Distinct ‘pattern of autofluorescence’ of acute ischemic stroke patients’ skin and fingernails: A novel diagnostic biomarker for acute ischemic stroke

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Abstract

Early diagnosis of stroke is critical for therapeutic efficacy. Our study was designed to test our hypothesis that altered ‘Pattern of Autofluorescence (AF)’ may be a novel diagnostic biomarker for acute ischemic stroke (AIS). The major findings of our study include: First, the green AF intensity of the AIS Group in their fingernails and most regions of their skin examined is significantly higher than that of the Healthy group, the Low-Risk Group, the High-Risk Group, and the Groups of Ischemic Stroke Patients during Recovery Phase. Second, the AF increases of the AIS patients are asymmetrical: The AF intensity of their left hands is significantly different from that of their right hands. Third, ROC analyses show that both the sensitivity and the specificity of the AF-based diagnostic approach for AIS are greater than 0.86, when AF intensity is used as the sole factor for the analyses. Fourth, the number of the regions with increased AF intensity is highly positively correlated with the probability that the person examined is a AIS patient. Moreover, there are distinct differences among the ‘Pattern of AF’ of the patients of AIS, lung cancer as well as myocardial ischemia, stable coronary artery disease and Parkinson’s disease. Our study has further suggested that the AF increases in the skin and the fingernails of AIS patients may result from oxidative stress-induced alterations of keratins. Collectively, our study has indicated that the characteristic ‘Pattern of AF’ of AIS patients could become a novel diagnostic biomarker for the disease.

Keywords: Stroke; Pattern of Autofluorescence; Asymmetry; Skin; Diagnosis.

Introduction

Stroke is one of the leading causes of death and disability around the world. The only FDA-approved drug tissue plasminogen activator (tPA) for treating ischemic stroke has very limited use, mainly due to its short therapeutic window (15): The drug must be administered within 4.5 hours after the incidence of ischemic stroke, otherwise such major detrimental effects as hemorrhagic transformation ensue. However, it usually takes greater than 4.5 hrs for a stroke patient to get doctor's diagnosis. Therefore, early diagnosis has become a key factor that determines the therapeutic effectiveness for the disease. To solve this crucial problem, it becomes increasingly valuable to establish approaches that can conduct diagnosis at patients' homes non-invasively and efficiently, although it appears to be an exceedingly challenging task.

The AF (AF) of advanced glycation products (AGEs) of skin has shown great promise for non-invasive diagnosis of diabetes (13). Our recent study has reported that UV-induced epidermal green AF, which is originated from UV-induced keratin 1 proteolysis, can be used as a novel biomarker for predicting UV-induced skin damage (9). Keratins, together with melanin, NADH and FAD, are major epidermal fluorophores (1,14). It is noteworthy that the AF of AGEs in skin has profoundly different excitation wavelength compared with that of keratins' AF: The excitation wavelength for keratins' AF is 488 nm (9,14), while the excitation wavelength for AGEs' AF is 300 - 420 nm (10,11). Since detection of AF is non-invasive and relatively simple, it is of great clinical and scientific significance to further investigate

the potential of AF as biomarkers for diseases.

Our recent study has found that the oxidative stress induced by UVC is causative to the increased epidermal AF of mouse ears by inducing keratin 1 proteolysis (12). As we proposed in the article, since there is increased oxidative stress in the blood and urine of ischemic stroke patients (4,5,16) and animal models of cerebral ischemia (6), oxidative stress may also induce increases in the epidermal AF of the patients of ischemic stroke, which might become a novel biomarker for non-invasive and efficient diagnosis of ischemic stroke. Moreover, a large number of studies have indicated that oxidative stress plays a critical pathological role in ischemic brain injury (3,7,18). Therefore, it is necessary to determine if there are increases in the epidermal AF of ischemic stroke patients.

There has been little study on the AF of human fingernails. Since there is increased oxidative stress in the plasma of ischemic stroke patients (4,5,16) and animal models of cerebral ischemia (6), the oxidative stress may also enhance the AF of the nails' keratins of ischemic stroke patients. Thus, it is also warranted to determine if there are increases in the fingernails' AF of acute ischemic stroke (AIS) patients. Moreover, it has been reported that there is increased oxidative stress in the plasma of genetic stroke-prone rats (6), suggesting that the persons in high risk to have stroke may also increased oxidative stress, which may induce increased AF of the fingernails and certain regions of the skin. Therefore, increased AF of fingernails might also become a predictive biomarker for stroke.

In our current study, we tested our hypothesis that AIS patients may have

distinct ‘Pattern of AF’ in their skin and fingernails, which may become both a novel diagnostic biomarker and a predictive biomarker for AIS. Our study has provided first evidence supporting this hypothesis, which has indicated that the ‘Pattern of AF’-based diagnostic approach for AIS have high sensitivity and specificity. Our study has also indicated distinct differences among the ‘Pattern of AF’ of the patients of AIS, lung cancer, myocardial ischemia, stable coronary artery disease, and Parkinson’s disease (PD).

Methods and materials

Human subjects

The study was conducted according to a protocol approved by the Ethics Committee of Shanghai Fifth People’s Hospital Affiliated to Fudan University. The human subjects in our study were divided into six groups: Group 1: The group of healthy persons; Group 2: The group of Low-Risk persons; Group 3: The group of High-Risk persons - the persons who were clinically diagnosed as ‘the patients in high risk to have ischemic stroke’ by the neurologists of the Department of Neurology, Shanghai Fifth People’s Hospital Affiliated to Fudan University; Group 4: The group of AIS patients who had ischemic stroke within 7 days; Group 5: The group of ischemic stroke patients in recovery phase (1-3 months), who have had ischemic stroke for 1 – 3 months; and Group 6: The group of ischemic stroke patients in recovery phase (> 3 months), who have had ischemic stroke for more than 3 months. The age of Group 1, Group 2, Group 3, Group 4, Group 5 and Group 6 is 64.11 ± 1.07 ,

66.53 $\pm$ 0.58, 67.33 $\pm$ 0.90, 64.09 $\pm$ 1.12, 62.16 $\pm$ 1.39, 65.38 $\pm$ 1.29 years of old, respectively. The ages of all of the subjects range from 50 - 80 years of old.

Determination of the Autofluorescence of Skin and Fingernails

A portable AF imaging equipment was used to detect the AF of the fingernails and certain regions of the skin of the human subjects. The excitation wavelength is 485 nm, and the emission wavelength is 500 - 550 nm. For all of the human subjects, the AF intensity in the following seven regions on both hands, i.e., fourteen regions in total, was determined, including the Index Fingernails, Ventroforefingers, Dorsal Index Finger, Centremetacarpus, Dorsal Centremetacarpus, Ventribrachium and Dorsal Antebrachium.

Determinations of the AF spectrum of fingernails and skin

The fingernails' clippings of AIS patients and High-Risk persons were collected. The spectra of the fingernails' AF was determined by using a two-photon fluorescence microscope (A1 plus, Nikon Instech Co., Ltd., Tokyo, Japan). The excitation wavelength was 760 nm, and the emission wavelength ranged from 420 to 640 nm. The signal was detected each 10 nm. After the imaging, the images were analyzed automatically.

By using a portable equipment for AF spectrum determinations, we determined the spectra of the AF of the 12 regions of the skin of the Healthy Group, the High-Risk Group and the AIS Group. The excitation wavelength is 445 nm.

The emitting light at the wavelength that is longer than 450 nm was detected.

Statistical analyses

All data are presented as mean $\pm$ SEM. Data were assessed by one-way ANOVA, followed by Student - Newman - Keuls *post hoc* test, except where noted. *P* values less than 0.05 were considered statistically significant.

Results

1. The AF intensity of the fingernails and most regions of the skin of the AIS Group is significantly higher than that of the Healthy Group, the Low-Risk Group, the High-Risk Group, and the Groups of Ischemic Stroke Patients during Recovery Phase

For each person of the Healthy Group, the Low-Risk Group, the High-Risk Group, the AIS Group, the Group of Ischemic Stroke Patients during Recovery Phase (1 – 3 months after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke), we determined the green AF intensities of two index fingernails and twelve regions of the skin. Our study showed that in Dorsal Index Finger (Fig. 1A), Ventroforefinger (Fig. 1B), Dorsal Centremetacarpus (Fig. 1C), Centremetacarpus (Fig. 1D), and Index Finger Nails (Fig. 1E), the AF intensity of AIS group is significantly higher than that of all of the other five groups. In contrast, at right Dorsal Ventriantebrachium, there is no significant difference among the AF intensities of the AIS Group, the High-Risk Group, and the Group of

Ischemic Stroke Patients during Recovery Phase (1 – 3 months after stroke) (Fig. 1F).

At left Dorsal Ventriantebrachium, there is no significant difference between the AF intensity of the AIS Group and that of the High-Risk group (Fig. 1F). At left Ventriantebrachium, the only group that has significantly lower AF intensity than that of the AIS group is the Low-Risk group (Fig. 1G), while at right Ventriantebrachium, the AF intensity of the AIS group is significantly higher than that of all of the other five groups (Fig. 1G).

2. The AF asymmetry of the fingernails and most regions of the skin of the AIS Group is significantly higher than that of the Healthy Group, the Low-Risk Group, the High-Risk Group, and the Groups of Ischemic Stroke Patients during Recovery Phase

We determined the ‘asymmetry of AF intensity’, which is defined by us as ‘a significant difference (in absolute value) between the same region at the left or right side of the body’. Our study showed that at Dorsal Index Finger (Fig. 2A), Ventroforefinger (Fig. 2B), Centremetacarpus (Fig. 2C), Dorsal Ventriantebrachium (Fig. 1D), and Ventriantebrachium (Fig. 2E), the AF asymmetry of the AIS Group is significantly higher than that of all of the other five groups. In contrast, at the Index Fingernails, the AF asymmetry of the AIS Group is significantly higher than that of the other groups, except the Group of Ischemic Stroke Patients during Recovery Phase (1 – 3 months after stroke) (Fig. 2F). At Dorsal Centremetacarpus (Fig. 2G), The AF asymmetry of the AIS Group is significantly higher than that of the other

groups, except the High-Risk group and the Group of Ischemic Stroke Patients during Recovery Phase (1 – 3 months after stroke).

3. The Healthy Group, the Low-Risk Group, the High-Risk Group, the AIS Group, and the Groups of Ischemic Stroke Patients during Recovery Phase have profound differences in the percentage of the people with one or more regions with increased AF intensity

We found that the percentage of the people with one or more regions with increased green AF is 14%, 26%, 44%, 88%, 47% and 32% for the Healthy Group, the Low-Risk Group, the High-Risk Group, the AIS Group, the Group of Ischemic Stroke Patients during Recovery Phase (1–3 months after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke), respectively (Fig. 3A). The percentage of the people with three or more regions with increased green AF is 5%, 4%, 17%, 64%, 22% and 8% for the Healthy Group, the Low-Risk Group, the High-Risk Group, the AIS Group, the Group of Ischemic Stroke Patients during Recovery Phase (1–3 months after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke) (Fig. 3A). We also found that in all of the regions examined, markedly higher percentage of the AIS patients have increased AF intensity, compared with each of the other five groups (Fig. 3B).

4. The Healthy Group, the Low-Risk Group, the High-Risk Group, the AIS

Group, and the Groups of Ischemic Stroke Patients during Recovery Phase have profound differences in the percentage of the people with one or more regions with increased AF asymmetry

We found that the percentage of the people with one or more regions with AF asymmetry is 16%, 31%, 36%, 81%, 46% and 32% for the Healthy Group, the Low-Risk Group, the High-Risk Group, the AIS Group, the Group of Ischemic Stroke Patients during Recovery Phase (1–3 months after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke), respectively (Fig. 4A). The percentage of the people with two or more regions with AF asymmetry is 3%, 10%, 11%, 53%, 20% and 6% for the Healthy Group, the Low-Risk Group, the High-Risk Group, the AIS Group, the Group of Ischemic Stroke Patients during Recovery Phase (1–3 months after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke), respectively (Fig. 4A). We also found that in all of the regions examined, markedly higher percentage of the AIS patients have increased AF asymmetry, compared with each of the other five groups (Fig. 4B).

5. The number of the regions with increased AF intensity is highly positively correlated with probability that the examined subject is a AIS patient

Statistical analyses among the seventeen basic biological parameters of the persons in the Low-Risk Group, the High-Risk Group and the AIS Group were conducted (Table 1). The biological parameters that have a *P* value less than 0.2 in

the statistical analyses were used as the major potential diagnostic factors for further SASS software-based logistic regression analyses, which were conducted to determine the relationship between the factors and the probability that the examined subject is an AIS patient (Table 2). While our Univariate logistic regression analyses showed that each of the twelve factors had an unadjusted *P* value less than 0.05, our Multivariate logistic regression analyses showed that only two of the factors had an adjusted *P* value less than 0.05: The homocysteine concentrations in the plasma had a *P* value of 0.032, while ‘Number of the regions with increased AF intensity’ had a *P* value less than 0.001 (Table 2).

We also conducted logistic regression analyses to determine the diagnostic value of the AF intensity of each of the position examined, the number of the regions with increased AF intensity, and the number of the regions with AF asymmetry (Table 3). Our analyses have indicated that all of these factors, except the AF intensity at the Dorsal Antebrachium, had a adjusted *P* value less than 0.05 (Table 3). In particular, the following five factors in Table 3 had an adjusted *P* value less than 0.0001: The number of the regions with increased AF intensity, the number of the regions with AF asymmetry, the AF intensity at right Ventroforefinger, the AF intensity at left Ventroforefinger, and the AF intensity at Centremetacarpus.

6. Both of the AF intensity-based diagnostic approach and the AF asymmetry-based diagnostic approach have achieved high sensitivity and specificity in AIS diagnosis

We have conducted ROC analysis on the data of the AF intensity and the AF asymmetry of these groups. Our analysis have indicated that both of the AF intensity-based diagnostic approach and the AF asymmetry-based diagnostic approach have achieved high sensitivity and specificity in AIS diagnosis: For comparisons between the Healthy Group and the AIS Group, our ROC analyses show that the AUC is 0.91 and 0.85, when AF intensity or AF asymmetry is used as the sole factor for the analyses, respectively (Fig. 5A). For comparisons between the Low-Risk Group and the AIS Group, our ROC analyses show that the AUC is 0.89 and 0.79, when AF intensity or AF asymmetry is used as the sole factor for the analyses, respectively (Fig. 5B). For comparisons between the High-Risk Group and the AIS Group, our ROC analyses show that the AUC is 0.82 and 0.77, when AF intensity or AF asymmetry is used as the sole factor for the analyses, respectively (Fig. 5C). The sensitivity and the specificity for each cut off value was shown in Table 4.

7. The ‘Patter of AF’ of the AIS patients is distinctly different from that of the other major diseases including untreated lung cancer patients

We have conducted the comparisons between the AF intensity of AIS patients and that of untreated lung cancer patients (Table 5). While the AF intensity of the AIS Group at each of the 14 regions examined was significantly higher than that of the Healthy Group, the AF intensity of the Untreated Lung Cancer Group was significantly higher than that of the Healthy Group only at 9 of the 14 regions examined (Table 5). Moreover, at 11 regions of the 14 regions examined of the AIS

Group, there are greater than two folds of the increase in the AF intensity compared with the Healthy Group (Table 5). In contrast, at only 5 regions of the 14 regions examined of Untreated Lung Cancer Group, there are greater than two folds of the increase in the AF intensity compared with the Healthy Group (Table 5).

We have also conducted the comparisons between the AF asymmetry of the AIS patients and that of untreated lung cancer patients (Table 6). While the AF asymmetry of the AIS Group at each of the 7 regions examined was significantly higher than that of the Healthy Group, the AF asymmetry of the Untreated Lung Cancer Group was significantly higher than that of the Healthy Group at only 4 of the 7 regions examined (Table 6). Moreover, at 6 regions of the 7 regions examined of the AIS Group, the value of the AF asymmetry was greater than 3 (Table 6). In contrast, at only 3 regions of the 7 regions examined of the Untreated Lung Cancer Group, the value of the AF asymmetry was greater than 3 (Table 6).

We have further conducted the comparisons between the AF intensity of AIS patients and that of the patients of myocardial infarction, stable coronary artery disease or PD. We have also conducted the comparisons between the AF asymmetry of AIS patients and that of the patients of myocardial infarction, stable coronary artery disease or PD. We have found that each of these diseases have distinct properties in its AF intensity and AF asymmetry (data not shown), as we observed in the comparisons of these AF properties of the AIS patients and the Untreated Lung Cancer Patients. These analyses have suggested that each of these major diseases have its unique 'Pattern of AF'.

8. Determinations of the AF spectrum of the skin of AIS patients have suggested that the altered AF is originated from keratins

By using a portable equipment for AF spectrum determinations, we determined the spectra of the AF of the 12 regions of the skin of the Healthy Group, the High-Risk Group and the AIS Group. We found that of the AF of all of the skin regions examined have highly similar spectra (Fig. 6), which match that of keratins. Moreover, the skin of all of the groups examined, including the Healthy Group, the High-Risk Group and the AIS Group, also has highly similar AF spectrum, which also matches that of keratins (Fig. 6).

9. Determinations of the AF spectrum of the fingernails' clippings of AIS patients have suggested that the altered AF is originated from the keratins of their fingernails

By using a two-photon fluorescent microscope, we analyzed the AF spectrum of the fingernails' clippings of the AIS patients and the High-Risk persons. Our study have indicated that the AF spectrum matches that of keratins (1,14) (Fig. 7). Since keratins are key components of fingernails (2), this observation has suggested that the increased fingernails' AF of the AIS patients could result at least partially from the increased keratins' AF of the fingernails.

Discussion

The major findings of our study include: First, the green AF intensity of the

Group of AIS in their fingernails and most regions of their skin examined is significantly higher than that of the Healthy group, the Low-Risk Group, the High-Risk Group, and the Groups of Ischemic Stroke Patients during Recovery Phase. Second, the AF increases of the AIS patients are asymmetrical. Third, ROC analyses show that the AUC is 0.91 and 0.85, respectively, when AF intensity or AF asymmetry is used as the sole factor for the analyses. Fourth, the number of the regions with increased AF intensity is highly positively correlated with the probability that the person examined is an AIS patient. Moreover, there are distinct differences among the ‘Pattern of AF’ of the patients of AIS, lung cancer, acute myocardial ischemia, stable coronary artery disease and PD. Our study has further suggested that the AF increases in the fingernails and the skin of AIS patients may result from oxidative stress-induced alterations of keratins. Collectively, our study has indicated that AIS patients have significant asymmetric AF increases in their fingernails and certain regions of their skin, which has formed a characteristic ‘Pattern of AF’. This ‘Pattern of AF’ could become a novel diagnostic biomarker for AIS.

One of the most important and challenging tasks for stroke treatment is to realize early diagnosis of stroke. Considering that the time for both traveling from patients’ homes to hospitals and diagnosis is likely over 4.5 hrs, a key strategy to realize early diagnosis could be diagnosis at patients’ homes non-invasively and efficiently, immediately after occurrence of stroke’s symptoms. Our current study has provided the first evidence suggesting that AIS patients may be diagnosed at home by determining the AF of the fingernails and certain regions of their skin, which

has shown significant sensitivity and specificity in AIS diagnosis: ROC analyses show that both the sensitivity and the specificity are greater than 0.86, when AF intensity is used as the sole factor for the analyses. We expect that the sensitivity and specificity of our approach for diagnosis of AIS would be significantly enhanced in the future, with both increases in the regions examined and applications of both artificial intelligence and big data science in our future studies.

Our studies have suggested that each major disease could have their unique ‘Pattern of AF (PA)’, which is defined as the unique combinations of several properties of the AF of each disease, including the locations of the skin with increased AF intensity, extent of the increases in the AF intensity, AF asymmetry, and structural properties of the AF. The Table 4 and Table 5 have indicated the both AIS and untreated lung cancer have their distinct PA. The following is several examples indicating the unique PA of AIS, myocardial infarction, stable coronary artery disease and PD: 1) There is AF asymmetry at index fingernails and only two regions of the skin of PD patients, while there is AF asymmetry at all examined regions of AIS patients; 2) both the AF intensity and AF asymmetry at the Centremetacarpus of PD patients is significantly lower than those of AIS patients; and 3) while the AF intensity and AF asymmetry of both SCRD and MI is higher than that of healthy and low-risk persons in a few regions, AIS patients have significantly higher AF intensity and AF asymmetry than the healthy and Low-Risk group in virtually all regions examined.

Since the AF imaging device used in our studies is portable and inexpensive, which can provide non-invasive diagnosis of multiple major diseases with satisfactory

precision, the device and its associated technology may become the first home-used device that can diagnose major diseases in history. It is reasonable to expect that with the increases in both the regions of skin examined for AF and applications of big data science and artificial intelligence (AI), we would discover increasingly precise and characteristic PA of most major diseases. Based on these increasingly precise PA, we would achieve increasingly greater precision in diagnosis of the diseases.

A key question regarding our findings is: Why are there significant increases in the green AF in certain regions of skin of the AIS patients? We have found that the oxidative stress induced by UVC mediates the increase in the epidermal green AF of mouse ears by inducing keratin 1 proteolysis (9). A number of studies have shown that there is increased oxidative stress in the blood and urine of AIS patients (4,5,16) and animal models of cerebral ischemia (6). Therefore, we propose that the oxidative stress may induce increases in the epidermal AF of the AIS patients by inducing keratin 1 proteolysis, which may become a novel biomarker for non-invasive and efficient diagnosis of AIS. Future studies are needed to further test the validity of this proposal.

Our study has also obtained the following highly interesting observation: AIS patients have significantly greater AF asymmetry in the skin and fingernails than that of the other five groups. To our knowledge, this is the first report regarding the asymmetry of the AF of human body. We propose that this AF asymmetry may be accounted for by the following two potential mechanisms: First, the pathological changes in one side of the brains of the AIS patients can cause significant pathological

alterations on one side of the patients' body, which is exhibited by the asymmetric increases in the AF intensity of their skin and fingernails; and second, before the incidence of ischemic stroke, the patients have had significant pathological alterations on one side of their body, which can be indicated by the asymmetric AF increases of their skin and nails. Future studies are necessary to test the validity of these two proposals, which may provide highly valuable information for understanding the pathogenesis of ischemic stroke. Based on our observations regarding the increased AF in the fingernails of the AIS patients, it is highly likely that before the incidence of ischemic stroke, the patients have had significant pathological alterations on one side of their body, which can be indicated by the asymmetric AF increases in their skin and nails.

Our current study found that AIS patients have significantly higher AF intensity in virtually each region of the skin examined, compared with that of the ischemic stroke patients during recovery phase. It has been reported that oxidative stress is higher in the plasma and urine of AIS patients up to the 7th day after the stroke incidence (16). Thus, we propose that the oxidative stress in the body of the AIS patients, which could be higher than that of the stroke patients during the recovery phase, may account for the differences in the AF intensity between these two groups of stroke patients.

Another key question regarding our findings is: Why are there significant increases in the green AF in the fingernails of AIS patients? The responses to this question include: First, our AF spectrum study on the fingernails has suggested that

the AF results at least partially from the keratins in the fingernails. Keratins are well-established fluorophores (1,14), which are also the main components of human nails (2): It was reported that human nail plate is composed of approximately 10% - 20% soft epithelial keratins (48K/56K and 50K/58K keratin pairs), with the remainder being hard α -keratin (8); and second, our previous study has shown the oxidative stress induced by UV can produce increased epidermal green AF (9). Since there are multiple disulfide bonds in α -keratin (2), it is reasonable to expect that oxidative stress may significantly affect both the structure and fluorescent properties of α -keratin, leading to increased AF of α -keratin. Based on these two pieces of information, we propose that the increased AF of the fingernails may result from alterations of fingernails' keratins, which are induced by oxidative stress or other stress signals. Future studies are needed to further test validity of this proposal.

One of the key findings of our study is that there is significant variability in the extent of the increase in the AF intensity at each region of the AIS patients' skin examined. There is also significant variability in the AS asymmetry at the regions of the AIS patients' skin examined. The major scientific question regarding these findings is: Why is there the significant variability in the AF at the various regions of the AIS patients' skin? We propose that these regions with altered AF are just the regions of Blockage ('Yu Du') in Chinese medicine. According to Chinese medicine, there are certain regions of the skin and the tissues under the skin which are essentially linked with the tissues or organs in the disease state. These skin and the tissues under the skin would show distinct pathological changes named Blockage ('Yu

Du’) that is both a major source for diagnostic information and a major therapeutic target for the diseases. We have obtained certain pieces of information to support this proposal (data not shown), while extensive studies are needed to further test the validity of this proposal. If this proposal is proven true, our study would provide revolutionary information for elucidating the nature of Blockage (‘Yu Du’) – a pivotal concept in Chinese medicine for both diagnosis and treatment of diseases. In other words, our future study may demonstrate that our AF imaging is the first approach that can provide imaging of the areas of Blockage (‘Yu Du’), thus providing the first quantifiable and reproducible judgment on Blockage (‘Yu Du’) in history. These breakthroughs may lead to transformation of Chinese medicine, which may enable Chinese medicine to become highly quantifiable and reproducible.

Our study has found that the fingernails of the AIS Group show significant and asymmetric increases in the AF, compared with the Healthy Group, the Low-Risk Group, and the High-Risk Group. The growth of the fingernails should occur days or even months before our examination. Therefore, it is reasonable to propose that the asymmetric increases in the fingernails’ AF should occur before the incidence of AIS, since the AIS patients had stroke only 1 - 7 days ago. Therefore, we propose that ‘asymmetric increases in the fingernails’ AF’ may also become a novel predicative biomarker for AIS incidence. The asymmetric increases in the AF may become a warning sign for AIS incidence, which requires medical intervention to prevent stroke incidence.

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Figure Legends:

Fig. 1. The AF intensity of the fingernails and most regions of the skin of the AIS Group is significantly higher than that of the Healthy Group, the Low-Risk Group, the High-Risk Group, the Group of Ischemic Stroke Patients during Recovery Phase (1–3 months after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke). (A) At Dorsal Index Finger, the AF intensity of AIS group is significantly higher than that of all of the other five groups. (B) At Ventroforefinger, the AF intensity of AIS group is significantly higher than that of all of the other five groups. (C) At Dorsal Centremetacarpus, the AF intensity of AIS group is significantly higher than that of all of the other five groups.; (D) At Centremetacarpus, the AF intensity of AIS group is significantly higher than that of all of the other five groups. (E) At Index Fingernails, the AF intensity of AIS group is significantly higher than that of all of the other five groups. (F) At right Dorsal Ventriantebrachium, there is no significant difference among the AF intensities of the AIS Group, the High-Risk Group, and the Group of Ischemic Stroke Patients during Recovery Phase (1 – 3 months after stroke). At left Dorsal Ventriantebrachium, there is no significant difference between the AF intensity of the AIS Group and that of the High-Risk group. (G) At left Ventriantebrachium, the only group that has significantly lower AF intensity than that of the AIS group is the Low-Risk group, while at right Ventriantebrachium, the AF intensity of the AIS group is significantly higher than that of all of the other five groups. The number of subjects in the Healthy Group, the Low-Risk Group, the High-Risk group, the AIS

Group, the Group of Ischemic Stroke Patients during Recovery Phase (1–3 months after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke) is 37, 144-146, 63-66, 66-69, 58-59, 49-50, respectively. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 2. The AF asymmetry of the fingernails and most regions of the skin of the AIS Group is significantly higher than that of the Healthy Group, the Low-Risk Group, the High-Risk Group, the Group of Ischemic Stroke Patients during Recovery Phase (1–3 months after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke). (A) At Dorsal Index Finger, the AF asymmetry of the AIS Group is significantly higher than that of all of the other five groups. (B) Ventroforefinger, the AF asymmetry of the AIS Group is significantly higher than that of all of the other five groups. (C) At Centremetacarpus, the AF asymmetry of the AIS Group is significantly higher than that of all of the other five groups. (D) At Dorsal Ventriantebrachium, the AF asymmetry of the AIS Group is significantly higher than that of all of the other five groups. (E) At Ventriantebrachium, the AF asymmetry of the AIS Group is significantly higher than that of all of the other five groups. (F) At Index Fingernails, the AF asymmetry of the AIS Group is significantly higher than that of the other groups, except the Group of Ischemic Stroke Patients during Recovery Phase (1 – 3 months after stroke). (G) At Dorsal Centremetacarpus, The AF asymmetry of the AIS Group is significantly higher than that of the other groups, except the High-Risk

Group and the Group of Ischemic Stroke Patients during Recovery Phase (1 – 3 months after stroke). The number of subjects in the Healthy Group, the Low-Risk Group, the High-Risk group, the AIS Group, the Group of Ischemic Stroke Patients during Recovery Phase (1–3 months after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke) is 37, 144-146, 63-66, 66-69, 58-59, 49-50, respectively. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Fig. 3. The Healthy Group, the Low-Risk Group, the High-Risk Group, the AIS Group, and the Groups of Ischemic Stroke Patients during Recovery Phase have profound differences in the percentage of the people with one or more regions with increased AF intensity. (A) We found that the percentage of the people with one or more regions with increased green AF is 14%, 26%, 44%, 88%, 47% and 32% for the Healthy Group, the Low-Risk Group, the High-Risk Group, the AIS Group, the Group of Ischemic Stroke Patients during Recovery Phase (1–3 months after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke), respectively. The percentage of the people with three or more regions with increased green AF is 5%, 4%, 17%, 64%, 22% and 8% for the Healthy Group, the Low-Risk Group, the High-Risk Group, the AIS Group, the Group of Ischemic Stroke Patients during Recovery Phase (1–3 months after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke). (B) In all of the regions examined, markedly higher percentage of the AIS patients have increased AF intensity, compared with each of the other five groups. The

number of subjects in the Healthy Group, the Low-Risk Group, the High-Risk group, the AIS Group, the Group of Ischemic Stroke Patients during Recovery Phase (1–3 months after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke) is 37, 146, 66, 69, 59, and 50, respectively.

Fig. 4. The Healthy Group, the Low-Risk Group, the High-Risk Group, the AIS Group, and the Groups of Ischemic Stroke Patients during Recovery Phase have profound differences in the percentage of the people with one or more regions with increased AF asymmetry. (A) The percentage of the people with one or more regions with AF asymmetry is 16%, 31%, 36%, 81%, 46% and 32% for the Healthy Group, the Low-Risk Group, the High-Risk Group, the AIS Group, the Group of Ischemic Stroke Patients during Recovery Phase (1–3 months after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke), respectively. The percentage of the people with two or more regions with AF asymmetry is 3%, 10%, 11%, 53%, 20% and 6% for the Healthy Group, the Low-Risk Group, the High-Risk Group, the AIS Group, the Group of Ischemic Stroke Patients during Recovery Phase (1–3 months after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke), respectively. (B) In all of the regions examined, markedly higher percentage of the AIS patients have increased AF asymmetry, compared with each of the other five groups. The number of subjects in the Healthy Group, the Low-Risk Group, the High-Risk group, the AIS Group, the Group of Ischemic Stroke Patients during Recovery Phase (1–3 months

after stroke), and the Group of Ischemic Stroke Patients during Recovery Phase (> 3 months after stroke) is 37, 146, 66, 68, 59, and 50, respectively.

Fig. 5. Both of the AF intensity-based diagnostic approach and the AF asymmetry-based diagnostic approach have achieved high sensitivity and specificity in AIS diagnosis. (A) For comparisons between the Healthy Group and the AIS Group, our ROC analyses show that the AUC is 0.91 and 0.85, when AF intensity or AF asymmetry is used as the sole factor for the analyses, respectively. (B) For comparisons between the Low-Risk Group and the AIS Group, our ROC analyses show that the AUC is 0.89 and 0.79, when AF intensity or AF asymmetry is used as the sole factor for the analyses, respectively. (C) For comparisons between the High-Risk Group and the AIS Group, our ROC analyses show that the AUC is 0.82 and 0.77, when AF intensity or AF asymmetry is used as the sole factor for the analyses, respectively. The number of subjects in the Healthy Group, the High-Risk group, and the AIS Group is 37, 66, and 68, respectively.

Fig. 6. Determinations of the AF spectrum of the skin of AIS patients have suggested that the altered AF of the skin is originated from the keratins. The AF of all of the skin regions examined of the AIS patients has highly similar spectrum, which matches that of keratins. Moreover, the AF of the examined skin's regions of all of the groups, including the Healthy Group (A), the High-Risk Group (B) and the AIS Group (C), also have highly similar spectrum, which matches that of keratins.

Excitation wavelength is 445 nm. The emitting light at the wavelength that is longer than 450 nm was detected. The number of subjects in the Healthy Group, the High-Risk Group, and the AIS Group is 37, 66, and 68, respectively.

Fig. 7. Determinations of the AF spectrum of the fingernails' clippings of the AIS patients have suggested that the increased AF of the fingernails is originated from the keratins of their fingernails. The AF spectrum of the fingernails' clippings of the AIS patients and High-Risk persons matches that of keratins. N = 3. Excitation wavelength is 760 nm using a Nikon two-photon fluorescence microscope.

Table legends:

Table 1. Statistical analyses among the seventeen basic biological parameters of the persons in the Low-Risk Group, the High-Risk Group and the AIS Group.

The number of subjects in the Healthy Group, the High-Risk Group, and the AIS Group is 37, 66, and 68, respectively.

Table 2. The probability that the examined subject is an AIS patient is significantly correlated with only two of the factors examined. Univariate logistic regression analyses showed that each of the twelve factors had an unadjusted *P* value less than 0.05, while Multivariate logistic regression analyses showed that only two of the factors had an adjusted *P* value less than 0.05: The homocysteine concentrations in the plasma had a *P* value of 0.032, while ‘Number of the regions with increased AF intensity’ had a *P* value less than 0.001. All of the subjects in the High-Risk Group (*N* = 26) and the AIS Group (*N* = 33) were hospitalized patients.

Table 3. The number of the regions with increased AF intensity is highly correlated with probability that the examined subject is an AIS patient. Logistic regression analyses were conducted to determine the diagnostic value of the AF intensity of each of the position examined, the number of the regions with increased AF intensity, and the number of the regions with AF asymmetry. All of these factors, except the AF intensity at the Dorsal Antebrachium, had an adjusted *P* value less than 0.05. All of the subjects in the High-Risk Group (*N* = 26) and the AIS Group (*N* =

33) were hospitalized patients.

Table 4. Roc analyses show the sensitivity and the specificity of the AF intensity-based diagnostic approach or the AF asymmetry-based diagnostic approach for each cut off value.

Table 5. The comparisons between the AF intensity of the AIS patients and that of untreated lung cancer patients. The AF intensity of the AIS Group at each of the 14 regions examined was significantly higher than that of the Healthy Group, while the AF intensity of the Untreated Lung Cancer Group was significantly higher than that of the Healthy Group only at 9 of the 14 regions examined. At 11 regions of the 14 regions examined of the AIS Group, there are greater than two folds of the increase in the AF intensity compared with the Healthy Group. In contrast, at only 5 regions of the 14 regions examined of Untreated Lung Cancer Group, there are greater than two folds of the increase in the AF intensity compared with the Healthy Group.

Table 6. The comparisons between the AF asymmetry of the AIS patients and that of untreated lung cancer patients. The AF asymmetry of the AIS Group at each of the 7 regions examined was significantly higher than that of the Healthy Group, while the AF asymmetry of the Untreated Lung Cancer Group was significantly higher than that of the Healthy Group at only 4 of the 7 regions examined. At 6 regions of the 7 regions examined of the AIS Group, the value of the

AF asymmetry was greater than 3. In contrast, at only 3 regions of the 7 regions examined of the Untreated Lung Cancer Group, the value of the AF asymmetry was greater than 3.

—○— AF Intensity, $A = 0.91$
- -△- - AF asymmetry, $A = 0.85$

—○— AF Intensity, $A = 0.82$
- -△- - AF Asymmetry, $A = 0.77$

- Dorsal Index Finger
- Ventroforefinger
- Centremetacarpus
- Dorsal Centremetacarpus
- Ventriantebrachium
- Dorsal Antebrachium
- Index Fingernail

- Dorsal Index Finger
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- Dorsal Antebrachium
- Index Fingernail

— High-Risk Group
— Acute Ischemic Stroke Group

Factors	Low and High-risk Group (n=127)	AIS Group (n=63)	P Value
Male	44 (34.65%)	51 (80.95%)	0.140
Age	66.75±0.62	65.03±1.07	<0.0001
Hypertension	78 (61.42%)	48 (76.19%)	0.044
Diabetes	27 (21.26%)	25 (39.68%)	0.008
hs-CRP (mg/L)	2.95±0.95	2.7±0.4	0.861
ALT (U/L)	17.84±0.77	17.52±1.39	0.830
AST (U/L)	19.81±0.54	18.36±1.02	0.170
Scr (μmol/L)	67.3±1.29	77.16±2.23	<0.0001
UA (μmol/L)	304.53±7.65	305.37±12.17	0.951
Cystatin C (mg/L)	0.98±0.02	1.02±0.03	0.262
Triglyceride (mmol/L)	4.4±0.09	4.1±0.09	0.035
Total Cholesterol (mmol/L)	1.35±0.06	1.44±0.09	0.404
HDL (mmol/L)	1.38±0.03	1.1±0.04	<0.0001
LDL (mmol/L)	3.06±0.26	2.72±0.09	0.414
FBG (mmol/L)	5.48±0.1	6.35±0.32	0.003
HCY (μmol/L)	13.12±0.55	18.83±1.78	0.003
GHb (%)	5.8±0.07	6.73±0.22	<0.0001

hs-CRP: hypersensitive C-reactive protein; ALT: alanine aminotransferase; AST: aspartic aminotransferase; Scr: serum creatinine; UA: uric acid; HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; FBG: fasting blood-glucose; HCY: homocysteine; GHb: GHB: glycosylated hemoglobin; categorical variables: number (percentage%); continuous variables: mean ± sem

Factors	Univariate		Multivariate	
	OR (95%CI)	Unadjusted <i>P</i> Value	OR (95%CI)	Adjusted <i>P</i> Value
Male	8.017 (3.874,16.592)	0.14	1.983 (0.62,6.336)	0.248
Age	0.97 (0.932,1.01)	<0.0001	0.967 (0.908,1.029)	0.29
Hypertension	0.497 (0.252,0.983)	0.044	1.669 (0.536,5.192)	0.377
Diabetes	0.41 (0.212,0.794)	0.008	2.223 (0.436,11.336)	0.337
AST (U/L)	0.966 (0.918,1.015)	0.17	1.003 (0.943,1.067)	0.92
Scr (μmol/L)	1.04 (1.018,1.062)	<0.0001	1.008 (0.973,1.045)	0.654
Triglyceride (mmol/L)	0.69 (0.489,0.974)	0.035	0.945 (0.516,1.731)	0.854
HDL (mmol/L)	0.102 (0.037,0.28)	<0.0001	1.067 (0.208,5.474)	0.938
FBG (mmol/L)	1.333 (1.102,1.614)	0.003	1.855 (0.936,3.68)	0.077
HCY (μmol/L)	1.081 (1.027,1.137)	0.003	1.074 (1.006,1.147)	0.032
GHb (%)	1.765 (1.353,2.301)	<0.0001	1.132 (0.717,1.788)	0.595
Number of the Regions with Increased AF Intensity	2.339 (1.823,3.001)	<0.0001	2.148 (1.592,2.898)	<0.0001

AST: aspartic aminotransferase; Scr: serum creatinine; HDL: high-density lipoprotein cholesterol; FBG: fasting blood-glucose; HCY: homocysteine; GHb: glycosylated hemoglobin;

Factors		OR (95%CI)	Adjusted P Value
AF of Dorsal Index Finger	R	1.075 (1.025,1.127)	0.003
	L	1.051 (1.007,1.098)	0.023
AF of Ventroforefinger	R	1.079 (1.041,1.118)	<0.0001
	L	1.098 (1.048,1.15)	<0.0001
AF of Centremetacarpus	R	1.108 (1.036,1.186)	0.003
	L	1.201 (1.093,1.321)	<0.0001
AF of Dorsal Antebrachium	R	1.02 (0.968,1.076)	0.453
	L	1.077 (1.018,1.141)	0.01
AF of Ventriantebrachium	R	1.182 (1.066,1.309)	0.001
	L	1.054 (1.006,1.104)	0.028
AF of Index Fingernail	R	1.035 (1.006,1.064)	0.017
	L	1.027 (0.999,1.056)	0.057
Number of the Regions with Increased AF Intensity		2.148 (1.592,2.898)	<0.0001
Numer of the Regions with Asymmetry AF Intensity		2.25 (1.489,3.399)	<0.0001

	Cutoff >	Sensitivity	95% CI	Specificity	95% CI
Healthy Group vs AIS Group					
Intensity	0.5	0.8841	0.7843 to 0.9486	0.8649	0.7123 to 0.9546
	1.5	0.7826	0.6669 to 0.8729	0.9459	0.8181 to 0.9934
	2.5	0.6377	0.5131 to 0.7501	0.9459	0.8181 to 0.9934
Asymmetry	0.5	0.7971	0.6831 to 0.8844	0.8378	0.6799 to 0.9381
	1.5	0.5217	0.3980 to 0.6435	0.973	0.8584 to 0.9993
Low-risk Group vs AIS Group					
Intensity	0.5	0.8841	0.7843 to 0.9486	0.7397	0.6607 to 0.8088
	1.5	0.7826	0.6669 to 0.8729	0.8699	0.8043 to 0.9198
	2.5	0.6377	0.5131 to 0.7501	0.9589	0.9127 to 0.9848
Asymmetry	0.5	0.7971	0.6831 to 0.8844	0.6918	0.6101 to 0.7655
	1.5	0.5217	0.3980 to 0.6435	0.8973	0.8362 to 0.9413
High-risk Group vs AIS Group					
Intensity	0.5	0.8841	0.7843 to 0.9486	0.5606	0.4330 to 0.6826
	1.5	0.7826	0.6669 to 0.8729	0.7424	0.6199 to 0.8422
	2.5	0.6377	0.5131 to 0.7501	0.8333	0.7213 to 0.9138
Asymmetry	0.5	0.7971	0.6831 to 0.8844	0.6364	0.5087 to 0.7513
	1.5	0.5217	0.3980 to 0.6435	0.8939	0.7936 to 0.9563

Factors		AIS Group (n=69)	Lung Cancer Group (n=49)
AF of Ventroforefinger	R	3.63±0.34 ^{###}	1.24±0.21
	L	2.88±0.20 ^{###}	0.75±0.06
AF of Index Fingernail	R	3.01±0.30 ^{###}	1.12±0.13
	L	3.11±0.28 ^{###}	1.32±0.14
AF of Dorsal Antebrachium	R	1.95±0.35 [#]	1.77±0.43
	L	2.19±0.26 ^{###}	1.92±0.23 ^{##}
AF of Ventriantebrachium	R	1.65±0.16 ^{##}	1.56±0.16 ^{##}
	L	2.26±0.37 [#]	1.65±0.25 [#]
AF of Centremetacarpus	R	2.20±0.24 ^{###}	2.77±0.227 ^{###}
	L	2.83±0.33 ^{###}	2.68±0.35 ^{###}
AF of Dorsal Index Finger	R	3.59±0.41 ^{###}	1.60±0.12 ^{###}
	L	3.63±0.53 ^{###}	2.17±0.19 ^{###}
AF of Doral Centremetacarpus	R	2.42±0.26 ^{###}	4.02±0.52 ^{###}
	L	1.89±0.24 ^{##}	2.03±0.25 ^{##}
Comparisons of the AF intensity of the healthy group, the acute ischemic stroke(AIS) group and lung cancer group (Student t-test, two tail).			

Factors	ALS Group (n=69)	Lung Cancer Group (n=49)
Asymmetry of Ventroforefinger	4.17±0.55 ^{###}	1.16±0.35
Asymmetry of Index Fingernail	3.53±0.47 ^{###}	1.43±0.27
Asymmetry of Ventriantebrachium	2.97±0.64 [#]	2.27±0.57
Asymmetry of Centremetacarpus	6.28±0.96 ^{###}	4.72±0.72 ^{###}
Asymmetry of Dorsal Index Finger	5.20±0.84 ^{###}	2.25±0.32 ^{##}
Asymmetry of Dorsal Centremetacarpus	3.08±0.52 ^{##}	4.74±0.78 ^{###}
Asymmetry of Dorsal Antebrachium	3.38±0.72 [#]	3.29±0.98 [#]

Comparisons of the AF asymmetry of the healthy group, the acute ischemic stroke(ALS) group and lung cancer group (Student t-test, two tail).