JPP JOURNAL OF PROTEINS AND PROTEOMICS
7(2), 2016, pp. 101-106

Research Article

COMPARATIVE EVALUATION OF SEVEN ISOFORMS OF SERUM SIRTUINS AS PROTEIN MARKER FOR FRAILTY

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Abstract: Frailty is a complex clinical state in elderly population and is associated with adverse health outcomes. No diagnostic biomarker has been identified for frailty so far. In our previous study we had shown that SIRT1 and SIRT3 are associated with low circulating levels in case of frail, even after adjusting various confounders. As sirtuin (SIRT) proteins are the family of seven isoforms, so quantifying the levels of SIRT4-7 would further expand our knowledge regarding the role of sirtuins in frailty. The aim of this study was to estimate the level of other sirtuins (SIRT4-7) in frail and assess their potential utilization as protein marker to detect frailty in early stage. Level of SIRT4-7 in serum of 166 frail and non-frail subjects were determined using surface plasmon resonance technology and western blot experiments. In the cross sectional study, it was observed that the levels of SIRT5, SIRT6 and SIRT7 were significantly lower in case of frail as compared to non-frail individuals. After multivariable regression analysis only SIRT7 level was found to be significantly lower in case of frail. Receiver operating characteristic (ROC) analysis revealed that SIRT5 and SIRT6 were able to detect in the frail individuals with moderate accuracy. It can be summarized that after analysis of seven isoforms of sirtuin in serum with frailty, SIRT1 and 3 can be used as a potential protein marker for frailty and other SIRTs 2,4,5,6,7 are not strongly associated with frailty.

***Keywords*:** Frailty; sirtuin; surface plasmon resonance; geriatrics

Introduction

Frailty is a common geriatric syndrome characterized by age-related decline in several inter-related physiological systems, depletion of homeostatic reserves and increased vulnerability to sudden health status changes triggered by minor stressor events (Clegg *et al.,* 2013). These changes occur with normal aging, however in case of frailty the changes starts to develop in an accelerated manner. Thus, an important perspective to understand the frailty includes the involvement of various pathways which can regulate aging. One such pathway includes sirtuins- a family of histone deacetylase which had been shown to regulate longevity in various model organisms like *Saccharomyces cerevissae* (Kaeberlein *et al*., 1999), *Caenorhabditis elegnas* (Tissenbaum and Guarente, 2001), *Drosophila* (Rogina and Helfand, 2004) and mice (Herranz *et al*., 2010). In mammals, sirtuins family includes seven members namely SIRT1-SIRT7 (Guarente and Franklin, 2011). In our previous study levels of serum SIRT1, 2 and 3 were evaluated in frail and non frail subjects. The significantly lower circulating levels of SIRT1 and SIRT3 in case of frail subjects as compared to non-frails was observed even after adjusting various confounders like age, gender, diabetes mellitus,

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Received: March 15, 2016
Accepted: May 6, 2016
Published: May 10, 2016

hypertension, Mini Mental State Examination scores (MMSE Scores) and number of comorbidities. Receiver operating characteristic (ROC) analysis was performed and it was observed that only SIRT1 and SIRT3 can be utilized as a potential protein marker to detect frailty (Kumar *et al.*, 2014). The role of SIRT1 and SIRT3 is well known in countering oxidative stress, the accumulation of which is a major force behind the onset of frailty. Although SIRT1-7 belongs to same class of protein family, the differences in terms of various characteristics like cellular localization, enzymatic activity and target proteins (Haigis and Sinclair, 2010) make it necessary to quantify the levels of all sirtuins. Further, it would expand our knowledge regarding the involvement of all serum sirtuins in frailty. It would provide information whether all the sirtuins are involved in frailty or only SIRT1 and SIRT3 are involved. Serum is an easily accessible medium as compared to tissue, more so in case of the elderly frail patients and will help in detecting the disease in an early stage. It is known that many proteins circulated in the human blood by leakage, diffusion or otherwise across the cells and capillary membranes (Fried *et al.*, 2001). Hence, circulating levels of other sirtuins namely SIRT4-7 were evaluated in the present study.

Materials and Methods

Study groups - One hundred sixty patients over the age of 60 years visiting Geriatric Medicine Outpatient Department of AIIMS, New Delhi, were included in this cross sectional study. The study was approved by the Institute Ethics Committee (IESC/T-270/01.07.2011) and all participants provided written informed consent. Frailty was diagnosed by using Fried's criteria [9] which were based on the presence of five measurable characteristics.

Estimation of SIRT4-7 level in the study group using surface plasmon resonance (SPR)- BIAcore 2000 (Pharmacia Biosensor AB, Sweden) was used for all labelfree real-time monitoring of target bimolecular interactions. Rabbit anti human SIRT4 IgG, Mouse anti human SIRT5 IgG, Rabbit anti human SIRT6 IgG and Rabbit anti human SIRT7 IgG (Santa Cruz Biotechnology, CA) were used. IgG were immobilized on three different flow cells of CM5 sensor chip separately via amine coupling. Six different known concentrations of pure SIRT4 (1.31, 1.87, 2.66, 4.01, 5.35 and 6.69 ng/µl), SIRT5 (2.45, 3.5, 4.97, 7.49, 10.00 and 12.49 ng/µl), SIRT6

(3.5,5,7.1,10.7,14.28 and 17.85 ng/µl) and SIRT7 (5.25, 7.5, 10.65, 16.05, 21.42 and 26.78 ng/µl.) were passed over respective immobilized antibody. Standard graphs were prepared by plotting a graph between concentrations and corresponding RU values obtained. Serum samples were diluted with HBS-EP buffer (10 mm HEPES pH 7.4, 150 mm NaCl, 3 mm EDTA, 0.005% polysorbate 20) and allowed to run over immobilized antibodies. The concentrations of SIRT4-7 were determined from respective standard curves.

Estimation of SIRT4-7 level in the study group using western blot experiments- To further confirm the presence of SIRT 4-7, 12 serum samples from frail and 12 from non-frail subjects were prepared by removing major interfering proteins using Albumin OUT columns (G-Biosciences, St. Louis. Total protein concentration was determined using (bicinchoninic acid assay (BCA) using bovine serum albumin as standards. 50 µg of total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis, protein was transferred to polyvinylidenedifluoride (PVDF) membranes (MDI Membrane Technologies, India). The membranes were then blocked in 5% nonfat dry milk prepared in TBS (10 mM Tris pH 7.5, 150 mM NaCl) for 2 hours and subsequently incubated with primary antibodies diluted with TBS at 4°C overnight. After washing with TBS-T (20 mM Tris pH7.5, 500 mM NaCl, 0.05% Tween 20), membranes were incubated with HRP-conjugated secondary antibodies; Goat Anti-Mouse IgG (1:5000; GenScript, Piscataway, NJ,USA) or Goat Anti-Rabbit IgG (1:5000, GenScript) at room temperature for 1 hour. After washing with TBS-T, bands were visualized using enhanced chemiluminescent system (Pierce ECL Western Blotting Substrate; Thermo Scientific, Rockford, IL, USA). Quantification of band intensity was performed using Quantity-one1-D-analysis software (Bio- Rad Laboratories, Hialeah FL, USA).

Results

Baseline data

Out of 166 subjects recruited, 78 (47%) were classified as non-frail and 88 (53%) as frail. It was also observed that higher mean age (p=0.0001) and lower mean MMSE score (p=0.0005) were found to be significantly associated with frailty (Table 1).

Serum level determined by SPR

The standard curve of SPR experiment (Figure 1) for SIRT4,5,6,7 showed the change in RUs with different concentration of sirtuin. The linear increase of RU with the increase of concentration showed the specificity and sensitivity of sirtuin proteins. The cross check were also done and no response were obtained. The concentration of serum SIRT4, 5, 6 ,7 were determined from the respective standard curves. The serum levels of SIRT5 (non-frail – 8.71 ± 3.48 ng/µL; frail – 7.25 ± 3.13 ng/µL; p=0.005), SIRT6 (non-frail – 9.82 ± 3.64 ng/µL; frail – 8.36 ± 3.33 ng/µL; p=0.008) and SIRT7 (non-frail – 19.26± 3 ng/µL; frail – 16.96 ± 3.33 ng/µL; p=0.009 ng/µL) were significantly lower in frail as compared to non-frail (Figure 2A-2D). However, in case of SIRT4, no significant difference was found between frail and non-frail. In multivariable regression analysis, only lower SIRT7 level were significantly associated with frailty after adjusting age, gender, diabetes mellitus, hypertension, cognitive status (MMSE scores) and number of comorbidities (Table 1).

ROC analysis

ROC analysis was carried out to determine the optimum diagnostic cut-off value. The area under curve for SIRT5 was 0.63 at a cut-off value of <7.82 ng/µL which can detect frailty with sensitivity of 67.05% and specificity of 64.10% (Figure 3A). The area under curve for SIRT6 was 0.65 at a cut-off value of <7.82 ng/µL which can detect frailty with sensitivity of 61.36% and specificity of 64.10% (Figure 3B). The area under curve was 0.57 for SIRT7, hence cut-off values were not determined (Figure 3C). In case of SIRT4 the ROC analysis was not performed as the difference between frail and non-frail was non-significant.

Western blot

The results obtained in case of western blot experiment were in accordance with the SPR results and lower levels of SIRT5, 6 and 7 were observed in case of frail as compared to non-frail (Figure 4A-D).

Discussion

Frailty is a phenotypical expression of a collapsing system which manifests as lack of muscle

Table 1
Comparative analysis of baseline data and SIRT4-7 concentrations in frail and non- frail subjects

		Non-frail	*Frail*	*p-value*
N		78	88	
Age (years), mean±SD		73.64±9.73	79.06±8.13	0.0001
Male, n (%)		50 (64.10)	44 (50.00)	0.067
BMI, mean±SD		20.86±3.76	20.23±4.29	0.318
MMSE, mean±SD		26.91±2.91	25.02±3.79	0.0005
Hypertension, n (%)		24 (30.77)	37 (42.05)	0.133
Diabetes Mellitus, n (%)		9 (11.54)	27 (30.68)	0.003
SIRT4 (ng/µL, mean±SE)	Unadjusted	7.01±0.23	7.56±0.22	0.082
	Adjusted	6.99±0.32	7.58±0.23	0.119
SIRT5 (ng/µL, mean±SE)	Unadjusted	8.71±0.39	7.25±0.33	0.005
	Adjusted	8.53±0.41	7.41±0.38	0.065
SIRT6 (ng/µL, mean±SE)	Unadjusted	9.82±0.41	8.36±0.35	0.008
	Adjusted	9.88±0.43	8.31±0.40	0.63
SIRT7 (ng/µL, mean±SE)	Unadjusted	19.26±0.70	16.96±0.53	0.009
	Adjusted	19.08±0.68	17.11±0.64	0.05

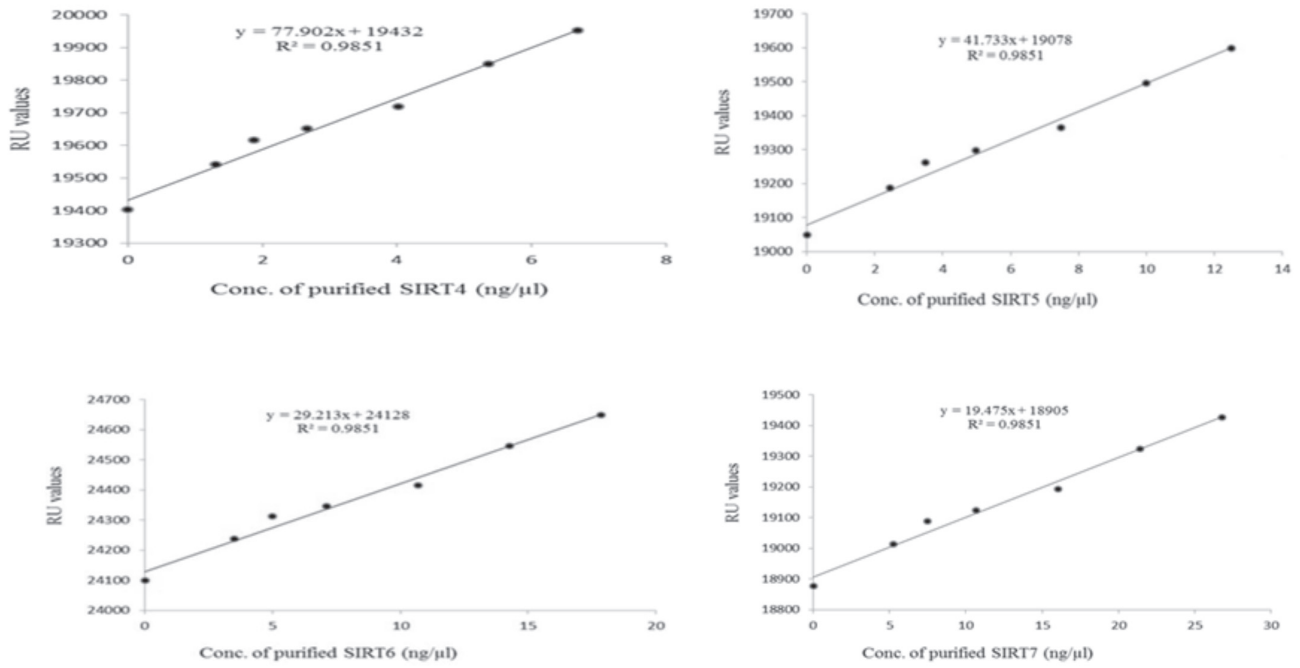

Figure 1: **Standard curve plotted between corresponding RU obtained by passing different concentrations of SIRT4, SIRT5, SIRT6 and SIRT7 over respective antibody**

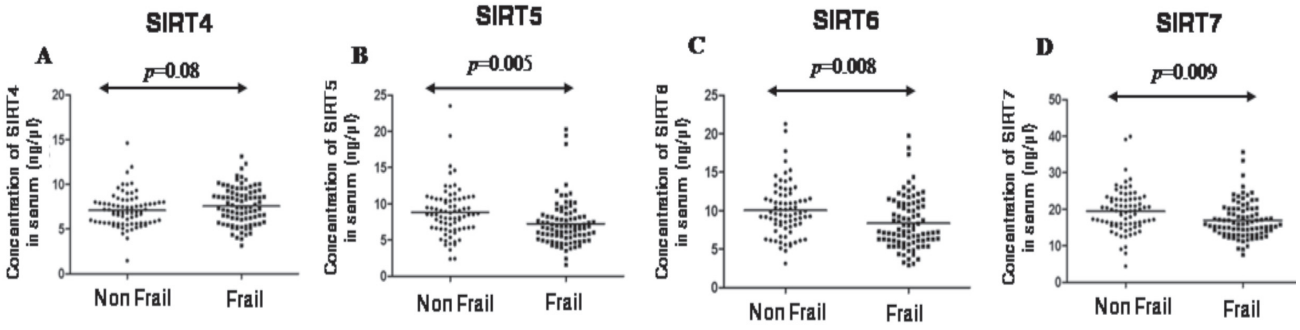

Figure 2: **Scatter diagram showing the concentrations of SIRT4** (A)**, SIRT5** (B)**, SIRT6** (C) **and SIRT7** (D) **in frail and non-frail subjects.**

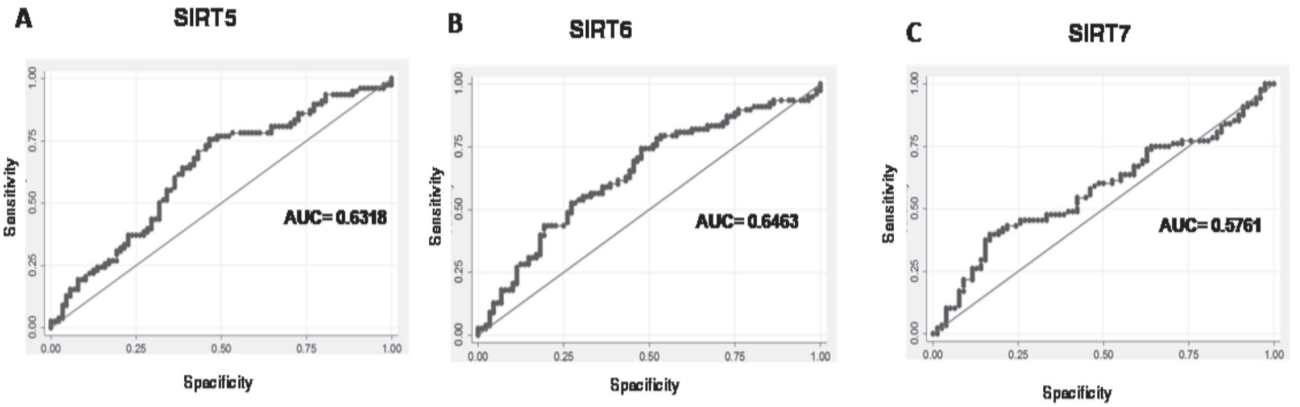

Figure 3: **ROC analysis showing the area under curve for SIRT5** (A)**, SIRT6** (B) **and SIRT7** (C) **to distinguish frail from non-frail subjects.**

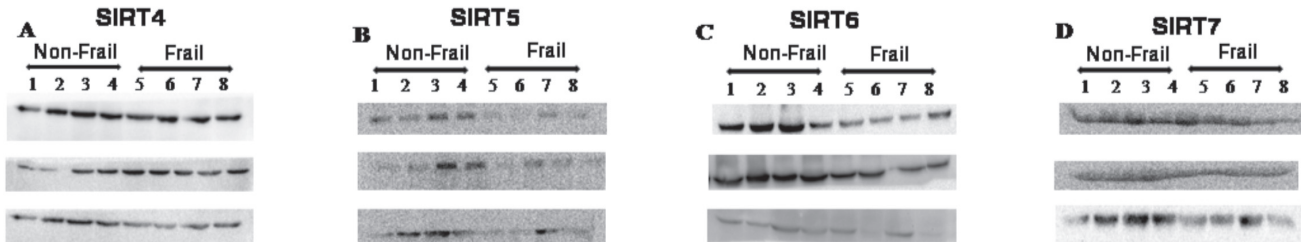

Figure 4: **Western blots showing the level of SIRT4** (A), **SIRT5** (B), **SIRT6** (C) **and SIRT7** (D) **in frail and non-frail subjects. 3 western blots were performed for each protein and each blot consisted of 4 non frail (lane -4) and 4 frail (lane 5-8). Hence, total 12 frail and 12 non-frail samples were run for western blot**

strength, exhaustion, unintentional weight loss, lack of desire to function, low physical activity, slow speed of movement and being bed bound. The "syndrome of frailty" is a concept rather than a disease and objective diagnostic parameters are still evolving (Rockwood, 2005).

Objective parameter for diagnosis of frailty is often difficult as there is no typical phenotype of this state. There are several diagnostic criteria related to physical performance tests, but the criteria proposed by Fried *et al.* (2001) is mostly used in clinical practice and research. These definition suits North American and European populations with well established health care system. However, for Indian population there are several practical difficulties in using these tools because of social cultural reasons and lack of information on health status in childhood and adulthood. To obviate these difficulties there is a need for developing a new tool, which uses the principles of the above criteria but obviates the disadvantages, useful for older Indians (Dey *et al.*, 2001).

Frailty may develop in the setting of chronic inflammation, dementia, heart failure and other chronic debilitating diseases. Activation of the inflammatory system is considered as one of the most prominent pathophysiological features of frailty and can be relied upon as a biological marker that can be used for early diagnosis of frail and especially pre-frail older patients. Inflammatory biomarkers for diagnosis of frailty identified till date are: white blood cell count, fibrinogen, retinol binding protein, albumin, D dimmer, factor VIII, tumour necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6) and C-reactive protein (CRP), Interleukin-1(secreted from senescent cells), Cyclooxygenase-2 (joint inflammatory marker), Interleukin-8 (neutrophil attractants), MCP -1 (Monocyte chemo attractant) etc (Ferrucci *et al.*, 2001). However, these markers are nonspecific and often used for inflammatory diseases. As the exact pathway that leads to the state of frailty is still not clear, there is no diagnostic biological test available till date. The condition needs to be detected earlier so that further progression to dependency can be prevented. In this context, attempts have been made to develop biomarkers for early detection of frailty. Owing to the strong association of sirtuin with aging, sirtuins were chosen as they had shown to regulate the lifespan in number of lower model organisms. Seven isoforms of sirtuins differ in cellular localization as SIRT1 and SIRT6 are predominately found in the nucleus, SIRT2 is predominantly located in the cytoplasm, SIRT3, SIRT4 and SIRT5 are localized to the mitochondria and SIRT7 is located within the nucleolus. On the basis of catalytic activity, SIRT1, SIRT2, SIRT3, SIRT5 and SIRT7 exhibit NAD^+ dependent deacetylase activity while SIRT4 and SIRT6 are ADP ribosyltransferases. Further, all the sirtuins have been shown to act upon different target proteins (Haigis and Sinclair, 2010). So, it might not be possible that all the sirtuins are involved in frailty. Our previous study had indicated towards the utilization of SIRT1 and SIRT3 as protein marker for frailty (Kumar *et al.*, 2014). Since there are seven members of sirtuin protein family, it is necessary to determine the role of other sirtuins in frailty. Therefore, in the present study, we assessed the potentiality of other sirtuins namely SIRT4, 5, 6 and 7 to diagnose frailty in early stage. The present study involved SPR technology which was further confirmed by traditional method like Western Blot. SPR is a label free real time assay and its advantage over

other immunological methods such as ELISA, lies in its reusability. The high sensitivity and specificity of the technology in detecting sirtuin proteins was also established. In the present study It was observed that though the level of other sirtuins are low in frail compare to non frail subjects, but none of them can be a strong protein marker candidate as reflected by multivariable regression analysis and ROC analysis compare to SIRT1 and 3. The same results were obtained in case of western blotting experiments. Thus, it can be said owing to the differences in their localization, enzymatic activity and targets not all sirtuins were found to be associated with frailty. However, significant lower circulating levels of SIRT1 and SIRT3 in case of frail even after adjusting various confounders was observed in our previous study (Kumar *et al.*, 2014). In the present study we assessed the potential of other sirtuins namely SIRT4, 5, 6 and 7 to diagnose frailty in early stage.

It was observed owing to the differences in their localization, enzymatic activity and targets not all sirtuins were found to be associated with frailty. The study thus provided further insight into the role of sirtuin proteins in frailty and strengthened the potentiality of SIRT1 and SIRT3 as a protein marker to detect frailty.

Acknowledgements

Authors acknowledge research section of AIIMS for proving financial support for purchasing chemicals and Council for Scientific and Industrial Research (CSIR) and Indian Council of Medical Research (ICMR) for the fellowships of Rahul Kumar and Rashmita Pradhan, respectively.

Abbreviations

SIRT, Sirtuin; SPR, Surface Plasmon Resonance; ROC, Receiver operating characteristic

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