Original Article

Changes in the Cutaneous Nerve Fiber Staining and Distribution of PGP9.5 in Clinically Uninvolved Skin in Leprosy Patients after Completion of Multidrug Therapy and Assessing PGP9.5 as a Marker of Treatment Response

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Abstract

Background: Subclinical involvement of nerves may sometimes be present much before the overt clinical manifestations become apparent. Protein gene product (PGP) 9.5, a ubiquitin-C-terminal hydrolase, has been widely used as a marker to study the involvement of peripheral nerve fibers in many diseases. **Aim and Objectives:** To evaluate the change in cutaneous nerve fiber staining and distribution from pre-treatment and post completion of multidrug therapy through the expression of PGP9.5 and to assess PGP9.5 as a marker of treatment response. **Materials and Methods:** In this prospective single-center observational study, skin biopsy was taken in patients with leprosy, having areas of nerve function impairment (NFI), based on findings of nerve conduction studies (NCSs), but not having lesions or impaired tactile or thermal impairment clinically. The thin nerve fiber density in the clinically normal skin in areas supplied by nerve showing changes of sensory neuropathy was evaluated to study the density of the fibers. A second biopsy was taken at the end of treatment from a site near the previous site to assess the changes in intra-epidermal nerve fiber staining and distribution. **Results:** Thirty-three patients were recruited in the present study (24 males and 9 females). Pre-treatment, 27 patients had abnormal NCSs, while six patients did not have any evidence of neuropathy on NCSs. Staining for nerve fibers using PGP9.5; in the epidermis was positive in five patients pre-treatment and 11 patients post treatment ($P = 0.181$). Staining in the dermis revealed positivity in 14 pre-treatment, which increased to 18 post treatment ($P = 0.342$). Adnexae showed positivity in five patients pre-treatment and increased to 17 post treatment ($P = 0.005$). **Conclusion:** A reduced PGP9.5 staining in the epidermal, dermal, and adnexal regions was seen in leprosy patients, which improved post treatment. Thus, PGP9.5 may serve as a marker of NFI and treatment response.

Keywords: *Intraepidermal nerve fiber density, leprosy, nerve conduction study, PGP9.5*

Introduction

Peripheral nerve involvement is the hallmark feature of leprosy. Subclinical involvement of nerves may sometimes be present much before the overt clinical manifestations become apparent. Nerves of various calibers may be involved in leprosy, ranging from destruction of the minute unmyelinated twigs in a leprosy patch rendering lesional hypoesthesia to compression, axonal degeneration, and demyelination of larger myelinated peripheral nerve trunks.[1] Nerve conduction studies (NCSs) help in the diagnosis of large nerve involvement, whereas altercations in sympathetic skin response (SSR) usually occur with the involvement of smaller nerve fibers in the epidermal compartment. Protein gene product (PGP) 9.5 is a cytoplasmic protein with a molecular weight of 27 kD and is present in the cells of the nervous system and neuroendocrine systems. It acts as a ubiquitin-C-terminal hydrolase and is involved in the processing of ubiquitinated proteins. It has been widely used as a marker to study the involvement of peripheral nerve fibers in disease processes such as leprosy, diabetes, HIV, and other small-fiber neuropathies.[2-4] In a previous study, PGP9.5 positive fibers were evaluated in lepra reaction and post-reactional state and no significant difference was found.[5] However, no study

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has been conducted to evaluate the pre- and post-treatment PGP9.5 staining of nerve fibers in leprosy. Thus, this study was done to evaluate the change in the cutaneous nerve fiber staining and distribution after the recommended duration of World Health Organization multidrug therapy (WHO-MDT) through the expression of PGP9.5 and assess PGP9.5 as a marker of treatment response in such cases.

Materials and Methods

This was a prospective observational study carried out in the Departments of Dermatology, Venereology, and Leprology; Histopathology; and Neurology at a tertiary care center from July 2015 to December 2018. The study was approved by Institute Ethics Committee (ethical approval number- PGI/IEC/2015/1366). Diagnosis of leprosy was based on the clinical and histopathological features consistent with leprosy. A detailed history, examination (cutaneous and neurological), slit-skin smear, and skin biopsy were done in all the patients pre and post treatment. The tactile sensitivity of the lesions was assessed by Semmes-Weinstein monofilaments. SSR and standardized NCSs were carried out at the beginning and end of the completion of treatment by using a portable electrophysiologic device. SSR represents a potential generated in skin sweat glands. It originates from the activation of the reflex arc with different kinds of stimuli. SSR for the detection of autonomic dysfunction was performed in both the hands and feet, and the parameters assessed were mean latency and mean amplitude; an increase in latency or decreased amplitude was suggestive of impaired SSR. The ulnar, median nerve, common peroneal, posterior tibial nerve, and sural nerves of both sides were studied. Both motor and sensory nerve responses were assessed. Then, we selected the areas that had nerve function impairment (NFI) based on findings of NCSs but that did not have a skin lesion or impaired tactile or thermal impairment clinically. After complete cutaneous and neurological evaluation, a skin biopsy (4-mm punch biopsy) was performed from the area selected above. The thin nerve fiber density in the clinically normal skin in areas supplied by nerve showing changes of sensory neuropathy was evaluated to study the density of the fibers. A second biopsy was taken at the end of treatment (MDT) from a site near the previous site to assess the changes in cutaneous nerve fiber staining and distribution. Lesional skin biopsy was obtained in patients with normal pre-treatment NCS and SSR studies.

Processing of skin biopsy

Immunohistochemistry

The antibodies used for the immunohistochemistry (IHC) included S-100 and anti-PGP9.5 as primary antibodies and biotin-conjugated secondary antibodies (Novacastra Laboratories Ltd., United Kingdom) as secondary antibody. Tissue sections were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin blocks following a standard protocol. Each paraffin block was cut into 5-µm sections and applied to pre-coated slides with poly-L-lysine. Sections were fixed in a hot air oven at 42°C for 30 minutes. The paraffin sections were deparaffinized in xylene using four changes of 15 minutes each. Sections were hydrated gradually through graded alcohols by washing in 100%/90%, 70%, and 50% ethanol for 15 minutes each, followed by washing in de-ionized H_2O for 1 minute with stirring. Deparaffinized sections were dipped in retrieval buffer and exposed to microwave fixation at 750 W for 10 minutes to increase immunogenicity. Then, they were washed with de-ionized water for 1 minute.

The slides were incubated for 45 minutes in 1% hydrogen peroxide in methanol to quench endogenous peroxidase activity and were then washed in phosphate-buffered saline (PBS), pH 7.2 thrice for 5 minutes each. The slides were incubated with primary antibody for 1 hour at room temperature or overnight at 4°C. The optimal antibody concentration was determined by titration as per the manufacturer's instructions. The slides were then washed with three changes of PBS for 5 minutes each. Incubation for 30 minutes was done with a biotin-conjugated secondary antibody (Novacastra Laboratories Ltd, United Kingdom) as per the manufacturer's instructions. Washing was done with three changes of PBS for 5 minutes each, followed by incubation in 3,3′-Diaminobenzidine, buffer till the desired stain intensity developed (1–2 minutes). Individual slides were then monitored to determine the proper development time. Then, sections were dipped in de-ionized H_2O for 15 seconds. Counter-stain with hematoxylin for 60 seconds, followed by immediate washing with tap water. Sections were air-dried; then, 1–2 drops of permanent mounting medium (di-n-butyl phthalate in xylene) were added, and the sections were covered with a glass coverslip and observed under the light microscope.

Morphometry

Two measurements were done: (1) the intensity of the IHC of an intra-epidermal nerve fiber (IENF), and (2) dermal and adnexal nerve fiber staining (brown color) and distribution pattern. The images were assessed and analyzed. Only fibers above the basal cell layer were assessed to minimize the erroneous counting of unwanted structures.

Results

Patient population

Thirty-three patients were recruited in the present study (24 males and 9 females). The diagnoses were tuberculoid leprosy (TT, n = 1), borderline tuberculoid (BT, n = 10), borderline-borderline (BB, n = 4), borderline lepromatous (BL, n = 3), lepromatous leprosy (LL, n = 12), and pure neuritic leprosy (PNL, n = 3). The mean ± SD age was 40.4 ± 14.4 years (range: 16–66 years). The mean duration of illness was 5.2 ± 3 years (range: 3–18 years).

Fifteen patients had zero bacillary index (BI), while in 18 patients who were smear positive, the mean BI was 3.61 ± 0.85.

Clinically appreciable nerve thickening was noticed in 27 patients, while six patients (1 in the TT spectrum and 5 in the BT spectrum) had lesional hypoesthesia/anesthesia in the absence of nerve thickening. Seventeen patients had a grade-1 deformity (sensory loss only), four had a grade-2 deformity (motor weakness and/or visible manifestations of sensory loss), and 12 had no deformity. Two patients received multidrug therapy-paucibacillary regimen (MDT-PBR), whereas the rest of the 31 received the multibacillary regimen (MDT-MBR). In addition, eight patients received prednisolone. The type of leprosy, presence, type of reaction, mean bacillary index (BI), and clinical features of patients are summarized in Table 1.

Neurological symptoms and NCS findings

The neurological symptoms experienced by the patients are listed in Table 2. Glove and stocking hypoesthesia/anesthesia was the most common type observed in the present study. The findings of NCSs about the type of neurological symptoms are also summarized in Table 2. Importantly, of the seven patients with no neural complaints, four patients had multiple nerve involvement (1 BT with type-1 reaction, 1 BL, and 2 LL), two had a single nerve involvement (1 BT with type-1 reaction and 1 LL), and one had no nerve involvement on NCSs (BT without reaction). Similarly, four patients had grade-2 deformity clinically; though NCSs demonstrated the involvement of motor components in 18 patients.

Pre- and post-treatment NCS, SSR, and staining for PGP9.5 in skin

Twenty-one patients (21/33, 63.3%) reported some degree of clinical improvement after completion of treatment, whereas 11/33 (33.3%) patients had no clinical improvement. One patient who had no NFI did not develop any new NFI during or after the completion of treatment. Prior to treatment, 27 patients had abnormal NCSs, while six patients did not have any evidence of neuropathy on NCSs. Post treatment, 25 patients had abnormal NCSs, while eight did not have any evidence of neuropathy on NCSs (P = 0.479, Mc Nemar test). Of all, 24 patients had non-reactive SSR pre treatment, while 18 had non-reactive SSR post treatment (P = 0.148, Mc Nemar test, Table 2).

Staining for nerve fibers was tested using PGP9.5; in the epidermis, it was positive in five patients pre treatment and in 11 patients post treatment (P = 0.181). Staining in the dermis [Figure 1] revealed positivity in 14 pre treatment, which increased to 18 post treatment (P = 0.342). Adnexae showed positivity in five patients pre treatment and increased to 17 post treatment (2-tailed P = 0.005 [1-tailed P = 0.002, OR = 0.142, Mc Nemar Test]). Detailed attributes of PGP9.5 staining and their distribution in different leprosy subtypes pre and post treatment are mentioned in Table 3.

On comparing IENF staining intensity with NCSs, epidermal staining was absent in 28/33 (84.8%) patients pre treatment, while abnormal NCS results were observed in 27/33 (81.8%) patients (P = 0.74). Similarly, on comparing absent epidermal staining with abnormal NCS results post treatment, no significant difference was observed (22/33 vs. 25/33; P = 0.41).

Discussion

Leprosy is an infectious disease affecting the skin and peripheral nerves, resulting in increased morbidity and physical deformities. "Early detection improves prognosis" is a general axiom in medicine, and in leprosy, delay in detection is strongly associated with an increased risk of neural impairment at diagnosis.[6,7] In addition, NFI already present at diagnosis is a strong predictor of the risk of

Table 1: Baseline clinicodemographic data of the patients

n=33					
Parameter	**Mean**	**SD**	**Range**	**Median**	**IQR**
Age (years)	40.42	14.38	16–66	38	21
Total duration of illness (years)	5.17	3.03	3–18	4	2
Bacillary index (BI)	1.97	1.93	0–5	2	4
TT	**BT**	**BB**	**BL**	**LL**	**PNL**
n=1	*n*=10 (5 with type-1 lepra reaction)	*n*=4 (1 with type-1 lepra reaction)	*n*=3	*n*=12	*n*=3 (10 with type-2 lepra reaction)
Mean±SD BI (median, IQR)					
0	0	2.75±2.21 (3, 2.75)	3±1 (3, 1)	3.75±0.62 (4, 1)	0
Nerve thickening (*n*=27)					
n=0	*n*=5 (all in BT with type-1 reaction)	*n*=4	*n*=3	*n*=12	*n*=3
Deformities (*n*=21)					
n=0	Grade 1, *n*=2	Grade 1, *n*=4	Grade 1, *n*=1	Grade 1, *n*=9	Grade 1, *n*=1
	Grade 2, *n*=1 (all in BT with type-1 reaction)		Grade 2, *n*=1		Grade 2, *n*=2

Table 2: Summary of neurological symptoms and nerve conduction findings in the study patients

Pre- and post-treatment patterns of NCS							
Pre-treatment patterns of NCS (abnormal NCS recorded in *n*=27)							
Non-recordable	**Axonal sensorimotor neuropathy**	**Axonal sensory neuropathy**	**Axonal motor neuropathy**	**Demyelinating sensorimotor neuropathy**	**Demyelinating sensory neuropathy**	**Demyelinating motor neuropathy**	**Small fiber neuropathy**
n=6 (5 LL, 1 BT with type-1 reaction and neuritis)	*n*=14 (3 BT in type-1 reaction [2 with neuritis and 1 without neural symptoms], 2 BB, 1 BL, 5 LL, 3 PNL)	*n*=3 (1 BB, 1 LL, 1 BT in type-1 reaction)	*n*=1 (1 BL)	-	-	-	*n*=0 (small fiber neuropathy alone) *n*=3 (combined axonal sensorimotor and small fiber neuropathy, these are not mentioned under the column of axonal sensorimotor neuropathy; 1 BB, 1 BL, 1 LL)
Post-treatment pattern of NCS (abnormal NCS recorded in *n*=25)							
Non-recordable	**Axonal sensorimotor neuropathy**	**Axonal sensory neuropathy**	**Axonal motor neuropathy**	**Demyelinating sensorimotor neuropathy**	**Demyelinating sensory neuropathy**	**Demyelinating motor neuropathy**	**Small fiber neuropathy**
-	*n*=3 (1 BL, 2 BT with type-1 reaction and neuritis)	*n*=4 (1 BB, 1 LL, 2 BT with type-1 reaction without neuritis)	-	*n*=1 (1 PNL)	-	-	*n*=16 (small fiber neuropathy alone; 9 LL, 2 BL, 2 BB, 2 PNL, 1 BT with type-1 reaction and neuritis) *n*=1 (combined axonal sensorimotor and small fiber neuropathy; 1 BB)

	Patients with abnormal readings pre-treatment, *n*	**Patients with abnormal readings post-treatment, *n***	***P* (Mc Nemar difference test)**
NCS	27	25	0.479 (2-tailed), 0.239 (1-tailed)
SSR	24	18	0.148 (2-tailed), 0.074 (1-tailed)

LL: Lepromatous leprosy, BL: Borderline lepromatous leprosy, BB: Mid-borderline leprosy, BT: Borderline tuberculoid leprosy, TT: Tuberculoid leprosy, PNL: Pure neuritic leprosy, NCS: Nerve conduction studies, SSR: Sympathetic skin response

further immunological reactions or episodes of sensory or motor neuropathy.[8,9]

NCSs are a non-invasive tool to assess peripheral nerve involvement in leprosy. The advantage of NCSs is the quantitative observations. However, the demerits of this are the costly setup and expertise required in carrying out the investigation.

In this study, NCS was abnormal in 81.8% of patients at baseline (pre treatment). Superficial peroneal nerve was the most common to be involved in 57.5% of patients, followed closely by the sural nerve in 54.5% of patients. This is contrary to the study by Vashisht *et al.*, where the posterior tibial nerve was most commonly involved.[10] Median and ulnar nerves were involved in 27.2% and 24.2% of patients, respectively, in our study. However, no statistically significant difference was noted in NCS parameters pre and post treatment.

Skin biopsy samples can demonstrate the selective degeneration of somatic unmyelinated fibers, that convey pain and thermal sensations. These fibers cannot be observed in routine neurophysiological tests. Investigators in cross-sectional studies have concluded that NCSs are very useful and would potentially detect pre-clinical neuropathy. Skin biopsy can also provide diagnostic information when

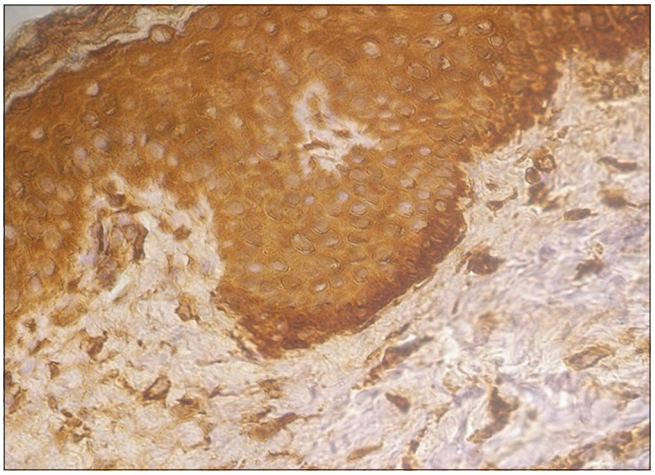

Figure 1: Image showing immunohistochemistry staining with PGP9.5 having dermal staining in a skin biopsy of a leprosy patient

Table 3: Pre- and post-treatment positivity of PGP9.5 is detailed among different leprosy subgroups in different skin sites (dermis, adnexal, and epidermis)

Type of leprosy	Site in skin biopsy specimen	Number of patients with positive staining pre-treatment	Number of patients with positive staining post-treatment	2-tailed *P* of Mc Nemar test (1-tailed *P*, odds ratio)
LL, *n*=12	Dermis	7	10	0.248 (0.124, 0)
	Adnexae	2	9	0.023 (0.011, 0)
	Epidermis	1	7	0.077 (0.038, 0.14)
BL, *n*=3	Dermis	1	0	1 (0.5, 0)
	Adnexae	0	2	0.479 (0.239, 0)
	Epidermis	0	1	1 (0.5, 0)
BB, *n*=4	Dermis	2	3	1 (0.5, 0)
	Adnexae	0	1	1 (0.5, 0)
	Epidermis	0	0	-
BT, *n*=10	Dermis	4	4	0.617 (0.308, 1)
	Adnexae	3	2	1 (0.5, 2)
	Epidermis	4	1	0.248 (0.124)
Without type-1 reaction, *n*=5	Dermis	1	1	-
	Adnexae	1	2	1 (0.5, 0)
	Epidermis	1	1	-
With type-1 reaction, *n*=5	Dermis	3	3	0.617 (0.308, 1)
	Adnexae	2	0	0.479 (0.239, -)
	Epidermis	3	0	0.248 (0.124)
TT, *n*=1	Dermis	0	1	-
	Adnexae	0	1	-
	Epidermis	0	1	-
PNL, *n*=3	Dermis	0	0	-
	Adnexae	0	2	0.479 (0.239, 0)
	Epidermis	0	1	1 (0.5, 0)
Combined for all types of leprosy	Dermis	14	18	0.342 (0.171, 0.428)
	Adnexae	5	17	0.005 (0.002, 0.142)
	Epidermis	5	11	0.181 (0.09, 0.4)

LL: Lepromatous leprosy, BL: Borderline lepromatous leprosy, BB: Mid-borderline leprosy, BT: Borderline tuberculoid leprosy, TT: Tuberculoid leprosy, PNL: Pure neuritic leprosy

there is little or no clinical evidence of neuropathy. The minimal invasiveness of skin biopsy makes it a useful tool not only in clinical practice but also for monitoring the progression of neuropathy in trials of neuroprotective treatments. The range of applications of skin biopsy has recently been expanded to include autonomic neuropathies and immune-mediated and inherited demyelinating neuropathies. In addition, the correlation of skin biopsy findings with the overall clinical picture and with the results of neurophysiological examinations has provided important insights into the pathogenesis and features of neuropathic pain in peripheral neuropathies.[11]

Another advantage of the skin biopsy for IENF density is that its location can be chosen based on the patient's signs and symptoms, and it can be conducted in regions where nerve conduction tests cannot be performed, such as the trunk and fingers. IENFs can spontaneously regenerate, in parallel with sensory recovery, after nerve injury; for example, in diabetic truncal neuropathy.[12] These findings provided insights into the pathogenesis of these common neuropathies and suggested the need for early neuroprotective interventions. In patients with impaired glucose tolerance, drastic lifestyle changes resulted in partial recovery of IENF density and sural SNAP amplitude and reduction in pain.[11] Similarly, skin nerves were shown to regenerate in steroid-responsive neuropathy.[13]

We observed significantly increased staining for nerve fibers by PGP9.5 post treatment in the adnexal tissue in patients of the lepromatous leprosy spectrum. However, no statistically significant difference was found in the number and intensity of pre and post-treatment staining of PGP9.5 in the epidermis and dermis of lepromatous leprosy patients. These findings were similar to the previous study, where the patients with non-lepromatous spectrum also did not have any significant difference in PGP and NGFr immunostained fibers before or after MDT.[14] The pretreatment epidermal staining of PGP9.5 correlated well with the NCS findings. Thus, IENF density can be used as an auxiliary tool to NCS for the identification of NFI. Positive epidermal staining of PGP9.5 was observed in significantly more patients post treatment, while there was no difference in NCS findings pre and post treatment. As SSR improvement was seen in a significant proportion of patients post treatment, increased staining post treatment

may suggest nerve regeneration. Previous studies suggested that nerve fibers regenerate slowly in leprosy, and nerve regeneration has been observed in the nerve trunks of the lower limbs of leprosy patients.[15] However, the process is inefficient because of endoneurial fibrosis, and this regenerative process appears to be functionally ineffective as the sensory impairment persists after treatment.[15]

Limitations

The small sample size is the major limitation of our study with a short duration of follow-up. In addition, the measurement of IENF density was not done. Another limitation of our study is that the lesional skin biopsy for PGP9.5 staining was done in PB leprosy patients only and not in MB leprosy patients; this could be assessed in future studies to evaluate the role of PGP9.5 as a marker of treatment response.

Conclusion

Our findings showed a reduced PGP9.5 staining in the epidermal, dermal, and adnexal regions in leprosy patients, which improved post treatment. Thus, PGP9.5 can be used in the follow-up of leprosy patients to assess nerve regeneration.

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Conflicts of interest

There are no conflicts of interest.

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