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MYCN-non-amplified metastatic neuroblastoma with good prognosis and spontaneous regression: A molecular portrait of stage 4S

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ABSTRACT

Stage 4 neuroblastoma (NB) are heterogeneous regarding their clinical presentations and behavior. Indeed infants (stage 4S and non-stage 4S of age <365 days at diagnosis) show regression contrasting with progression in children (>365 days). Our study aimed at: (i) identifying age-based genomic and gene expression profiles of stage 4 NB supporting this clinical stratification; and (ii) finding a stage 4S NB signature. Differential genome and transcriptome analyses of a learning set of *MYCN*-non amplified stage 4 NB tumors at diagnosis ($n = 29$ tumors including 12 stage 4S) were performed using 1Mb BAC microarrays and *Agilent* 22K probes oligo-microarrays. mRNA chips data following filtering yielded informative genes before supervised hierarchical clustering to identify relationship among tumor samples. After confirmation by quantitative RT-PCR, a stage 4S NB's gene cluster was obtained and submitted to a validation set ($n = 22$ tumors). Genomic abnormalities of infant's tumors (whole chromosomes gains or loss) differ radically from that of children (intra-chromosomal rearrangements) but could not discriminate infants with 4S from those without this presentation. In contrast, differential gene expression by looking at both individual genes and whole biological pathways leads to

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Abbreviations: NB, neuroblastoma; infants, patients <365 days of age at diagnosis of NB; children, patients >365 days of age at diagnosis; stage 4S NB, metastatic NB of neonate or infant with a very special clinical presentation (small primary and hepatic, skin nodules, bone marrow involvement without bone metastasis; [1 yr$^-$] stage 4 NB, metastatic NB of infant without stage 4S NB clinical criteria;; [1 yr$^+$] stage 4 NB, metastatic NB of children; Q-RT PCR, quantitative reverse transcribed PCR; *S.B.I.M.E.*, Searching Biological Interpretation of Microarrays Experiments' software; DI, DNA index.

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a molecular stage 4S NB portrait which provides new biological clues about this fascinating entity.

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1. Introduction

Neuroblastoma (NB), the most frequent solid tumor of very early childhood (for reviews Schwab et al., 2003; Brodeur, 2003) is diagnosed as a disseminated disease (stage 4) in about 60% of the cases. Unlike stage 4 in the other malignant diseases of childhood or the adult, stage 4 NB at diagnosis elicit three distinct clinical patterns, based on disease distribution and the age of the patients (Evans et al., 1971). On the one hand, there are the infants (<365 days of age) with stage 4S (2–5% of all NB) and the similarly young stage 4 without 4S features hereafter termed "[1 yr^{-}] stage 4" who make up 5–10% of the NB population. On the other hand there are the stage 4 children (≥365 days of age) comprising 45% of all NBs who will hereafter be termed "[1 yr^{+}] stage 4". These three clinical aspects of stage 4 NB differ in their respective malignant behaviors and associated-prognosis: good for stage 4S and [1 yr^{-}] stage 4 (5 years-EFS >80%), and dismal for [1 yr^{+}] stage 4 (5 years-EFS of around 30%) despite intensive treatment including high-dose chemotherapy and hematopoietic stem-cell transplantation (Matthay et al., 1999; Valteau-Couanet et al., 2005). Noteworthy is the fact that 12 months of age or older had been the criterion for making management decisions for stage 4 NB. Recently, however, an age cutoff of 18 months has been proposed, but so far no molecular genetic criteria are available to uphold this empirical age-based stratification (London et al., 2005).

The so-called 4S stage (for special) is a very rare clinical entity first described by D'Angio et al. (1971). This form of disseminated NB usually affects neonates and infants in the first few months of life. There can be rapid tumor growth often followed by a spontaneous slower tumor regression. Stage 4S is usually diagnosed by an adrenal tumor of small size, associated with widespread disease in the skin and liver with modest bone marrow involvement, or any combination of these features. In stage 4 patients, extensive bone marrow involvement is common and associated more frequently in older patients with bone lesions.

As originally formulated by Brodeur (1995), and further refined by others (Lastowska et al., 2001; Vandesompele et al., 2005), a genetic classification of NB tumors – localized and metastatic as a whole – has been proposed. Type 1 tumors are hyperdiploid due to a whole chromosome set gains and losses resulting from mitotic disjunction, and have a good prognosis. By contrast, tumors of types 2, 3 and 4 display a spectrum of progressively more dismal outcome. They are mainly diploid (except a few tetraploid), and manifest genomic instability with specific structural chromosomal alterations. These include 17q gain and 11q loss without 1p deletion for type 2; 17q gain (Meddeb et al., 1996) and 11q loss with 1p deletion for type 3; and 1p deletion, 17q gain and *MYCN* amplification (Schwab et al., 1983; Seeger et al., 1985) and a few other alterations for type 4.

DNA index and *MYCN* genomic content are routinely used as criteria for determining prognosis and treatment for infants with stage 4 NB (Bourhis et al., 1991a,b; Ambros et al., 2003). Regarding *MYCN*-non-amplified [1 yr^{+}] stage 4, hyperdiploidy (George et al., 2005) as well as the combination of 1p and 11q deletions (Attiyeh et al., 2005) have been recently demonstrated to be helpful for prognosis.

If DNA microarray technologies (Sturn et al., 2002) may improve prediction of NB outcome (Oberthuer et al., 2006), they may also help to approach the issues raised by the clinical and biological stage 4 NB heterogeneity and, in particular, the spontaneous NB regression of stage 4S. This study was designed, first, to gather and catalog stage 4 NB, age-based genomic and gene expression profiles aiming towards a possible biological rationale for clinical stratification according to the patient's age at diagnosis. Second it aims at obtaining a stage 4S NB molecular portrait that could lead to new biological insights on this intriguing clinical presentation.

2. Results

2.1. *Genomic features of children and infants with non-amplified-MYCN stage 4 NB*

DNA index analysis of 25 tumor patients (Table 1) yielded mostly two DI peaks in infants and one DI peak in children. Infants stage 4 showed a wide distribution of DI values (over 18 cases, nine hyperdiploid, seven diploid and two tetraploid) while [1 yr^{+}] stage 4 NB were all diploid (6/7) or tetraploid (Table 2). Interestingly, the three stage 4S regressing tumors showed a DI of 1.00.

Out of the 29 tumors that composed the training set, gDNA of 28 was suitable for a BAC-CGH microarrays analysis. Among stage 4S tumors, one diploid (# 10) showed a normal profile without any detectable abnormalities (hereafter designated as "flat") while the 11 others elicited significant genomic abnormalities. Our stage 4 NB series clearly displays an increasing and gradual accumulation of genetic alterations (including whole and intra-chromosomal alterations) from infants to children. Importantly, chromosomes that showed whole loss or gain in hyperdiploid tumors were those eliciting intra-chromosomal loss or gain in near-diploid tumors, as already reported (Tomioka et al., 2003). Infant stage 4 tumors showed an excess of whole chromosomes changes (loss or gain) contrasting with a significant accumulation of intra-chromosomal alterations chromosomal abnormalities in children ($p < 0.0003$; Mann–Whitney test). Gains of 1q, 6p25–21, and 17q as well as loss of 3p, 8p23 and 11q13.3–25 significantly characterize the [1 yr^{+}] stage 4 tumors (Supplementary Table 1). Similar data were obtained using Agilent-oligo 244karray (data not shown).

Table 1 – Summary of patients and tumor specimens characteristics

	N°	Stage	Sex	Age (days)	Tumor tissue	Tissue origin	DNA index	DNA-Ploidy	Major S phase (%)	SPF class	Evolutive Phase at diagnosis	Chemo-therapy post-diagnosis	Follow-up (months/post-diagnosis)	Present Status (point May 2008)
	1	4s	F	36	PT	Abdominal	1.2	HD	11.9	3	Stable	No	120	A
	2	4s	M	37	MN	Hepatic	1.45, 1.00	HD	nd	nd	Stable	No	57	A
	3	4s	M	46	MN	Hepatic	1.18, 1.00	HD	5.8	2	Progression	No	43	A
	4	4s	M	47	MN	Skin	1.97	T	10.2	3	Progression	CT	1	Deceased
	5	4s	F	47	MN	Hepatic	1.00, 1.67	D	nd	nd	Unknown	CT	107	A
	6	4s	M	85	PT	Abdominal	1.55, 1.00	HD	2.0	1	Stable	No	18	A
	7	4s	F	99	MN	Skin	1.00, 1.79	D	nd	nd	Stable	CT	67	Deceased
	8	4s	M	108	MN	Hepatic	1.00	D	11.0	3	Stable	CT	44	Deceased
	9	4s	M	187	MN	Skin	1.46, 1.00	HD	11.0	3	Progression	CT	60	A
	10	4s	F	277	MN	Hepatic	1.00, 1.41	D	0.6	1	Regression	No	36	A
	11	4s	M	285	MN	Hepatic	1.00, 1.16	D	6.5	2	Regression	No	132	A
Infants	12	4s	F	289	PT	Abdominal	1.00	D	0.4	1	Regression	No	194	A
	13	4	F	39	MN	Hepatic	1.53, 1.00	HD	3.0	1	Progression	CT	74	A
	14	4	M	58	PT	Paravertebral	1.31, 1.00	HD	16.0	3	Progression	CT	204	A
	15	4	M	80	PT	Thoracic	1.57, 1.00	HD	nd	nd	Progression	CT	102	A
	16	4	F	141	PT	Abdominal	nd	nd	nd	nd	Progression	CT	60	A
	17	4	M	166	PT	Abdominal	nd	nd	nd	nd	Stable	CT	156	A
	18	4	M	302	PT	Abdominal	1.98, 1.00	T	11.0	3	Progression	CT	72	A
	19	4	F	305	PT	Abdominal	1.33,1.00	HD	9.0	3	Progression	CT	6	Deceased*
	20	4	M	353	MN	Parotidian	1.00, 2.16	D	nd	nd	Progression	CT	96	A
	21	4	M	369	MN	Mediastinal	nd	nd	nd	nd	Progression	CT	120	A
	22	4	F	375	PT	Abdominal	1.00	D	nd	nd	Progression	CT	48	A
	23	4	F	488	PT	Abdominal	nd	nd	nd	nd	Progression	CT	180	A
	24	4	M	843	PT	Abdominal	1.00	D	nd	nd	Progression	CT	204	A
Children	25	4	M	881	PT	Abdominal	1.00	D	10.0	3	Progression	CT	2	Deceased
	26	4	M	1226	PT	Abdominal	1.90, 1.00	T	15.0	3	Stable	CT	8	Deceased**
	27	4	M	1433	PT	Abdominal	1.09	D	15.0	3	Progression	CT	8	Deceased
	28	4	F	1978	PT	Abdominal	1.10	D	14.2	3	Progression	CT	48	Deceased
	29	4	F	5063	MN	Clavicular	1.16 + 1.00	D	12.2	3	Progression	CT	78	Deceased

F: female; M: male; PT: primary tumor; MN: metastatic nodule; D: diploid or near-diploid, HD: hyperdiploid, T: tetraploid or near tetraploid; nd: not determined; A: alive. DNA flow cytometry (Chassevent et al., 2001) led to DNA Index (DI) tumor values classified as diploid (1.00 < DI < 1.17), hyperdiploid (1.18 < DI < 1.85) and tetraploid range (DI > 1.86) (Bourhis et al., 1995a). In the DNA index column, when two peaks are measured, the major peak (>50% of area under the two G1 peaks) is underscored.

* Not CT responding, renal stenosis at necroscopy.

** Not DOD.

Table 2 – Stage 4 series genotypes

Type Tumor #	Flat	Atypical	1	2	3	Profiles remarks
1			○			
2			○			
3			○			
4					■	
5			●			
6			○			
7				●		
8		●				−1p, +2p, +17q
9			○			
10	●					
11		●				−1p, +2p
12			○			
13			○			
14			○			
15			○			
16					×	
17						CGH, DI, nd
18				■		
19			○			
20			●			
21				×		
22				●		
23			×			
24				●		
25					●	
26				■		
27				●		
28				●		
29				●		

Tumors which harbored a few unclassifiable genomic anomalies in the Lastowska's classification consisted in genotype "flat" or "atypical", in contrast to those fitting with genotypes 1, 2 and 3 criteria. Symbols are related to DNA index value (DI): ○, hyperdiploid; ●, diploid; ■, tetraploid. × means that DI value was not determined. nd: not done. Tumors #: 1–12, 4S; 13–20, [1 yr$^-$]; 21–29, [1 yr$^+$].

According to the current genetic classification (Lastowska et al., 2001; Vandesompele et al., 2005), 25 tumors were of types 1, 2 and 3. Three infant tumors, however, displayed either no genomic alteration ("flat", $n = 1$), or abnormalities that did not fit any of the three already proposed genetic types (hereafter designated as "atypical"; $n = 2$) (Table 2). Remarkably, "flat", "atypical" and type 1 were significantly found in stage 4 infants (15/19 vs 1/9, $p = 0.001$); conversely, there was a great excess of types 2 and 3 in [1 yr$^+$] stage 4 tumors (8/9 vs 4/19).

Finally, in considering results of genomic analysis in either the stage 4S or [1 yr$^-$] stage 4 cases whatsoever the array CGH technology used and the DI value, no specific genomic abnormalities discriminated these two clinical infantile types. Thus we searched for differential gene expression by looking at both individual genes and whole biological pathways in using a new software, *Searching Biological Interpretation of Microarrays Experiments*, or *S.B.I.M.E* (Kauffmann et al., 2008).

2.2. *Children stage 4 NB transcript profiling*

The 29 tumors of the learning set included the three clinical classes of stage 4 tumors: stage 4S, $n = 12$, [1 yr$^-$] stage 4, $n = 8$; and children ([1 yr$^+$] stage 4, $n = 9$). Transcriptome analysis was performed using the Agilent 22K long-oligo chips with the pooled tumors signals as reference values. A classification of stage 4 NB was obtained with a set of 144 genes that were differentially regulated across the 29 tumors (Supplementary Figures 1a,b). The [1 yr$^+$] stage 4 NB gene classifier (Supplementary Table 2) shows a significant differential up-regulation of a set of transcription factors, cell adhesion molecules and several melanoma antigens.

2.3. *Stage 4S NB transcripts profiling*

A class prediction analysis (BRB software) could discriminate 4S from the non-4S' cohort in a set of 124 genes (Supplementary Table 3) that are differentially regulated across the 29 tumors with a 76–97% correct classification (not shown). A hierarchical clustering with this set of genes shows the tight homogeneity of the 12 stage 4S (Figure 1a, Supplementary Figure 2). Transcript levels of 54 top-ranked (differentially up or down-regulated) genes of this 4S cluster were assessed by Q-RTPCR, 70% of them showing Pearson's correlation coefficients >0.6 (not shown). Combination of gene cluster with the *S.B.I.M.E.* software – designed to pick up, by statistical testes, the functions, pathways or interaction networks of interest, directly from all the information provided by microarray experiments (Kauffmann et al., 2008) – revealed evidence of differentially regulated biological activities in stage 4S NB (Supplementary Tables 4a,b). Among genes related to neuroectodermal functions the gamma-aminobutyric acid (GABA) receptor rho 2 was up-regulated (Figure 2a). Stage 4S tumors also elicited a differential transcript up-regulation of genes (Supplementary Table 3) involved in inflammatory, and immune response: SERPIN5 (Zou et al., 1994), COLEC11 (Stuart et al., 2006) and RAG1 (Gellert, 2002). Similarly, there were found to be differentially activated (Figures 2b,c) the stress-inducible ULBP3 (Eleme et al., 2004) as well as the pro-inflammatory cytokine TSLP1 (Soumelis and Liu, 2004). Three transcription factors were also found to be differentially up-regulated (Figures 2d–f): NFYA (Jin et al., 2001), HMGB1 or HBP1 (Berasi et al., 2004; Scaffidi et al., 2002) and PBX3 (Monica et al., 1991). Strikingly, HBP1 appears to be differentially induced in the three regressing stage 4S tumors (Figure 2f).

Stage 4S tumors also displayed a transcript level up-regulation of genes linked to mitochondrial matrix enzymes required for long chain fatty acids degradation: thiolases (HADHB) (Figure 2g), hydratases, and dehydrogenases (acyl-CoA dehydrogenase, ACADM) (not shown). The differential up-regulation of both cytoplasmic (MGST1, CDO1) and active mitochondrial detoxication processes (PRDX3, ALDH3A2, ALDH2, CLU) also support an intense mitochondrial activity (Supplementary Figure 3).

To identify reliably the genes expressed differentially between infants with stage 4S and [1 yr$^-$] stage 4 disease, transcriptomes of these two clinical subtypes were compared. The BRB class prediction analysis led to a set of 45 genes (80–95%, correct classification rate) and a corresponding heatmap was obtained (Figure 1b, Supplementary Figure 4). This stage 4S NB classifier (Table 3), with fold change from −3.1 to 4.5, showed 19 genes that were common with the previous 4S vs non-stage 4S gene set (42%), including NFYA, PBX3 and GABRR2, all genes being significantly validated by QRT-PCR (not shown). A test set

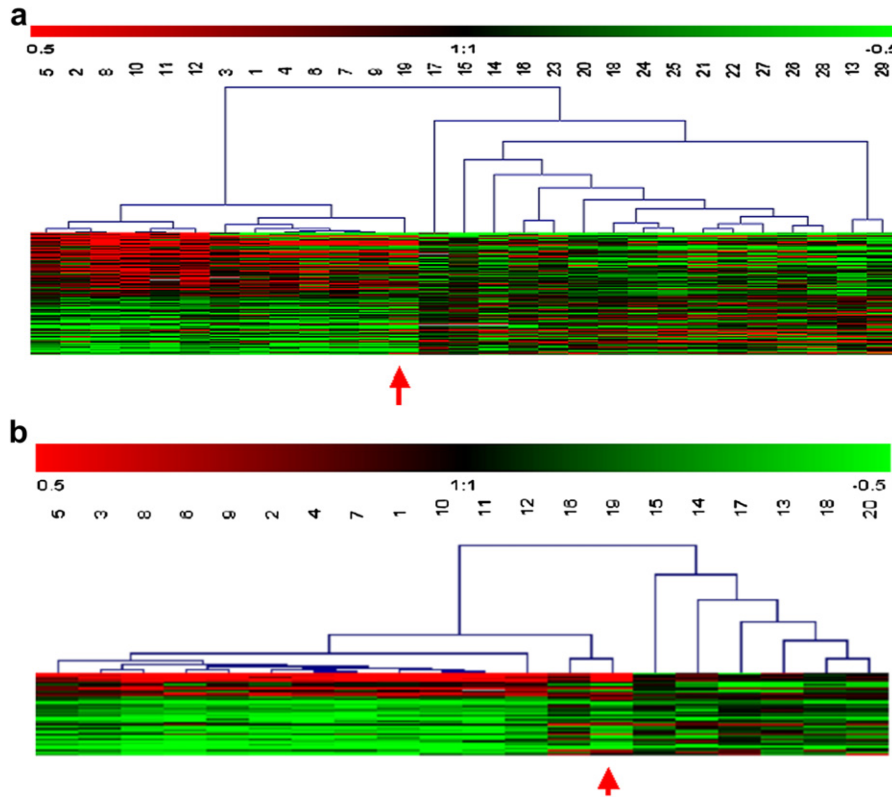

Figure 1 – Supervised hierarchical clustering of stage 4 NB comparing: (a) stage 4S NB to non-stage 4S NB (124 significant genes), (b) stage 4S to [1 yr^{-}] stage 4 NB (45 significant genes). Each row represents a single gene and each column a tumor. Tumors #: 1–12, 4S; 13–20, [1 yr^{-}]; 21–29, [1 yr^{+}]. As shown in the color bar, red and green indicate up and down expressions, respectively, black no change, and gray no data available. Red arrow points #19 [1 yr^{-}] stage 4 NB tumor with a 4S' profile suggesting an atypical stage 4S clinical presentation.

of this stage 4S classifier was then analyzed by QRT-PCR using 22 distinct tumors obtained at diagnosis from infants *MYCN*-non-amplified stage 4 NB including stage 4S ($n' = 12$), [1 yr^{-}] stage 4 ($n'' = 10$). This validation analysis yielded a significant correlation with four genes namely GABRR2, NFYA, DRHS8 and AP4E1 ($p < 0.05$, Student's test).

When applied to common biological databases, *S.B.I.M.E* tool indicates active lipid metabolism in infants stage 4 (not shown) and an active citric acid cycle in stage 4S tumors, as assessed by differential increases in transcript levels of HMGCoA lyase (HMGCL) and acetyl-CoA C-acetyltransferase (ACAT1), fumarase, FH and glutamate dehydrogenase GLUD1 and GLUD2 (Supplementary Table 4). Moreover, *S.B.I.M.E* data indicate that [1 yr^{-}] stage 4 NB shows Ras-independent pathway in NK cell-mediated cytotoxicity and increase T cell response activity (Table 4).

3. Discussion

3.1. *Genomic characteristics of non-amplified-MYCN stage 4 NB*

Non-hyperdiploid stage 4 tumors in patients older than 12 months of age exhibit an excess of intra-chromosomal alterations, in contrast to the mostly hyperdiploid infantile stage 4 patients aged under 12 months whose tumors show whole chromosome changes (losses or gains). Strikingly, among the 20 tumors in infants, one showed a ''flat'' profile and two others were ''atypical'' regarding to the Brodeur–Lastowska–Vandesompele's classification (Brodeur, 1995; Lastowska et al., 2001; Vandesompele et al., 2005). Significantly, chromosomal types of infant tumors are type 1, ''flat'', or ''atypical'', while childhood tumors exclusively show types 2 or 3. The other 3 tetraploid tumors of the series fall into types 2 or 3, in agreement with a progressive disease as reported (Bourhis et al., 1991a,b). The combination of DI values for the four genotypes do not, however, improve discrimination between infants and children (Table 2). Noteworthy, two among three infants with tumors showing intra-chromosomal alterations followed a fatal course. One of these two tumors was type 3 (case # 4) and the other (case # 8) was of an atypical type very close to type 3 (-1p and $+17$q without containing -11q). This latter child died of disease 3 years after a marked regression of both the primary tumor and the hepatomegaly and despite careful monitoring over 2 years. This case indicates that intra-chromosomal alterations did not develop *de novo* with time but were present at diagnosis. Today, *MYCN* genomic content is the only criterion used to eliminate type 4, but is of no help for prognosis of infants with *MYCN*-non-amplified stage 4 disease. On the basis of the genomic data here presented, we propose a systematic genotyping to improve management of infants with stage 4 NB. Such a pangenomic CGH array stratification is feasible and easily generated from

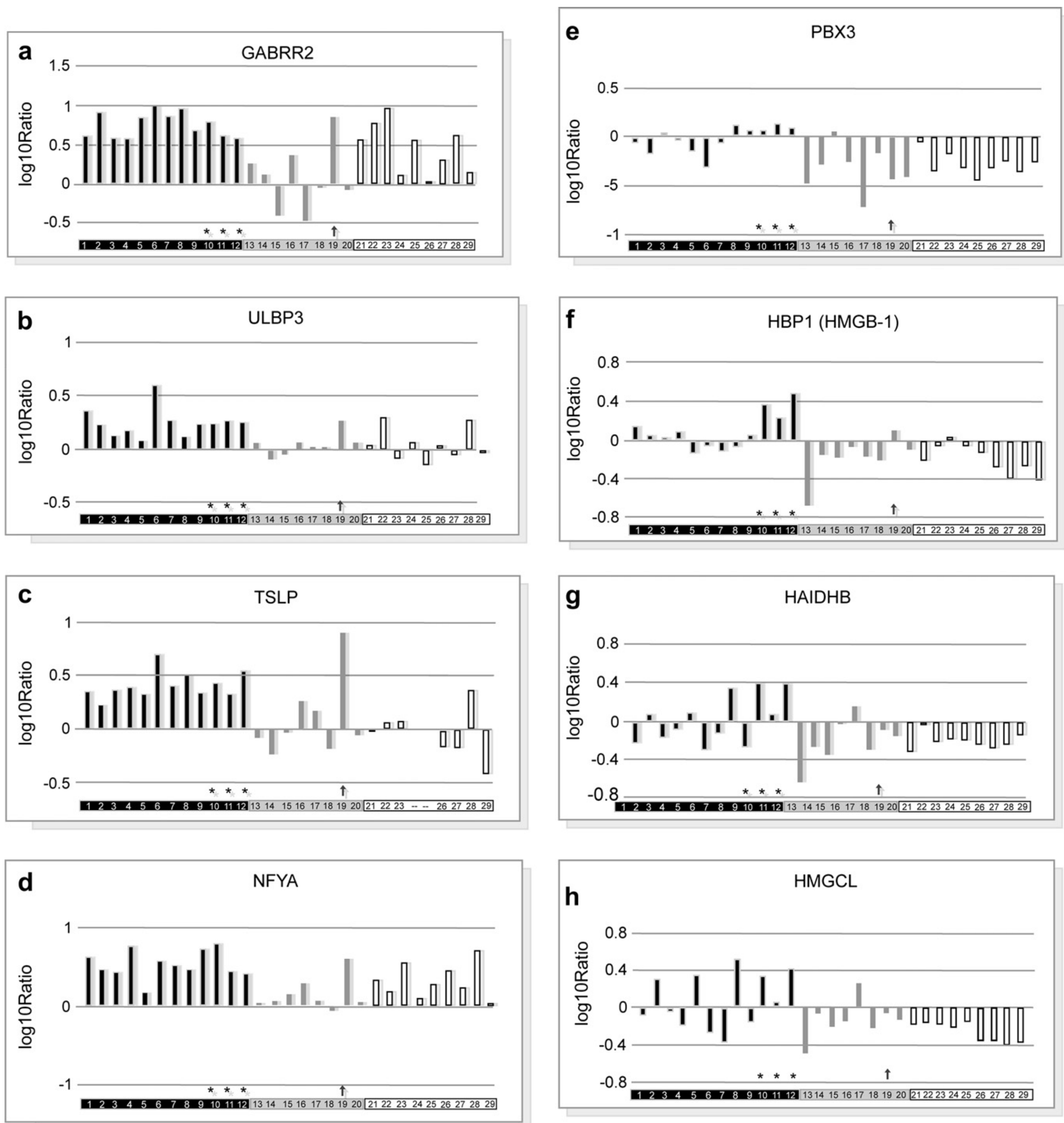

Figure 2 – Expression profiling of top genes selected using gene classifier and *S.B.I.M.E.* analysis in each individual tumor of the patients cohort. For a given gene, the value of each individual bar of histogram represents the log ratio of the tumor expression signal as compared to that of the pool. Student's test yielded 2 *p* values, respectively 4s *vs* non-4s and 4s *vs* [1 yr^-], as indicated in brackets. Tumors #: 1–12, 4S (blue bars); 13–20, [1 yr^-] (yellow bars); 21–29, [1 yr^+] (hatched bars). # 10, 11 & 12 tumors are in regression (*). Red arrow points #19 [1 yr^-] stage 4 NB tumor. a: GABRR2 [7 10^{-4}; 10^{-4}], b: ULBP3 [4 10^{-4}; 3 10^{-3}]; c: TSLP1[3 10^{-4}; 1.5 10^{-2}]; d: NFYA [9 10^{-4}; 3 10^{-4}]; e: PBX3 [4 10^{-5}; 6 10^{-4}]; f: HBP1 [4 10^{-4}; 7 10^{-3}]; g: HADHB [9 10^{-3}; 7 10^{-2}]; h: HMGCL [4 10^{-3}; 10^{-1}].

gDNA obtained from fine needle-biopsies at diagnosis. This would yield genotypes that would be used for clinical decision-making, as currently demonstrated by an European study carried out on a very large cohort of all NB patients on one hand (Janoueix et al., in press) as well as specifically in infants NB (Schleiermacher et al., 2007, submitted). A "flat" genotype or one showing whole chromosome changes (type 1) would call for careful observation alone or minimal therapy. Intra-chromosomal rearrangements (types 2 and 3 and "atypical" with pejorative genetics markers, i.e., −1p, +17q) would demand active monitoring and a therapeutic strategy similar to that adopted for *MYCN*-amplified NB (type 4).

Table 3 – Differentially expressed genes ($n = 45$) between stage 4S and [1 yr$^-$] stage 4 neuroblastoma

Symbol	Title	Accession number	Loc chrom	Fold change
Immune response				
IFNG	Interferon gamma	NM_000619	12q14	−1.6
SERPINI1	Serine protein 1	NM_005025	3q26.1	−1.6
RAG2	Recombination activating gene	NM_000536	11p13	−1.8
BC039725	Protein with an immunoglobulin (Ig) domain	BC039725	–	−2.0
BRDG1	B cell receptor downstream signaling 1	NM_012108	4q13.2	−3.0
Transcription factors				
NFYA	Nuclear transcription factor Y alpha	NM_002505	6p21.3	2.5
PBX3	Pre-B-cell leukemia transcription factor 3	NM_006195	9q33–q34	2.1
Signaling				
LOC123722	Hypothetical protein	ENST00000334574	15q25.2	2.8
PTP4A1	Pr. tyrosine phosphatase type IVA member1	NM_003463	6q12	2.0
TNFAIP2	Tumor necrosis factor alpha-induced protein2	NM_006291	14q32	−1.6
AP4E1	Adaptor-related pr. complex 4 subunit epsilon1	CR749604	15q21.2	−1.8
FLJ35220	Member of the endonuclease V family	A_23_P32433	17q25.3	−1.8
RASGRP1	RAS guanyl releasing protein	NM_005739	15q15	−1.8
SLC36A2	Solute carrier family 36 member 2	NM_181776	–	−1.9
CX62	Connexin 62	NM_032602	6q15–q16	−2.0
PART1	Prostate androgen-regulated transcript 1	NM_016590	5q12.1	−2.2
EGFL6	EGF-like-domain, multiple 6	NM_015507	Xp22	−2.4
Metabolism				
DHRS8	Dehydrogenase/reductase 2(RetSDR2)	NM_016245	4q22.1	2.3
GPR133	G protein-coupled receptor 133	NM_198827	12q24.33	1.7
A_23_P61288	–	A_23_P61288	–	−1.6
BOC	Brother of CDO	NM_033254	3q13.2	−1.8
KLRF1	Killer cell lectin-like receptor F member 1	NM_016523	12p12.3–13.2	−2.0
Muscle development				
CFL2	Cofilin 2	NM_021914	14q12	1.8
CASQ2	Calsequestrin 2	NM_001232	1p13.3–p11	−1.6
ITGA7	Integrin alpha 7	NM_002206	12q13	−1.8
MYL1	Myosin, light polypeptide 1	NM_079422	2q33–q34	−2.0
Synaptic transmission				
GABRR2	Gamma-aminobutyric acid receptor rho 2	NM_002043	6q14–q21	4.5
KCNMB1	b1subunit calcium-activated K+ channel	NM_004137	5q34	−1.5
Unknown function				
A_23_P15851	Unknown function	A_23_P158513	–	2.5
BTNL8	Similarity with butyrophylin Btn1a1	NM_024850	5q35.3	2.4
MGC50721	Hypothetical protein	NM_173806	16p12.1	2.0
X102	X102 protein	ENST00000327992	15q11.2	1.4
C6orf165	Chromosome 6 open reading frame 165	NM_178823	6q15	−1.6
FLJ36198	Hypothetical protein	NM_173801	11q12.1	−1.6
MGC26816	Hypothetical protein	NM_152613	22q13.2	−1.6
FLJ20825	Unknown function	AK000832	20q13.33	−1.6
C9orf52	Chromosome 9 open reading frame 52	NM_152574	9p22.3	−1.7
R7BP	Similarity with D13Bxg1146e	BX640900	–	−1.7
KIAA0125	Hypothetical protein	NM_014792	14q32.33	−1.7
PRO1257	Hypothetical protein	AF116629	1q24.1	−1.7
CMYA3	Cardiomyopathy associated A3	AL833291	2q24.3	−1.7
FLJ23550	Hypothetical protein	NM_025063	1q24.3	−1.8
C15orf25	Chromosome 15 open reading frame 25	A_23_P58157	15q15.1	−2.3
MGC45438	Unknown function	NM_152459	16p13.3	−3.1

Top-ranked genes of the stage 4S NB cluster (up- and down-regulated). Genes listed are statistically significant (t-test p-value <0.001) as reported by BRB Array Tools after combination of the arrays within Rosetta Resolver® system for gene expression data analysis. Genes are classified by biological function, as reported in the 22K Agilent annotation. Functional annotation was performed using the Online Mendelian Inheritance in Man (OMIM). Fold change is calculated as the 4S/[1 yr$^-$] stage 4 ratio. Negative fold change means the log(Cy5 labeled [1 yr$^-$]/Cy3 labeled pool) ratio is higher than that of 4S. Underlined genes are common with those of the 4S vs non-4S genes' set.

3.2. *Molecular features of stage 4S NB*

Except Dein, a single novel gene with high expression in stage 4S NB (Voth et al., 2007), no genes set identifying this subtype of disease has been reported so far. Our transcriptome analysis confirms the marked difference of genomic alterations within stage 4 NB between infants and children and thus validates the age-based clinical classification. However a small number

Table 4 – Immune response functional analysis discriminating stage 4S ($n = 12$) from [1 yr^{-}] stage 4 ($n = 8$) according to *S.B.I.M.E.* tool

	Ng	Ngc	Ns	%	Z	P	Genes
BioCarta							
Ras-independent pathway in NK cell-mediated cytotoxicity	17	17	4	23.5	4.84	1.32E-06	PTPN6 $\Downarrow$ SYK $\Downarrow$ LAT $\Downarrow$ KLRC1 $\Downarrow$
T cytotoxic cell surface molecules	12	10	2	20	3.25	1.14E-03	CD28 $\Downarrow$ THY1 $\Downarrow$
E2F1 destruction pathway	9	9	2	22.2	3.24	1.20E-03	SKP2 $\Uparrow$ CUL1 $\Uparrow$
T helper cell surface molecules	12	10	2	20	3.15	1.63E-03	CD28 $\Downarrow$ THY1 $\Downarrow$
Role of Tob in T cell activation	15	12	2	16.7	3.1	1.91E-03	IFNG $\Downarrow$ CD28 $\Downarrow$
Gene ontology – biological process							
Defense response	98	77	7	9.1	3.13	1.73E-03	IFNG $\Downarrow$ MX2 $\Downarrow$ IFNA21 FLJ10979 $\Uparrow$ PTPRCAP $\Downarrow$ CD84 $\Downarrow$ SP140 $\Downarrow$
Humoral immune response	28	25	3	12	2.7	6.86E-03	CD28 $\Downarrow$ BRDG1 $\Downarrow$ LTF $\Downarrow$
Integrin-mediated signaling pathway	45	39	4	10.3	2.61	9.03E-03	ITGB7 $\Downarrow$ ITGA7 $\Downarrow$ ITGB3 $\Downarrow$ SYK $\Downarrow$
Gene ontology – molecular function							
MHC class I receptor activity	13	11	2	18.2	2.85	4.36E-03	KLRF1 $\Downarrow$ ULBP3 $\Uparrow$
4s < [1 yr^{-}] stage 4	$\Downarrow$						
4s > [1 yr^{-}] stage 4	$\Uparrow$						

Using ANOVA, only functional categories with a p-value <0.01 were selected according to the Z-score (comparison of the observed percentage to a randomized set of theoretical percentages). Each row corresponds to a category found by *S.B.I.M.E.* Kauffmann et al. (2008), ordered by increasing p-value. "Ng" = genes number in the functional category. "Ngc" = genes number present in the data set. "Ngc" > "Ng" if there are several oligonucleotides for the same gene on the array. "Ns" = number of significantly differentially expressed genes. "%" is the percentage of genes significantly expressed (Ns/Ngc $*$ 100). "P" = p-value associated to the Z-score calculation, i.e., the probability to find this functional category significant by chance. Following this column, the *S.B.I.M.E.* output file normally presents all the genes (not shown).

of [1 yr^{+}] stage 4 tumor specimens at diagnosis prevents us from validating the proposed 18-month cut-off age for discriminating between high risk and non-high risk NB (London et al., 2005). A recent study of *MYCN*-non-amplified stage 4 NB shows that tumors transcriptome profiling can identify subgroups with different outcomes (Asgharzadeh et al., 2006).

The combination of genes clusterings with the *S.B.I.M.E.* data provides a molecular stage 4S NB portrait in facets regarding neuro-ectodermal embryogenesis in agreement with recent findings (Fischer et al., 2006), mitochondrial and fatty acids metabolism activities, transcription factors expression and inflammatory/immune response.

Differential up-regulation of PBX3 and GABRHO2 receptor transcript levels were observed. The homeodomain transcription factor PBX3 is expressed at high levels in the developing nervous central system including the medulla oblongata involved in respiration control. PBX3-deficient mice die, within a few days of birth, from central respiratory hypoventilation syndrome (Rhee et al., 2004). Remaining to be elucidated is the role PBX3 plays relative to PHOX2B, the genetic determinant of the Ondine syndrome and proposed as being involved in NB oncogenesis (Trochet et al., 2004). Similarly, the channel GABRHO2 receptor, a Ca2+ current inhibitor *via* G-protein-coupled mechanisms in sympathetic neurons (Filippov et al., 2000), might represent a trophic factor involved in neuroblast development. These evidence suggest stage 4S not *per se* a "metastatic" disease, but rather multifocal dysregulated proliferations of embryonal neuroblasts.

A differential increase of acetyl-CoA carboxylase alpha transcript levels in infants compared to children likely indicates lipogenesis activation. Differential up-regulation of fatty acids catabolism with key enzymes activation, clearly pinpoints fatty acid β-oxidation (FAβ0) activation in stage 4S. Moreover transcriptome data point out the involvement of various metabolisms that might tolerate an excessive FAβ0 and subsequent acetyl-CoA overproduction as well as differential up-regulation of mitochondrial activity of both cytoplasmic and mitochondrial detoxication processes. Stage 4S tumor cells thus appear as very highly energy consuming cells, able to sustain an active growth with subsequent associated detoxification mechanisms. The active lipogenesis and fatty acid catabolism must be considered in the context of the milk diet of infants during the first months of life.

The transcriptional up-regulation of NFY-A would reflect a compensatory DNA repair process at work. As for the transcription regulator HBP1, known to be a repressor of p47 phox (Jin et al., 2001), it can also be an endogenous immune adjuvant (Rovere-Querini et al., 2004) able to trigger inflammation, when released by dead cells (Scaffidi et al., 2002). In this respect, we observed HBP1 differential overexpression in the three regressing stage 4S tumors showing a diploid DNA index, a low S phase fraction, and many necrotic foci (not shown).

In humans, the NK cells cytotoxic receptor NKG2D may be activated after recognition by the ULBP3 ligand that is up-regulated in tumor cells (Pende et al., 2002). Not restricted to NK cells, NKG2D expression also concerns subsets of γ/δ T cells, particularly $CD8^{+}$T cells. Moreover a down-regulation of KLRF1, the inhibitor of NK activation via MHC class I cells, is found (Table 4) favoring NK cells as instrumental in stage 4S. The up-regulation of TSLP1, a cytokine necessary for NK activation indicates an inflammatory process and subsequent cell "stress" (Gasser and Raulet, 2006). Such an immunity monogram reflects tumor-infiltrating NK cells.

Altogether, from our data one may speculate the following tumor model for stage 4S. Firstly residual

undifferentiated neural crest progenitors would proliferate using various energy sources, in particular fatty acids, given the high milk diet of the neonate. Secondly subsequent active β-oxidation would generate oxygen-reactive species possibly leading, during cell division, to whole chromosome loss and gains. Thirdly, once all compensatory mechanisms of metabolism are overridden, such genome instability cannot be repaired. In this context, an inflammatory and NK-mediated response of the host, a kind of host ''intolerance'' against malignant neuroblasts, would then be triggered and lead to tumor regression. Molecular determinants of such a proliferation-regression oscillation remain to be found.

4. Experimental procedures

4.1. Patients, tumor tissues and procedures

Snap frozen tumors samples (tru-cut needle or open biopsy) were collected at diagnosis from these patients. These tumors were stringently selected as containing more than 70% of malignant tumor cells and *MYCN*-non-amplified ($\leq$3 copies/haploid genome) (Ambros et al., 2003). Experiments were done from 35-μm thick tumor slices flanked by 5 μm histological controls showing immature malignant neuroblasts population higher than 70%; out of 81 tumor specimens of patients with *MYCN*-non-amplified stage 4 NB patients, 29 were available for genome and transcriptome analyses in a tumors set composed of stage 4S ($n = 12$), [1 yr^{-}] stage 4 ($n = 8$), [1 yr^{+}] stage 4 ($n = 9$). Pertinent clinical and biological features of these patients are presented in Table 1.

DNA flow cytometry (Chassevent et al., 2001) permitted measurement of DNA index (DI) tumor values. Both genomic DNA (gDNA) and total RNA were purified from the tumor slices in using appropriate Qiagen extraction kits (Qiagen S.A., France).

4.2. Genome and transcriptome analyses

Tumor and reference genomic DNAs (900 ng each), after differentially Cy3/Cy5-dCTP labeling, were hybridized to 1 Mb resolution pan-genomic DNA microarrays from *Spectral Genomics* (Inc., Houston, TX, USA). Scanning and images processing allowed fluorescent ratio normalization across all array elements in order to compensate for differences in the whole DNA genomic content between test and diploid reference DNAs, and for differences in labeling efficiencies.

Cy5-labeled purified cRNA from each sample (500 ng) was mixed with the same amount of the Cy3-labeled reference cRNA (a pool of the 29 cRNA tumor patients samples) and hybridized onto 22K oligonucleotide microarrays from *Agilent* (Inc, Palo Alto). Lack of human neonates and infants pertinent control tissue led to the use of a pool as reference that further allowed optimization of expression variations. Each microarray contains 20,172 distinct probes, corresponding to 16,300 unique genes. Raw data in the *Resolver*™ software (Weng et al., 2006) for all elements across all 29 samples were deposited at the *European Bioinformatics Institute* (http://www.ebi.ac.uk/arrayexpress/), accession number E-TABM-119 (Brazma et al., 2003). Genes transcript levels of the top up- or down-regulated genes were measured by Quantitative RT-PCR (QRT-PCR) using the Applied Biosystems 7900 HT Microfluidic card.

4.3. Statistical analysis

Agilent Feature Extraction Software (version A.6.1.1) quantified the fluorescent image intensity and normalized data using the background subtraction method. Following log transformation and intensity-dependent (LOWESS) normalization, data were imported for management, quality control and analysis. The arrays corresponding to each patient (two or three replicates) were then combined using a weighted average method as defined in the *Resolver*™ software; data were transferred to BrB Array Tools to perform the class prediction analyses (http://linus.nci.nih.gov/BRB-ArrayTools.html). Minimum fold change filtering and missing values filtering finally selected 8553 genes.

The class prediction module uses six learning methods and a leave-one-out cross validation over the patients (not shown). Significantly regulated genes were those whose tumor expression was different in the two groups considered ($p < 0.001$, Student's t-test p-value). The statistical significance test of the cross-validated misclassification rate (based on 2000 random permutations) provides an additional p-value for each misclassification rate. The BrB selected genes were classified by biological function relative to the Gene Ontology annotations (Ashburner et al., 2000).

A new software, Searching Biological Interpretation of Microarrays Experiments (*S.B.I.M.E.*), was designed to pick up, by statistical testes, the functions, pathways or interaction networks of interest, directly from all the information provided by microarray experiments (Kauffmann et al., 2008).

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Appendix A. Supplemental material

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