Dear Dr. Puebla,

We appreciate the reviews of our manuscript and have responded to each of the issues raised by the referees. As instructed, we have uploaded the revised version entitled “Medulloblastoma outcome is adversely associated with overexpression of EEF1D, RPL30, and RPS20 on the long arm of chromosome 8.” Each of the referees’ comments and our responses are detailed below.

Dr. De Preter noted several major and discretionary revisions that we have addressed and responded to in our manuscript.

• **Major Compulsory Revision 1** - “Page 11: The authors performed (or show) survival analysis on a small subset of cytogenetic changes that have been identified. Please provide also the p-values for OS and EFS log-rank test for all other cytogenetic changes, e.g. in Table 2.”  

  -We have now listed the p values for overall survival (OS) and progression-free survival (PFS) log-rank test for all CNA in Table 2 on p.26.
Major Compulsory Revision 2- “Page 15: I don't understand why you can not perform multivariate analysis on all CNAs in order to find the CNA with highest prognostic significance.”

As suggested, we performed multivariate analysis of significance for 8q gain with respect to clinical variables that are widely accepted as prognostically significant: age relative to 3 years old and metastatic stage at diagnosis, and the degree of primary resection. As described in the revised text (on p.12 of the Results and in the Methods sections), our results confirm the prognostic significance of 8q gain for OS and PFS (p=0.013 and p=0.003, respectively). Further details are shown in the Supplemental Tables below.

**SUPPLEMENTAL DATA**

Multivariate Analysis of 8q Gain for Overall Survival Controlled for Other Prognostic Variables.

<table>
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<th>Variables in the Equation</th>
<th>B</th>
<th>SE</th>
<th>Wald</th>
<th>df</th>
<th>Sig</th>
<th>Exp(B)</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CGH8q</td>
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<td>1</td>
<td>.464</td>
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</table>

<table>
<thead>
<tr>
<th>Variables in the Equation</th>
<th>B</th>
<th>SE</th>
<th>Wald</th>
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<tbody>
<tr>
<td>Progression-free Survival</td>
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<td>.510</td>
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</table>

It is common to test if factors of interest are significant for prognosis in univariate analysis before applying them in multivariate analyses such as a Cox proportional hazard model. Since we detected 8q gain as the only prognostically significant CNA, we did not have other CNAs to test in a multivariate model controlling for other factors.

As suggested, we also performed multivariate Cox model analysis for all CNAs detected at greater than 7% frequency together as a group. We confirmed the 8q gain is significantly associated with overall survival (p = 0.003) in a multivariate analysis with other CNAs.

We have elected to reserve these Tables as Supplemental Data as mentioned on p.12. Together, these multivariate analyses further support our conclusion that gain of 8q is significantly associated with clinical outcome in our series of medulloblastoma patients. Nonetheless, we have added the following caveat in the Discussion on p.16:

“Due to the relatively low frequency of 8q gain, its prognostic significance requires confirmation in studies with larger sample size.”

**Major Compulsory Revision 3**- “Table 4: The header of column 5 is not clear enough: mention that this is the ratio with CNA/without CNA. Do the values in column 6 represent the expression ratio of samples with versus without CNA, or not? If so: how can you explain the high value for MYC, but not significant and is the statistical comparison of 2 samples with CNA compared to 10 samples without CNA reliable? If not: what does these data tell us?”

-We have clarified Table 4 on p. 28 to indicate that the ratios in Column 5 reflect relative expression levels in tumors with vs. without a given CNA as determined by microarray analysis. The ratios in Column 6 also reflected relative expression levels in tumors with vs. without a given CNA, but by qRT-RTPCR. This column has been deleted since we agree with the Reviewer that such comparison of two tumors to 10 others holds limited statistical significance and thus provides little additional information. As described on p.13, one of the two specimens
with 8q gain had 8q amplification and a markedly elevated expression level, which skewed the average and the high variance is reflected in the t-test value. These values and their correlation with microarray data were originally provided simply to indicate concordance between the two assay methodologies as indicated by the high correlation values ($R^2$) as noted on p.13.

**Major Compulsory Revision 4**- “Page 13: Have you used a statistically based algorithm in order to identify the genes that are differentially expressed in the 2 subgroups, e.g. SAM?”
-We considered just such an approach, but given the constraints of our CGH dataset, we finally did not employ SAM in order to minimize the risk of false discovery balanced against the risks of false negatives. Because of the limited number of cases with expression data that displayed 8q gain, permutation procedures such as in SAM (while certainly a valid approach) might be too stringent and may lack sufficient power to detect differences. In addition, since we tested the expression of fewer candidate genes (i.e. those mapped to 8q), the need for controlling multiple testing is less urgent than if we were testing all of the genes represented on the microarray. For these reasons, we employed a parametric t-test implemented in dCHIP software to identify differentially expressed 8q candidate genes. Afterwards, since our main goal was to identify only those prognostically significant genes mapped to 8q, we further tested their significance by log-rank testing, as noted on p.14.

**Major Compulsory Revision 5**- “Comment on the fact that the small expression changes that you notice for the three genes in CNA samples compared to non-CNA samples might only be due to dosage effect (more copies of the genes give slightly higher expression) and are possibly not involved in medulloblastoma oncogenesis.”
-We agree that gene dosage effects due to gain or loss of large chromosomal regions can certainly result in minor expression changes. For this reason, we analyzed our list of differentially expressed candidate genes for clinical significance in a larger group of tumor specimens (the majority of which did not exhibit the CNA in question). Although the distinct possibility remains that in those tumors with 8q gain display overexpression of 8q-mapped genes as a result of dosage effects, the clinical significance of those genes reflects many more tumors without 8q gain. That is, overexpression of $EEF1D$, $RPL30$, and $RPS20$, regardless of 8q status, are adversely associated with clinical outcome. Together, these data support a hypothesized role for $EEF1D$, $RPL30$, and $RPS20$ in medulloblastoma growth. We have added comments to this effect on p.14.

The following Discretionary Revisions are also addressed in the revised manuscript.

**Discretionary Revision 6**- “Page 8: Why do you compare the qRT-PCR results with the U133Plus2.0 data, and not with the HuGeneFL microarray data?”
-Unfortunately, we were unable to perform expression profiling of recent specimens on old HuGeneFL microarrays and older specimens are no longer available for repeat analysis on U133Plus2.0 microarrays, as noted on p.14.

**Discretionary Revision 7**- “Page 17: Please give more explanation about the possible role of the genes with ribosomal function in cancer.”
-We have included the following additional text in the expanded Discussion section (pp.16-17) to address the potential function of $EEF1D$, $RPL30$, and $RPS20$ in medulloblastoma growth:

These candidate genes represent a novel group involved in translational regulation and have not been previously associated with outcome in medulloblastoma. Although $RPL30$ expression was previously reported to be associated with classic histology, the smaller expression differences precluded prior detection and association
with survival [Pomeroy]. In that study, we employed a two-class comparison of array profiles based on an unsupervised clustering algorithm (self-organizing map) to define a multi-gene predictor of adverse outcome. The multi-gene predictor included several other ribosomal genes, but did not include $\textit{EEF1D}$, $\textit{RPL30}$, or $\textit{RPS20}$. In the present study, we also found overexpression of these candidate genes in medulloblastoma samples that did not display 8q gain, suggesting alternate mechanisms of induction that may nonetheless contribute to tumor phenotype and clinical outcome. All three candidate genes are involved in ribosomal functions: $\textit{RPS20}$ encodes a component of the 60S ribosomal subunit, and $\textit{RPL30}$ part of the 40S subunit. The $\textit{EEF1D}$ protein contributes to delivery of t-RNA to ribosomes. The overexpression of $\textit{EEF1D}$ has been associated with advanced tumor stage in gastrointestinal carcinomas and $\textit{EEF1D}$ reportedly displays oncogenic properties \textit{in vitro} [Joseph; Lei; Ogawa]. This supports the hypothesis that increased expression of ribosomal genes confers a growth advantage [Ruggero].

Indeed, accumulating evidence indicates that aberrant regulation of ribosomes, their components, and their functions can be linked to cellular transformation. Several oncogenes and tumor suppressor genes, including $\textit{MYC}$ and $\textit{MYCN}$, regulate the expression of rRNA and ribosomal proteins [Ruggero]. Furthermore, ribosomal proteins and translation factors directly regulate protein synthesis. Not surprisingly, cancer cells display increased metabolism and protein synthesis, which requires upregulated ribosomal proteins and rRNA [Ruggero]. Influencing ribosomal biogenesis is one of the possible mechanisms by which cellular growth controls can be disrupted, resulting in increased proliferation. It remains unclear precisely how deregulation of rRNA and ribosomal functions are involved in tumor formation or progression. Nonetheless, our results suggest that better appreciation of the relative contribution of $\textit{EEF1D}$, $\textit{RPL30}$, $\textit{RPS20}$, and their associated regulatory mechanisms will impact our understanding of medulloblastoma biology. The identification of these three candidate genes indicates that specific mechanisms of ribosomal biosynthesis and translational regulation are certainly worthy of future study in medulloblastoma.

- **Discretionary Revision 8**- “Table 1 will be more clear when you put significant p-values in Italics or Bold.” -We have made the suggested changes to Table 1 on p.25.

Dr. Castresana recommended the following minor revisions to the text, figures and legends; all of which have been made as suggested.

- **Minor Essential Revision 1**- “Check whether the only reference quoted in the Abstract should be quoted in a long form, or following the directions for quotations along the manuscript.” -We have changed the reference to numerical format on p.2.

- **Minor Essential Revision 2**- “In Methods-Patient samples: line 4: the word 'were' is written twice, and only once is needed.” -We have made the change on p.6.

- **Minor Essential Revision 3**- “In Methods-Patient samples: line 6: 'subject' might be better spelled as 'subjected'.” -We have made the suggested correction on p.6

- **Minor Essential Revision 4**- “Change figures 3 and 4 in the following way: Figure 3 should be the one on 'Gain of 2p, and Figure 4 should deal on 'Gain of 8q'. If not, the manuscript will contain important mistakes, like on page 11 (third and fourth paragraphs when quoting Figures 3 and 4), and on page 27, in lines 1 and 2, when quoting the Figures as well.”
-We have made the correction by switching **Figures 3 and 4** on p.31, and changing the **Figure Legend** on p.23.

- **Minor Essential Revision 5**- “Discussion, page 17, first paragraph: the authors should make an effort to describe the main principles of the hypothesis on ribosome protein production and cancer development. Essentially, reinforcing some of the principles described in reference number 34, and including some notes of references 35-37. They can create one more paragraph commenting on these important points.”
  -Please see above excerpt from additions to the **Discussion** on pp. 16-17.

- **Minor Essential Revision 6**- “Conclusions: lines 5 and 6: the word 'expression' should be mentioned. It is important to underline that it is the expression of those genes and not just gains at 8q, which is adversely (mention also this word) associated to overall survival.”
  -We have made the correction as suggested on p. 18.

- **Minor Essential Revision 7**- “Page 26 Legend to Figure 1: it should be more clear and concise. It mixes the explanation of the figure together with the interpretation of results.”
  - Line 1: change '71 medulloblastoma' for '71 medulloblastomas'.
  - Please, erase '...showed relevance of 8q gain for overall survival'.
  - Also erase from 'For example, seven of 71....' to '...24 others to identify candidate genes'. In that way, the figure legend will just contain a description of the experimental approach followed.” -We have changed the **Figure Legend** on p.23.

- **Minor Essential Revision 8**- Page 28, Table 1. **In Histology of the 71 tumors, the authors show 32, 22, 17 and 3 tumors, which make a total of 74 tumors, instead of 71. The mistake should be corrected.”** -We have made the correction to **Table 1** on p.25.

Please note that we have also added our research funding sources in the **Acknowledgements** section. On behalf of the authors, I would like express our appreciation for the constructive criticisms of the Referees. We trust that these revisions adequately address their concerns and hope they strengthen our manuscript. We welcome any additional comments or questions.

Sincerely yours,

John Y. H. Kim, MD, PhD