Biological and clinical behavior of disseminated neuroblastoma has remarkably variable range from spontaneous (stage 4S) to fatal tumor progression (stage 4). In the present manuscript, Lavarino and co-workers focus their study on 35 infant disseminated neuroblastomas (25 stage 4 and 10 stage 4S). Authors perform allelic analyses of chromosomes 1p, 11q, 14q, 17q on 31 samples, and gene expression profiling of 8 stage 4S and 12 stage 4 (<12 months) / 19 stage 4 (<18 months) / 9 stage 4 (<18 months, MYCN not amplified) tumors. 

The aim of this work is to identify specific genetic abnormalities in order to provide novel prognostic markers that are able to predict accurately the outcome of patients suffering from metastatic neuroblastoma.

Authors describe allelic abnormalities of some neuroblastoma-related loci and patterns of genes differentially expressed in stage 4 and 4S tumors and they conclude that such chromosomal abnormalities and gene expression profiles associated with each infant NB subgroup can be used to distinguish spontaneously regressing stage 4S from infant stage 4 NB.

Most of findings mentioned in the paper are not particularly novel. Authors use an allelic analysis approach focused on chromosomes already known as aberrant DNA regions related to neuroblastoma tumor behavior (1p, 2p, 3p, 11q, 14q, and 17q). Moreover, it has been already reported distinctive gene expression signatures with strong prognostic effect in various cohorts also including infant patients with disseminated neuroblastoma (Spitz R. et al., Genes, Chromosomes & Cancer 2006; Fisher M. et al. Clin Cancer Res 2006; Mosse Y. et al. Genes, Chromosomes & Cancer 2007)

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 Center in 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?
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?

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.

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.

5) Authors include MYCN amplified tumors in their analyses. It is known that MYCN amplification is observed in a fraction (30-35%) of unfavorable 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.

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.

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.

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?

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?

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.

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, LOC392666, NAV3 and S100 genes.

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
39157_at: Homo sapiens, RET finger protein-like 3
1842_at: Homo sapiens, oncogene TLS/CHOP, fusion activated
1838_g_at: Homo sapiens, RAS-like protein TC21
723_s_at: Homo sapiens, small nuclear ribonucleoprotein, polypeptide C, alt. splice 2

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.

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)
- 31494_at: hypothetical protein MGC29898
- 35087_at: CUG triplet repeat, RNA binding protein 2 (CUGBP2)
- 37060_at: cDNA FLJ42813 FIS, clone BRCAN2012355
- 39750_at: cluster INCL W61005:ZD29A11.S1 Homo sapiens cDNA, 3'end/clone=IMAGE
- 296_at: tubulin, beta
- 32926_at: hypothetical protein FLJ43663
- 297_g_at: tubulin, beta
- 723_s_at: small nuclear ribonucleoprotein, polypeptide C, alt. splice 2
- 1515_at: RAD2

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

6) In Supplementary Table 2C Authors miss to annotate 1842_at Probe set. According to the Gene ID Conversion Tool it corresponds to “oncogene TLS/CHOP, fusion activated”. 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;
   - 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;
   - 32049_f_at corresponds to “transcription elongation factor B (SIII), polypeptide 3” (TCEB3) and not to “cDNA FLJ42849 fis, A-BRHIP2004902” as reported.

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; 38307_at and 38308_g_at to NCDN; 32780_at and 32781_f_at to DST; 33458_r_at and 911_s_at to CALM2).

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).

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).

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.

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.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:

I declare that I have no competing interests