ACN
ANNALS OF CLINICAL NEUROPHYSIOLOGY

REVIEW

Ann Clin Neurophysiol 2018;20(1):3-11
https://doi.org/10.14253/acn.2018.20.1.3

Skin biopsy: an emerging method for small nerve fiber evaluation

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Skin biopsy and staining the specimens with immuno-reactive markers has been proven to be a useful method to demonstrate the pathologic status of small nerve fibers. Quantification of intraepidermal nerve fiber density using anti-protein gene product 9.5 antibody is a standard method to diagnose small fiber neuropathy. Skin biopsy also makes it possible to differentiate the nerve fibers according to their function by using different markers. Quantification of dermal structures with different types of nerve fibers could reveal the pathophysiologic mechanism of the disease state.

Key words: Skin, Biopsy; Small fiber neuropathy; Epidermis, Nerve fiber

Received: December 26, 2017
Revised: December 28, 2017
Accepted: December 28, 2017

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INTRODUCTION

Nerve conduction study has been the gold standard to diagnose peripheral neuropathy, but it only reveals the function of myelinated nerve fibers. Small nerve fibers that are thinly myelinated or unmyelinated nerve fibers consist of somatic sensory and autonomic nerve fibers and specialized tests are needed to measure the function of the small nerve fibers.

The skin is densely innervated by sensory and autonomic nerve fibers, which are mainly small nerve fibers. Sensory fibers convey pain and somatic sensation from the epidermal and dermal layers to the dorsal root ganglia. Sudomotor, vasomotor, and pilomotor nerve fibers innervate the sweat gland, cutaneous vessels, and arrector pili muscles in the dermal layer. Since the pan-axonal marker protein gene product 9.5 (PGP 9.5) enables visualization of all nerve fibers within the epidermal and dermal layer,[1] several methods have been developed to stain and quantify innervation of the epidermal and dermal layers.[2,3] The European Federation of Neurological Societies (EFNS) suggested a standard way to perform skin biopsy and to quantify the intraepidermal nerve fiber density (IENFD) using bright-filed microscopy. They also recommended to use IENFD as a standard diagnosing tool for small fiber neuropathy (SFN).[4,5]

http://www.e-acn.org
pISSN 2508-691X
eISSN 2508-6960

NORMAL ANATOMY

The skin is composed of epidermis and dermis, which are demarcated by the basement membrane. Nociceptive C-fibers extend vertically to the epidermis from the sub-epidermal neural plexus through the basement membrane (Fig. 1). The sensory nerve fibers in the epidermis are C-fibers only, C-fibers and Aδ-fibers co-exist in the sub-epidermal layer. C-fibers transmit slow pain and warm sensation and Aδ-fibers transmit sharp pain and cold impulses.[6]

The skin is divided into hairy and glabrous skin according to the hair. Almost parts of the body are covered with hairy skin and usual biopsy site for IENFD analysis is the hairy skin. The hairy skin includes blood vessels, hair follicles, arrector pili muscles, and sweat glands (Fig. 2). The glabrous skin is located at the palmar and plantar area, and it contains the Meissener corpuscle and Merckel cell which are innervated by thinly myelinated nerve fibers.

Sweat glands are located 3-5 mm below the epidermal layer and connect to the skin surface through the sweat duct. Hair follicles extend from the deep dermal tissue through the epidermal layer and are anchored to the dermal tissue by arrector pili muscles (Fig. 3).[7] Upper blood vessel plexuses are located at superficial dermal layer and lower blood vessel plexuses are located at deep dermal layer. Capillaries project vertically to the epidermis from the upper blood vessel plexuses.[8]

Dermal structures are mainly innervated by autonomic nerve fibers and the nerve fibers have particular reactivity to the different antibodies according to their function. The nerve fibers innervating the blood vessels and arrector pili muscles are adrenergic sympathetic fibers. They are immuno-reactive to tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH).[6,9] Sweat glands innervated by cholinergic sympathetic fibers, which have immuno-reactivity to vasointestinal peptide (VIP) (Fig. 4).[9] PGP 9.5 is pan-axonal marker so all of these sensory and autonomic nerve fibers are positive to PGP9.5.

QUANTIFICATION OF INTRAEPIDERMAL NERVE FIBERS

Intraepidermal nerve fibers (IENF) are sensory C-fibers. The EFNS recommends quantifying IENFD as main method of diagnosis of painful small fiber neuropathy.[4,5]

Method of skin biopsy and staining the specimens

Skin biopsy is most commonly performed using 3 mm disposable punch after topical anesthesia with lidocaine. There have been no serious complications of skin biopsy, however excessive bleeding might occur especially if the patient has coagulopathy or takes anticoagulant. Distal leg (10 cm above the lateral malleolus) and proximal thigh (20 cm be-

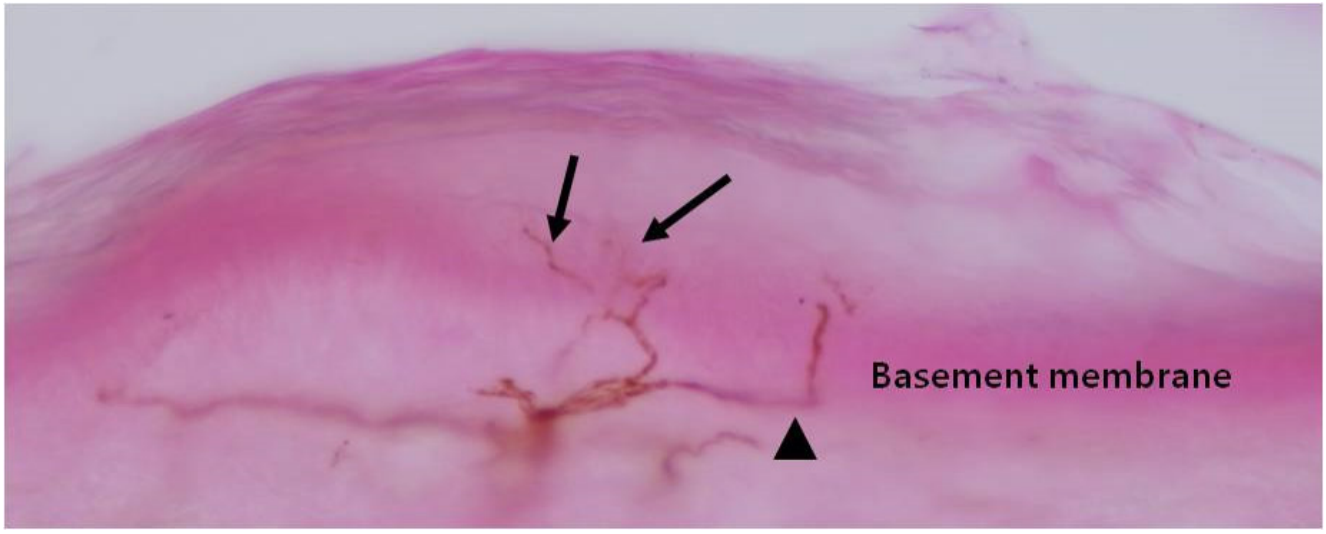

Fig. 1. Nerve fibers of epidermis and superficial dermis. Epidermal nerve fibers (arrows) originate from the subepidermal neural plexus (arrowhead) and travel vertically to the epidermal surface. (anti-PGP 9.5 antibody for nerve fiber).

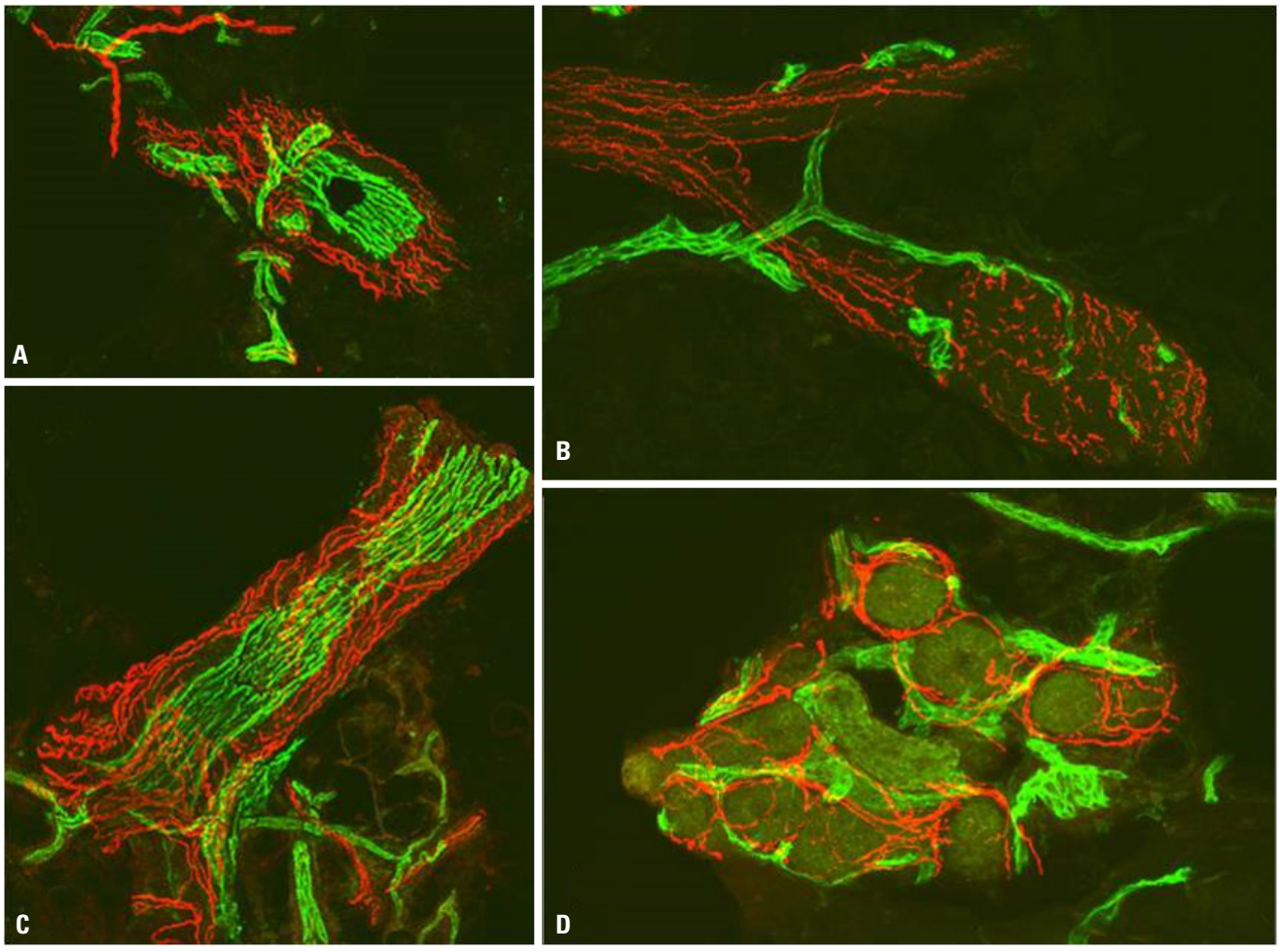

Fig. 2. Dermal structures stained with anti-PGP 9.5 antibodies for nerve fibers (red) and anti-CD31 antibodies for blood vessels (green). (A, C) Deep dermal vessels are densely surrounded by innervating nerve fibers. (B) The nerve fibers innervating arrector pili muscle run parallel with muscle fibers. Intervening blood vessels were also showed. (D) Sweat gland and intervening capillaries. Sweat gland is innervated densely and complicatedly.

low from the anterior iliac spine) are recommended as the biopsy sites for detecting length-dependent polyneuropathy.[4]

The obtained skin specimen is fixed in cold fixative for up to 24 hours at 4°C. Most studies have used 2% paraformaldehyde-lysine periodate (2% PLP) for bright-field microscopy and Zamboni's fixative (2% paraformaldehyde and picric acid) for indirect immunofluorescence with confocal microscopy.[5] Formalin fixation seemed to cause a more fragmented appearance of nerve fibers, however it did not affect the measurement of the innervation density.[10,11] The specimen is kept in a cryoprotective solutions for one night after fixation, and serially cut with a freezing microtome or cryostat in 50 μm thickness. After staining for PGP 9.5, nerve fibers can be quantified using bright-field microscopy or confocal microscopy (Fig. 5). Bright-field microscopy method dose not differentiate the nerve fibers by their function, however it is easier and faster than confocal microscopy method. Confocal microscopy method uses multiple antibodies at once so can discriminate the nerve fibers by their function. Bright-field microscopy is mainly used for diagnosing SFN in clinical practice and confocal microscopy is usually used for research.[4,12-14]

Quantification of intraepidermal nerve fiber density

Single nerve fibers crossing epidermal-dermal junction should be counted at high magnification (i.e., ×40) in at least 3 non-consecutive sections, excluding secondary branching

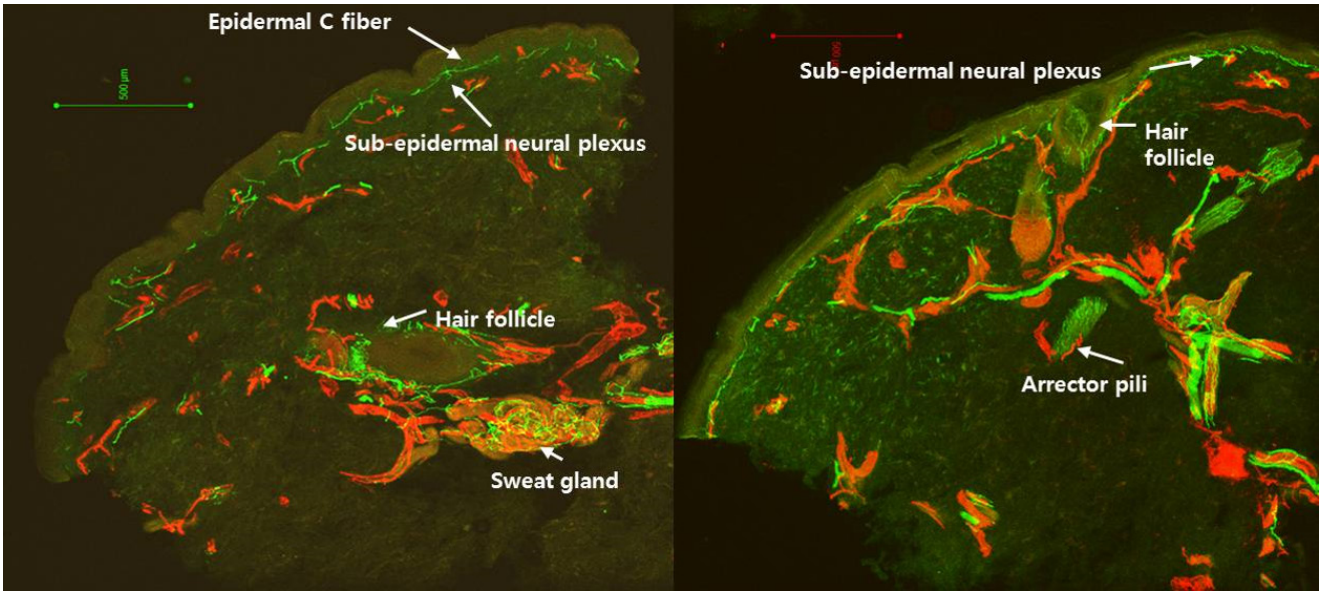

Fig. 3. Samples of stained skin biopsy tissue. Epidermal C fiber and sub-epidermal neural plexus are located at the superficial dermis. Hair follicle travels from the deep dermis to the superficial dermis and arrector pili muscles are located near the hair follicle. Sweat glands are located in the deep dermis and innervated densely (anti-PGP 9.5 antibodies for nerve fibers [green] and anti-CD31 antibodies for endothelia [red]).

and fragments from quantification (Fig. 6). The length of the section should be measured and IENFD is calculated as total IENF counts divided by length of the section (IENF/mm).[4,5]

IENFD is higher in women than men and decreases with aging. Table 1 summarizes the reported normal IENFD according to age and gender using bright-field microscopy.[4,15]

Applications and limitations of IENFD

Quantification of IENFD is the main method to diagnose painful SFN.[5,10,16-21] Several studies recently applied IENFD quantification in axonal neuropathy, vasculitic neuropathy, and hereditary neuropathy.[22-24] Comparison the IENFD of symptomatic side with that of symmetric healthy side is a useful way to diagnose asymmetric or focal neuropathy.[25] Skin biopsy is a minimally invasive method and repeated biopsy causes no complication. Repeated biopsy could help to determine the effectiveness of treatment and the progression of disease. It is also used for comparison of other specialized tests for small nerve fibers. For example, skin specimen could be obtained at the same site after quantitative sensory testing or sudomotor testing, and comparison of these two tests might reveal the pathophysiologic mechanism.

There are several limitations of quantification of IENFD. IENFD is normal in some symptomatic SFN which is originated from the functional disability[16] and quantification of IENFD would not reveal the cause of neuropathy.

QUANTIFICATION OF DERMAL INNERVATION

Dermal structures, such as sweat glands and arrector pili muscles, are innervated by autonomic nerve fibers. Quantifying innervation of these structures enables to determine autonomic involvement in idiopathic SFN, hereditary neuropathy, inflammatory neuropathy and so on. Sweat glands and arrector pili muscles distribute randomly in the dermal layer so quantifying dermal structures needs more tissue sections than IENFD quantification.[7]

Pilomotor nerve fibers distribute longitudinally following the regular course of muscle fibers (Fig. 2B). To quantify the nerve fibers innervating arrector pili muscles, draw a vertical line to the nerve fibers at first and then count the nerve fibers intersecting the line. The results are expressed as the ratio between the number of the nerve fibers and the width of the arrector pili muscle (nerve fiber number/mm).[26] The pilomotor nerve fiber density (PNFD) was decreased in diabetic patients compared with healthy controls.[26] The arrector pili muscles are innervated by adrenergic fibers, so it will

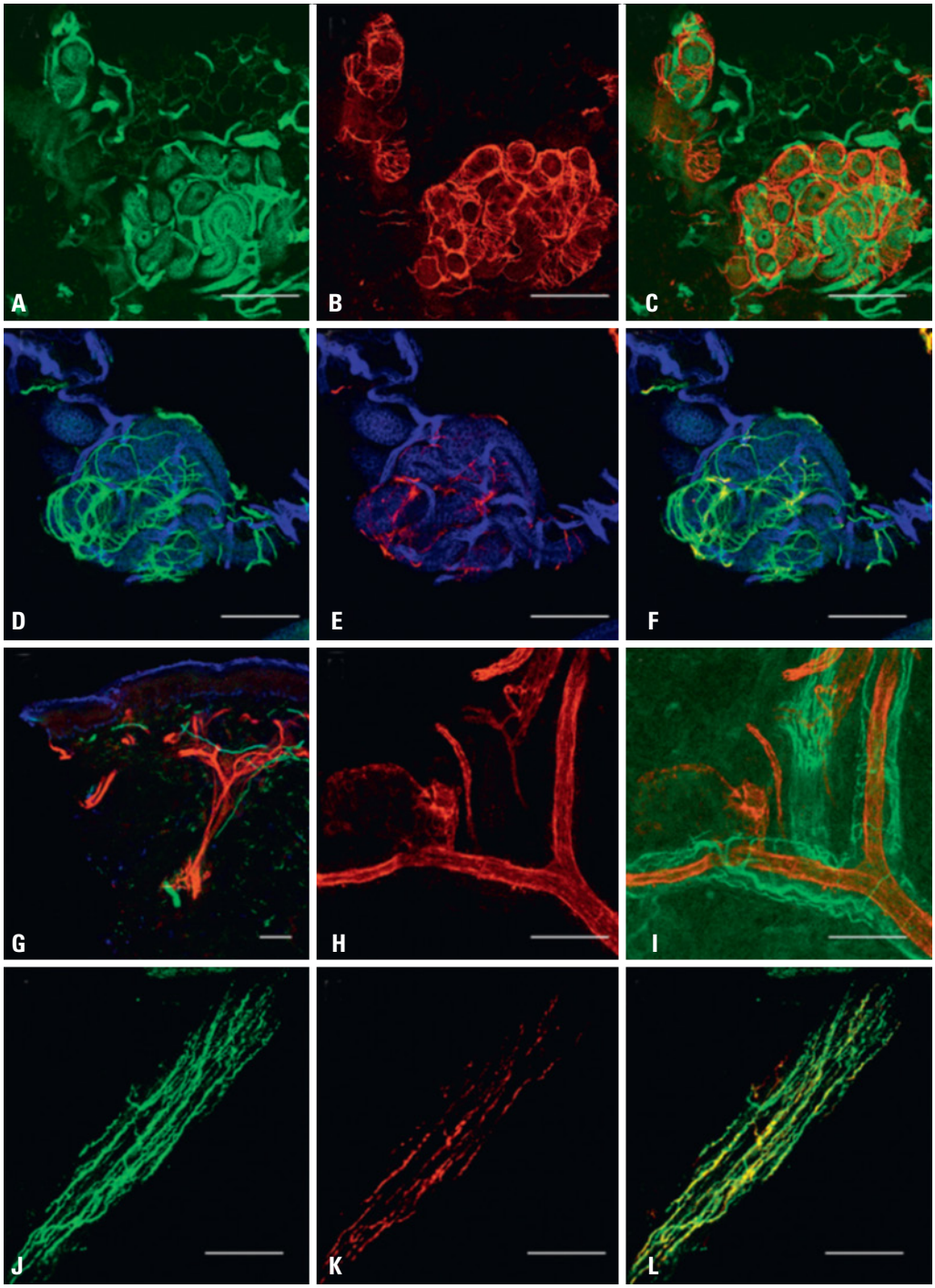

Fig. 4. Example of images obtained by immunohistochemical staining. (A-C) A sweat gland. Endothelia of capillaries are stained with anti-CD31 antibody in green (A). Nerve fibers innervating sweat gland are stained with anti-PGP 9.5 antibody in red (B). Merged image (C). (D-F) A sweat gland and endothelia (anti-CD31 antibody, blue). Sympathetic cholinergic fibers are stained with anti-VIP antibody in green (D) and sympathetic adrenergic fibers are stained with anti-TH antibody in red (E). Merged image (F). (G-I) Cutaneous blood vessels. Endothelia are stained wit anti-CD31 antibody in red and nerve fibers are stained with anti-PGP 9.5 antibody in green. (J-L) Arrector pili muscles are innervated with sympathetic adrenergic fibers (anti-TH antibody, J) and sympathetic cholinergic fibers (anti-VIP antibody, K). Merged image (L). Originally adapted from Wang et al.[7]

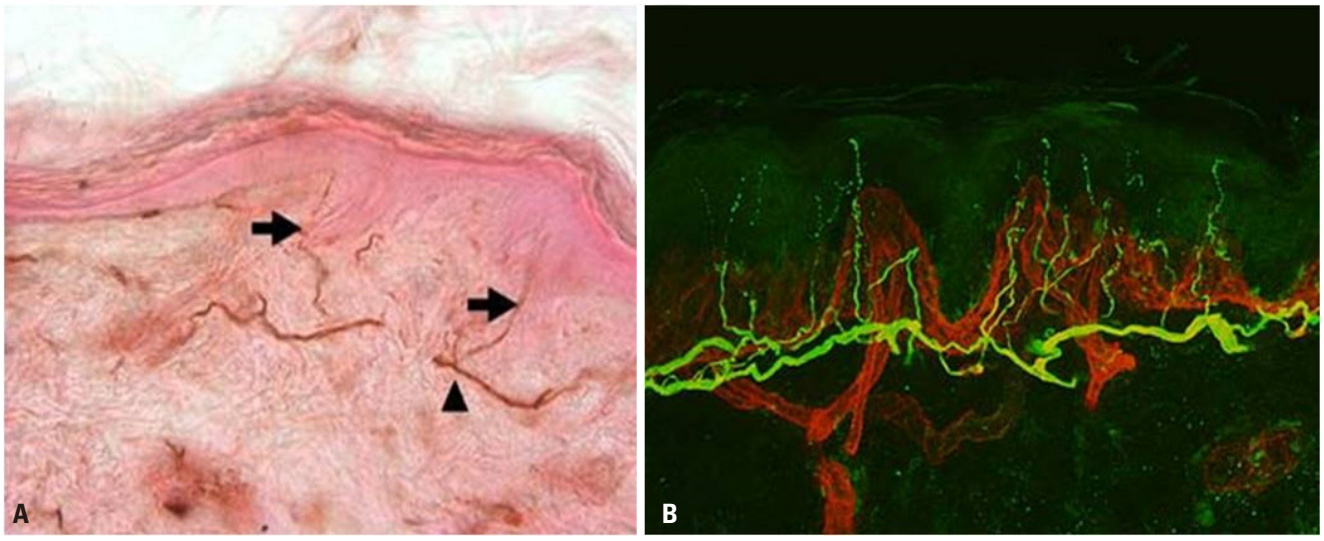

Fig. 5. Dermal and epidermal nerve fibers stained with anti-PGP 9.5 antibodies. (A) Bright-field microscopy finding. Intraepidermal nerve fibers (arrows) cross from the sunepidermal neural plexus (arrowhead) to the epidermis. (B) Confocal microscopy showing nerve fibers (in green) and blood vessels and basement membrane (in red).

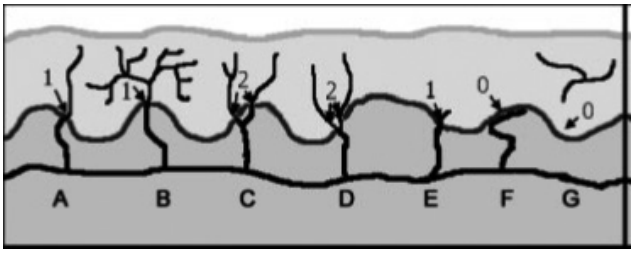

Fig. 6. Intraepidermal nerve fiber counting rule. Diagram of skin innervations: nerves (black), basement membrane (dark grey), dermis (medium grey), and epidermis (light grey). Nerve fibers which cross the basement membrane are only counted as one nerve fiber. Nerve fibers which branches after crossing the basement membrane or which resides only in the epidermis should be excluded when count the nerve fibers. The epidermal nerve fiber branches before crossing the basement membrane, it should be counted as two fibers. Originally adapted from Lauria et al.[4]

be useful to determine the abnormality of the adrenergic nerve fibers.

Sweat glands are made up of sweat gland tubules that are intertwined with capillaries and nerve fibers (Fig. 2D). There have been several ways to quantify the sweat glands innervation without standardized method due to the complicated structures.[27] Semi-quantitative analysis of sweat gland innervation was developed first, which scores the degree of sweat gland innervation semi-quantitatively on a 5-point scale from 0 (no identifiable nerve fibers) to 4 (normal nerve fiber density). This method has shown poor inter- and intra-reviewer reliability especially in relatively mild degree neuropathy.[28-30] Manual and automated quantification are more recent ways to quantify the sweat gland innervation.[30,31] In manual quantification, standardized grid of circles is placed over the sweat gland image and intersecting nerve fibers with the circle are counted. The results are expressed as the number of circles intersected out of the total number of circles. This method showed high inter- and intra-rater reliability.[31] Sweat gland nerve fiber density (SGNFD) by this method was decreased significantly in diabetic patients compared with healthy controls and correlated well with standardized neuropathy examination scores and IENFD.[31] The limitation of this method is it takes a lot of time and effort. In automated quantification, nerve fibers of sweat gland are highlighted using a histogram based segmentation tool automatically and the results are expressed as the percent area of nerve fibers within the area of interest. Automated method had a high inter- and intra-reviewer reliability and correlated well with other parameters, such as IENFD and standardized neuropathy examination score.[30] However, this method has larger variability and greater overlap of confidence interval between the diseased state and control subjects than manual method. So the diagnostic discrimination is superior in manual method than automated method and automated method is better for screening or epidemiologic studies because of the speed of

Table 1. Normal values of intraepidermal nerve fiber density at the ankle

Age (yr)	Females (n = 97)		Males (n = 91)	
	0.05 quantile values per age span	Median values per age span	0.05 quantile values per age span	Median values per age span
20-29	6.7	11.2	5.4	9.0
30-39	6.1	10.7	4.7	8.4
40-49	5.2	9.9	4.0	7.8
50-59	4.1	8.7	3.2	7.1
60-69	3.3	7.9	2.4	6.3
≥70	2.7	7.2	2.0	5.9

the analysis. SGNFD is proposed as an additive tool to diagnose diabetic SFN recently.[32]

Quantification of vascular innervation is unsolved issue until now. Cutaneous blood vessels are composed of upper blood vessel plexuses and lower blood vessel plexuses and nerve distribution is different from upper and lower blood vessel plexuses. They are distributed diffusely, so it is difficult to quantify the innervation density of cutaneous blood vessel. Furthermore, the innervation of the capillary remains unclear. A manual method to quantify blood vessel innervation in the superficial dermal layer was reported recently. This method will provide pathophysiologic clues in many disease states involving vasculature.

OTHER APPLICATIONS OF SKIN BIOPSY

α-synuclein staining

Many studies have tried to find the α-synuclein deposition in the easily accessible tissue to develop diagnostic method at the early stage and therapeutic methods in idiopathic Parkinson disease (IPD). Since α-synuclein deposits were found at the colonic mucosa, it has been shown that IPD involves the peripheral nerves at very early stage of the disease.[33-36] Since decreased IENFD at the early stage of IPD was reported,[37] α-synuclein deposition in the nerve fibers, which innervate the sweat gland and the arrector pili muscle, were found.[38] A recent study described phosphorylated α-synuclein deposition which could be a pathophysiological marker of IPD in the skin.[39] These studies reporting IPD pathology in the easily accessible skin promote further research about the pathophysiologic mechanism of IPD.

Pathophysiologic mechanism

Ross syndrome is characterized by tonic pupil, decreased deep tendon reflex, and segmental hypohidrosis. The syndrome has clinical similarity with Homes-Adie syndrome. Some researchers proposed that they represent other clinical manifestation with same pathophysiologic mechanism.[40] However, Nolano et al.[19] found a difference between Ross syndrome and Homes-Adie syndrome using the skin biopsy. Patients with Ross syndrome had no cholinergic fibers at all in contrast to patients with Homes-Adie syndrome who showed cholinergic reactivity. Skin biopsy will be helpful to clarify the pathophysiologic mechanism.

CONCLUSIONS

The skin is densely innervated by sensory fibers of epidermis and autonomic fibers of dermal structures. The skin is easily accessible and skin biopsy is minimally invasive than sural nerve biopsy. Quantification of epidermal nerve fibers and dermal structures innervation has been highlighted for diagnosing SFN, which needs a specialized test for diagnosis. In addition special antibodies for nerve function (for example TH, VIP, DβH) and pathophysiologic causes (for example α-synuclein) will make it possible to research the pathophysiologic mechanism of the disease.

Acknowledgement

This article is translated from the original that was published in the Korean Journal of Clinical Neurophysiology (the former official journal of Korean Society of Clinical Neurophysi-

ology) on December 2015 (article titled "Skin biopsy: Emerging method for small nerve fiber evaluation").

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