (0.38)	159 (0.40)	0.56	0.91 (0.66-1.25)	56 (0.36)	197 (0.42)	0.25	0.80 (0.55-1.17)	121 (0.38)	151 (0.41)	0.36	0.87 (0.64-1.18)	115 (0.38)	153 (0.41)	0.57	0.91 (0.67-1.25)
HACE1 *rs4336470*	91 (0.32)	116 (0.29)	0.31	1.19 (0.85-1.65)	62 (0.42)	135 (0.28)	0.00	1.92 (1.31-2.82)	104 (0.35)	108 (0.27)	0.02	1.46 (1.05-2.02)	104 (0.32)	103 (0.28)	0.30	1.19 (0.86-1.65)
LIN28B *rs17065417*	22 (0.08)	35 (0.09)	0.78	0.92 (0.53-1.61)	19 (0.13)	36 (0.07)	0.04	1.83 (1.02-3.30)	26 (0.09)	32 (0.08)	0.74	1.10 (0.64-1.88)	27 (0.08)	30 (0.08)	0.89	1.04 (0.60-1.79)

Moreover, it was evaluated potential pairwise interaction effects among the studied SNPs. No evidence of epistasis was detected through this analysis.

To further investigated the cumulative effects of the eight susceptibility loci on NBL development in Italians the total number of risk alleles carried by individual subjects was used as predictor.

The distribution of risk alleles carried in both cases and controls followed a normal distribution, but with a shift toward a higher number of risk alleles in the cases (Figure 10a).

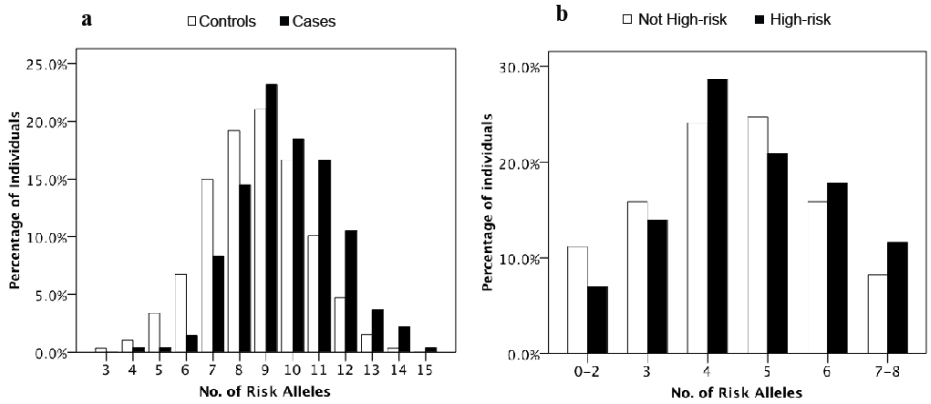

Figure 10. Distribution of risk alleles in healthy controls and NBL cases for the identified NBL (a) and aggressive disease phenotype (b) susceptibility loci.

As shown in figure 11a, individuals with multiple risk alleles had higher risk of developing NBL compared to those with 0-7 risk alleles of the eight variants, in a dose-dependent manner with increasing number of risk variant alleles (p-trend=8.55x10-13, OR=1.35, 95% CI:1.24-1.46). We also tested for the cumulative effects of the four genetic variants (rs6939340, rs6435862, rs7585356, rs110419) significantly found to be associated with clinically aggressive NBL in European Americans (Capasso et al. 2013). The distribution of risk alleles carried in both not high-risk and high-risk patients is shown in figure 10b. A shift toward a higher number of risk alleles in high-risk individuals was observed but it was not so evident in Italians. As shown in figure 11b, the ORs relative to the 0-3 risk alleles group increased with increasing number of risk alleles in Italians without reaching the threshold for statistical significance (p-trend=2.2x10-1, OR=1.11, 95% CI: 0.94-1.30),

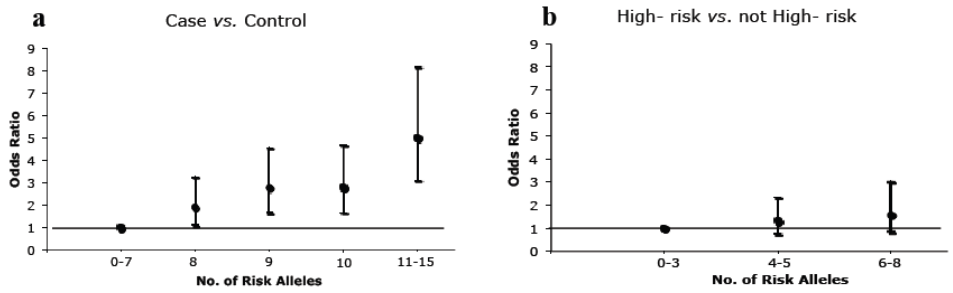

Figure 11. Plots showing the increasing ORs for a) NBL patients and b) high-risk patients with the increasing number of risk alleles for the identified loci. The vertical bars represent 95% CIs. The horizontal line denotes the null value (OR=1).

Replication study on IL-6 promoter polymorphism.

The allele frequencies for rs1800795 for NBL patients and controls are shown in table 5. The distribution of the genotypes was in agreement with HWE. The genotype CC was more significantly frequent in high-risk (p=0.03) and stage 3 and 4 patients (p=0.02). No significant association was found with NBL development.

Table 5. Allele frequencies for rs1800795 for NBL patients and controls.

	Genotypic frequencies						
	GG (%)	**GC (%)**	**CC (%)**	**[a]P**	**[a]OR (CI=95%)**	**[b]P**	**[b]OR (CI=95%)**
Cases	176 (0.54)	125 (0.38)	25 (0.08)	0.24	1.14 (0.92-1.43)	0.34	1.31 (0.75- 2.28)
Controls	295 (0.58)	184 (0.36)	32 (0.06)				
Age							
≥ 18 months	77 (0.50)	63 (0.41)	12 (0.08)	0.39	1.17 (0.82-1.65)	0.65	1.22 (0.52-2.87)
< 18 months	94 (0.56)	62 (0.37)	12 (0.07)				
Sex							
Male	92 (0.52)	72 (0.40)	14 (0.08)	0.41	1.16 (0.81-1.65)	0.52	1.34 (0.55–2.35)

Female	79 (0.56)	53 (0.38)	9 (0.06)				
INSS stage							
Stage 1 + 2	57 (0.55)	44 (0.43)	2 (0.02)	[c]0.21	1.29 (0.87-1.91)	[c]0.02	5.03 (1.13-22.46)
Stage 3 + 4	102 (0.53)	72 (0.37)	18 (0.09)				
Stage 4S	12 (0.52)	8 (0.35)	3 (0.13)				
Risk							
High-risk	73 (0.51)	55 (0.38)	16 (0.11)	0.08	1.36 (0.96-1.92)	0.03	2.51 (1.05-5.99)
Not high-risk	103 (0.57)	70 (0.38)	9 (0.05)				
MYCN							
Amplified	35 (0.48)	32 (0.44)	6 (0.08)	0.17	1.33 (0.88- 2.02)	0.33	1.66 (0.59-4.69)
Not Amplified	126 (0.57)	82 (0.37)	13 (0.06)				

[a]p-values and ORs from comparison of allelic frequencies

[b]p-values and ORs from comparison of genotype frequencies (GG/GC vs CC)

[c]p-values and ORs from comparison of Stage 1 + Stage 2 patients vs Stage 3 + Stage 4 patients

Then we examined OS of the genotyped patients samples to determine if the IL-6 SNP was predictive of NBL outcome. IL-6 SNP showed statistically significant differences ($p<0.05$) in predicting OS. Indeed, patients that were homozygous for the C allele had worse survival than patients who were homozygous and heterozygous for the G allele in all patients (Figure 12A) and not MYCN amplified (Figure 12B) patient sub-group. OS 5 years from the date of diagnosis was 85.2% (95% CI 0.83-0.87) for the group of patients carrying GG and GC genotype, compared with 67.2% (95% CI 0.55-0.79) for the group of patients carrying CC genotype (Figure 12A). This genetic association was independent from MYCN status, age and INSS stage (table 6). A

similar trend, without reaching the significance, was observed when the analysis was restricted to high-risk patients (Figure 12C and 12D).

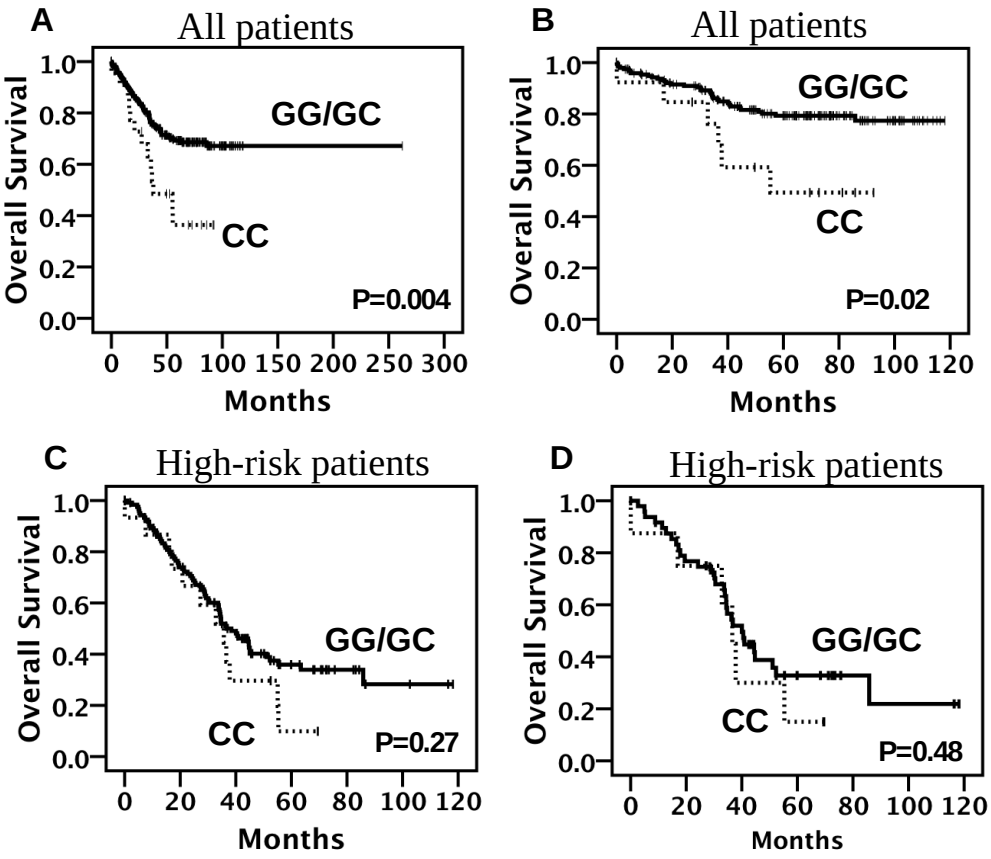

Figure 12. Kaplan-Meier curves for OS rates. OS rates were compared between CC and any G (GC or CC) for the SNP IL-6 -174 in (A) all NB and (B) not *MYCN* amplified patients, and (C) in high-risk NB and (D) not *MYCN* amplified patients.frequencies for rs1800795 for NBL patients and controls.

Table 6. Test for independent statistical significance of -174 IL-6 SNP after adjustment for NBL risk factors.

Model	HR (95% CI)	P
A.		
Age (≥18 months vs <18 months)	4.18 (2.55-6.84)	<0.0001
-174 IL6 SNP (CC vs GC/GG)	1.90 (1.05-3.43)	0.03
B.		
MYCN (amplified vs not amplified)	3.85 (2.48-5.98)	<0.0001
-174 IL6 SNP (CC vs GC/GG)	1.95 (1.03-3.68)	0.04
C.		
INSS Stage (4 vs 1-2-3-4s)	9.06 (5.11-16.08)	<0.0001
-174 IL6 SNP (CC vs GC/GG)	1.90 (1.05-3.42)	0.03

The *in silico* analysis of LCLs demonstrated that the CC genotype correlated with high level of IL-6 expression (p=2.61x10-5, Figure 13A). Subsequently, qRT-PCR of mRNAs from tumor specimens was performed and the results showed that the levels of IL-6 mRNA were higher in CC carriers than in GG and GC carriers (p=0.27, Figure 13B) without reaching the significant threshold.

When we verified the association of IL-6 gene expression with outcome disease in two independent sets of patients, we found that the increase of the mRNA expression was significantly associated with lower OS and progression of disease (Figure 13C, 13D and 13E).

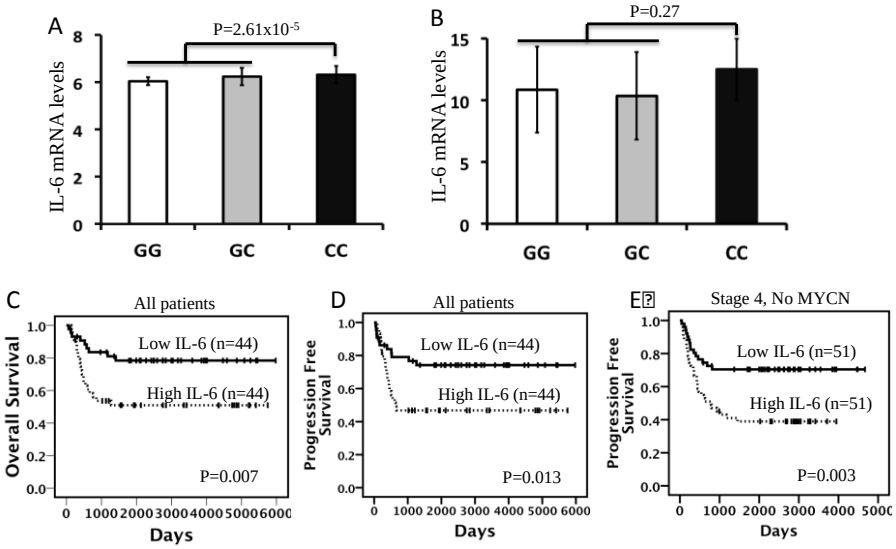

Figure 13. SNP-gene expression and IL-6 gene expression-outcome correlations. *In silico* and qRT-PCR analysis of IL-6 mRNA expression in (A) 198 LCLs and (B) 31 NBL tumors, respectively, stratified according to the SNP IL-6 -174. Kaplan-Meier analysis is shown, with individuals grouped by median of expression of IL-6 for OS and Progression Free Survival rates in (C and D) 88 NBL patients and (E) 102 INSS stage 4 patients with MYCN not amplified.

Discussion.

In this study, a number of GWAS-identified NBL susceptibility loci in Italian NBL patients and healthy controls have been replicated. All SNPs at the BARD1 locus showed a strong association while the association with SNPs in LINC00340, LMO1, DUSP12, HSD17B12, HACE1, LIN28B was more moderate. SNPs at the DDX4 and IL31RA locus showed a trend towards association with NBL, but did not reach statistical significance.

Interestingly, SNP rs7585356 located 3' downstream of BARD1 was found to be an additional and independent risk factor for NBL. These findings strengthen the role of BARD1 as NBL susceptibility gene. Indeed, it has already demonstrated that the genetic variant rs6435862 was strongly associated with high-risk NBL (Capasso et al. 2009) and correlated with high expression of the oncogenic BARD1β isoform (lacking exon 2 and 3), that led to an increased tumorigenicity of NBL cell lines (Bosse et al. 2012). Recently, NBL SNP association at the BARD1 locus has been replicated in African-Americans while limited association has been found at LINC00340 and LMO1 in the same population (Latorre et al. 2012).

In this study it has been demonstrated that rs7585356, independently from rs6435862, was associated with NBL and also influenced full length BARD1 isoform expression in NBL cell lines. Based on these data, it is evident that more than one disease-contributing BARD1 variant may exist.

Moreover, the less frequent genotype of SNP rs7585356 showed a protective role in NBL development and a correlation with increased expression of the full length BARD1 isoform. This suggests an onco-suppressor role of BARD1 in the biology of neuroblastic malignant transformation, as also recently indicated for colon tumorigenesis (Sporn et al. 2011).

The genetic association analysis of clinical NBL phenotypes demonstrated that only BARD1 SNP rs7585356 showed a significant association with advanced INSS stage and age>18 months. This lack of association to the other SNPs is due to insufficient statistical power because of the limited sample size.

These findings indicate that NBL could arise from some as yet unknown combination of relatively common SNPs that can cumulatively increase the risk of a neuroblastic malignant transformation. No combinations of genotypes at the eight SNPs showed evidence of interaction.

One of the major goals of this work was to determine the cumulative effect of these variants on NBL risk demonstrating that although individual susceptibility alleles only moderately increase the risk of NBL, the risk becomes substantial when risk alleles are combined.

The other task of my study was the replication of the association study for a SNP within IL-6 polymorphism. It has been demonstrated that Italian NBL patients homozygous for the C allele had a worse outcome than patients homozygous or heterozygous for the G allele. No association was found when the analysis was restricted to high-risk patients. Moreover, it has been identified a genetic association between IL-6 CC genotype and high-risk phenotype and INSS stage 3 and 4. In contrast, a recent study by Lagmay et al. (2009) reported the genotype GG of the SNP (-174 G>C) in promoter as responsible of a worse outcome in patients with high-risk NBL. Results from previous studies concerning the effect of IL-6 -174 on expression, cancer risk or survival are contradictory (Belluco C et al. 2003; Boiardi L et al. 2006; Bruunsgaard H et al. 2004; Jones KG et al. 2001; Fishman D et al. 1998; Landi S et al. 2003). In this genetic study, compared to that of Lagmay et al. (2009), a greater number of affected children have been analyzed, in addition to a pretty large sample of healthy individuals.

Subsequently, we evaluated the impact of the SNP on IL-6 gene expression in LCLs and NBL patients. The C allele has been described to

correlate with increased IL-6 serum levels (Jones KG et al. 2001; Fishman D et al. 1998). Other studies have associated the C allele with lower IL-6 serum levels (Belluco C et al. 2003; Boiardi L et al. 2006; Bruunsgaard H et al. 2004; Jones KG et al. 2001; Fishman D et al. 1998). My results showed an alteration in IL-6 mRNA expression that results increased in subjects homozygous for the C allele. These findings are intriguing because a strong association between high levels of IL-6 and poor outcome in two gene expression array datasets composed of NBL patients was noted. However, given contradictory results, more investigations are needed to establish the effective genotype of the SNP in IL-6 promoter associated with clinical outcome of NB.

These data suggest a role for the SNP in influencing transcription and synthesis of IL-6 and support the idea that IL-6 could be a marker of NBL progression. Indeed, IL-6 seems to play a role in chemioresistance. Ara et al. (2009) has demonstrated the protective effect of IL-6 on drug-induced apoptosis in NBL. Retinoic acid, currently used for NBL therapy, seems to inhibit cell growth by down-modulation of the expression of IL-6 receptor and the secretion of IL-6 (Sidell N et al. 1991). Moreover, IL-6 pathway can be targeted by the monoclonal antibody Tocilizumab used as therapy for rheumatoid arthritis (Paul-Pletzer K 2006). This suggests the possibility that the targeting of IL-6 pathway could be a therapeutic strategy also for NBL.

Although these findings, a large proportion of the heritability of NBL remains undefined. Future studies will focus on discovering other common and rare disease susceptibility variants and epistatic effects at known risk alleles. Probably, when a greater number of risk variants will be identified a more robust genetic score could be built which may allow subgroups of patients at different risks of developing NBL to be better distinguished.

Conclusions.

Conclusions.

The replication of genetic association findings remains the golden standards for results validation.

This study confirms that the majority of risk loci identified in European American children also affect susceptibility to NBL in Italian children. Moreover, it demonstrates that BARD1 is the most significant genetic contributor to NBL risk, exhibiting two independent risk variants.

This work also shows that the identified NBL susceptibility loci individually have a moderate effect size but when are combined may increase risk of NBL substantially.

Another important result shows that eight independent NBL-associated common genetic variants (rs6939340, rs6435862, rs7585356, rs110419, rs1027702, rs11037575, rs4336470, rs17065417) have been discovered and validated. Moreover, common variation within BARD1 may also influence BARD1 mRNA expression and maybe it influences BARD1 splicing.

This study also supports the role of IL-6 in NBL as marker of disease progression, but additional studies are needed to clarify the role of the SNP rs1800795 (-174 G>C) in predicting the clinical outcome of patients with NBL and to define its function in affecting transcription and synthesis of IL-6. Indeed the biological effect of this SNP in relation to promotion of cancer progression is consistent with the observed decreased survival time. These findings suggest that the SNP IL-6 -174 may be useful marker for NBL prognosis. Given the contradictory results between this and the previous study on SNP IL-6 -174 more independent confirmatory evaluations are needed.

However these findings are just a “small piece” of the whole “puzzle” and further studies are needed. So, my next steps will be the characterization of the role of the SNPs within BARD1. Moreover, I planned to sequence the

whole BARD1 gene in one hundred both somatic and germ line DNA by high-throughput sequencing to define if acquired mutations are associated to NBL.

Another task may be the study of the gene-gene interactions between the predicted interactors of BARD1 (CDK2, BRCA2, PTN, UBE2D3, RBBP8) and BARD1 to understand their role in NBL progression and development.

All these tasks should lead us to better understand the events responsible of NBL progression and development.

Aknowledgements.

Aknowledgments.

Un doveroso e sentito grazie al Prof. Iolascon che mi ha permesso di frequentare il suo gruppo di lavoro permettendomi di imparare tanto.

Un grazie particolare a Mario, che ha supervisionato e supportato il mio intero PhD. Grazie per tutto l'aiuto che mi hai dato e per avermi insegnato cosa vuol dire essere un ricercatore.

Un grazie a tutti i ragazzi del laboratorio: un grazie a Robi Rù, a Imma, a Robi Asci, a Rosaria, a Fara, a Carla, a Concetta, per esserci sempre state quando ne avevo bisogno, per tutte le risate che mi avete fatto fare e per avermi insegnato tutte qualcosa; un grazie a Gina, che mi ha insegnato più di tutti come approcciare a questo lavoro; un grazie a Francesca, a MarySun, a Flora, a Lucia, a Marianna, a Ilaria, a Mariateresa, a Frank, per tutti i bei momenti passati insieme e per essere sempre stati disponibili.

Un grazie ai miei due "apprendisti", Piero e Giovanni, per avermi fatto ridere anche nei miei momenti più tristi e per avermi insegnato ad insegnare qualcosa (spero!). Un grazie a Giovanni per esserci sempre stato per qualunque sfogo, favore, caffè e per tutto l'aiuto che mi hai dato (prima che la bioinformatica ti rapisse). Un grazie a Piero, per essere stato sempre carino nonostante il mio caratteraccio, per cercare di insegnarmi ad essere meno orgogliosa (fatica sprecata), per avermi ascoltata (anche se le cose te le devo ripetere sempre 2 volte), per essere sempre disponibile quando ne ho bisogno e per avermi fatto ricredere su alcune mie convinzioni (avete avuto i vostri ringraziamenti "carini", ora però non vi atteggiate troppo!).

Un grazie ad AntoSnellina perché mi ascolti sempre, non importa cosa tu stia facendo, per me tu ci sei sempre… grazie per supportarmi-sopportarmi e per essermi così amica.

Un grazie a Sandra, che ha condiviso con me gioie e dolori dell'essere una PhD Student e che mi ha sempre spronato a non farmi mettere i piedi in testa.

Un grazie ai miei amici "acquisiti" ma che ormai fanno parte della mia famiglia: Diego, Riccardo, AngeloLuca e Grazia perchè lo so che posso sempre contare su di voi qualunque cosa accada.

Un grazie alle migliori amiche che si possano desiderare: a Silvia, per capirmi al volo soltanto guardandomi; ad Ale, che mi ha insegnato ad avere fiducia in me stessa e a battermi per i miei sogni; a Vale, che mi ha insegnato a credere in me stessa e a non smettere mai di sperare; a Dani, la persona più buona e dolce che io conosca.

Un grazie ai miei cugini, a Cristina, la mia sorella maggiore e a Fabrizio, il mio fratellino... voi riuscite ad incoraggiarmi con poche parole e non ci sono parole per dirvi quanto vi voglio bene.

Un grazie alla famiglia Zadra, a Carmela, a Rossella, a Ermanno, per avermi accolto come parte della famiglia da subito e per non farmi sentire mai fuori posto e un grazie ai tre monelli, Martina, Giovanni e Silvia, per la gioia immensa che mi danno.

Un grazie a Pierpetto, "il cognato preferito" di tutti e mio fratello acquisito, che riesce sempre a farmi ridere quando ne ho bisogno, che ascolta ogni mio sfogo e che è sempre disponibile per tutto.

Un grazie a Claudietta, la mia sorellina, la luce dei miei occhi… tu forse sei quella che più di tutti mi sprona ad inseguire i miei sogni, non credo si possa avere una sorella migliore.

Un grazie ai miei genitori, che mi hanno insegnato che la vita non è sempre facile, che è "tutto un equilibrio sopra la follia", e che è necessario battersi per ciò in cui si crede.

Un grazie a mio padre e a Lorella, per esserci sempre, non importa il dove o l'ora.

Un grazie a mia madre, che crede in me e continua a supportarmi nonostante le difficoltà.

Un grazie ad Enzo, l'amore della mia vita, la mia anima gemella… grazie per amarmi e per avermi insegnato ad amare, per avere fiducia in me, per non smettere mai di sostenermi… sei il mio faro nella tempesta, sempre e per sempre.

References.

References

Ara T, Song L, Shimada H, Keshelava N, Russell HV, Metelitsa LS, Groshen SG, Seeger RC, DeClerck YA. **Interleukin-6 in the bone marrow microenvironment promotes the growth and survival of neuroblastoma cells.** Cancer Res 2009;69:329-337.

Asgharzadeh S, Pique-Regi R, Sposto R, Wang H, Yang Y, Shimada H, Matthay K, Buckley J, Ortega A, Seeger RC. **Prognostic significance of gene expression profiles of metastatic neuroblastomas lacking MYCN gene amplification**. J Natl Cancer Inst 2006;98(17):1193-1203.

Baer R, Ludwig T. **The BRCA1/BARD1 heterodimer, a tumor suppressor complex with ubiquitin E3 ligase activity.** Curr Opin Genet Dev. 2002;12(1):86-91.

Barrett JC, Fry B, Maller J, Daly MJ. **Haploview: analysis and visualization of LD and haplotype maps**. Bioinformatics 2005;21:263-5.

Belluco C, Olivieri F, Bonafè M, Giovagnetti S, Mammano E, Scalerta R, Ambrosi A, Franceschi C, Nitti D, Lise M. **-174 G.C polymorphism of interleukin 6 gene promoter affects interleukin 6 serum level in patients with colorectal cancer.** Clin Cancer Res 2003;9:2173–2176.

Boiardi L, Casali B, Farnetti E, Pipitone N, Nicoli D, Cantini F, Macchioni P, Bajocchi G, Catanoso MG, Pulsatelli L, Consonni D, Salvarani D. **Relationship between interleukin 6 promoter polymorphism at position -174, IL-6 serum levels, and the risk of relapse/recurrence in polymyalgia rheumatica.** J Rheumatol 2006;33:703–708.

Bosse KR, Diskin SJ, Cole KA, Wood AC, Schnepp RW, Norris G, Nguyen le B, Jagannathan J, Laquaglia M, Winter C, Diamond M, Hou C, Attiyeh EF, Mosse YP, Pineros V, Dizin E, Zhang Y, Asgharzadeh S, Seeger RC, Capasso M, Pawel BR, Devoto M, Hakonarson H, Rappaport EF, Irminger-Finger I, Maris JM. **Common variation at BARD1 results in the expression of an oncogenic isoform that influences neuroblastoma susceptibility and oncogenicity.** Cancer Res 2012;72:2068-78.

Bown N, Cotterill S, Lastowska M, O'Neill S, Pearson AD, Plantaz D, Meddeb M, Danglot G, Brinkschmidt C, Christiansen H, Laureys G, Speleman S, Nicholson J, Bernheim A, Betts DR, Vandesompele J, Van Roy N. **Gain of chromosome arm 17q**

and adverse outcome in patients with neuroblastoma. N Engl J Med 1999;340(25):1954-61.

Brodeur GM, Hayes FA, Green AA, Casper JT, Wasson J, Wallach S, Seeger RC. **Consistent N-myc copy number in simultaneous or consecutive neuroblastoma samples from sixty individual patients.** Cancer Res 1987;47(16):4248-53.

Brodeur GM, Pritchard J, Berthold F, Carlsen NL, Castel V, Castelberry RP, De Bernardi B, Evans AE, Favrot M, Hedborg F. **Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment.** J Clin Oncol. 1993;11:1466-77.

Brodeur GM, Seeger RC, Barrett A, Berthold F, Castelberry RP, D'Angio G, De Bernardi B, Evans AE, Favrot M, Freeman AI. **International criteria for diagnosis, staging, and response to treatment in patients with neuroblastoma.** J Clin Oncol. 1988;6:1874-81.

Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM. **Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage.** Science 1984;224(4653):1121-24.

Brodeur GM, Seeger RC. **Gene amplification in human neuroblastomas: basic mechanisms and clinical implications**. Cancer Genet Cytogenet 1986;19(1-2):101-11.

Brodeur GM. **Neuroblastoma: biological insights into a clinical enigma**. Nat Rev Cancer 2003;3(3):203-16.

Bruunsgaard H, Christiansen L, Pedersen AN, Schroll M, Jørgensen T, Pedersen BK. **The IL-6 -174G.C polymorphism is associated with cardiovascular diseases and mortality in 80-year-old humans.** Experimental Gerontology 2004;39:255–261.

Capasso M, Avvisati RA, Piscopo C, Laforgia N, Raimondi F, de Angelis F, Iolascon A. **Cytokine gene polymorphisms in Italian preterm infants: association between interleukin-10 -1082 G/A polymorphism and respiratory distress syndrome**. Pediatr Res. 2007;61(3):313-7.

Capasso M, Devoto M, Hou C, Asgharzadeh S, Glessner JT, Attiyeh EF, Mosse YP, Kim C, Diskin SJ, Cole KA, Bosse K, Diamond M, Laudenslager M, Winter C, Bradfield JP, Scott

RH, Jagannathan J, Garris M, McConville C, London WB, Seeger RC, Grant SF, Li H, Rahman N, Rappaport E, Hakonarson H, Maris JM. **Common variations in BARD1 influence susceptibility to high-risk neuroblastoma.** Nat Genet. 2009;41(6):718-23.

Capasso M, Diskin S, Totaro F, Longo L, De Mariano M, Russo R, Cimmino F, Hakonarson H, Tonini GP, Devoto M, Maris JM, Iolascon A. **Replication of GWAS-identified neuroblastoma risk loci strengthens the role of BARD1 and affirms the cumulative effect of genetic variations on disease susceptibility**. Carcinogenesis 2013.

Caron H, van Sluis P, de Kraker J, Bokkerink J, Egeler M, Laureys G, Slater R, Westerveld A, Voute PA, Versteeg R. **Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma**. N Engl J Med 1996;334(4):225-30.

Cecchetto G, Mosseri V, De Bernardi B, Helardot P, Monclair T, Costa E, Horcher E, Neuenschwander S, Toma P, Rizzo A, Michon J, Holmes K. **Surgical risk factors in primary surgery for localized neuroblastoma: the LNESG1 study of the European International Society of Pediatric Oncology Neuroblastoma Group.** J Clin Oncol 2005; 23(33):8483-89.

Chen Y, Takita J, Choi YL, Kato M, Ohira M, Sanada M, Wang L, Soda M, Kikuchi A, Igarashi T, Nakagawara A, Hayashi Y, Mano H, Ogawa S. **Oncogenic mutations of ALK kinase in neuroblastoma.** Nature 2008;455(7215):971-74.

Cimmino F, Spano D, Capasso M, Zambrano N, Russo R, Zollo M, Iolascon A. **Comparative protomic expression profile in all-trans retinoic acid differentiated neuroblastoma cell line.** J Proteome Res. 2007;6(7):2550-64.

Diskin SJ, Capasso M, Schnepp RW, Cole KA, Attiyeh EF, Hou C, Diamond M, Carpenter EL, Winter C, Lee H, Jagannathan J, Latorre V, Iolascon A, Hakonarson H, Devoto M, Maris JM. **Common variation at 6q16 within HACE1 and LIN28B influences susceptibility to neuroblastoma.** Nat Genet. 2012;44(10):1126-30.

Egler RA, Burlingame SM, Nuchtern JG, Russell HV. **Interleukin-6 and soluble interleukin-6 receptor levels as markers of disease extent and prognosis in neuroblastoma.** Clinical Cancer Research 2008;14:7028–34.

Fishman D, Faulds G, Jeffery R, Mohamed-Ali V, Yudkin JS, Humphries S, Woo P. **The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis**. J Clin Invest 1998;102:1369-76.

Gehring M, Berthold F, Edler L, Schwab M, Amler LC. **The 1p deletion is not a reliable marker for the prognosis of patients with neuroblastoma**. Cancer Res 1995;55(22):5366-69.

Guo C, White PS, Weiss MJ, Hogarty MD, Thompson PM, Stram DO, Gerbing R, Matthay KK, Seeger RC, Brodeur GM, Maris JM. **Allelic deletion at 11q23 is common in MYCN single copy neuroblastomas.** Oncogene 1999;18(35):4948-57.

Gurney JG, Ross JA, Wall DA, Bleyer WA, Severson RK, Robison LL. **Infant cancer in the U.S.: histology-specific incidence and trends, 1973 to 1992**. J Pediatr Hematol Oncol. 1997;19(5):428-32.

Heikkilä K, Ebrahim S, Lawlor DA. **Systematic review of the association between circulating interleukin-6 (IL-6) and cancer**. European Journal of Cancer 2008;44:937-945.

Hirano T. **Interleukin 6**. In: A. Thomson editor, The Cytokine Handbook. London: Academic Press; 1997. p.197-228.

Holm K, Melum E, Franke A, Karlsen TH. **SNPexp - A web tool for calculating and visualizing correlation between HapMap genotypes and gene expression levels**. BMC Bioinformatics 2010;11:600.

Hutchins D, Steel CM. **Regulation of ICAM-1 (CD54) expression in human breast cancer cell lines by interleukin 6 and fibroblast-derived factors**. International Journal of Cancer 1994;58:80-84.

Irminger-Finger I, Leung WC, Li J, Dubois-Dauphin M, Harb J, Feki A, Jefford CE, Soriano JV, Jaconi M, Montesano R, Krause KH. **Identification of BARD1 as mediator between proapoptotic stress and p53-dependent apoptosis.** Mol Cell. 2001;8(6):1255-66.

Janoueix-Lerosey I, Lequin D, Brugieres L, Ribeiro A, de Pontual L, Combaret V, Raynal V, Puisieux A, Schleiermacher G, Pierron G, Valteau-Couanet D, Frebourg T,

Michon J, Lyonnet S, Amiel J, Delattre O. **Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma.** Nature 2008;455(7215):967-70.

Janoueix-Lerosey I, Schleiermacher G, Michels E, Mosseri V, Ribeiro A, Lequin D, Vermeulen J, Couturier J, Peuchmaur M, Valent A, Plantaz D, Rubie H, Valteau-Couanet D, Thomas C, Combaret V, Rousseau R, Eggert A, Michon J, Speleman F, Delattre O. **Overall genomic pattern is a predictor of outcome in neuroblastoma**. J Clin Oncol 2009;27(7):1026-33.

Jefford CE, Feki A, Harb J, Krause KH, Irminger-Finger I. **Nuclear-cytoplasmic translocation of BARD1 is linked to its apoptotic activity.** Oncogene 2004;23(20):3509-20.

Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ, de Bakker PI. **SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap.** Bioinformatics 2008;24:2938-9.

Jones KG, Brull DJ, Brown LC, Sian M, Greenhalgh RM, Humphries SE, Powell JT. **Interleukin-6 (IL-6) and the prognosis of abdominal aortic aneurysms.** Circulation 2001;103:2260–2265.

Kaneko Y, Kanda N, Maseki N, Nakachi K, Takeda T, Okabe I, Sakurai M. **Current urinary mass screening for catecholamine metabolites at 6 months of age may be detecting only a small portion of high-risk neuroblastomas: a chromosome and N-myc amplification study.** J Clin Oncol 1990;8(12):2005-13.

Lagmay JP, London WB, Gross TG, Termuhlen A, Sullivan N, Axel A, Mundy B, Ranalli M, Canner J, McGrady P, Hall B. **Prognostic significance of interleukin-6 single nucleotide polymorphism genotypes in neuroblastoma: rs1800795 (promoter) and rs8192284 (receptor).** Clin Cancer Res 2009;15:5234-5239.

Landi S, Moreno V, Gioia-Patricola L, Guino E, Navarro M, de Oca J, Capella G, Canzian F; Bellvitge Colorectal Cancer Study Group. **Association of common polymorphisms in inflammatory genes interleukin (IL)6, IL8, tumor necrosis factor alpha, NFKB1, Interleukin promoter polymorphisms and prognosis in colorectal cancer and peroxisome proliferator-activated receptor gamma with colorectal cancer.** Cancer Res 2003;63:3560–3566.

Latorre V, Diskin SJ, Diamond MA, Zhang H, Hakonarson H, Maris JM, Devoto M. **Replication of neuroblastoma SNP association at the BARD1 locus in African-Americans.** Cancer Epidemiol Biomarkers Prev 2012;21:658-63.

Linet MS, Ries LA, Smith MA, Tarone RE, Devesa SS. **Cancer surveillance series: recent trends in childhood cancer incidence and mortality in the United States**. J Natl Cancer Inst 1999;91(12):1051-58.

London WB, Castleberry RP, Matthay KK, Look AT, Seeger RC, Shimada H, Thorner P, Brodeur G, Maris JM, Reynolds CP, Cohn SL. **Evidence for an age cutoff greater than 365 days for neuroblastoma risk group stratification in the Children's Oncology Group.** J Clin Oncol 2005;23(27):6459-65.

Look AT, Hayes FA, Shuster JJ, Douglass EC, Castleberry RP, Bowman LC, Smith EI, Brodeur GM. **Clinical relevance of tumor cell ploidy and N-myc gene amplification in childhood neuroblastoma: a Pediatric Oncology Group study.** J Clin Oncol 1991;9(4):581-91.

Maris JM, Hogarty MD, Bagatell R, Cohn SL. **Neuroblastoma**. Lancet 2007;369:2106-20.

Maris JM, Mosse YP, Bradfield JP, Hou C, Monni S, Scott RH, Asgharzadeh S, Attiyeh EF, Diskin SJ, Laudenslager M, Winter C, Cole KA, Glessner JT, Kim C, Frackelton EC, Casalunovo T, Eckert AW, Capasso M, Rappaport EF, McConville C, London WB, Seeger RC, Rahman N, Devoto M, Grant SF, Li H, Hakonarson H. **Chromosome 6p22 locus associated with clinically aggressive neuroblastoma.** N Engl J Med. 2008;358(24):2585-93.

Maris JM, Weiss MJ, Guo C, Gerbing RB, Stram DO, White PS, Hogarty MD, Sulman EP, Thompson PM, Lukens JN, Matthay KK, Seeger RC, Brodeur GM. **Loss of heterozygosity at 1p36 independently predicts for disease progression but not decreased overall survival probability in neuroblastoma patients: a Children's Cancer Group study.** J Clin Oncol 2000;18(9):1888-99.

Martinsson T, Sjoberg RM, Hedborg F, Kogner P. **Deletion of chromosome 1p loci and microsatellite instability in neuroblastomas analyzed with short-tandem repeat polymorphisms.** Cancer Res 1995;55(23):5681-86.

Monclair T, Brodeur GM, Ambros PF, Brisse HJ, Cecchetto G, Holmes K, Kaneko M, London WB, Matthay KK, Nuchtern JG, von Schweinitz D, Simon T, Cohn SL, Pearson AD; INRG Task Force. **The International Neuroblastoma Risk Group (INRG) staging system: an INRG Task Force report**. J Clin Oncol 2009;27(2):298-303.

Mosse YP, Laudenslager M, Khazi D, Carlisle AJ, Winter CL, Rappaport E, Maris JM. Germline **PHOX2B mutation in hereditary neuroblastoma.** Am J Hum Genet 2004;75(4):727-30.

Mossè YP, Laudenslager M, Longo L, Cole KA, Wood A, Attiyeh EF, Laquaglia MJ, Sennett R, Lynch JE, Perri P, Laureys G, Speleman F, Kim C, Hou C, Hakonarson H, Torkmani A, Schork NJ, Brodeur GM, Tonini GP, Rappaport E, Devoto M, Maris JM. **Identification of ALK as a major familial neuroblastoma predisposition gene.** Nature 2008;455(7215):930-35.

Nguyen le B, Diskin SJ, Capasso M, Wang K, Diamond MA, Glessner J, Kim C, Attiyeh EF, Mosse YP, Cole K, Iolascon A, Devoto M, Hakonarson H, Li HK, Maris JM. **Phenotype restricted genome-wide association study using a gene-centric approach identifies three low-risk neuroblastoma susceptibility Loci**. PLoS Genet. 2011;7(3):e1002026.

Oberthuer A, Berthold F, Warnat P, Hero B, Kahlert Y, Spitz R, Ernestus K, Konig R, Haas S, Eils R, Shwab M, Brors B, Westermann F, Fisher M. **Customized oligonucleotide microarray gene expression-based classification of neuroblastoma patients outperforms current clinical risk stratification.** J Clin Oncol 2006;24(31):5070-78.

Oberthuer A, Kaderali L, Kahlert Y, Hero B, Westermann F, Berthold F, Brors B, Eils R, Fischer M. **Subclassification and individual survival time prediction from gene expression data of neuroblastoma patients by using CASPAR**. Clin Cancer Res 2008;14(20):6590-6601.

Ohira M, Oba S, Nakamura Y, Isogai E, Kaneko S, Nakagawa A, Hirata T, Kubo H, Goto T, Yamada S, Yoshida Y, Fuchioka M, Ishii S, Nakagawara A. **Expression profiling using a tumor-specific cDNA microarray predicts the prognosis of intermediate risk neuroblastomas.** Cancer Cell 2005;7(4):337-50.

Olshan AF, De Roos AJ, Teschke K, Neglia JP, Stram DO, Pollock BH, Castelberry RP. **Neuroblastoma and parental occupation**. Cancer Causes Control. 1999;10(6):539-49.

Olshan AF, Smith J, Cook MN, Grufferman S, Pollock BH, Stram DO, Seeger RC, Look AT, Cohon SL, Castelberry RP, Bondy ML. **Hormone and fertility drug use and the risk of neuroblastoma: a report from the Children's Cancer Group and the Pediatric Oncology Group.** Am J Epidemiol. 1999;150(9);930-8.

Osajima-Hakomori Y, Miyake I, Ohira M, Nakagawara A, Nakagawa A, Sakai R. **Biological role of anaplastic lymphoma kinase in neuroblastoma.** Am J Pathol 2005;167(1):213-22.

Paul-Pletzer K. **Tocilizumab: blockade of interleukin 6 signaling pathway as a therapeutic strategy for inflammatory disorders.** Drugs Today (Barc) 2006;42:559-76.

Plantaz D, Vandesompele J, Van Roy N, Lastowska M, Bown N, Combaret V, Favrot MC, Delattre O, Michon J, Benard J, Hartmann O, Nicholson JC, Ross FM, Brinkschmidt C, Laureys G, Caron H, Matthay KK, Feuerstein BG, Speleman F. **Comparative genomic hybridization (CGH) analysis of stage 4 neuroblastoma reveals high frequency of 11q deletion in tumors lacking MYCN amplification.** Int J Cancer 2001;91(5):680-86.

Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC. **PLINK: a tool set for whole-genome association and population-based linkage analyses**. Am J Hum Genet 2007;81:559-75.

Schleiermacher G, Raynal V, Janoueix-Lerosey I, Combaret V, Aurias A, Delattre O. **Variety and complexity of chromosome 17 translocations in neuroblastoma**. Genes Chromosomes Cancer 2004;39(2):143-150.

Schwab M, Alitalo K, Klempnauer KH, Varmus HE, Bishop JM, Gilbert F, Brodeur G, Goldstein M, Trent J. **Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour**. Nature 1983;305(5931):245-48.

Schwab M, Westermann F, Hero B, Berthold F. **Neuroblastoma: biology and molecular and chromosomal pathology.** Lancet Oncol 2003;4(8):472-80.

Seeger RC, Brodeur GM, Sather H, Dalton A, Siegel SE, Wong KY, Hammond D. **Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas.** N Engl J Med 1985;313(18):1111-16.

Sidell N, Taga T, Hirano T, Kishimoto T, Saxon A. **Retinoic acid-induced growth inhibition of a human myeloma cell line via down-regulation of IL-6 receptors.** Journal of Immunology 1991;146:3809–14.

Simons AM, Horwitz AA, Starita LM, Griffin K, Williams RS, Glover JN, Parvin JD. **BRCA1 DNA-binding activity is stimulated by BARD1.** Cancer Res. 2006;66(4):2012-18.

Spitz R, Betts DR, Simon T, Boensch M, Oestreich J, Niggli FK, Ernestus K, Berthold F, Hero B. **Favorable outcome of triploid neuroblastomas: a contribution to the special oncogenesis of neuroblastoma**. Cancer Genet Cytogenet 2006;167(1):51-56.

Spitz R, Hero B, Ernestus K, Berthold F. **Deletions in chromosome arms 3p and 11q are new prognostic markers in localized and 4S neuroblastoma.** Clin Cancer Res 2003;9(1):52-58.

Spitz R, Hero B, Westermann F, Ernestus K, Schwab M, Berthold F. **Fluorescence in situ hybridization analyses of chromosome band 1p36 in neuroblastoma detect two classes of alterations.** Genes Chromosomes Cancer 2002;34(3):299-305.

Spitz R, Oberthuer A, Zapatka M, Brors B, Hero B, Ernestus K, Oestreich J, Fischer M, Simon T, Berthold F. **Oligonucleotide array-based comparative genomic hybridization (aCGH) of 90 neuroblastomas reveals aberration patterns closely associated with relapse pattern and outcome**. Genes Chromosomes Cancer 2006;45(12):1130-42.

Sporn JC, Hothorn T, Jung B. **BARD1 expression predicts outcome in colon cancer**. Clin Cancer Res 2011;17:5451-62.

Totaro F, Cimmino F, Pignataro P, Acierno G, De Mariano M, Longo L, Tonini GP, Iolascon A, Capasso M. **Impact of interleukin-6 promoter polymorphism and gene expression interleukin-6 level on childhood cancer neuroblastoma**. Cancer Epidemiology, Biomarkers & Prevention 2013. [submitted]

Trochet D, Bourdeaut F, Janoueix-Lerosey I, Deville A, de Pontual L, Schleiermacher G, Coze C, Philip N, Frebourg T, Munnich A, Lyonnet S, Delattre O, Amiel J. **Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma**. Am J Hum Genet 2004;74(4):761-64.
van Limpt V, Schramm A, van Lakeman A, Sluis P, Chan A, van Noesel M, Baas F, Caron H, Eggert A, Versteeg R. **The Phox2B homeobox gene is mutated in sporadic neuroblastomas.** Oncogene 2004;23(57):9280-88.

Wang K, Diskin SJ, Zhang H, Attiyeh EF, Winter C, Hou C, Schnepp RW, Diamond M, Bosse K, Mayes PA, Glessner J, Kim C, Frackelton E, Garris M, Wang Q, Glaberson W, Chiavacci R, Nguyen L, Jagannathan J, Saeki N, Sasaki H, Grant SF, Iolascon A, Mosse YP, Cole KA, Li H, Devoto M, McGrady PW, London WB, Capasso M, Rahman N, Hakonarson H, Maris JM. **Integrative genomics identifies LMO1 as a neuroblastoma oncogene**. Nature 2011;469(7329):216-20.

Wang Q, Diskin S, Rappaport E, Attiyeh E, Mosse Y, Shue D, Seiser E, Jagannathan J, Shusterman S, Bansal M, Khazi D, Winter C, Okawa E, Grant G, Cnaan A, Zhao H, Cheung NK, Gerald W, London W, Matthay KK, Brodeur GM, Maris JM. **Integrative genomics identifies distinct molecular classes of neuroblastoma and shows that multiple genes are targeted by regional alterations in DNA copy number.** Cancer Res 2006;66(12):6050-62.

Wei JS, Greer BT, Westermann F, Steinberg SM, Son CG, Chen QR, Whiteford CC, Bilke S, Krasnoselsky AL, Cenacchi N, Catchpoole D, Berthold F, Schwab M, Khan J. **Prediction of clinical outcome using gene expression profiling and artificial neural networks for patients with neuroblastoma**. Cancer Res 2004;64(19):6883-91.

White PS, Maris JM, Beltinger C, Sulman E, Marshall HN, Fujimori M, Kaufman BA, Biegel JA, Allen C, Hilliard C, Valentine MB, Look AT, Enomoto H, Sakiyama S, Brodeur GM. **A region of consistent deletion in neuroblastoma maps within human chromosome 1p36.2-36.3.** Proc Natl Acad Sci U S A 1995;92(12):5520-24.

White PS, Thompson PM, Seifried BA, Sulman EP, Jensen SJ, Guo C, Maris JM, Hogarty MD, Allen C, Biegel JA, Matise TC, Gregory SG, Reynolds CP, Brodeur GM. **Detailed molecular analysis of 1p36 in neuroblastoma.** Med Pediatr Oncol 2001;36(1):37-41.

Yang TP, Beazley C, Montgomery SB, Dimas AS, Gutierrez-Arcelus M, Stranger BE, Deloukas P, Dermitzakis ET. **Genevar: a database and Java application for the analysis and visualization of SNP-gene associations in eQTL studies**. Bioinformatics 2010;26:2474-6.

Original papers.

Carcinogenesis vol.00 no.00 p.1 of 7, 2012
doi:10.1093/carcin/bgs380
Advance Access publication December 7, 2012

Replication of GWAS-identified neuroblastoma risk loci strengthens the role of *BARD1* and affirms the cumulative effect of genetic variations on disease susceptibility

Mario Capasso[1,2,*], Sharon Diskin[3–5], Francesca Totaro[1,2], Luca Longo[6,7], Marilena De Mariano[6,7], Roberta Russo[1,2], Flora Cimmino[1,2], Hakon Hakonarson[4,8,9], Gian Paolo Tonini[7], Marcella Devoto[4,9–11], John M.Maris[3–5] and Achille Iolascon[1,2]

[1]Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli "Federico II", Naples, Italy, [2]CEINGE - Biotecnologie Avanzate, Naples, Italy, [3]Division of Oncology and Center for Childhood Cancer Research, The Children's Hospital of Philadelphia, Philadelphia, PA, [4]Department of Pediatrics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, [5]Abramson Family Cancer Research Institute, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, [6]Terapia Immunologica, IRCCS AOU San Martino-IST, National Cancer Research Institute, Genoa, Italy, [7]Italian Neuroblastoma Foundation, Genoa, Italy, [8]The Center for Applied Genomics, The Children's Hospital of Philadelphia, Philadelphia, PA, [9]Division of Genetics, The Children's Hospital of Philadelphia, Philadelphia, PA, [10]Department of Biostatistics and Epidemiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA and [11]University of Rome "La Sapienza", Department of Molecular Medicine, Rome, Italy

*To whom correspondence should be addressed. Tel: +390813737889; Fax: +390813737804;
Email: capasso@ceinge.unina.it

Several neuroblastoma (NB) susceptibility loci have been identified within *LINC00340*, *BARD1*, *LMO1*, *DUSP12*, *HSD17B12*, *DDX4*, *IL31RA*, *HACE1* and *LIN28B* by genome-wide association (GWA) studies including European American individuals. To validate and comprehensively evaluate the impact of the identified NB variants on disease risk and phenotype, we analyzed 16 single nucleotide polymorphisms (SNPs) in an Italian population (370 cases and 809 controls). We assessed their regulatory activity on gene expression in lymphoblastoid (LCLs) and NB cell lines. We evaluated the cumulative effect of the independent loci on NB risk and high-risk phenotype development in Italian and European American (1627 cases and 2575 controls) populations. All NB susceptibility genes replicated in the Italian dataset except for *DDX4* and *IL31RA*, and the most significant SNP was rs6435862 in *BARD1* ($P = 8.4 \times 10^{-15}$). *BARD1* showed an additional and independent SNP association (rs7585356). This variant influenced *BARD1* mRNA expression in LCLs and NB cell lines. No evidence of epistasis among the NB-associated variants was detected, whereas a cumulative effect of risk variants on NB risk (European Americans: $P_{trend} = 6.9 \times 10^{-30}$, Italians: $P_{trend} = 8.55 \times 10^{13}$) and development of high-risk phenotype (European Americans: $P_{trend} = 6.9 \times 10^{-13}$, Italians: $P_{trend} = 2.2 \times 10^{-1}$) was observed in a dose-dependent manner. These results provide further evidence that the risk loci identified in GWA studies contribute to NB susceptibility in distinct populations and strengthen the role of *BARD1* as major genetic contributor to NB risk. This study shows that even in the absence of interaction the combination of several low-penetrance alleles has potential to distinguish subgroups of patients at different risks of developing NB.

Introduction

Neuroblastoma (NB) is a neuroendocrine tumor, arising from any neural crest element of the sympathetic nervous system. Despite its relative low incidence accounting for <1000 cases in the USA and 150 cases in Italy per year, NB accounts for 15% of childhood cancer mortality (1). Currently, clinical trials stratify patients into four prognostic subgroups with expected very low risk, low risk, intermediate risk, and high risk of death from disease, and 16 pretreatment designations (2). Stratifying patients according to risk subgroups represent an important strategy to choose an appropriate and effective therapy. However, children with severe clinical course and widespread metastases categorized as 'high risk' have survival rates <35% despite aggressive and intensive therapies (1).

Recent genome-wide association studies (GWAS) demonstrated that common single nucleotide polymorphism (SNP) alleles were associated with NB. Some of these SNP associations were particularly enriched in the high-risk group for long intergenic non-protein coding RNA 340 (*LINC00340*) (3), BRCA1-associated RING domain 1 (*BARD1*) (4), and LIM domain only 1 (*LMO1*) (5), and in the low-risk group for dual specificity phosphatase 12 (*DUSP12*), hydroxysteroid (17-beta) dehydrogenase 12 (*HSD17B12*), DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (*DDX4*) and interleukin 31 receptor A (*IL31RA*) (6). Additional SNPs within HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1 (*HACE1*) and lin-28 homolog B (*C. elegans*) (*LIN28B*) have been found to be associated with NB but not with disease phenotype (7). These findings suggested that in NB common variants affect both tumor initiation and malignant progression.

All of the NB GWAS have been performed in European American population samples. Some associated SNPs at *LINC00340* (1), *BARD1* (4), and *LMO1* (5) have been replicated in a North European sample from the UK and in an African American sample (8). It is well known that European Americans form a structured population due to historical immigration from diverse source populations, and this can create false-positive genetic associations (9). Furthermore, among Europeans there is a consistent and reproducible distinction in SNP frequencies between ''northern'' and ''southern'' population groups (10). Based on these observations, it is evident that confirmatory studies are needed through replication in diverse populations to validate the GWAS findings.

In this study, we assessed the association of NB with eight SNPs (rs6939340 and rs4712653 located in *LINC00340*, and rs6435862, rs3768716, rs7585356, rs2070094, rs2229571 and rs1048108 located in *BARD1*) in a southern European population sample composed of 370 cases and 809 controls of Italian origin. In addition, we extended to this larger group the study of rs110419 and rs4758051 (*LMO1*), rs1027702 (*DUSP12*), rs11037575 (*HSD17B12*), rs2619046 (*DDX4*) and rs10055201 (*IL31RA*) already genotyped in a smaller sample (5,6). The results of the Italian case-control study were compared with those of the European American GWAS composed of 1627 cases and 2575 controls (6).

In our previous studies, we demonstrated that rs110419 in *LMO1* (5), rs6435862 in *BARD1* (11), and rs17065417 in *LIN28B* (7) are functional regulatory variants and are involved in NB tumorigenicity. In this study, we tested for SNP–gene expression associations in lymphoblastoid (LCL) and NB cell lines. Finally, we evaluated the cumulative effect of each GWAS identified and replicated genetic variants on NB risk and development of high-risk phenotype, and their potential interactions (epistasis), in both European American and Italian populations.

Materials and methods

Study subjects

This study consisted of 370 NB patients and 809 cancer-free controls of Italian origin. Case subjects were defined as children with a diagnosis of NB or ganglioneuroblastoma and collected through the Italian Neuroblastoma Group. The eligibility criterion for genotyping was the availability of DNA. All

Abbreviations: CI, confidence interval; GWA, genome-wide association; LCLs, lymphoblastoid cell lines; LD, linkage disequilibrium; NB, neuroblastoma; OR, odds ratio; SNPs, single nucleotide polymorphisms.

control subjects were recruited from Italian blood donor centers. Eligibility criteria for control subjects were Italian origin, availability of DNA, no serious underlying medical disorder, including cancer.

In addition, this study included a GWAS dataset of 1627 NB patients registered through the North American-based Children's Oncology Group (COG) and 2575 cancer-free children of self-reported Caucasian ancestry who were recruited and genotyped by the Center for Applied Genomics at the Children's Hospital of Philadelphia (CHOP). European American cases and controls have been described in detail in a previous publication (6). Case subjects were defined as children with a diagnosis of NB or ganglioneuroblastoma and registered through the Children's Oncology Group. The blood samples from the patients with NB were identified through the NB biorepository of the Children's Oncology Group for specimen collection at the time of diagnosis. The eligibility criterion for genome-wide genotyping was the availability of 1.5 µg of DNA of high quality from a tumor-free source such as peripheral blood or bone marrow mononuclear cells that were uninvolved with a tumor. Control subjects were recruited from the Philadelphia region through the Children's Hospital of Philadelphia Health Care Network, including four primary care clinics and several group practices and outpatient practices that included well-child visits. Eligibility criteria for control subjects were European ancestry as determined by self-report or parental report, availability of 1.5 µg of high-quality DNA from peripheral-blood mononuclear cells, and no serious underlying medical disorder, including cancer.

Main clinical and biological characteristics of the patients, including age, stage of the disease (International Neuroblastoma Staging System), and v-myc myelocytomatosis viral related oncogene, neuroblastoma derived amplification status, are shown in Table I. Samples were assigned into two risk groups (not high risk and high risk) based on the Children's Oncology Group risk assignment (1). This study was approved by the Ethics Committee of the Medical University of Naples. The participants of this study gave written informed consent.

SNP selection and genotyping

We selected for genotyping 14 genetic variants that have previously been associated with NB in European American children. We included the two most significant SNPs identified in *LINC00340* (intronic rs6939340 and rs4712653) (1), in *BARD1* (intronic rs6435862 and rs3768716) (4) and in *LMO1* (intronic rs110419 and intergenic rs4758051) (5) as previously reported. In addition, we genotyped the putative functional variation rs7585356 located 3′ downstream of *BARD1*, and its coding variants rs2070094, rs2229571 and rs1048108 (4). We also analyzed four SNPs (intergenic rs1027702 near *DUSP12*, intronic rs11037575 in *HSD17B12*, rs2619046 in *DDX4* and rs10055201 in *IL31RA*) recently found to be associated with low-risk NB (6). The Italian DNA samples were genotyped using SNP Genotyping Assay on 7900HT Real-time PCR system (Applied Biosystems). To monitor quality control, three DNA samples per genotype were genotyped by Sanger sequencing (3730 DNA analyzer, Applied Biosystems) and included in each 384-well reaction plate; genotype concordance was 100%. To confirm genotypes, we sequenced 20 samples chosen randomly from cases and controls; concordance between genotypes was 100%. Primer sequences are available on request. To test NB risk variants cumulative effect, we also used genotype data of rs4336470 (*HACE1*) and rs17065417 (*LIN28B*) already demonstrated to be associated with NB in this Italian case-control sample (7). The European American DNA samples were genotyped using the Illumina Infinium II BeadChip, HumanHap550 v1 and v3 array (Illumina) according to methods detailed elsewhere (3,4). The quality control analyses for this GWAS dataset are described in detail elsewhere (6).

SNP–gene expression correlation analysis

The influence of SNPs on gene expression was evaluated using two web tools, SNPexp v1.2 (http://app3.titan.uio.no/biotools/tool.php?app=snpexp) (12) and Genevar v3.1.0 (http://www.sanger.ac.uk/resources/software/genevar/) (13). SNPexp calculates correlation between HapMap genotypes and gene expression levels in LCLs using linear regression under an additive model. For this analysis, 198 unrelated HapMap3 subjects were chosen. In Genevar, a linear regression (under an additive model) was used to calculate the SNP–gene expression correlations performing a matched co-twin analysis. The expression and genotype data were obtained from LCLs of 170 individuals separate in two sets, Twin 1 (81 subjects) and Twin 2 (89 subjects). This analysis permits immediate replication and validation of the identified SNP–gene expression associations.

Genotyping of NB cell lines

For routine maintenance, cells were grown in RPMI 1640 (Sigma–Aldrich, R8758) complete media or Dulbecco's modified Eagle's medium (Sigma–Aldrich, D6546) complete media containing 10% fetal bovine serum (Sigma–Aldrich, F7524), 1X Penicillin–Streptomycin (Sigma–Aldrich, P0781) and 2 mM L-Glutamine (Sigma–Aldrich, G7513). NB cell lines were genotyped for the *HSD17B12*-SNP rs11037575 and for the *BARD1*-SNP rs7585356. Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI). DNA (50 ng) from NB cell lines was used as a template, and PCR amplicons were generated using Promega PCR Master Mix. The DNA primer sequences were 'rs11037575' *forward*: 5′-GGTGGGTGTGCATTCTTTT-3′; *reverse*: 5′-TACTTGCCTTTGGCCCATTA-3′; and 'rs7585356' *forward*: 5′-GTTCATGAGGAACCAAACTGG-3′; *reverse*: 5′-TGGAGGCAGAAGTTG GTGAT-3′. Each amplicon was isolated on 1% agarose gel and purified using QIAamp purification kit (Qiagen). Finally, each amplicon was sequenced using 3730 DNA analyzer (Applied Biosystems). The NB cell lines and genotypes are reported in the Supplementary Table 1, available at *Carcinogenesis* Online.

Table I. Clinical characteristic of study sample

	Italians	European Americans	
	Cases		
	n = 370	*n* = 1627	*P*[a]
Variable	Number (%)	Number (%)	
Age			
>=18 months	170 (46.8)	744 (52.9)	0.04
<18 months	193 (53.2)	662 (47.1)	
Unknown	7	221	
Sex			
Male	203 (56.1)	865 (53.2)	0.31
Female	159 (43.9)	762 (46.8)	
Unknown	8	—	
INSS Stage			
4	145 (40.2)	605 (43.2)	0.30
1,2,3,4s	216 (59.8)	795 (56.8)	
Unknown	9	227	
MYCN			
Amplified	80 (24.0)	233 (17.3)	0.005
Not amplified	254 (76.0)	1111 (82.7)	
Unknown	36	283	
Risk			
High risk	212 (57.3)	595 (43.7)	0.000003
Not high risk	158 (42.7)	767 (56.3)	
Unknown	—	265	
	Controls		
	n = 809	*n* = 2572	
Variable	Number (%)	Number (%)	
Sex			
Male	458 (56.6)	1325 (51.5)	0.01
Female	351 (42.3)	1247 (48.4)	
Age (mean years; SD)[b]	10.1 (6.4)	9.4 (5.6)	0.002

[a]Statistical comparison of clinical variables between the two case and control groups.
[b]Age data were not available for 359 European Americans

Gene expression of NB cell lines

RNA was isolated from NB cell lines using the TRIzol reagent (Invitrogen Life Technologies). Two hundred nanograms of total RNA was reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative RT–PCR (qRT–PCR) using Power SYBR Green PCR Master Mix (Applied Biosystems) was performed to evaluate the gene expression of *HSD17B12* and *BARD1*. Samples were amplified on an Applied Biosystems 7900HT Sequence Detection System using standard cycling conditions and data collected and analyzed by $2^{-\Delta ct}$ method as described in our previous article (14). β-Actin gene was used as housekeeping gene. Primers overlapping the exon–exon junction were used. Primer sequences were as follows: '*HSD17B12*' *forward*: 5′-TTGTAGATTTCTTCTCTCAGTG-3′; *reverse*: 5′-GCAGGACACTCTGCACAAA-3′; '*BARD1*' *forward*: 5′-GAC AACTGGACAGCATGATTCAA-3′; *reverse*: 5′-TTGTTTCCTGCATCATTA AACAAAC-3′. Primers used for *HSD17B12* and *BARD1* were specifically designed to detect the cDNA full lenght isoforms.

Genotype imputation on chromosome 2q35 data

As SNPs rs7585356, rs2070094, rs2229571 and rs1048108 of *BARD1* were not included in Illumina HumanHap550 arrays, genotype imputation was performed in the European American GWAS dataset using PLINK software v1.06 (15). The HapMap genotypes (release 23) of CEU subjects, downloaded from the HapMap database (http://www.hapmap.org), were used as reference. A total of 1071 SNPs of the 2q35 band included in the arrays were used to perform the imputation analysis.

Statistical analysis

Hardy–Weinberg equilibrium was evaluated using the goodness-of-fit chi-square test in control subjects. Two-sided chi-square tests were used to

evaluate differences in the distributions of allele frequencies between all patients and controls, and only high-risk patients and controls. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to assess the relative disease risk conferred by a specific allele. We defined replication as a $P \leq 0.05$ with a consistent direction of association. We did not correct for multiple tests because the analyzed genes were previously reported associated to NB in multiple independent datasets (3–6,8). Student's *t*-test was used to compare the differences in the mRNA expression levels between NB cell lines stratified according to genotype. Linkage disequilibrium (LD) and haplotype analysis were performed using the web-site tool SNAP v2.2 (http://www.broadinstitute.org/mpg/snap/index.php) (16) and Haploview v4.2 software (17). We assessed the cumulative effects of the independent significant risk loci on NB initiation and phenotype, by counting the number of risk alleles in each subject and modeling the summary variable categorically in logistic regression analysis. The alleles with higher frequency in cases than controls for each SNP were defined as 'risk alleles', and genotypes were coded as 0, 1 or 2 according to the number of risk alleles. Cumulative risk scores were calculated for all SNPs and patients were grouped into categories based on the number of risk alleles 0–7, 8, 9, 10, 11–15 for case-control comparison, and 0–3, 4–5, 6–8 for case-only (high-risk versus not high-risk phenotype comparison). The risk categories were created keeping similar percentage of individuals in each group. For the case-only comparison of high-risk against not high-risk phenotype, we created only three risk categories to have more statistical power as the number of SNPs and patients was lower than in the case-control group. ORs were calculated comparing the groups defined by varying number of risk alleles to the group with the lowest number of risk alleles (0–7 for the case-control analysis and 0–3 for the case-only analysis). A *P* value of ≤0.05 was considered as statistically significant. This analysis was performed using IBM SPSS 19.0 software. Gene–gene interaction was tested using logistic regression additive model by adding an interaction term between the genotypes from all pair of SNPs investigated. This analysis was performed by PLINK software v1.06 (15).

Results

Table I shows the clinical parameters of 370 and 1627 NB patients with Italian and European American origin, respectively. The allele frequencies of all selected genetic variants among cases and controls and their association with NB and high-risk phenotype are shown in Table II and Supplementary Table 2, available at *Carcinogenesis* Online. Because SNPs rs7585356, rs2070094, rs2229571 and rs1048108 were not included in the European American study, we performed genotype imputation at 2q35 (Supplementary Table 3, available at *Carcinogenesis* Online). The most significant imputed SNP was rs17487827, which is in strong LD with genotyped markers rs3768716 and rs6435862 ($r^2 = 0.95$ and 0.60, respectively in 1000 Genomes Project data). The observed genotype frequencies among the control subjects were in agreement with Hardy–Weinberg equilibrium, except for rs2070094 ($P < 0.001$) in Italian children. This SNP was excluded from further analyses. Table III summarizes the characteristics of the studied SNPs.

In the Italian case-control study, significant differences of allele distributions were observed for all SNPs except rs4758051 (*LMO1*), rs2619046 (*DDX4*) and rs10055201 (*IL31RA*). SNP rs6435862 within *BARD1* was the most significant (Table II, $P = 8.4 \times 10^{-15}$). All SNPs showed a similar pattern of association to NB in Italian and European American children. All SNPs of *LINC00340* and *BARD1*, and one of *LMO1* were significantly associated with high-risk NB in both populations (Table II and Supplementary Table 2, available at *Carcinogenesis* Online).

We then tested for SNP–gene expression associations at all 14 SNPs and at 2 previously reported SNPs (7). The analysis of gene expression variation using genome-wide expression arrays from LCLs of 198 unrelated HapMap individuals demonstrated that SNPs rs7585356 (*BARD1*),

Table II. Association of 16 previously identified SNPs with neuroblastoma in Italian children

SNP ID	Allele[a]	Italians					SNP–gene expression associations						
		Case genotypes	High-risk genotypes	Control genotypes	Case MAF	High-risk MAF	Control MAF	P^b	OR[b] (95% CI)	P^c	OR[c] (95% CI)	P HapMap[d]	P Twin1[e]	P Twin2[e]
LINC00340 (rs6939340)	A/G	74/162/103	29/71/48	196/390/175	0.54	0.56	0.49	0.01	1.25 (1.04–1.50)	0.01	1.37 (1.06–1.76)	N/A	N/A	N/A
LINC00340 (rs4712653)	T/C	78/155/108	31/62/57	199/376/197	0.54	0.59	0.50	0.05	1.20 (1.00–1.44)	5.3×10^{-3}	1.43 (1.11–1.83)	N/A	N/A	N/A
BARD1 (rs6435862)	T/G	120/155/69	49/67/33	423/312/49	0.43	0.45	0.26	8.4×10^{-15}	2.10 (1.73–2.53)	1.2×10^{-10}	2.28 (1.76–2.94)	0.50	4.8×10^{-6}	3.0×10^{-4}
BARD1 (rs3768716)	A/G	155/153/50	60/74/20	473/289/45	0.35	0.37	0.23	2.9×10^{-9}	1.78 (1.50–2.16)	6.3×10^{-7}	1.91 (1.48–2.48)	0.16	5.0×10^{-4}	4.0×10^{-4}
BARD1 (rs7585356)	G/A	204/120/19	94/51/7	370/305/94	0.23	0.21	0.32	2.0×10^{-5}	0.63 (0.51–0.78)	2.2×10^{-4}	0.57 (0.43–0.77)	3.0×10^{-4}	1.0×10^{-4}	7.7×10^{-8}
BARD1 (rs2070094)	G/A	219/83/24	103/33/9	419/247/109	0.20	0.18	0.30	1.8×10^{-6}	0.59 (0.47–0.73)	2.0×10^{-5}	0.50 (0.36–0.68)	N/A	N/A	N/A
BARD1 (rs2229571)	G/C	165/124/61	74/53/24	238/363/208	0.35	0.33	0.48	7.1×10^{-9}	0.58 (0.49–0.70)	2.5×10^{-6}	0.54 (0.42–0.70)	N/A	1.0×10^{-3}	6.8×10^{-6}
BARD1 (rs1048108)	C/T	156/98/28	69/39/10	296/355/99	0.27	0.25	0.37	5.0×10^{-5}	0.64 (0.52–0.79)	3.9×10^{-4}	0.57 (0.42–0.78)	N/A	N/A	N/A
LMO1 (rs110419)	G/A	84/152/87	36/62/39	271/370/133	0.50	0.51	0.41	5.0×10^{-5}	1.46 (1.21–1.76)	2.0×10^{-3}	1.50 (1.16–1.94)	0.24	0.03	0.60
LMO1 (rs4758051)	A/G	114/156/70	47/72/23	246/405/141	0.44	0.42	0.43	0.94	1.00 (0.84–1.21)	0.57	0.93 (0.72–1.20)	0.34	0.20	0.22
DUSP12 (rs1027702)	C/T	141/156/52	61/67/22	258/385/128	0.37	0.37	0.42	0.05	0.83 (0.69–1.00)	0.14	0.83 (0.64–1.06)	4.0×10^{-7}	0.42	0.27
HSD17B12 (rs11037575)	T/C	123/168/52	52/74/21	322/379/84	0.40	0.39	0.35	0.03	1.23 (1.02–1.47)	0.13	1.22 (0.94–1.57)	1.1×10^{-8}	7.2×10^{-6}	2.0×10^{-4}
DDX4 (rs2619046)	G/A	180/130/25	78/60/8	456/300/44	0.27	0.26	0.24	0.19	1.15 (0.93–1.41)	0.52	1.10 (0.83–1.46)	0.30	0.24	0.69
IL31RA (rs10055201)	A/G	199/123/20	90/54/7	475/264/36	0.24	0.23	0.22	0.26	1.13 (0.91–1.40)	0.75	1.05 (0.78–1.41)	0.68	0.26	0.41
HACE1 (rs4336470)	C/T	176/136/38	63/68/18	348/329/103	0.30[f]	0.35	[f]0.34	[f]0.06	[f]0.71 (0.52–0.97)	0.84	1.03 (0.79–1.33)	0.02	0.23	0.60
LIN28B (rs17065417)	A/C	298/48/5	126/22/2	600/155/8	0.08[f]	0.09	[f]0.11	[f]0.03	[f]0.83 (0.68–1.00)	0.20	0.75 (0.49–1.16)	N/A	0.92	0.14

MAF, minor allele frequency.
[a]Major/minor Alleles; Italians: 370 cases and 809 controls.
[b]*P* values and ORs from comparison of case versus control.
[c]*P* values and ORs from comparison of high risk versus control.
[d]*P* values from SNP–gene expression correlation using 198 unrelated HapMap subjects (SNPexp web tool).
[e]*P* values from SNP–gene expression correlation using 170 subjects (Genevar web tool).
[f]Data as reported in Diskin *et al.* 2012.
N/A: Not available.

Table III. Features of analyzed SNPs

n	SNP ID	Chromosome band	Function	Allele[a]	Italian dataset SNP type	EA dataset SNP type
1	*LINC00340* (rs6939340)	6p22	—	A/G	Genotyped	Genotyped
2	*LINC00340* (rs4712653)	6p22	—	T/C	Genotyped	Genotyped
3	*BARD1* (rs6435862)	2q35	Intronic	T/G	Genotyped	Genotyped
4	*BARD1* (rs3768716)	2q35	Intronic	A/G	Genotyped	Genotyped
5	*BARD1* (rs7585356)	2q35	3′downstream	G/A	Genotyped	Imputed
6	*BARD1*[b] (rs2070094)	2q35	Exon 6 (Val507Met)	G/A	Genotyped	—
7	*BARD1* (rs2229571)	2q35	Exon 4 (Arg378Ser)	G/C	Genotyped	Imputed
8	*BARD1* (rs1048108)	2q35	Exon 1 (Pro24Ser)	C/T	Genotyped	Imputed
9	*LMO1* (rs110419)	11p15	Intronic	G/A	Genotyped	Genotyped
10	*LMO1* (rs4758051)	11p15	Intergenic	A/G	Genotyped	Genotyped
11	*DUSP12* (rs1027702)	1q23.3	Intergenic	C/T	Genotyped	Genotyped
12	*HSD17B12* (rs11037575)	11p11.2	Intronic	T/C	Genotyped	Genotyped
13	*DDX4* (rs2619046)	5p15.2-p13.1	Intronic	G/A	Genotyped	Genotyped
14	*IL31RA* (rs10055201)	5p15.2-p13.1	Intronic	A/G	Genotyped	Genotyped
15	*HACE1* (rs4336470)	6q16.3	Intronic	C/T	Genotyped[c]	Genotyped
16	*LIN28B* (rs17065417)	6q21	Intronic	A/C	Genotyped[c]	Genotyped

[a]Major/minor alleles. [b]Excluded as not in agreement with Hardy–Weinberg equilibrium.
[c]Already genotyped in Diskin *et al*. 2012.

rs1027702 (*DUSP12*), rs4336470 (*HACE1*) and rs11037575 (*HSD17B12*) affected expression of the respective genes (Table II). These results were confirmed in 170 additional LCLs only for rs7585356 (*BARD1*) and rs11037575 (*HSD17B12*) when we performed a matched co-twin analysis, which permits immediate replication and validation of expression quantitative trait loci discoveries (Table II, Supplementary Figure 1, available at *Carcinogenesis* Online). These analyses demonstrated a high reproducibility of results for these two putative regulatory variants, whereas the other SNPs did not show the same results in different populations. Therefore, a qRT–PCR gene expression analysis was performed only for rs7585356 and rs11037575. The mRNA expression of full length *BARD1* isoform was significantly higher in NB cell lines heterozygous at rs7585356 (AG) (Figure 1a). A trend toward association between high mRNA levels of full length *HSD17B12* isoform and presence of the risk allele C was observed for rs11037575 without reaching the threshold for statistical significance (Figure 1b).

To select SNPs independently associated with NB among the 13 replicated ones, we performed a LD analysis using data from 1000 Genomes Project (http://www.broadinstitute.org/mpg/snap/index.php). SNPs with a $r^2 > 0.10$ with the most significant SNP within each gene were removed (Supplementary Table 4, available at *Carcinogenesis* Online). Consequently, eight SNPs (rs6939340 (*LINC00340*), rs6435862 and rs7585356 (*BARD1*), rs110419 (*LMO1*), rs1027702 (*DUSP12*), rs11037575 (*HSD17B12*), rs4336470 (*HACE1*) and rs17065417 (*LIN28B*) were selected for further analyses. Of note, the LD and haplotype analyses demonstrated that SNPs rs6435862 and rs7585356 of *BARD1* were located in two independent genetic loci (Supplementary Figure 2, available at *Carcinogenesis* Online). To confirm the independence of their association with NB risk, we conducted a logistic regression analysis by adjusting for either of the two SNPs, and found that both SNPs still remained significant in European Americans (rs6435862: $P = 1.8 \times 10^{-10}$, OR = 1.25, 95% CI: 1.11–1.40; rs7585356: $P = 0.04$, OR = 0.88, 95% CI: 0.78–0.99) and Italians (rs6435862: $P = 1.7 \times 10^{-4}$, OR = 1.97, 95% CI: 1.60–2.43; rs7585356: $P = 0.04$, OR = 0.80, 95% CI: 0.64–0.99).

The association of these eight independent risk variants and NB stratified by clinical stage (stage 4 versus not stage 4), MYCN status (amplified versus not amplified), risk assessment (high risk versus not high risk), and age at diagnosis (age ≥18 months and <18 months) was further evaluated among Italian and European American cases. As shown in Table IV, in the European American sample the genetic variants of *LINC00340* (3), *BARD1* (4) and *LMO1* (5) confirmed the association with more clinically aggressive phenotypes, as already demonstrated in the previous articles. In the Italian patients, these SNPs showed the same trend for association, but only rs7585356

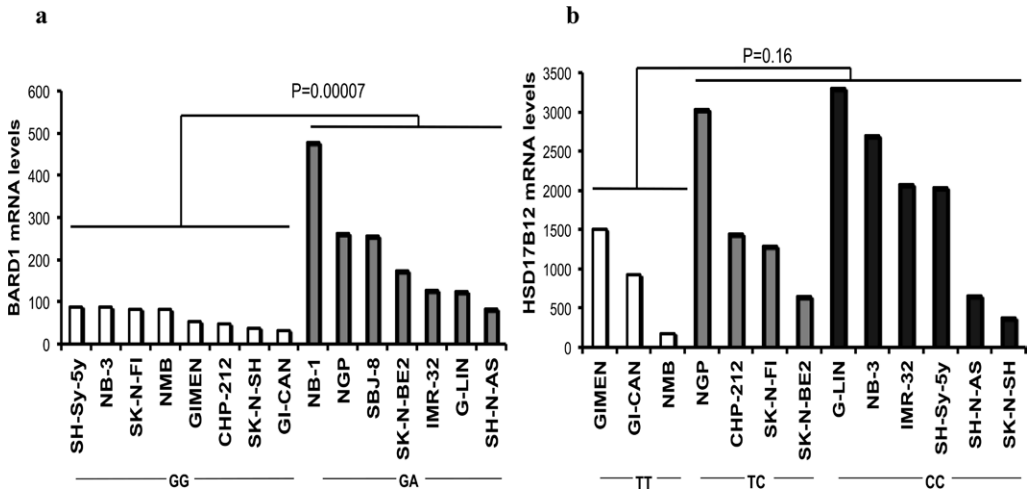

Fig. 1. qRT–PCR analysis to test the SNP–gene expression correlation in neuroblastoma (NB) cell lines. (**a**) mRNA expression of full length *BARD1* isoform in NB cell lines stratified according to rs7585356 genotype. No NB cell lines carried the genotype AA. (**b**) mRNA expression of full length *HSD17B12* isoform in NB cell lines stratified according to rs11037575 genotype.

Table IV. Association of the eight genetic variants with pathologic characteristics of neuroblastoma

SNP ID	Stage 4 N (%)	Not Stage 4 N (%)	*P*	OR (95% CI)	Stage 4 N (%)	Not Stage 4 N (%)	*P*	OR (95% CI)	MYCN Amp N (%)	Not MYCN Amp N (%)	*P*	OR (95% CI)	MYCN Amp N (%)	Not MYCN Amp N (%)	*P*	OR (95% CI)
LINC00340 (rs6939340)	155 (0.56)	206 (0.54)	0.52	1.11 (0.81–1.51)	561 (0.60)	648 (0.54)	$9.0x10^{-4}$	1.29 (1.11–1.51)	89 (0.57)	245 (0.54)	0.44	1.15 (0.80–1.67)	226 (0.62)	924 (0.54)	2.0×10^{-3}	1.38 (1.13–1.70)
BARD1 (rs6435862)	119 (0.43)	169 (0.43)	0.95	1.01 (0.74–1.38)	516 (0.40)	358 (0.30)	$7.2x10^{-8}$	1.54 (1.31–1.80)	75 (0.47)	187 (0.41)	0.17	1.29 (0.90–1.85)	170 (0.41)	644 (0.33)	1.7×10^{-3}	1.39 (1.13–1.70)
[a]*BARD1* (rs7585356)	52 (0.19)	98 (0.25)	0.04	0.67 (0.46–0.98)	729 (0.25)	845 (0.32)	$2.6x10^{-4}$	0.71 (0.58–0.85)	35 (0.23)	106 (0.23)	0.92	1.02 (0.66–1.58)	293 (0.22)	1205 (0.31)	8.3×10^{-4}	0.64 (0.49–0.83)
LMO1 (rs110419)	131 (0.51)	187 (0.50)	0.98	1.01 (0.73–1.38)	576 (0.59)	654 (0.54)	$2.8x10^{-3}$	1.26 (1.46–1.08)	71 (0.49)	230 (0.52)	0.60	0.91 (0.62–1.32)	208 (0.55)	961 (0.56)	0.58	0.94 (0.77–1.16)
DUSP12 (rs1027702)	104 (0.38)	154 (0.38)	0.99	0.99 (0.73–1.37)	528 (0.34)	413 (0.33)	0.56	1.05 (0.89–1.23)	59 (0.37)	179 (0.38)	0.81	0.96 (0.66–1.39)	749 (0.36)	168 (0.33)	0.24	1.13 (0.92–1.40)
HSD17B12 (rs11037575)	106 (0.38)	159 (0.40)	0.56	0.91 (0.66–1.25)	753 (0.43)	524 (0.48)	0.03	0.84 (0.72–0.98)	56 (0.36)	197 (0.42)	0.25	0.80 (0.55–1.17)	1042 (0.44)	206 (0.47)	0.29	0.90 (0.73–1.10)
HACE1 (rs4336470)	91 (0.32)	116 (0.29)	0.31	1.19 (0.85–1.65)	484 (0.29)	351 (0.31)	0.33	0.92 (0.78–1.09)	62 (0.42)	135 (0.28)	0.00	1.92 (1.31–2.82)	667 (0.30)	138 (0.30)	0.78	0.97 (0.78–1.21)
LIN28B (rs17065417)	22 (0.08)	35 (0.09)	0.78	0.92 (0.53–1.61)	133 (0.08)	92 (0.08)	0.46	0.90 (0.68–1.19)	19 (0.13)	36 (0.07)	0.04	1.83 (1.02–3.30)	169 (0.09)	43 (0.08)	0.24	1.23 (0.87–1.75)
SNP ID	High risk, *n* (%)	Not high risk, *n* (%)	*P*	OR (95% CI)	High risk, *n* (%)	Not high risk, *n* (%)	*P*	OR (95% CI)	Age >= 18 months, *n* (%)	Age < 18 months, *n* (%)	*P*	OR (95% CI)	Age >= 18 months, *n* (%)	Age < 18 months, *n* (%)	*P*	OR (95% CI)
LINC00340 (rs6939340)	176 (0.55)	192 (0.53)	0.60	1.08 (0.80–1.47)	556 (0.60)	622 (0.54)	$1.3x10^{-3}$	1.29 (1.10–1.50)	160 (0.55)	203 (0.55)	0.91	1.02 (0.75–1.39)	677 (0.58)	540 (0.54)	0.01	1.21 (1.04–1.40)
BARD1 (rs6435862)	142 (0.44)	151 (0.41)	0.45	1.12 (0.83–1.52)	439 (0.41)	410 (0.31)	$2.1x10^{-7}$	1.52 (1.30 - 1.78)	134 (0.45)	155 (0.41)	0.24	1.20 (0.88–1.64)	516 (0.39)	358 (0.31)	6.4×10^{-5}	1.37 (1.18–1.61)
BARD1[a] (rs7585356)	65 (0.21)	93 (0.24)	0.36	0.84 (0.59–1.21)	722 (0.24)	817 (0.32)	$1.4x10^{-4}$	0.69 (0.57–0.84)	59 (0.19)	93 (0.26)	0.02	0.64 (0.44–0.92)	873 (0.27)	704 (0.32)	5.2×10^{-3}	0.77 (0.64–0.92)
LMO1 (rs110419)	150 (0.51)	176 (0.50)	0.92	1.02 (0.74–1.38)	575 (0.60)	628 (0.53)	$5.9x10^{-4}$	1.31 (1.12–1.52)	140 (0.52)	179 (0.49)	0.25	1.20 (0.88–1.65)	694 (0.59)	539 (0.53)	4.31×10^{-3}	1.24 (1.07–1.44)
DUSP12 (rs1027702)	123 (0.38)	137 (0.37)	0.72	1.06 (0.78–1.44)	517 (0.34)	403 (0.34)	0.86	1.01 (0.86- 1.19)	116 (0.38)	142 (0.34)	0.87	1.03 (0.75–1.40)	438 (0.34)	507 (0.33)	0.58	1.04 (0.89–1.22)
HSD17B12 (rs11037575)	121 (0.38)	151 (0.41)	0.36	0.87 (0.64–1.18)	717 (0.44)	526 (0.47)	0.18	0.90 (0.77- 1.05)	115 (0.38)	153 (0.41)	0.57	0.91 (0.67–1.25)	611 (0.46)	675 (0.46)	0.75	0.97 (0.84–1.13)
HACE1 (rs4336470)	104 (0.35)	108 (0.27)	0.02	1.46 (1.05–2.02)	455 (0.30)	351 (0.30)	0.89	0.98 (0.84–1.17)	104 (0.32)	103 (0.28)	0.30	1.19 (0.86–1.65)	404 (0.30)	440 (0.31)	0.66	0.96 (0.82–1.13)
LIN28B (rs17065417)	26 (0.09)	32 (0.08)	0.74	1.10 (0.64–1.88)	127 (0.07)	89 (0.08)	0.44	0.89 (0.67–1.18)	27 (0.08)	30 (0.08)	0.89	1.04 (0.60–1.79)	105 (0.08)	120 (0.08)	0.90	1.02 (0.77–1.34)

Data from Italian population are colored in grey.
[a]Imputed in the US population.

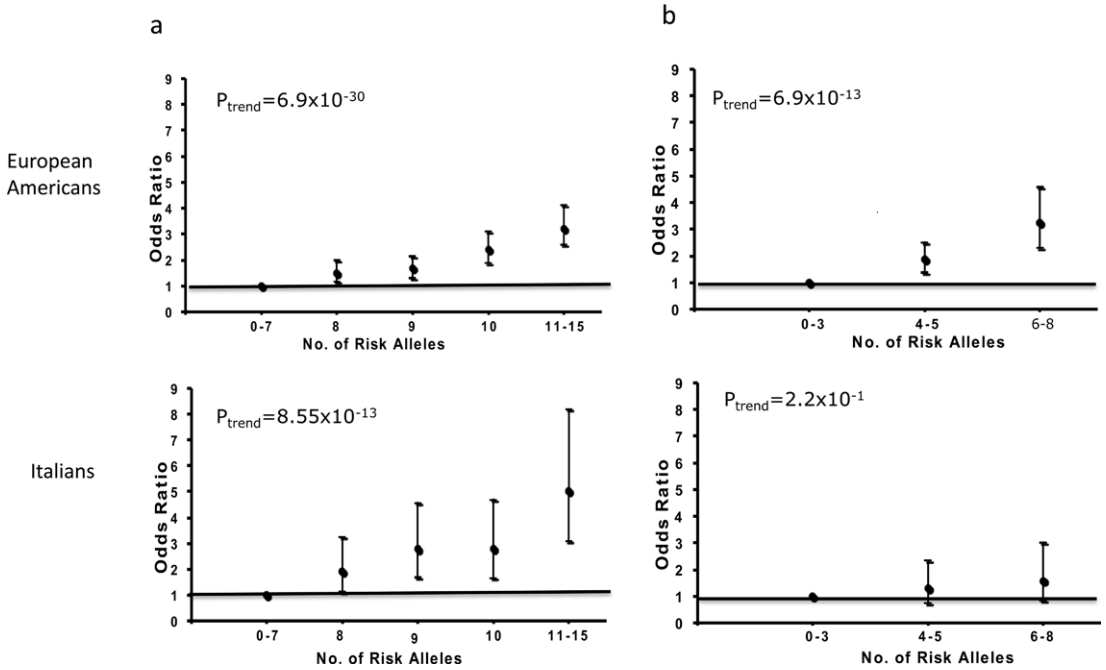

Fig. 2. Odds ratio for (**a**) neuroblastoma patients and (**b**) high-risk patients according to increasing number of risk alleles in Italian and European American cases for the identified risk loci (a) rs6939340, rs6435862, rs7585356, rs110419, rs1027702, rs11037575, rs4336470 and rs17065417; (b) rs6939340, rs6435862, rs7585356 and rs110419). The vertical bars represent 95% confidence intervals. The horizontal line denotes the null value (OR = 1).

resulted to be significantly associated with stage 4 and age ≥18 months ($P = 0.04$, OR = 0.67, 95% CI = 0.46–0.98 and $P = 0.02$, OR = 0.64, 95% CI = 0.44–0.92).

We evaluated potential pairwise interaction effects among the studied SNPs. No evidence of epistasis was detected through this analysis in both populations (Supplementary Table 5, available at *Carcinogenesis* Online). We further investigated the cumulative effects of the eight susceptibility loci on NB development in both populations using as predictor the total number of risk alleles carried by individual subjects. In European Americans, the distribution of risk alleles carried in both cases and controls followed a normal distribution, but with a shift toward a higher number of risk alleles in the cases. This result was confirmed in Italian population (Supplementary Figure 3a, available at *Carcinogenesis* Online). As shown in Figure 2a, European American individuals with multiple risk alleles had higher risk of developing NB compared with those with 0–7 risk alleles of the eight variants, in a dose-dependent manner with increasing number of risk alleles ($P_{trend} = 6.9 \times 10^{-30}$, OR = 1.23, 95% CI: 1.19–1.28). We were able to confirm this result in Italian ($P_{trend} = 8.55 \times 10^{-13}$, OR = 1.35, 95% CI: 1.24–1.46). We also tested for the cumulative effects of the four genetic variants (rs6939340, rs6435862, rs7585356 and rs110419) significantly found to be associated with clinically aggressive NB subgroups in European Americans (Table IV). The distribution of risk alleles carried in both not high-risk and high-risk patients is shown in Supplementary Figure 3b, available at *Carcinogenesis* Online. A shift toward a higher number of risk alleles in high-risk individuals was observed in European Americans but it was less evident in Italians. As shown in Figure 2b, the OR relative to the 0–3 risk alleles group significantly increased with increasing number of risk alleles in European Americans ($P_{trend} = 6.9 \times 10^{-13}$, OR = 1.38, 95% CI: 1.26–1.51). The same trend was confirmed in Italians without reaching the threshold for statistical significance ($P_{trend} = 2.2 \times 10^{-1}$, OR = 1.11, 95% CI: 0.94–1.30).

Discussion

In this study, we set out to replicate a number of GWAS-identified NB susceptibility loci in Italian NB patients and healthy controls. All SNPs at the *BARD1* locus showed a strong association, whereas the association with SNPs in *LINC00340*, *LMO1*, *DUSP12*, *HSD17B12*, *HACE1* and *LIN28B* was more moderate. SNPs at the *DDX4* and *IL31RA* locus showed a trend toward association with NB, but did not reach statistical significance. Interestingly, SNP rs7585356 located 3′ downstream of *BARD1* was found to be an additional and independent risk factor for NB. These findings strengthen the role of *BARD1* as NB susceptibility gene. Indeed, we have already demonstrated that the genetic variant rs6435862 was strongly associated with high-risk NB (4) and correlated with high expression of the oncogenic *BARD1*β isoform (lacking exons 2 and 3), that led to an increased tumorigenicity of NB cell lines (11). Recently, NB SNP association at the *BARD1* locus has been replicated in African Americans, whereas limited association has been found at *LINC00340* and *LMO1* in the same population (8). In this study, we have demonstrated that rs7585356, independently from rs6435862, was associated with NB and also influenced full length *BARD1* isoform expression in NB cell lines. Based on these data, it is evident that more than one disease-contributing *BARD1* variant may exist. Of note, the less frequent genotype of SNP rs7585356 showed a protective role in NB development and a correlation with increased expression of the full length *BARD1* isoform. This suggests an oncosuppressor role of *BARD1* in the biology of neuroblastic malignant transformation, as also recently indicated for colon tumorigenesis (18). Given the complex structure of *BARD1* that displays diverse domains (RING, ANK and BRCT), this gene has been indicated to have multiple functions (19). These functions might be regulated by the expression of differentially spliced isoforms. So, it is reasonable to hypothesize that common and rare variants might affect the expression of distinct *BARD1* isoforms that in turn might have tumor suppression and oncogenic functions in NB. Further studies are needed to address this hypothesis.

Our genetic association analysis of clinical NB phenotypes confirmed that four SNPs at *LINC00340* (rs6939340), *BARD1* (rs6435862 and rs7585356) and *LMO1* (rs110419) conferred an increased risk for high-risk phenotype in European American patients. In Italian patients, only *BARD1* SNP rs7585356 showed a significant association with advanced INSS stage and age older than 18 months. We hypothesize that this lack of association to the other SNPs is due to insufficient statistical power because of the limited sample size. Indeed, the trend toward association with clinically aggressive phenotypes was the same for all four variants in European American and Italian patients.

This article shows that eight independent NB-associated common genetic variants (rs6939340, rs6435862, rs7585356, rs110419, rs1027702, rs11037575, rs4336470, rs17065417) have been validated in an Italian population. These findings indicate that NB could arise from some as yet unknown combination of relatively common SNPs that can cumulatively increase the risk of a neuroblastic malignant transformation in fetal or early childhood development. No pairwise combinations of genotypes at the eight SNPs showed evidence of interaction, defined as deviation from additivity of allelic effects at separate loci. One of the major goals of this work was to determine the cumulative effect of these variants on NB risk. Performing separate genetic analyses in two independent populations, we have demonstrated that although individual susceptibility alleles only moderately increase the risk of NB, the risk becomes substantial when risk alleles are combined. When we tested for their cumulative effect, we found that children carrying 10–15 risk alleles had a 3.2-fold for European Americans and 5-fold for Italians increased risk of developing NB compared with those who carried 0–7 risk alleles, indicating the importance of the combined independent risk loci in neuroblast carcinogenesis. We observed similar results when the same analysis was performed on high-risk patients using the four genetic variants associated with clinically aggressive phenotypes (rs6939340, rs6435862, rs7585356 and rs110419). Indeed, European American children with NB carrying 6–8 risk alleles had 3.3-fold increased risk of developing high-risk NB compared with those who carried 0–3 risk alleles. In Italian patients the same risk was increased 1.6-fold, but it did not reach statistical significance.

One limitation of our study is the relative small number of Italian cases and controls. This probably affects some of the observed results. For instance, we speculate that the lack of association between some of SNPs and clinical conditions and the not statistically significant result of the risk allele cumulative analysis of high-risk patients are probably due to the limited power of the sample. However, in both analyses the trend toward association was similar in both Italian and European American populations. Surely, additional confirmatory studies are needed in larger populations with diverse origins to further validate the hypothesis that heritable DNA variation influences the clinical course of the disease.

Our study suggests that the heritable DNA variation that influences the initiation of NB and the clinical course of the disease has the potential to predict disease and more interestingly to predict high-risk NB. However, a large proportion of the heritability of NB remains undefined. Future studies will focus on discovering other common and rare disease susceptibility variants and epistatic effects at known risk alleles. We hypothesize that when a greater number of risk variants will be identified a more robust genetic score could be built, which may allow subgroups of patients at different risks of developing NB to be better distinguished. Several findings have demonstrated that NB is a type of tumor genetically and phenotypically heterogeneous (20). We think that the discriminatory power of combined risk alleles analysis will improve with time because the number of reproducibly associated variants is likely to increase rapidly with increasing number of cases and controls, as we have already demonstrated in our recent articles (5,7). We have planned to increase prominently the number of cases from three diverse populations (European Americans, Italians and British individuals) within the next 5 years through a multicentric collaborative project. A genetic risk score that includes more risk alleles could be relevant in improving the current risk models of NB adding more prediction power to the well-known clinical and genetic markers such as age, INSS stage, MYCN status. However, to assess the applicability of SNPs in the risk classification of NB, large prospective cohort studies will be needed.

In summary, our study confirms that the majority of risk loci identified in European American children also affect susceptibility to NB in Italian children and demonstrates compelling evidence of *BARD1* as the most significant genetic contributor to NB risk, exhibiting two independent risk variants. This work also shows that the identified NB susceptibility loci individually have a moderate effect size but when combined may increase risk of NB substantially.

Supplementary material

Supplementary Tables 1–5 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

Funding

Associazione Italiana per la Ricerca sul Cancro (10537), MIUR- FIRB Ricerca in Futuro (RBFR08DWQ3 to M.C.); Fondazione Italiana per la Lotta al Neuroblastoma (to M.C., F.C., L.L., M.D. and G.P.T.); Associazione Oncologia Pediatrica e Neuroblastoma (to M.C. and F.C.).

Conflict of Interest Statement: None declared.

References

1. Maris,J.M. (2010) Recent advances in neuroblastoma. *N. Engl. J. Med.*, **362**, 2202–2211.
2. Cohn,S.L. *et al.* (2009) The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. *J. Clin. Oncol.*, **27**, 289–297.
3. Maris,J.M. *et al.* (2008) Chromosome 6p22 locus associated with clinically aggressive neuroblastoma. *N. Engl. J. Med.*, **358**, 2585–2593.
4. Capasso,M. *et al.* (2009) Common variations in BARD1 influence susceptibility to high-risk neuroblastoma. *Nat. Genet.*, **41**, 718–723.
5. Wang,K. *et al.* (2011) Integrative genomics identifies LMO1 as a neuroblastoma oncogene. *Nature*, **469**, 216–220.
6. Nguyen,l.e.B. *et al.* (2011) Phenotype restricted genome-wide association study using a gene-centric approach identifies three low-risk neuroblastoma susceptibility Loci. *PLoS Genet.*, **7**, e1002026.
7. Diskin,S.J. *et al.* (2012) Identification of a new neuroblastoma susceptibility locus at 6q16 implicating HACE1 and LIN28B. *Nat. Genet.*, **44**, 1126–1130.
8. Latorre,V. *et al.* (2012) Replication of neuroblastoma SNP association at the BARD1 locus in African-Americans. *Cancer Epidemiol. Biomarkers Prev.*, **21**, 658–663.
9. Price,A.L. *et al.* (2008) Discerning the ancestry of European Americans in genetic association studies. *PLoS Genet.*, **4**, e236.
10. Seldin,M.F. *et al.* (2006) European population substructure: clustering of northern and southern populations. *PLoS Genet.*, **2**, e143.
11. Bosse,K.R. *et al.* (2012) Common variation at BARD1 results in the expression of an oncogenic isoform that influences neuroblastoma susceptibility and oncogenicity. *Cancer Res.*, **72**, 2068–2078.
12. Holm,K. *et al.* (2010) SNPexp: a web tool for calculating and visualizing correlation between HapMap genotypes and gene expression levels. *BMC Bioinformatics*, **11**, 600.
13. Yang,T.P. *et al.* (2010) Genevar: a database and Java application for the analysis and visualization of SNP-gene associations in eQTL studies. *Bioinformatics*, **26**, 2474–2476.
14. Capasso,M. *et al.* (2007) Cytokine gene polymorphisms in Italian preterm infants: association between interleukin-10 -1082 G/A polymorphism and respiratory distress syndrome. *Pediatr. Res.*, **61**, 313–317.
15. Purcell,S. *et al.* (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.*, **81**, 559–575.
16. Johnson,A.D. *et al.* (2008) SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap. *Bioinformatics*, **24**, 2938–2939.
17. Barrett,J.C. *et al.* (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics*, **21**, 263–265.
18. Sporn,J.C. *et al.* (2011) BARD1 expression predicts outcome in colon cancer. *Clin. Cancer Res.*, **17**, 5451–5462.
19. Irminger-Finger,I. *et al.* (2006) Is there more to BARD1 than BRCA1? *Nat. Rev. Cancer*, **6**, 382–391.
20. Capasso,M. *et al.* (2010) Genetics and genomics of neuroblastoma. *Cancer Treat. Res.*, **155**, 65–84.

Received August 14, 2012; revised November 9, 2012; accepted November 30, 2012

Impact of interleukin-6 promoter polymorphism and gene expression interleukin-6 level on childhood cancer neuroblastoma

Francesca Totaro[1,2], Flora Cimmino[1,2], Piero Pignataro[2], Giovanni Acierno[1,2], Marilena De Mariano[3,4], Luca Longo[3,4], Gian Paolo Tonini[5], Achille Iolascon[1,2], Mario Capasso[1,2]

[1]Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli "Federico II", Naples, Italy

[2]CEINGE - Biotecnologie Avanzate, Naples, Italy

[3]Translational Oncopathology, IRCCS AOU San Martino-IST, National Cancer Research Institute, Genova, Italy

[4]Italian Neuroblastoma Foundation, IRCCS AOU San Martino-IST, National Cancer Research Institute, Genova, Italy

[5]Laboratory of Neuroblastoma, Onco/Hematology Department SDB University of Padua, Pediatric Research Institute, Padua, Italy.

Corresponding author: Mario Capasso, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli "Federico II", CEINGE Biotecnologie Avanzate, Via G. Salvatore 482, Naples 80145, Italy. Tel: +390813737889; Fax: +390813737804, Email: capasso@ceinge.unina.it

Running Title: SNP *IL-6* -174 G>C and neuroblastoma outcome

Conflict interest statement: None declared.

Keywords: SNP, *IL-6* , neuroblastoma

Abstract

Background: Common variants in DNA may predispose to neuroblastoma (NB) onset and progression. The genotype GG of single nucleotide polymorphism (SNP) rs1800795 (-174G>C) in interleukin(IL)-6 promoter has been associated with lower survival in high-risk NB.

Methods: To validate and evaluate the impact of *IL-6* SNP rs1800795 on disease risk and phenotype, we analyzed an Italian population of 326 NB patients and 511 controls. We performed *in silico* and qRT-PCR analyses to evaluate the influence of the SNP on gene expression in 198 lymphoblastoid cell lines (LCLs) and in 31 NB tumors, respectively. Kaplan-Meier analysis was used to verify if *IL-6* gene expression is associated with patient survival.

Results: The SNP was not implicated in susceptibility to NB development. The results indicated instead the minor frequent genotype CC as significantly associated with a reduction of the overall survival, advanced stage, and high-risk phenotype. The *in silico* analyses (p=2.61x10^{-5}) and the qRT-PCR (p=0.27) showed similar trend indicating the genotype CC as correlated with increased level of *IL-6* expression. Kaplan-Meier analysis demonstrated that high levels of *IL-6* were associated with poor outcome of disease in two independent gene expression array datasets.

Conclusions: The biological effect of this SNP in relation to promotion of cancer progression is consistent with the observed decreased survival time.

Impact: This study suggests that the SNP *IL-6* -174 may be useful marker for NB prognosis. Given the contradictory results between this and the previous study on SNP IL-6 -174 more independent confirmatory evaluations are needed.

Introduction

Neuroblastoma (NB) is a neuroendocrine tumor, arising from any neural crest element of the sympathetic nervous system. NB accounts for more than 7% of malignancies in children and around 15% of pediatric oncologic deaths (1). In the last years we have demonstrated that common DNA variants located in diverse genes (*LINC00340*, *BARD1*, *LMO1*, *DUSP12*, *HSD17B12*, *DDX4*, *IL31RA*, *HACE1* and *LIN28B*) predispose to NB onset and progression by genome-wide association studies (GWAS) (2-8). These findings suggested that NB tumorigenesis could be the result of different genetic alterations and some these alterations could also influence the clinical outcome of disease.

Interleukin (IL)-6 is a pro-inflammatory cytokine that plays an active role in neoplasia, bone metabolism and iron homeostasis. Recent studies have demonstrated a role for *IL-6* in progression and development of multiple myeloma, colon cancer, melanoma, renal cancer, Hodgkin's disease, non-Hodgkin's lymphoma, prostate cancer, breast cancer (9). The increase of *IL-6* has been associated to the up-regulation of the expression of adhesion receptors on endothelial cells and to the production of growth factors. These events promoted the formation of favorable microenvironment for metastasis and suggested a role for IL-6 as a stimulator of metastasis (10-11). Moreover, it has been demonstrated that the peripheral blood levels of *IL-6* correlated with NB progression and development (12) and that *IL-6* promoted growth and survival of NB cells in the bone marrow (13). Interestingly, the studies by Lagmay et al. (14) reported the SNP *rs1800795* (-174 G>C) in *IL-6* promoter as associated with inferior clinical outcomes in patients affected by high-risk NB. Replication of genetic association findings remains the golden standards for results validation. In this study, we evaluate whether the association of the germ-line polymorphism *rs1800795* of *IL-6* with NB survival is confirmed in an Italian population. We also verify if this SNP is associated with NB initiation. Finally, we tested for SNP-gene expression association in

lymphoblastoid cell lines (LCLs) and in NB patients, and for the influence of *IL-6* gene expression on patient survival.

Materials and methods

DNA samples. The study consisted of 326 DNAs extracted from peripheral blood of NB patients collected through the Italian Neuroblastoma Group and 511 DNAs from cancer-free controls of Italian origin (mean age 9.67 ± 5.41 year). Clinical and biologic characteristics of the patients are shown in Table 1. Samples were assigned into two risk groups (not high-risk and high-risk) based on the COG risk assignment (1). Tumor specimens were collected at the onset of disease from 31 patients who were diagnosed with a primary NB between 1990 and 2006, and referred to the Gaslini Children Hospital, Genoa, Italy. The characteristics of tumors are reported in Supplementary Table S1. This study was approved by the Ethics Committee of the Medical University of Naples and informed consent was obtained by all children's legal guardians.

rs1800795 (-174 G>C) genotyping. DNA samples were screened for the SNP rs1800795 using RFLP mapping to identify each patient's genotype. The same RFLP mapping strategy described by Lagmay et al. (14) was used. Briefly, the method was PCR based and used primers that flank the SNP locus to produce an amplicon of 305 bp. The PCR products were gel purified on 2% agarose and digested with the DNA restriction endonuclease Nla-III. The DNA primer sequences used were as follows: forward-*ATGCCAAGTGCTGAGTCACTA*; reverse-*TCGAGGGCAGAATGAGCCTC*. For quality control, 10% of randomly selected samples containing both cases and controls were analyzed at second time by Sanger sequencing (3730 DNA analyzer, Applied Biosystems) without finding any discrepancies. Genomic DNA from NB and control samples was isolated using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI).

In silico SNP-gene expression correlation analysis. The influence of the SNP on gene expression was evaluated using a web tool, SNPexp v1.2 (http://app3.titan.uio.no/biotools/tool.php?app=snpexp) (15) as performed in our previous study (4).

SNPexp calculates correlation between HapMap genotypes and gene expression levels in LCLs using linear regression. For this analysis, 198 unrelated HapMap3 subjects were chosen.

In silico analysis of IL-6 gene expression-outcome correlation. Two independent sets of normalized gene expression data and clinical annotations were downloaded from the website R2, microarray analysis and visualization platform (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi):

1) Versteeg dataset composed of 88 NB samples;
2) Seeger dataset composed of 102 NB samples.

IL-6 gene expression analysis in primary tumors. RNA from 31 NB tissues using the TRIzol reagent (Invitrogen Life Technologies). Two thousand ng of total RNA was reverse transcribed into cDNA using iScript cDNA Synthesis Kit (BIORAD). To evaluate the gene expression of *IL-6*, quantitative real time PCR (qRT-PCR) was performed using Power SYBR Green Master Mix (Applied Biosystems). Samples were amplified on an Applied Biosystems 7900HT Sequence Detection System using standard cycling conditions and data were collected and analyzed by $2^{\wedge-\Delta ct}$ method as described in our previous paper (16). *HPRT* was used as housekeeping gene. Primers overlapping the exon-exon junction were used (*IL-6* forward-*TCTCCACAAGCGCCTTCGGT, IL-6* reverse-*TGGGGCAGGGAAGGCAGC; HPRT* for: *TGACACTGGCAAAACAATGCA, HPRT* rev: *GGTCCTTTTCACCAGCAAGCT*). The primers for *IL-6* were specific for the full length isoform.

Statistical analysis. Hardy-Weinberg equilibrium was evaluated using the goodness-of-fit chi-square test in control subjects. Two-sided chi-square tests were used to test for associations of the existence of polymorphism versus each other clinical factor, all patients, and only high-risk patients. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to assess the disease risk conferred by a specific allele or genotype. Overall Survival (OS) was calculated by Kaplan-Meier method to generate survival curves which were compared using a log-rank test. The Cox regression model was used to test for the independent predictive ability of the SNP after the adjustment for other significant factors: *MYCN* amplification, age, INSS stages. *P*-values of ≤ 0.05 were considered statistically significant.

Results

IL-6 SNP genotyping. Allele frequencies for rs1800795 for NB patients and controls are shown in Table 1. The distribution of the genotypes was in agreement with Hardy–Weinberg equilibrium. The genotype CC was more significantly frequent in high-risk (p=0.03) and stage 3 and 4 patients (p=0.02). No significant association was found with NB development.

Impact of the IL-6 SNP on OS. We examined OS of the genotyped patients samples to determine if the *IL-6* SNP was predictive of NB outcome. *IL-6* SNP showed statistically significant differences ($p < 0.05$) in predicting OS. Patients that were homozygous for the C allele had worse survival than patients who were homozygous and heterozygous for the G allele in all patients (Figure 1A) and not *MYCN* amplified (Figure 1B) patient sub-group. OS 5 years from the date of diagnosis was 85.2% (95% CI 0.83-0.87) for the group of patients carrying GG and GC genotype, compared with 67.2% (95% CI 0.55-0.79) for the group of patients carrying CC genotype (Figure 1A). This genetic association was independent from *MYCN* status, age and INSS stage (Supplementary Table S2). A similar trend, without reaching the significance, was observed when the analysis was restricted to high-risk patients (Figure 1C and 1D).

SNP-gene expression analysis. The *in silico* analysis of LCLs demonstrated that the CC genotype correlated with high level of *IL-6* expression (p=2.61x10^{-5}, Figure 2A). qRT-PCR of mRNAs from tumor specimens was performed. The levels of *IL-6* mRNA were higher in CC carriers than in GG and GC carriers (p=0.27, Figure 2B) without reaching the significant threshold.

IL-6 gene expression-outcome correlation. When we verified the association of *IL-6* gene expression with outcome disease in two independent sets of patients, we found that the increase of the mRNA expression was significantly associated with lower OS and progression of disease (Figure 2C, 2D, and 2E).

Discussion

Our recent findings have suggested that NB tumorigenesis could be the result of different genetic alterations that might also influence the clinical outcome of the disease (1-8). *IL-6* is crucial cytokine involved in several cellular pathways (9). Peripheral blood and bone marrow *IL-6* levels have been found to be elevated in patients with high-risk NB when compared with those with low and intermediate risk NB (12). We found that Italian NB patients homozygous for the C allele had a worse outcome than patients homozygous or heterozygous for the G allele. We found no association when the analysis was restricted to high-risk patients. We also identified a genetic association between *IL-6* CC genotype and high-risk phenotype and INSS stage 3/4. In contrast, a recent study by Lagmay et al. (14) reported the genotype GG of the SNP (-174 G>C) in promoter as responsible of a worse outcome in patients with high-risk NB. Results from previous studies concerning the effect of *IL-6* -174 on expression, cancer risk or survival are contradictory (17-22). In this genetic study compared to that of Lagmay et al. (14), we have analyzed a greater number of affected children, in addition to a pretty large sample of healthy individuals. The present study was particularly suitable for searching genetic association with NB as this Italian sample has been successfully used to validate GWAS-identified NB risk variants (4, 5, 7, 8). Moreover, we evaluated the impact of the SNP on *IL-6* gene expression in LCLs and NB patients. The C allele has been described to correlate with increased *IL-6* serum levels (20-21). Other studies have associated the C allele with lower *IL-6* serum levels (17-21). Our results showed an alteration in *IL-6* mRNA expression that results increased in subjects homozygous for the C allele. These findings are intriguing as we also found a strong association between high levels of *IL-6* and poor outcome in two gene expression array datasets composed of NB patients. However, given contradictory results, more investigations are needed to establish the effective genotype of the SNP *IL-6* -174 associated with clinical outcome of NB.

These data suggest a role for the SNP in influencing transcription and synthesis of *IL-6* and support the idea that *IL-6* could be a marker of NB progression. Indeed, *IL-6* seems to play a role in

chemioresistance. Ara et al. has demonstrated the protective effect of *IL-6* on drug-induced apoptosis in NB (13). Retinoic acid, currently used for NB therapy, seems to inhibit cell growth by down-modulation of the expression of *IL-6* receptor and the secretion of *IL-6* (23). Moreover, *IL-6* pathway can be targeted by the monoclonal antibody Tocilizumab used as therapy for rheumatoid arthritis (24). This suggests the possibility that the targeting of *IL-6* pathway could be a therapeutic strategy also for NB.

This article supports the role of *IL-6* in NB as marker of disease progression, but additional studies are needed to clarify the role of the SNP *rs1800795* (-174 G>C) in predicting the clinical outcome of patients with NB and to define its function in affecting transcription and synthesis of *IL-6*.

Grant Support

This study was supported by grants from Associazione Italiana per la Ricerca sul Cancro (10537), MIUR- FIRB Ricerca in Futuro (RBFR08DWQ3), Fondazione Italiana per la Lotta al Neuroblastoma and Associazione Oncologia Pediatrica e Neuroblastoma.

References

1. Capasso M, Diskin SJ. Genetics and genomics of neuroblastoma. Cancer Treat Res 2010; 155:65–84.
2. Maris JM, Mosse YP, Bradfield JP, Hou C, Monni S, Scott RH, et al. Chromosome 6p22 locus associated with clinically aggressive neuroblastoma. N Engl J Med 2008;358:2585-93.
3. Capasso M, Devoto M, Hou C, Asgharzadeh S, Glessner JT, Attiyeh EF, et al. Common variations in *BARD1* influence susceptibility to high-risk neuroblastoma. Nature Genetics 2009;41:718-23.
4. Wang K, Diskin S, Zhang H, Attiyeh EF, Winter C, Hou C, et al. Integrative genomics identifies LMO1 as a neuroblastoma oncogene. Nature 2011;469:216-20.
5. Nguyen LB, Diskin S, Capasso M, Wang K, Diamond MA, Glessner J, et al. Phenotype restricted genome-wide association study using a gene-centric approach identifies three low-risk neuroblastoma susceptibility loci. PLOS Genetics 2011;7:e1002026.
6. Bosse KR, Diskin S, Cole KA, Wood AC, Schnepp RW, Norris G, et al. Common variation at BARD1 results in the expression of an oncogenic isoform that influences neuroblastoma susceptibility and oncogenicity. Cancer Research 2012;72:2068-78.
7. Diskin SJ, Capasso M, Schnepp RW, Cole KA, Attiyeh EF, Hou C, et al. Common variation at 6q16 within HACE1 and LIN28B influences susceptibility to neuroblastoma. Nature Genetics 2012;44:1126-30.
8. Capasso M, Diskin S, Totaro F, Longo L, Mariano MD, Russo R, et al. Replication of GWAS-identified neuroblastoma risk loci strengthens the role of BARD1 and affirms the cumulative effect of genetic variations on disease susceptibility. Carcinogenesis 2013 (in press).
9. Heikkilä K, Ebrahim S, Lawlor DA. Systematic review of the association between circulating interleukin-6 (IL-6) and cancer. European Journal of Cancer 2008;44:937-945.

10. Hirano T. Interleukin 6. In: A. Thomson editor, The Cytokine Handbook. London: Academic Press; 1997. p.197-228.
11. Hutchins D, Steel CM. Regulation of ICAM-1 (CD54) expression in human breast cancer cell lines by interleukin 6 and fibroblast-derived factors. International Journal of Cancer 1994;58:80-84.
12. Egler RA, Burlingame SM, Nuchtern JG, Russell HV. Interleukin-6 and soluble interleukin-6 receptor levels as markers of disease extent and prognosis in neuroblastoma. Clinical Cancer Research 2008;14:7028–34.
13. Ara T, Song L, Shimada H, Keshelava N, Russell HV, Metelitsa LS, et al. Interleukin-6 in the Bone Marrow Microenvironment Promotes the Growth and Survival of Neuroblastoma Cells. Cancer Research 2009;69:329-337.
14. Lagmay JP, London WB, Gross TG, Termuhlen A, Sullivan N, Axel A, et al. Prognostic significance of interleukin-6 single nucleotide polymorphism genotypes in neuroblastoma: rs1800795 (promoter) and rs8192284 (receptor). Clinical Cancer Research 2009;15:5234-5239.
15. Holm K, Melum E, Franke A, Karlsen TH. SNPexp-A web tool for calculating and visualizing correlation between HapMap genotypes and gene expression levels. BMC Bioinformatics 2010;11:600.
16. Capasso M, Avvisati RA, Piscopo C, Laforgia N, Raimondi F, de Angelis F, et al. Cytokine gene polymorphisms in Italian preterm infants: association between interleukin-10 -1082G/A polymorphism and respiratory distress syndrome. Pediatric Research 2007;61:313-7.
17. Belluco C, Olivieri F, Bonafè M, Giovagnetti S, Mammano E, Scalerta R, et al. -174 G.C polymorphism of interleukin 6 gene promoter affects interleukin 6 serum level in patients with colorectal cancer. Clinical Cancer Research 2003;9:2173–2176.

18. Boiardi L, Casali B, Farnetti E, Pipitone N, Nicoli D, Cantini F, et al. Relationship between interleukin 6 promoter polymorphism at position -174, IL-6 serum levels, and the risk of relapse/recurrence in polymyalgia rheumatica. Journal of Rheumatology 2006;33:703–708.
19. Bruunsgaard H, Christiansen L, Pedersen AN, Schroll M, Jørgensen T, Pedersen BK. The IL-6 -174G.C polymorphism is associated with cardiovascular diseases and mortality in 80-year-old humans. Experimental Gerontology 2004;39:255–261.
20. Jones KG, Brull DJ, Brown LC, Sian M, Greenhalgh RM, Humphries SE, et al. Interleukin-6 (IL-6) and the prognosis of abdominal aortic aneurysms. Circulation 2001;103:2260–2265.
21. Fishman D, Faulds G, Jeffery R, Mohamed-Ali V, Yudkin JS, Humphries S, et al. The effect of novel polymorphisms in the interleukin-6 (IL-6) gene on IL-6 transcription and plasma IL-6 levels, and an association with systemic-onset juvenile chronic arthritis. Journal of Clinical Investigation 1998;102:1369-76.
22. Landi S, Moreno V, Gioia-Patricola L, Guino E, Navarro M, de Oca J, et al. Association of common polymorphisms in inflammatory genes interleukin (IL)6, IL8, tumor necrosis factor alpha, NFKB1, Interleukin promoter polymorphisms and prognosis in colorectal cancer and peroxisome proliferator-activated receptor gamma with colorectal cancer. Cancer Research 2003;63:3560–3566.
23. Sidell N, Taga T, Hirano T, Kishimoto T, Saxon A. Retinoic acid-induced growth inhibition of a human myeloma cell line via down-regulation of IL-6 receptors. Journal of Immunology 1991;146:3809–14.
24. Paul-Pletzer K. Tocilizumab: blockade of interleukin 6 signaling pathway as a therapeutic strategy for inflammatory disorders. Drugs Today (Barc) 2006;42:559-76.

Table 1. Characteristics of NB patients stratified per IL-6 -176 (G>C) SNP genotype

		Genotypic frequencies						
	n	**GG (%)**	**GC (%)**	**CC (%)**	**[a]P**	**[a]OR (CI=95%)**	**[b]P**	**[b]OR (CI=95%)**
Cases	326	176 (0.54)	125 (0.38)	25 (0.08)	0.24	1.14 (0.92-1.43)	0.34	1.31 (0.75- 2.28)
Controls	511	295 (0.58)	184 (0.36)	32 (0.06)				
Age								
≥ 18 months	152	77 (0.50)	63 (0.41)	12 (0.08)	0.39	1.17 (0.82-1.65)	0.65	1.22 (0.52-2.87)
< 18 months	168	94 (0.56)	62 (0.37)	12 (0.07)				
N. A.	6							
Sex								
Male	178	92 (0.52)	72 (0.40)	14 (0.08)	0.41	1.16 (0.81-1.65)	0.52	1.34 (0.55–2.35)
Female	141	79 (0.56)	53 (0.38)	9 (0.06)				
N. A.	7							
INSS stage								
Stage 1 + 2	103	57 (0.55)	44 (0.43)	2 (0.02)	[c]0.21	1.29 (0.87-1.91)	[c]0.02	5.03 (1.13-22.46)
Stage 3 + 4	192	102 (0.53)	72 (0.37)	18 (0.09)				
Stage 4S	23	12 (0.52)	8 (0.35)	3 (0.13)				
N. A.	8							
Risk								
High-risk	144	73 (0.51)	55 (0.38)	16 (0.11)	0.08	1.36 (0.96-1.92)	0.03	2.51 (1.05-5.99)
Not high-risk	182	103 (0.57)	70 (0.38)	9 (0.05)				
MYCN								
Amplified	73	35 (0.48)	32 (0.44)	6 (0.08)	0.17	1.33 (0.88- 2.02)	0.33	1.66 (0.59-4.69)
Not Amplified	221	126 (0.57)	82 (0.37)	13 (0.06)				
N. A.	32							

N.A. = not available

[a]p-values and ORs from comparison of allelic frequencies

[b]p-values and ORs from comparison of genotype frequencies (GG/GC vs CC)

[c]p-values and ORs from comparison of Stage 1 + Stage 2 patients vs Stage 3 + Stage 4 patients

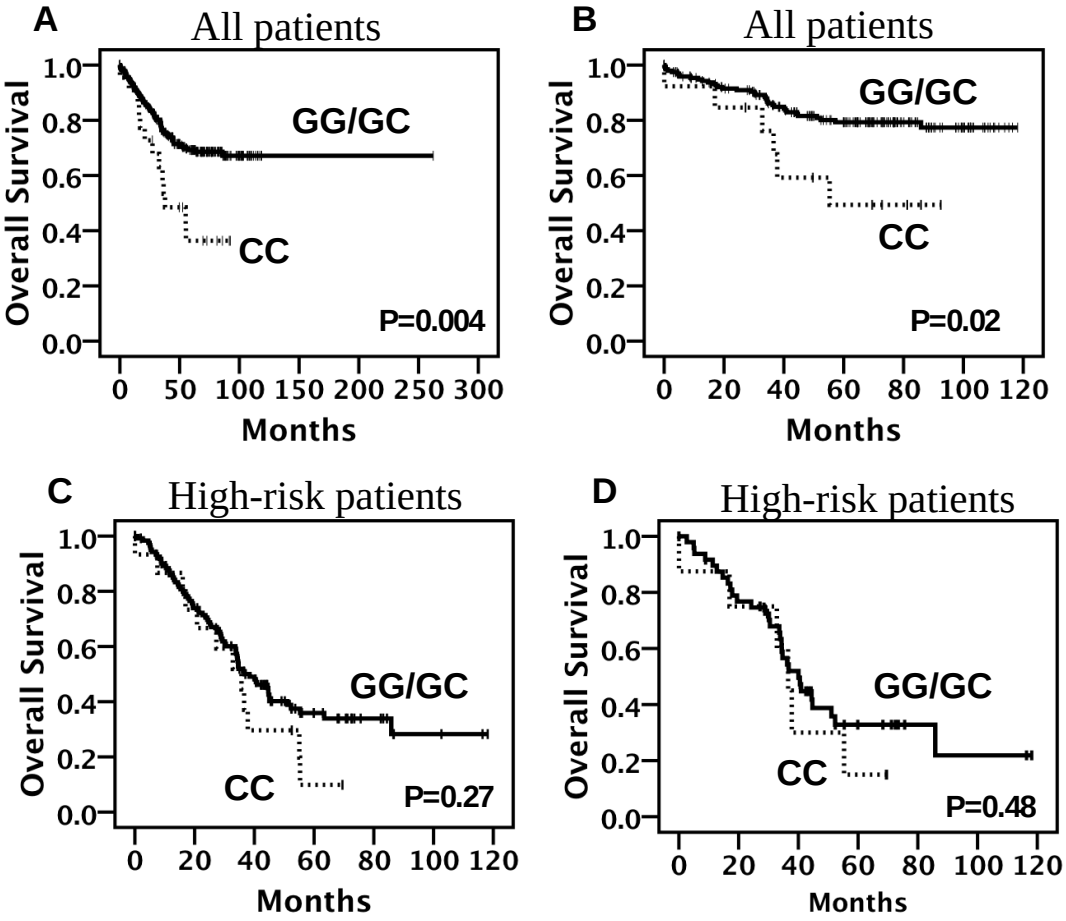

Figure 1. Kaplan-Meier curves for OS rates. OS rates were compared between CC and any G (GC or CC) for the SNP IL-6 -174 in (A) all NB and (B) not *MYCN* amplified patients, and (C) in high-risk NB and (D) not *MYCN* amplified patients.

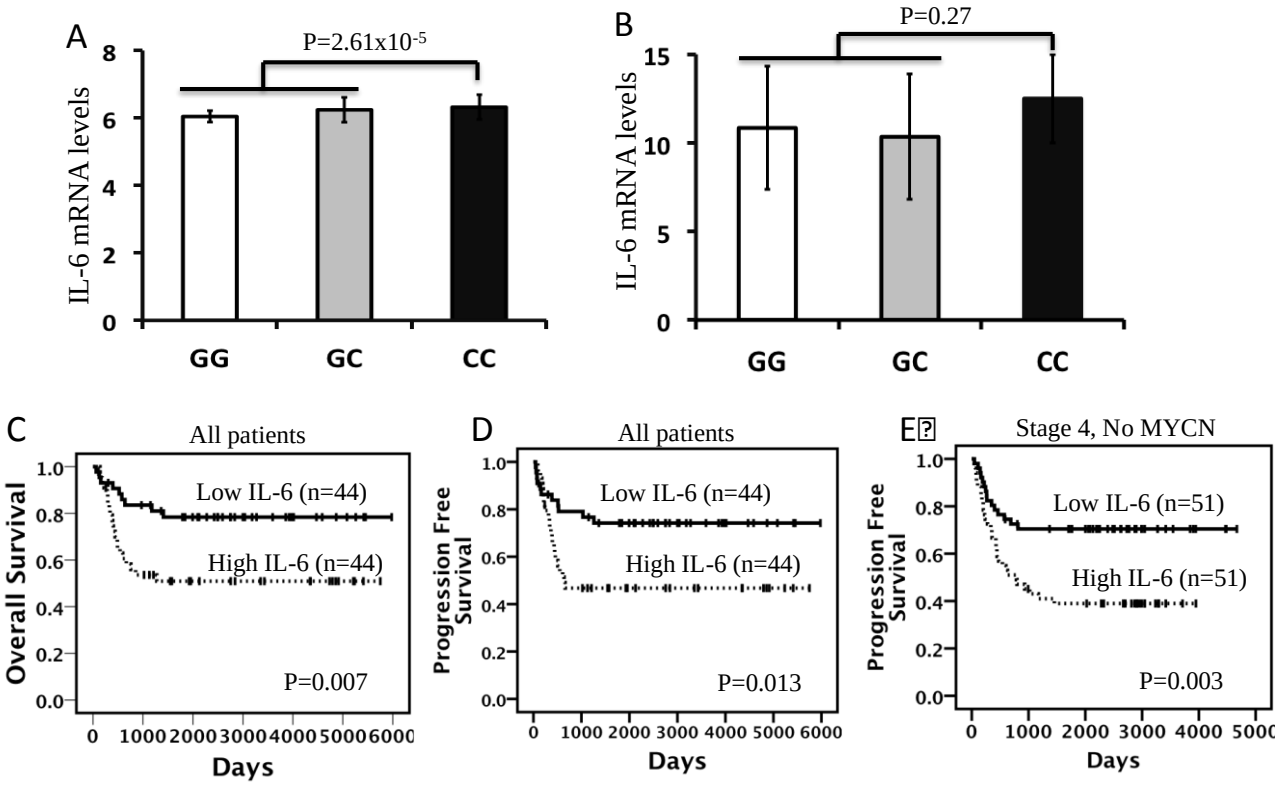

Figure 2. SNP-gene expression and *IL-6* gene expression-outcome correlations. *In silico* and qRT-PCR analysis of *IL-6* mRNA expression in (A) 198 LCLs and (B) 31 NB tumors, respectively, stratified according to the SNP *IL-6* -174. Kaplan-Meier analysis is shown, with individuals grouped by median of expression of *IL-6* for OS and Progression Free Survival rates in (C and D) 88 NB patients and (E) 102 INSS stage 4 patients with *MYCN* not amplified.

Supplementary Materials

Supplementary Table 1. Characteristics of 31 neuroblastoma tumors

ID	-174 IL-6 SNP genotypes	IL-6 mRNA levels	Sex	Age at diagnosis (months)	INSS Stage	MYCN status
1224	GG	7.56	Male	32	Stage 4	No AMP
1243	GG	8.08	Female	47	Stage 4	No AMP
1498	GG	8.65	Male	6	Stage 4	No AMP
1928	GG	13.52	Female	173	Stage 4	No AMP
1968	GG	15.28	Male	2	Stage 4	No AMP
2309	GG	11.51	Male	20	Stage 4	AMP
2372	GG	7.05	Male	80	Stage 4	No AMP
2388	GG	2.74	Female	36	Stage 4	No AMP
2397	GG	10.59	Female	7	Stage 4	No AMP
2437	GG	9.40	Female	45	Stage 4	No AMP
2484	GG	17.91	Female	6	Stage 4	No AMP
2576	GG	12.64	Female	32	Stage 4	No AMP
2578	GG	13.48	Female	23	Stage 4	Gain
2841	GG	12.20	Female	16	Stage 4	No AMP
2854	GG	15.06	Male	51	Stage 4	No AMP
3038	GG	15.06	Male	12	Stage 4	AMP
3055	GG	10.67	Male	17	Stage 4	AMP
1845	GC	5.99	Female	5	Stage 4	No AMP
2348	GC	7.08	Male	40	Stage 4	No AMP
2384	GC	12.00	Male	58	Stage 4	Gain
2568	GC	10.41	Female	35	Stage 4	No AMP
2599	GC	5.86	Female	48	Stage 4	No AMP
2622	GC	10.32	Male	49	Stage 4	No AMP
2633	GC	13.50	Male	37	Stage 4	No AMP
2692	GC	11.57	Female	116	Stage 4	No AMP
2852	GC	16.36	Male	50	Stage 4	Gain
931	CC	11.27	Female	7	Stage 4	N.A.
1124	CC	16.62	Male	58	Stage 4	No AMP
1275	CC	10.00	Female	9	Stage 4	No AMP
2497	CC	12.94	Male	48	Stage 4	Gain
2704	CC	11.68	Male	13	Stage 4	AMP

No AMP, not amplified; AMP, amplified

N.A. not available

Supplementary Table 2. Test for independent statistical significance of -174 IL-6 SNP after adjustment for neuroblastoma risk factors

Model	HR (95% CI)	P
A.		
Age (≥18 months vs <18 months)	4.18 (2.55-6.84)	<0.0001
-174 IL6 SNP (CC vs GC/GG)	1.90 (1.05-3.43)	0.03
B.		
MYCN (amplified vs not amplified)	3.85 (2.48-5.98)	<0.0001
-174 IL6 SNP (CC vs GC/GG)	1.95 (1.03-3.68)	0.04
C.		
INSS Stage (4 vs 1-2-3-4s)	9.06 (5.11-16.08)	<0.0001
-174 IL6 SNP (CC vs GC/GG)	1.90 (1.05-3.42)	0.03

Test for independent statistical significance of -174 IL-6 SNP after adjustment for NB risk factors.

HR, hazard ratio; CI, confidence interval.