Title: Fascin overexpression promotes neoplastic progression in Oral Squamous Cell Carcinoma

Authors:

Hunain Alam (hunainalam@gmail.com)
Amruta V Bhat (amrutabhate@gmail.com)
Prakash Gangadaran (prakashonmail@gmail.com)
Sharda S Sawant (ssssawant@actrec.gov.in)
Shimul Salot (ssshimul10@yahoo.com)
Lalit Sehgal (lalit.genetics@gmail.com)
Prerana P Dange (dange.prerana@gmail.com)
Devendra A Chaukar (dchaukar@gmail.com)
Anil K D&cruz (docdcruz@gmail.com)
Sadhna Kannan (skannant@actrec.gov.in)
Rajiv Gude (rgude@actrec.gov.in)
Shubhada Kane (drsvkane@rediffmail.com)
Sorab N Dalal (sdalal@actrec.gov.in)
Milind M Vaidya (mvaidya@actrec.gov.in)

Version: 6 Date: 3 January 2012

Author's response to reviews: see over
To,
The Associate Editor,
BMC Cancer,

Dear Prof. Roskelley,

Thank you for sending us the review of our manuscript “Fascin overexpression promotes neoplastic progression in OSCC”. We have addressed all the comments of the reviewers. Point-by-point rebuttal to these comments has been included. As you have suggested, we have answered the concerns raised by reviewer 1 regarding microscopy. We have changed the figure wherever we found it was necessary. We have also addressed the issue regarding reproducibility raised by reviewer 2 and modified the text related to EMT.

We have restructured the abstract according to editorial requirement. We have also included a separate CONCLUSIONS SECTION as the last section of the text.

I hope modifications made by us meet the requirements for the publication in BMC Cancer.

Thanking you,

Sincerely,

Milind M. Vaidya Ph.D.
KS115, ACTREC, CRI, Tata Memorial Centre
Kharghar Node, Navi Mumbai 410210.
Maharashtra, India
Email: mvaidya@actrec.gov.in
Phone #: 91-22-27405055
Answers to Reviewer's comments

Reviewer 1: Johanna Ivaska

Reviewer's report:
In their manuscript Alam and co-workers have investigated the function of fascin in OSCC cells. They show that overexpression of fascin in an OSCC cell line induces adhesion, migration, invasion and production of MMP2 in vitro. They also present some data suggesting that overexpression of fascin correlates with loss of E-cadherin and reduced cell-cell contacts in cells. They also show that cells overexpressing fascin proliferation more in vitro and in vivo in mouse xenografts. Finally they analyze fascin expression in over 200 clinical samples and show that high-levels of fascin correlate with poor prognosis and the occurrence of metastasis. The role of fascin in promoting cancer and invasion has been already demonstrated in several cancer types and fascin has been clearly linked with cancer progression, invasion and migration in several studies. However, this study contains still sufficient novelty to warrant publication in BMC cancer. They authors show for the first time that fascin may play a role in primary tumor development in OSCC. In addition, their clinical data shows that fascin levels correlate with disease free survival. The authors need to address some of the specific concerns related to the experimentation but with the requested revision this manuscript is suitable for publication.

Major compulsory revisions--

Minor essential revisions

1) The microscopy is not of sufficient quality. The GFP images are overexposed, fascin localizes everywhere (hence any claims related to co-localization are not warranted) and the localization of GFO in Figure 1 looks far better and more specific than localization of GFP-fascin. The panel labelling and the legend in figure 1 are incorrect and should be carefully revised. What are the arrows?

Ans: We agree with the reviewer’s comment we have modified the image using LSM10 software. In GFP-fascin transfected cells, Fascin appears both in cell cytoplasm and at the cell surface. In Figure1 we have tried to show that Fascin at the cell surface co localizes with F-actin based structures like filopodia which was not observed in GFP only transfected cells. We have modified the text related to co-localization of fascin with F-actin accordingly. We have corrected the legend for Figure 1. Arrows indicate filopodia and lamellipodia in Figure 1B and for colocalization in Figure 1C. This has been included in figure legend now.

2) Figure 2 the magnifications of all the wounds healing panels need to be the same. Now it looks like the top panel has a smaller magnification.

Ans: All the images shown in the wound healing assay are of the same magnification. As shown in supplementary material Figure S1A, fascin transfected cells undergo morphological changes, in terms of shape, size and spreading pattern, which has been discussed in results/discussion sections. Therefore, images of fascin transfected cells look bigger in size than GFP alone transfected cells under inverted microscope.
Reviewer 2: Monther Al-Alwan

Reviewer's report:

Fascin has been reported to correlate with aggressiveness and metastasis of various types of cancer, while the exact role of fascin in this process remains to be elucidated. In this manuscript, authors presented in vitro data supporting a role for fascin in OSCC invasion and metastasis as well as an in vivo data showing clinical significance of fascin in OSCC progression. This manuscript presented interesting and valuable results, and applied rational approaches. However, some concerns should be addressed.

Major Compulsory Revisions:

1- The author generated 2 stable clones and showed fascin overexpression by WB (Figure 1A). It was shown that there was less fascin overexpression in clone 2 than in clone 1, yet clone 2 showed more invasion and adhesion. Is fascin expression in clone 2 always lower than clone 1? If so, the author showed discuss the relationship between fascin expression in these clone and the observed function.

Ans: As pointed out by the reviewer, in Figure 1A fascin expression in clone 2 appear less than in clone 1. In repeat experiments of western blot analysis, we did not see appreciable difference in fascin overexpression in these two clones (additional figure included for reviewers per user).

2- In figure 3B, I see decrease levels of #6-intigrin, #4-intigrin and FAK in AW-fascin-1. The #-actin is saturated, so it is hard to judge whether this is a loading problem. Is this decrease reproducible?

Ans: Since we saw decrease in the levels of in α6-, β4-integrin and pFAK, only in AW-fascin-1 clone, we are not able to conclude that decrease in the levels of these proteins is due to fascin overexpression. Moreover, phenotype observed upon fascin overexpression may not be the effect of decrease in levels of these proteins. We have also provided the images with densitometry analysis for reviewers per user (Figure R1).

3- The association between fascin overexpression and decreased E-cadherin levels (Figure 3F) is interesting and demonstrate that it may be involved in EMT as suggested by authors. However, other EMT markers (slug, snail, vimentin) were not change at the mRNA levels. Since the author concluded fascin regulation of tumor progression is not the consequence of EMT in OSCC, they should examine these markers at the protein levels to make that conclusion as this regulation may occur at the protein level. They also showed reduced #-catenin levels, but the rational for its use has not been discussed.
Ans: We agree with the reviewers comment, although we see decrease in E-cadherin protein levels, we did not see much change in protein levels of other EMT marker vimentin. In addition we also analyzed the mRNA levels of other EMT markers such as snail and slug using RTPCR. There was no significant difference observed in these markers as well. Since we do not have antibodies for snail and slug, we are not able to do the western blot analysis for the same. Therefore we have modified the text accordingly.

We thank the reviewer for suggestion regarding our beta-catenin results. We have discussed the results now and have also included a figure for its cell surface staining (Figure S1C).

4- Page 11 line 12 (Figure S2A), if larger size image available it should be presented to clearly show the localization of fascin in the cell membrane and cytoplasm.

Ans: We have carried out immunohistochemistry showing fascin expression in OSCC. Since these images were taken on upright microscope, it is not possible to increase the magnification further without losing the resolution of the images.

5- I suggest that the last section “Correlation of combined expression of fascin, K8 and 4-integrin with clinicopathological parameters of the patients” to be summarized and merged with the previous section “Correlation of fascin expression with clinicopathological parameters of the patients”. As this building on your previous finding (J Cell Sci) and is not the main finding of the paper.

Ans: We agree with reviewer’s suggestion. We have summarized and merged the results of “Correlation of combined expression of fascin, K8 and 4-integrin with clinicopathological parameters of the patients” with previous section.

6- Page 16 last paragraph starting at line 20, the first 6 lines were written like results. This should be edited as discussion and not results.

Ans: We have modified the discussion according to reviewer’s suggestion.

Minor Compulsory Revisions:
1- OSCC should be spelled out in the title as “Oral Squamous Cell Carcinoma”

Ans: We have made the required change.

2- Page 2 last sentence in the results under Abstract “Fascin expression in OSCC showed statistically significant correlation with tumor stage (P=0.041), lymph node metastasis (P=0.001), differentiation (P=0.005), recurrence (P=0.038) and survival (P=0.004) of the patients.” Should be clarified into “Fascin expression in OSCC showed statistically significant correlation with increased tumor stage (P=0.041), increased lymph node metastasis (P=0.001), less differentiation (P=0.005), increased recurrence (P=0.038) and shorter survival (P=0.004) of the patients.”.

Ans: We have made the required change.

3- Many typo errors
a. Title of table S1- “an decrease” should be changed to “a decrease”.
Ans: We have made the required change.

b. Page 9 last sentence “We also observed colocalization of -catenin with fascin in as analysed by confocal microscopy (Figure 3E, F).” has some missing words and it should read “We also observed colocalization of #-catenin with fascin in fascin overexpressing cells as analyzed by confocal microscopy (Figure 3E, F).”.

Ans: We have made the required change.

c. Page 10 line 1, “Since, fascin” should be change to “Since fascin” with no comma.

Ans: We have made the required change.

d. Figure S4A “K1” should be changed into “K14”.

Ans: We disagree with the reviewer. In figure S4A, the labeling K1 is correct it is not K14. We have used β4-integrin and K1 in IF staining which are markers for basal and suprabasal layers respectively.

e. Page 16 line 8 “PI’3K” should be changed to “PI3K”.

Ans: We have made the required change.

f. Page 17 line 14 “i.e, higher the fascin expression poorer is the survival” should be removed as it just repeat the prior sentence.

Ans: We have made the required change.

4- Figure S4 should be switched with Figure S3 as it appeared before Figure S3 in the result section.

Ans: We have made the required change.

5- Figure S1C was presented, but was not discussed anywhere in the results.

Ans: We thank the reviewer for pointing out the mistake. We have referred and discussed the Figure S1A now.

6- Page 4 line 10 “could be correlated” should be changed to “correlated” by removing “could be” as this is only a correlation.

Ans: We have made the required change.

7- Page 9 line 1 “(P=0.05)” was indicated on the figure 2B as “P<0.05” and it should refer to Figure 2B and not 2A.

Ans: We have made the required change.

8- Short paragraph discussing the migration and invasion should be included in the method section.
Ans: We have included the paragraph related to migration and invasion in method section as suggested by the reviewer.

9- Page 12 line 7-9 “In addition, fascin expression significantly correlated with recurrence (Figure 6D; P = 0.038) whereas fascin non expression demonstrated statistically significant correlation with disease free survival (Figure 6C; P =0.013).” should be edited into “In addition, fascin expression significantly correlated with increased recurrence rate (Figure 6D; P = 0.038) and shorter disease free survival (Figure 6C; P = 0.013).”

Ans: We have made the required change.

10- Page 13 line 5, “could be associated” should be changed to “associated” by removing “could be” as this is only association.

Ans: We have made the required change.

Level of interest: An article of importance in its field Quality of written English: Needs some language corrections before being published Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:
I declare that I have no competing interest