Author's response to reviews

Title: Nuclear expression of Rac1 in cervical premalignant lesions and cervical cancer cells

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Author's response to reviews: see over
January 27th, 2012

Professor Sophie F Derchain
Journal Editorial Office
BioMed Central

Dear Professor Derchain,

I would like to thank you for your email dated 27/09/11, concerning the manuscript: Nuclear expression of Rac1 in cervical premalignant lesions and cervical cancer cells: implications for cell proliferation by Mendoza-Catalan, et al. (MS: 2897957275827158). Considering the content of your letter and the enclosed suggestions of the reviewers, we now submit a revised version of the manuscript in which we have made several changes. We have included data to address the concerns of the reviewers, although there are few points we were unable to address experimentally. In addition, we include specific arguments for each reviewer’s comment, where we discuss and try to clarify, as much as possible, each point raised by them. Our new version of the manuscript incorporates the changes bolded in the text. In addition, Figures were improved with additional images according to Reviewers suggestions.

We really appreciate the suggestions of both reviewers since they greatly improved our manuscript. We hope that now you would find our paper suitable for publication in your important Journal.

We thank you in advance,

Sincerely yours,

Dr. Eduardo Castañeda Saucedo
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Reviewer: LF Jave-Suarez

Point 1: Is the overexpression of RAC and TIAM1 also observed in cervical derived cell lines compared with control cells? Authors could address this question by utilizing westernblot assays.

To answer to reviewer concern about overexpression of Rac1 and Tiam1, we performed Western blot using total protein extracts from Hacat, C33A and SiHa cells. Figure 2 was modified to include the results of the WB experiments (panel C) and the following information was added in the results section: **Using Western blot analysis on whole-cell extracts from HaCat, C33A and SiHa cells, we found that Rac1 protein levels are similar in all cell lines (Figure 2D).**

In the materials and methods section we included the following paragraph: **For total protein extracts, cells were lysed with RIPA buffer (50 mM Tris-HCl pH 7.6, 160 mM NaCl, 0.5 mM EDTA/EGTA, 1% Triton X-100, 10% glycerol, 1 mM PMSF and 1 μg/ml leupeptin). Whole cell, cytoplasmic and nuclear proteins were separated by SDS-PAGE in 10% acrylamide gels, transferred to PVDF membranes and detected by Western blot using antibodies against alpha-tubulin, lamin B, Rac1 (all from Millipore) and Tiam1 (Santa Cruz Biotechnology).**

Point 2: The nuclear localization of RAC1 in SiHa cells described by the authors is unclear. Authors should demonstrate the nuclear localization of RAC1 in cell lines (Hacat, SiHa and C33) by westernblot.

We agree with Dr. Jave-Suarez that the presented immunocytochemistry data and the description of the results regarding the nuclear localization of Rac1 in cultured cells is unclear. To better demonstrate the subcellular localization of Rac1 in all the cell lines, we performed cellular fractionation and Western blotting experiments.

Figure 2 has been modified, a new panel (D) was added in which we show the results of the cellular fractionation and Western blotting experiments.

In the methods section of the abstract, the following sentence was added: **Nuclear localization of Rac1 in Hacat, C33A and Siha cells was assessed by cellular fractionation and Western blotting, in the presence or not of a chemical Rac1 inhibitor (NSC23766).**

In the results section of the abstract we included HaCat cells in the statement about the effect of the Rac1 inhibitor in cell proliferation, the new statement is: **Chemical inhibition of Rac1 resulted in reduced cell proliferation in HaCat, C33A and SiHa cells.**
In the material and methods section we included the following paragraph: "Cellular fractionation and Western blotting. Cells were seeded on petri dishes and incubated for 24h in the presence or absence of the Rac1 inhibitor NSC23766. Cells were washed with PBS and lysed in 500 µl of buffer A (10 mM HEPES, pH 9.7; 10 mM KCl, 0.1 M EDTA, 1 mM DTT; 0.5 mM PMSF plus protease inhibitors) directly on the plate and the protein lysate was transferred to a new microtube and centrifuged at 15 000 g for 3 min at 4°C. The cytoplasmic fraction (supernatant) was recovered in a new microtube and the pellet was resuspended in 150 µl of RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8). The supernatant (nuclear fraction) was transferred to a new microtube. For total protein extracts, cells were lysed with RIPA buffer (50 mM Tris-HCl pH 7.6, 160 mM NaCl, 0.5 mM EDTA/EGTA, 1% Triton X-100, 10% glycerol, 1 mM PMSF and 1 µg/ml leupeptin). Whole cell, cytoplasmic and nuclear proteins were separated by SDS-PAGE in 10% acrylamide gels, transferred to PVDF membranes and detected by Western blot using antibodies against alpha-tubulin, lamin B, Rac1 (all from Millipore) and Tiam1 (Santa Cruz Biotechnology).

In the results section, at the end of the second paragraph we added: "These observations were further confirmed using cellular fractionation and Western blot analyses. As shown in figure 2C, Rac1 was detected in the cytoplasmic fraction of the three cell lines, and in the nuclear fraction of C33A and SiHa cells, but not in the nuclear fraction of Hacat cells. Using Western blot analysis on whole-cell extracts from HaCat, C33A and SiHa cells, we found that Rac1 protein levels are similar in all cell lines (Figure 2D).

Point 3: The most important event during the development of cervical cancer is the infection with HPV, in this regard, overexpression of Rho-GTPases and RhoGEFs should to be a posterior event induced or not by the HPV oncoproteins. The authors demonstrate the overexpresion of RAC, TIAM1, RHOA and Beta-PIX, but did not observe a relation with HPV infection. However, they only looked for infection with high risk –HPVs. It is adventurous to conclude that HPV infection doesn’t play any role in the overexpression of these proteins. If that is true, what are the mechanisms that are inducing the overexpression of these proteins in L-SIL and H-SIL?

Regarding the possible role of HPV in inducing overexpression of Rho proteins, we found overexpression of Rho proteins in biopsies in which HR-HPV infection was not evident. However, as mentioned in the discussion section, the method we use for HPV detection does not detect all viral types and it is possible that some HPVs are present in those samples, affecting the expression of Rho proteins. Rho proteins and their GEFs have been found overexpressed in several types of cancers [1, 2], however the mechanism leading to the overexpression is not well understood. Expression of these proteins may be regulated at the transcriptional level or by epigenetic events, as no mutations or gene amplifications of the genes coding for these protein have been reported. In order to clarify this point, we have improved the fourth paragraph of the discussion section as follows: We found that the increased immunoreactivity of Rac1, RhoA and beta-Pix correlates with the histological diagnosis but not with HR-HPV infection. In contrast, Tiam1
immunoreactivity was associated with both histological diagnosis and HR-HPV infection. These observations suggest that altered expression of Tiam1, but not that of Rac1, RhoA and beta-Pix may be dependent of HR-HPV infection. However, further studies are needed in order to determine if increase levels of Rho proteins and their GEFs is induced directly by HPV oncoproteins or is the result of a secondary event related to the progression of the malignancy. Our data indicate that nuclear expression of Rac1 in cervical lesions may be independent of HR-HPV infection as not all HR-HPV positive samples have nuclear staining for Rac1. Moreover, both HPV-negative and HPV-positive cervical cancer derived cells have nuclear staining for Rac1. However, as mentioned above, it is possible that infection with other HPV types not detected by ISH technique we used in this work may affect the subcellular localization of Rac1. Moreover, ISH does not allow us to identify which HR-HPV type is present in the samples, and it is possible that infection with some HR-HPVs such as HVP16 and HPV18 will have a more dramatic effect on the expression of these proteins.

Point 4: How the authors could demonstrate that HPV oncoproteins are not playing a role in the expression of Rho-GTPases and RhoGEFs? If HaCaT cells express low levels of RAC1, what would happen with RAC1 if the expression of E6 or E7 is induced in these cells?

As mentioned in the answer to Point1 (see above), Western blot analysis with whole cell extracts from Hacat, C33A and Siha cells, showed that the protein levels of Rac1 in the three cell lines are very similar. One possible explanation to this result is that HPV infection does not directly affect Rac1 expression. However, this is not a direct demonstration on the effect of HPV oncoproteins in Rac1 expression. This could be addressed by transiently overexpressing E6 or E7 oncoproteins in HaCat cells or by comparing the Rac1 protein levels between wild-type keratinocytes and E6 or E7-expressing keratinocytes. However, for the moment we do not have the technical resources for performing these experiments.

Point 5: HaCaT cells are non-tumorigenic, however, the exogenous expression of HPV16 E6 protein turn these cells into tumorigenic. Would induce the overexpression of RAC1 the same effect in these cells?

Experimental evidences show that overexpression of a constitutively active form of Rac1 in mouse FB, enhance their ability to grow in reduced medium conditions. However, the FB overexpressing active Rac1 were unable to form tumors in athymic mice [3]. This indicates that Rac1 activation might not be enough to turn cells into tumorigenic. Therefore, we hypothesize that overexpression of wt-Rac1 in HaCat cells will not transform the cells in to tumorigenic, and that overexpression of an active for of Rac1 may contribute to the
transformed phenotype, but not to necessarily turn the cells tumorigenic. Unfortunately, at the moment we are unable to perform experiments to test this hypothesis.

Point 6: How explain the authors, why some samples without SIL showed moderate to strong immunoreactivity?

To address this concern, we included the following statement in the discussion section: We also found moderate-strong immunoreactivity of the five proteins in samples without SIL (Table 1). Recent evidences demonstrate infection with HR-HPVs in patients without SILs [4-6]. It is possible that some of the samples without SIL that showed moderate-strong reactivity are infected with HR-HPV. As mentioned above, we used ISH for the detection of HR-HPV infection. However this method has limitations as it detects only a subset of HR-HPV types. Further studies using more sensitive techniques such as PCR-RFLP or sequencing for the detection and typing of HPV infection will be required to answer to this concern.
Reviewer: Margaret E McLaughlin-Drubin

Point 1: The authors of J Cell Biol. 2008 May 5;181(3):485-96. Epub 2008 Apr 28. Rac1 accumulates in the nucleus during the G2 phase of the cell cycle and promotes cell division conclude that none of the commercially available antibodies reliably reports the subcellular localization of endogenous Rac1 in fixed cells. Given this major limitation, the authors should perform subcellular fractionation and western blotting to determine the localization of Rac1.

We agree with the reviewer regarding the reliability of the available antibodies for studying subcellular localization of Rac1. To address this concern, we performed subcellular fractionation and Western blotting. We have added the results of cellular fractionation in figures 2B and 3D and modified the manuscript as mentioned above in response to Dr. Jave-Suarez concerns (see above, response to point 2).

Point 2: HaCaTs (or preferably a normal cell type) should be treated with the Rac1 inhibitor to determine if the observations are merely a general effect of the inhibitor.

We have treated HaCat cell with the Rac1 inhibitor NSC23677 for 48h, and evaluated the effect on cell proliferation using chrystal violet assays. Figure 3 (panel D) was modified, we included a graphic summarizing the results of proliferation assays in HaCat, C33A and SiHa cells after 48h of treatment with the Rac1 inhibitor NSC23677.

The results section from the abstract was modified adding HaCat to the last phrase. In the results section the last sentence was changed by the following sentence “We next tested whether the chemical inhibition of Rac1 has an effect on the proliferation of HaCat, C33A and SiHa cells. We found that NSC23766 treatment resulted in a significant decrease in the proliferation of the three cell lines (Figure 3C).

The second paragraph from the discussion section was modified by adding: “Here we show that the nuclear localization of Rac1 in C33A and SiHa is not affected by treatment with the Rac1 inhibitor NSC23766. This data indicate that in these cells, the presence of Rac1 in the nucleus is not dependent on its activity. It has been shown that inactive Rac1 is present in the nucleus of colorectal cancer cells, where it associates with the transcription factor TCF-4 [27]. Interestingly, these authors demonstrated that activation of the Wnt signaling pathway induced the nuclear translocation of Tiam1, a Rac1-specific activator, in a complex with beta-catenin, and that once in the nucleus a beta-catenin/Tiam1/TCF4/Rac1 complex can be formed, resulting in the activation of Rac1 and transcriptional activation of Wnt target genes [27]. Wnt signaling pathway is
altered in cervical cancer, and therefore nuclear Rac1 may cooperate with this pathway to stimulate proliferation of cervical cancer cells”

Point 3: The authors of British Journal of Cancer (2011) 104, 324–331. doi:10.1038/sj.bjc.6606026 www.bjcancer.com Published online 7 December 2010

The HPV16 E6 binding protein Tip-1 interacts with ARHGEF16, which activates Cdc42 found that cells expressing HPV16 E6 had higher levels of Cdc42 activation. Is it possible that the overall expression levels of the GTPases and Rho-GEFs might not change while the activation level does change? This should be experimentally addressed.

We agree that activation levels might change while no changes in protein levels do. However, this is an experimentally challenging issue, as we will need to obtain protein extracts from fresh tumor samples in order to perform the GTPase activity assay, and we do not have possibility to obtain fresh tumor biopsies. An alternative approach will be to perform the activity assay in protein extracts from liquid cytology samples, however, as these samples are collected in a preservative solution, experimental validation needs to be done to establish the usefulness of the method.

References cited