Author’s response to reviews

Title: Acetylcholinesterase overexpression mediated by oncolytic adenovirus exhibited potent anti-tumor effect

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Author’s response to reviews: see over
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The Biomed Central Editorial Team

Object: MS:1942810311128359-Acetylcholinesterase overexpression mediated by oncolytic adenovirus exhibited potent anti-tumor effect

Thank you for consideration of our manuscript for publication in your journal. We have reviewed the above manuscript according to your reviewer’s comments.

Reviewer # 1 (Hildegard M. Schuller)

Major compulsory revisions:
The physiological function of AChE is to enzymatically degrade the neurotransmitter acetylcholine. In turn, this removes acetylcholine from cholinergic receptors, thus inhibiting intracellular functions stimulated by these receptor families. Whether or not AChE has tumor inhibiting effects in vitro therefore depends on the ability of the tumor cells under study to produce their own acetylcholine. The observed in vitro differences in susceptibility to AChE do not correlate well with the described expression levels of the enzyme in cancer tissue samples. This discrepancy is likely the reflection of variations in vitro in acetylcholine production. To support the author’s hypothesis, it is therefore important to measure intracellular and secreted acetylcholine in each of the in vitro systems. The use of fibroblast as in vitro controls is not a good choice. Normal epithelial cells of origin of the investigated cancers should be used instead.

This is a helpful suggestion. We detected the intracellular acetylcholine in various types of cells using an ACh determination ELISA Kit (new Figure S2). We also tried to measure the level of ACh in the medium, however, possibly due to the sensitivity of our method, we failed to get any result. We found that ACh production level of AGS cells appeared to be the highest one among the gastric epithelia cells (Figure S2A) and that of pancreatic BxPC-3 cells is relatively high (Figure S2B).

We used the normal gastric epithelial cell line GES-1 as in vitro control, together with the fibroblast cells (new Figure 2E & 3F).

Minor revision:
The term "9 types of cancerous tissue" is misleading and should be replaced throughout the manuscript by "cancer tissues from 9 different organ sites".

Thank you for this suggestion. Change made as indicated by the reviewer. According to another reviewer’s comments, we removed the non-gastric tissues-related data from Figure 1 & Figure S1. Because, in our study, the number of non-gastric samples is not enough (6 esophageal, 5 breast, 6 hepatic, 6 colonic, 6 rectal, 6 renal and 7 pancreatic tissues). The differences in histological features between lung tumour types (Martinez-Moreno et al., Carcinogenesis 27: 429-36, 2006) and kidney tumour classes (Muñoz-Delgado et al. FEBS J, 277: 4519-29, 2010)
may arise from the specific cell type from which each tumor class emanates and do not permit appropriate comparison of data in Figure 1 with previous observations. We will collect more patient samples for further study in the future.

Reviewer # 2 (CECILIO J. VIDAL)

Major Compulsory Revisions:

1- The authors make use of immunohistochemistry to determine differences in AChE expression between cancerous (CT) and non-cancerous (ANCT) gastric, esophageal, breast, hepatic, colonic, rectal, renal and pancreatic tissues. When discussing the reported immunolabelling data, the following points have to be considered: 1) AChE of normal tissues occurs in a range of molecular components, whose distribution could change by cancer; 2) the affinity of antibodies for AChE protein depends on the polymerization state, higher for AChE oligomers than monomers; and 3) cancer may alter the oligoglycans linked to AChE molecules with the same polymerization state and by this means affect the strength of the enzyme-antibody interaction. Uncertainty in the extent of antibody binding with monomeric, dimeric and tetrameric AChE cast serious doubts at the time to draw conclusions from the results of Figure 1. In fact, the reported unchanged AChE expression in liver tumours (p11, L-217) disagrees with previous data showing a drop of AChE activity and shorter disease-free survival (DFS) and overall survival (OS) rates of patients carrying low AChE activity hepatocarcinomas (Zhao et al. Hepatology, 35: 493-503, 2011). AChE activity levels in healthy and pathologic samples should be determined by the extent of substrate hydrolysis. Apart from the above methodological troubles, the elevated number of paired gastric pieces used (96) compared to the small number of non-gastric samples: 6 esophageal, 5 breast, 6 hepatic, 6 colonic, 6 rectal, 6 renal and 7 pancreatic tissues, the lack of statistical analysis of the data shown in Figure 1, and the differences in histological features between lung tumour types (Martínez-Moreno et al., Carcinogenesis 27: 429-36, 2006) and kidney tumour classes (Muñoz-Delgado et al. FEBS J, 277: 4519-29, 2010), which likely arise from the specific cell type from which each tumour class emanates, do not permit appropriate comparison of data in Fig. 1 with previous observations. So, the results regarding non-gastric tissues should be removed from both Fig. 1 and supplemental Fig. 1. In forthcoming studies, authors should employ a well-validated protocol (Muñoz-Delgado et al. FEBS J, 277: 4519-29, 2010), which includes measurement of AChE activity levels according to the Ellman method (1961), comparison of the activity levels in non-cancerous and cancerous pieces, and application of statistical analysis in order to draw solid conclusions.

Thanks for these suggestions. Change made as indicated by the reviewer. We have removed the non-gastric tissues from Figure 1 & supplemental Figure 1. In the future studies, we will investigate the AChE activity in large number of cancerous and non-cancerous tissues with the suggested method.
Animal experiments are well described but not so the cell experiments. So, a new subheading indicating cell infection conditions has to be added to the text. This may help understanding the reason for the increased AChE activity at 0 min in Figs. 2B (10 and 50 MOI Ad.AChE, cell lysate) and 2C (ZD55-AChE, medium and cell lysate).

Change made as indicated by the reviewer. Cells were infected with Ad.AChE or ZD55-AChE virus for 48 hours, and then were performed for AChE determination. The 0min in Figure 2B and 3B is the start time point for AChE determination, and we measured every 5 minutes for a total of 6 times. We add this as a new section in the Methods and also included in the Figure legends. New line 138-143, 538-540, 550-552.

Discretionary Revisions:
1-The potential clinical use of AChE against gastric cancer makes it necessary to know which kind of AChE protein (AChE-R, AChE-H or AChE-T) is responsible for the anti-tumour action. For this, authors have to undertake sedimentation experiments (Ruiz-Espejo et al. Breast Cancer Res Treat, 80: 105-14, 2003) in order to identify AChE components in cell extracts (culture medium, optional) of gastric AGS and NCI-N87 cell lines, non-infected (control) and infected with Ad.AChE and ZD55-AChE.

Thanks for the suggestion. The overexpressed AChE gene in this work is T form, and the functional form of AChE in Ad.AChE and ZD55-AChE infected cells should be AChE-T. We incorporated this into the Methods and the Discussion. New line 127, 332.

Minor Essential Revisions:
Page 6, Line 108, please write ...(labelling intensity x percentage of...).

Change made as indicated by the reviewer. New line 105.

P6, L-109, please add reference of the Millipore antibody, and, if possible, the peptide used for immunization.

We have already added the lot number of the antibody and the reference into the Methods. We were unable to get the information of the peptide for immunization. New line 106.

P7, L-139 write ...AChE determination

Change made as indicated by the reviewer. New line 144.

P7, L-128-29, please indicate the amplified DNA fragment and if it is expected that its transcription generates the three classes of 3’-spliced AChE mRNA variants (AChE-R, AChE-H and AChE-T transcripts).
We used AChE T form as the template in this study. We had added this information in the manuscript. **New line 127.**

P7, L-137, write ...AChE gene was identified by PCR assay using....

Change made as indicated by the reviewer. **New line 137.**

P8, L-157-59, please add catalogue number for identifying reliably all primary antibodies used.

Change made as indicated by the reviewer. **New line 171-174.**

P9, L-183, change need...to make ... needed.

Change made as indicated by the reviewer. **New line 198.**

P11, L-219, keep ANCT throughout the text (correct ACNTs).

Change made as indicated by the reviewer. **New line 228.**

P.14, The Discussion Section is too short. It must be extended to include data regarding the variation of AChE activity in lymph nodes, colon, kidney and other tissues. Further, the apparent long survival rate of patients with gastric tumours displaying high AChE activity should be compared with previous observations in liver adenocarcinoma. Bibliography with respect to the down-regulation of nAChRs by AChE must be added.

Thanks for the suggestion. We extended the Discussion Section and included the suggested topics. **New line 311-340.**

We correct the sentence “Other researches indicated that AChE can down-regulate nicotinic ACh receptors (nAChRs) activity which is pathologically over-activated in tumor” (old line 307) to “Other researches indicated that ACh stimulated the nicotinic acetylcholine receptor (nAChR) signaling which is pathologically over-activated in tumor” (**new line 351-353**), and included the reference [1].

**Fig. 4E, please correct PARP precursor (precursor).**

Change made as indicated by the reviewer. **Figure 4E.**