Author’s response to reviews

Title: c-MYC expression sensitizes medulloblastoma cells to radio- and chemotherapy and has no impact on response in medulloblastoma patients

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Author’s response to reviews: see over
Dear Prof. Dr. Norton
Dear PD Dr. Zips

Thank you for your e-mail of November 26, 2010 informing us of the potential acceptability of our paper "c-MYC expression sensitizes medulloblastoma cells to radio- and chemotherapy and has no impact on response in medulloblastoma patients" by von Bueren A et al. for publication in BMC Cancer pending suitable emendations in response to the reviewer's comments.

Please find our comments and changes listed below. Bold text indicates sentence from the paper. New additions and/or changes are underlined.

Following issues were addressed by the Reviewer 1:

**Comment 1:** Fig. 1-5: Statistics are missing; which differences are statistically significant? Statistical differences must be calculated from independent experiments, not from sixplicates or triplicates from one representative experiment.

**Response:** Figures 1-5 illustrate our evaluation/validation of our previous findings (von Bueren et al., BMC Cancer, 2009). siRNA-mediated down-regulation of c-MYC resulted in a decrease of apoptotic activity and sensitivity to radio- and some chemotherapeutic agents.

By using two medulloblastoma cell lines engineered to stably over-express c-MYC we were able to validate these results using different assay. We completed the statistical analyses and added a statement, methods section, page 11, statistical analysis, second sentence: **Differences between groups were examined for statistical significance using the unpaired Student's t-test.**

**Figure 1:** MTS assay (radio-sensitivity). We indicated which measurements are statistically different in figure 1. For specification we added a statement, figure legend section, page 26, figure 1, last sentence: **Student's t-test compared DAOY M2 and UW2282 M13/irradiated cells vs. DAOY V11 and UW228 V1/irradiated cells (***: P<0.001, **: P<0.01, *: P<0.05).**
Figure 2: MTS assay (chemo-sensitivity); statistical analysis has been extended as described previously (von Bueren et al., BMC Cancer, 2009 = Reference 8, and D’cunja J et al. Eur J Cancer. 2007). For clarification we indicated also the p-values in figure 2 (IC 50 and confidence intervals were already indicated) and we added a statement, figure legend section, page 26, figure 2, last sentence: Differences between the two curves are represented by P-values (P<0.001, P<0.01, P<0.05, n.s. not significant). We would like to mention that we have used many different treatment concentrations for each individual drug, increasing our confidence about the dose response curves.

Figure 3: We indicated which measurements are statistically different in figure 3 and we added a statement, figure legend section, page 27, figure 3, last sentence: Student’s t-test compared DAOY M2/treated cells vs. DAOY V11/treated cells (***: P<0.001, **: P<0.01, *: P<0.05).

Figure 4: (please refer to our response to comment 2).

Figure 5: We indicated which measurements are statistically different in figure 5 and completed the figure legend of figure 5, figure legend section, page 27, figure 5, last sentence: Student’s t-test compared DAOY M2/treated cells vs. DAOY V11/treated cells (***: P<0.001, **: P<0.01, *: P<0.05).

We agree with the reviewer that a statistical analysis of independent experiments would be preferable. However, our statistician (Prof. Dr. Seifert, Biostatistics Unit, University of Zurich) advised us not to measure statistical differences over two independent experiments as we would need (3)4-6 different experiments. Moreover, as the laboratory moved recently in another building, experimental conditions have changed, making comparisons between repetitions and former experiments more difficult. Our statistician does not advice to do so.

We feel confident about our results as c-MYC expression showed - using different assays (MTS assay and clonogenic survival assay) - comparable effect on cell sensitivity to chemo- and radiotherapy (figures 1-3). Induction of apoptosis has been investigated using three different methods (figure 4-6) as each of the methods to detect and measure apoptosis has its advantages and limitations. DNA fragmentation is a hallmark of apoptosis and was measured with Cell Death ELISA assay by quantification of histone complexed DNA fragments. Apoptotic cell death was also analyzed using propidium iodide to profile DNA content. Cells with lower DNA staining than that of G1 cells have been considered apoptotic. Finally, we assessed caspase 8 and 9 expression and cleavage using western blotting upon treatment with IR, cisplatin, and etoposide at 7 different treatment times (0, 3, 6, 12, 24, 48, and 72 hours). Assessing 7 different treatment times increases our confidence of the findings reported here. Moreover, these results – using medulloblastoma over-expressing c-MYC - confirm previous data using an approach to inhibit c-MYC expression in DAOY, D341, and D425 medulloblastoma cells.

As c-MYC expression appears not to have an impact on response in medulloblastoma patients, we think that additional in vitro experiments/repetitions will not provide a conclusive answer for the role of c-MYC in modulating treatment response. It needs to be tested whether c-MYC expression may modulate the response to treatment (IR and chemotherapy) in xenograft models and/or in a larger medulloblastoma patient cohort.

Comment 2: Fig. 4: How many independent experiments were performed? Standard deviations and statistics are missing.
Response: Figure 4 illustrates the treatment-induced cell cycle alterations in DAOY MB cells expressing different levels of c-MYC. The percentages of apoptosis, measured as hypodiploidity in flow cytometry, has also been determined. Two independent experiments have been performed with the treatment conditions explained in the figure legend (figure 4). Accordingly, we added the standard deviations in figure 4 and a statement in the figure legend section (figure 4), page 27, lines 6-7: The percentages of cells in G1, S, G2/M, and sub-G1 phases of the cell division cycle are indicated ± standard deviation (n=2).

Moreover, we clarified/specified our aim of this experiment. We added a statement in the results section, page 13, Irradiation-, cisplatin-, etoposide-, and doxorubicin- induced cell cycle alteration in DAOY MB cells expressing different levels of c-MYC, first sentence: To study whether treatment-induced effects on cell cycle progression and induction of apoptosis differ in DAOY cells, we exposed DAOY (wt, V11, and M2) MB cells to IR.

The major finding was that treatment-induced apoptosis was more evident in DAOY M2 (high c-MYC expressing) cells. Additional experiments to analyse induction of apoptosis - in DAOY medulloblastoma cells expressing different level of c-MYC - have been performed using different assays (Cell Death ELISA assay, caspase 8 and 9 expression and cleavage using western blotting; please refer to response to comment 1). Thus, we performed for proof of principle these experiments, showed reproducibility, and we validated the data using different assay.

Comment 3: Fig. 6: How many independent experiments were performed? What means IR dose/Cisplatin/Etoposide in hours, time after IR/ cisplatin/etoposide treatment (h)?

Response: Figure 6 illustrates an evaluation of caspase 8 and 9 expression and cleavage using western blotting at 7 different treatment time points.

For clarification we specified figure 6:

Figure 6A: Time of treatment (hours): IR, 6 B: Time of treatment (hours): cisplatin; 6 C: Time of treatment (hours): etoposide

Two independent experiments were performed. Accordingly, we added a statement in the figure legend section, page 28, figure 6, last sentence: Representative blots (two independent experiments) are shown.

Following issues were addressed by the Reviewer 2:

Comment 1: The title is relatively misleading. The phrase “Moderate effect . . .” does not adequately express observed differences between in vivo and in vitro effect of high MYCC mRNA expression on irradiation or cytostatic treatments.

Response: For clarification, we changed the title: c-MYC expression sensitizes medulloblastoma cells to radio- and chemotherapy and has no impact on response in medulloblastoma patients.
Minor Essential Revisions

Comment 1: Abstract, Results, line 5: I would prefer “similar in primary MB samples of responders …”

Response: For specification, we added this additional information. Abstract section, page 3-4, last 3 lines: *c-MYC mRNA expression was similar in primary MB samples of responders and non-responders* (Mann–Whitney U test, \( p = 0.50 \), ratio 0.49, 95% CI 0.008-30.0 and \( p = 0.67 \), ratio 1.8, 95% CI 0.14-23.5, respectively).

Comment 2: Abstract, Conclusion, line 1: “c-MYC sensitizes MB cells to some anti-cancer treatment in vitro.

Response: For clarification, we added this information, abstract section, page 4, conclusions, lines 1: *c-MYC sensitizes MB cells to some anti-cancer treatments in vitro.*

Comment 3: Methods, Part MB patients and therapy: It should be clarify that FFPE MB samples were pretreatment (i.e. not second surgery samples).

Response: For clarification, methods section, page 10, MB patients and therapy, second paragraph, last three lines: *All tumors - analyzed for c-MYC mRNA expression (n=68) - were resected from patients who had not received any treatment before. Postoperative residual tumor was evaluated by MRI or CT within 72 hours after surgery.*

Discretionary Revisions

Comment 1: page 15, line 19: the source of reference human cerebellum RNA should be Noted.

Response: We added the reference indicating the source of human cerebellum RNA, page 15, results section, Response of postoperative residual MB tumors to radio- and chemotherapy and outcome according to c-MYC mRNA expression, line 11: *The amount of c-MYC mRNA expression was related to human cerebellum [13]* (Reference 13 = Rutkowski et al., Clinical Cancer Research, 2007), and a cut-off value of 1 was chosen to define patient groups with high or low expression of c-MYC, as described elsewhere [13].

Comment 2: I would recommend using more than only two MB cell lines, and to make also transient transfections to exclude the possibility that observed effects could be caused by deregulation of other genes due to incorporation of plasmid with strong CMV promoter.

Response: We agree entirely with the concern that stably transfected cell lines may exhibit a phenotype derived from "off-target" effects of aberrant transcriptional deregulation derived from
constitutive activation from the inserted CMV promoter and that using additional MB cell lines over-expressing c-MYC would strengthen the observation we made. However, the identical experimental results were obtained using siRNA-mediated c-MYC down-regulation in D341, D425 and DAOY MB cells (two MB cell lines that over-express c-MYC due to gene amplification; von Bueren et al., BMC Cancer, 2009 = Reference 8). We feel that this reduces the risk that our results stem from changes in gene expression that are not related to c-MYC.

Additional changes: We added the sentence in the material and methods part, page 7, Human MB cell lines, lines 9-11: It has been shown that mRNA expression of DAOY M2 and UW228 M13 cells are comparable to those seen in the upper quartile of primary human MB tumors [23].

In summary, we think that changes according to the reviewer’s comments improved the quality and clarity of the paper. We hope you find the emended revision of our paper acceptable for publication in the BMC Cancer and we look forward to hearing from you in the near future regarding the disposition of this manuscript.

Yours sincerely,

André von Bueren