Author's response to reviews

Title: Alteration of protein expression pattern of vascular endothelial growth factor (VEGF) from soluble to cell-associated isoform during tumourigenesis

Authors:

Ratchada Cressey (ratchada@chiangmai.ac.th)
Onusa Wattananupong (onusa_37@hotmail.com)
Nirush Leartprasertsuke (nlerpra@med.cmu.ac.th)
Usanee Vinitketkumnuen (uvinitke@mail.med.cmu.ac.th)

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

Thank you for forwarding the reviewers’ comments following the peer review of our report entitled “Alteration of protein expression pattern of vascular endothelial growth factor (VEGF) from soluble to cell-associated isoform during tumourigenesis”.

Please find attached the revised manuscript for re-evaluation, which has addressed the comments from the reviewers. We have answered the specific comments in the same order that they were provided. The reviewer’s comments are in italic.

**Reviewer: Rainer Broll**

Comment 1: The authors claim in the title that there is an alteration of protein expression pattern of VEGF from soluble to cell-associated isoform during tumourigenesis. I can not duplicate this theory by reading the data of the study. They demonstrated only a significant change in VEGF isoform 189 (26 kDa) expression from early to late tumor stage in colorectal cancer and also but not significant in lung cancer. Besides this they found that VEGF 121 is more present in smaller tumors (<5 cm) than in tumors > 5 cm. This is not sufficient to prove their hypothesis.

In our manuscript, we presented representative Western blots to demonstrate that the 18 kDa VEGF protein, which is believed to be the soluble VEGF121 was equally expressed in both normal tissues and tumour tissues of colorectal tissues (Figure 1). In lung tissue, this soluble VEGF isoform was expressed in normal tissues and not in tumor tissues. In contrast, for VEGF165 (semi-soluble and cell-associated isoform) and VEGF189 (the cell-associated isoform) the blots clearly demonstrated that these proteins were predominately expressed in both lung and colorectal tumor tissues. Based on this observation, we only analyzed the relationship between the expression of VEGF189 and VEGF165 in tumor tissues to the pathological features of the tumors. Our analysis revealed that there was a significant change in VEGF isoform 189 (26 kDa) expression from early to late tumor stage in colorectal cancer and also in lung cancer (after being re-analyzed).

The reviewer’s comment that “VEGF 121 is more present in smaller tumors (<5 cm) than in tumors > 5 cm”, is incorrect as we reported that VEGF165, not VEGF121, expression was increased in tumor tissues in comparison to the normal adjacent tissues and its presence was significantly correlated with smaller tumors (≤5 cm), than in tumors > 5 cm.
Based on these results we concluded that the cell-associated VEGF189 isoform appears to play a major role during cellular transformation as its presence in tumors was positively correlated with tumor progression. Although the secretable isoform VEGF165 was only found in tumor tissue its presence was not positively correlated with tumor progression and VEGF121 was predominately found in normal tissues Therefore, measurement of circulating VEGF in serum may have limited use as a tumor marker.

**Comment 2:** The second question, whether there is a relationship between the expression pattern of VEGF isoforms and the level of the circulating VEGF in serum could not be answered, and they did not find any correlations to pathological features nor to VEGF isoforms. Furthermore, it is important to consider that authors used an ELISA assay which recognizes only VEGF165 but not VEGF 121 and 189 in serum. For this reason a direct comparison between VEGF protein expression in tumor samples and VEGF serum levels is not useful.

For the measurement of circulating VEGF in serum we utilized two VEGF antibodies (cat. no MAB293 and AB293NA) purchased from R&D Systems to set up Capture ELISA. These antibodies were obtained from animals immunized with recombinant human vascular endothelial cell growth factor 165 amino acid isoform (rhVEGF165). However, due to a common epitope both of these antibodies also recognized rhVEGF121 in addition to rhVEGF165 (Datasheet: R&D Systems). For this reason, the Capture ELISA was able to recognize both soluble forms of VEGF (VEGF121 and VEGF165), which are expected to be secreted and detectable in the blood steam. Although these antibodies may not recognize VEGF189 it is not expected to be detectable in the blood circulation as this isoform retains cationic residues from both exon 6 and 7 and thus is tightly cell associated. The reported results demonstrating no relationship between the expression pattern of VEGF isoforms and the level of the circulating VEGF in serum is not surprising taking into consideration our results showing that only the VEGF189 was associated with tumor progression and not the detectable soluble isoforms.

**Comment 3:** Furthermore, authors "assume/believe" that protein bands with molecular weight of 18 and 26 kDa correspond to VEGF121 and VEGF189. They have to prove it as it was done with the 23 kDa protein (VEGF165).

During the time period of sample analysis the only commercially available human recombinant VEGF protein was VEGF165 from R&D Systems and we agree that this is an essential control to include in our Weston Blot analysis. Recently, a human recombinant VEGF121 protein has been available from R&D System and to our knowledge still no recombinant VEGF189 is available. Ideally, a recombinant protein for each isoform would be needed to definitely prove each band but this problem is not uncommon in publications dealing with detecting VEGF isoform by Western blotting as the inclusion of recombinant VEGF protein as a positive control has not been possible in several publications (Nishimura et al, 2002, Cytokine, 18(4), 191-198). Therefore, with the limited recombinant proteins available, using the recombinant VEGF165 to identify VEGF165 in tissue samples and to relate the other VEGF isoforms to this band is a reasonable assumption.
**Comment 4:** There is no information about the percentage of tumor tissue in a tumor sample. However, this is of importance, because this can have an affect on the VEGF expression profile. Therefore it must be at least 80%.

We agree with the reviewer’s comment that 80% of the samples tested should be tumor cells. For our samples, we are confident that there were more than 80% of tumor cells in each tumor sample, as the pathological procedure was designed to maximize the percentage of tumor cells in the sample. For example, an entire tumor mass was surgically removed from cancer patient which was then trimmed by a pathologist in order to remove the surrounding normal tissues and the center of the tumor (around 200-300 mg) without any sign of necrosis was subjected to cell homogenate preparation for Western blot analysis.

**Comment 5:** There is no information in the paper, why only sera from 56 cancer patients were examined and not from all 94 patients.

Serums were collected from cancer patients prior to the surgical removal of the tumor and were shared between the blood-baking department of Maharaj Nakorn Chiang Mai hospital and our research team. The priority of the collected serum was for pretransfusion testing, and in many cases following these tests insufficient sample was available to be used in our study.

**Comment 6:** We know that recent trauma, surgery, pregnancy or diseases like rheumatoid arthritis elevates the VEGF serum level. Was this excluded in the group of healthy volunteers? Were both groups (tumor patients and volunteers) comparable regarding age and gender? Data should be demonstrated.

The answer to both these questions is yes. Rheumatoid arthritis patient or healthy volunteer with history of recent trauma, surgery, pregnancy, (within 1 month) menstruation (within 1 week) were excluded from our study. [This information has been added to the methods section on page 6]

The age range of healthy volunteer (n=47) was 51±10.9 (mean±SD) years and cancer patients (n=56) was 58±12.5 years. The healthy volunteers were comprised 27 males and 20 females, and the cancer patients were composed 24 males and 32 females. Statistical analysis using Mann-Whitney test showed that there was no significant difference in the level of circulating VEGF between both genders. [We have already added this part of the data into the result section, Table 3]

**Comment 7:** Detailed information about the VEGF-antibodies used in the western blot and also their dilution has to be demonstrated.
We agree with the reviewer and we have added this information regarding the VEGF-antibodies used in the western blot and their dilution in the method section, page 7.

**Reviewer: Pan-Chyr Yang**

**Comment 1:** The author did not correlate the expression pattern to clinical outcome such as overall survival or metastasis in both types of cancer.

We agree that this is an interesting analysis to add to our manuscript. We have correlated the expression pattern to metastasis and have included this analysis into the result section, Table 1 and Table 3. However, we did not get enough data to correlate VEGF expression survival to the overall survival of the patients.

**Comment 2:** In patients with lung cancers, are the expressions of VEGF isoform or circulatory VEGF different in different histology type (Adenocarcinoma vs squamous ca)?

In our samples, 9 Adenocarcinoma and 9 squamous ca were analyzed. Following the reviewer’s comment we have investigated the expression of the VEGF isoforms and circulatory VEGF levels with regard to histology type and found no significant differences between them.

**Comment 3:** Are the VEGF isoforms expression or circulatory VEGF affected by gender or smoking

We analyzed the circulating level and expression pattern of VEGF compared between the two genders and found no significant difference between the two groups. [We have already added this part of the data into the result section, Table 1 and Table 3]

We did not note if the volunteers were smokers or non-smokers. Nevertheless, due to the low numbers of Thai women smokers in comparison to males we believe that if smoking does affect the circulating level and tissue expression pattern of VEGF a difference would be observed between genders. Previous reports on the effect of smoking on VEGF level have been inconsistent, although, smoking has been reported to increase VEGF expression in mice (Ye Y-N et al, 2004, Toxicology, 203, 179-188), the effect was not pronounced in humans (Schmidt-Lucke C et al, Int J of Cardiology, 2005, 100, 207-212).

**Comment 4:** Can the circulatory VEGF levels be used to monitor the treatment response? Are the VEGF level decreased after surgery?

We did not follow the VEGF level in the patients after their operation.

We thank you for your careful review.
Yours sincerely,

Ratchada Cressey

Division of Clinical Chemistry,
Faculty of Associated Medical Science
Chiang Mai University,
Thailand, 50200