Author's response to reviews

Title: Alterations in PTEN and PIK3CA in colorectal cancers in the EPIC Norfolk study: associations with clinicopathological and dietary factors

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Version: 2 Date: 17 January 2011

Author's response to reviews: see over
Dear BMC Cancer Editorial Team,

Thank you for your email letter of 12 January 2011, regarding manuscript 2752371634733623.

Please find enclosed the revised manuscript accompanying this letter entitled “Alterations in PTEN and PIK3CA in colorectal cancers in the EPIC Norfolk study: associations with clinicopathological and dietary factors” as a resubmission for consideration for publication in BMC Cancer. The manuscript was first submitted to BMC Cancer for review on 3rd November 2010. Editorial and referees’ comments, as part of the peer review process, were received on 12th January 2011 and are shown below in green font. The authors’ responses to the referees’ comments are set out below in blue font and the changes made to the manuscript are in black font marked with yellow highlight (these same alterations have been made in the accompanying revised manuscript file and are similarly marked with yellow highlight there).

Referee 1.

1. However, all the data and conclusions of the papers can be modified (or confirmed, of course) if the Authors investigate other regions of PTEN mutational status. In fact, according to the literature and to the data included in the COSMIC database, there are other exons of the PTEN gene that are altered in a consistent number of cases: for example, exons 5, 6 and 9. Therefore, the Authors must include the analysis of these additional regions to their data.

We are in agreement with the referee that other published studies have shown PTEN mutations occurring in exons 5, 6 and 9. However, in our PTEN mutation analysis we analysed exons 7 and 8 of PTEN as there are several papers published in the literature describing PTEN exons 7 and 8 as possessing a higher prevalence of mutations than other exons of the gene. This analysis revealed very few PTEN mutations in these exons. We reasoned that if the most frequently mutated exons 7 and 8 harboured very few genetic
mutations in this series of colorectal cancers, then one would expect even fewer mutations in other exons elsewhere in the gene, such that further mutation detection studies by sequencing would yield extremely few additional mutations and hence this strategy was not pursued. Consequently, we are unable to present data on the prevalence of mutations in other exons of the \textit{PTEN} gene. In order to acquire the most comprehensive overview of \textit{PTEN} alterations in the CRC cohort, an immunohistochemistry (IHC) protocol was used instead to examine abnormalities of protein expression. This approach has several inherent advantages. First, this type of testing bypassed the need for PCR and sequencing analysis of all 9 exons of the \textit{PTEN} gene or of analysis of the \textit{PTEN} cDNA which differs by only 18bp from the \textit{PTEN} pseudogene transcript. Second, IHC testing served as a read-out of the effects of all alterations affecting \textit{PTEN} (including gene mutations, promoter methylation, copy number changes such as gene deletion, or miRNA changes). Furthermore, loss of PTEN expression, as a result of any of these alterations, was predicted to provide a group of tumours large enough to perform statistical testing with a reduced likelihood of encountering type 2 errors in the tests. This reasoning was not adequately described in the originally submitted manuscript and as such a section describing this has been added to the \textit{Discussion} section.

It has been reported that the majority of \textit{PTEN} mutations in CRC occur in exons 7 and 8 [15], which in our cohort harboured mutations in only 2.2% of CRC samples, in keeping with published studies [13]. Following this, we performed an immunohistochemical (IHC) analysis of \textit{PTEN} protein expression as there are advantages of such an approach. First, IHC analysis of tissue samples circumvents the requirement for PCR based sequencing analyses of all 9 exons of \textit{PTEN} or of the analysis of \textit{PTEN} cDNA, which is identical to the non-translated transcript of the \textit{PTEN} pseudogene except for 18 bp [22]. Second, expression analysis by IHC allows for all factors, including mutation, promoter methylation, miRNA alterations or \textit{PTEN} gene copy number changes, that affect \textit{PTEN} protein expression levels in colorectal tumours to be assessed in one assay with direct comparison.
of normal, adenoma and adenocarcinoma cells. Third, this integrative assay identified a large enough group of CRCs with consistently detected PTEN abnormalities to allow meaningful statistical testing of associations with dietary and lifestyle factors.

2. Second major point: the frequency of PIK3CA mutation. As the Authors found also a low number of KRAS mutations, it is possible that they did not properly select the tissue for DNA extraction. Can the Authors give much more details about guidelines they followed for DNA extraction? Did they follow the suggestions of Van Krieken and colleagues published on Virchows Archiv?

As noted, in our sample set there were relatively low prevalences of both K-RAS and PIK3CA mutations. However, we consider that these estimates of gene mutation frequencies are accurate based on several lines of evidence. First, analysis of these same DNA samples identified a higher that usual prevalence of BRAF mutations (Naguib et al. 2010), which were not identified in DNA from matched blood samples or normal mucosa samples derived from non-cancerous tissues from the same individuals. This is highly suggestive that the DNA samples analysed contained sufficient quality and quantity of DNA from the tumours for mutation detection. Second, in other analyses performed in our laboratory on the same tumour derived DNA samples, a mutation frequency in the APC gene mutation cluster region of 33% was observed (Gay et al., in preparation). This prevalence of mutation is very similar to that described in the COSMIC database (36%). Furthermore, as described in this manuscript, MSI analysis of the same tumour DNA identified 17.1% of the CRC as microsatellite unstable, a figure slightly above the expected ~15% prevalence for colorectal cancer. Taking these data into account, the relatively low mutation frequencies observed for K-RAS and PIK3CA appear to be accurate for this cohort of colorectal cancers. The originally submitted manuscript did not adequately describe these data which confirm the validity of the scientific protocols used in this study, hence an appropriate addition has been made to the Discussion section.

PIK3CA mutations in exons 8, 9 and 20 were observed in 7.0% of cancers. This frequency is at the lower end of the range of previously reported frequencies of 10-20% [3-6] and may be due to the restricted region of analysis used (exons 8, 9 and 20). It has been reported that 70-80% of all PIK3CA mutations are located in the
helical and kinase domains in these 3 exons [2, 3, 7] and as such, restricting analysis to these regions may have led to some mutations in other exons not being identified. In our previous analyses we also observed that 22% of the cancers in our study cohort possessed K-RAS mutations, lower than the 30-40% expected prevalence of this mutation type [20]. Several observations suggest that these low mutation frequencies are accurate for this CRC cohort and not the result of technical shortcomings. First, our previous analysis of BRAF mutations in this same sample set demonstrated a higher than expected prevalence of this mutation (15.9%). Second, in a similar study performed in our laboratory analysing mutations in the APC gene mutation cluster region, a mutation frequency of 33% was observed [Gay et al., in preparation], in keeping with the mutation frequency of 36% described in the COSMIC database [www.sanger.ac.uk/cosmic]. Finally, our analyses identified that 17% of the tumours in our dataset demonstrated an MSI phenotype, a level slightly above the expected ~15% prevalence of microsatellite instability in CRC [21]. As the same DNA samples were used across all analyses, these observations suggest that the observed low mutation frequencies in PIK3CA and K-RAS are a characteristic of this study cohort.

In addition to these observations, the isolation of tumour DNA was performed with great care such that ‘contamination’ of normal DNA in tumour samples was minimised. An experienced consultant gastrointestinal pathologist analysed all tumour samples and
identified regions of tumour tissue and normal tissue for harvesting. In several instances, human tissue samples were available as part of the EPIC Norfolk study in which the proportion of viable neoplastic cells (excluding necrotic tissue) was limited to a very small region of the sample. In these cases, the samples were omitted from testing and not included in this study. Only samples in which good quality viable tumour tissue could be appropriately collected for extraction of high quality DNA in sufficient quantities using our methodology were included. The approach used here is similar to that of van Krieken et al. In order to clarify the process by which tumour tissue was obtained from FFPE blocks and DNA extracted, a substantial addition to the Materials and Methods section has been included.

**Tissue processing and DNA extraction**

Formalin fixed, paraffin embedded human tissue samples, biopsied from the caecum, proximal colon, distal colon and rectum were processed for DNA extraction. Ten, 4µm sections were cut from each block using a Microm HM 325 microtome (Thermo Scientific, Basingstoke, UK). A single section of tissue from each block was stained and used as a template for identification of adenocarcinoma, adenoma and normal tissue regions within each sample. To stain, sections underwent four, 15 minute washes: two in xylene followed by 2 in 100% ethanol. Four, 5 minute sequential washes in 95%, 80%, 70% and 40% ethanol, followed by rinsing in deionised water, completed the rehydration of the section. The sections were then immersed in Harris Haematoxylin Solution (Sigma-Aldrich, Gillingham, UK) for 30 seconds. The sections were then rinsed in tap water and stained with 1% Eosin Solution (Solmedia, Romford, UK) for 5 seconds. Following a final rinse with tap water to remove excess stain, cover slips were mounted using DePeX Mounting Medium Gurr (BDH)
Laboratory Supplies, Lutterworth, UK). Template slides were analysed by a consultant gastrointestinal histopathologist (MJA) using a light microscope and tissue regions corresponding to normal/tumour regions were identified and marked on the slide. Subsequently, the remaining 9 slides were dehydrated and cells from normal, adenoma or adenocarcinoma regions were carefully microdissected using a sterile scalpel. Tissue was collected starting 2 mm away from the normal/tumour tissue boundary, as indicated on the template slide, in order to minimize collection of non-tumour tissue in adenoma or adenocarcinoma samples.

**DNA extraction**

Isolated tissue was digested in 240 µl of Buffer PKD with 10 µl of Proteinase K (both obtained from RNEasy kits, QIAGEN, Valencia, USA). Samples were agitated at 150 rpm at 55°C for 4-6 days with the level of tissue digestion checked after 3 days: those which still had visible amounts of tissue had a further 10 µl of Proteinase K added for the remainder of the incubation. Samples were then incubated at 80°C for 15 minutes in order to partially reverse formaldehyde modification of the nucleic acids and to denature any residual protein. These samples were then used directly, without further purification, for PCR amplification.
3. Last point: the PTEN expression is made by comparing tumoral cells with surrounding endothelial and non-neoplastic cells. In our opinion, it is better to use as comparison the normal mucosa. The Authors should re-evaluate PTEN expression on the basis of this suggestion.

We agree with the reviewer that the most accurate assessment of PTEN immunohistochemical staining in adenocarcinoma in the colorectum is achieved when comparing staining patterns with the normal mucosa (non-cancerous epithelial cells). In the originally submitted manuscript, this was in fact the methodology used! However, this important point was not adequately described. For the large majority of cases, tissue biopsies or large surgical specimens were resected, sectioned and stained and on the same slide neoplastic and non-neoplastic epithelial cells were visible. In our assessment, we did indeed use regions of normal mucosa as a reference against which PTEN expression levels in adenocarcinoma cells were assessed. This methodology was not described in the originally submitted manuscript, hence a section has been added to the Materials and Methods section clearly explaining the methods by which PTEN expression assessment was performed.

Furthermore, in order to highlight this, changes have been made to annotate the image of Figure 4 along with alterations to the legend for Figure 4. This figure exemplifies this methodological technique as it allows the appropriate comparison between normal non-cancerous epithelium and adenocarcinoma cells in the way that the reviewer describes. Figure 4 clearly shows that the staining pattern in entrapped normal (non-cancerous) epithelial cells is different to that of stromal fibroblasts, lymphoid cells and endothelial cells. In the text of the manuscript, we have emphasised the strongly positive PTEN staining in stromal fibroblasts, lymphoid cells and endothelial cells compared with the weaker positivity in normal epithelial cells and the negativity in certain colorectal cancers. Furthermore, these stromal cells demonstrate successful IHC staining in slides in which the majority of cells are PTEN negative tumour cells. A paragraph of text describing PTEN protein expression assessment has been added to the Materials and Methods section. The image of Figure 4 has been modified to indicate the location of normal, non-cancerous (N.C.) epithelial cells and the legend for Figure 4 has been altered accordingly.

PTEN protein expression was classified as negative if over 50% of the tumour cells present demonstrated loss of expression. In order to assess PTEN expression status in cancer cells, the PTEN expression levels in normal mucosa on the same slide were used as a reference. Due to the difference in PTEN staining patterns between stromal fibroblasts, lymphoid cells and endothelial cells in the gut, these stromal cells were used only as an internal control to assess successful IHC staining, not as a reference for comparison with cancer cell PTEN expression levels.
Figure 4: Immunohistochemical analysis showing loss of PTEN expression in a colorectal adenocarcinoma. A: Colorectal adenocarcinoma showing loss of PTEN expression by immunohistochemistry (x100). The cancer cells show lack of expression, whereas stromal lymphoid and fibroblastic cells demonstrate high levels of PTEN expression in the nucleus with reduced but evident cytoplasmic staining. B: A different region of the same adenocarcinoma magnified (x200) to reveal some foci of entrapped normal non-cancerous epithelial cells [N.C.] showing PTEN expression in both nuclear and cytoplasmic compartments with weaker expression than nuclear PTEN expression in the lymphoid and stromal cells.

Referee 2.

The article is of interest by confirming in a large cohort of CRC patients the pattern of occurrence of such molecular alterations and their association with clinicopathological features. Even more interesting are the data regarding the association between PTEN alterations and dietary factors, showing no impact of dietary influences, with the exception of lower levels of LDL. The study is scientifically accurate and clearly written, making the paper fully comprehensive to the reader. I have no revisions to ask for this paper and I suggest publication in BMC Cancer.

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

Referee 2 made no suggestions for revisions to the manuscript.

Referee 1 also stated that “The paper is well written, the experiments nicely conceived. The data support the conclusions.”

The authors are pleased to note the positive and supportive comments from both referees and are grateful to the editorial team and to both referees for their helpful and constructive comments, which we have addressed and this has led to a significant improvement of the manuscript, in particular the responses to the comments made by Referee 1 have improved both the clarity and substance of the paper.

Following completion of these amendments we hope that the revised manuscript is considered suitable for publication in BMC Cancer.

Yours sincerely,

Adam Naguib & Mark Arends (on behalf of the authors)