Reviewer's report

Title: Verification of genes differentially expressed in neuroblastoma tumours; a study of potential tumour suppressor genes.

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Reviewer: Matthias Fischer

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Comments to the authors

This study describes the identification of transcripts down-regulated in neuroblastoma with unfavourable clinical courses. To identify putative candidates, the microarray technology was applied to a total of six neuroblastoma samples of either favourable or unfavourable outcome. A large set of transcripts were then evaluated by real-time RT-PCR using micro-fluid cards in five of the initial tumors and in additional twelve samples. A set of twelve genes were examined in a second verification cohort of 13 tumors by TaqMan assays. Finally, the sequence and the methylation status of one candidate gene (POU4F2) were determined to examine possible causes for its down-regulation in unfavourable neuroblastoma.

This study provides an elaborated strategy to identify transcripts down-regulated in adverse neuroblastoma, although the number of tumors analyzed in this study is limited. In general, the results and conclusions are in good agreement with the results of previous studies. However, there are several major and a number of minor critical points that have to be addressed prior to publication.

Major comments

1. The characteristics of the tumor samples used for identification of candidate genes by microarray analyses should be explained in more detail. It has to be noticed that two of the three favourable tumors were from stage 3 disease, one of which had in addition a diploid karyotype and the other an unbalanced loss of 1p. All these features are not “favourable” markers of neuroblastoma. On the other hand, these tumors were derived from patients <12 months, for which spontaneous regression has been described even in stage 3 disease. Thus, to help the reader to assess whether the biology of these tumors may represent a favourable neuroblastoma phenotype, clinical information about the treatment and the duration of follow-up of these two patients should be added. Moreover, the characteristics of the third tumor of the favourable subset should be included in table 1.

In addition, the majority (9/15) of unfavourable tumors of the whole cohort showed amplification of MYCN. It is well known that MYCN amplified tumors (constituting about one third of unfavourable neuroblastoma) exhibit a specific global gene expression profile that is different from other unfavourable neuroblastoma. Taking these facts together, the reviewer suggests that the
conclusions of this study may primarily refer to transcriptomic differences of MYCN amplified vs. non-amplified tumors, which should be discussed in the manuscript.

2. Comparing the QPCR results obtained from verification groups 1 and 2, one can observe a large difference in the fold-change of expression levels for several genes (e.g., POU4F2: 1517 fold-change in group 1, <4 fold-change in group 2). Although these discrepancies may be due to sampling differences, it is also possible that they result from a technical bias due to the distinct methods applied in the two groups. To rule out the latter possibility, the authors should re-analyze the expression of (some exemplary) genes in verification group 1 using the individual TaqMan assays utilized for verification group 2.

3. For the summary of the microarray results, the authors refer to reference 20 (Wilzen et al. 2008). In addition, they use this reference to explain their normalization strategy for QPCR analyses. There is, however, no journal name given in the references section, and the article cannot be found in PubMed. Thus, if the referenced manuscript has not been published so far, the microarray results and the development of the QPCR normalization strategy should be given in detail in the present study.

Minor comments
1. Gene aliases should be given in italics throughout the manuscript.

2. Punctuation of the manuscript should be reviewed (e.g., replace semicolon by comma at several positions of the manuscript).

3. Abstract, page 3: There is no clear evidence that real-time RT-PCR reveals more robust results than microarray-based techniques. If the authors do not provide arguments for this assertion, such statements should be avoided.

4. Introduction, page 4: Staging of localized neuroblastoma is based upon the local disease extension and degree of resection, not on tumour localization.

5. Introduction, page 4: According to recent publications, ALK is affected by mutations in about 10% of neuroblastoma cases and by amplification in about 5% of the cases.

6. Material and methods, page 11, paragraph Sequencing analysis: Replace “cleansed” by “cleaned”.

7. Results, page 13, and Discussion, page 15: According to table 3, the fold-change of SLC35E2 was larger than TFAP2B in verification group 2.

8. Discussion, page 15: In the sentence “In the first verification group …”, please replace “has” by “have”.

9. Discussion, page 16: The reviewer suggests to modify the sentence “In a larger whole-genome expression study …” as follows: “..., preliminary data shows CNTNAP2 to be one of the top-ranked genes differing between groups,
and follow up of those findings is ongoing.”

10. Discussion, page 16: In the sentence “It has recently been suggested ...” please add “in” before “an approach very similar to this study”.

11. Discussion, page 17: In the sentence “POU4F2 is a multi-functional protein ...” please add “in” before “the involvement of different forms of malignancies ...”.

12. Conclusions, page 18: The construction of the first sentence of this paragraph should be revised.

13. Conclusions, page 18: The reviewer suggests to modify the sentence “These transcripts are potential markers ...” in “Down-regulation of these transcripts is a potential marker of tumour progression”.


15. Legends to figures, figure 1: Replace “fourteen genes” by “twelve genes”.

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

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests.