Image Anal Stereol 2025;44:105-110 doi: 105566/ias.3145
Original Research Paper

EVALUATING THE RESULTS OF CD31 AND CD34 EXPRESSION ON SKIN VESSEL ENDOTHELIAL CELLS AFTER RADIOTHERAPY BY STEREOLOGICAL METHOD

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(Received January 27, 2024; revised June 21, 2025; accepted June 23, 2025)

ABSTRACT

Skin damage is one of the most frequent side effects during radiotherapy in patients with cancer diseases. In this study, the stereological method was used to assess the level of CD31 and CD34 expression on the skin microvascular endothelial cells after radiotherapy. We collected thirty radiation-ulcer skin samples, each of which has three studied regions: area of ulcer, adjacent area and unwounded area. We found that the surface density (Sv) and length density (Lv) of blood vessel with CD31 and CD34 positive decreased significantly from center to outside of the ulcer. We conclude that stereological method could be used to evaluate the results of CD31 and CD34 expression on skin microvascular endothelial cells after radiotherapy. Moreover, the success of our study may permit to following application of this morphological quantitative method for analysis of further immunohistochemistry studies.

Keywords: CD31; CD34; immunohisto-chemistry; radiation-induced ulcer; radiotherapy; stereology.

INTRODUCTION

Necrosis and other structural morphological changes of skin (perivascular fibrosis, embolism, and hemorrhage) are frequent side effects of radiation therapy (Majeed, H., Gupta, V. , 2023). Moreover, vascular damage due to unwanted effects of radiation treatment is one of the most important factors that lead to some consequences such as atrophy, fibrosis, and necrosis of human skin (Kameniet *et al*., 2024; Quarmby *et al*., 1999).

Immunohistochemistry method for expression of CD31 and CD34 on the vascular endothelial cell membranes has been used widely to evaluate the degree and role of microvascular damage after radiation. CD31 is gold-standard for endothelium cell integrity and inflammation. Loss of CD31 staining indicates endothelium cell detachment, a hallmark of acute injury. Altered functional staining reflects endothelium cell activation or early damage (Pober and Sessa, 2007). At the same time, CD34 is a marker of angiogenesis and tissue repair. Increased CD34 vessels signify angiogenesis, that is why CD34 expression is gold standard for quantifying angiogenesis (Vermeulen *et al.* ,2002). Therefore, combined use CD31 and CD34 provides a complete picture of vascular injury (Ribatti *et* al. 2007).

However, high subjectivity level in qualitative or semi-quantitative assessment of immunohistochemical reactions was considered to be one of main factors that leads to an increase in both systemic and statistical errors of the studies (Bencze *et al*., 2021).

Quantitative morphological method based on system stereology is the new concept in science, especially in the studies of human tissue section. This procedure helps to obtain 3D characteristics of tissue based on stereological assessment on "2D" tissue slides which increases the objectivity and minimizes error of the study (Avtandilov, 1990; Howard and Reed, 2004). Therefore, based on applying stereological methods in evaluating the results of immunohistochemistry, our study aims to estimate the level of CD31 and CD34 expression on the skin microvascular endothelial cells after radiotherapy in cancer patients.

MATERIALS AND METHODS

Skin samples

We resected thirty radiation-induced chest skin samples from post-radiotherapy patients during period of October 2013 to September 2017 at the Vietnam National Burn Hospital. The median time of the skin ulcer appearance was 7.5 years (range from 1 to 31 years). Each skin sample has three examined regions: area of

ulcer (zone 1), adjacent area (zone 2), and unwounded area (zone 3) (Fig.1).

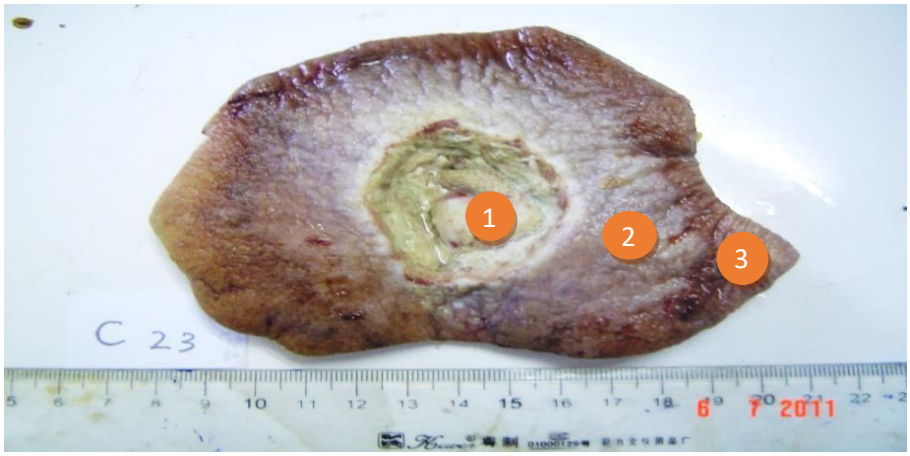


Fig. 1: *Skin sample with three zones of the radiation-induced ulcer: (1) area of ulcer, (2) adjacent area, and (3) unwounded area*.

Samples processing

The vertical uniform random (VUR) skin sections were prepared for stereological analysis. All the samples (about 10 x 8 x 2 cm) were fixed in a 10% - formalin solution before being transferred to the Pathology Department, where each sample was dissected perpendicularly to the surface of the skin. Each skin sample was divided into 3 parts according to 3 regions: area of ulcer (zone 1), adjacent area (zone 2), and unwounded area (zone 3), followed by preparation of paraffin-embedded tissue blocks (3 blocks for each zone of 1 sample). For each block, we cut two four-micrometer thin sections (perpendicularly to the surface of the skin) to perform an immunohistochemical reaction with CD31 and CD34 antibodies using the ABC standard immunohistochemistry staining method.

Digital images

Stained tissue slides prepared for microscopy were imaged using a CCD Canon camera connected to the computer. Five images (one in the center, the others in the four corners of section) were taken randomly for each section at 400 x magnification.

Stereological analysis of immunohistochemical reaction

The ImageJ software (American National Institutes of Health) was used to optima the stereological analysis (Fig.2).

The following stereological parameters have been obtained:

- The surface density (Sv) of CD31, CD34 positive vessel was obtained using the equation:

SV= 2I/L (Avtandilov, 1990; Howard and Reed, 2004)

L: the total length of the horizontal and vertical line.

I: the total number of intersections between lines and the CD 31, CD34 positive vessel.

Relative error (RE) was calculated by formula:

RE = K*t/I1/2.100%; (K= 0,45; t = 1,96) (Avtandilov, 1990)

- The length density (Lv) of CD 31, CD34 positive vessel was obtained using the equation:

LV= 2 Q/A (Avtandilov, 1990; Howard and Reed, 2004)

A: the area of micro field

Q: the number of micro vessels in the area of micro field

Relative error (RE) was calculated by formula:

RE = K*t/Q1/2.100%; (K= 0,45; t = 1,96) (Avtandilov, 1990)

Statistical analysis

Area and length densities of each zone were calculated by appropriate equations, summing up counts from all of the digital images (30 samples x 15 images/sample). Zone vessel densities were compared with each other by using T-Test calculator for 2 independent means with a two-tailed hypothesis, significance level was 0,05 ($p<0.05$).

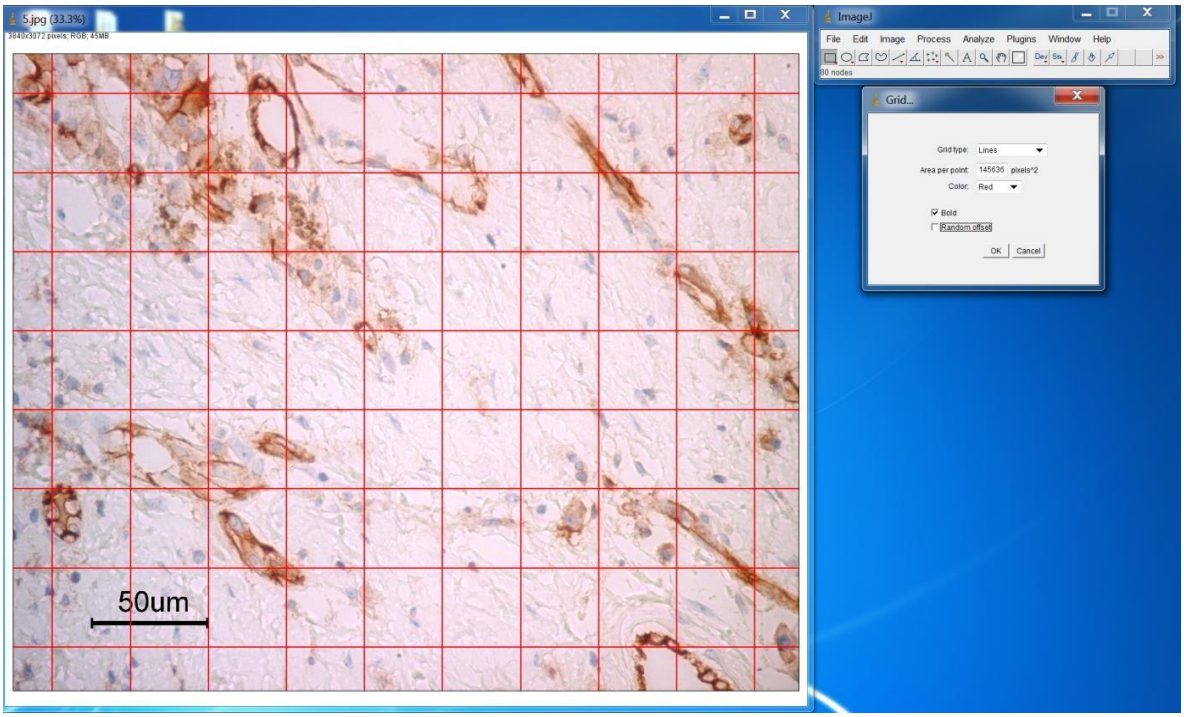


Fig. 2: *Use of ImageJ software with the superimposed grid for stereological analysis of immunohistochemical reaction in skin sections: 400x- magnification Jpeg images; Grid type: lines; Area per point: 145636 pixel2 (8x10 grid).*

RESULTS

CD31,CD34 expression

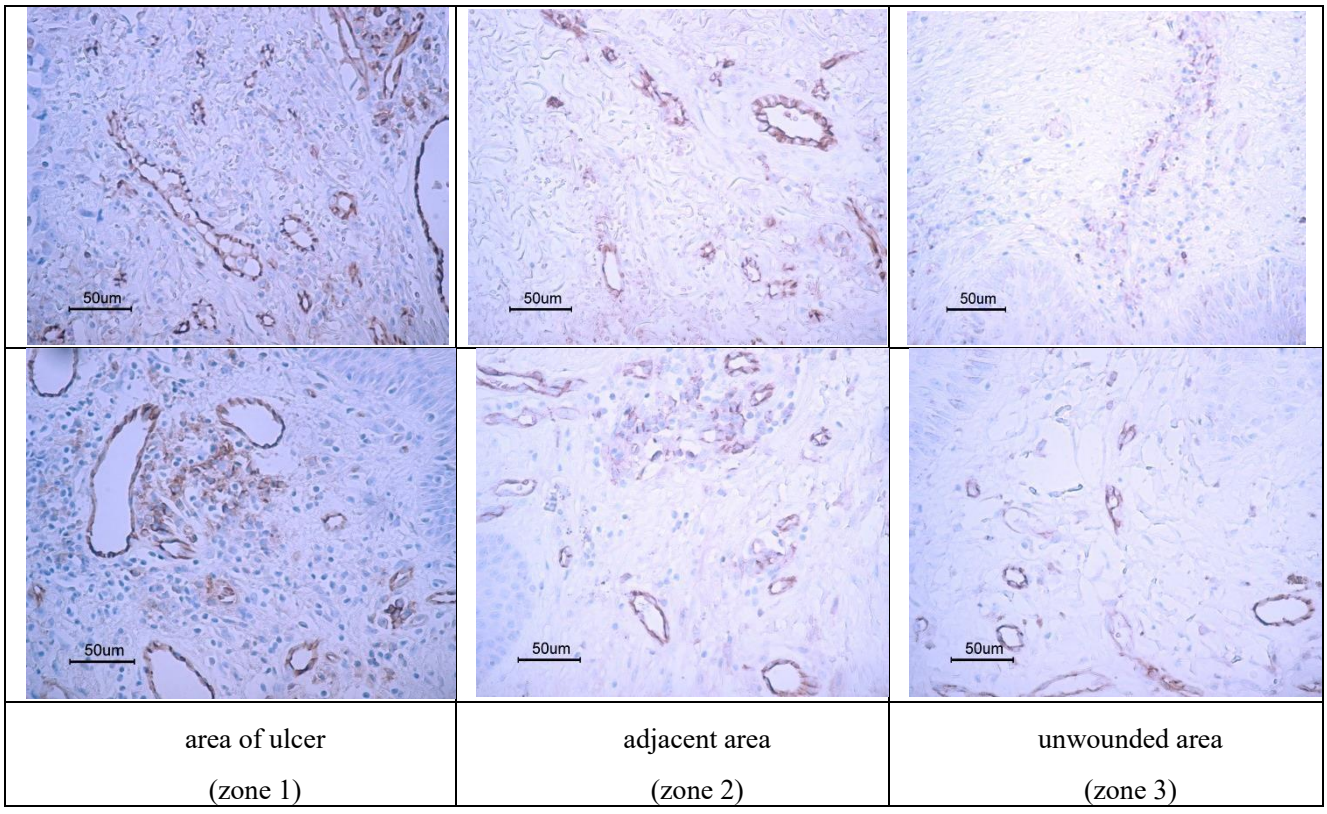
All of the sections from 3 skin regions were positive for CD31 and CD34, but with different values of surface density (Sv) and length density (Lv) of CD 31, CD34 positive vessels (table 1).

For CD31, both Sv and Lv were decreased gradually from zone 1 to zone 3 with high statistic significant ($p<0.01$) for value difference of Sv or Lv in two nearby zones (Zone 1 *vs.* zone 2 and zone 2 *vs.* zone 3) (Fig.3). Most of RE indexes were under 5% except the RE index of Lv in zone 3 (was 5.01%).

For CD34, the values of Sv and Lv showed a similar trend with CD31 immunoactivity (Fig.4). From zone 1 to zone 3, the values were down (from 0.29 mm^{-1} to 0.22 mm^{-1} for Sv and from 1.08 mm^{-2} to 0.76 mm^{-2} for Lv). Although when compare for value difference of Sv or Lv in two zones, only the Lv difference between zone 1 and zone 2 was statistically significant with $p<0,01$, remain p-values were higher than 0.05. All of the relative error indexes (RE) were below 5%.

Table 1: *The surface density (Sv) and length density (Lv) of CD 31, CD34 positive vessels*

	CD31						CD34					
	Sv (mm^{-1})	RE (%)	p	Lv (mm^{-2})	RE (%)	p	Sv (mm^{-1})	RE (%)	p	Lv (mm^{-2})	RE (%)	p
Zone 1	0.53	1.97%	<0.01	0.9835	3.51	<0.01	0.29	2.57	0.28	1.0792	3.24	<0.01
Zone 2	0.29	2.66%		0.7003	4.16		0.25	2.79		0.854	3.64	
Zone 3	0.27	3.43%	<0.01	0.4762	5.01	<0.01	0.22	2.93	0.078	0.7611	3.85	0.087



area of ulcer (zone 1) | adjacent area (zone 2) | unwounded area (zone 3)

Fig. 3: *The CD31 expression on the skin microvascular endothelial cells after radiotherapy in different zones of ulcer (400 x)*

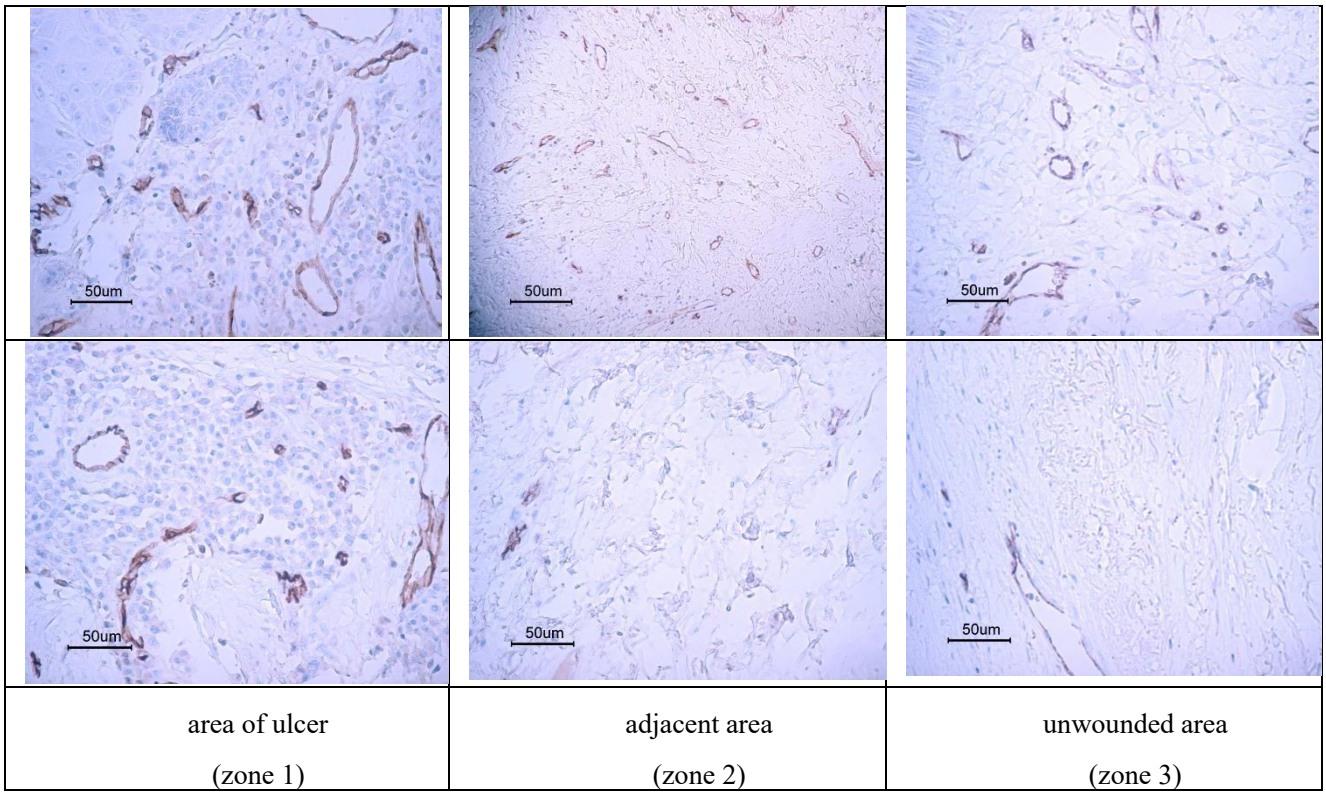


Fig. 4: *CD34 expression on the skin microvascular endothelial cells after radiotherapy in different zones of ulcer (400 x).*

DISCUSSION

Results of our study illustrate the advantage of using stereological method for estimating immunohistochemical reactions in tissue slides via analysis of area and length densities of CD31, CD34 positive blood vessels. It is a novel application in comparison with the current popular semi-quantitative method.

In this study, we decided to quantify both the area and length densities of blood vessels for testing the accuracy of our applied method. The result of area density analysis was similar to that of length density in skin tissue samples after radiotherapy, which was positive for CD31 or CD34. The expression of these markers increases gradually from the margin to the center of the ulcer. Moreover, our results in assessment of CD31 and CD34 expression level were similar with previous studies (Chin, 2015).

One of the most important requirements when applying morphological quantitative methods based on stereology analysis, is ensuring its strict systematicity from study design to measurement method, as well as statistical analysis of the results. All of these stages must be matching methodical guidance since it will directly affect the accuracy of the results. To meet these requirements, we collected a relatively lager number of biopsy samples (from 30 patients) to ensure representativeness. We designed the study as a single-blind that all stages of sample processing, digital images obtain and stereological analysis of immunohistochemical reaction have been performed by different specialists who did not know the information about this research. At the same time, the process of selecting the field for the calculation was also conducted randomly with 4 peripheral images and 1 image in the center of the section. Owing to our investigation's compliance, the results of our study may consider to be accurate and reliable.

One of the advantages of the stereology is not only to provide quantitative data but also to indicate its relative error, that not only allows the researchers to assess results accuracy but also help researchers in study design with early established level of accuracy (i.e. relative error). On the other hand, the quantitative results from stereological analysis could help to choose more statistical methods (continuous variables, ranking variables and list variables) for analysis of the study results.

CONCLUSION

Along with the development of science and technology, it is important to apply more quantitative methods to evaluate the results of scientific research.

Morphological quantification based on stereological analysis has been considered to be a perspective method to increase objectivity and reliability in evaluating the morphological characteristics of biological tissues. The stereological results are milestones for further application of the 4.0 scientific and technological revolution towards "digitalization" and "automation" not only in medical research but also in clinical morphology diagnosis. Our success in estimating the level of CD31 and CD34 expression on the skin microvascular endothelial cells after radiotherapy permits to following application of this morphological quantitative method for evaluating the results of further immunohistochemistry studies.

ACKNOWLEDGEMENTS

We are grateful to Professor Dung N.T, President of Vietnam Society of Pathology and Cytology, for his skillful assistance.

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