Author's response to reviews

Title: Up-regulation of expression and lack of 5CpG island hypermethylation of p16 INK4a in HPV-positive cervical carcinomas.

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Author's response to reviews:

We resubmit our revised manuscript entitled "Up-regulation of expression and lack of 5'CpG island hypermethylation of p16 INK4a in HPV-positive cervical carcinomas." We have incorporated all specific comments into the new version of the manuscript. We used for the language corrections Russian professional copyediting service. If it is insufficient, we are ready to use services that you recommend for final version of the manuscript.

We have made the following changes.

Specific comments of Reviewer 1

Dear Dr. Nam Hoon Cho,

Thank you for helpful revision of our manuscript.

We have made the following changes:

Specific comment 1
We included the results of MSP analysis that we had earlier (5 cervical cancer cell lines, 26 squamous cell carcinomas of uterine cervix. Among them there are 23% MSP-positive carcinomas). Our results of MSP analysis of the commonly examined region of the p16 INK4a 5'CpG island with the commonly used set of primers are in close agreement with findings of other studies (including the publication that we cite at your request, Kang S et al., 2006; 24% with primers of Dr.Herman). Then methylation status of half of tumor and normal samples and 5 cell lines were verified by bisulfite-based DNA sequencing.

Specific comment 2

We added to fif.1 some examples of partially methylated CpG sites in tumors and normal tissues. We indicated the numbers of partially methylated sites in carcinomas in table 1.

Specific comment 3

We significantly have selected the commonly examined region of the p16 INK4a 5'CpG island and commonly used set of primers (Herman J. 1996) for analysis by MSP. Our aim was to confirm previous results and to evaluate methylation status of this commonly examined region by another technique. To avoid false negative results, the region examined by genomic bisulfite sequencing has been extended and data have been presented in first version (fif.1). Examined region included 7 CpG sites more than usually (it is exactly the same region that has been examined by MSP in the publication that we cite at your request, Kang S et al., 2006) We did not extend further coding region because it was known that the p16 transcription can occur even in the presence of a relatively heavy methylation of CpG sites surrounding the ATG initiation codon. On the contrary, methylation of critical CpG sites located around putative multiple sites of the transcription initiation (Hara E et al., 1996, Mol Cell Biol, 16, 859-867) significantly down-regulates the p16 INK4a promoter activity ("References" ref.4 Gonzalgo et al., 1998; ref. 31 Song S et al., 2000). Earlier we examined methylation status of this critical CpG sites and we added these findings in new version.

Specific comment 4

We agree with the opinion that p16 cytoplasmic staining is controversial and classified as unspecific as well as specific staining in diverse studies. We cite both types of studies. Therefore we control mRNA level and draw a conclusion about p16 overexpression according to the transcriptional activation. But we have paid attention to strong correlation of the transcriptional activation with p16 cytoplasmic staining in carcinomas. (We do not touch a situation with CIN that may be quite different). We do not doubt in specificity of the p16 antibody E6H4: 1) There is no cytoplasmic staining in normal cervical epithelium in our hands and in the hands of the researcher who developed the p16-specific antibody E6H4 [Klaes et al., 2001, - ref 17]. 2) There is no cytoplasmic staining in the case of the complete absence of mRNA in carcinoma No1. 3) We controlled specific character of staining including into every set of slides normal cervical epithelium.

We only call not to ignore the accumulation of the p16 cytoplasmic staining in carcinomas and further
investigated specificity of the p16ink4a cytoplasmic staining in parallel with the transcriptional activation. These studies may help to avoid discrepancies in interpretation of the p16ink4a immunoreactivity. We have included such sentences in "Discussion".

Specific comment 5

We corrected the mistake and replaced "...with persistent HR-HPV infection and up-regulated.." by "...HR-HPV-positive cancer cells with up-regulated p16ink4a expression." ("Conclusions", last phrase).

Specific comment 6

We included the publication in "Diag Mol Pathol "(2006 15:74-82, Kang et al) in spite of the fact that it dealt with CIN. In first version of our manuscript we significantly selected for citation only the publication that examined the p16 methylation by MSP with commonly used set of primers (Herman et al., 1996) in HR-HPV-positive invasive cervical squamous cell carcinomas (I-IV FIGO stages). This selection allows us to compare our results with previous data correctly. There are much more publications that deal with p16 methylation status in adenocarcinomas, displasia, plasma of cervical cancer patients and used in situ MSP-PCR and etc. Situation with these objects may be quite different. We cannot cite and discuss all of them due to journal limitation of reference number.

Specific comments of Reviewer 2

Dear Dr. Duenas-Gonzalez ,

Thank you for helpful revision of our manuscript.

We have made the following changes:

In first version of our manuscript we significantly selected for citation only the publication that examined the p16 methylation by MSP with commonly used set of primers (Herman et al., 1996) in HR-HPV-positive invasive cervical squamous cell carcinomas (I-IV FIGO stages). This selection allows us to compare our results with previous data correctly. Now we have included our results of MSP analysis of the same region p16 INK4a with the same set of primers in 26 invasive SCC (I-III FIGO stages). They are in close agreement with cited publication ("Results", first 7 lines). We have clearly designated our selection for citing in the text (p 11, 4-7 lines from bottom). We discussed our and previous MSP-data together with our results.
Specific comments of Reviewer 3

Dear Dr. Zwerschke,

Thank you for your favourable revision of our manuscript.

We corrected spelling mistakes and reference 27 (24 in new version).

With best regards

On behalf of co-authors

Natalia Kisseljova