Author's response to reviews

Title: Argonaute proteins: potential biomarkers for human colon cancer

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

Thank you for your letter on August 9 concerning our manuscript “Expression of EIF2C1-4 and PIWIL1-4 in human colon carcinoma with tissue microarray” (Manuscript number: 5451233982679361), together with the comments from reviewers.

We have revised the manuscript in accordance with the reviewers’ comments, and carefully proofread the manuscript to minimize grammatical and spelling errors.

Here below are our responses to the reviewers’ comments, along with the indication of the location of the revision.

Thank you and all the reviewers for the kind advice. We hope that the revised manuscript can meet requirement for publication in BMC Cancer.

Best Regard,

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Response to the concerns of Reviewer 1

Comment 1: A serious rewriting of the article is needed. Many sentences are grammatically incorrect (e.g. in Abstract section, Conclusion paragraph: First sentence "Argonaute proteins are overexpression in colon cancer…", many similar mistakes are all over the article and need to be corrected.

Respond: We have realized our mistake and carefully proofread the manuscript to minimize grammatical and spelling errors.

Comment 2: The article title might need to be changed to reflect the article findings instead of just stating what was done.

Respond: In accordance with the reviewer’s suggestion, the article title has been changed to “Argonaute proteins: potential biomarkers for human colon cancer”.

Comment 3: In Materials and Methods: A table with clinical and demographic data of all 75 patients might be added.

A second table displaying the peptides that were used for antibody production must be added to the article as well as a Western Blot showing specificity of the generated antibodies.

Respond: The reviewer’s comments are very helpful and important. As suggested by the reviewer, Table 1 with clinicopathaological date of all cases, Table 2 with amino acid sequences of peptides, and Figure 1 with Western blot analysis of polyclonal antibodies have been added in the revised manuscript accordingly.

Comment 4: First paragraphs in "Discussion" need to be shortened as they are repetition of sections from the "Introduction".

Respond: In accordance with the reviewer’s suggestion, we have deleted the relevant passage since they are repetition of sections from the "Introduction".
Response to the concerns of Reviewer 2

Comment 1:
The authors do not provide clinical data of their colon cancer patients at all. The manuscript needs data for: age, sex, histological type, histological grade, Duke stage, lymph node stage, distant metastases, observation time, survival. Therefore it is not possible to evaluate their conclusions as f. e. significant correlation between positive expression of EI2C2, EIF2C3, EIF2C4, and PIWIL4 and lymph node metastasis.

Respond: The reviewer’s comment is very important. Here we have two points should to be stated carefully.

1) As suggested by the reviewer, Table 1 about the clinicopathological parameters of patients has been added in the revised manuscript accordingly.

2) Relationships between clinicopathological parameters and Argonaute proteins expression were statistically analyzed using Spearman's rank correlation coefficient. As shown in Table 4, there was no statistical difference among each protein expression and age, sex, histological grade, lymph node status or Duke’s stage. The positive expression of EIF2C2 (r = 0.268, P < 0.05), EIF2C3 (r = 0.269, P < 0.05), EIF2C4 (r = 0.242, P < 0.05) and PIWIL4 (r = 0.301, P < 0.01) in colon cancer was associated with the presence of distant metastasis. According to this explanation, we revised our manuscript in the “Results” sections (page 8, paragraph 2).

Comment 2:
Furthermore, since it is not described which histological types of colon lesions were studied a correlation between histological types and expression of EIF2C1 and PIWIL2 can not be stated.

Respond: The sentence of “The influence of each variable on histotype of colonic lesion was assessed by logistic regression analysis” was incorrectly stated in the original manuscript. In fact, we would assess the effects of eight variables (EIF2C1-4 and PIWIL1-4) on colon cancer risk by a forward multiple logistic regression. The results revealed that the positive expressions of EIF2C1 (OR = 3.071, P = 0.005) and
PIWIL2 (OR = 7.392, \( P < 0.001 \)) were associated with a significantly increased risk of colon cancer (Data are shown in Table 5).

According to this explanation, the relevant changes have been made in the “Result” section and “Discussion” section (page 8, paragraph 3 and page 11, paragraph 2). We are grateful to the reviewer for pointing out our error.

Comment 3:

On page 5 it is reported that each antibody was validated by ELISA and Western blot analysis, however, no reference is given or any figure is shown. The authors have to show at least Western blots for each protein. What were the positive and negative controls?

Respond: According the reviewer’s comment, we have carefully revised the “Results” part of our manuscript, and confirmed the specificity of antibodies in detail (page 7, paragraph 2). Nonimmune rabbit serum served as a negative control. Figure 1 with Western blot analysis of polyclonal antibodies against Argonaute proteins has been added in the revised manuscript accordingly.
Response to the concerns of Reviewer 3

Comment 1: The authors should provide the detailed information on specific peptides used as antigens, namely amino acid sequences for each antigen.

Respond: The reviewer’s comments are very helpful and important. As suggested by the reviewer, Table 2 with amino acid sequences of immunogens for preparation of antibodies against human Argonaute proteins has been added in the revised manuscript accordingly.

Comment 2: The specificity of the eight antibodies needs to be shown: evidence with both Western blots and immunohistology.

Respond: Following the reviewer’s comment, we have carefully revised the “Results” part of our manuscript, and confirmed the specificity of antibodies in detail (page 7, paragraph 2). Western blot analysis showed that these purified antibodies recognized the bands at expected molecule mass corresponding to each Argonaute member, respectively. In contrast, no band was detected with preimmune rabbit serum. Furthermore, immunohistochemical analysis showed predominantly cytoplasmic staining in most tumor tissues but very weak or absent staining in normal human tissues. Figure 1 with Western blot analysis of polyclonal antibodies against Argonaute proteins has been added in the revised manuscript accordingly.

Comment 3: Impaired expression of SND1 and several miRNAs in colon cancer has been reported. The related ref. should be cited and the potential relationships among SND1, miRNAs, EIF2C1-4 and PIWIL1-4 should be discussed.

Respond: As suggested by the reviewer, the potential relationships among SND1, miRNAs, and Argonaute proteins have been analyzed in the “Discussion” section (page 12, paragraph 1). The important papers (Paukku K et al., 2008; Tsuchiya N et al., 2007) have been cited as reference 30 and 31, and added to the “Reference” section.

Comment 4: In general, the writing is clear and understandable. However the authors need to be more precise and professional.

Respond: The reviewer’s comments are very helpful. We have realized our mistake
and carefully proofread the manuscript to minimize grammatical and spelling errors.
Response to the concerns of Reviewer 4

Comment 1: The assays are dependant on polyclonal antibodies generated from in house peptides from the Ago’s or PIWIs. The validation of the respective antibodies is not shown. The authors claim there are no available recombinant argonaute proteins. This is not true the Ago’s 1-4 are available online from AddGene. The authors need to validate that their antibodies are actually recognizing the proteins they claim they are recognizing. How do we know that the antibodies aren’t hitting some other target?

Respond: Following the reviewer’s comment, we have carefully revised the “Results” part of our manuscript, and confirmed the specificity of antibodies in detail (page 7, paragraph 2). Western blot analysis showed that these purified antibodies recognized the bands at expected molecule mass corresponding to each Argonaute member, respectively. In contrast, no band was detected with preimmune rabbit serum. Furthermore, immunohistochemical analysis showed predominantly cytoplasmic staining in most tumor tissues but very weak or absent staining in normal human tissues. Table 2 with amino acid sequences of peptides and Figure 1 with Western blot analysis of polyclonal antibodies have been added in the revised manuscript accordingly.

Comment 2: On page 7 the authors discuss western blot data for their derived antibodies but no western blot was shown or figure for which this claim is attached.

Respond: The reviewer’s comments are very helpful and important. As suggested by the reviewer, Figure 1 with Western blot analysis of polyclonal antibodies against Argonaute proteins has been added in the revised manuscript accordingly.

Comment 3 and 5: The enrichment of each Ago or PIWI should be validated from the tissue samples using qRT-PCR. It is possible to isolate RNAs from embedded tissue samples. A correlation of Ago or PIWI RNA with enriched protein by validated antibodies would bolster the data.

The authors should attempt to validate to what extent the Agos or PIWI are involved in cancer progression. One way to do this would be to suppress the respective Agos or
PIWIs in colon cancer cell lines and determine what effect this has on the colon cancer cells division, metastasis, or overall fidelity. One could easily knockout the Agos or PIWIs using U1 adapters or antisense ODNs. Such experiments would assist in supporting the notion that the Agos or PIWIs are somewhat functional in cancer cell fidelity.

**Respond:** We agree with the reviewer that mRNA level of Argonaute proteins should be measured by qRT-PCR and the effect of Argonaute proteins could be assessed by gene knockout in the study, which might further improve the quality of the study. However, RNA isolated from formalin-fixed paraffin-embedded (FFPE) samples remains challenging: the RNA is heavily degraded, nucleic acids are cross-linked to proteins, and base modifications are introduced during the fixation process. No consensus or standardized isolation method has emerged thus far. Therefore, we decide to obtain RNA from cell lines and/or fresh samples instead of FFPE samples. Further studies are underway to examine expression of EIF2C or PIWI on mRNA level by qRT-PCR analysis and determine the effect of Agronaute proteins by direct up-regulation or down-regulation of EIF2C or PIWI expression using antisense adenovirus or RNA interference techniques. In addition, several previous studies (Lee et al., 2006; Liu et al., 2006; Taubert et al., 2007) using qRT-PCR reported the elevated expression of Agronaute family at the RNA level in several human tumor entities. Thus, we believe the data from our study are reliable although we would apply qRT-PCR to our future studies.

**Comment 4:** The authors discuss Agos and PIWI in the context of cancer stem cells. There should be an analysis of stem cell factor enrichment correlating with the Ago enrichment. An attempt to delineate the cancer tissue as distinctly different and maybe stem like with increased expression of the Agos or PIWI would be beneficial.

**Respond:** Following the reviewer’s comment, an analysis of cancer stem cell correlating with increased expression of Argonaute proteins has been included in the “Discussion” section (page11, paragraph 2). In near future plan, we will devote our major efforts to this subject.
**Comment 6:** One page 4 the authors refer to RNA-mediated gene quelling. This is not terminology used in mammalian cells but rather applicable to chromosomal control found in fungi.

**Respond:** We have changed “RNA-mediated gene quelling” to “RNA-mediated gene silencing”.

**Comment 7:** In the abstract the authors state “Argonaute proteins are overexpression in colon cancer…”. This should read “Argonaute proteins are overexpressed in colon cancer…”

**Respond:** The mistake has been corrected in the revised manuscript. We are grateful to the referee for pointing out our error.
Reference


