Author's response to reviews

Title: Specific Gene Expression Profiles and Unique Chromosomal Abnormalities are Associated with Regressing Tumors Among Infants with Dissiminated Neuroblastoma.

Authors:

Cinzia Lavarino (clavarino@fsjd.org)
Nai-Kong V Cheung (cheungn@mskcc.org)
Idoia Garcia (igarcia@fsjd.org)
Gema Domenech (gema.domenech@h1.org.es)
Carmen de Torres (cdetorres@hsjdbcn.org)
Miguel Alaminos (malaminos@histolii.ugr.es)
Jose Rios (jose.rios@uab.es)
William L Gerald (geraldw@mskcc.org)
Brian Kushner (kishnerb@mskcc.org)
Mike LaQuaglia (laquaglia@mskcc.org)
Jaume Mora (jmora@hsjdbcn.org)

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Author's response to reviews: see over
Reviewer’s report

Title: Specific Gene Expression Profiles and Unique Chromosomal Abnormalities are associated with Regressing Tumors Among Infants with Dissiminated Neuroblastoma.

Following one of the reviewers’ suggestions, the manuscript title has been modified.

Now: SPECIFIC GENE EXPRESSION PROFILES AND CHROMOSOMAL ABNORMALITIES ARE ASSOCIATED WITH INFANT DISSEMINATED NEUROBLASTOMA

Reviewer 1

Major Compulsory Revisions

1) Authors state that the 35 tumor samples included in the present study were collected from 1987 to 2000 at Memorial Sloan Kettering Cancer Center (MSKCC). This means that in 13 years, a mean of only 3 cases (35/13) were collected at the MSKCC per year. Since the Memorial Sloan Kettering Institute is one of the major cancer centers in the US, someone could ask whether the samples presented here might have been selected in some way. Which criterion did Authors follow to collect tumor samples?

We report only the infant cases treated and followed at MSKCC exclusively according to our restricted minimal or no therapy approach. Infants are a rare subgroup of neuroblastomas. MSKCC is certainly one of the major cancer centers in the US for neuroblastoma management, however, most referred cases are high-risk, often pre-treated patients, making the recruitment of specimens at diagnosis difficult. Furthermore, sufficient and adequate tumour tissue samples (snap frozen samples with >70% viable tumour cell content) to perform molecular studies are not always available for all patients. This is especially true for stage 4s patient for which, as reported in material and methods, since 1987 a conservative approach has been adopted at MSKCC for stage 4s NB, based on minimal or no therapy and surgery only when required.

2) It is known that neuroblastoma tumors show high tissue heterogeneity and that reliability of results can be affected by the presence of infiltrating normal cells. Authors state “Stage 4 tumors were predominantly unfavorable histology cases, whereas all stage 4S tumors were histologically favorable”. Does it mean that stage 4 tumors were stroma-poor neuroblastomas and stage 4S were predominantly stroma-rich ones? Do all tumor samples show at least 60% tumor content? Can Authors exclude any biases due to massive presence of Schwann stromal cells and non-malignant infiltrating cells in the tissues?

All infant disseminated (4 and 4s) neuroblastomas are undifferentiated or poorly-differentiated tumors, therefore, are stroma-poor tumors. According to the INPC (Shimada system) prognostic histological classification, infants less than 1.5 years of age with undifferentiated or poorly differentiated tumors and low or intermediate MKI are classified as histologically favourable tumors. Therefore the differences between stage 4 or 4s cases are mainly derived from their MKI ratio. The presence of stroma is, by definition, excluded in this subset of disseminated infant neuroblastoma.

All tumour-specimens were evaluated by the same pathologist (WG) to assess tumour cell content; only tumours with > 70% neuroblastic cells were included in the study. This concept has now been included in material and methods (pg. 4).

3) Authors do not specify whether the study has been performed on primary tumors and/or metastases. I would recommend to specify if metastases or primary tumors have been analyzed.
The study has been performed using primary tumour samples. It has now been specified in material methods text (pg. 4).

4) Authors correctly state that up to now “given the low incidence of stage 4S neuroblastoma few expression profiling studies with reduced cohorts have been reported”, but the sample size of the present study is too small in any case to allow identification of recurrent rearrangements and to talk about significant correlations between molecular abnormalities of tumors and clinical parameters of patients. This was recognized by the Authors, yet in the Title and in the Discussion section they try to draw definitive conclusions. Therefore I would recommend not to indicate percentages of alterations and especially Authors must emphasize that this is a pilot study.

Following the reviewers suggestions we have performed the following modifications:

**Title:**

*Now:* SPECIFIC GENE EXPRESSION PROFILES AND CHROMOSOMAL ABNORMALITIES ARE ASSOCIATED WITH INFANT DISSEMINATED NEUROBLASTOMA.

**Discussion section.** We have removed percentages (pg. 14)

*Now:* “Our results confirm this major genetic subdivision, infant stage 4 MYCN NA NB exhibit a lower incidence of 1p36 chromosomal losses, and higher 11q LOH associated with 14q LOH with respect to infant MYCN A NB, significantly associated with distal 1p36 allelic loss”.

**Conclusions**

*Now:* “According to our preliminary results, distinct chromosomal aberrations may be reflected in gene expression profiles associated with spontaneously regressing or aggressive infant NB, and thus, with the distinct clinical behaviour. The identification of gene expression profiles associated with subgroups of infant disseminated NB warrants further investigation to identify sets of gene useful for better stratification of infant neuroblastoma. The potential to use this information must be tested in larger, prospective, cooperative trials.

5) Authors include MYCN amplified tumors in their analyses. It is known that MYCN amplification is observed in a fraction (30-35%) of unfavourable neuroblastomas and it results in distinctive effects on the tumor’s transcriptome, as Authors previously reported (Alaminos M, Mora J, Cheung NK, et al. Genome wide analysis of gene expression associated with MYCN in human neuroblastoma. Cancer Res 2003). Therefore, I would suggest to exclude MYCN amplified stage 4 tumors from the study because their contribute may give a bias.

The reviewer is right in raising this concern because in one of the pair-wise analyses carried out (stage 4s vs. stage 4 < 18m) we do see a less distinct expression pattern; one of the reasons could be to the presence of MYCN amplified cases. In fact, in the last analysis reported in the article, amplified stage 4 cases were excluded. Specifically, in this study we performed three pair-wise comparison analyses, first between stage 4s and stage 4 <12months, in this case owing to the small number of cases, stage 4 NB were analyzed together without distinction of MYCN gene status. Interestingly, the results of this analysis showed that all stage 4 tumours segregate together and displayed a distinct expression profile with respect to stage 4s NB. Furthermore, amplified stage 4 tumours were found mixed with non amplified stage 4 cases, displaying similar expression patterns. We then performed the same analysis shifting the age at diagnosis of stage 4 NB <18months, first including all stage 4 cases (both amplified as well as non amplified). This analysis was aimed at comparing the same NB subgroups of the first comparison, abovementioned, but introducing a different age cut-off for stage 4. All stage 4 tumours segregated together but with a less definite expression pattern within stage 4 tumours. Although amplified cases again segregated with the rest of stage 4, it could be due, as suggested by the reviewer, to the contribution of MYCN amplified cases. We therefore carried out a third pair-wise analysis where MYCN amplified stage 4 tumours were excluded from
comparison analysis with stage 4s. This last comparison showed a clear differential expression between the NB subgroups in study. Taken together, we think that despite the small number of cases and the enclosure of MYCN amplified cases, all three analyses provide interesting results.

6) Authors conclude that “the existence of a specific gene expression profile associated with 4S biology and the potential to use this information for better risk stratification should be tested in larger, prospective, cooperative trials”. Actually, Authors have not tested their classifier in an independent set of patients and the clinical relevance of their findings remains elusive: the application of rigid statistical methodologies to generate a predictor and testing of sufficiently large independent sets of tumors are necessary factors for the integration of gene expression-based prediction into clinical trials. An enlargement of cohort size could improve substantially this pilot study, in order to validate, in an independent test set of tumors, the accuracy of the gene expression-based outcome prediction proposed by the Authors. Moreover, a much larger number of cases would allow to estimate EFS and OS of patients with a favorable and an unfavorable gene signature.

Yes, our study represents a first approach or pilot study, as defined by the reviewer. In fact, here we have not proceeded with the identification of a specific gene-signature associated with infant NB outcome. The aim of this study was to investigate the existence and association of chromosomal alterations and differential gene expression amongst the subgroups of infant disseminated NBs, enabling to further understand of their distinct clinical behaviour. The identified differential gene expression profiles may be a useful source from which prognostic predictor candidates can emerge.

The conclusions have been modified. Now: “According to our preliminary results, distinct chromosomal aberrations may be reflected in gene expression profiles associated with spontaneously regressing or aggressive infant NB, and accordingly, with the distinct clinical behaviour. The identification of gene expression profiles associated with subgroups of infant disseminated NB warrants further investigation to identify sets of gene useful for better stratification of infant neuroblastoma. The potential to use this information must be tested in larger, prospective, cooperative trials.”

7) Methods used in the study do not look fully appropriate. As regarding genome analyses, Authors applied an allelic approach that gives limited information on patterns of gains and losses of selected loci (1p36, 1p34-p31, 1p22, 2p24.3 (MYCN), 11q, 14q, 17q). The manuscript would be greatly improved by performing high-resolution wide-genome analyses (i.e. MLPA or array-Comparative Genomic Hybridization), able to detect both structural and numerical chromosome aberrations. Moreover, the use of the B-Cell maturation factor (BCMA) gene (mapping at chromosome 16p13) as reference gene in real-time PCR assay for MYCN gene (mapping at 2p24.3) copy number quantification is questionable. It is known that favorable neuroblastomas are associated to numerical DNA changes and the use of a reference gene located on another chromosome may give false information. Therefore, the use of a reference gene mapping at the opposite arm respect to MYCN (i.e. MSTN mapping at 2q32.2) would permit to distinguish MYCN gains from numerical aberrations of chromosome 2.

We have noticed that in material and methods this point has not been clearly explained, we have thus inserted additional information and a reference.

Gene copy number quantification was performed as reported by De Preter, et al. Quantification of MYCN, DDX1, and NAG Gene Copy Number in Neuroblastoma Using a Real Time Quantitative PCR Assay. Mod Path 2002; 15(2):159-166. Specifically, the gene copy number was evaluated using the comparative C_T method using the following formula:

\[ 2^{-\Delta\Delta C_T} = \frac{(1 + E)^{-\Delta C_{text}}} {(1 + E)^{-\Delta C_{reference}}} \]
E = efficiency of the PCR reaction (set at default value 0.95)
ΔC_{T_{\text{gene}}} = difference in threshold cycle value between test sample and calibrator sample for the gene under investigation (test gene)
ΔC_{T_{\text{ReferenceGene}}} = difference in threshold cycle value between test sample and calibrator sample for reference gene.

As reported, this method uses the difference in C_{T} values between test and calibrator samples without requiring the construction of standard curves. The authors of this article show that both methods provide identical results.

The choice of the reference gene was also based on the abovementioned article. De Preter et al. report two reference genes, BCMA and SDC4, localized in chromosomal region that rarely show genetic abnormalities in NB (16p13 and 20q13, respectively). This choice is based on previous genomic hybridization results obtained on a large cohort of NB tumors. In their study they assess the validity of these genes as appropriate reference genes by determining the copy number ratio BCMA/SDC4 in DNA samples of leukocytes, NB tumors and cell lines, and confirm their validity as appropriate disomic reference genes in NB.

This article reported that the two reference genes, BCMA and SDC4, are localized in chromosomal regions that rarely show genetic abnormalities in NB (16p13 and 20q13, respectively). In our previous study on NB, results obtained using aCGH confirmed that chromosome 16 rarely displays chromosomal abnormalities, yet, in our experience, this was not so for chromosome 20. For this reason SDC4 gene was discarded as reference gene. Before using BCMA as reference gene we determined the copy number ratio (BCMA_{NB tumor test sample} / BCMA_{placenta calibrator sample}) between our NB tumors and the normal calibrator sample, in this case placenta samples. The observed ratio measured was equal to the ratio measured was equal to 1.0016; (tumour DNA 1.0012 ± 0.13 SD)/(placenta DNA 0.9996 ± 0.05). This validation was also performed with a set of normal peripheral blood samples as an alternative for the placenta samples (BCMA_{NB tumor test sample} / BCMA_{blood calibrator sample}). The ratio measured was 1.0019, values of blood samples ranged from 0.992 to 1.003. We think these results confirm the validity of BCMA as appropriate reference gene in our set of NB tumors.

Following reviewers’ suggestions “disomic reference gene” has modified to “reference gene”.

Finally, Authors carried out gene expression profiling using Affymetrix GeneChip U95 set. This is a “previous-generation GeneChip array” (as defined by Affymetrix web site) containing probe sets interrogating approximately 54,000 UniGene clusters derived from Build 95 of UniGene. Since on April 2001 Affymetrix released GeneChip Sets created from the Build 133 UniGene database, may Authors discuss the scientific rationale of still applying such old platform?

The reviewer is right, this is a previous generation array, yet, results obtained using the Affymetrix GeneChip U95 have recently been reported by several premier groups in NB investigation; amongst others:
Fujita T et al. JNCI 2008; 100 (3): 940-949.

8) Authors simply list genes differentially expressed in stage 4 and 4S tumors and perform a Gene Ontology annotation. The combination between expression data of the mentioned candidate genes and detailed genome information would strengthen the authors' aim to identify biological markers useful for accurate clinical classification of infant NB patients. In the manuscript Authors report results of Real-Time quantification for 6 of the differentially expressed genes. Which criterion did Authors follow to deeply investigate such small set of candidate genes?

Q-PCR expression analysis was performed as final proof to validate the expression differences observed in the microarray analysis.
We selected genes located at chromosomal regions of interest, chromosome 1p36 and chromosome 17 and that have been previously implicated in neuroblastoma biology. Among these, of particular interest are: CHD5 (1p36.31) Chromodomain helicase DNA-binding protein 5, recently reported a potential tumour suppressor and described with high expression in low risk NBs, in our case in stage 4s; PMP22 (17p11.2), protein comprised in peripheral nervous system myelin and in our case found with gene expression consistently higher in stage 4s NBs; NME1, nucleoside diphosphate (NDP) kinases located at 17q21.3, is one of the few genes located on chromosome 17 that showed higher expression levels in stage 4 NBs both < 12months as well as in stage 4 MYCN NA. As reported in our manuscript, three nucleoside diphosphate (NDP) kinases, two located on 17q23.2, NME1 and NME2, and NME4 on 16p13.3, exhibited increased expression levels in infant stage 4. The incremented expression of NME1 and NME2 has been previously associated with 17q gain, unfavorable NB and MYCN overexpression (45). Our results thus, support the assumption of a major role of nucleoside diphosphate (NDP) kinases in tumorigenesis of infant unfavorable NB.

9) The manuscript does not adhere to the standards for reporting and data deposition. Raw expression data and annotations should be deposited in a public database (such as GEO or ArrayExpress), that complies with the MIAME (Minimal Information About a Microarray Experiment) guidelines.

The public access of the data is being made available.

Minor Essential Revisions:

1) In Supplementary Table 2A the Probe set ID and the Gene title corresponding to PPP1R14BP1 and S100 genes are missing, respectively. Chromosomal locations are not reported for HIST1H3D, LOC 392666, NAV3 and S100 genes.

PPP1R14BP1: this probe set ID is not missing. 38618_at probe set recognizes both LIMK2 and PPP1R14BP1.

297_g_at: named as S100, has now been changed to TUBB, tubulin beta.

Chromosomal locations of HIST1H3D, LOC392666, NAV3 and S100 genes have been added.

2) In Supplementary Table 2A Authors miss to annotate 5 probe sets (namely, 297_g_at, 39157_at, 1842_at, 1838_g_at, 723_s_at). By using the Gene ID Conversion Tool, freely available at http://david.abcc.ncifcrf.gov web site, it is possible to annotate such probe sets as follows:

297_g_at: Homo sapiens, tubulin beta: Gene title, gene symbol and chromosomal location have been added.

39157_at: Homo sapiens, RET finger protein-like 3: Gene title, gene symbol and chromosomal location have been added.

1842_at: Homo sapiens, oncogene TLS/CHOP, fusion activated: Gene title, gene symbol and chromosomal location have been added.

1838_g_at: Homo sapiens, RAS-like protein TC21: Gene title, gene symbol and chromosomal location have been added.

723_s_at: Homo sapiens, small nuclear ribonucleoprotein, polypeptide C, alt. splice 2: Gene title, gene symbol and chromosomal location have been added.
3) In Supplementary Table 2B the Probe set ID and the Chromosomal locations corresponding to Acyl-CoA synthetase long-chain family member, AKR1C2 and LOC731709 genes are missing.

Acyl-CoA synthetase: probe set ID 33881_at recognizes ACSL3, located in 2q34-q35.

AKR1C2: AKR1C1 and AKR1C1 share both probe set ID and chromosomal location.
LOC731709: has been removed from the Table since it has been remove from Affymetrix Gene expression Query web site.

4) In Supplementary Table 2B Authors miss to annotate 10 Probe sets (namely 38396_at, 31494_at, 35087_at, 37060_at, 39750_at, 296_at, 32926_at, 297_g_at, 723_s_at, 1515_at). According to the Gene ID Conversion Tool such probe sets correspond to:

- 38396_at: microtubule-associated protein 1B (MAP1B): Gene title, gene symbol and chromosomal location have been added.
- 31494_at: hypothetical protein MGC29898: Gene title, gene symbol and chromosomal location have been added.
- 35087_at: CUG triplet repeat, RNA binding protein 2 (CUGBP2): Gene title, gene symbol and chromosomal location have been added.
- 37060_at: cDNA FLJ42813 FIS, clone BRCAN2012355: Gene title has been added.
- 39750_at: cluster INCL W61005:ZD29A11.S1 Homo sapiens cDNA, 3'end/clone=IMAGE: Gene title has been added.
- 296_at: tubulin, beta: Gene title, gene symbol and chromosomal location have been added.
- 32926_at: hypothetical protein FLJ43663: Gene title, gene symbol and chromosomal location have been added.
- 297_g_at: tubulin, beta: Gene title, gene symbol and chromosomal location have been added.
- 723_s_at: small nuclear ribonucleoprotein, polypeptide C, alt. splice 2: Gene title, gene symbol and chromosomal location have been added.
- 1515_at: RAD2: Gene title and gene symbol have been added.

5) In Supplementary Table 2C the Probe set ID and the Chromosomal location corresponding to SMN2 gene are missing.

SMN1 and SMN2, both share the Probe set ID and the Chromosomal location.

6) In Supplementary Table 2C Authors miss to annotate 1842_at Probe set. According to the Gene ID Conversion Tool it corresponds to “onco gene TLS/CHOP, fusion activated”.

Gene title, gene symbol and chromosomal location have been added.

Moreover, in Table 2C Authors report some wrong annotations, i.e.:
- 36260_at corresponds to “protein geranylgeranyltransferase type I, beta subunit
(PGGT1B)” and not to “mRNA from chromosome 5q21-22, clone:357Ex” as reported;

Gene title has been changed, and gene symbol and chromosomal location have been added.

- 40530_at corresponds to “amyloid beta precursor protein-binding, family B, member 2” (APBB2) and not to “Full-length cDNA clone CS0DC015YK09 of Neuroblastoma Cot 25-normalized of Homo sapiens” as reported;

Gene title has been changed, and gene symbol and chromosomal location have been added.

- 32049_f_at corresponds to “transcription elongation factor B (SIII), polypeptide 3” (TCEB3) and not to “cDNA FLJ42849 fis, A-BRHIP2004902” as reported.

We obtain the reported name, cDNA FLJ42849 fis, A-BRHIP2004902, when using Affymetrix web site, but “transcription elongation factor B (SIII), polypeptide 3” (TCEB3) on the http://david.abcc.ncifcrf.gov web site.

7) Authors erroneously report the number of differentially expressed genes identified applying different multiple testing corrections.

In Supplementary Table 2A Authors list 237 probe sets, corresponding to 232 annotated sequences and not 233 genes as reported in the text and Supplementary data legend (39762_at and 33288_i_at probes both correspond to ZNF263; 1980_s_at and 33415_at probes both correspond to NME2; 38907_at and 38908_g_at to NCDN; 32780_at and 32781_f_at to DST; 33458_r_at and 911_s_at to CALM2).

Supplementary Table 2A has been modified following the reviewers’ corrections:

Reviewed: 233 probe sets, corresponding to 230 annotated sequences. There are 12 probe sets that correspond to 6 genes (see table below) but also 4 probe sets that recognize two genes each (see table below).

<table>
<thead>
<tr>
<th>Probe Set</th>
<th>Gene Name</th>
<th>Chromosomal Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>33458_r_at</td>
<td>calmodulin 2 (phosphorylase kinase, delta)</td>
<td>CALM2 2p21</td>
</tr>
<tr>
<td>911_s_at</td>
<td>calmodulin 2 (phosphorylase kinase, delta)</td>
<td>CALM2 2p21</td>
</tr>
<tr>
<td>32780_at</td>
<td>dystonin</td>
<td>DST 6p12-p11</td>
</tr>
<tr>
<td>32781_f_at</td>
<td>dystonin</td>
<td>DST 6p12-p11</td>
</tr>
<tr>
<td>296_at</td>
<td>Homo sapiens, tubulin beta</td>
<td>TUBB 6p21.33</td>
</tr>
<tr>
<td>297_g_at</td>
<td>Homo sapiens, tubulin beta</td>
<td>TUBB 6p21.33</td>
</tr>
<tr>
<td>38307_at</td>
<td>neurochondrin</td>
<td>NCDN 1p34.3</td>
</tr>
<tr>
<td>38308_g_at</td>
<td>neurochondrin</td>
<td>NCDN 1p34.3</td>
</tr>
<tr>
<td>1980_s_at</td>
<td>non-metastatic cells 2, protein (NM23B) expressed in NME2</td>
<td>17q21.3</td>
</tr>
<tr>
<td>33415_at</td>
<td>non-metastatic cells 2, protein (NM23B) expressed in NME2</td>
<td>17q21.3</td>
</tr>
<tr>
<td>33288_i_at</td>
<td>zinc finger protein 263</td>
<td>ZNF263 16p13.3</td>
</tr>
<tr>
<td>33289_f_at</td>
<td>zinc finger protein 263</td>
<td>ZNF263 16p13.3</td>
</tr>
<tr>
<td>41055_at</td>
<td>KIAA0363 protein similar to KIAA0363</td>
<td>LOC392666 7p13</td>
</tr>
<tr>
<td>31693_f_at</td>
<td>histone 1, H2ad histone 1, H3d</td>
<td>HIST1H2AD HIST1H3D 6p21.3</td>
</tr>
<tr>
<td>1243_at</td>
<td>damage-specific DNA binding protein 2, 48kDa</td>
<td>DDB2 11p12- p11</td>
</tr>
<tr>
<td></td>
<td>LIM homeobox 3</td>
<td>LHX3 9q34.3</td>
</tr>
<tr>
<td>38618_at</td>
<td>LIM domain kinase 2 protein phosphatase 1, regulatory (inhibitor) subunit 14B pseudogene 1</td>
<td>LIMK2 22q12.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPP1R14BP1 22q12</td>
</tr>
</tbody>
</table>
In Supplementary Table 2B Authors list 231 probe sets, corresponding to 221 annotated sequences and not 224 genes as reported in the in the text and Supplementary data legend (33458_r_at and 911_s_at both correspond to CALM2; 32780_at and 32781_f_at to DST; 41720_r_at and 41719_i_at to FADS1; 38095_i_at and 38096_f_at to HLA-DPB1; 36773_f_at and 36878_f_at to HLA-DQB1; 656_at and 41524_at to INPP1; 1478_at and 1479_g_at to ITK; two probe sets to LOC731709; 37287_at and 37286_at to NRCAM; 296_at and 297_g to tubulin beta).

Supplementary Table 2B has been modified following the reviewers’ corrections: Reviewed: 224 probe sets, corresponding to 220 annotated sequences. 20 probe sets correspond to 10 genes (see table below), but 7 other probe sets recognize two genes each (see table below).

<table>
<thead>
<tr>
<th>Probe Set</th>
<th>Description</th>
<th>Chromosome</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>33458_r_at</td>
<td>Calmodulin 2 (phosphorylase kinase, delta)</td>
<td>CALM2</td>
<td>2p21</td>
</tr>
<tr>
<td>911_s_at</td>
<td>Calmodulin 2 (phosphorylase kinase, delta)</td>
<td>CALM2</td>
<td>2p21</td>
</tr>
<tr>
<td>32780_at</td>
<td>Dystonin</td>
<td>DST</td>
<td>6p12.1</td>
</tr>
<tr>
<td>32781_f_at</td>
<td>Dystonin</td>
<td>DST</td>
<td>6p12.1</td>
</tr>
<tr>
<td>41720_r_at</td>
<td>Fatty acid desaturase 1</td>
<td>FADS1</td>
<td>11q12.2-q13.1</td>
</tr>
<tr>
<td>41719_i_at</td>
<td>Fatty acid desaturase 1</td>
<td>FADS1</td>
<td>11q12.2-q13.1</td>
</tr>
<tr>
<td>1478_at</td>
<td>IL2-inducible T-cell kinase</td>
<td>ITK</td>
<td>5q31-q32</td>
</tr>
<tr>
<td>1479_g_at</td>
<td>IL2-inducible T-cell kinase</td>
<td>ITK</td>
<td>5q31-q32</td>
</tr>
<tr>
<td>656_at</td>
<td>Inositol polyphosphate-1-phosphatase</td>
<td>INPP1</td>
<td>2q32</td>
</tr>
<tr>
<td>41524_at</td>
<td>Inositol polyphosphate-1-phosphatase</td>
<td>INPP1</td>
<td>2q32</td>
</tr>
<tr>
<td>36773_f_at</td>
<td>Major histocompatibility complex, class II, DQ beta 1</td>
<td>HLA-DQB1</td>
<td>6p21.3</td>
</tr>
<tr>
<td>36878_f_at</td>
<td>Major histocompatibility complex, class II, DQ beta 1</td>
<td>HLA-DQB1</td>
<td>6p21.3</td>
</tr>
<tr>
<td>37287_at</td>
<td>Neuronal cell adhesion molecule</td>
<td>NRCAM</td>
<td>7q31.1-q31.2</td>
</tr>
<tr>
<td>37286_at</td>
<td>Neuronal cell adhesion molecule</td>
<td>NRCAM</td>
<td>7q31.1-q31.2</td>
</tr>
<tr>
<td>297_g_at</td>
<td>Tubulin, beta</td>
<td>TUBB</td>
<td>6p21.33</td>
</tr>
<tr>
<td>296_at</td>
<td>Tubulin, beta</td>
<td>TUBB</td>
<td>6p21.33</td>
</tr>
<tr>
<td>38095_i_at</td>
<td>Major histocompatibility complex, class II, DP beta 1</td>
<td>HLA-DPB1</td>
<td>6p21.3</td>
</tr>
<tr>
<td>38096_f_at</td>
<td>Major histocompatibility complex, class II, DP beta 1</td>
<td>HLA-DPB1</td>
<td>6p21.3</td>
</tr>
<tr>
<td>38095_i_at</td>
<td>Similar to HLA class II histocompatibility antigen, DP(W4) beta chain precursor</td>
<td>LOC731709</td>
<td>6p21.3</td>
</tr>
<tr>
<td>38096_f_at</td>
<td>Similar to HLA class II histocompatibility antigen, DP(W4) beta chain precursor</td>
<td>LOC731709</td>
<td>6p21.3</td>
</tr>
<tr>
<td>41243_at</td>
<td>Solute carrier family 35, member E2</td>
<td>SLC35E2</td>
<td>1p36.33</td>
</tr>
<tr>
<td>33881_at</td>
<td>Full-length cDNA clone CS0DF008YJ12 of Fetal brain of Homo sapiens (human) Acyl-CoA synthetase long-chain family member 3</td>
<td>ACSL3</td>
<td>2q34-q35</td>
</tr>
<tr>
<td>38095_i_at</td>
<td>Major histocompatibility complex, class II, DP beta 1 similar to HLA class II histocompatibility antigen, DP(W4) beta chain precursor</td>
<td>HLA-DPB1 LOC731709</td>
<td>6p21.3</td>
</tr>
<tr>
<td>38096_f_at</td>
<td>Major histocompatibility complex, class II, DP beta 1 similar to HLA class II histocompatibility antigen, DP(W4) beta chain precursor</td>
<td>HLA-DPB1 LOC731709</td>
<td>6p21.3</td>
</tr>
<tr>
<td>Probe Set</td>
<td>Description</td>
<td>Genes</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>32805_at</td>
<td>aldo-keto reductase family 1, member C1 (dihydriol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)</td>
<td>AKR1C1 AKR1C2</td>
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</tr>
<tr>
<td>41152_f_at</td>
<td>ribosomal protein L36a</td>
<td>LOC72936</td>
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<tr>
<td>38618_at</td>
<td>LIM domain kinase 2</td>
<td>LIMK2</td>
<td></td>
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<tr>
<td>ST4 MYCN NA</td>
<td>survival of motor neuron 1, telomeric</td>
<td>SMN1 SMN2</td>
<td></td>
</tr>
</tbody>
</table>

In Supplementary Table 2C Authors list 108 probe sets, corresponding to 107 annotated sequences and not 108 genes as reported in the in the text and Supplementary data legend (486_at and 487_g_at probe sets both correspond to CASP9).

Supplementary Table 2C has been modified following the reviewers' corrections:
Reviewed: 107 probe sets, corresponding to 107 annotated sequences. Even if 486_at and 487_g_at probe sets correspond to CASP9, probe set 37313_at recognizes two genes, SMN1 and SMN2.

8) I would suggest to avoid reporting the number of cases analyzed by each technique in Materials and Methods section (i.e. page 5 line 2, page 7 line 4). Such data should be provided only in Results.

As requested by the reviewer we have reported the number of cases analyzed only in the Results section.

9) Results section, “Patient and tumor characteristics”: this paragraph is very detailed and lengthy and it should be added as supplementary data, since such informations are not further discussed in the manuscript.

We think that this paragraph aids the understanding of “Patient and tumour characteristics” in the Result section.

Reviewer 2

Major remarks:
1. The gene expression profiling was done on the Affymetrix U95 platform (needs to be specified further: 95A, 95Av2, or all A through E arrays?). This platform is outdated and is no more available for some time. Since one of the authors (M. Alaminos) has been involved in a similar study using the same array platform (Alaminos et al., Cancer Res. 63:4538-4546, 2003), it could be suspected that the gene expression profiles presented here are partially or fully identical to the ones reported on earlier. While this would not totally preclude publication of this manuscript, it is still important information that should be given. Furthermore, the authors need to provide public access to their data; it
cannot be retrieved from the CAarray database, at least not by public access. Furthermore, the identifier of the data record needs to be given.

The reviewer is right and this manuscript describes the information obtained in our original microarray database regarding specifically the infant subgroup. Much information was obtained from those platforms that had not been fully investigated and lots of interesting information can be drawn from those datasets, for instance, when put together with other techniques like chromosomal aberration analysis or genomic DNA studies. The public access of the data is being made available.

2. The section on 'Gene expression data analysis' in the Methods part contains a number of flaws. The authors describe several methods for 'cut-off Family-wise error applied to select significant genes' (p.6, 2nd paragraph). From the methods that are described, however, i.e. step-down permutation, false discovery rate (FDR), and no type-I error adjustment, only the first controls the family-wise error rate (FWER). In fact, FWER and FDR are opposing, mutually exclusive concepts in statistics. Furthermore, analysis without adjustment for multiple testing (the 'raw' method) is not appropriate at all since the number of false positives will be unacceptably high just by random effects. It is also notable that the authors describe three methods but present results only on one, namely the 'raw' method. The 'raw' results should only be included for reference, but lists of differentially expressed genes may only be determined based on thresholds of FDR or FWER. The exact threshold (the authors name three: 0.01, 0.05 and 0.1) should be explicitly named.

We agree with the reviewer that methods without adjustment for multiple testing are not appropriate to select significant genes. Various screening approaches have been carried out to identify sets of differentially expressed genes; adjusting the type-I error for multiple tests (Step-down permutation (SDP), and False Discovery Rate (FDR), and with no type-I error adjustment (Raw method). Yet, only analyses performed with the RAW method yield sets of differentially expressed genes capable of clearly distinguishing amongst subgroups. This is potentially due to the small cohort of cases analyzed and may have taken to an overestimation of the differentially expressed genes. However, even though this study represents a first approach, the results suggest that infant stage 4 and stage 4s NB harbour distinct gene expression profiles, and these are associated with specific chromosomal alterations reported recurrently in NB tumours. This proves that the percentage of false positives in the final set of genes analysed is small enough not bias the result.

The rest of the statistical analysis was performed with adjustment for multiple analyses. In our text, the reason for using this procedure was not clearly explained; the text has now been modified (p. 6, see below).

"Genes with high variability within samples were selected, for screening purposes, by pair-wise comparison analyses performed by adjusting the type-I error for multiple tests (Step-down permutation (SDP) (24), and False Discovery Rate (FDR) (25), and with no type-I error adjustment (Raw method). The cut-off Family-wise error applied to select significant genes by means of the T-test for independent data, a univariate screening supervised procedure, were equivalent for all three methods: <0.1, <0.05 and <0.01. The aim of this screening procedure was to provide multiple sets of genes for hierarchical clustering analyses. The hierarchical clustering was performed using Ward’s algorithm with normalised data and the square of Euclidean distance as metrics; analyses were performed for the differentially expressed genes selected in the previous step, taking into account the relationship between gene expressions. Fisher’s exact test and 95% bilateral confidence interval using Wilson method were used to evaluate the proportion with which chromosomes were represented in the selected gene sets in comparison to chromosome representation within the Affymetrix GeneChip U95Av2. Statistical analyses were performed using SAS 9.1 and JMP 5.1 (SAS Institute Inc) for Windows and CIA 2.1.1".
3. There are a number of studies that addressed the same questions than the authors, namely Berwanger et al., Cancer Cell 2:377-386, 2002, Schramm et al., Oncogene 24:7902-7912, 2005, and Fischer et al., Clin. Cancer Res. 12:5118-5128, 2006. The findings presented here need to be compared in detail with the results from these previous studies.

Thanks for the interesting suggestions to improve our discussion of results. Few reports have been published on this subject and amongst these none perform a study exclusively on infant disseminated NB, two age cut-off (<12 and <18 months). In these studies, the cohort of stage 4 NB analyzed included all age patients, yet, as reported in literature infant stage 4 NB have a better prognosis than older children with stage 4 NB (Schmidt et al. J Clin Oncol 18(6):1260-1268, 2000).

In our text, we referred to some of these studies like the Schramm et al paper. Certainly, the Fischer et al manuscript is worth adding to the discussion. Both Berwanger et al., and Schramm et al. papers failed to identify a gene expression pattern characteristic of stage 4s versus stage 4 (all ages). Both studies included a small sample set of cases (9 each) and that was one of the reasons subsequent papers argued about their results. The paper by Fischer et al is clearly the largest but uses SAGE technology to identify the relevant set of genes and this extract was from only 8 cases (5 stage 4s and 3 stage 4 > 12 months). Afterwards a validation Q-PCR analysis was performed in a large group of cases (total of 76 samples) and identified from an arbitrary group of 41 selected genes, 18 which consistently discriminated between stage 4 and 4s. The conflicting findings of a discriminating profile by the group of Fischer et al compared to the previous mentioned reports was argued because of the SAGE technology or the sample size. They found overexpression of genes involved in neuronal differentiation as important genes in stage 4s tumours compared to stage 4. Among the genes identified by Fischer et al, Dystonin (DST) on chromosome 6p, and MAP7 on chromosome 6q were also identified in our list of genes (Table 2, supplementary data). Our report identifies a distinct gene expression profile between stage 4 and 4s using the same U95 Affymetrix microarray technology as Schramm et al., in agreement with the report of Fischer et al, but adds regional chromosomal expression patterns correlating with specific genomic abnormalities for each group of tumours. Correlations between genomic abnormalities and expression profiles have recently been reported in many tumour types including neuroblastoma by our group (Lavarino et al, BMC Med Genomics 2008), and are relevant to deciphering the intricacies of tumorigenesis.

4. The authors include tumors with MYCN amplification (MNA) from stage 4 into their analysis. As MNA will affect the expression of thousands of genes, this will significantly distort the gene expression profiles of stage 4 tumors. As a result, it is obvious from Additional File 2 that the lists for the comparisons: 4 (with MNA) versus 4S compared to: 4 (without MNA) versus 4S fail to show large overlap: only 38 probe sets are found in common among all three lists, and substantial numbers (96 probe sets for 4 vs. 4S < 12 months, 90 for 4 vs. 4S < 18 months, and 46 for 4 without MNA vs. 4S < 18 months) are only found in one of the three comparisons. The overlap between these analyses needs to be described, e.g. by a Venn diagram. The lack of overlap might be due to two different effects: first, the number of tumors investigated is small, thus the study lacks power to detect large proportions of the differentially expressed genes, and, second, the presented lists are highly likely to contain large numbers of false positives, see my comment #2 above.

In this study, we performed three distinct pair-wise comparisons, taking into account different parameters (different age cut-off and MYCN amplification) in each comparison. As a result, and considering, as suggested also by the reviewer, the small number of cases available for the
analyses, we did not expect a complete overlap of the identified gene list.

The Venn diagram shows how the highest proportion of overlap is found between the comparison 4s vs. 4 <12m and 4s vs. 4 <18m (124 genes, approximately 53-55% of genes), analyses which included MYCN amplified cases. This further confirms that MYCN amplification influences gene expression profiles. Yet, it is interesting to note in the hierarchical clustering, how amplified stage 4 tumours segregated intermingled with non amplified stage 4 cases, showing common gene profiles.

Given these results, we performed a third comparison (4s vs. 4 <18m MYCN NA) where amplified cases were excluded. Approximately 45-47% of the differentially expressed genes identified here overlapped either with one or the other of the abovementioned comparisons.

Taken together, as reported by the reviewer, 38 probe sets were found overlapping among all three gene lists.

The reviewer is right in raising this concern. Although our study strongly suggests that infant stage 4 and stage 4s NB harbour distinct gene expression profiles, the small cohort of cases may have taken to an overestimation of the differentially expressed genes. This study provides further insights into infant disseminated biology, but we are conscious that this information must be tested in larger, prospective, cooperative trials, as reported in the text.

As a whole, we think that despite the small number of cases and the enclosure of MYCN amplified cases, all three analyses provide interesting results.

This comparison of overlapping gene has now been included in the text (pg. 11); the Venn diagram together with gene lists as Supplementary Figure 2.

Minor remarks:

1. Were all samples obtained prior to chemotherapy? Samples obtained after initial chemotherapy should be excluded since cytotoxic treatment is likely to considerably affect the gene expression of many genes.

The study has been performed using only primary NB samples obtained at diagnosis. It has now been specified in material methods text (pg. 4).

2. The meaning of 'Hierarchical cluster analyses (...) using a multivariate unsupervised method, taking into account the relationships between gene expressions' (p.6, 2nd paragraph) is unclear. The authors need to report the distance metrics used (e.g., Euclidean distance, Manhattan distance or Correlation distance), and the algorithm for hierarchical clustering (single linkage, average linkage, complete linkage or Ward's).
This information has now been included in the text.

Now: "The hierarchical clustering was performed using Ward’s algorithm with normalised data and the square of Euclidean distance as metrics; analyses were performed for the differentially expressed genes selected in the previous step, taking into account the relationship between gene expressions."

3. How was the adjustment to FDR for the results from Fisher’s exact test performed (p. 7, 1st par.)? How many genomic regions were tested for association with the gene list?

Adjusted p-values are calculated using the step-up procedure of Benjamini and Hochberg (1995). Setting a cut-off at 0.05 for the adjusted p-value will control the False Discovery Rate (FDR) at level 0.05.

This point has been further detailed in the manuscript (p. 7 1st par.):

Now: “Overrepresented GO annotations were determined statistically by Fisher’s exact test; p-value cut-off was set at 0.05. P-values from Fisher’s exact test were adjusted for multiple testing (False Discovery Rate) using the Benjamini-Hochberg step-up procedure; cut-off was set at 0.05 for the adjusted p-value.”

4. In the discussion, p. 14, the authors speculate on prognostic relevance of their findings. They would need to demonstrate that the found gene expression changes are independent of other prognostic factors. It might turn out, e.g. that the differential expression of NME1 and NME2 is driven by some other factor (e.g. increases with age, or is driven by MNA) and thus is not itself causing tumor progression. In fact, with the current experimental design it is not possible to discriminate accidental changes driven by aberrant expression of some transcription factors from causal dysregulation that actively promotes aggressive behavior of tumors.

The aim of this study was to investigate the existence and the association of chromosomal alterations and differential gene expression amongst the subgroups of infant disseminated NB, enabling to further understand of their distinct clinical behaviour. The identified differential gene expression profiles may be a useful source from which genes, candidates of being prognostic predictors, can emerge. In fact, here we have not proceeded with the identification of a specific gene-signature associated with infant NB outcome being this a first approach or pilot study.

In the discussion, the conclusions have been modified as follows:

“According to our preliminary results, distinct chromosomal aberrations may be reflected in gene expression profiles associated with spontaneously regressing or aggressive infant NB, and accordingly, with the distinct clinical behaviour. The identification of gene expression profiles associated with subgroups of infant disseminated NB warrants further investigation to identify sets of gene useful for better stratification of infant neuroblastoma. The potential to use this information must be tested in larger, prospective, cooperative trials.”