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Capillary Dimension Measured by Computer Based Digitalized Image in Patients with Systemic Sclerosis

Hyun-Sook Kim
Division of Rheumatology, Department of Internal Medicine, College of Medicine, The Chosun University of Korea, Gwangju Republic of Korea

1. Introduction

Systemic sclerosis (SSc) is an autoimmune disease characterized by fibrotic vasculopathy and excessive organ fibrosis. Endothelial and vascular damage are main leading disability in SSc. The goal of treatment lies in the prevention of excessive fibrosis affecting major organs such as lung, esophagus or skin, and in minimizing microvascular injury to lessen the deterioration in quality of life. Raynaud's phenomenon is the clue of early diagnosis that present vascular damage developing in the preclinical stage of SSc in 80-90% of patients. Nailfold capillaroscopy (NFC) is a easily accessible diagnostic tool in secondary Raynaud's phenomenon. Examination of the nailfold capillaries can reveal the nature and extent of microvascular pathology in patients with SSc. Several prominent nailfold capillary changes, for example, megacapillary, capillary hemorrhage, loss in capillary distribution, is distinctively apparent in SSc.

The main cytokines that induce fibrosis from fibroblasts and endothelial cells are transforming growth factor-β (TGF-β), interleukin-1 (IL-1), endothelin-1 (ET-1), tumor necrosis factor-α (TNF-α). Especially, ET-1 was reported to play an important role in vasoconstriction, stimulation of fibroblasts growth incurring serious complications of SSc, such as, pulmonary fibrosis or pulmonary hypertension. These aberrant biochemical processes may involve systemic microcirculation and, thus, cause diffuse vascular abnormalities.

2. Optimal NFC parameter in SSc

Nailfold capillary is represent microcirculation (Figure 1). NFC is a well-established imaging technique widely used for diagnostic purposes in rheumatology as well as in other diseases, to assess features of microcirculation in vivo. Nailfold videocapillaroscopy enables the study of several aspects of capillary vessels, including morphology, distribution, density and blood flow. For Significant correlation between the results of NFC and microvascular injury and lung involvement was reported. Therefore, severity of the disease or response to the treatment could be estimated. Recently, researches on new factors in NFC, that would enable earlier

detection of change in clinical conditions and microvascular injury with computer-based digitalied analysis, are underway. Despite these advantages, there is some debate regarding the optimal parameter in assessing microvascular injury reflecting clinical status.

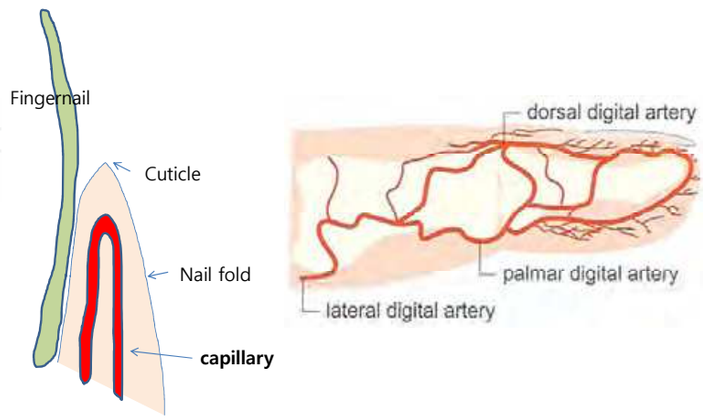

Fig. 1. Scheme of nailfold capillary

2.1 Study design

We carried out this study to define the value of optimal NFC parameter in patients diagnosed as SSc, by investigating correlation between clinical manifestations and plasma ET-1 which are known to reflect disease activity.

2.1.1 Patients and investigations of clinical manifestations

Sixty patients were randomly selected from those fulfilling American College of Rheumatology criteria for SSc and whom visited outpatient clinic. 30 healthy controls were chosen from adults with no known medical history, and 23 disease controls were chosen from the patients with connective tissue disorders other than SSc. The disease control group consisted of 14 systemic lupus erythematosus (SLE), 4 primary Sjogren's syndrome (pSS), 2 dermatomyositis (DM), 2 mixed connective tissue disease (MCTD) and 1 rheumatoid arthritis (RA) patient.

All patients and controls underwent NFC and blood sampling. After removing plasma from blood, the sample was stored at -70˚C freezer (ULT-1386-5D-40) in order to measure ET-1. Evaluation of patient group's clinical manifestations was done with following exams; manometry and gastrofiberscopy to discover gastric involvement, pulmonary function test (PFT) and chest HRCT to discover pulmonary fibrosis, echocardiography to discover pulmonary hypertension, urinalysis and kidney sonography to discover renal involvement. Symptomatically, the presence of arthralgia, arthritis and digital ulceration was investigated. Assessment of skin sclerosis was done by an experienced rheumatologist, converting the severity into score using Modified Rodnan Score (MRS) (range : 0-51). Antinuclear antibody (ANA), Anti scl-70 antibody, Anti centromere antibody, extracellular nuclear antigen (ENA) was measured by immunoblot method.

2.1.2 Nailfold capillaroscopy

Patients were kept inside the procedure room for a minimum of 15 minutes before the nailfold analysis can be performed, to adapt to the room temperature of 20–25 ℃. Each

subject seated with dorsum of hand facing upwards, and with halogen lightings illuminating upon the nails coated with immersion oil, under nailfold microscopy. The NFC examination was performed by an experienced examiner without the patient's clinical information and nailfolds of second, third and fourth digits of both hands were observed with light microscope (Olympus SZ-PT, Japan) under 100 times and 400 times magnification (Figure 2). All microphotographs were transmitted to computer by digital camera (Polaroid, USA) and after saving the images, enhancement was done by color filtering using Adobe Photoshop® ver. 7.0 for analysis. Quantitative analysis was done by counting total number of capillaries and number of capillaries with deletion, which were observed within 3mm width of the central part of digits. The results were recorded in average value of 6 digits. Also, an experienced rheumatologist measured the apical limb width and capillary width of 3 capillary rings located at the center, where resolution is the finest, directly from computer screen, from all 6 digits (Figure 3). Previous reports have suggested that activity of SSc is in strong correlation with capillary width and apical limb width, but not capillary length. Therefore, we presumed that measuring capillary dimension at fixed capillary length (25um) could be a new parameter. We defined capillary dimension as the sum of pixel numbers of the area set by measuring capillary boundary at capillary length of 25 um. (1cm on x 400) Adobe Photoshop® ver. 7.0 was used in this process (Figure 4).

Fig. 2. Nailfold capillaroscopy is a safe and noninvasive tool that possible to detect the progression of the morphological changes.

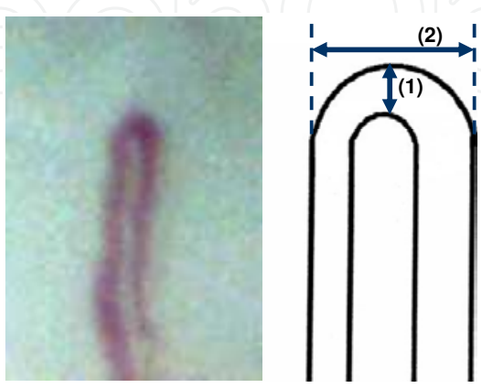

Fig. 3. Scheme of capillary loop measurements (× 400). (1) Apical limb width, (2) Capillary width.

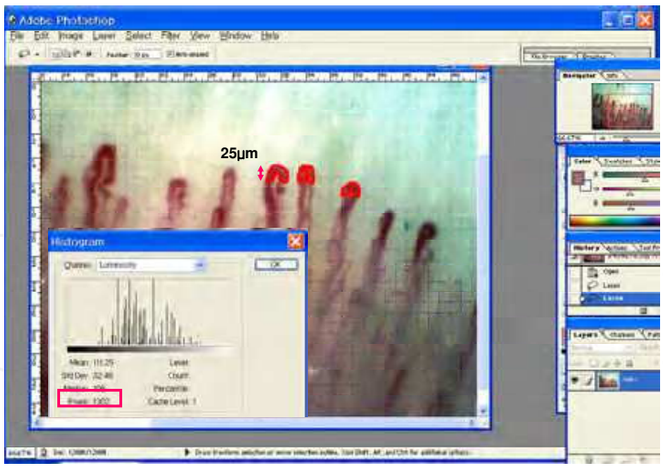

Fig. 4. The method of calculating "the sum of pixel number" with Adobe Photoshop® version 7.0 as reflect of capillary dimension: A perpendicular length of 25um tangent to internal limit of a capillary loop transverse segment defined the transverse segment area.

2.1.3 Measurement of plasma endothelin-1 and Statistical analysis

Plasma concentration of ET-1 was measured with ELISA (enzyme-linked immunosorbent assay) method. 500 uL of plasma ET-1 in EDTA tube was evenly mixed with 750ul of extraction solvent (acetone : 1N HCl :Water (40 : 1 : 5)) and then microcentrifuged. The supernant was dried down for 7 hours using speedVac concentrator and ET-1 ELISA kit (R&D, Minneapolis, MN, USA) was used for measurement. The results were interpreted using a microplate reader set to 450 nm as reference wavelength. The results were expressed in median values and inter-quartile range. SPSS ver. 17.0 program for Windows (SPSS, Chicago, IL) was used for statistical analysis. Mann-Whitney test was used for the comparison of NFC parameters and continual variables of ET-1, and Chi-square test and Fisher's exact test was used for the comparison of clinical manifestations, between the SSc patient group and the control groups. Spearman's correlation coefficient was used to express the relationship between MRS, measurement parameters of NFC, and plasma levels of ET-1 in SSc. All results were interpreted to be statistically significant when p value < 0.05.

2.1.4 Results

There were 30 people of diffuse type of systemic sclerosis (dSSc), and 30 people of limited type (lSSc). The mean age of 60 patients is 47 years old (12 - 66 years); 52 people are women (84%), the mean prevalence period is 4 years (0.1-18 years), which is similar in the sexual ratio and the age compared to the control groups. 57 people (95%) of SSc have Raynaud phenomena and it was more frequent than the disease control group. Raynaud phenomenon period is 12 months (1-120 months), statistically longer than the disease control group, which is 6 months (3-24months). Among the clinical manifestations, dysfunction due to gastrointestinal sclerosis, pulmonary fibrosis, arthralgia, arthritis, and digital ulcer were significantly frequent in SSc (Table 1). The difference in clinical patterns due to the subtypes of SSc was significantly elevated in the outbreak frequency of MRS high scores, gastrointestinal dysfunction, pulmonary fibrosis, and digital ulcer in the dSSc than the lSSc. On the contrary, there was no difference in Raynaud phenomenon, pulmonary hypertension, renal diseases, arthralgia, and arthritis. Statistically, autoantibody such as ANA, anti scl-70 antibody, ENA showed more positive incidence in the dSSc (Table 2).

	Healthy Controls	Disease control*	SSc
	(N =30) (%)	(N =23) (%)	(dSSc=30, lSSc=30) (%)
Female sex , n (%)	30 (100)	23 (100)	52 (83.87)
Age, yrs, median (IQR)	30 (24-53)	41 (18-57)	47 (12-66)
Disease duration, yrs, median (IQR)		5 (0.5-10)	4 (0.1-18)
Raynaud's phenomenon	0 (0)	10 (43.48)	57 (95.0)
Duration of Raunaud's ph, months, median (IQR)		6 (3-24)	12 (1-120)
GI manifestation	0 (0)	0 (0)	29 (48.33) #
Pulmonary fibrosis	0 (0)	2 (8.70)	33 (55.0) #
Pulmonary Hypertension	0 (0)	2 (8.70)	3 (5.0)
Renal disease	0 (0)	1 (4.35)	3 (5.0)
Arthralgia	0 (0)	11 (47.83)	45 (75.0) #
Arthritis	0 (0)	2 (8.70)	14 (23.33) #
Digital ulceration	0 (0)	5 (21.74)	27 (45.0) #

Table 1. Clinical characteristics of control and patients with SSc.

	Diffuse type of SSc	Limited type of SSc	*p*- value
	(N =30) (%)	(N=30) (%)	
Clinical			
Raynaud's phenomenon	28 (93.33)	29 (96.67)	NS
Modified Rodnan score, IQR	10.5 (4-33)	4 (2-18)	$p<0.05$*
GI manifestation	19 (63.33)	10 (33.33)	$p<0.05$*
Pulmonary fibrosis	19 (63.33)	14 (46.67)	$p<0.05$*
Pulmonary Hypertension	1 (3.33)	2 (6.67)	NS
Renal disease	1 (3.33)	2 (6.67)	NS
Arthralgia	23 (76.67)	22 (73.33)	NS
Arthritis	7 (23.33)	7 (23.33)	NS
Digital ulceration	17 (56.67)	10 (33.33)	$p<0.05$*
Serological positivity			
ANA (>1:160)	30 (100)	24 (80.0)	$p<0.05$*
Anti scl-70 Ab	18 (60.0)	12 (40.0)	$p<0.05$*
Anticentromere Ab	3 (10.0)	4 (13.33)	NS
ENA	15 (50.0)	6 (20.0)	$p<0.05$*

Table 2. Clinical and serological finding according to type of SSc

2.1.5 Results of NFC parameter

In healthy control, nailfold capillary looks like comb-like patten with minor disorganization and tortousity (Figure 5). Not like uniformly ordered capillary shape in the healthy control group, there was statistical significance in the smaller number of capillaries within 3mm, increased deletion numbers, apical limb with, capillary width, capillary hemorrhage and dimension of capillary loop in SSc patient group.

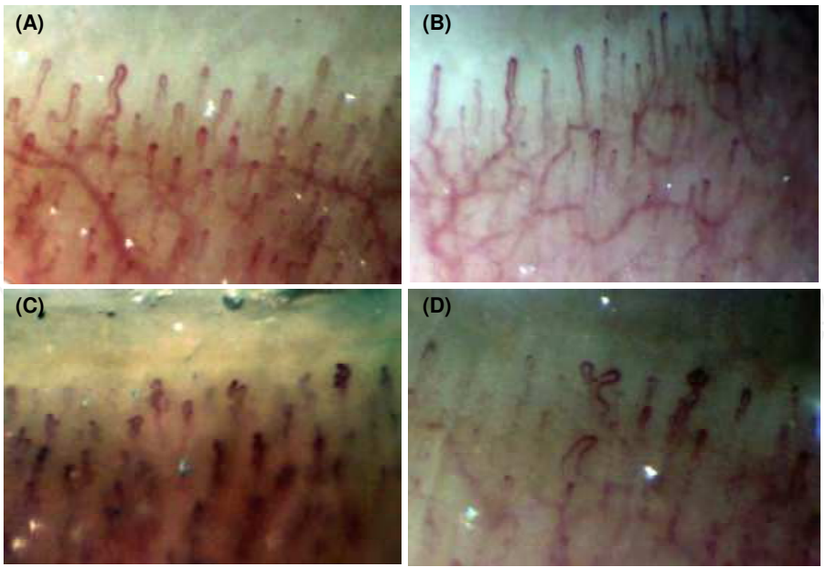

Fig. 5. The example of NFC finding (x400): (A)(B) In healthy control, the field of observation is rather uniform as comb-like appearance. (C)(D) Minimal change of capillary dilatation and increased tortousity are also seen even in healthy control.

The nailfold capillaroscopic pattern of early SSc is characterized by the massive dilatation and presence of giant capillaries (homogeneous and symmetrical capillary enlargement over 10 times compared with normal pattern) and microhemorrages (Figure 6-A,B) (early to active pattern). At the late phase of SSc progresses into fibrosis, the capillaroscopic pattern most likely reflects the effects of capillary destruction, loss of capillaries, and avascular areas are observed along with ramifications and bushy capillaries (Figure 6-C,D) (late pattern).

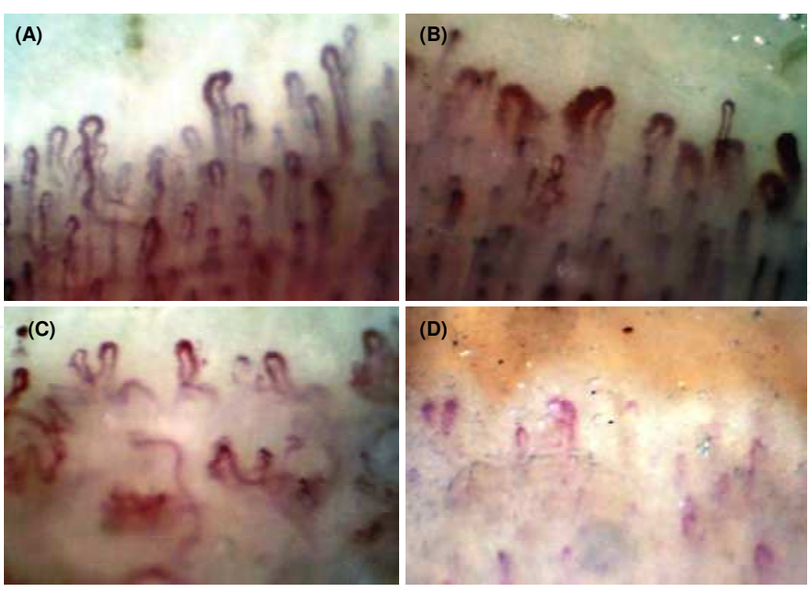

Fig. 6. The example of NFC finding (x400): (A)(B) In early to active phase of SSc, typically many giant capillary and microhemorrage is noted. (C)(D) At the late phase, ramified capillaries and avascular areas are observed along with bushy capillaries.

Above all, the capillary dimension presented as the sum of pixel numbers per 25 um capillary length was 1097, which showed the biggest difference; 516 from the healthy control group, 561 from disease control group (p <0.001) (Table3-1). Median and range of plasma ET-1 of the SSc patient group, normal control group and disease control group are on Table 3-1. The SSc patient group and disease control group had higher plasma ET-1 level than normal control group.

	Healthy Controls (N =30)	Disease control (N =23)	SSc (N =30)
NFC feature, median (IQR)			
No. of loop (in 3mm)	21.8 (16-28)	20 (13.5-25)	11.8 (1-19.5)*
Deletion No. of loop	0.5 (0-2)	1 (0.5-5.5)	3 (0-13)*
Apical width (um)	9.2 (6.7-13.3)	9.5 (6.7-11.8)	14.23 (7.9-30)*
Capillary width (um)	19.6 (12.5-26.7)	25 (15-49.7)	37.5 (15-100.9)*
Capillary dimension#	515.9 (357-566)	561.0(420-920)	1096.7(312.6-2134)*
Endothelin-1(pg/ml), median (IQR)	1.7 (0.5-2.5)	2.1 (1.2-4.1)#a	2.4 (0.9-6.3)#b

#Capillary dimension is presented by the sum of pixel number
IQR: Inter-quartile range, median(range: minimum-maximum)
* : p <0.05 is significant value
#a : p <0.05 between disease control and heatrhy control group
#b : p <0.05 between SSc and heatrhy control group

Table 3-1. Nailfold capillary microscopic feature and plasma endothelin-1 level

Other than that the capillary dimension of the dSSc was significantly wider than that of the lSSc, there was no difference between the two subtypes. There was no difference in the levels of plasma ET-1 among the subtypes of SSc patient group (Table 3-2).

	dSSc (N =30)	lSSc (N =30)	p- value
NFC feature, median (IQR)			
No. of loop (in 3mm)	10.1 (3-30)	13.5(3.5-23)	NS
Deletion No. of loop	3 (0.5-13)	3 (0-8)	NS
Apical width (um)	14.75(7.5-35)	12.75(8-30)	NS
Capillary width (um)	40.25(8-100.75)	35.13(21.68-88.35)	NS
Capillary dimension#	1312.7(313-2097)	965.1(527-2156)	p<0.05*
Endothelin-1 (pg/ml), median (IQR)	2.63(0.86-4.54)	2.21(1.09-6.31)	NS

Table 3-2. Nailfold capillary microscopic feature and plasma endothelin-1 between diffuse and limited type in patients with Systemic sclerosis.

The NFC parameters did not differ in the presence of pulmonary fibrosis, renal diseases, arthralgia and arthritis in SSc. However, the number of capillaries in 3mm and capillary deletion had statistically significant smaller in gastrointestinal dysfunction and pulmonary hypertension. When there is a digital ulcer, all parameters of NFC showed significant

disparity, especially in number of capillary deletions (p <0.01) and capillary dimension (p <0.001) (Table 4).

Comparison of plasma ET-1 of SSc patient group divided according to clinical characteristics showed notably high in the group with digital ulcer and pulmonary hypertension (p < 0.01, p < 0.05) (Table 4). It should be noted that plasma ET-1 is statistically proportional to MRS. Moreover, there was a statistic correlation between the level of plasma ET-1 and the capillary dimension in NFC (Table 5).

	Clinical manifestation						
	G-I	Pul. fibrosis	Pul. HTN	Renal dis.	Arthralgia	Arthritis	Digital ulcer
NFC feature							
No. of loop (in 3mm)	*p* <0.01*	NS	*p* <0.01*	NS	NS	NS	*p* <0.05*
Deletion No. of loop	*p* <0.05*	NS	*p* <0.01*	NS	NS	NS	*p* <0.01*
Apical width	NS	NS	NS	NS	NS	NS	*p* <0.05*
Capillary width	NS	NS	NS	NS	NS	NS	*p* <0.01*
Capillary dimension#	NS	NS	NS	NS	NS	NS	*p* <0.001*
Endothelin-1	NS	NS	*p* <0.05*	NS	NS	NS	*p* <0.01*

#Capillary dimension is presented by the sum of pixel number
G-I:abnormal finding in manometry including of GERD (gastro-esophageal reflux disease)
Pul. fibrosis: Pulmonary fibrosis
Pul. HTN: Pulmonary Hypertension
Renal dis.: Renal disease
NS: not significant
p <0.05* is significant value

Table 4. Correlation of clinical manifestations with NFC parameters and cytokines in patients with SSc.

	Endothelin-1
Skin hardness (MRS)	*p* <0.05*
NFC feature	
No. of loop	NS
Deletion No. of loop	NS
Apical width	NS
Capillary width	NS
Capillary dimension#	*p* <0.05*

MRS: modified rodnan score
SSc: Systemic sclerosis
#Capillary dimension is presented by the sum of pixel number
NS: not significant
p <0.05* is significant value by Spearmam's correlation.

Table 5. Correlation of plasma endothelin-1 with skin hardness and NFC parameters in patients with SSc

The level of plasma ET-1 and the capillary dimension were notably correlated in all of healthy, disease control group and SSc patient group (Rs = 0.82 / p<0.001, Rs = 0.83 / p<0.001, Rs = 0.31 / p<0.05) (Figure 7). The results suggest that computer-based microscopic analysis of NFC is a useful method that potentially provides information on organ involvement and plasma ET-1.

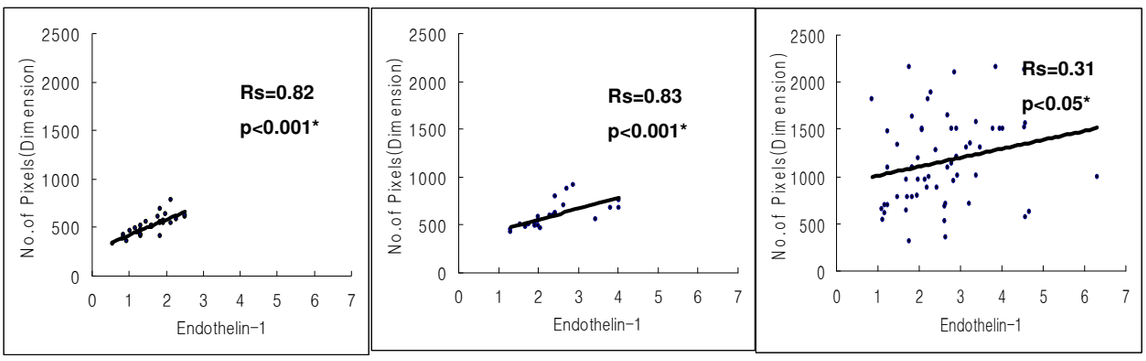

Fig. 7. Relationship between plasma Endothelin-1 and capillary dimension; Capillary dimension showed strong correlation with the level of endothelin-1 in (A) healthy control, (B) disease control, (C) SSc (p <0.05* is significant value by Spearmam's correlation).

3. Conclusion

NFC is a non-invasive, relatively inexpensive modality in diagnosing secondary Raynaud's phenomenon and it detects characteristic changes of SSc. NFC is able to indirectly evaluate vascular function of the connective tissue disorder. Recent researches reported the correlation between the NFC change and the occurrence of gastrointestinal invasion, pulmonary fibrosis, portal hypertension; thereby there is increasing the possibilities of the early detection of internal organ invasion or the marker for the follow-up after treatment based with digitalized computer-based analysis. Many clinical findings and plasma cytokines was compared with traditionally used NFC parameter, which to observe the number of capillaries and deletions in 3mm, apical limb width and the capillary width itself; however, we suggest that a computer can generate a more powerful relation which predicts the capillary dimension presented as the sum of pixel number in 25um of length.

Previously anderson et al. asserted that capillary dimension could be a new parameter for Raynaud phenomenon; there were differences in capillary dimension according with diabetic's vasospastic symptoms. Also in our study, the capillary dimension positively correlates with the apical limb width and capillary width in healthy control group. In disease control and SSc group, the capillary dimension negatively correlates with the number of capillary and showed positive correlations to the rest parameters, which advocates as a new optimal parameter. The capillary dimension also is illustrated statistic correlation to MRS which is the distinctive symptom in SSc. Additionally, the capillary dimension increases the most with the incidence of digital ulcer, which best reflects the activity of the disease in general. Not only in SSc, capillary dimension represented an authentic correlation to plasma ET-1 level in both the healthy control and the disease control group. For those reasons, the capillary dimension can be a factor that speaks for plasma ET-1, regardless of the disease types.

The endothelial cell or the smooth muscle cell secretes ET-1, which is a strong vasoconstrictor related to the onset of numerous diseases. ET-1 is known to the main cytokine which causes capillary deletion and directly to the development of fatal diseases such as pulmonary fibrosis and portal hypertension in SSc. As like in other studies, in SSc, plasma ET-1 level was increased than the healthy control group, but there was no statistic difference with the disease control group. The reason is that ET-1 is also increased in other connective tissue disorders that involve microvessel disruption due to vasoconstriction. Scala et al. emphasized the research and control of essential cytokines that overproduces or causes unbalance in extracellular materials in connective tissue cells, among many causes of fibrosis, is adequate for pathophysiologic approach to the disease and to prevent the progress of the disease. On the other hand, it is troublesome to measure the changes of cytokines all the time and the relationship to internal organ invasion is uncertain.

From this study, plasma ET-1 is elevated in SSc than the healthy control group proportionately to MRS, and meaningfully high in digital ulcer and pulmonary hypertension. For that reason, ET-1 could be considered to be closely related to the disease progression and severity of SSc. In our study, the capillary dimension is the best reflects of plasma ET-1 in the NFC parameters. Consequently, capillary dimension using computer pixel number is able to assume according to increasing in the plasma ET-1 and disease activity. Capillary dimension maybe a powerful parameter, could be advantageous for early diagnosis of complications as a result of organ involvement, and for the regular follow-ups to assessments of the treatment in the patients with SSc.

4. Acknowledgment

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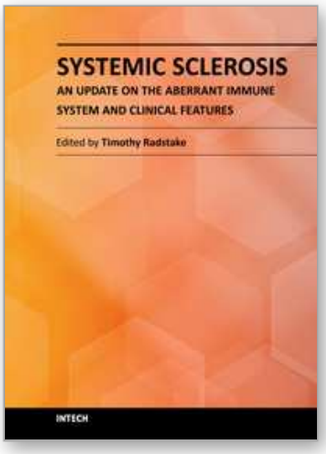

Systemic Sclerosis - An Update on the Aberrant Immune System and Clinical Features

Edited by Dr Timothy Radstake

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Systemic sclerosis (SSc), or often referred to as Scleroderma (tight skin), is characterized by an exaggerated formation of collagen fibers in the skin, which leads to fibrosis. Accumulating evidence now points toward three pathological hallmarks that are implicated in Ssc, the order of which has yet to be determined: endothelial dysfunction, autoantibody formation, and activation of fibroblasts. This current book provides up-to-date information on the pathogenesis and clinical features of this severe syndrome. It is our hope that this book will aid both clinicians and researchers in dealing with patients with this clinical syndrome. In addition, we hope to shed more light on this rare and severely disabling syndrome, ultimately leading to better research and successful therapeutic targeting.

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Phone: +86-21-62489820
Fax: +86-21-62489821