

METHODOLOGY

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Assessment of vascular endothelial growth factor in formalin fixed, paraffin embedded colon cancer specimens by means of a well-based reverse phase protein array

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Abstract

Background: Vascular endothelial growth factor (VEGF) is a critical pro-angiogenic factor, found in a number of cancers, and a target of therapy. It is typically assessed by immunohistochemistry (IHC) in clinical research. However, IHC is not a quantitative assay and is rarely reproducible. We compared VEGF levels in colon cancer by IHC and a quantitative immunoassay on proteins isolated from formalin fixed, paraffin embedded tissues.

Results: VEGF expression was studied by means of a well-based reverse phase protein array (RPPA) and immunohistochemistry in 69 colon cancer cases, and compared with various clinicopathologic factors. Protein lysates derived from formalin fixed, paraffin embedded tissue contained measurable immunoreactive VEGF molecules. The VEGF expression level of well differentiated colon cancer was significantly higher than those with moderately and poorly differentiated carcinomas by immunohistochemistry ($P = 0.04$) and well-based RPPA ($P = 0.04$). VEGF quantification by well-based RPPA also demonstrated an association with nodal metastasis status ($P = 0.05$). In addition, the normalized VEGF value by well-based RPPA correlated ($r = 0.283$, $P = 0.018$). Furthermore, subgroup analysis by histologic type revealed that adenocarcinoma cases showed significant correlation ($r = 0.315$, $P = 0.031$) between well-based RPPA and IHC.

Conclusions: The well-based RPPA method is a high throughput and sensitive approach, is an excellent tool for quantification of marker proteins. Notably, this method may be helpful for more objective evaluation of protein expression in cancer patients.

Keywords: Vascular endothelial growth factor, Formalin-fixed paraffin-embedded, Colon cancer, Immunohistochemistry, Reverse-phase protein array

Background

The vascular endothelial growth factor (VEGF), one of the key regulators of angiogenesis, is a major target for anti-angiogenic therapy. VEGF overexpression has been associated with vessel invasion [1,2], tumor progression and poor prognosis in several tumor entities, including colorectal cancer [3,4]. It is reported that there is a correlation

between VEGF expression and microvessel density [5,6]; therefore, the precise quantification of VEGF expression of clinical specimens is an invaluable factor for understanding clinical outcome, pathophysiological processes or therapeutic responses. In diagnostic pathology setting, tissue samples are mainly preserved as formalin fixed, paraffin embedded (FFPE) tissue blocks for histological examination. Currently, VEGF expressions of clinical FFPE tissue specimens are routinely assessed by immunohistochemistry, however quantification is limited.

Immunohistochemistry has been used as an adjunct diagnostic method as well as a validation tool of candidate

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biomarkers for a variety of cancers. Unfortunately, immunohistochemistry is labor intensive and stained specimens must be evaluated by a pathologist one at a time. In addition, several difficult issues remained in evaluation of immunohistochemistry, including the subjective of determination of intensity [7]. Furthermore, immunohistochemical studies with manual scoring are not a quantitative measurement but rather a qualitative assessment. Although image analysis overcomes some of these issues, it lacks normalization to account for differences in tissue handling and processing [8,9]. In order to overcome these limitations of immunohistochemistry, a number of proteomic based technologies have been developed and evaluated in clinical research fields. Although these techniques are generally superior in expression profiling and quantitation of protein changes associated with disease states, each has significant limitations including specimen handling [10]. Many of these methods require frozen tissues or native proteins of starting material required, such limiting their application to FFPE tissue application [11]. Recent advances in techniques for extracting proteins from FFPE tissue sections have been facilitating tissue protein profiling in the clinical proteomics, with varying degrees of success [12,13]. We described a proteomic profiling method which can be applicable to routine clinical FFPE tissue specimens. It is based on combined technologies as a new protein extraction method and a novel protein array platform. We have demonstrated that the array is reliable and a stable proteomic profiling platform for clinical research areas [14].

Here we show that protein expressional assessment by this protein-profiling platform, coupled with protein extraction, correlated with immunohistochemical measurement; therefore, suggesting that it can be used as a substitute method for conventional immunohistochemistry of FFPE tissue specimens. The new proteomic profiling method reported here is, especially in secreted proteins, a sensitive and specific method capable of efficiently unraveling molecular profiles associated with disease status or clinical outcome.

Results

Clinicopathologic characteristics of patients

The characteristics of the cases are summarized in Table 1. The ages of the patients ranged from 26 to 89 years (mean, 68.6 years). Thirty-nine patients were men and 30 were women. Six cases were T1 tumors, 9 T2, 43 T3, and 11 T4. Twenty-two patients (32%) had nodal metastasis associated with the primary tumor. Microscopic vascular invasion was identified in 13% of patients (9 cases).

Immunoreactivity of protein extracted from FFPE tissue specimens

Prior to application of the well-based reverse phase VEGF quantitation, we examined protein quality to determine

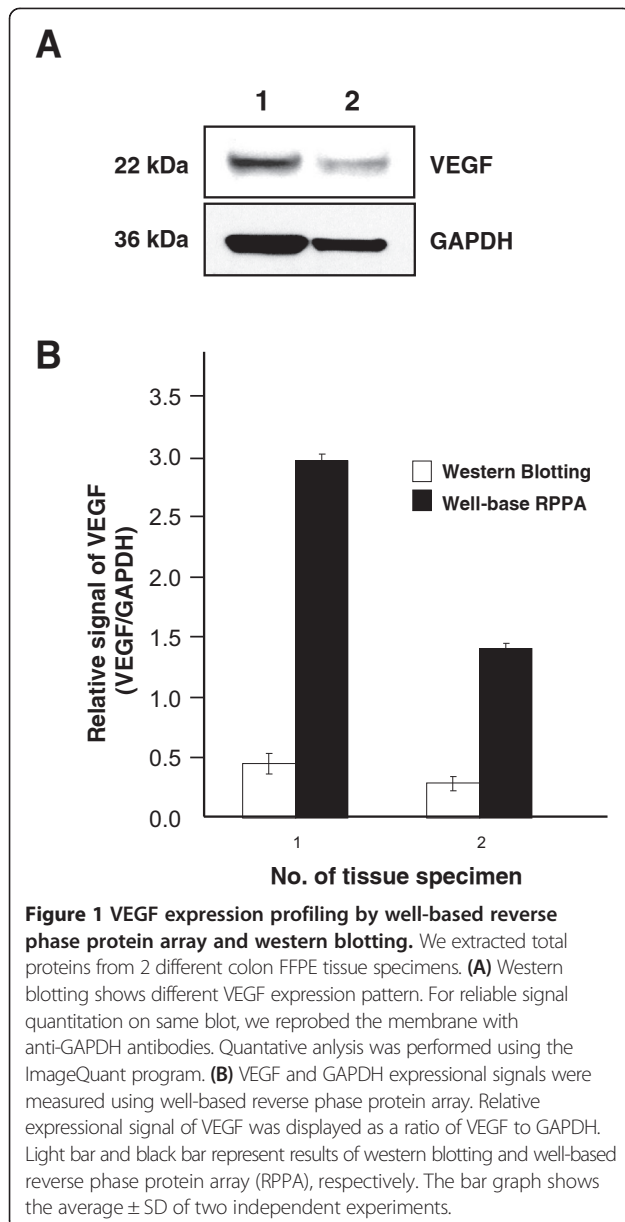
Table 1 Characteristics of cases

Factor	Characteristics	No. of cases (%)
Gender	Male	39 (56.5%)
	Female	30 (43.5%)
Differentiation	Well	3 (4.3%)
	Moderately	31 (44.9%)
	Poorly	35 (50.7%)
Histologic type	Adenocarcinoma	46 (66.7%)
	Mucinous carcinoma	23 (33.3%)
T classification	T ₁	6 (8.7%)
	T ₂	9 (13.0%)
	T ₃	43 (62.3%)
	T ₄	11 (15.9%)
N classification	N ₀	34 (50.0%)
	N ₁	22 (32.4%)
	N ₂	12 (17.6%)
Vascular invasion	Absent	60 (87.0%)
	Present	9 (13.0%)

whether the proteins extracted from archival FFPE human colon specimens were of sufficient quality for well-based reverse-phase protein array (RPPA). We extracted measurable immunoreactive proteins that demonstrated specific signal of the predicted molecular weight by immunoblotting analysis including VEGF and GAPDH, respectively (Figure 1A). Having the immunoreactive proteins, we evaluated whether a well-based RPPA is compatible for VEGF assessment in clinical specimens. Internal control (GAPDH) signals of clinical specimens were strongly correlated with input amount of protein ($R^2 = 0.9992$). Subsequently, we are also confirmed high sensitivity and linearity of using anti-VEGF ($R^2 = 0.9597$), with dynamic range from 0.03 μg to 1.0 μg (Additional file 1: Figure S1). As shown in Figure 1B, relative VEGF signals in two different colon tissues were measured and the ratio of VEGF to GAPDH was calculated. Although there is similar expression pattern between well-based RPPA and immunoblotting, the expression level of novel protein array has a better dynamic range than immunoblotting.

VEGF immunohistochemical staining

To determine whether VEGF immunohistochemical expression is associated with clinicopathologic factors in colon cancer, we assessed VEGF expression patterns by semiquantitative analysis of immunohistochemical staining. Representative images of VEGF immunohistochemical staining are illustrated in Figure 2. Results of VEGF immunohistochemistry analysis are summarized in Table 2. Mean histo-score of well differentiated colon cancer (20.0) was significantly higher than those with moderately (10.9) and poorly differentiated (9.1) carcinomas ($P = 0.04$, ANOVA



post-hoc Duncan, Figure 3A). However, there were no VEGF expression differences when evaluated with respect to other clinicopathologic factors.

VEGF quantitation by well-based RPPA

To assess quantitative VEGF scoring, we performed the well-based RPPA with human colon FFPE tissue specimens. Results of VEGF quantitation by the well-based RPPA are summarized in Table 2. Mean relative VEGF expression of well differentiated colon cancer (4.2) was significantly higher than those with moderately (2.9) and poorly differentiated (2.5) carcinomas ($P = 0.04$, ANOVA post-hoc Duncan, Figure 3B). VEGF expression was higher in female patients (3.1) than male patients (2.4, $P = 0.03$). Cases with nodal status N0 (no lymph node metastasis) showed a higher VEGF histo-score (3.1) than what patients with N1 (2.5) or N2 (2.3, $P = 0.05$) showed. There was no VEGF expression difference based on other clinicopathologic factors.

Correlation of VEGF quantitation based on input amount of protein as well as histo-score of VEGF and novel VEGF quantitation assay are summarized on Figure 4. There was an excellent concordance of novel VEGF quantitation assay between 500 ng and 1000 ng of input protein ($P < 0.0001$, $r = 0.79$, Pearson's correlation, Additional file 1: Figure S2). In addition to the comparison of VEGF quantitation based on input protein, we compared VEGF quantitation based on a new assay with classic "semi-quantitation" of VEGF immunohistochemical expression based on "Histo-score". Notably, unnormalized VEGF value by well-based RPPA showed negative correlation ($r = -0.350$, $P = 0.003$, Figure 4A) with immunohistochemical assessment, whereas the normalized VEGF value based on GAPDH displayed statistically meaningful correlation ($r = 0.283$, $P = 0.018$, Figure 4B). Furthermore, the adenocarcinoma type showed significantly better correlation ($r = 0.283$, $P = 0.018$, Figure 4C) with immunohistochemical measurement than that of mucinous adenocarcinoma type ($r = -0.086$, $P = 0.703$, Figure 4D) by histological subgroup analysis.

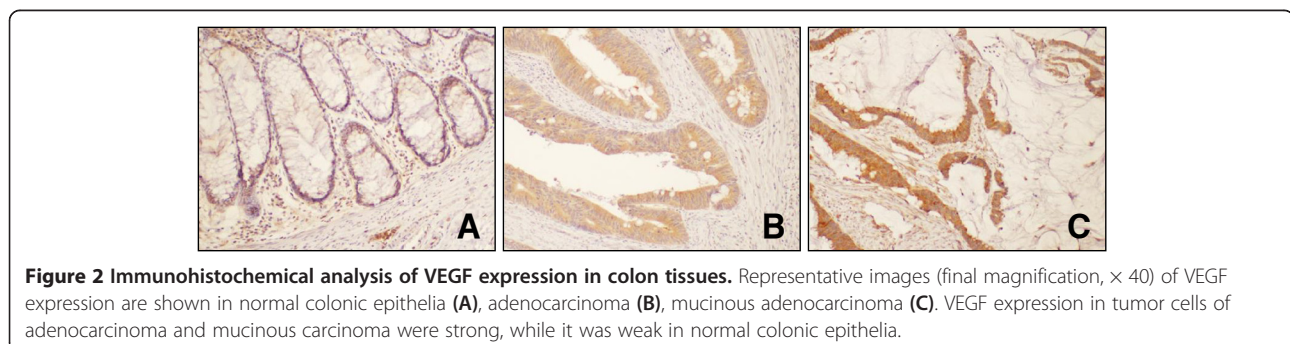


Table 2 Results of VEGF expression by immunohistochemical staining and well-based Reverse Phase Protein Array (RPPA)

Factor	Characteristics	Quantitation method			
		IHC		Well-based RPPA	
		VEGF ^a	P-value	VEGF ^b	P-value
Gender	Male	8.7	0.07	2.4	0.03*
	Female	12.5		3.1	
Differentiation	Well	20.0	0.04*	4.2	0.04*
	Moderately	10.9		2.9	
	Poorly	9.1		2.5	
Histologic type	Adenocarcinoma	9.6	0.27	2.8	0.29
	Mucinous carcinoma	11.9		2.5	
T classification	T ₁	8.0	0.24	2.1	0.10
	T ₂	6.1		2.9	
	T ₃	11.9		3.0	
	T ₄	9.1		2.3	
N classification	N ₀	12.4	0.16	3.1	0.05
	N ₁	8.3		2.5	
	N ₂	8.3		2.3	
Vascular invasion	Absent	10.3	0.93	2.8	0.60
	Present	10.0		2.6	

^aHisto-score was generated as the product of intensity times area.

^bAfter normalization with GAPDH level, relative expressional signals were represented as a ratio.

*Significant at the level of $P < 0.05$.

Discussion

Angiogenesis is one of the hallmarks of cancer and has recently become the target of therapeutic approaches in oncology. Vascular endothelial growth factor (VEGF) was found to be associated with angiogenesis [15-17]. Due to the VEGF expression's correlation with metastasis and mortality, anti-VEGF therapy shows promise to suppress tumor progression [18]. Based on its importance in regulating

tumor angiogenesis, VEGF expression has been assessed in tumor samples via detection of gene amplification or mutation, qualitative RT-PCR, immunohistochemistry, fluorescence *in situ* hybridization, and enzyme-linked immunosorbent assays (ELISA). In clinical research areas, ELISA and immunohistochemistry are commonly employed as VEGF detection assays coupled with serum and FFPE tissue specimens, respectively. VEGF serum

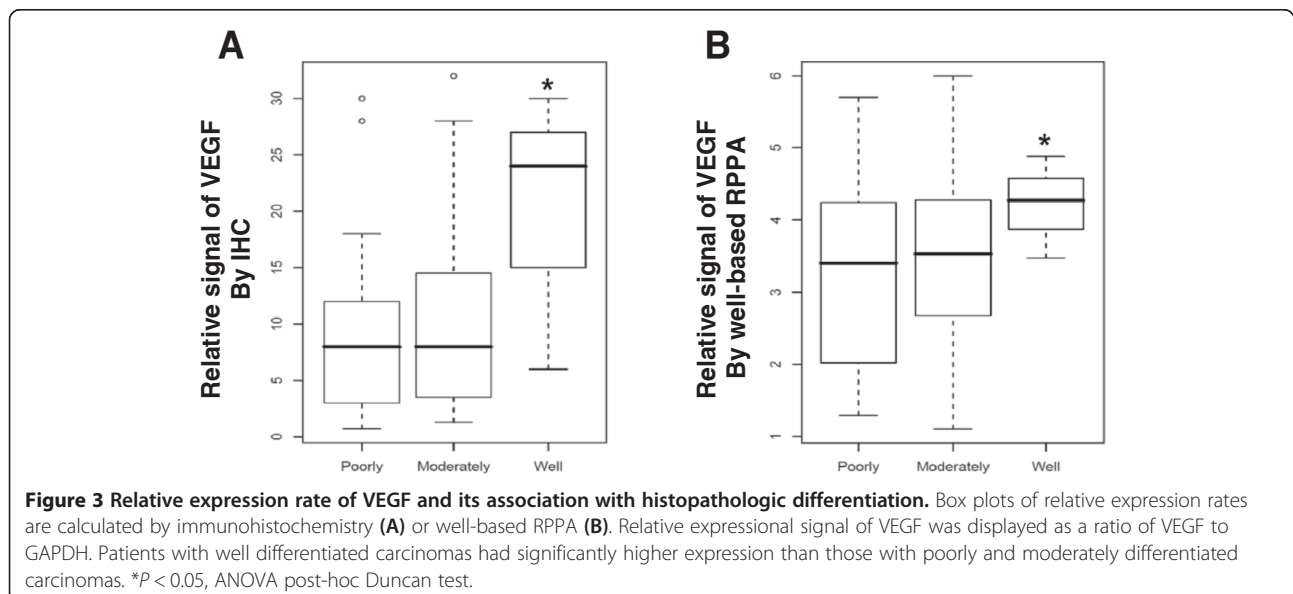


Figure 3 Relative expression rate of VEGF and its association with histopathologic differentiation. Box plots of relative expression rates are calculated by immunohistochemistry (A) or well-based RPPA (B). Relative expressional signal of VEGF was displayed as a ratio of VEGF to GAPDH. Patients with well differentiated carcinomas had significantly higher expression than those with poorly and moderately differentiated carcinomas. * $P < 0.05$, ANOVA post-hoc Duncan test.

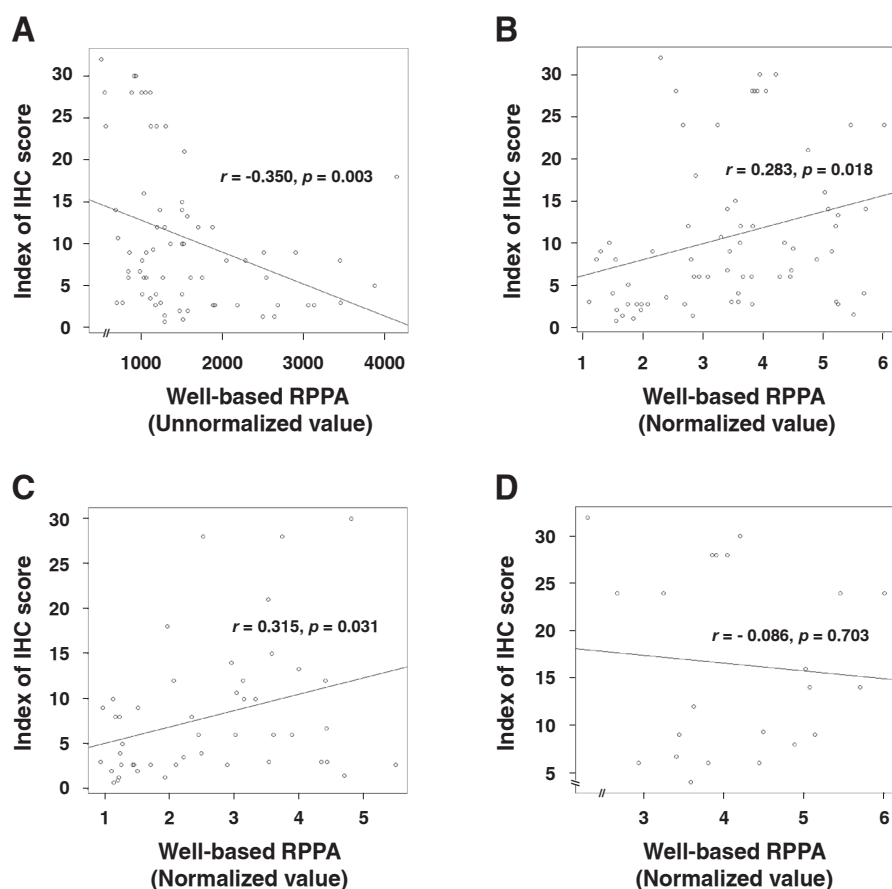


Figure 4 Reliability of well-based RPPA and its correlation with immunohistochemistry. Scatter diagrams are presented by unnormalized VEGF value vs. immunohistochemical VEGF score (A) and normalized VEGF value vs. IHC VEGF score (B). There was a statistically meaningful correlation between expressional signals in the normalized VEGF value by well-based RPPA and immunohistochemical VEGF score ($r = 0.283, P = 0.018$) whereas the unnormalized VEGF signal was expressed negative correlation ($r = -0.350, P = 0.003$). By histologic subgroup analysis, adenocarcinoma type (C) was only showed good correlation ($r = 0.315, P = 0.031$) with IHC assessment compare to that of mucinous adenocarcinoma ($r = -0.086, P = 0.703$, D).

levels are generally reported to correlate with the tumor burden and seem to associate with the overall survival of patients with colorectal cancer [19,20]. Werther et al. [21] showed a significant reduced overall survival in patients with high levels of serum VEGF (>533 pg/ml) compared with patients with serum values below this threshold. On the other hand, studies using VEGF immunohistochemistry in FFPE tumor samples are less clear in terms of the prognostic value of VEGF. Ferroni et al. [22] observed a significant association between colorectal tumors with advanced stage and higher VEGF expression by immunohistochemistry. In contrast, Lee et al. [4] and Khorana et al. [23] showed VEGF expression was not associated with survival. There are also conflicting reports between VEGF expression and tumor differentiation. High levels of VEGF expressions were significantly associated with poorly differentiated tumors in colorectal carcinomas [24,25] and soft tissue sarcomas [26]. In this study, however, VEGF expression was elevated in well differentiated tumors in

comparison with poorly differentiated ones by immunohistochemistry ($P = 0.04$) and protein array ($P = 0.04$). Similar findings were observed in other studies on colorectal carcinoma [27,28] and esophageal squamous cell carcinoma [29]. Overall the conflicting results of prior studies may be explained by a lack of a standard immunohistochemical method, different standard of interpretation, or differences in studies' patient populations. Therefore, these problematic environments of VEGF expression assessment have caused some disparity in the reports of VEGF expression as a prognostic marker. Immunohistochemistry, although providing excellent localization, lacks quantification without sophisticated instrumentation and normalization in chromogenic applications.

As a validation study, we employed adenocarcinoma and mucinous carcinoma histologic types for the well-based RPPA assay. Although the adenocarcinoma subgroup showed a good correlation between the well-based RPPA and IHC, the mucinous carcinoma subgroup

showed a poor correlation. This difference is due to the cellularity of the samples, where the IHC, un-normalized is an estimate based on a very small number of cells, while the well-based RPPA is a normalized measure. Notably, there is no correlation between tumor cellularity and VEGF expressional signals by well-based RPPA (Spearman's $\rho = 0.088$, $P = 0.471$) whereas the IHC values shown a statistical correlation with the cellularity (Spearman's $\rho = 0.700$, $P < 0.0001$). To date, all immunohistochemical studies of VEGF quantitation in colon cancer tissues are limited to tumor areas and do not reflect the contribution of VEGF in adjacent tissue. Since VEGF is a secreted protein, its presence not only in tumor cells but also in surrounding matrix and cellular membranes has hampered its analysis by immunohistochemistry [30,31]. Therefore, lysates of whole sections would better represent the complex VEGF expression pattern.

Formalin fixation results in protein cross-linking. Moreover, the protein quality of FFPE tissue is impacted by additional pre-analytical variable factors including warm ischemia, fixation time and tissue processing conditions [32]. Although it is typical for extracted proteins from FFPE tissue to show a "smear" on SDS-PAGE, the extent of the smear is related to the specimen processing conditions, with greater "smearing" indicates different degradation levels for each tissue sample. In this study, we used GAPDH protein signal as an internal control to measure the protein quality from each tissue sample. The GAPDH protein has a relatively small molecular weight and is relatively well-reserved in FFPE tissue [14]. In this context, the VEGF signal was double normalized with total amount of protein and the GAPDH signal. By employing the normalization tool by GAPDH, our methodology has an advantage that can evaluate VEGF value without the risk of low reliability and poor validity in the current immunohistochemical VEGF assessment.

Conclusion

In conclusion, we revealed a better correlation and less scattering using the novel protein array methodology in tissue lysates of FFPE tissue. In addition, we showed the new approach could be used for protein profiling analysis in FFPE tissue, with quantification and normalization tools. As a research tool, this method offers an additional great tool for assessment of VEGF expression in translational medicine.

Methods

Patients and tumor samples

The study subjects were composed of 69 patients with surgically resected colon cancer at RWTH Aachen University, Aachen, Germany. Medical records were reviewed to obtain data including age, gender of patients, cancer stage, tumor differentiation, cell type, lymphovascular invasion

and lymph node metastasis. Tissue samples were collected from patients who had signed informed consent forms, which was approved by the institutional review board of the RWTH Aachen University Hospital. This study was additionally approved by the Office of Human Subjects Research at the NIH. Information on post-operative radiation and/or chemotherapy, and outcome of patients were not available.

Protein extractions from FFPE tissue sections

Protein extraction from two 10 μm FFPE tissue sections was performed as previously described [14]. This methodology has the advantage of being compatible with archival tissue and does not require deparaffinization. Briefly, sections were trimmed of excess wax and homogenized using a Disposable Pellet Mixer in 200 μl protein extraction solution [1x high pH Antigen retrieval buffer (pH 9.9) (Dako, Carpinteria, CA), 1% NaN_3 , 1% SDS, 10% glycerol and protease inhibitor (1 tablet/25 ml, Roche)], followed by incubation for 15 min at 115°C within a pressure cooker (Dako). After incubation, the tissue lysates were centrifuged at 13,000 rpm for 30 min at 4°C. The supernatants were collected and stored at -20°C. Total protein concentrations were measured with the BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL).

SDS-PAGE and western blotting

The protein extracted from FFPE tissue sections was resolved by 4-12% NuPAGE® Novex Bis-Tris polyacrylamide gel, and electroblotted to nitrocellulose membrane using iBlot™ Dry Blotting System (Invitrogen, Carlsbad, CA). The membranes were blocked with 5% nonfat dry milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h, washed, and subsequently incubated overnight at 4°C in TBST with 5% BSA containing the following antibodies; anti-VEGF (NeoMarkers, Fremont, CA, 1:50) and anti-GAPDH (Calbiochem, Gibbstown, NJ, 1:5000). Specific molecules were detected with horseradish peroxidase-labeled anti-mouse antibodies (Chemicon International, Temecula, CA) and enhanced with SuperSignal Chemiluminescence kit (Pierce Biotechnology). Signals were detected on KODAK BIOMAX MR X-ray film (Kodak, Rochester, NY). Quantitative analysis of the western blotting was performed using ImageQuant (Ver. 5.2, Molecular Dynamics, Sunnyvale, CA).

VEGF quantitation by well-based reverse phase protein array

VEGF expression signals from archival FFPE tissues were measured as previously reported [14]. Briefly, five microliters of protein extract from FFPE tissue specimen, at pre-determined protein concentrations, were added to Meso Scale Discovery (MSD, Gaithersburg, MD) Multi-Spot™ plates (MA2400 96 HB Plate). The plate was allowed to

dry at room temperature for 90 min, and the plates were subsequently further incubated for 30 min at 37°C. The antigen-coated plates were preincubated with 5% BSA in PBST for 60 min at RT before primary antibody reactions. Anti-VEGF (Neomarkers) and anti-GAPDH (Calbiochem) were diluted 1:200 and 1:1000 with 5% BSA in PBST, and then incubated overnight at 4°C. After washing with PBST, the plates were incubated for 1 h with goat anti-mouse SULFO-TAG™ antibodies at a dilution of 1:1000 (0.5 µg/ml) with 5% nonfat milk in PBST. The plates were then aspirated and washed three times with PBST. Finally, MSD-T read buffer was added to the plates and they were read on the MSD Sector Imager 2400 reader (Meso Scale Discovery). BSA coated wells were included on each plate as a control for non-specific binding effects. The values from non-specific wells were subtracted from all standards samples to calculate actual value. Two independent experiments were performed with triplicates.

Immunohistochemistry and scoring

Tissue sections were deparaffinized and rehydrated in xylene and serial alcohol solutions, respectively. Endogenous peroxidase was blocked by incubation in 3% H₂O₂ for 10 min. The antigen retrieval was performed in a steam pressure cooker with prewarmed antigen retrieval buffer pH 9 (Dako) at 95°C, for 20 min. To minimize non-specific staining, sections were incubated with protein block (Dako) for 15 min. Anti-VEGF antibody (Neomarkers, 1:50) were incubated for 60 min at RT. After secondary incubation of Envision + kit/horseradish peroxidase (Dako), substrate chromogen was added and specimens were lightly counterstained with hematoxylin.

Protein expression for tumour cells was measured over the full tissue specimen using the weighted histo-score method [33]. The weighted histo-score grades staining intensity as 0 (negative), 1+ (weak), 2+ (moderate), or 3+ (strong), then multiplication of percentage of tumor cells within each category. Furthermore, three additional different areas were scored by percentage and intensity: normal colon epithelium, adjacent tissue and surrounding extracellular matrix. Two independent pathologists (TB, SMH) scored slides blindly and separately. For those cases with discrepancy amongst pathologists, a decision was made based on the consensus opinion.

Statistical analysis

Statistical analyses were performed using SPSS (version 19, Chicago, IL) and R (<http://www.r-project.org>). Comparison of means was performed using Student's *t*-test, Mann-Whitney, or one-way ANOVA tests. Linear regression analysis was performed to examine the relationship of VEGF expression by immunohistochemical staining and VEGF quantitation by well-based RPPA. A *p*-value less than 0.05 was considered statistically significant.

Additional file

Additional file 1: Figure S1. Protein expression profiling by well-based RPPA. **Figure S2.** Reliability of well-based RPPA for VEGF assessment in FFPE tissue lysates.

Abbreviations

ELISA: Enzyme-linked immunosorbent assays; FFPE: Formalin-fixed and paraffin-embedded; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IHC: Immunohistochemistry; RPPA: Reverse phase protein array; VEGF: Vascular endothelial growth factor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

J-YC and SMH conceived the study and devised the experimental design for novel protein assays. J-YC, TB, and DK performed experiments. S-MH, S-HE, HC, TB, J-YC and SMH performed data analysis for experiments and clinical records. J-YC, TB and S-MH drafted the final version of the manuscript and figure legends. SMH revised the figures, added critical content to the discussion and was responsible in revising all portions of the submitted portion of the manuscript. All authors read and approved the final manuscript.

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