Author's response to reviews

Title: Over-expression of COX-2 mRNA in colorectal cancer

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Author's response to reviews: see over
Dear Dr Curtin/Manibo,

Thank you very much for your comments on our manuscript # 1320549047101787: Over-expression of COX-2 mRNA in colorectal cancer, by Hennie Roelofs et al., and for offering the opportunity to submit a revised version.

We revised the paper according to most of the comments of editors and reviewers as follows:

Editorial Requirements: Please state in the revised manuscript the full name of the ethics committee that approved your study.
At page 7 we added this information.

Referee 1.
1. The authors argue that tissue weight is a superior choice over individual housekeeping genes as a normalization strategy. This appears to be based on the fact that this normalization method yielded the highest percentage of tumors with upregulated PTGS2 expression, rather than a careful comparison of the precision and accuracy of the different normalization strategies. Without such data, it is impossible to say whether the tissue weight is a superior normalization strategy and thus such a conclusion is not supported by the data offered.

The accuracy of weighing the tissue in the 10-50 mg range at our Mettler AC100 weighing device is 0.2-0.6%. Data on the accuracy of real time quantitative PCR were obtained from Pfaffl et al. and are ranging between 0.7-5.3%. This information is now added in Materials and Methods (page 7) and Discussion (page 11) sections.

2. The authors also argue that tissue weight is a simpler normalization strategy compared to B2M or GAPDH, due to the variable expression of these two genes. However, the authors do not provide any information on the variability of the weight measurements and how that may impact their findings.

The large variability of GAPDH expression in human colon epithelium samples was already described by Bustin SA. These data are now given and discussed in the Discussion section at page 12.

3. No true replicate samples were analyzed, and no %CV was reported for any assay. Thus the accuracy and precision of the measurements generated are not known as the error associated with the weighing procedure, the reverse-transcriptase procedure, and inter-assay variability are not captured for any method.

These data are now given at pages 7 and 11.

4. The author only looked at two housekeeping genes, and did not look at the combination of multiple reference genes, using algorithms such as geNorm.
Based on the results of studying 13 endogenous control genes, de Kok et al state that: **Ideally, the gene transcript number is corrected for the number of cells analyzed.** Since the number of cells analyzed directly correlated to tissue weight (more tissue, more cells) we only compared the COX-2 data normalized against tissue weight, with a very limited number of two (very commonly used) household genes.

5. One alternative to using housekeeping genes that has been suggested in the literature is to measure cDNA input through the use of fluorescein-labeled oligonucleotides such as Oligreen (Invitrogen), and use that value as a normalizer (Rhinn H, Scherman D, Escriou V (2008) One-step quantification of single-stranded DNA in the presence of RNA using Oligreen in a real-time polymerase chain reaction thermocycler. Anal Biochem 372: 116–118.) This would have been a useful and potentially more objective measurement. We feel that the much more simpler method, by just weighting the tissue, is more accurate, since only a very small variation (0.2-0.6%) was found in applying this method.

Minor Essential Revisions
1. No discussion in the methods section of the type of balance used to weigh the tissues, or mention of the accuracy/precision of such weight measurements is included. This missing information is now added at page 7.

2. No discussion of the limitation of tissue weight as a normalization strategy are presented. For instance, this is not a useful strategy for formalin fixed tissue, and thus irrelevant for large epidemiological studies using clinical blocks. Some discussion on tissue weight as normalization is now added at page 11. We do not agree with the referee that the simple and accurate method of weighing cannot be used for formalin tissue! Formalin tissue slices can simply be weighed and further processed for DNA or RNA isolation, without any problem.

3. The authors should use the accepted HUGO gene symbol- PTGS2 when they are discussing mRNA expression of COX-2. At page 2 we now also mentioned the HUGO gene name PTGS2 coding for COX-2.

Referee 2.
1. The authors performed qPCR to detect the mRNA level of COX-2 relative to tissue weight or the mRNA levels of two housekeeping genes in 60 paired CRC tissues. They did not mention the quality of mRNAs extracted from tissues. Moreover, it is too simple for experimental design. The changes of COX-2 protein levels should be shown by Western blotting in the same CRC tissues. We used standard procedures for mRNA isolation, commercially available and commonly used, which yields good quality of RNA. We disagree with the referee that Western blotting should be used to demonstrate COX-2 levels in the tissues. We performed Western blotting on human colon tissues with 3 commercially available monoclonal antibodies against COX-2, but all antibodies tested showed numerous a-specific COX-2 protein bands, which made it impossible to reliably screen for COX-2 expression, by using this technique.
2. The clinicopathological analyses of COX-2 in 60 paired CRC tissues should be shown in a table. The clinicopathological data of the patients (is that what the referee wants?) are available and are presented in Table 1. In the results section we presented the data on possible associations of COX-2 expression levels with gender, tumor localization, tumor grade or lymph node metastases.

3. The figure of COX-2 expression in CRC tissues is not intuitive. Figures with bar may be better. We do not understand how we can present the three normal/tumor levels of 60 different patients in one bar graph figure.

Minor essential revisions
1. There are many grammar and careless mistakes. Some sentences are hard to read. Authors should have the manuscript edited by a native English speaker with scientific experience. We fully agree with the referee that many mistakes with respect to grammar and style were present. We now carefully re-edited the whole manuscript and made many corrections throughout the whole paper.

Referee 3.

Major Compulsory Revisions
1. The authors found COX2 mRNA expression normalized by tissue weight showed the best correlation with tumor when compared to normalization by other house-keeping genes. Was this due to the fact that some house-keeping gene expression also changed in tumor tissues? Yes, housekeeping gene expression of GAPDH was shown to be strongly varying, even in normal colon tissue. We now addressed this point in the Discussion section at page 12.

2. Can absolute quantification method be used to measure COX2 mRNA level instead of relying on normalization? When having established COX-2 mRNA levels in paired normal and malignant colon tumor tissue, normalization is essential in order to able to make a correct comparison between the levels present in both tissues.

3. Did COX1 mRNA expression change in tumor tissues? COX1 is a very good control to compare to COX2. That is a good suggestion; however unfortunately we did not perform qPCR of COX-1 mRNA levels.

4. Western blot or immunostaining to confirm up-regulation of COX2 protein expression is important supporting data to show. We tried this; however see remarks under point 1 of referee 2.

5. The authors found COX2 expression level was independent of tumor grade or lymph node metastases. Could the authors further comment on the role of COX2 during colorectal cancer development? And whether patients with up-regulated COX2 expression or not respond differently to COX2 inhibitor treatment? As stated on page 12 in the Discussion section: “this suggests that over-expression of COX-2 is lower in colorectal adenomas compared with carcinomas whereas expression is increasing somewhere in the stage between adenoma and carcinoma”; we feel that over-expression of
COX-2 in colorectal cancer is a relatively late event, occurring near the end of the adenoma-carcinoma sequence.
The last question of the referee is extremely interesting, but unfortunately we do not have an answer. We could hypothesize that patients with high COX-2 expression in their malignant tissue would respond to specific COX-2 inhibitors and patients with low or absent expression would not, but this remains to be confirmed!

I hope that this work will now be acceptable for publication in BMC Gastroenterology.

With kind regards,

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