

Alteration of phospholipids in the blood of patients with Duchenne muscular dystrophy (DMD): in vitro, high resolution ^{31}P NMR-based study

Niraj Kumar Srivastava^{1,2} · Somnath Mukherjee² · Neeraj Sinha³

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Abstract In vitro, high-resolution ^{31}P NMR (Nuclear Magnetic Resonance) spectroscopy-based analysis of phospholipids in serum is well recognized in leukemia, lymphoma, non-hematological cancers and renal cell carcinoma. In context of these studies, phospholipids were analyzed in blood of thirty-two ($n = 32$) patients with Duchenne muscular dystrophy (DMD) (Age, Mean \pm SD; 8.0 ± 1.6 years) and sixteen ($n = 16$) healthy subjects (Age, Mean \pm SD; 8.6 ± 2.3 years). Quantity of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and lyso-phosphatidylcholine (Lys-PC) was significantly higher ($p < 0.05$) in DMD patients as compared to healthy subjects. There were no significant differences ($p > 0.05$) observed for the quantity of phospholipids in blood of gene deletion positive cases of DMD as compared to negative gene deletion cases of DMD. Quantity of phospholipids in negative gene deletion cases of DMD patients as well as DMD cases with positive gene deletion was significantly higher ($p < 0.05$) as compared to normal individuals. The present study distinguishes the patients with DMD from the healthy subjects on the basis of the quantity of

phospholipids in blood. These observations may be useful in future for the development of new diagnostic method of DMD.

Keywords Phospholipids · ^{31}P NMR spectroscopy · Muscular dystrophy · DMD · Blood · Phosphatidylcholine · Phosphatidylethanolamine · Phosphatidylinositol · Phosphatidylserine

Abbreviations

DMD	Duchenne muscular dystrophy
EMG	Electromyography
CK	Creatine kinase
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine
Lys-PC	Lyso-phosphatidylcholine
EDTA	Ethylene Diamine Tetra Acetate
PCR	Polymerase chain reaction
Pm	Primer
MDP	Methylene di phosphonic acid
MLPA	Multiplex ligation-dependent probe amplification

✉ Niraj Kumar Srivastava
nirajsuprabhat@gmail.com

¹ Department of Neurology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Raebareli Road, Lucknow 226014, India

² Present Address: School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India

³ Center of Biomedical Research, Sanjay Gandhi Postgraduate Institute of Medical Sciences Campus, Raebareli Road, Lucknow 226014, India

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked disorder, it is the most common muscular dystrophy in children, present in early childhood and characterized by proximal muscle weakness and calf hypertrophy in affected boys. It occurs 1 in 3500 live male births. This disease is

caused by the mutation in dystrophin gene located at Xp21.2 that codes for dystrophin protein, an important structural component of muscle cell [1].

Diagnosis of DMD is carried out on the basis of clinical symptoms (muscle wasting and weakness, difficulty in walking and standing, and frequent fall) and, estimation of CK (Creatine kinase) activity and gene mutation analysis. Most of the children suffering from DMD develop proximal muscle weakness by 5 years of age and are unable to walk by the time they reach 8–12 years of age [1, 2]. Estimation of CK levels in serum is a simple and inexpensive method to detect muscular dystrophies, but this test is not specific since, levels of CK are increased in all types of myopathies [1]. Activity of serum CK in DMD patients can be 50–100 times higher as compared to normal levels even at birth, even before the disease becomes clinically evident [1]. Gene mutation analysis of these patients can be performed using peripheral blood and the diagnosis can be established in most of the cases [3, 4]. Around 30 % DMD cases are reported negative when diagnosed using gene mutation analysis due to point mutation (single base substitution in the gene) in the dystrophin gene [4]. Multiplex PCR is a commonly used method for DMD diagnosis in India, which targets about 18–32 exons of the dystrophin gene to identify complete exons deletions. Multiplex PCR is mostly qualitative or semi-quantitative and serves for the exons in the hot spot region [5]. MLPA (multiplex ligation-dependent probe amplification) has facilitated more reliable and quicker quantitative deletion of the complete dystrophin gene containing 79 exons to study the deletions and duplications. Multiplex PCR method permits the detection of approximately 98 % of deletions, which accounts for 65 % of all mutations [5, 6]. Following multiplex PCR by MLPA increases the percentage of patients with a precise diagnosis to 75 %. MLPA-based diagnostic method is used to diagnose DMD in USA, China and several European countries [6, 7]. Diagnosis using immunohistochemistry is a crucial diagnostic method for cases which are negative gene mutation cases of DMD and exclusion of the congenital muscular dystrophy [8], McLeod syndrome [9, 10], miscellaneous myopathic and neurogenic disorders, such as the autosomal recessive form of limb girdle muscular dystrophy [11], primary alpha- and gamma-sarcoglycanopathies [12, 13], spinal muscular atrophy [14] and in Charcot-Marie-Tooth disease type 1A [15].

Nuclear Magnetic Resonance (NMR) spectroscopy is a versatile technique that can be used in a broad series of discipline. The best known medical applications of NMR are in vivo magnetic resonance imaging and spectroscopy, but in vitro NMR spectroscopy of body fluids such as blood plasma or serum has also been used to diagnose inborn errors of metabolism [16–20]. Various authors have studied

different individual blood components to find out any abnormalities that could be specific to DMD cases. Analysis of the biochemical, morphological and biophysical characteristics of red blood cell (RBC) membrane demonstrated an alteration in both myotonic dystrophy and DMD [21]. Higher levels of free fatty acids and ketone bodies were detected in serum of DMD patients [22]. Studies of lipid profile in dystrophic chicken and mouse model showed that high levels of phospholipids are present in the serum [23–25]. In vitro, ^{31}P NMR spectroscopy-based assessment of phospholipid metabolism is gaining importance among investigators of tumor pathophysiology and systemic cancer effects [26–31]. ^{31}P NMR spectroscopy-based analysis of serum phospholipids in leukemia, lymphoma, renal cell carcinoma and other non-hematological cancers are of immense clinical importance [26–28]. These studies were the basis for NMR spectroscopy-based analysis of the phospholipids in the whole blood of DMD patients.

The present study was performed to evaluate quantitative changes in phospholipids (PL), i.e. phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and lyso-phosphatidylcholine (LyPC) in whole blood of DMD patients as compared to normal individuals.

Materials and methods

Blood samples ($n = 32$) of suspected DMD cases (Age, Mean \pm SD; 8.0 ± 1.6 year; sex, male) were collected from outpatient clinics and inpatients wards at department of neurology Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India. Approval from Institute's ethical committee was obtained before beginning of this study. Blood samples ($n = 16$) of healthy male volunteers (Age, Mean \pm SD; 8.6 ± 2.3 year) were used as control. These samples were collected in EDTA (Ethylene diamine tetra acetate) coated vials and stored at -80°C . The collected blood sample from each individual was two milliliters. Experimental analysis of blood samples was performed within a week from the time of collection.

Chemicals

All reagents were procured from the Sigma-Aldrich, USA.

Methods

Clinical examination

Diagnosis of suspected DMD patients was done at Neurology OPD (out-patients department), Sanjay Gandhi

Postgraduate Institute of Medical Sciences, Lucknow. Patients were examined on the basis of clinical symptoms, signs and family history. Patients complained about their muscular wasting and weakness, difficulty in running or getting up from the ground and frequent falls or toe-walking. They also told that the beginning of symptoms took place in between the age of 3–5 years. Patients were examined for the well-known Gower's sign (weakness of knee and hip extensors results in the classical Gower's sign when the child attempts to rise from the floor) and valley sign along with calf muscle hypertrophy [1, 32]. Valley sign was helpful to diagnose chair bound patients as well as those patients, who did not have prominent calves [33] (Fig. 1a, b).

Measurement of CK (creatine kinase) activity

The measurement of plasma CK activity is important for the diagnosis of muscle diseases such as muscular dystrophy, polymyositis, and muscle trauma [34, 36]. In present study, plasma CK activity was measured by the method of Rosalki et al. [36].

Gene mutation analysis

DNA was extracted by salting out method from 1.0 ml blood using standard protocols [3, 4, 37]. The concentration of DNA samples was determined and integrity of DNA in the samples was confirmed by 1.0 % (w/v) agarose gel electrophoresis. Gene mutation (deletion) analysis was

performed by multiplex PCR using 21 pairs of primers (Pm 3, 4, 6, 8, 12, 17, 19, 34, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 55 and 60) of the dystrophin gene [8, 9]. The 10 μ l aliquot of amplified products was loaded on 2 % agarose gel and electrophoresis run was performed. After complete separation of bands in gel electrophoresis, the gel was stained and bands were visualized under UV transilluminator.

Immunohistochemical analysis

Muscle biopsy of suspected DMD patients, who showed the negative result for gene mutation analysis, was performed for further confirmation of the diagnosis. Immunohistochemical staining of the dystrophin protein was carried out on the muscle specimen using standard protocol [38].

^{31}P NMR spectroscopy-based analysis of blood

Processing of blood samples Frozen blood samples were processed before performing the experiments of ^{31}P NMR spectroscopy. The required reagents for processing were Lysis buffer (Sucrose, Triton X-100, MgCl_2 and Tris), proteinase K buffer (NaCl and EDTA) and SDS (Sodium dodecyl sulfate) (10 %; pH= 7.2). All these reagents and frozen blood were mixed together for 2–3 min with the help of cyclomixer and then the whole mixture was incubated at 37.5 °C for 30 min. Processed samples were lyophilized. Lyophilized sample were re-dissolved in 0.5 ml

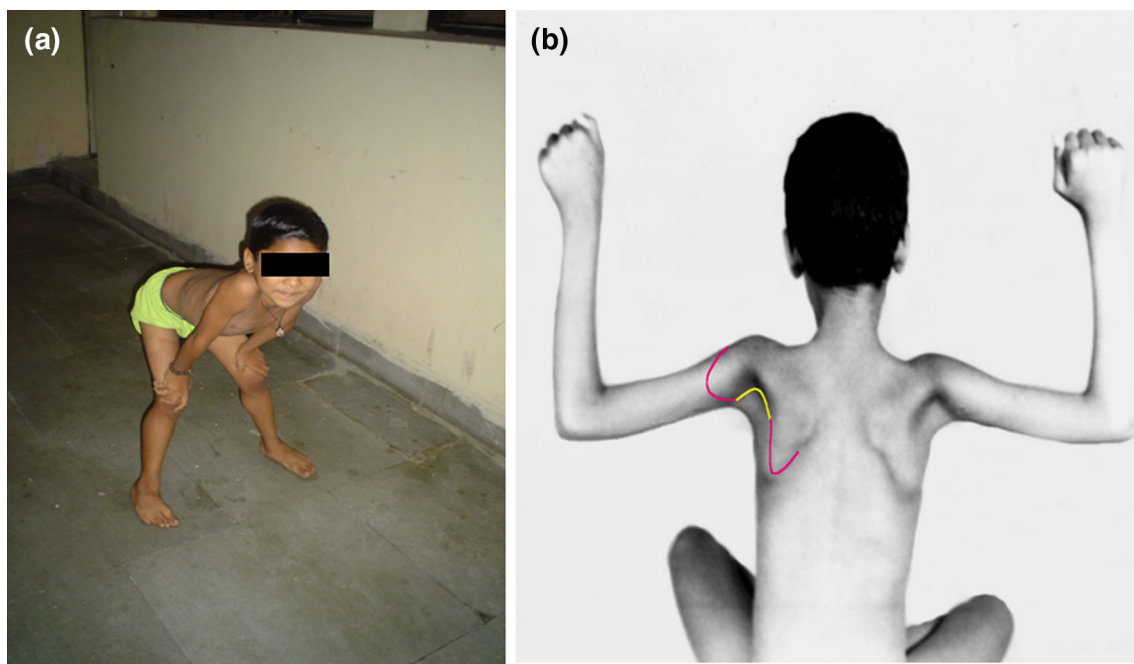


Fig. 1 Clinical signs in DMD patients **a** Gower's maneuver or sign, **b** valley sign—a valley (yellow) between the two mounts (red)

deuterated water and transferred to 5 mm NMR tube. ^{31}P NMR spectroscopy experiments were performed on Bruker Avance 400 MHz spectrometer (Bruker Biospin, Zurich, Switzerland) at 25 °C temperature.

Quantification of phospholipids Quantification of phospholipids is based on the fact that the NMR signal intensity is proportional to the total amount of the chemical constituents present in the sample. NMR peak intensity can be calculated relative to the intensity of reference peak with the known concentration. Errors associated with the quantification are minimized by performing the experiment for all the samples at uniform experimental conditions [39, 40].

NMR spectroscopy-based precise and accurate quantification also depends upon two experimental factors: (1) “quantitative experimental conditions”, including selection of appropriate parameter such as relaxation delay and pulse sequence and (2) selection of suitable post acquisition processing parameters for the optimization of spectral integration [41].

^{31}P NMR spectroscopy parameters

One-dimensional ^{31}P NMR experiment was performed on processed blood using single pulse sequence. The parameters used were:—spectral width: 10,000 Hz; time domain size: 16 K; number of scans: 1000; relaxation delay: 10 s; line broadening: 10.00 Hz. These parameters were optimized and used to achieve the standard conditions for accurate quantification.

T1 (spin–lattice relaxation) measurement experiment for the relaxation delay of different phospholipids

Phosphorus T1 measurement experiment were performed on the whole processed blood (in deuterated water) with MDP (methylene di phosphonic acid) capillary with standard inversion recovery pulse sequence. The T1 value for PC, PE, PI, PS and LysPC were 1.32, 4.47, 5.4, 5.8 and 1.88 s, respectively. Relaxation time was measured by “Inversion Recovery Method” [42]. Quantification of the phospholipids can be measured by taking into the effect of incomplete relaxation during re-cycle delay. When the relaxation time is known, this can be used to predict the optimum pulse sequence cycle time for accurate quantification.

The amount of a phospholipid is calculated from the ratio of its peak area to that of the internal standard by the equation:

$$\frac{M_t^P}{M_t^S} = \frac{M_\infty^P [1 - \exp(-\tau/T_1^P)]}{M_\infty^S [1 - \exp(-\tau/T_1^S)]}$$

where superscripts P and S refer to the phospholipid and internal standard, respectively. The ratio of fully relaxed magnetization M_∞^P/M_∞^S represents the true relative molar quantities of phosphorous in the lipid and the standard, and t is the repetition time [43].

Assignment of the phospholipids

Assignment of the phospholipids (PC, PE, PI, PS and LysPC) was confirmed by one-dimensional ^{31}P NMR spectroscopy, using single pulse sequence, was performed in non-polar solvents (chloroform alone and with combination of chloroform and methanol) as well as on standard phospholipids in aqueous media [D2O with combination of Lysis buffer, proteinase K buffer and SDS (pH = 7.2)]. Assignments were also confirmed by literature [43, 44].

Quantitative evaluation of the phospholipids in blood samples (in duplicate)

Twelve blood samples of healthy individuals of different age groups were divided into two equal parts (0.5 ml by volume) and processed through above described method. One-dimensional ^{31}P NMR spectroscopy-based experiment was performed on both parts of the processed blood samples and quantity of phospholipids (PC, PE, PI, PS and LysPC) was calculated. Both parts of the samples were close together on the basis of the quantity of PC, PE, PI, PS and LysPC. Uniform conditions were applied in sample processing and performing the NMR experiment to eliminate the possible errors.

Statistical analysis

Quantitative data of PC, PE, PI, PS and LysPC was not normally distributed. In this regard, median of PC, PE, PI, PS and LysPC in DMD and healthy subjects was compared by Mann–Whitney test for independent groups. The p value less than 0.05 was considered significant. The data management and analysis were carried out using statistical software SPSS version 15.0.

Results

Clinical examination

All suspected cases of DMD ($n = 32$) were clinically considered in the frame of DMD on the basis of clinical examinations, including symptoms, signs and family

history. After preliminary examinations, confirmatory tests of DMD were done.

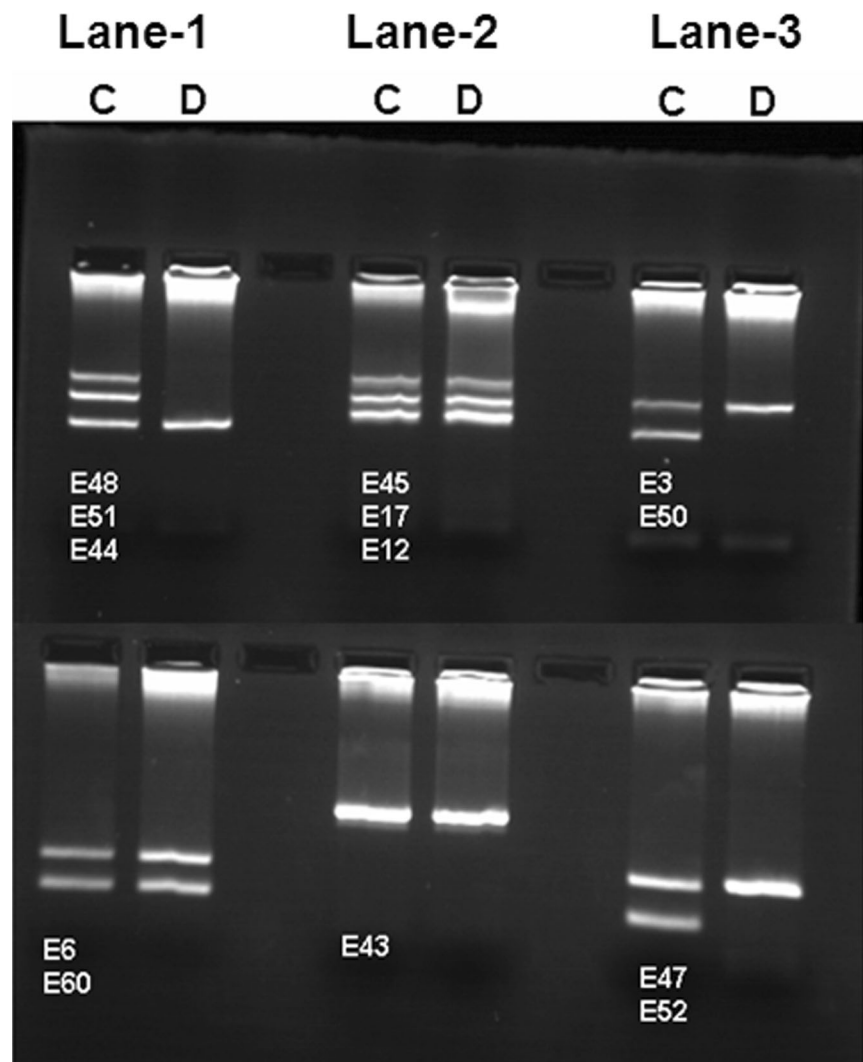
Measurement of CK (creatine kinase) activity

Activity of CK was measured for sixteen healthy subjects with the median value of 123.5 IU/L and situated in the range of 20–200 IU/L. Median value of CK activity was found 8529 IU/L with the range of 1183–29000 IU/L for clinically recognized thirty-two DMD patients.

Gene mutation analysis

Gene mutation analysis showed positive gene deletion in twenty-two cases of clinically recognized DMD patients. Deletion in exons 45–52 was observed in eighteen cases and four cases demonstrated only single deletion in exons 50 and 52. Negative results were observed in ten cases of clinically recognized DMD patients (Fig. 2).

Fig. 2 Electrophoretic pattern in *Lane-1*, showed the deletion in exons 48 and 51, *Lane-2* showed no deletion in exons and *Lane-3* showed the deletion in exons 3 and 52 (*C* control, *D* disease)



Immunohistochemical analysis

Immunohistochemical analysis of clinically recognized ten cases of DMD patients (showed negative result for gene mutation analysis) was performed on muscle biopsy specimens for dystrophin protein. Dystrophin protein was absent in the muscle of DMD patients (Fig. 3).

³¹P NMR spectroscopy-based analysis of blood

One-dimensional ³¹P NMR spectra of processed blood of DMD patients and normal subjects were obtained (Fig. 4). Quantitative analysis of these spectra showed that the phospholipids [PC, PE, PI, PS and LysPC] in the blood of DMD patients were significantly higher ($p < 0.05$) as compared to healthy/normal subjects (Table 1). There were no significant differences observed for the phospholipids in the blood of negative gene deletion cases of DMD patients as compared to DMD cases with positive gene deletion

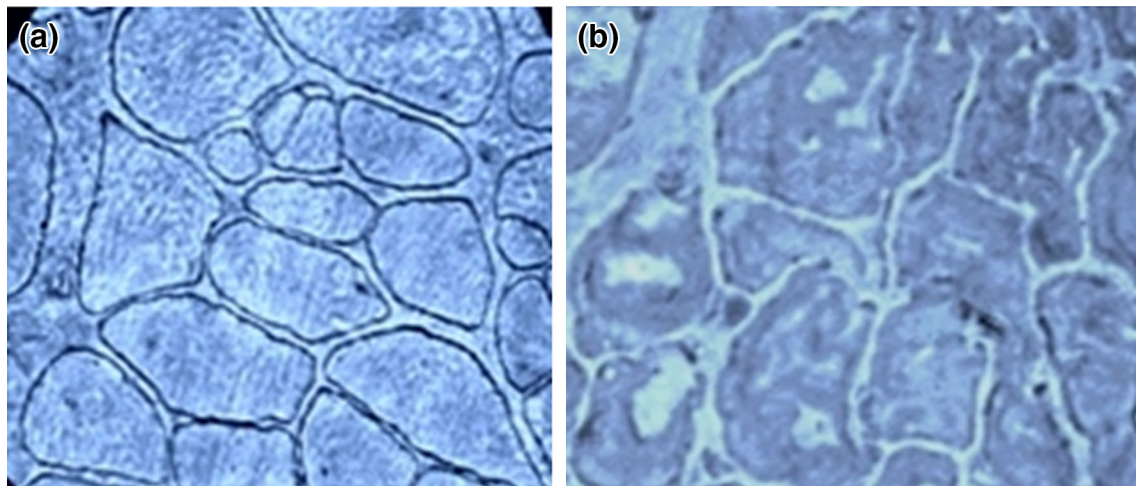
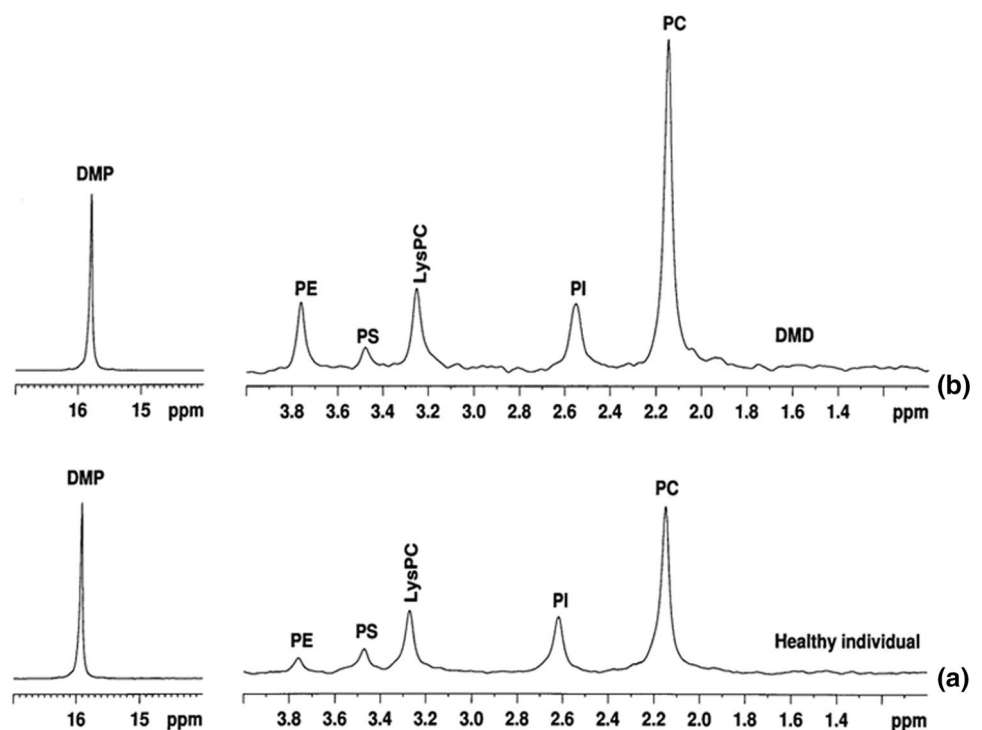


Fig. 3 Dystrophin staining showed **a** presence of dystrophin in normal muscle, **b** completely absent of dystrophin in DMD muscle

Fig. 4 ^{31}P NMR spectra of the whole blood of DMD patients and healthy subjects showed the phospholipid components (*PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PS* phosphatidylserine, *Lys-PC* Lyso-phosphatidylcholine)



($p > 0.05$) (Table 2). Quantity of phospholipids in negative gene deletion cases of DMD patients as well as DMD cases with positive gene deletion was significantly higher ($p < 0.05$) as compared to normal individuals (Table 3).

Discussion

Preliminary diagnosis of DMD patients were based on symptoms, family history and clinical signs (Gower's sign and valley sign). Creatine Kinase estimation, gene

mutation analysis and immunohistochemical analysis were done as confirmatory tests.

Quantitative alteration of phospholipids in blood samples of confirmed DMD patients were done using ^{31}P NMR spectroscopy. Quantity of PC, PE, PI, PS and LysPC in blood of DMD patients was significantly higher as compared to normal subjects. The present finding is supported by various previous reports. The abnormal erythrocyte membrane in DMD patients has been reported earlier [21, 45–49]. There were biochemical, morphological and biophysical basis of alteration observed in red blood cell

Table 1 Evaluation of the quantity of phospholipids classes in DMD patients vs. healthy subjects

Phospholipids (PL)	DMD patients (<i>n</i> = 32, mmol/l, Median, range; min–max)	Normal individuals (<i>n</i> = 16, mmol/l, Median, range; min–max)	Mann–Whitney test
Phosphatidylcholine (PC)	1.38 (0.28–15.41)	0.45 (0.24–1.58)	<i>p</i> < 0.05, significant difference
Phosphatidylinositol (PI)	0.25 (0.05–2.68)	0.11 (0.01–0.28)	<i>p</i> < 0.05, significant difference
Lysophosphatidylcholine (LysPC)	0.26 (0.11–3.48)	0.17 (0.04–0.32)	<i>p</i> < 0.05, significant difference
Phosphatidylserine (PS)	0.07 (0.02–3.85)	0.03 (0.01–0.13)	<i>p</i> < 0.05, significant difference
Phosphatidylethanolamine (PE)	0.22 (0.02–2.60)	0.07 (0.01–0.20)	<i>p</i> < 0.05, significant difference

Table 2 Evaluation of the quantity of phospholipids classes in gene deletion positive cases of DMD patients vs. negative gene deletion cases of DMD patients

Phospholipids (PL)	DMD patients (Gene deletion positive cases, <i>n</i> = 22, mmol/l, Median, range; min–max)	DMD patients (gene deletion negative cases, <i>n</i> = 10, mmol/l, Median, range; min–max)	Mann–Whitney test
Phosphatidylcholine (PC)	1.36 (0.28–14.30)	1.28 (0.24–15.41)	<i>p</i> > 0.05, Insignificant
Phosphatidylinositol (PI)	0.23 (0.05–2.68)	0.21(0.04–2.58)	<i>p</i> > 0.05, Insignificant
Lysophosphatidylcholine (LysPC)	0.24 (0.11–3.08)	0.27 (0.15–3.48)	<i>p</i> > 0.05, Insignificant
Phosphatidylserine (PS)	0.06 (0.02–3.79)	0.07(0.02–3.85)	<i>p</i> > 0.05, Insignificant
Phosphatidylethanolamine (PE)	0.20 (0.02–2.54)	0.18(0.02–2.60)	<i>p</i> > 0.05, Insignificant

Table 3 Evaluation of the quantity of phospholipids classes in gene deletion positive cases of DMD patients, negative gene deletion cases of DMD patients as compared to normal subjects

Phospholipids (PL)	DMD patients (gene deletion positive cases, <i>n</i> = 22, mmol/l, Median, range; min–max)	DMD patients (gene deletion negative cases, <i>n</i> = 10, mmol/l, Median, range; min–max)	Normal individuals (<i>n</i> = 16, mmol/l, median, range; min–max)	Mann–Whitney test
Phosphatidylcholine (PC)	1.36 (0.28–14.30)	1.28 (0.24–15.41)	0.45 (0.24–1.58)	<i>p</i> < 0.05, significant difference
Phosphatidylinositol (PI)	0.23 (0.05–2.68)	0.21 (0.04–2.58)	0.11 (0.01–0.28)	<i>p</i> < 0.05, significant difference
Lysophosphatidylcholine (LysPC)	0.24 (0.11–3.08)	0.27 (0.15–3.48)	0.17 (0.04–0.32)	<i>p</i> < 0.05, significant difference
Phosphatidylserine (PS)	0.06 (0.02–3.79)	0.07 (0.02–3.85)	0.03 (0.01–0.13)	<i>p</i> < 0.05, significant difference
Phosphatidylethanolamine (PE)	0.20 (0.02–2.54)	0.18 (0.02–2.60)	0.07 (0.01–0.20)	<i>p</i> < 0.05, significant difference

(RBC) membrane of DMD patients [21]. Structural abnormality of erythrocyte membranes is a result of its tendency to form abnormally oriented vesicles [45]. Abnormality in the structure of erythrocyte membrane in these patients was also confirmed by freeze-fracture studies [46]. Erythrocyte from DMD patients is fragile and interactions between skeletal proteins and/or between membrane and skeleton components may contribute to the alterations of erythrocyte membranes in DMD [47]. This is another evidence of an organizational abnormality in DMD erythrocyte membranes. Decreased water permeability of erythrocyte membranes in patients with DMD also provides the evidence of its abnormality [48]. DMD erythrocyte plasma membrane abnormality is also proved by the

alteration in ion transport and in various enzymatic activities [49].

Alteration of phospholipids in blood of DMD patients have been reported earlier. Elevated level of plasma phospholipid was found in the serum of animals undergoing dystrophies in their muscles [24, 25]. Serum phospholipid was also found prominent in patients with DMD [50]. Hunter et al. [46] reported increased amount of PC in outer leaflet of erythrocytes membrane of DMD as compared to normal/control subjects. A study by Piperi et al. [51] showed abnormal fatty acid composition and disorganization of erythrocyte membrane in patients with DMD.

Collective findings of these two groups confirm that structural abnormality of erythrocyte membrane is related

to the alteration of phospholipid composition. The major constituent of lipid bilayer of membrane is phospholipids [52] that are altered in blood plasma and plasma membrane of red blood cell. There are very few reports on alteration of different classes of phospholipid in blood of DMD patients. The present study attempts to cover the lacunae.

There are several other remarkable reasons responsible for the higher level of phospholipid classes in blood of DMD patients as described in the present results. During degeneration of the muscle membrane, phospholipids are released in the blood pool where they get accumulated. Muscle regeneration enhances synthesis of phospholipid [52]. Increased rate of synthesis of phospholipids and breakdown of muscle membrane may be responsible for increase level of phospholipids in blood of DMD patients [52, 53]. An earlier study demonstrated that increased rate of phospholipid metabolism in gastrocnemius muscle and sciatic nerves of dystrophic mouse model is due to elevated activity of phospholipases A [54]. Elevated activity of phospholipases A in muscles is also responsible for enhanced amount of phospholipids in the blood because higher activity of phospholipases A is also reported in human erythrocytes from patients with DMD [55].

There was no significant difference in different phospholipid classes in whole blood of negative gene deletion cases of DMD patients (diagnosis was confirmed by the method of immunohistochemistry on muscle tissue) as compared to DMD cases with positive gene deletion. The phospholipid classes in whole blood were elevated in negative gene deletion cases of DMD patients and DMD cases with positive gene deletion as compared with normal individuals. Elevated levels of different phospholipids in whole blood of negative gene deletion cases of DMD were similar to the positive gene deletion cases of DMD. The results of this study conclusively prove that ^{31}P NMR spectroscopy-based quantitative comparison of different classes of phospholipids is more reliable and sensitive method as compared to gene mutation analysis for diagnosing DMD.

Conclusion

The present study distinguishes the patients with DMD from the healthy subjects on the basis of quantity of phospholipids in blood. These observations may be useful in future for the development of new diagnostic method of DMD.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Standard Ethical committee of SGPGIMS, Lucknow was approved the research work and provided the permission to take the blood samples.

Informed consent A written consent was obtained from all the patients.

References

- Emery AEH (2000) Muscular dystrophy: the facts. Oxford University Press, Oxford
- Yiu EM, Kornberg AJ (2008) Duchenne muscular dystrophy. *Neurol India* 56:236–247
- Chamberlain JS, Gibbs RA, Ranier JE, Caskey CT (1990) Multiplex PCR for the diagnosis of Duchenne muscular dystrophy. PCR protocols. A guide to methods and application. Academic Press, San Diego, pp 272–281
- Chaturvedi LS, Mukherjee M, Srivastava S, Mittal RD, Mittal B (2001) Point mutation and polymorphism in Duchenne/Becker Muscular Dystrophy (D/BMD) patients. *Exp Mol Med* 33:251–256
- Murugan S, Chandramohan A, Lakshmi BR (2010) Use of multiplex ligation-dependent probe amplification (MLPA) for Duchenne muscular dystrophy (DMD) gene mutation analysis. *Indian J Med Res* 132:303–311
- Potnis-Lele M (2012) Genetic etiology and Diagnostic strategies for Duchenne and Becker Muscular Dystrophy: a 2012 update. *Indian J Basic Appl Med Res* 2:357–369
- Lalic T, Vossen RH, Coffa J, Schouten JP, Guc-Scekic M, Radivojevic D, Djuricic M, Breuning MH, White SJ, den Dunnen JT (2005) Deletion and duplication screening in the DMD gene using MLPA. *Eur J Hum Genet* 13:1231–1234
- Mihran OT (1997) Clinical pediatric orthopedics: the art of diagnosis and principle of management. McGraw-Hill publishers, p 404
- Peng J, Redman CM, Wu X, Song X, Walker RH, Westhoff CM et al (2007) Insights into extensive deletions around the XK locus associated with McLeod phenotype and characterization of two novel cases. *Gene* 392:142–150
- Ho MF, Monaco AP, Blonden LA, van Ommen GJ, Affara NA, Ferguson-Smith MA et al (1992) Fine mapping of the McLeod locus (XK) to a 150–380-kb region in Xp21. *Am J Hum Genet* 50:317–530
- van der Kooij AJ, Barth PG, Busch HF, de Haan R, Ginjaar HB, van Essen AJ et al (1996) The clinical spectrum of limb girdle muscular dystrophy. A survey in The Netherlands. *Brain* 119:1471–1480
- Eymard B, Romero NB, Leturcq F, Piccolo F, Carrié A, Jeanpierre M et al (1997) Primary adhalinopathy (alpha-sarcoglycanopathies): clinical, pathologic, and genetic correlation in 20 patients with autosomal recessive muscular dystrophy. *Neurology* 48:1227–1234

13. Merlini L, Kaplan JC, Navarro C, Barois A, Bonneau D, Brasa J et al (2000) Homogeneous phenotype of the gypsy limb-girdle MD with the gamma- sarcoglycan C283Y mutation. *Neurology* 54:1075–1079
14. Ratnagopal P, Puvandendran K, Lee YS (1991) Calf hypertrophy in spinal muscular atrophy. *Singapore Med J* 32:448–450
15. Krampitz DE, Wolfe GI, Fleckenstein JL, Barohn RJ (1998) Charcot-Marie-Tooth disease type 1A presenting as calf hypertrophy and muscle cramps. *Neurology* 51:1508–1509
16. Leuzzi V, Mastrangelo M, Battini R, Cioni G (2013) Inborn errors of creatine metabolism and epilepsy. *Epilepsia* 54:217–227
17. Mochel F, Barritault J, Boldieu N, Eugène M, Sedel F, Durr A, Seguin F (2007) Contribution of in vitro NMR spectroscopy to metabolic and neurodegenerative disorders. *Rev Neurol (Paris)* 163:960–965
18. Sperl W (1997) Diagnosis and therapy of mitochondriopathies. *Wien Klin Wochenschr* 109:93–99
19. Mochel F, Engelke UF, Barritault J, Yang B, McNeill NH, Thompson JN, Vanderver A, Wolf NI, Willemsen MA, Verheijen FW, Seguin F, Wevers RA, Schiffmann R (2010) Elevated CSF N-acetylaspartylglutamate in patients with free sialic acid storage diseases. *Neurology* 74:302–305
20. Wevers RA, Engelke U, Heerschap A (1994) High-resolution ¹H-NMR spectroscopy of blood plasma for metabolic studies. *Clin Chem* 40:1245–1250
21. Plishker GA, Appel SH (1980) Red blood cell alterations in muscular dystrophy: the role of lipids. *Muscle Nerve* 3:70–81
22. Nishio H, Wada H, Matsuo T, Horikawa H, Takahashi K, Nakajima T et al (1990) Glucose, free fatty acid and ketone body metabolism in Duchenne muscular dystrophy. *Brain Dev* 12:390–402
23. Chio LF, Peterson DW, Kratzer FH (1972) Lipid composition and synthesis in the muscles of normal and dystrophic chickens. *Can J Biochem* 50:267–272
24. Kwok CT, Kuffer AD, Tang BY, Austin L (1976) Phospholipid metabolism in murine muscular dystrophy. *Exp Neurol* 50:362–375
25. Kwok CT, Austin L (1978) Phospholipids composition and metabolism in mouse muscular dystrophy. *Biochem J* 176:15–22
26. Nydegger UE, Butler RE (1972) Serum lipoprotein levels in patients with cancer. *Canc Res* 32:1756–1760
27. Ruiz-Cabello J, Cohen JS (1992) Phospholipid metabolites as indicators of cancer cell function. *NMR Biomed* 5:226–233
28. Engan T (1996) Magnetic resonance spectroscopy of blood plasma lipoproteins in malignant disease: methodological aspects and clinical relevance. *Anticancer Res* 16:1461–1472
29. Podo F, de Certaines JD (1996) Magnetic resonance spectroscopy in cancer: phospholipid, neutral lipid and lipoprotein metabolism and function. *Anticancer Res* 16:1305–1316
30. Kuliszkiewicz-Janus M, Janus W, Baczyński S (1996) Application of ³¹P NMR spectroscopy in clinical analysis of changes of serum phospholipids in leukemia, lymphoma and some other non-haematological cancers. *Anticancer Res* 16:1587–1594
31. Sullentrop F, Moka D, Neubauer S, Haupt G, Engelmann U, Hahn J et al (2002) ³¹P NMR spectroscopy of blood plasma: determination and quantification of phospholipid classes in patients with renal cell carcinoma. *NMR Biomed* 15:60–68
32. Schott S, Hahn J, Kurbacher C, Moka D (2012) (31)P and (1) h nuclear magnetic resonance spectroscopy of blood plasma in female patients with preeclampsia. *Int J Biomed Sci* 8:258–263
33. Pradhan S (2002) Valley sign in Duchenne muscular dystrophy: importance in-patient with inconspicuous calves. *Neurol India* 2:184–186
34. Okinaka S, Kumagai H, Ebashi S, Sugita H, Momoi H, Toyokura Y et al (1961) Serum creatine phosphokinase activity in progressive muscular dystrophy and neuromuscular disease. *Arch Neurol* 4:520
35. Eshchar J, Zimmerman HJ (1967) Creatine phosphokinase in disease. *Am J Med Sci* 253:272–282
36. Rosalki SB (1967) An improved procedure for serum creatine phosphokinase determination. *J Lab Clin Med* 69:696–705
37. Olerup O, Zetterquist H (1992) HLA-DR typing by PCR amplification with sequence specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens* 39:225–235
38. Ramos-vara JA (2005) Technical aspects of immunohistochemistry. *Vet Pathol* 42:405–426
39. Tofts PS, Wray S (1988) A critical assessment of methods of measuring metabolite concentrations by NMR spectroscopy. *NMR Biomed* 1:1–10
40. Meyerhoff DJ, Karczmar GS, Matson GB, Boska MD, Weiner MW (1990) Non-invasive quantitation of human liver metabolites using image-guided ³¹P magnetic resonance spectroscopy. *NMR Biomed* 3:17–22
41. Ernst RR, Bodenhausen G, Wokaun A (1992) Principles of nuclear magnetic resonance in one and two dimensions, Oxford Science Publications
42. Metz KR, Dunphy LK (1996) Absolute quantification of tissue phospholipids using ³¹P NMR spectroscopy. *J Lipid Res* 37:2251–2264
43. London E, Feigenson GW (1979) Phosphorus NMR analysis of phospholipids in detergents. *J Lipid Res* 20:408–412
44. Niebrój-Dobosz (1982) The distribution of inside-out and right-side-out erythrocyte membrane vesicles in Duchenne progressive muscular dystrophy. *J Neurol* 3:228
45. Wakayama Y, Hodson A, Pleasure D, Bonilla E, Schotland DL (2004) Alteration in erythrocyte membrane structure in Duchenne muscular dystrophy. *Ann Neurol* 4:253–256
46. Hunter MI, Lao Mio Sam, de Vane PJ (1983) Is erythrocyte membrane phospholipid organization abnormal in Duchenne muscular dystrophy? *Clin Chim Acta* 128:69–74
47. Matsumura T, Takahashi M, Nakamori M, Saito T, Nozaki S, Fujimura H et al (2004) Erythrocyte from Duchenne muscular dystrophy is fragile. *Rinsho Shinkeigaku* 44:695–698
48. Serbu AM, Marian A, Popescu O, Pop VI, Borza V, Benga I et al (1986) Decreased water permeability of erythrocyte membranes in patients with Duchenne muscular dystrophy. *Muscle Nerve* 9:243–247
49. Scarlato G, Meola G, Silani V, Manfredi L, Bottiroli G, Zanella A (2009) Erythrocyte spectrofluorometric abnormalities in Duchenne patients and carriers: a new approach to carrier detection. *Acta Neurol Scand* 59:262–269
50. Srivastava NK, Pradhan S, Mittal B, Gowda GA (2010) High resolution NMR based analysis of serum lipids in Duchenne muscular dystrophy patients and its possible diagnostic significance. *NMR Biomed* 23:13–22
51. Piperi C, Papanagiotou A, Kalofoutis C, Zisaki K, Michalaki V, Tziraki A et al (2004) Altered long chain fatty acids composition in Duchenne muscular dystrophy erythrocytes. *In Vivo* 18:799–802
52. David LN, Michael MC (2002) Lehninger, principle of biochemistry, 3rd edn. Worth Publishers, New York, pp 368–381
53. Shull RL, Alfin-Slater RB (1958) Tissue lipids of dystrophia muscularis, a mouse with inherited muscular dystrophy. *Proc Soc Exp Biol Med* 2:403–405
54. Jato-Rodriguez JJ, Hudson AJ, Strickland KP (1974) Triglycerides metabolism in skeletal muscle from normal and dystrophic mice. *Biochim Biophys Acta* 348:1–13
55. Iyer SL, Katyare SS, Howland JL (1976) Elevated erythrocyte phospholipase A associated with Duchenne and myotonic muscular Dystrophy. *Neurosci Lett* 2:103–106