Comparison of manual and digital microvascular density counting of RECK expression in glioma

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Abstract

A microvascular density (MVD) counting method for RECK expression, using a digital image analysis tool, has advantages over manual counting by microscope. 30 glioma cases with RECK staining were photographed at a magnification of 200x high power field and the photographs in RGB images were analyzed, and stained vessels were captured and were counted automatically. MVD with RECK expression using digital image analysis tool showed comparable result to those of manual method. RECK intensity expression could show linear correlation with grades of glioma by digital method, which was superior compared to manual method. The present method is recommended to researchers undertaking MVD study for glioma.

Keywords: glioma, RECK, microvascular density, digital image counting

Running Title: Microvascular Density Digital Counting in Glioma

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Introduction

Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) acts as a membrane-anchored metalloproteinase regulator [1-4] that is down-regulated in many cancer cells.[5] RECK also said to cause death in RECK-deficient mice around embryonic day 10.5 with their vascular network arrested at the stage of primary capillary plexus, suggesting angiogenesis rather than vasculogenesis is affected in these animals.[2] It is still unclear, however, why embryonic angiogenesis fails to proceed in the absence of RECK while tumor angiogenesis is suppressced by RECK. Noda et al found abundant expression of RECK in the cells associated with blood vessels undergoing rapid remodeling in the mouse implantation chambers, suggesting that RECK in vascular remodeling which may involve nonsprouting mechanisms such as intussusception and pruning.[6] Our preliminary study (unpublished data) showed that RECK gave a positive vessel staining in glioma, and that the microvascular density (MVD) values found correlated with glioma grades.

Microvascularity may be assessed quantitatively by counting the number of vascular profiles, MVD, in immunohistochemically stained tissue section. MVD is considered an important marker for angiogenesis, which in turn is responsible for local growth and metastasis in tumors.[7] Increased MVD has been shown in primary tumor and metastases of various tumors.[8] In glioma, MVD count has been reported to be associated with stage and tumor progression, although somewhat contradictory results were found.[9, 10] However, there has not been any study of vessel staining using RECK in glioma.

Digital counting (DC) has been proposed by several authors.[11-15] It is supposed to help the person responsible for cell counting in having efficient work. However, DC for MVD in glioma has never been performed before. In this study, we would like to

analyze the technique of digital MVD counting and its reliability, for routine use in the future.

Materials and Methods

Immunohistochemical study

This study was approved by the local Ethical Committee of Shinshu University School of Medicine under registration number of 1321. Thirty patients diagnosed with glioma from Shinshu University Hospital were included in this study. Glioma grades were extracted from the medical records. Glioma tissues were stained with RECK (R&D Systems, Inc.). RECK expressions using a polyclonal antibody for RECK (R&D Systems, Inc.) were studied in formalin-fixed and paraffin-embedded tissue samples of glioma. Tissue samples were washed free of fixative with phosphate-buffered saline (PBS, pH 7.4), exposed to 0.3% H2O2 in PBS for 5 minutes to inactivate endogenous peroxidase, then pretreated with trypsin solution 37°C for 30 minutes. The sections were then reacted with primary antibody overnight in a humidity chamber at 4°C. Dilution of primary antibodies was 1:400 for anti-RECK IgG. Polyclonal goat IgG antibody, as the secondary antibody for RECK, was applied to the sections for 30 minutes. The color was developed with 0.02% 3-3` diaminobenzidine tetrahydrochloride and 0.006% H2O2 in PBS. The slides were counterstained with Mayer's hematoxylin. Between steps, the slides were washed three times in PBS. H&E staining served as controls. For vessel staining comparison, RECK endothelial staining was compared to that of CD34 (Beckman Coulter, Inc, Fullerton, California), which is considered as an established endothelial staining. Manual and digital counting of RECK expressions were compared.

Manual and Digital Counting Methods

Manual counting was performed by NNR and YL. 3 photographs taken from the software, Biorevo BZ-9000 (Keyence, Osaka, Japan), were used as the same area counted for both manual and digital counting. The counting was done under 200x high power field, and MVD was scored as the number of vessels found in the field. Score of 3 areas from each slide was averaged. Results were confirmed in each experiment by at least two observers. Result of those manual counting was compared to the digital ones.

DC of RECK MVD was performed using Biorevo BZ-9000 microscope (Keyence, Osaka, Japan). Each slide was scanned microscopically and 3 representative fields, with large numbers of RECK-positive cells, were photographed at a magnification of 200x high power field. Vessels with any detectable staining, i.e., above background levels, were considered positive. Hue range of 50 was chosen initially and one representative stained cell was pointed, and the rest with the same range was automatically recognized and was masked with red color. The "Analyze Particles" command automatically counted the number of objects, and the counts were displayed in the "Info" window. The threshold was then lowered to 15 to obtain the remaining cells with less staining intensity. The program then automatically counted the number of RECK-positive and negative cells. Results were shown on the computer monitor and interactive correction was performed if required. The MVD staining index was defined as the number of RECK-positive vessels found in the photographed area. Intensity was defined as saturation of the stained samples, according to the respective desaturated and saturated markings, which discriminated objects of interest from surrounding background, desaturated or less hue being the lowest (0) to saturated or full hue as the highest (255). Intensity of the stained vessels

was also examined. Brightness level and white balance of all slides were standardized, and intensity score was obtained from the `Info` window of the masked-stained areas. Subsequently, the image and data could be converted into TIFF and excel data, respectively, for analysis. Scanning and analysis time were approximately 10-15 minutes each slide.

Statistical Analysis

PASW v. 18.0 for MAC (SPSS, Chicago, IL, USA) was used for the statistical analysis. Correlations between the results obtained by the different methods to quantify MVD and intensity of the staining between different observers were calculated using paired T-test. To calculate whether the difference between the two measurements was related to the magnitude of the measurement, the difference between the two methods was plotted against the standard, as suggested by Bland and Altman.[16]

Results

Characteristics of the glioma cases

In total 30 gliomas were included in the study. They consisted of 6 samples of grade 2, 16 of grade 3, and 8 of grade 4. Observers were blinded to the grades during counting. Thirty specimens were stained with RECK, and all showed strong staining of the microvessels. No staining was found in the tumor cells or cytoplasm. It was also confirmed with CD34 staining, an established endothelial marker, that RECK showed similar pattern of staining to that of CD34 (Figure 1). Microvascular density was counted using the standard method,[22] and was compared manually and digitally.

Microvascular density with RECK

Intra and Inter-observer analysis of manual counting

Manual counting of 30 slides (3 areas each) of RECK stained vessels took approximately 22.5 to 23 hours to complete for each individual observer (approximately 15 min per section). Intra and inter-observers variations were slightly found in the repeated counting of MVD (Figure 2), however, it did not show any significant difference either in intra-observer one, two, or inter-observers ($p=0.698$, 0.119, 0.137, respectively). The same sections (photographs) were later used for digital counting.

Comparison with digital counting

The method allowed rapid counting of the stained vessels. DC of 30 slides (3 areas each) took approximately 7.5 to 10 hours to complete (approximately 10 min per area). RECK gave a strong staining intensity in all samples, allowing easy identification during tagging process. Vessels appeared in dark brown from DAB coloring. The stained vessels were tagged from a brightfield image (RGB photograph) by setting the initial hue range of 50 (scale of 0-255). All visible vessels could be easily tagged (marked) with addition of minor adjustment, such as enhancing the margin of each tag, or sharpening the image (Figure 3).

Consistent result was found in the repeated DC, as shown in Bland and Altman graph in Figure 5. The discordance between the manual and digital methods was minimal in images less than 100 vessels/image, however, it was clearer in images containing more than 100 positive vessels/image. Nevertheless, variation found did not show any significant difference ($p=0.404$).

Intensity of the stained vessels was also automatically calculated, and was measured in the scale of nanometer. Average intensity of the staining was 96.44 (range: 77.3-120) with DC, whereas manually, all slides could only be evaluated as having strong intensity without any exact value. Intensity value calculated with DC disclosed linear correlation with grades of glioma (p=0.01, Table 1).

Discussion

Lack of RECK expression has been noticed in several cancers, and it is correlated with poorer prognosis.[23] Our previous study in skull base chordoma also showed concordance result.[24] However, in glioma, we found out that RECK was not expressed in glioma cells, and was the same findings through out all grades. It was not even found in normal brain cell (unpublished data). It was found in endothelial cell of vessels with increasing tendency in accordance to its grade. RECK was found in maternal and embryonic vessels in mice,[6] and endothelial cells in osteosarcoma.[25] Recent studies have demonstrated the importance of stable vessels to sustain adequate tumor blood supply.[26]

This novel role as angiogenesis marker, especially in glioma, interested us in finding out the MVD value of RECK-stained specimens. Increased vascularity and angiogenesis occur in support of actively proliferating tumor cells and thus blood vessel parameters may have a potential application as diagnostic and prognostic indicators. Takeuchi et al in their glioblastoma study explained the role of transformation and proliferation of smooth muscle cells, which may involve in neovascularization or in angiogenesis.[27] In this study, we performed MVD counting of RECK stained vessels, and we compared between the manual and DC.

Several studies have been conducted computer-assisted image counting [11-15] for

quantification of cells in immunohistochemically stained sections in different compartments of the human body, including the central nervous system,[20, 21] as well as in malignant tumors.[28-30] However, to our knowledge, there has been no report on the comparison between manual and DC for the quantification of vessel density in glioma.

Role of RECK in angiogenesis of glioma is a new finding, therefore before we could it to the present digital counting, we also performed CD34 immunohistochemical staining, an established endothelial marker, to confirm the staining found in RECK samples. Our result supported that RECK showed a positive endothelial staining, similar to those of CD34, however, the difference between the two staining is not discussed in the present study; it will be published in a separate article. DC method (tagging method) employed in RECK-stained vessels was based on the brown color appearance resulted from DAB coloring, in which vessels made a halo or donut-like shape. Vessel or nuclear staining, compared to membrane or cytoplasmic staining, is easier to be conducted using digital method as explained by Lejeune et al. They found that the automated method represents an objective and accurate quantification system for nuclear marker. The concordance of the results compared to manual method was approximately 90%, whereas membrane staining was more difficult.[11] The RECK staining in our glioma cases had a clear margin to the surrounding unstained cells. It provided easy counting, where consistent DC result was obtained, whereas with manual method even though variance between observers existed, there was no significant difference found. Therefore with the same simple algorithm, consistent MVD results could be obtained.

The major advantage of DC compared with manual counting is the simplicity of the tagging method, which made it easy to use even for beginners, combined with the

reduction in time necessary to complete the analysis compared with manual method. Another important advantage is the standardization of the analysis, especially in intensity count. In manual counting, staining intensity could only be classified as weak, mild or strong staining. Using DC, staining intensity is expressed as an absolute number, providing an advantage over the subjective scales. In our study, intensity values showed correlation with glioma grades.

DC also has some limitations. In some instances, the computer is not able to interpret differences in staining intensity between different sections. For example, aggregates of vessel in thick-cut sections might be seen by the computer as a single object. Standardized protocols in cutting or staining procedures must be followed to prevent these irregularities. Secondly, observers have to be trained to be able to use the digital system, which includes a learning curve, in order to avoid intra- and inter-observer variability. Thirdly, the system is quite expensive, preventing it from being available widely. Nevertheless, with the advantages it offers, the reliability and reproducibility of the system, DC for MVD will not only be useful glioma, but perhaps in other diseases as well.

Conclusion

Digital MVD counting with the commercially available software is a valid method for the digital vessel density counting. Although improvements related to the speed and overall assessment of image complexities are warranted, the use of our automatic method offers clear advantages over the manual method in terms of improved precision, reliability and reproducibility of RECK immunostaining in glioma. Further improvements in the simplicity will be needed for easier application on a routine base.

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Conflict of Interest

All authors have no financial, commercial, legal, or professional relationship with other organizations or with the people working with us that may exert an influence on this research. Therefore, we have disclosed any conflict of interest in the making of this paper.

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Figure Legends

Figure 1. Various expressions of RECK and CD 34 in glioma

RECK (A,C,E,G) and CD 34 (B,D,F,H) are expressed in endothelial cells in glioma. A,B) are showing distribution of RECK and CD 34 around a necrotic core of glioma (10x). C,D) are showing typical pattern of tumor microvessels, slid-like or small round shape vessels (20x). E,F) are showing the enlarged or irregular shapes of vessels mostly found in higher grades of glioma (20x). G,H) are showing a highly dense tumor vessels found in high grade glioma (20x).

Figure 2. Intra and interobservers difference of RECK MVD staining manual counting.

A) is showing observer one variations during manual counting of RECK staining, B) is showing observer two variations during manual counting of RECK staining, and C) is showing inter-observer variations during manual counting of RECK staining. Variations are slightly seen, but no significant difference is observed.

Figure 3. Steps in performing digital counting of RECK stained-vessels.

A) is showing the original image of RECK stained-vessels of glioma, B) is showing the automatic tagging of the stained vessels, C) is the numbers put on the tagged vessels, D) enlarged area of C, showing the automatically set numbers on the microvessel.

Figure 4. Differences in manual and digital counting of RECK MVD staining.

A) is showing variations between manual and digital counting of RECK staining, and

B) is showing minimal variations during digital counting of RECK staining. Variations are slightly seen especially in images containing more than 100 positive vessels/image, but no significant difference is observed.

Table 1. Distribution of mean intensity values based on glioma grades

WHO Grading	Number of Cases	Mean Intensity
II	6	86.66 (range: 77.3-99.3)
III	16	96.12 (range: 82.5-120)
IV	8	104.45 (range: 98-117.3)
Total	30	96.44 (77.3-120)

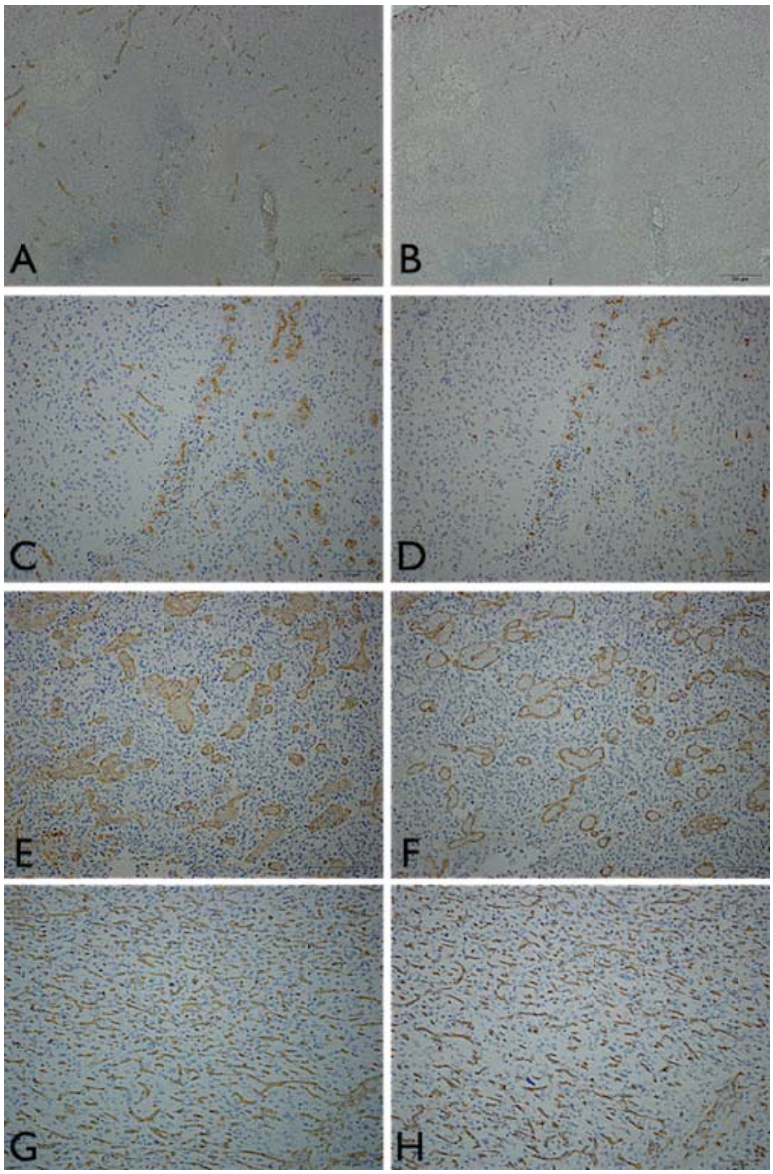

Figure 1.

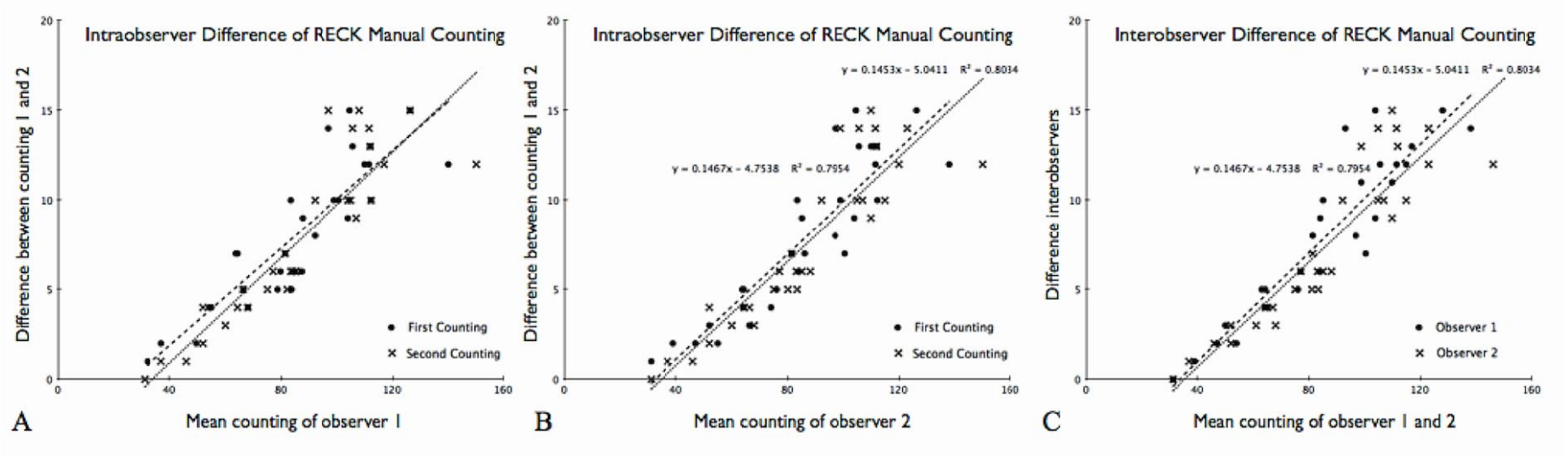
Intraobserver Difference of RECK Manual Counting
Difference between counting 1 and 2
Mean counting of observer 1
First Counting
Second Counting
A
Intraobserver Difference of RECK Manual Counting
y = 0.1453x - 5.0411 R² = 0.8034
y = 0.1467x - 4.7538 R² = 0.7954
Difference between counting 1 and 2
Mean counting of observer 2
First Counting
Second Counting
B
Interobserver Difference of RECK Manual Counting
y = 0.1453x - 5.0411 R² = 0.8034
y = 0.1467x - 4.7538 R² = 0.7954
Difference interobservers
Mean counting of observer 1 and 2
Observer 1
Observer 2
C

Figure 2

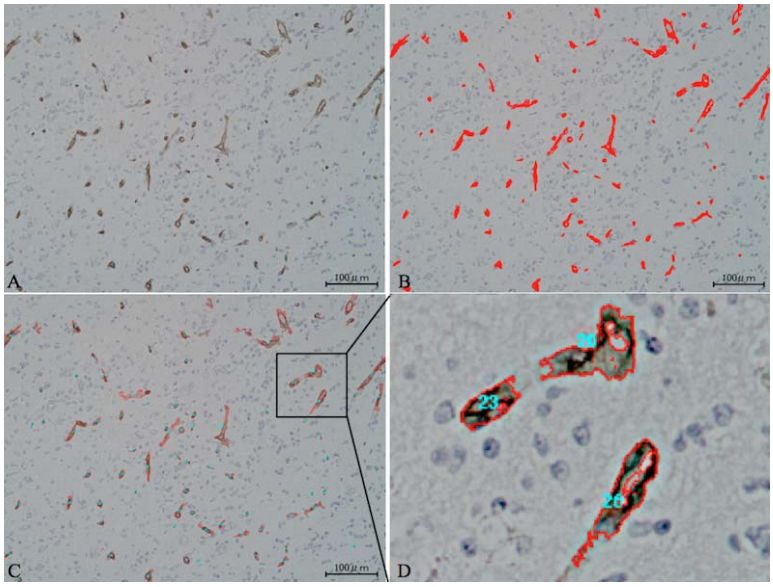

Figure 3.

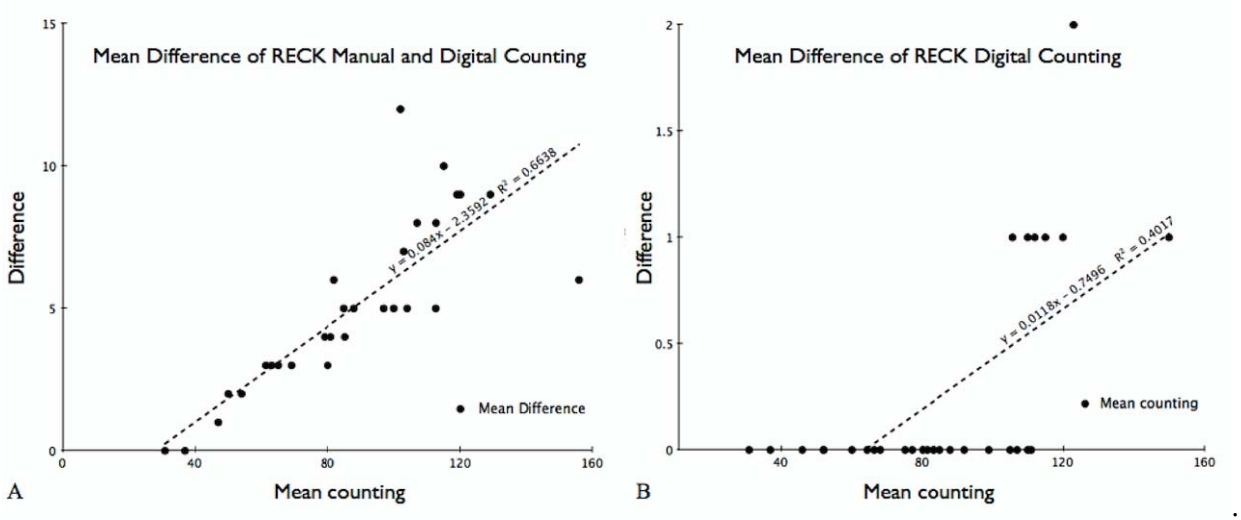
Mean Difference of RECK Manual and Digital Counting
Difference
Mean counting
y = 0.084x - 2.3592
R² = 0.6638
Mean Difference
A
Mean Difference of RECK Digital Counting
Difference
Mean counting
y = 0.0118x - 0.7496
R² = 0.4017
Mean counting
B

Figure 4.