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Letter to the Editor

Association of glucose 6-phosphate dehydrogenase (G6PD) 3'UTR polymorphism with vitiligo and *in vitro* studies on G6PD inhibition in melanocytes

Letter to the Editor,

Vitiligo is an acquired depigmentation disorder caused due to loss of functional melanocytes from the skin. Oxidative stress is the initial event during the course of vitiligo [1]. Glucose 6-phosphate dehydrogenase (G6PD) is involved in the oxidizing stress defence. Previous studies have demonstrated decreased G6PD levels in vitiligo [2,3]. *G6PD* 3'UTR polymorphism (rs1050757) was found to be associated with decreased G6PD activity [4]. However, there is no report on the status of *G6PD* polymorphisms in vitiligo. We aimed to investigate the association of *G6PD* polymorphisms, G6PD activity and perform genotype-phenotype correlation analysis in Gujarat vitiligo patients (n = 366) and controls (449). We also examined the *in-vitro* effect/s of G6PD inhibition by 6-aminonicotinamide (6-ANAD) on normal human melanocytes (NHM), immortalized normal human melanocytes (PIG1) and immortalized melanocytes derived from vitiligo patient (PIG3V). Methodology is described in 'Supporting information'.

Earlier, we have reported increased LPO and decreased *G6PD* mRNA levels in vitiligo patients [3,5,6]. In the present study vitiligo patients showed significantly decreased G6PD activity as compared to controls (Mean ± SEM: 0.784 ± 0.052 *vs.* 1.314 ± 0.059; $p < 0.0001$; Fig. 1A). Further, NSV (Mean ± SEM: 0.649 ± 0.033) and SV (Mean ± SEM: 0.733 ± 0.082) patients showed significant decrease in G6PD activity as compared to controls ($p < 0.0001$ and $p = 0.0001$ respectively). The analysis based on disease progression demonstrated that active vitiligo patients (Mean ± SEM: 0.601 ± 0.030) exhibited significant decrease in G6PD activity as compared to stable (Mean ± SEM: 0.793 ± 0.082) as well as controls ($p = 0.016$ and $p < 0.0001$ respectively; Fig. 1A). Overall, these results support the oxidative stress hypothesis of vitiligo.

The impact of exonic polymorphisms on the stability and function of G6PD was predicted using *in silico* bioinformatics tools (Table S1). However, genotyping results demonstrated that six among eight addressed exonic polymorphisms of *G6PD i.e.*, rs1050827, rs11555344, rs5030870, rs34233392, rs34193178, rs2230036 were mono-allelic in Gujarat population (Appendix AFigs. S1 and S2). While, *G6PD* rs5030868 and rs2230037 polymorphisms were not associated with vitiligo (Table 1). In contrast, genotype and allele frequencies for *G6PD* 3' UTR A/G (rs1050757) differed significantly between patients and controls ($p = 0.0002$ and $p = 0.0002$ respectively). In particular, the minor allele 'G' was more frequent in patients as compared to controls ($p = 0.0002$; Table 1). Further, 'GG' and 'AG' genotypes were found to be significantly associated with vitiligo ($p = 0.014$ and $p < 0.0001$ respectively). Both control as well as patient populations were under HWE for *G6PD* 3' UTR polymorphism ($p = 0.222$ and $p = 0.199$ respectively). These results indicate the association of *G6PD* 3'UTR polymorphism with Gujarat vitiligo patients.

The genotype–phenotype correlation analyses for *G6PD* activity and LPO levels were performed with respect to *G6PD* 3' UTR A/G genotypes. Individuals with GG and AG genotypes showed significant decrease in G6PD activity compared to individuals with AA genotype (Mean ± SEM: 0.693 ± 0.078 and 0.834 ± 0.064 *vs.* 1.052 ± 0.077; $p = 0.018$ and $p = 0.049$ respectively; Fig. 1B). Interestingly, individuals with GG and AG genotypes showed significant increase in LPO levels as compared to individuals with AA genotype (Mean ± SEM: 264.9 ± 27.13 and 229.7 ± 10.04 *vs.* 201.2 ± 8.887; $p = 0.016$ and $p = 0.034$ respectively; Fig. 1C). In addition, patients with GG ($p = 0.039$) and AG ($p = 0.012$) genotypes showed early age of onset compared to patients with AA genotype (Fig. 1D). These results suggest a possible role of rs1050757G in development and progression of vitiligo. rs1050757 is located in the 35bp AG-rich region of *G6PD* 3'UTR [3]. Amini and Ismail [3] have reported that the transition of A to G caused significant alterations in the secondary structure of *G6PD* transcript creating two more binding sites for hsa-miR-1238 and hsa-miR-877* where, rs1050757 G is located inside the 'seed region' of these miRNAs. The new conformation of mRNA might place some sequences in the open structures such as loop or arc; therefore, these sequences are no longer involved in base pairing and they are accessible to miRNAs. Previously we have shown miR-1 mediated *G6PD* regulation in vitiligo [3]. The present study speculates possible miRNA mediated mRNA degradation and/or translational repression of *G6PD* resulting into decreased *G6PD* activity in vitiligo patients.

To study the effect of G6PD inhibition on melanocytes, NHM, PIG1 and PIG3V melanocytes were treated with different concentrations of 6-ANAD (0.5, 1, 2, 4 & 5 mM) and viability was observed after 24 h (n = 3). The cells showed significant decreased viability at 0.5 mM 6-ANAD [NHM: $p = 0.0007$, 70.55 ± 1.03%; PIG1: $p = 0.014$,

Abbreviations: G6PD, glucose 6-phosphate dehydrogenase; 6-ANAD, 6-aminonicotinamide; miRNA, microRNA; 3'UTR, 3'-untranslated region; SNP, single nucleotide polymorphism; AV, active vitiligo; NSV, non-segmental vitiligo; SV, segmental vitiligo; LPO, lipid peroxidation; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism; ARMS-PCR, amplification refractory mutation system-restriction fragment length polymorphism; CAT, catalase; GPX1, glutathione peroxidase 1; EDN1, endothelin 1; HSP60, heat shock protein 60; HSP70, heat shock protein 70; SERP1, stress-associated endoplasmic reticulum protein 1; SIRT1, sirtuin 1; TYR, tyrosinase; POLH, polymerase (DNA directed), eta; EZR, ezrin; LAMP1, lysosome-associated membrane protein 1; PRDX3, peroxiredoxin 3; TRPM1, transient receptor potential cation channel, subfamily M, member 1; TYRP1, tyrosinase-related protein 1; MITF, microphthalmia-associated transcription factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

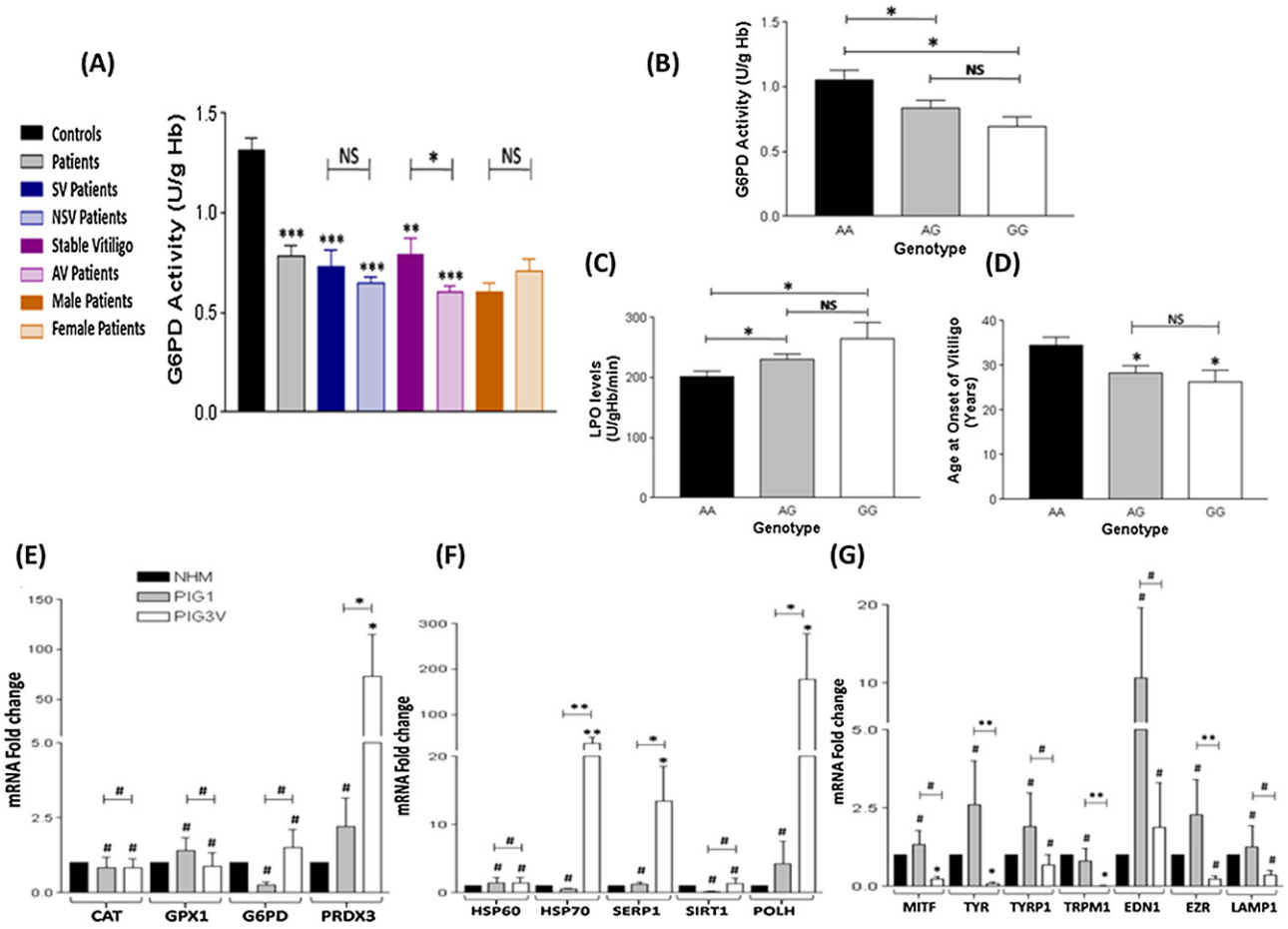

Fig. 1. (A) G6PD activity in vitiligo patients: Vitiligo patients showed significantly decreased G6PD activity in erythrocytes, as compared to controls ($p < 0.0001$). SV, NSV, stable and AV patients showed significantly decreased G6PD activity, as compared to controls ($p = 0.0001$, $p < 0.0001$, $p = 0.004$ and $p < 0.0001$ respectively). AV patients showed significantly decreased G6PD activity, as compared to stable ($p = 0.016$). No difference in G6PD activity was observed for SV *vs.* NSV ($p = 0.294$) and male *vs.* female patients ($p = 0.120$). [AV: Active Vitiligo; NSV: Non-segmental vitiligo; SV: Segmental vitiligo]; **(B–D) Genotype-phenotype correlation analysis for G6PD** 3' **UTR A/G polymorphism: (B) G6PD activity:** Individuals with GG and AG genotypes showed significantly decreased G6PD activity compared to individuals with AA genotype ($p = 0.018$ and $p = 0.049$ respectively). **(C) LPO levels:** Individuals with GG and AG genotypes showed significantly increased LPO levels as compared to individuals with AA genotype ($p = 0.016$ and $p = 0.034$ respectively). **(D) Age of onset:** Patients with the GG ($p = 0.039$) and AG ($p = 0.012$) genotypes showed early age at onset compared with AA genotype. However, there was no significant difference in age of onset between vitiligo patients with AG and GG genotypes ($p = 0.572$); **(E–G) Gene expression profiles of primary NHM, PIG1 and PIG3V cells after 24 h of 0.5 mM 6-ANAD treatment: (E) Anti-oxidants:** PIG3V cells showed significantly increased expression of PRDX3 as compared to NHM and PIG1 cells. There was no difference observed in the expression of CAT, GPX1, and G6PD among NHM, PIG1, and PIG3V cells. **(F) Stress-related genes:** PIG3V cells showed significantly increased expression of HSP70, SERP1, and POLH as compared to NHM and PIG1 cells. There was no difference observed in the expression of HSP60 and SIRT1 among NHM, PIG1, and PIG3V cells. **(G) Melanocyte specific genes:** PIG3V cells showed significantly decreased expression of MITF, TYR and TRPM1 as compared to NHM. Also, PIG3V cells showed significantly decreased expression of TYR, TRPM1 and EZR as compared to PIG1 cells. There was no difference observed in the expression of TYRP1, EDN1 and LAMP1 among NHM, PIG1 and PIG3V cells [$^{*}p < 0.05$; $^{**}p < 0.01$; #$p > 0.05$ or non-significant].

$69.40 \pm 6.15\%$; PIG3V $p = 0.0006$, $58.93 \pm 3.95\%$ (mean $\pm$ SEM)] as compared to respective untreated cells (Fig. 1C). mRNA expression levels of anti-oxidant genes, stress related genes and melanocyte specific genes were monitored in NHM, PIG1 and PIG3V cells upon 0.5 mM 6-ANAD treatment (24 h). PIG3V cells showed significantly increased expression of *PRDX3* as compared to NHM ($p = 0.017$) and PIG1 cells ($p = 0.027$; Fig. 1E). PIG3V cells showed significantly increased expression of *HSP70, SERP1* and *POLH* as compared to NHM ($p = 0.004$, $p = 0.018$ and $p = 0.012$ respectively; Fig. 1F) and PIG1 cells ($p = 0.002$, $p = 0.034$ and $p = 0.039$ respectively). The PIG3V cells showed significantly decreased expression of *MITF, TYR* and *TRPM1* as compared to NHM ($p = 0.048$, $p = 0.046$ and $p = 0.013$ respectively; Fig. 1G). Also, PIG3V cells showed significantly decreased expression of *TYR, TRPM1* and *EZR* as compared to PIG1 cells ($p = 0.004$, $p = 0.028$ and $p = 0.009$ respectively). For detailed results, please see 'Supporting information'. Overall, these results showed that PIG3V melanocytes were more susceptible to 6-ANAD as suggested by decreased cell viability as compared to PIG1 and NHM. Further, PIG3V melanocytes showed significantly decreased expression of *CAT, MITF, TYR, TRPM1* and/ *EZR* as compared to NHM in response to 6-ANAD, indicating impaired homeostasis of vitiligo melanocytes. Whereas, expressions of *PRDX3, HSP70, SERP1* and *POLH* were elevated in PIG3V cells, suggesting their protective role during oxidative stress induced by G6PD inhibition. For detailed discussion, please see 'Supporting information' file.

In conclusion, the present study suggests genetic and biochemical association of *G6PD* 3'UTR rs1050757 polymorphism with vitiligo in Gujarat population. In addition, the melanocytes from individuals with vitiligo are more sensitive to G6PD inhibition

Table 1
Distribution of genotype and allele frequencies for *G6PD* polymorphisms in Gujarat vitiligo patients and controls.

SNP	Genotype or Allele	Controls (n = 449)	Patients (n = 366)	*p* value[a] for Association	OR	95% CI
3'UTR A/G (rs1050757)	AA	239 (0.53)	142 (0.39)	R	1	
	AG	170 (0.38)	181 (0.49)	<0.0001	1.792	1.334–2.407
	GG	40 (0.09)	43 (0.12)	0.014	1.809	1.122–2.919
	A	648 (0.72)	465 (0.64)	R	1	
	G	250 (0.28)	267 (0.36)	0.0002	1.488	1.207–1.836
Exon 6 C/T (Ser218Phe; rs5030868)	CC	444 (0.99)	361 (0.986)	R	1	
	CT	3 (0.006)	4 (0.011)	0.515	1.640	0.364–7.377
	TT	2 (0.004)	1 (0.003)	0.689	0.615	0.055–6.813
	C	891 (0.99)	726 (0.99)	R	1	
	T	7 (0.01)	6 (0.01)	0.928	1.052	0.351–3.145
Exon 11 C/T (Tyr467Tyr; rs2230037)	CC	446 (0.993)	363 (0.992)	R	1	
	CT	3 (0.007)	3 (0.008)	0.801	1.229	0.246–6.126
	TT	0 (0.0)	0 (0.0)	–	–	–
	C	895 (0.99)	729 (0.99)	R	1	
	T	3 (0.003)	3 (0.004)	0.802	1.228	0.247–6.103

n: number of subjects; R: reference group; OR: Odds Ratio; CI: Confidence Interval.
[a] Vitiligo Patients *vs.* Controls using chi-squared test with 2 × 2 contingency table.

compared to normal melanocytes. Therefore, vitiligo may result from an insufficient response to oxidative stress and impaired G6PD levels.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2018.12.001.

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Mohmmad Shoab Mansuri, Mala Singh, Shahnawaz D. Jadeja, Rasheedunnisa Begum*
Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara 390002, Gujarat, India

* Corresponding author.
E-mail address: rasheedunnisab@yahoo.co.in (R. Begum).

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Letter to the Editor

Simple cell culture media expansion of primary mouse keratinocytes

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Keratinocytes are the majority cell population in the epidermis and protect the body from the environment by forming stratified layers with successively differentiated keratinocytes. The study of keratinocyte biology has yielded many important insights, both through *in vivo* mouse models, and *in vitro* culture systems. Given the highly dynamic genetic systems available for *in vivo* mouse investigation, it is important to culture primary mouse keratinocytes derived from these models in isolation for further mechanistic study *in vitro*. Despite the need, culturing mouse primary keratinocytes has been difficult.

With existing protocols for the isolation and culture of mouse keratinocytes (MKC) [1–3], cells often do not passage consistently, and often lose features of undifferentiated basal layer cells. Moreover, many protocols in the literature are inconvenient because of either the use of feeder cells, or the use of a large repertoire of individual distinct supplements such as growth factors. Previously, Chapman et al. found that Y-27632, a Rho kinase inhibitor, robustly enhances proliferative capacity and maintains original characteristics of human keratinocytes [4]. However, the effect of Y-27632 on culturing primary mouse keratinocytes has not been reported. Given the deficiencies in current common techniques, our goal was to test simple cell culture media preparations using the Y-27632 to streamline primary cultures of mouse keratinocytes.

Rho kinases (ROCKs) are the first downstream mediator of the GTP-binding protein RhoA and have pleiotropic functions including the regulation of cellular contraction, migration, morphology, polarity, and cell division [5]. ROCKs exert their functions by phosphorylating different substrates such as myosin light chain (MLC), LIM kinase (LIMK), and phosphatase and tensin homologue