Title

Morphological study of the phrenic nerve to determine a reference value for the myelinated fiber density in elderly individuals

Short title

Phrenic nerve morphology in the elderly

Author names

Atsuko Motoda, MD,[1,2,3] Tomoyasu Matsubara, MD, PhD,[1,3] Zen-ichi Tanei, MD, PhD,[1] Yasuhiro Sakashita, MD, PhD,[1,2] Mikihiro Yamazaki, MD,[1] Ito Kawakami, MD, PhD,[1] Renpei Sengoku, MD, PhD,[1] Tomio Arai, MD, PhD,[2] Hirofumi Maruyama, MD, PhD,[3] Yuko Saito, MD, PhD,[1] Shigeo Murayama, MD, PhD,[1,4,*]

*This author is the corresponding author.

Affiliations

[1] Department of Neuropathology (the Brain Bank for Aging Research), Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology, Tokyo, Japan

[2] Department of Pathology, Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology, Tokyo, Japan

[3] Department of Clinical Neuroscience and Therapeutics, Hiroshima University Graduate School of Biomedical and Health Sciences, Hiroshima, Japan

[4] The Brain Bank for Neurodevelopmental, Neurological and Psychiatric Disorders, United Graduate School of Child Development, Osaka University, Osaka, Japan

ABSTRACT

Phrenic nerves (PNs) play an important role in respiration; however, very few morphological studies have assessed them. This study aimed to provide control reference values, including the density of large and small myelinated PN fibers, for future pathological studies. We assessed a total of nine nerves from eight cases among consecutive autopsy cases registered to the Brain Bank for Aging Research between 2018 and 2019 (5 men and 3 women, mean age 77.0 ± 7.0 years). The nerves were sampled distally, and their structures were analyzed using semi-thin sections stained with toluidine blue. The mean and standard deviation of the density of each myelinated fiber of the PN was 6908 ± 1132 fibers/mm^2 (total myelinated fiber), 4095 ± 586 fibers/mm^2 (large diameter myelinated fiber; diameter ≥ 7 μm), and 2813 ± 629 fibers/mm^2 (small diameter myelinated fiber; diameter < 7 μm). There was no correlation between myelinated fiber density and age. This study provides the density measurement of the human PN myelinated fiber, and these findings can be used as reference values for the PN in elderly individuals.

KEY WORDS

aging, elderly, morphological, myelinated fiber, phrenic nerve

ABBREVIATIONS

ALS: amyotrophic lateral sclerosis, PN: phrenic nerve

INTRODUCTION

The phrenic nerve (PN) mainly carries motor fibers to the diaphragm, and sensory and autonomic fibers to the diaphragm, pleura, and peritoneum.[1] It plays an essential role in respiration and airway clearance.[1] Thus, impairment of the PN leads directly to respiratory failure. In fact, amyotrophic lateral sclerosis (ALS) has been shown to cause denervation of the PN along with lower motor neuron degeneration.[2] Similarly, other neurodegenerative diseases that involve the spinal motor neurons may also cause PN denervation to various degrees.[3] Moreover, peripheral neuropathy can involve the PN.[4,5] Thus, assessing PN degeneration is essential in determining the pathogenesis and cause of death. However, biopsies of PNs cannot be performed, and their physiological importance and morphological characteristics remain unclear.

A previous study by Bradley et al.[2] compared the PNs among ALS patients with normal controls. However, these normal controls were mainly young individuals, and normal values for the elderly, on whom the majority of autopsies are conducted, remain unclear. Moreover, normal values for the density of the PN myelinated fibers have never been determined. The number of nerve fibers, an index examined in previous reports on the PN, depends on the diameter of the nerve sampled and whether the nerve bundles are branched; thus, depending on the situation, density measurements can be ideal for evaluation that leads to less variation.[6-8]

In this study, we examined morphological characteristics of the PNs of neurologically normal controls using a consecutive autopsy series. This study aimed to provide fundamental data on the PN, which can serve as baseline data for future studies of neurological disorders affecting the nerve.

MATERIALS AND METHODS

Ethics

Our study was approved by the ethics committee of the Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology (approval number: No.71). Written informed consent was obtained from the patients' families prior to the autopsy. This study was performed in accordance with the principles of the Declaration of Helsinki.[9]

Participants and settings

This study included consecutive patients autopsied at the Tokyo Metropolitan Geriatric Hospital and Institute of Gerontology between September 2018 and September 2019. We defined potentially eligible patients who underwent autopsy with craniotomy registered to the Brain Bank for Aging Research. The exclusion criteria were as follows: (1) having mediastinal states unsuitable for sampling; (2) assessment difficulty due to postmortem autolysis or deformation; (3) having a condition involving lower motor neurons; or (4) having the peripheral neuropathy pathologically confirmed in the sural nerve or PN.

Clinical information

Clinical information was collected retrospectively from medical records, including sex, age, height, body weight, clinical symptoms of neuropathy, abnormal sensation or paralysis with no other proven cause, and systemic risk factors of neuropathy (e.g., metabolic disease and collagen disease).

Neuropathological evaluation

The PNs, which were 2 cm in length and within 10 cm from their point of entry into the

diaphragm, were sampled bilaterally during autopsy. The sampled nerves were prepared for orthodox evaluation of the peripheral nerves.[10] Briefly, the nerves were fixed in 2.5% glutaraldehyde in a phosphate buffer overnight and then additionally fixed in 2% osmium tetroxide. The post-fixed samples were embedded in epoxy resin, and 1-μm-thick semi-thin cross sections were stained with toluidine blue. We evaluated the PN with a light microscope (Eclipse Ni, Nikon, Tokyo, Japan), and photographed the whole fascicle using a digital camera (DS-Ri, Nikon, Tokyo, Japan) connected to a microscope with a 20× objective lens. The number of myelinated fibers and the endoneurial area were measured using Image-Pro Plus version 7.0 (Media Cybernetics Inc., Rockville, MD, USA). In addition, the outer diameter of the myelin sheath was defined as the diameter of myelinated fibers, and the maximum diameter perpendicular to the long axis was measured. The fibers were then divided into subgroups of small (< 7 μm) and large (≥ 7 μm) diameter myelinated fibers according to their diameters, based on established sural nerve measurement protocols.[7] The neuropathological analysis of the brain, spinal cord, and sural nerve was also performed as previously reported.[11-18] These pathological findings were confirmed by a consensus of two experienced neuropathologists (YS and SM).

Statistical analysis

The results are expressed as mean ± standard deviation. The Spearman correlation coefficient was used to evaluate correlations. The Wilcoxon rank sum test was used for non-normally distributed continuous variables (when the PNs were collected bilaterally, we used the mean value of both sides as the measured value for the test). All analyses were performed using JMP Pro 15 (SAS Institute Inc., Cary, NC, USA). The statistical significance level was set at $p < 0.05$.

Data availability statement

The present study's datasets and full study protocol are available from the corresponding author on reasonable request.

RESULTS

Baseline characteristics

Autopsies were performed on 36 consecutive cases. We excluded 14 cases whose mediastinal states were unsuitable for sampling and 8 cases whose nerves were difficult to assess due to postmortem autolysis or deformation. Subsequently, we performed a pathological examination that included the PNs and excluded 2 cases with neurodegenerative diseases that may have involved the lower motor neurons, and 4 cases with peripheral neuropathy pathologically confirmed in the sural nerve or PNs determined by experienced neuropathologists (Figure 1). No cases showed demyelinating neuropathy. Eventually, we included 8 cases that were considered to have normal PNs for final analysis. The mean age of the cases at death was 77.0 ± 7.0 years (range: 66–86 years); 62.5% (n = 5) were male and 37.5% (n = 3) were female. Of the 8 cases, 62.5% (n = 5) had risk factors of neuropathy (diabetes, n=3; hypothyroidism, n=1; CREST syndrome, n=1). The mean postmortem interval was 14.3 h (range: 2.1–41.9 h). The clinical characteristics of the 8 cases are summarized in Supplemental Table 1.

PN morphological analysis

Those cases with neuropathological changes in the spinal cord or sural nerves were excluded as mentioned above. Eight cases of both sides of PNs were evaluated, but if one side presented with poor fixation or considerable sampling artifacts, only the other relatively preserved side was selected for the study.

One of the eight cases was suitable for assessment bilaterally, five for the left side only, and two for the right side only. In total, nine PNs from eight cases were examined. Eight of these nine PNs had a single fascicle each; one nerve, the right PN in Case 1, had two adjacent fascicles. The morphological parameters of the PN myelinated fibers are shown in Table 1. The mean and standard deviation of the density of each myelinated fiber of the PN was 6908 ± 1132 fibers/mm^2 for total myelinated fiber, 4095 ± 586 fibers/mm^2 for large diameter myelinated fiber, and 2813 ± 629 fibers/mm^2 for small diameter myelinated fiber. The mean and standard deviation of myelinated fiber density on the left and right side, respectively, was 7163 ± 1332 fibers/mm^2 and 6397 ± 323 fibers/mm^2 for total myelinated fiber, 4236 ± 626 fibers/mm^2 and 3813 ± 468 fibers/mm^2 for large diameter myelinated fiber, and 2927 ± 731 fibers/mm^2 and 2584 ± 358 fibers/mm^2 for small diameter myelinated fiber. The individual values for the density of the myelinated fibers are shown in Figure 2A. In all cases, the histograms of the density of each nerve diameter were bimodal, with peaks of approximately 4 μm and 10 μm. The mean large/small diameter myelinated fiber ratio was 1.49 ± 0.26 (range: 1.15–1.95). Typical microscopic images and histograms of the density of the PN are shown in Figure 2B–2D.

Relationship between myelinated fiber density and clinical information

Myelinated fiber density did not correlate with age in the total myelinated fiber (correlation coefficient [r] = -0.32, p = 0.40), in the large diameter myelinated fiber (r = -0.15, p = 0.70), or in the small diameter myelinated fiber (r = 0.10, p = 0.80).

There were no significant differences in total myelinated fiber density, in large diameter myelinated fiber density, or in small diameter myelinated fiber density (p = 0.79, 0.57, 0.79,

respectively) between cases with and without diabetes. There were also no significant differences in total, large diameter, and small diameter myelinated fiber density (p = 1.00, 1.00, 1.00, respectively) between cases with and without any risk factors of neuropathy.

DISCUSSION

The present study revealed the parameters of myelinated fibers of the PNs in the elderly for the first time. The parameters calculated in this study, especially the mean and standard deviation of the density of myelinated fibers, can serve as reference values for the PNs of the elderly population that do not have overt disorders that affect the peripheral nerve.

The PN myelinated fiber density did not differ significantly with age, and the bimodal distribution characteristics of the fiber diameter were also preserved. Our study showed these characteristics in an elderly population, and a previous study by Bradley et al.[2] which included mainly younger individuals (49.0 ± 19.8 years, range: 11–67 years) also saw a similar trend of a bimodal pattern with a predominance of large diameter fibers, although they did not show myelinated fiber density data. Due to the limitation of the sample size, future studies on different populations are required to confirm that the values of the PN myelinated fiber density obtained from the present study can be applied without considering age.

Mean PN myelinated fiber density appeared to be higher on the left side than on the right side, with a similar trend in the one case with bilateral sampling. Suratos et al. reported that the right side of the PN tended to have a larger cross-sectional area than the left side in 28 normal control cases.[19] A left-right difference would be significant from an anatomical point of view; however, a histological study of 35 formalin-fixed human cadavers reported no significant left-right differences in PN surface area and myelination area.[20] The cross-sectional area in ultrasonography is considered equivalent to the endoneurial area histologically; thus, data regarding left-right PN differences appear to be contradictory. Since

the sample size in our study was small, further studies would be required to clarify this aspect.

Having risk factors of neuropathy did not necessarily affect the density of the PN myelinated fibers. In the elderly, comorbidities, such as diabetes, are not rare. A few cases of phrenic neuropathy with diabetes have been reported;[21-23] however, most of the neuropathy associated with these conditions will show a pattern of polyneuropathies, which affect length dependently and sensory dominantly.[24] Fortunately, the PN does not have the characteristics that make it vulnerable to polyneuropathy. Such anatomical features of the PNs may have less influence on density values, even with risk factors of neuropathy.

As the main limitation, the sample size to be able to evaluate the PN was small. Nevertheless, even a small number of cases were able to reveal the results with little variability, although future studies on different populations with larger numbers of cases are needed to confirm these findings. In addition, some degree of autolysis (swelling of myelinated fibers and loss of the myelin sheath) of the PNs collected during autopsy occurred, and cases with a high degree of autolysis had to be excluded from this study. This may occur because the PN is located in the center of the body, where body temperature remains high despite the immediate cooling of the corpse. This condition may have affected the measurement of the myelinated fibers, but it is unavoidable since the nerve cannot be biopsied. Therefore, it is noted here as a limitation of the pathological search method.

In conclusion, we report the human PN myelinated fiber density, and these findings can be used as a reference value for the PN of elderly individuals. Future studies on larger and different patient populations are needed to confirm these findings.

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FIGURE LEGENDS

Figure 1. Study flow diagram.

Figure 2. PN myelinated fibers of neurologically normal elderly individuals. Individual values for the density of the myelinated fibers (A). The boxplot indicates the median and 25% and 75% quartiles, with whiskers representing the minimum and the maximum value of the data set. Representative microscopic images in low-power field (B), high-power field (C), and the diameter histogram (D). Scale bars: 100 μm (B), 10 μm (C).

Figure 1

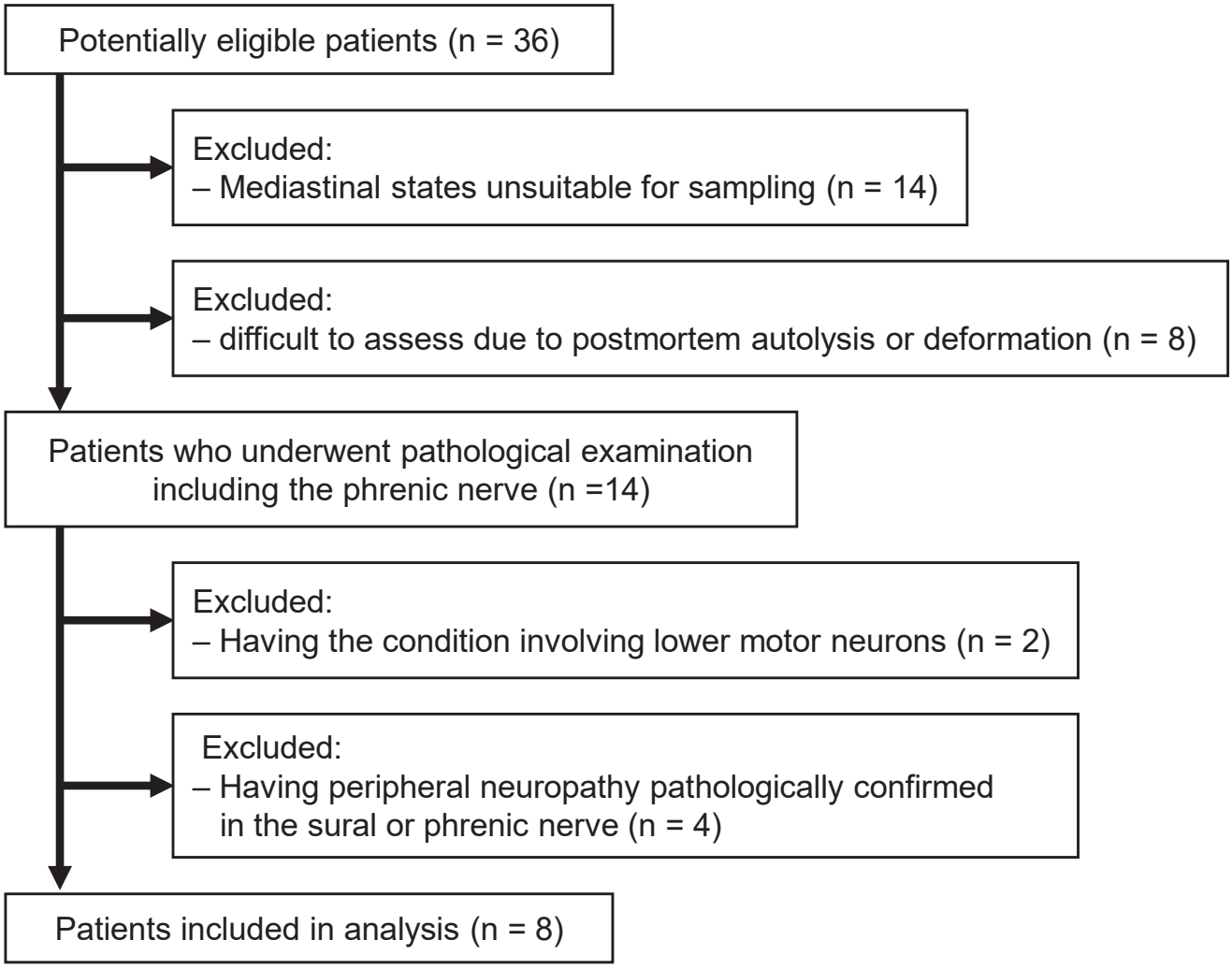
Potentially eligible patients (n = 36)
Excluded:
– Mediastinal states unsuitable for sampling (n = 14)
Excluded:
– difficult to assess due to postmortem autolysis or deformation (n = 8)
Patients who underwent pathological examination including the phrenic nerve (n =14)
Excluded:
– Having the condition involving lower motor neurons (n = 2)
Excluded:
– Having peripheral neuropathy pathologically confirmed in the sural or phrenic nerve (n = 4)
Patients included in analysis (n = 8)

FIGURE 2

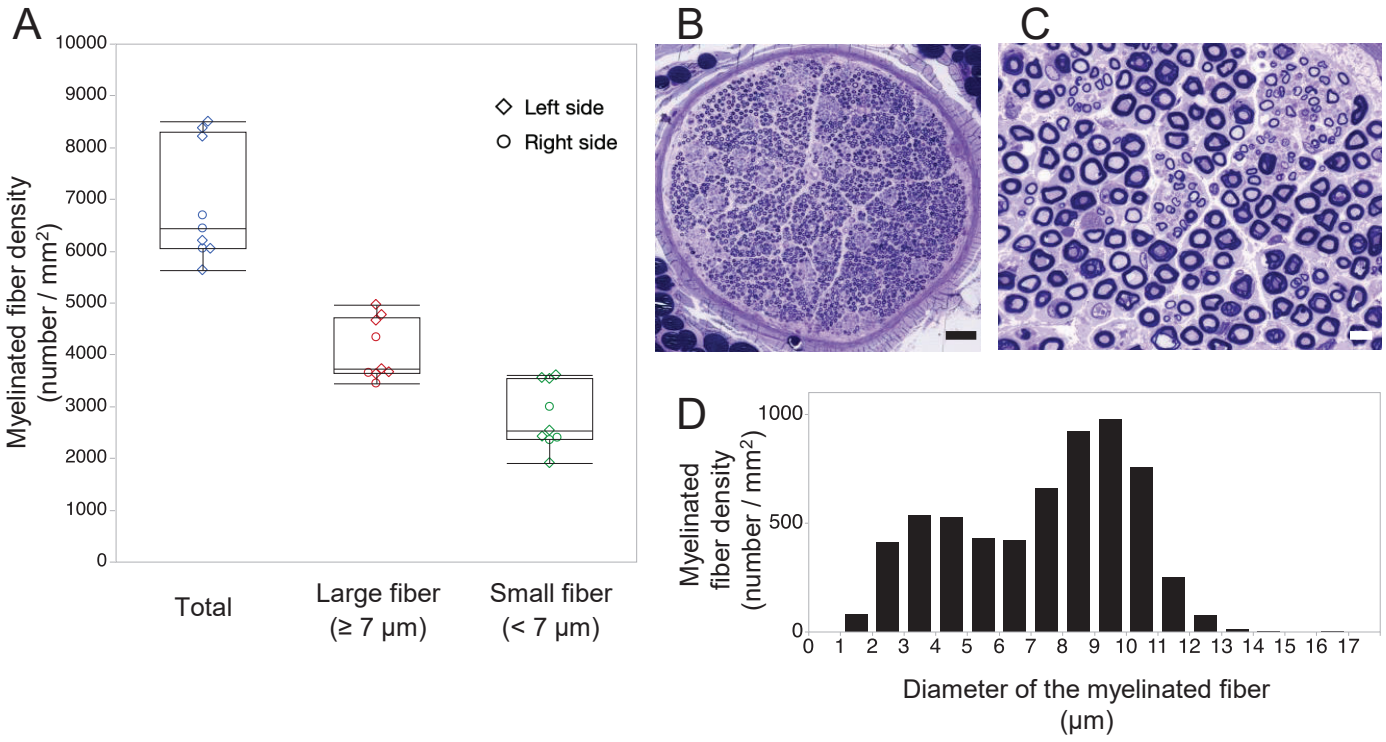
A
B
C
D
Myelinated fiber density (number / mm²)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Left side
Right side
Total
Large fiber (≥ 7 μm)
Small fiber (< 7 μm)
Myelinated fiber density (number / mm²)
1000
500
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Diameter of the myelinated fiber (μm)

Table 1 The morphological parameters of the phrenic nerve myelinated fibers

Case No.	Sex	Age at death (years)	Side	Density of myelinated fiber (/mm2)			Number of myelinated fiber			Large/small fiber ratio
				Total	Large diameter (≥ 7 μm)	Small diameter (< 7 μm)	Total	Large diameter (≥ 7 μm)	Small diameter (< 7 μm)	
1	M	66	L	8502	4966	3536	3857	2253	1604	1.40
			R	6695	4340	2355	3249	2106	1143	1.84
2	M	69	R	6054	3653	2401	3084	1861	1223	1.52
3	M	74	L	6048	3626	2421	3237	1941	1296	1.50
4	M	76	L	8214	4661	3554	3072	1743	1329	1.31
5	M	81	R	6442	3447	2996	2230	1193	1037	1.15
6	F	81	L	6203	3665	2537	2953	1745	1208	1.44
7	F	83	L	8376	4770	3607	3212	1829	1383	1.32
8	F	86	L	5634	3725	1909	2408	1592	816	1.95
Mean ± SD		77.0 ± 7.0		6908 ± 1132	4095 ± 586	2813 ± 629	3034 ± 480	1807 ± 304	1227 ± 222	1.49 ± 0.26

F: female, L: left, M: male, R: right, SD: standard deviation

Supplemental table 1: Baseline characteristics

Case No.	Height (m)	Body weight (kg)	Body mass index (kg/m^2)	Symptom of neuropathy	Systemic risk factor of neuropathy	Neurological disease	Cause of death	Postmortem interval (h)
1	1.6	39	15.2	Absent	Diabetes	Alzheimer's disease	Pneumonia	41.9
2	1.57	38	15.4	Absent	None	Cerebral hemorrhage	Respiratory failure	11.3
3	1.76	43	13.9	Absent	None	Cerebral hemorrhage	Cerebral hemorrhage	2.1
4	1.54	37	15.6	Absent	Hypothyroidism	Cerebral infarction	Pneumonia	3.7
5	1.7	37	12.8	Absent	Diabetes	Alzheimer's disease	Pneumonia	4.6
6	1.54	33	13.9	Absent	Diabetes	Cerebral infarction	Cerebral infarction	36.5
7	1.3	23	13.6	Absent	None	Alzheimer's disease	Pneumonia	5.8
8	1.47	25	11.6	Absent	CREST syndrome	Argyrophilic grain disease	Myocardial infarction	8.5