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Original Article

Changes in the spectral index of skin-surface laser Doppler signals of nude mice following the injection of CT26 tumor cells

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Abstract: This study investigated microcirculatory-blood-flow responses in nude mice following the injection of CT26 tumor cells by analyzing the frequency content of skin blood-flow signals recorded on the skin surface. CT26 cells were injected subcutaneously (10^4/100 μl) into the right back flank of each 7-week-old mouse. Three-minute laser Doppler flowmetry (LDF) signals were measured in 60 nude mice. The data sequences were obtained at 1, 2, and 3 weeks after injecting CT26 cells. Mouse tissue samples were cut into sections and examined microscopically to determine the condition of cancer metastasis. Spectral analysis performed after 1 week revealed a significant decrease in the relative energy contribution of the endothelium-related frequency band, and significant increases in those of the myogenic and respiration-related frequency bands of the LDF signals in the metastasis group (n=12). To the best of our knowledge, this is the first study demonstrating the feasibility of evaluating metastasis in animal subjects based on changes in noninvasively measured LDF parameters. Changes in the LDF spectral indexes can be attributed to differences in the microcirculatory regulatory activities. The present measurements performed on the skin surface provide a noninvasive and real-time method for evaluating the microcirculatory responses induced by implanting CT26 tumor cells.

Keywords: Tumor, laser Doppler, spectral analysis, CT26

Introduction

Cancer is the leading cause of death in most countries, and there is an urgent need to improve its diagnosis and therapy. Metastasis-the spread of cells from the primary neoplasm to distant organs, and their relentless growth-is a fatal step in the progression and therefore the most fearsome aspect of cancer [1]. It is critical to develop early detection techniques for metastasis in order to minimize the threat posed by cancer.

Tumor blood vessels are an abnormal morphology [2], and both angiogenesis and vasculogenesis can occur in tumor tissue. Tumor blood vessels have irregular diameters; they are fragile, leaky, and hence are characterized by abnormal blood flows [3]. These abnormalities in the tumor vessels contribute to the abnormal microenvironment of tumors, and also to tumor growth and metastasis [4]. For example, it has been suggested that poor tumor vessel quality (e.g., defects in pericytes and irregularities in the tumor endothelial cell lining surrounding the vessel protrusions [5]) can result in tumor cells leaking into the adjacent vessels, and so this could be a potential prognostic indicator of tumor progression or perhaps even metastasis [3]. The induced chaotic pattern of blood flow may alter the endothelial shape, size, and differentiation, perhaps via the aberrant expression of flow-mediated transcription factors [6]. Detecting abnormalities in the microcirculatory blood flow (MBF) in the vascular beds of tumor tissue may therefore aid the development of an index for the detection and monitoring tumor growth and metastasis.

Various new techniques have been developed for measuring the blood flow in tumors. For

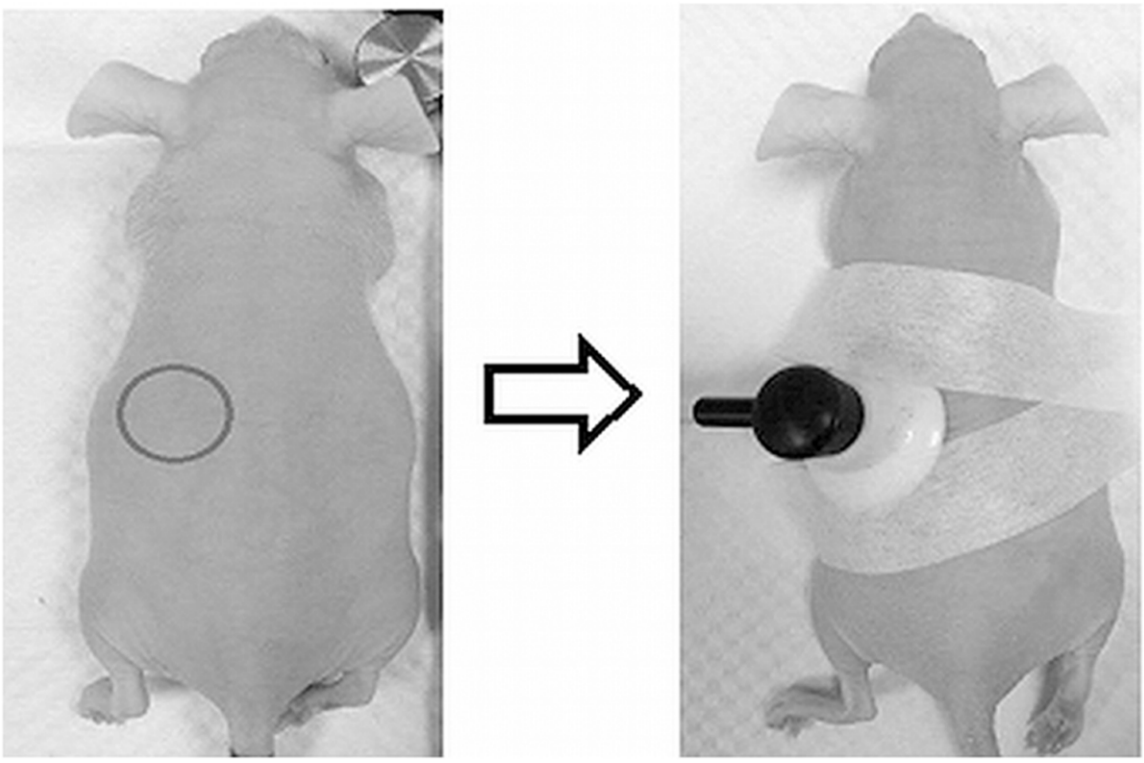

Figure 1. Photograph showing the placement of the LDF probe.

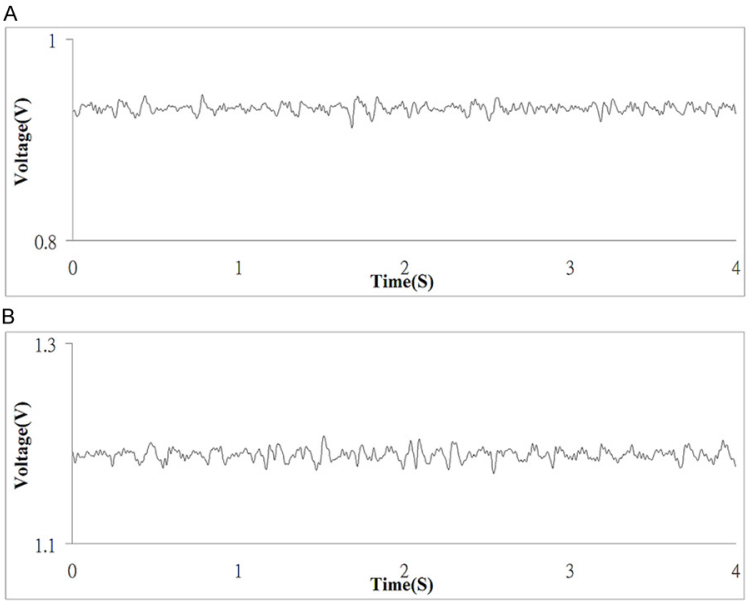

Figure 2. Typical LDF waveforms in the (A) metastasis group and (B) non-metastasis group.

example, imaging techniques such as scanning electron microscopy [7] have been used to evaluate morphological abnormalities of the vascular tree. Laser Doppler flowmetry (LDF) is a widely-used technique for monitoring the MBF response, with advantages such as allowing noninvasive and real-time measurements. Time-domain beat-to-beat waveform analysis of LDF signals has been used to study the MBF characteristics in subjects with various diseases, such as diabetes [8] and stroke [9]. In the frequency domain, spectral analysis has also been applied to LDF signals to study the activities of various regulatory mechanisms (e.g., endothelial function and myogenic responses) in local vascular beds in diseases [10] or following various types of stimulation [11, 12].

Colon cancer is one of the most common and deadliest types of cancer worldwide, with more than 1.47 million new cases diagnosed in 2012 [13]. CT26 is a mouse colon carcinoma cell line that is widely used in studies of colon cancer, and it can induce metastasis in the liver and

lung [14]. The present study performed skin-surface LDF measurements on nude mice injected with CT26 cells subcutaneously into the right flank. Liver and lung tissue samples were collected, stained, and then examined microscopically to evaluate the metastasis condition. Spectral analysis was applied to the acquired LDF data sequences, and the relative energy contribution (REC) of each frequency band was calculated, with the aim of comparing the regulatory activities at the local vascular beds following the injection of CT26 cells. The present findings may be pertinent to the development of a monitoring index for cancer metastasis.

Materials and methods

Animal preparation and experimental setup

The experiment protocol was approved by the Institutional Animal Care and Use Committee of Taipei Medical University. Mice were housed in accordance with the animal care guidelines of the National Laboratory Animal Center (Taipei, Taiwan), and all of the in vivo experiments were carried out in the accordance with the guidelines of the Institutional Animal Care and Use Committee of Taipei Medical University. Four-week-old male nude mice were purchased from BioLASCO Taiwan (http://www.biolasco.com.tw/index.php/en/introduction). The mice were housed in groups of five in stainless-steel cages under a 12-h/12-h light/dark cycle at a temperature of 20-22°C. After acclimatizing for 1 week, mice were anesthetized intraperitoneally with tribromoethanol (Avertin® 0.0125% with 0.0125% tert-amyl alcohol [v/v] in 130 μl/10 g PBS) for LDF measurements once per week.

The LDF signals were obtained from the middle-left back flank. LDF (VP1 probe; MBF3, Moor Instruments, UK; measurement site is shown in **Figure 1**; typical LDF waveforms were shown in **Figure 2**) was used to measure the MBF flux with a time constant of 0.001 s, a cut-off frequency of 14.9 kHz, and a sampling frequency of 40 Hz. At least 1 minute was allowed for stabilization before LDF measurements, mice were put on few sheets of paper towel and gently fixed by permeable plastic surgical adhesive tapes. The laser operating wavelength and out put power were 780 nm and less than 1.6 mW. The signals were connected to an analog-to-digital converter card (PCI-9111DG, ADlink Technology) operating at a sampling rate of 1024 Hz.

CT26 cells were injected subcutaneously (10^4/100 μl) into the right back flank of each 7-week-old mouse. From 1 week after injecting cancer cells, the body weight and tumor volume (calculated as 0.5 × length × width^2) were measured every week.

A 3-minute data sequence was acquired during each measurement. The data sequences were designated as follows: M0, 1 week after CT26 tumor cells were injected (no visible tumor); M1, 2 weeks after CT26 cells were injected (most mice had a visible tumor); and M2, 3 weeks after CT26 cells were injected.

Liver and lung tissue samples were collected from mice, fixed with 4% paraformaldehyde, and embedded in paraffin. The samples were cut into sections (5 μm thick) and stained with hematoxylin and eosin. The sections were examined microscopically to assess the cancer metastasis.

Data analysis

To determine the beat-to-beat LDF waveform, the two neighboring minimal points were used to identify the cut points in the LDF flux signal to define each pulse. Mean microcirculatory blood flow (MMBF) was defined as average of mean value for all the pulses in the 3-minute sequence. Values of the coefficient of variance of MMBF for all the pulses within a 3-minute LDF data sequence were then calculated to evaluate the beat-to-beat MBFV parameters (MMBFCV).

In the present spectral analysis, wavelet transform with Morlet mother wavelet was applied to the measured LDF signals to improve the low-frequency resolution. The average values of all LDF signals were removed before further analysis. In the LDF spectrum of human subjects, periodic oscillations with five characteristic frequency peaks can be noted within 0.0095-1.6 Hz, with the positions of these peaks falling within the following frequency bands: 0.0095-0.02, 0.02-0.06, 0.06-0.15, 0.15-0.4, and 0.4-1.6 Hz [15, 16]. Since the average HR of the mice was around 6.54 Hz, whereas that of human is around 1.2 Hz, the five frequency bands were set for the mice as 0.052-0.109, 0.109-0.327, 0.327-0.818, 0.818-2.180, and

Table 1. Body weights (in grams) of the mice

	Time point	Mean	SEM	*n*
Metastasis group	M0	28.93	0.53	12
	M1	29.85	0.61	
	M2	31.81	0.64	
Nonmetastasis group	M0	28.39	0.29	48
	M1	29.38	0.31	
	M2	31.70	0.46	

The metastasis group contained mice with CT26 tumor metastasis when they were sacrificed, and the nonmetastasis group contained mice without metastasis. M0, 1 week after CT26 cancer cells were injected (no visible tumor); M1, 2 weeks after CT26 cells were injected (most mice had a visible tumor); M2, 3 weeks after CT26 cells were injected.

Table 2. Tumor volumes (in cubic millimeters) of the mice

	Time point	Mean	SEM	*n*
Metastasis group	M1	72.49	31.04	12
	M2	1245.94	428.49	
Nonmetastasis group	M1	108.26	17.17	48
	M2	1736.81	194.80	

2.180-8.720 Hz (defined as FR1-FR5, respectively; each frequency band was uniformly divided into ten scales for wavelets). These five bands are suggested to be influenced by the endothelial activity of the vessel wall, the neurogenic activity of the vessel wall, the intrinsic myogenic activity of vascular smooth muscle, the respiration, and the heartbeat, respectively [15, 16]. The energy density within each frequency band (from FR1 to FR5) was calculated, and the relative energy contribution (REC) was defined as the ratio between the total energy density within each band and the total energy density of the entire spectrum from 0.052 to 8.720 Hz.

Statistics

All statistical analyses were carried out using SPSS, version 13.0. The differences between groups in the fundamental physiological were tested with t-test. Changes in the LDF parameters were tested with paired t-test. The level of significance was defined as $P<0.05$; all *P*-values were two-sided hypotheses.

Results

Tables 1 and **2** list the body weights and tumor volumes in the metastasis and nonmetastasis groups. The body weight and tumor volume increased with time in both groups. The body weight did not differ significantly between the two groups at any time point; it minimizes the possible interference effects on the tumor volume caused by different body weights.

There were significant differences in the spectral index of LDF signals between the metastasis ($n=12$) and nonmetastasis ($n=48$) groups in the present study. Changes in the spectra of the blood-flow signals in the two groups are compared in **Figure 3**. In the metastasis group, the REC of FR1 was significantly decreased during M1, and then significantly increased during M2. The REC of FR3 were significantly increased during M1, and appeared to be increased during M2 compared to that during M0. The REC of FR4 was significantly increased during M1. There were no significant changes in any frequency band in the nonmetastasis group.

The present results also revealed significant differences in the beat-to-beat LDF variability index between the metastasis and nonmetastasis groups. The responses of beat-to-beat LDF parameters are compared in **Figure 4**. MMBFCV in the metastasis group appeared to be lower during M1 than during M0, and higher during M2 than during M1 ($0.05<P<0.10$ by two-tailed paired *t*-test). As a comparison, there were no any significant changes in the nonmetastasis group.

Discussion

Changes in the LDF spectrum induced by cancer have been studied previously [17]. Although a significant association was noted by those authors, the frequency bands were not clearly defined in that study. The frequency bands associated with microcirculatory regulatory activities were defined in the present study. As conjectured below, these could be at least partly attributed to the changes in the microcirculatory regulatory activities induced by the injected tumor cells; it may be helpful to understand the changes in the underlying microcirculatory regulatory mechanisms induced by the development of tumor tissue.

The REC of FR1 was decreased during M1 in the metastasis group, and this is suggested to be associated with the endothelial function in

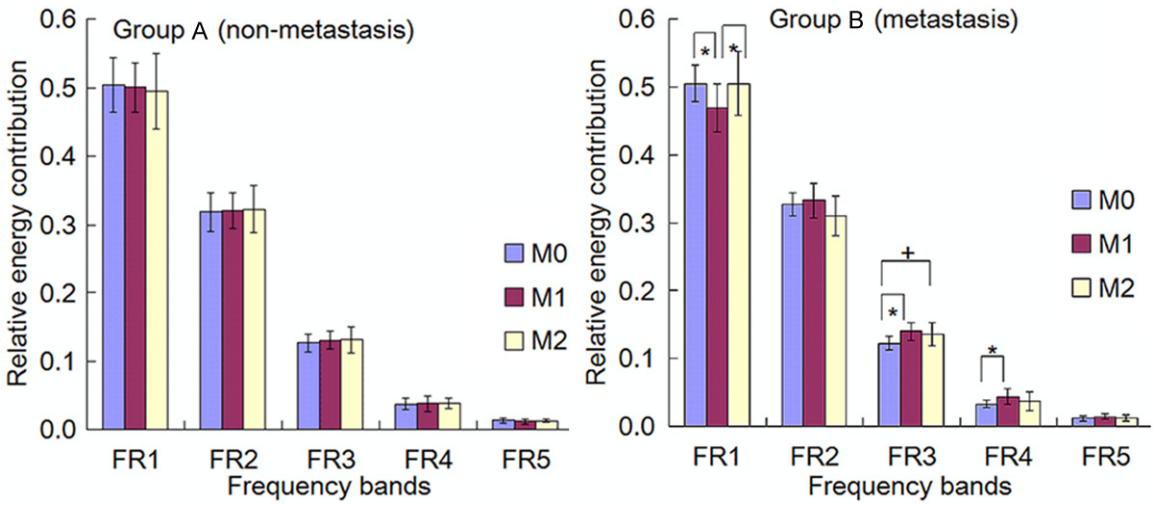

Figure 3. Changes in RECs in the LDF spectra. The REC of each frequency band was defined as the total energy density within a particular frequency band divided by the total energy density of the entire spectrum from 0.052 to 8.720 Hz. "*" and "+" indicate $P<0.05$ and $P<0.1$, respectively, by two-tailed paired *t*-test. There were significant changes in FR1, FR3, and FR4 in the metastasis group, whereas there were no significant changes in the nonmetastasis group.

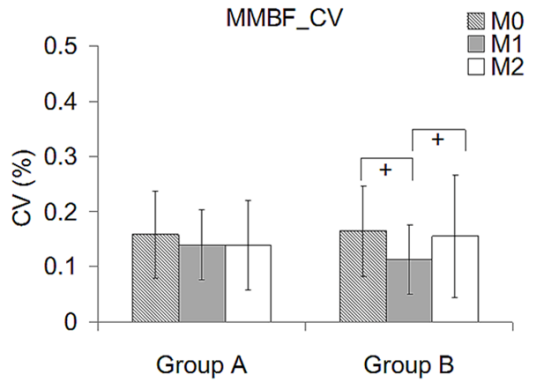

Figure 4. Comparison of beat-to-beat LDF indexes between groups. Data are mean and SD values. "+" indicates $0.05<P<0.1$ by two-tailed paired *t*-test.

the microcirculatory regulatory activities. The present noted decrease in the REC of FR1 could therefore be attributed to the vascular endothelial dysfunction induced by the tumor cells.

The endothelium was once thought of as the "cellophane wrapper" of the vascular tree, with no specific functions other than affording selective permeability to water and electrolytes [18]. It has subsequently been found that the endothelium performs several critical functions, including regulating the passage of nutrients, oxygen, and other solutes from the bloodstream into tissues, regulating the flow of blood by maintaining a nonthrombogenic surface, and controlling the trafficking of leukocytes into and out of the tissues [3].

Endothelial dysfunction is a cause of many diseases, such as hypertension and hyperlipidemia, and is also a consequence of many diseases, such as diabetes and cancer. Endothelial dysfunction is the initial step in the pathogenesis of cancer [18]. The present findings for the REC of FR1 can be partly attributed to the induction of endothelial dysfunction. Endothelial cell migration is an essential component of angiogenesis, and the underlying processes require the integration of signals elicited by chemotactic, haptotactic, and mechanotactic stimuli, and are associated with the activation of intracellular pathways that result in cytoskeleton remodeling [18]. Two putative mechanisms underlying metastasis have recently received significant attention: (1) an epithelial-to-mesenchymal transition and (2) tumor microenvironment interactions; and these two mechanisms may work synergistically to direct the progression of metastasis [4]. Epithelial-to-mesenchymal transition has been suggested as possible mechanism underlying the initiation of cancer progression during staged metastasis [19-21]. It is believed that the migratory characteristics acquired by the transition to a mesenchymal-like state enable the invasive capabilities of cancer cells. The secretion of vascular endothelial growth factor was noted to

induce endothelial cell proliferation, migration, and survival, which lead to tumor angiogenesis [18].

Morphologically, tumor endothelial cells have irregular shapes and sizes. For example, small endothelial gaps and larger openings are suggested to be responsible for the hemorrhage and plasma leakage observed in most tumors. Tumor endothelial cells lack the normal hierarchical arrangement from arteries to arterioles to capillaries [2, 7], and can also be associated with stiffer vessels, defects in pericytes, and higher vessel densities. Irregularities in the tumor endothelial cell lining surrounding these vessel protrusions will impair the blood flow, resulting in hypoxia and hypoperfusion [3]. These abnormalities in the tumor endothelium contribute to tumor growth and metastasis; abnormalities in the blood vessels themselves are also a major contributor to the abnormal microenvironment in tumors. The resultant fluctuation in the red-blood-cell flux can exert significant effects on the tumor interstitial PO_2 and the hypoxic fraction of tumors [17].

Furthermore, a dynamic interaction is suggested to exist between cancer cells and the host microenvironment that supports the growth and spread of cancer cells. Cancer cells from the primary tumor must first gain access to the circulatory system, and then they invade the secondary tissue and reestablish organizational growth as a solid secondary tumor [4]. This implies that the injected tumor cells may induce the MBF response at a site other than the injection site itself. This re-establishment procedure can therefore help to explain why changes in the LDF spectral index can be noted at a distant site (as in the present measurement design).

Figure 4 reveals that MMBFCV was decreased during M1 in the metastasis group, which is a similar trend to that of the REC of FR1. MMBFCV was defined as the variation of the MBF supply, and has been suggested to reflect the local microcirculatory regulatory activities [8, 9]. The present findings for MMBFCV hence imply that changes in the local microcirculatory regulatory activities can be partly attributed to changes induced in the endothelial function.

Other than FR1, the RECs of FR3 and FR4 were also noted to be significantly changed (increased) in the metastasis group. The RECs of FR3 and FR4 have been suggested to be associated with the regulatory activities of the myogenic response and baroreflex, respectively [15, 16]. It has been reported that an elevated blood pressure (BP) is associated with an increased cancer risk overall in men and with a higher risk of cancer-related death in both men and women. It was further suggested that a high BP is important for the progression of certain tumors [22]. It is possible that the BP was higher in the metastasis group in the present study. Local vessels can be stretched more by the increased BP, and this could increase the activity of the myogenic response, which could partly explain the increase in the REC of FR3 in the metastasis group. A higher BP can also increase the intensity of the baroreflex for restoring the BP value, and thus could increase the REC of FR4. Similar with FR1, the RECs of FR3 and FR4 can therefore aid the monitoring of cancer disease processes. However, one limitation of the present study was that BPs were not measured; such measurements should therefore be included in future animal studies.

To the best of our knowledge, this is the first study demonstrating the feasibility of evaluating the possibility of metastasis in animal subjects using changes in noninvasively measure LDF parameters. The analysis of the frequency content of the blood-flow signals revealed significant changes in LDF spectral indexes (the RECs of FR1, FR3, and FR4) in the metastasis group. Changes in the LDF spectral indexes can be attributed to differences in the microcirculatory regulatory activities between groups. The present measurements performed on the skin surface provide a noninvasive and real-time method for evaluating the microcirculatory responses induced by injecting CT26 tumor cells.

One important finding of this study is that changes in the LDF spectral index and MMBFCV took place during M1 in the metastasis group, which was prior to the time point at which the metastasis condition was evaluated and the tumor size measurements at M2. This implies that this LDF index may be used to aid the prediction of tumor progression. The present findings further illustrate that a spectral index of the LDF signal measured at a distant site can still change significantly following the growth of injected CT26 cells. This would help to improve the user-friendliness of future applications

involving the monitoring of tumor disease progression. Future studies should validate the responses in LDF spectral parameters for different tumor types in order to further verify the practical applicability of the present findings.

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Disclosure of conflict of interest

None.

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