Author’s response to reviews

Title: c.1810 C>T Polymorphism of NTRK1 Gene is associated with reduced Survival in Neuroblastoma Patients

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Author’s response to reviews: see over
Dear Dr Bucceri,

Please find below our response to the remarks and comments of the reviewers to the manuscript ref. MS: 359432080275473 entitled “c.1810C>T polymorphism of NTRK1 gene is associated with reduced survival in neuroblastoma patients” by Lipska BS, Drożyńska E, Scaruffi P, Tonini GP, Iżycka-Świeszewska E, Ziętkiewicz S, Balcerska A, Perek D, Chybicka A, Biernat W and Limon J. We are very grateful for competent and substantive comments of the reviewers. We hope that after thoughtful revision of our responses the manuscript will eventually be accepted for publishing in BMC Cancer journal. Below please find point-by-point response to the comments of the reviewers.

Sincerely,

On the behalf of the authors

Beata S. Lipska MD, PhD

**REVIEWER 1 - Kate Matthay (7624894682879337_comment.pdf)**

**REMARK 1.** It is of concern that the controls were germline DNA from peripheral blood, while all the cases were from primary tumors. The investigators should at least verify in some of the cases that the SNP variant was germline, particularly since no link found to incidence of neuroblastoma. There are many cooperative tumor banks in the US and Europe with both germline and tumor DNA.
In accordance to the request of the Editor and both Reviewers we have performed a nested study of the corresponding germline DNA for selected cases positive for the presence of the c.1810T allele. An RFLP analysis was performed (see Fig A. below) and the findings were confirmed by direct sequencing. In all analyzed cases we have identified presence of the c.1810T polymorphism at constitutional level. This, along with the results of our statistical analyses, suggests that detection of c.1810T allele may be of prognostic significance for the eventual outcome of the disease but is not related to the incidence of the NB. Plausibly, c.1810T allele modifies activity of the TrkA receptor and therefore may result in a more aggressive phenotype of the disease.

In the revised version of the manuscript in the Results section following sentence has been added:

*A nested study of the corresponding germline DNA confirmed presence of the SNPs at constitutional level.*

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**FIGURE A.**

RFLP analysis of the *NTRK1* c.1810C>T polymorphism (*Eco130I*). All of the analyzed cases were found heterozygote for *NTRK1* c.1810C>T polymorphism. The results were confirmed by direct sequencing.

**LEGEND:** T1-T3 DNA from tumor samples; C1-C3 corresponding constitutional DNA (peripheral blood); K+ positive control (heterozygote c.1810CT); K- negative control (homozygote c.1810TT); M – size marker.
REMARKS 2 and 3. The patient population differs widely from the two sites, Poland and Italy; the Italian group consists mainly of low stage, younger patients, but, oddly, has a higher proportion of unfavorable histology (Table 1). The reasons for the discrepancy in the patient population and the reason for the high proportion of UH in the otherwise more favorable Italian group should be clarified. The possible effect of the skewed population on the overall analysis should be addressed in the Discussion.

It has to be underlined that the submitted work is a retrospective analysis of the patients whose data were collected by two national tumor banks over the period of 20 years (Italian group) and 9 years (Polish group). Since NB is a relatively rare disease (10.9 cases per million children yearly) in order to collect sufficient number of cases the patients have to be enrolled into the study over a long period of time. On the other hand, over the years treatment protocols, histopathological classification etc are being constantly modified. In our work, in order to overcome this limitation, patients representing two European populations were enrolled in the study after initial validation of their correspondence using the Cochran-Mantel-Haenszel analysis. Histopathological assessment of the two groups differed. The Polish group was re-evaluated in 2007 in accordance to the revised version of INPC, while a portion of oldest samples from the Italian group was no longer available for such reassessment. We are aware that the divergences between the two populations and the fact that the study was retrospective might decrease the power of the study.

In accordance with the aforementioned due sentences have been added to the Discussion section: The additive value of c.1810T allele to the current clinical prognostic markers should be however assessed on a prospective, more coherent, group of patients. In the current study patients were enrolled in two countries over 20 years, thus they received a range of treatment protocols. Also, histopathological assessment was modified over the time and not all the samples could be assessed in accordance to the revised INPC. The above presented discrepancy in the patient population and the fact that the study was retrospective might however result in some limitation of the power of study.
**REMARK 4.** Results for aberrations of 11q are not provided, and these would be very interesting in understanding whether the SNPs in TRK in the younger patients are independently prognostic in the MYCN non-amplified group.

We agree with the Reviewer that, in view of the finding that c.1810T allele is the most informative in the group of youngest patients with no MYCN amplification, it might be interesting to see whether there is an association with 11q aberrations, which are recently recognized as an adverse marker in MYCN non-amplified patients (Attiyeh et al. Chromosome 1p and 11q deletions and outcome in neuroblastoma. *N Eng J Med* 2005, **352**:2243-53). Unfortunately, 11q status was not available either for Polish or for Italian patients. For the Polish group we had data on the occurrence of chromosome 17q trisomy (n=55 cases), however no statistically significant correlation with clinical outcome or c.1810T allele was found in relation to this parameter.

**REMARK 5.** It is unusual that the stage 4 vs not-4 is not significant in multivariate analysis, and one wonders if this relates to the skewed population in the Italian group. On the other hand, stage 4 was significant for recurrence; please address this in discussion.

In the univariate analysis we have identified stage 4 (p=0.0001; 5yOS 48.9%) along with MYCN amplification (p=0.0001; 5yOS 46.2%), age >18months (p=0.0006 5yOS 56.0%) and c.1810T allele (p=0.02 5 5yOS 26.3%) as prognostic markers of reduced survival (log-rank test). 1p deletion was at the borderline with p=0.052.

Then, we have performed the multivariate analysis in which we have assessed a number of Cox hazard models. Firstly, a model inclusive of NTRK1 genotypes and adjusted for classical risk factors (7 variables – Table 2) was assessed.

Secondly, in the backward elimination we have reduced number of variables excluding those not significant in order to obtain a minimal set of meaningful parameters which would provide most accurate prognostic model. Stage 4 vs. earlier stages was the first variable removed from the model based on a lack of significance. The most probable explanation for this finding is a strong association between stage and MYCN amplification (43% of patients with stage 4 harbored MYCN amplification in their tumors.
versus only 22% of patients with earlier stages; p=0.001 by Fisher’s exact test) and a strong association between stage and age<18 months (only 25% of patients with stage 4 were under 18 months versus 72% of patients with earlier stages; p=0.006 by Fisher’s exact test). We did not observe an association between MYCN amplification and c.1810T polymorphism (p=1.00 by Fisher’s exact test) or between age and c.1810T polymorphism (p=1.00 by Fisher’s exact test). Taken together, we believe that c.1810T provides additive information to MYCN amplification status and age of the patient allowing for finest prediction of the eventual outcome of the disease. The sentence explaining lack of significance of stage in the multivariate model due to associations with other prognostic variables was added to the manuscript.

In the revised version of the manuscript following sentences have been added:

1. In the Results section: *Inclusion of the information on stage of the disease did not improve the model (data not shown). This finding is probably due to a strong association of stage and MYCN amplification (43% of patients with stage 4 harbored MYCN amplification in their tumors versus only 22% of patients with earlier stages; p=0.001 by Fisher’s exact test) and a strong association between stage and age<18 months (25% of patients with stage 4 were under 18 months versus 72% of patients with earlier stages; p=0.006 by Fisher’s exact). We did not observe an association between MYCN amplification and c.1810T allele (p=1.00 by Fisher’s exact test) or between age and c.1810T allele (p=1.00 by Fisher’s exact test).*

2. In the Discussion section: *From the analyses of the Cox hazard regression models it appears that c.1810T provides additive information to MYCN amplification status and age of the patient allowing for finest prediction of the eventual outcome of the disease. Interestingly, the stage of the disease was less informative, and could be omitted in the model based on minimal set of predictive parameters. The lack of prognostic significance of stage is most probably due to strong associations with other prognostic variables – MYCN amplification and age. These associations were not observed for c.1810C>T polymorphism.*

**REMARK 6.** Despite the clinical and biological differences in the two groups of patients, there was no significant difference in the MAF frequency; this should be explained.
MAF did not differ significantly between Polish, Italian and control groups. Also data retrieved from HapMap project showed no differences of MAF in four representative human populations (CEPH, Nigerian, Chinese, Japanese). We propose that the presence of c.1810T allele is not related to the predisposition to neuroblastoma, however if present in a patient it may modify the course of the disease. Presence of amino acid substitution may result in the worse functioning of the TrkA receptor (its TK domain) and thus poorer transmission of the signal to differentiate/ regress – yet our working hypothesis requires further experimental studies for confirmation.

**REMARK 7.** *With the very small number of patients in the group with the 1810CT, TT (n=12) it is difficult to state with confidence that this minor allele is not associated with other risk factors and also to do a multivariable analysis!*

We are aware of the limitations of our study and in various sections of our manuscript we were very reluctant to draw definite conclusions.

In our study c.1810T allele has been detected in 8.7% (12) of sporadic tumors (n=169) what is a similar frequency to the mutations observed for the two known NB susceptibility genes (*ALK* = 12.4% [ref 24], *PHOX2b* = 4.3% [ref. 25]). These studies were conducted on a similar number of cases: ref. 24 n=194; ref. 25 n=69. Unfortunately, the relative rarity of the disease is the limiting factor for the size of the analyzed group what we have tried to overcome by performing a joined study on two European populations.

**REMARK 8.** *This work should be verified with a GWAS or larger number of patients.*

We are aware that the presented study is a pilot one and should be confirmed on a larger group of patients in a prospective study. We also hope that our data will be confirmed in a genome-wide association study yet currently such a study is out of the scope and possibilities of our group.

**Minor Comments 1.** *The number of patients in each group in the Kaplan Meier curves must be indicated in the legend, hard to read on the figure.*

The figure has been corrected in accordance with the Reviewer’s suggestion.
REMARK 1. *It does not appear clearly in the text how frequently this allele was present in the control population. The authors state in the conclusion that this polymorphism does not predispose to neuroblastoma development but I do not understand on which data this statement is based.*

The data on the frequency of the analyzed SNPs in the control population are presented in detail in Table 2 – Electronic supplementary material. In this table not only the frequencies of the SNPs are summarized, but also results of the comparison of their incidence in the analyzed groups of patients vs controls are given. There were no statistically significant differences in MAF between patients and unaffected controls. Since we did not observe higher incidence of c.1810C>T *NTRK1* polymorphism in the patient population we presume that the polymorphism does not predispose to NB development.

In order to clarify our findings in the Results section of the manuscript following sentence has been added: *There were no statistically significant differences in MAF between patients and unaffected controls (Table 2 Electronic supplementary material).*

REMARK 2: *In addition, a nested study of constitutional DNA in patient with the polymorphism present in the tumor should be added to ascertain if the polymorphism is not constitutional.*

Please refer to our response to Remark 1 by Reviewer 1.

REMARK 3. *Surprisingly, stage 4 does not appear to be prognostic in the multivariate analysis. The authors should provide an explanation for that.*

Please refer to the answer to the Remark 5 of the Reviewer 1.

REMARK 4. *Finally, some indications on the treatment received by the patients should be provided, at least as per protocol in order to allow the readers to judge if their results could be generalized.*
Since the patients were enrolled over the period of 20 years (Italian group) and 9 years (Polish group) there was a discrepancy on the treatment protocols. For instance in the Italian group 45 cases were treated by Infant protocol, 30 cases by NB92, 15 by Lneseg ad Interim and a few underwent NB97, NB85, NB79, Unresectable and NB-AR-01 therapies. We understand that such a discrepancy may influence the power of the study, yet still the presence of the SNP was independent (random) of the protocol and thus the effect of the treatment could bias our study both in favorable and unfavorable way. We are aware however that the results of our study should be confirmed in a new, prospective study carried on a more coherent group of patients (see also response to Remarks 2-4 of the second Reviewer).

In view of the above in the revised version of the manuscript following sentences have been added to the Discussion section: The additive value of c.1810T allele to the current clinical prognostic markers should be however assessed on a prospective, more coherent, group of patients. In the current study patients were enrolled in two countries over 20 years, thus they received a range of treatment protocols.