Author's response to reviews

Title: Apoptosis inhibitor-5 overexpression is associated with tumor progression and poor prognosis in patients with cervical cancer

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Version: 3
Date: 25 June 2014

Author's response to reviews: see over
Dear Editor,

Thank you for your efforts with our manuscript, entitled "Apoptosis inhibitor-5 overexpression is associated with tumor progression and poor prognosis in patients with cervical cancer" (Manuscript ID: 1063127652123972). We appreciate reviewers’ comments and advices on improving the manuscript. Attached is a revised version of the manuscript. Our specific responses to the reviewer’s questions and comments are attached.

Please feel free to contact us with any questions.

Truly,

/s/

Stephen M. Hewitt, M.D., Ph.D.
Chief, Tissue Array Research Program,
LP, CCR, NCI, NIH
We would like to thank the reviewers’ constructive comments that have allowed us to significantly improve the manuscript. We have provided a point-by-point description of the changes below and we highlighted them as red in the revised version of the manuscript.

Response to Reviewer 1
This study reports the API5 effect on cell growth in vitro as well as its in vivo expression and prognostic value in a relatively large collection of cervical cancer specimens. Overall the study appears sound and brings important information about API5 and its potential role in cancer. I have several points which need to be addressed before the article is accepted for publication.

We thank the reviewer for this positive comment.

1. Background: I do not think there is enough evidence identifying API5 as key molecule in tumor progression.

We have revised and added references in the background section as follows:

[On page 4-5, Background, Line 79-96]
Many oncogenes and tumor suppressor genes have been discovered and implicated in the regulation of apoptosis. Among these anti-apoptotic proteins, apoptosis inhibitor-5 (API5), also called anti-apoptosis clone 11 (AAC11), or fibroblast growth factor-2-interacting factor (FIF), is a nuclear protein. API5 expression has been shown to prevent apoptosis after growth factor deprivation [10]. The mechanism by which API5 prevents apoptosis is poorly understood, but Morris et al. recently showed that its anti-apoptotic action appears to be mediated by the negative regulation of transcription factor E2F1-induced apoptosis [11]. Furthermore, a recent study revealed that API5 contributes to E2F1 transcriptional activation of cell cycle-associated genes [12]. API5 has been reported to be up-regulated in multiple cancer cell lines, some metastatic tumor within lymph node tissues, and B cell chronic lymphoid leukemia [10, 11, 13-15]. However, there is no clear evidence showing API5 role in tumor progression of cervical cancer. Immune escape has been demonstrated as important in tumor progression especially in virus induced tumor such as cervical cancer. In this context, our recent study showed that API5 acts as an immune escape gene by rendering tumor cells resistant to apoptosis triggered by tumor antigen-specific T cells. This effect was associated with pERK-dependent degradation of a pro-apoptotic molecule, BIM [16]. In this report, we aimed at investigating the clinical significance of API5 and its relationship with phosphorylated ERK1/2 (pERK1/2) in development and progression of cervical cancer.

2. Fig. 1C: A phase contrast image showing cytoplasm should be included. More cells should be shown including those with cytoplasmic API5 staining as mentioned in text. The endogenous API5 signal in CaSki cells should also be demonstrated by ICC figure.

We appreciate the reviewers’ constructive comments. We have obtained new data including confocal differential interference contrast (DIC) images and cytoplasmic API5 of pEGFP-
transfected HeLa cells. We have modified the figure (Fig. 1C) in the revised paper. We have revised the results section and Figure legends section as follows:

[On page 11, Results, Line 245]
As shown in Figure 1C, we observed the dominant localization of API5 in nucleus although cytoplasmic API5 (indicated by arrowheads) was observed in small population of the transfected HeLa cells (less than 8%).

[On page 30, Figure Legends, Line 619-622]
(C) Confocal fluorescent microscopy was used to further evaluate the distribution of API5 in HeLa cells 24 hr after transfection of pEGFP-API5. DAPI fluorescent dye was used for a nuclear counterstaining. Magnified images of boxed areas are shown in the lower panels. Arrowheads indicate cytoplasmic EGFP-API5 in the transfected HeLa cells.

We have analyzed the endogenous API5 in CaSki cells by using confocal microscope as the reviewer suggested. The result was newly inserted into supplementary Fig 1 and legend, and we have revised results section accordingly as follows:

[Supplementary Figure Legend]
Supplementary Fig 1. Localization of endogenous API5 in CaSki cells. Confocal fluorescent microscopy was used to evaluate the distribution of endogenous API5 in CaSki cells. The cells were fixed, permeabilized, and then immunostained with anti-API5 antibody (Santa Cruz, USA; H-300, 1:250) at 4°C for overnight. After washing with PBS, the cells were further incubated with Alexa Flour 488-conjugated goat anti-rabbit IgG (Invitrogen) for 1 h at room temperature, followed by washing with PBS, and then analyzed by confocal fluorescent microscopy. DAPI fluorescent dye was used for a nuclear counterstaining.

[On page 11, Results, Line 246-248]
We also observed a similar localization pattern of endogenous API5 in CaSki cells after immunofluorescence staining (Supplementary Fig. 1).

3. In Fig. 2A it seems that only the upper form of API5 is downregulated following the siRNA treatment. This should be discussed because the authors consider both bands to be API5. Forced API5 expression leads to upregulation of both bands in 293T cells whereas only upper band is upregulated in CaSki cells. Also the increase of API5 signal in 293T cells is only marginal despite the fact that these cells usually express high levels of transgenic proteins after routine transfection. Authors should provide more data in order to clarify these discrepancies.

We thank the reviewer for pointing out these errors. There were non-specific bands on the previous western blot images due to low quality of API5-specific Ab. We have put new western blot images in the revised manuscript.

hAPI5 have four protein isoforms (NP_001136402, NP_006586, NP_001136403, NP_001230676). Among them, molecular weight of API5 isoform 1 and 2 is 57 kDa and 55 kDa, respectively. These isoforms are observed as the upper form of API5 in our WB data.
Molecular weight of isoform 3 is 49 kDa, and detectable as the lower form of API5 in the WB data. Isoform 4 of 35 kDa was not detected as a main band in our experimental system. Since we have cloned API5 isoform 2 in order to establishment of API5-overexpressing cell lines, it is reasonable that ectopic expression of it leads to increase of only upper band between both bands of API5. On the other hand, API5 siRNA used in this study targets all types of API5, so that both bands of API5 is down-regulated following siRNA treatment. Thus, the new data (FIG. 2A) of western blot images showing the correct expression patterns of API5 was replaced with the previous data of those in the revised paper.

4. Fig. 2B, C: The data on B imply that majority of 293T and CaSki cells were transfected. No information is given regarding the transfection efficiency. C, showing pictures of stained cell colonies would strengthen the results.

We established API5-overexpressing stable cell lines by Zeocin™ selection, indicating transfection efficiency of practically ca. 100%. We have clarified it in “Materials and Methods” section of the revised manuscript.

[On page 8, Materials and Methods, Line 162-164] Stable transfected lines were generated by transfecting no insert (pcDNA3) and pdDNA3-API5 vectors, selected and maintained in the presence of appropriate concentrations of Zeocin™ (Invitrogen).

As a request of the reviewer, we have newly performed colony formation assay. The Fig. 2C in the original manuscript was changed to new data including images of stained cell colonies in the revised paper.

5. Methods: Authors state that tissue culture media in colony formation assay was changed every 7 days. This does not look like a proper cultivation practice. Why was not the media replaced every 2-3 days as usual?

We used only 500 cells per well in 4 ml of media on 6 well cell culture system. It is obvious that there was no limitation of nutrients for forming colony in our culture conditions. We have clarified our culture conditions in the “Materials and Methods” section of the revised manuscript.

[On page 8, Materials and Methods, Line 176-178] The stable cell lines and siRNA transfected cells (500 cells/well) were plated onto 6 well tissue culture dishes and incubated for 2 weeks to allow colonies to develop. Media (4 ml/well) was replaced every 7 days.

6. Fig. 2A: No time frame is mentioned for the RNAi experiment done in HeLa cells. How long the RNAi-mediated API5 downregulation persisted in cells, considering that the colony forming assay took 2 weeks.

RNAi was induced 2-3 days and continued 5-7 days after transfection of siAPI5 in our cell culture system. Knock-down of API5 started to wane 5-7 days after transfection. Expression of
API5 was recovered 10-14 days after transfection. Thus, RNAi was maintained for 10-14 days after transfection of siAPI5. On the basis of these observations, we believe that RNAi-mediated API5 down-regulation persisted during colony forming assay. We have clarified it in “Materials and Methods” section of the revised manuscript.

[On page 8, Materials and Methods, Line 172-173]
RNAi was maintained 10-14 days after transfection of the siRNAs [18].

7. Sample numbers stated in abstract should reflect the numbers actually interpreted in the study, not total numbers.

Originally, we started immunohistochemical staining with 192 primary cervical cancer cases but we lost some tissue cores during sectioning and staining procedures. In the manuscript, we do not include the cases which were missing cores in both API5 and pERK1/2, which is why our actual interpreted number is 173.

Noted and corrected

9. Lines 338 and 342: Colonogenicity does not appear to be the right term here.

We appreciate the reviewer for his comment. We revised the manuscript as follows:

[On page 15, Discussion, Line 334-339]
Overexpressed API5 mediated an increase of cell proliferation in CaSki cell line. On the other hand, inhibition of API5 expression by API5 siRNA gene knockdown resulted in significant inhibition of cell growth in HeLa cells. These results demonstrated that API5 overexpression is closely linked to cancer cell proliferation, suggesting that API5 cloud contributes to the development of cervical cancer.

10. Authors should provide stronger link between API5 and ERK activation, since it is not clear why the clinical section of the paper analyzes ERK activation together with API5 expression. I wonder if the ERK activation increased/decreased in cultured cells along with modulation of API5 levels by overexpression and RNAi. Also, the discussion lacks comparison of ERK expression data obtained here with those already published. I assume this is not the first study addressing the ERK expression in cervical carcinoma.

We have found that API5 increases FGF2 production and secretion resulting in, activation of ERK through FGF2/FGFR1 pathway. This work is in press at Cancer Research. We have included these findings in the revised manuscript as follows:

[On page 5, Background, Line 87-96]
API5 has been reported to be up-regulated in multiple cancer cell lines, some metastatic tumor within lymph node tissues, and B cell chronic lymphoid leukemia [10, 11, 13-15]. However, there is no clear evidence showing API5 role in tumor progression of cervical cancer. Immune
escape has been demonstrated as important in tumor progression especially in virus induced tumor such as cervical cancer. In this context, our recent study showed that API 5 acts as an immune escape gene by rendering tumor cells resistant to apoptosis triggered by tumor antigen-specific T cells. This effect was associated with pERK-dependent degradation of a pro-apoptotic molecule, BIM [16]. In this report, we aimed at investigating the clinical significance of API5 and its relationship with phosphorylated ERK1/2 (pERK1/2) in development and progression of cervical cancer.

[On page 16, Discussion, Line 356-359]
Furthermore, we recently demonstrated that API5 activates ERK through FGF/FGFR1 pathway [16]. In agreement with this study, we observed that API5 expression levels were positively associated with ERK1/2 phosphorylation both in high grade CIN and cancer specimens.

11. Legend to Fig. 1B: Is Lamin B a H2B, please clarify.
We have revised the figure 1 legend to clarify the “index issue” in the revised manuscript as follows:

[On page 30, Figure legends, Line 615-622]
Figure 1 API5 expression and its localization in various cervical cancer cell lines. (A) Characterization of API5 expression in various cervical cancer cell lines by western blot analysis. (B) Nuclear and cytoplasmic fractions from HeLa cells were analyzed by western blot analysis. Calnexin and Lamin B1 were used as an index for cytosolic or nuclear fraction, respectively. (C) Confocal fluorescent microscopy was used to further evaluate the distribution of API5 in HeLa cells 24 hrs after transfection of pEGFP-API5. DAPI fluorescent dye was used for a nuclear counterstaining. Magnified images of boxed areas are shown in the lower panels. Arrowheads indicate cytoplasmic EGFP-API5 in the transfected HeLa cells.

12. Legend to Fig. 2C: is the data shown an average from triplicate in one experiment, or from three independent experiments? Please clarify.
We appreciate the reviewer for pointing out our mistake. We have clarified this point by including the following sentence in the revised manuscript.

[On page 30, Figure legends, Line 628-630]
(C) Colony formation: 500 cells were plated in 6 well plates and cultured for 2 weeks and formed colonies were stained with crystal violet. Data depicted as mean+s.e.m. from one representative experiment performed in triplicate.

Response to Reviewer 2
The manuscript by Cho et al., have investigated API5 expression in cervical cancer, and the clinical significance of API5 and its relationship with phosphorylated ERK1/2 (pERK1/2) in development and progression of cervical cancer. The manuscript is interesting. However, the data on cell lines has been mixed with clinical data and thus cell line data falls short of important findings/conclusions. Thus, the manuscript needs some major
revisions. Most importantly, the text needs major revisions from grammar point of view.

We would like to thank the reviewer for the helpful comments, and apologize for the failure in proof reading and are grateful for her suggestions. Below please find our point-by-point responses to the reviewer’s comments.

1. There is a lot of confusion in cell lines. Abstract: Line 53. HEK 293 is not a cervical cancer cell line and now it is not considered to be non-tumorigenic. You could repeat with human PBMCs or HaCaT cells.

We apologize for the use of confusing expressions about HEK293 cell lines. We have revised the “cervical cancer cell line issue” through the whole text in the revised manuscript. We understand the reviewer’s concern about HEK293 cells representing non-tumorigenic cells however there are some reports for HEK293 tumorigenicity. For example, Shen et al was claimed tumorigenicity of HEK293 (Shen et al., Biologicals 34:263). However, they verified clearly HEK293 cells cultured for long time more than 70 passages were developed tumors in nude mice. Lower passage of HEK293 didn’t show tumorigenicity (Shen et al., Biologicals 34:263). Based on Shen et al report, we purchased HEK293 cells from ATCC directly and used relatively young HEK293 cells (lower passage 10-15). In terms of control cells, the reviewer recommended HaCaT or PBMC cells instead of HEK293 cells. HaCaT is also transformed and immortalized cell line, and also reported the tumorigenic conversion by elevated temperature (Boukamp et al., Oncogene 18: 5638). In addition, most of all cell histology of PBMC is substantially different with cervical cancer. Resting lymphocytes are maintained through a cellular program that suppresses apoptosis (Chiravuri et al., J Immunol 163:3092). It suggests PBMC would not be an appropriate control for the studies of apoptosis related genes. Furthermore, HEK293 is still used for non-cancer cell line comparing other cancer cell lines (Taherian A et al., BMC Cancer 13:293). HEK293 is also widely used for in vitro as an ideal cell carrier system for functional test of cancer related genes (Yamamoto T et al., Nat Commun 5:3480).

2. In methods section, H1299 cells are mentioned in experimental protocol when there is no expt. done with it.

We thank the reviewer for pointing out our mistake. The word of “H1299” in the submitted manuscript was changed to the word of “HeLa” in the revised manuscript.

3. Why the term various cervical cancer cell lines is used when only two have been used for in vitro experiments. Why SiHa was not taken? Why only HeLa was used for siRNA studies and not CaSki?

We have adopted the reviewer’s comment. Fig 1A was changed to new western blotting data including API5 expression in SiHa cells as well as, other cervical cancer cell line, C33A in the revised manuscript. As shown in new figure, expression of endogenous API5 was most profound in HeLa and C33A while that in CaSki and SiHa was similar to HEK293.
cells. That is a reason why HeLa cells were selected for siRNA-mediated knock-down experiments. We have modified the revised manuscript accordingly as followed

[On page 11, Results, Line 236-238]
As shown in Figure 1A, API5 was detected as doublet bands, as has been reported in mammals [13]. Expression of API5 was most profound in HeLa and C33A while that in CaSki and SiHa was similar to non-tumorigenic HEK293 cells.

4. When expression of API5 in CaSki is same as HEK 293, how do you see it in localization studies (data not mentioned)? How is it explained?

We used pEGFP-C1 encoding EGFP-API5 to confirmation of intracellular localization of API5 in CaSki cells. Localization pattern in CaSki cells looked much similar with that in HEK 203 cells. To now, any alanine substitutions on known-phosphorylation sites did not affection the dominant nuclear localization of API5 except NLS deletion from API5. Further study is needed for understanding API5 localization.

5. Discussion part has lot of spelling errors. Proper references are not given at relevant places e.g. lines 347-349

We carefully proof read and revised the manuscript.

[On page 15-16, Discussion, Line 340-349]
Epidermal Growth Factor (EGF), Insulin Growth Factor 1 (IGF-1) and Vascular Endothelial Growth Factor (VEGF) are known to regulate cervical cancer cell proliferation and invasiveness which play key roles in determining the high-risk factors and lead to recurrence and mortality [21]. The expression of growth factor receptor is also increased in cervical cancer tissues and cancer cells. EGF Receptor (EGFR) is related to HPV infection as EGFR cytoplasmic expression increases with increasing grade of CIN [22]. IGF-1 receptor (IGF-1R) expression level is elevated in cervical cancer cell cultures [23]. However, the clinical utility of EGFR expression as a biomarker for prognosis or for treatment of cervical cancer is not defined, as normal cervical epithelium also expresses EGFR at various levels [24], and this expression is not correlated with the HPV type [22].

References in Response to Reviewers

