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Enzymatic Changes in Red Blood Cells of Diamond-Blackfan Anemia

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Diamond-Blackfan anemia is a congenital bone marrow failure syndrome characterized by red blood cell (RBC) aplasia with varied malformations in infants. Elevated activity of adenosine deaminase (ADA) has been considered as a useful biomarker of Diamond-Blackfan anemia, and ADA assay has been shown to be more sensitive than genetic diagnosis. Approximately, 80% of the examined patients showed elevated ADA activity, whereas genetic tests of ribosome subunit genes identified mutations in approximately 60% of the patients. We previously reported that reduced glutathione (GSH) levels in RBCs may serve as a biomarker of Diamond-Blackfan anemia. In this study, to confirm the universality of our data, we extended the analysis to seven RBC enzymes and GSH of 14 patients with Diamond-Blackfan anemia and performed a cross-analysis study using enzyme activity assay and recently reported proteome data. Statistical analysis revealed that both data exhibited high similarity, upregulation in the hexokinase and pentose-phosphate pathway, and downregulation in glycolytic enzymes such as phosphofructokinase and pyruvate kinase, in the RBCs obtained from the subjects with Diamond-Blackfan anemia. The only discrepancy between enzyme activity and proteome data was observed in glucose-6-phosphate dehydrogenase (G6PD), as increased G6PD activity showed no relation with the significant elevation in protein levels. These results suggest that our enzymatic activity data of Diamond-Blackfan anemia are universal and that the enzymatic activation of G6PD via a hitherto-unveiled mechanism is another metabolic feature of RBCs of Diamond-Blackfan anemia.

Keywords: Diamond-Blackfan anemia; glycolysis; oxidative stress; proteome; red blood cell enzymes
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Introduction

Diamond-Blackfan anemia (DBA) is an extremely rare form of congenital bone marrow failure syndrome diagnosed at a frequency of several to tens of cases per million births (Ohga et al. 2004; Vlachos et al. 2008). The pathogenesis of DBA has been extensively studied, and genetic analysis has revealed the existence of gene mutations in ribosome subunit proteins (RPs). Thus far, 20 different ribosome gene mutations have been identified (Da Costa et al. 2018) since the first observation of a mutation in the *RPS19* gene (Draptchinskaia et al. 1999).

Although translation is an essential physiological step in gene expression, the phenotype of DBA caused by RP gene mutations is mainly limited to anemia owing to the disturbance occurring in erythroid differentiation/maturation (Narla and Ebert 2010). The RP gene mutation destroys the ribosome assemblies and induces the migration of ribosomal protein L11 (RPL11) and/or RPL5 from the cytoplasm into the nucleus. The subsequent binding of RPL11/RPL5 to MDM2 results in the inhibition of MDM2-mediated suppression of p53, eventually leading to cell cycle arrest mediated by p53 activation (Gazda et al. 2008; Zheng et al. 2015). Additionally, hemoglobin (Hb) production, the major protein produced in RBCs, depends on the coordinated synthesis of heme and globin (Doty et

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al. 2015), and defects in the translation of the globin chains account for the accumulation of free heme in RBC cytoplasm, leading to cell death mediated by reactive oxygen species derived from excess heme accumulation.

RBC adenosine deaminase (ADA) activity has served as a useful biomarker in DBA diagnosis since the first report authored by Glader et al. (1983), and high RBC ADA activity is listed as one of the supporting criteria recently published by the US DBA registry group (Vlachos et al. 2008). We determined ADA activity in Japanese DBA cases and found that approximately 20% of the patients showed no elevation despite being diagnosed with DBA based on clinical symptoms (Utsugisawa et al. 2016), blood/bone marrow findings, and RP gene mutation analysis. Only 60% of the DBA cases showed RP gene mutations, suggesting the need for establishing a more sensitive and specific screening test in clinics.

Reduced glutathione (GSH) is the most important antioxidant present in red blood cells. GSH is biosynthesized via a two-step reaction involving γ-glutamylcysteine synthetase (GC-S) and glutathione synthetase (GSH-S). GSH is converted to oxidized glutathione (GSSG) by the enzymatic reaction of glutathione peroxidase (GSH-Px) to prevent peroxidation of proteins and lipids in erythrocytes, and GSSG is reduced to GSH by the action of glutathione reductase (GR). The GR coenzyme, NADPH, is produced by the pentose phosphate pathway via enzymatic reactions by glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD). In cases of deficiencies of GC-S, GSH-S, or G6PD, a decrease in GSH level has been observed, i.e. a marked decrease in GC-S and GSH-S deficiencies, and a slight decrease in G6PD deficiency.

We have reported that GSH level is elevated in most DBA cases examined, and that the simultaneous measurement of ADA and GSH activities enables the diagnosis of DBA (Utsugisawa et al. 2016). Subsequent enzymatic analysis showed an elevation in G6PD, 6PGD, and GSH-Px levels, but GC-S and GSH-S activity remained unchanged. Based on these observations, we suggest that the activation of the pentose-phosphate pathway, but not the upregulation in the *de novo* synthesis of GSH, is attributed to the high GSH content, a characteristic of RBCs derived from patients with DBA. Additionally, high GSH-Px activity may be reflected in the increased requirement for detoxification of hydrogen peroxide (Utsugisawa et al. 2016).

Pesciotta et al. (2015) recently reported data involving proteome analysis using Hb-free cytosolic proteins and showed that several protein levels increased in RBCs, including those of ADA and Hb subunit gamma, both of which are known to be upregulated in DBA (Pesciotta et al. 2015). These authors also identified the previously undescribed upregulated/downregulated proteins and suggested that the pattern of alterations in cytosolic protein levels in DBA was similar to that reported after the elicitation of inflammatory responses. The proteome analysis led to the acquisition of comprehensive data including those on the cytosolic enzymes assayed, thereby enabling the performance of a cross-analysis that confirmed the data obtained in our enzyme-based study. The metabolic changes occurring in RBCs in response to DBA have been also discussed.

Materials and Methods

Patient samples

Fourteen patients with DBA and fourteen control subjects were included in the study. All clinical samples were obtained with informed consent from 14 pediatric and/or hematology departments throughout Japan. The participants included patients aged < 16 years. Written informed consent was obtained from the legal guardian. To reduce contamination arising from transfused RBCs, blood sampling was performed after a minimum interval of 4 weeks following the previous RBC transfusion. Gene mutations in the enrolled patients have been previously confirmed. This study was approved by the Ethics Committee of Tokyo Women's Medical University Graduate School of Medicine.

Analysis of RBC enzyme activities

We determined GSH content (Miwa et al. 1989) and the activities of the following RBC enzymes by adopting the standard method using a spectrophotometer (Beutler et al. 1977) : ADA, G6PD, 6PGD, GSH-Px, HK, PFK, and PK. All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) or Boehringer-Mannheim (Mannheim, Germany).

Cross-analysis

We performed a cross-analysis using our enzymatic data and the proteome data described by Pesciotta et al. (2015) We selected data on five patients with DBA and six normal subjects from the study reported by Pesciotta et al. (2015), as one of the six patients exhibited responses to the treatment. Isozymes are tissue (cell)-specific enzymes expressed in the form of HK, PFK, and PK. Since *HK1*, *PFKM*, *PFKL*, and *PKLR* are expressed in erythrocytes, respectively, we examined the variability of the isozymes in erythrocytes of DBA patients. We calculated the relative activity and content of the following RBC cytosolic enzymes: ADA, HK-1, PFK (muscle- and liver-type [PFKM and PFKL, respectively]), PK isoenzymes R/L (PKLR), G6PD, and 6PGD. Statistical analyses were performed using the Wilcoxon rank-sum test. P-values < 0.001 were considered significant.

Results

To deduce the enzymatic profile of RBCs and GSH contents in patients with DBA, we reanalyzed the cases of 14 patients diagnosed with DBA by investigating the mutations in RP genes (Table 1) and compared using the data derived from 14 normal subjects. As shown in a

Table 1. Reduced glutathione (GSH) and adenosine deaminase (ADA) of the Diamond-Blackfan anemia patients with ribosomal protein (RP) gene mutations.

Patient	RP genemutation	GSH (mg/gHb)	ADA (IU/gHb)
1	*RPL5*	106	2.97
2		92.0	5.09
3		97.0	5.91
4		100	3.09
5	*RPL11*	118	10.0
6		122	7.73
7		124	4.99
8	*RPL35a*	108	1.75
9		114	1.87
10	*RPS10*	103	2.96
11	*RPS19*	114	0.95
12		92.3	1.93
13		93.4	3.59
14		96.5	2.64

The reference values: GSH, 62.4-98.9 mg/gHb; ADA, 0.46-1.50 IU/gHb.

previous study (Utsugisawa et al. 2016), a significant elevation in the levels of GSH, ADA, G6PD, 6PGD, and GPX was observed. We also evaluated the levels of enzymes such as HK, PFK, and PK (Table 2). The comparison of the mean values between DBA subjects and controls revealed an elevation in the levels of GSH, ADA, G6PD, 6PGD, and GPX, as previously observed. The elevated activity of HK and decreased activity of PFK and PK were statistically significant (Fig. 1).

Based on the supplemental data reported in the relevant paper authored by Pesciotta et al. (2015), we found a significant increase in the protein level of ADA, HK1, and 6PGD. In contrast, a significant decrease in the levels of PFKL and PKLR was reported. PFKM, an isoenzyme expressed in RBCs, showed no significant changes (Table 3). The average and variance of protein amount and the area under the curve (AUC) based on logistic regression were calculated for the two groups. In the item with AUC of 1, the patient group and the control group were completely separable and data on HK1, PFKL, 6PGD, and PKLR were included in addition to ADA.

The protein level of ADA in the RBCs from the DBA patients was approximately three times higher than that in the RBCs derived from normal subjects in the cited article. In contrast, the enzymes involved in GSH synthesis, such as GCLS, GCLM, and GSH-S, showed no changes between the patient and control groups, as indicated in our previous report. Both enzymes from the pentose phosphate pathway, G6PD and 6PGD, showed a significant increase in their activities. However, only an increase in the protein amount of 6PGD was significant in this analysis.

Table 4 shows a summary of the cross-analysis of proteomic and enzymatic studies of RBC enzymes in DBA. Combined proteomic and enzymatic data analyses for 19 patients with DBA and 20 control subjects are shown. ADA, 6PGD, and HK showed elevated levels and enzymatic activities. PFK and PK showed decreased activity and protein levels. G6PD showed elevated enzyme activity, but no change in its protein levels was reported.

Consistent with previous reports (Utsugisawa et al. 2016), cases with RPL11 mutations presented with higher levels of ADA and GSH. G6PD, 6PGD, and HK levels tended to be higher in cases with the RPL5 mutation, but no such tendency was observed for the most common mutation, RPS19. For other mutations, the tendency could not be defined because of the small number of cases investigated (Fig. 2).

Discussion

Premature erythroid cell death is considered the primary cause of DBA (Dutt et al. 2011), and extensive studies have been undertaken to understand the underlying mechanism. The clinical diagnosis of DBA is gaining

Table 2. The activities of the red blood cell enzymes in Diamond-Blackfan anemia (DBA).

	DBA (mean ± SD, n = 14)	Control (mean ± SD, n = 14)	Reference value (/gHb)	DBA / Control	p-value
GSH	105.7 ± 11.1	82.3 ± 6.7	62.4-98.9 (mg)	1.28	< 0.001
ADA	4.0 ± 2.5	1.2 ± 0.3	0.46-1.50 (IU)	3.19	< 0.001
G6PD	11.1 ± 2.7	9.2 ± 1.2	6.20-9.70 (IU)	1.2	0.024
6PGD	13.5 ± 2.2	10.5 ± 1.0	7.58-11.3 (IU)	1.29	< 0.001
GSH-Px	45.2 ± 11.9	42.7 ± 5.0	30.1-52.7 (IU)	1.06	0.487
HK	2.5 ± 0.8	1.4 ± 0.2	0.84-1.94 (IU)	1.86	< 0.001
PFK	14.0 ± 1.7	18.3 ± 2.4	10.2-21.0 (IU)	0.76	< 0.001
PK	12.8 ± 3.8	19.3 ± 3.9	13.7-21.0 (IU)	0.67	< 0.001

GSH, reduced glutathione; ADA, adenosine deaminase; G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; GSH-Px, glutathione peroxidase; HK, hexokinase; PFK, 6-phosphofructokinase; PK, pyruvate kinase.

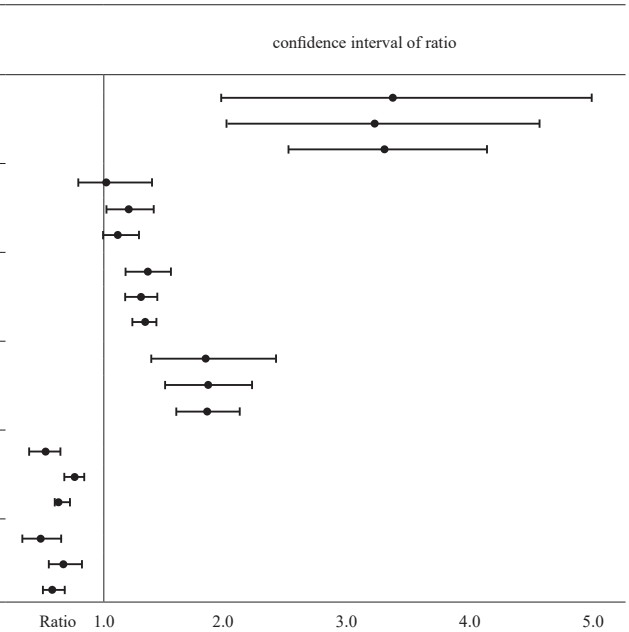
Table 3. The proteome analysis of the red blood cell enzymes in Diamond-Blackfan anemia (DBA).

Protein names	Gene names	DBA mean	Control mean	DBA/Control-mean ratio	p-value
Adenosine deaminase	*ADA*	3,350,100	1,004,465	3.33	0.008*
Glucose-6-phosphate 1-dehydrogenase	*G6PD*	13,717,400	13470450	1.01	0.930
6-phosphogluconate dehydrogenase, decarboxylating	*PGD*	92,231,700	6.84E+07	1.35E+00	0.008*
Glutathione peroxidase 1	*GPX1*	23,008,100	1.56E+07	1.48E+00	0.120
Hexokinase-1	*HK1*	2,706,940	1,471,817	1.83	0.008*
Glucose-6-phosphate isomerase	*GPI*	18,113,200	2.40E+07	7.56E-01	0.648
6-phosphofructokinase, muscle type	*PFKM*	2,497,760	3.50E+06	7.15E-01	0.055
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	154,874,000	1.24E+08	1.25E+00	0.022*
Phosphoglycerate kinase 1	*PGK1*	134,420,000	1.32E+08	1.02E+00	0.315
6-phosphofructokinase, liver type	*PFKL*	10,226,070	1.93E+07	5.30E-01	0.008*
Pyruvate kinase isozymes M1/M2	*PKM*	872,956	354,031.667	2.47E+00	0.522
Pyruvate kinase isozymes R/L	*PKLR*	14,960,450	3.08E+07	4.86E-01	0.008*
Glutamate cysteine ligase catalytic subunit	*GCLC*	36,084,100	4.36E+07	8.28E-01	0.235
Glutamate cysteine ligase regulatory subunit	*GCLM*	11,010,270	1.30E+07	8.49E-01	0.082
Glutathione synthetase	*GSS*	11,564,040	1.48E+07	7.79E-01	0.648
Adenylate kinase isoenzyme 1	*AK1*	45,354,600	6.33E+07	0.71	0.035

Representative data derived from the proteome analysis of red blood cell (RBC) enzymes reported by Pesciotta et al. (2015).
*p-values < 0.01 were considered statistically significant.

Table 4. Cross-analysis of proteome and activities of the red blood cell (RBC) enzymes in Diamond-Blackfan anemia (DBA).

			p-value	DBA/Control mean ratio	95% confidence interval upper limit	95% confidence interval lower limit
ADA	Proteome	ADA	0.008	3.34	1.94	4.95
	Enzyme	ADA	< 0.001	3.19	1.98	4.53
	Integrated	ADA/ADA	< 0.001	3.26	2.49	4.10
G6PD	Proteome	G6PD	0.930	1.02	0.79	1.39
	Enzyme	G6PD	0.024	1.20	1.02	1.39
	Integrated	G6PD/G6PD	0.097	1.11	0.99	1.28
6PGD	Proteome	PGD	0.008	1.35	1.17	1.55
	Enzyme	PGD	< 0.001	1.29	1.16	1.43
	Integrated	PGD/PGD	< 0.001	1.32	1.22	1.42
HK	Proteome	HK1	0.008	1.84	1.40	2.41
	Enzyme	HK	< 0.001	1.86	1.51	2.22
	Integrated	HK1/HK	< 0.001	1.85	1.60	2.11
PFK	Proteome	PFKL	0.008	0.53	0.41	0.66
	Enzyme	PFK	< 0.001	0.76	0.69	0.84
	Integrated	PFKL/PFK	< 0.001	0.65	0.62	0.74
PK	Proteome	PKLR	0.008	0.49	0.34	0.66
	Enzyme	PK	< 0.001	0.67	0.54	0.81
	Integrated	PKLR/PK	< 0.001	0.58	0.49	0.68



ADA, adenosine deaminase; G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; HK, hexokinase; PFK, 6-phosphofructokinase; PK, pyruvate kinase.

momentum through the consideration of clinical features, laboratory data, and genetic testing. Elevated ADA activity in RBCs was reported by Glader et al. (1983); it reportedly serves as a biomarker, which is more sensitive than the genetic testing of RP genes. The criteria proposed by the US DBA registry are useful in clinics (Vlachos et al. 2008). However, hematologists continue to face difficulties in the diagnosis of patients with atypical DBA when both ADA activity and RP gene tests show normal results. In our previous study, we demonstrated that GSH was a novel biomarker of DBA (Utsugisawa et al. 2016; Ichimura et al. 2017; Noguchi et al. 2017; Sonoda et al. 2018). Hence, the

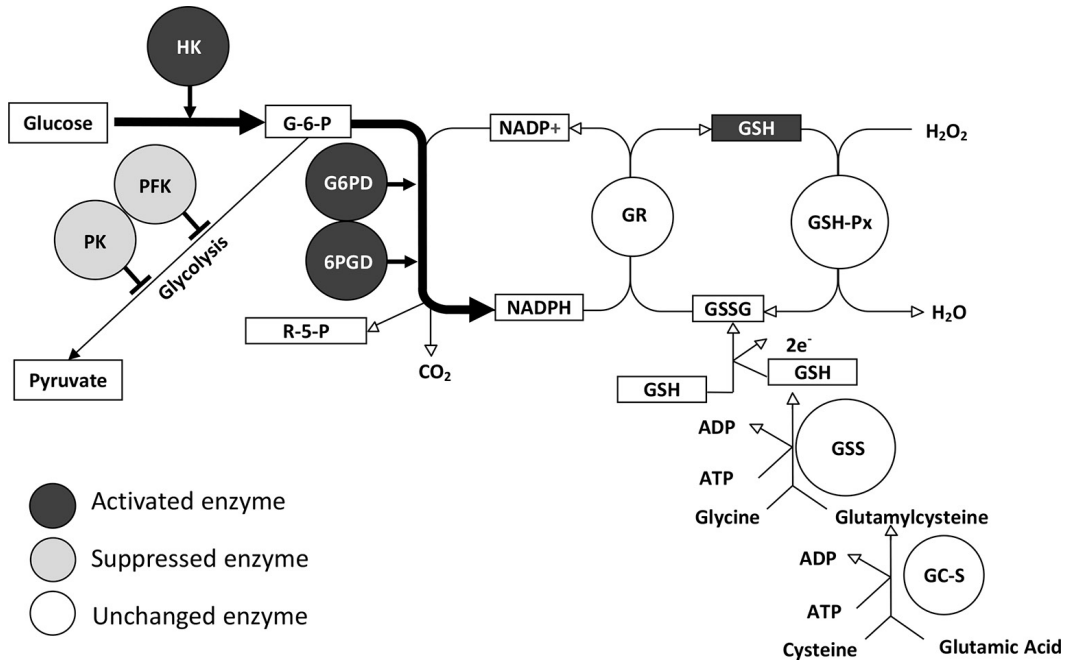


Fig. 1. Glycolytic repression and pentose phosphate pathway activation in Diamond-Blackfan anemia (DBA) erythrocytes. HK, hexokinase; PFK, 6-phosphofructokinase; PK, pyruvate kinase; G6PD, glucose-6-phosphate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; GR, glutathione reductase; GSH-Px, glutathione peroxidase; GSS, glutathione synthetase; GC-S, gamma glutamyl cysteine synthetase; GSH, reduced glutathione; GSSG, glutathione disulfide.

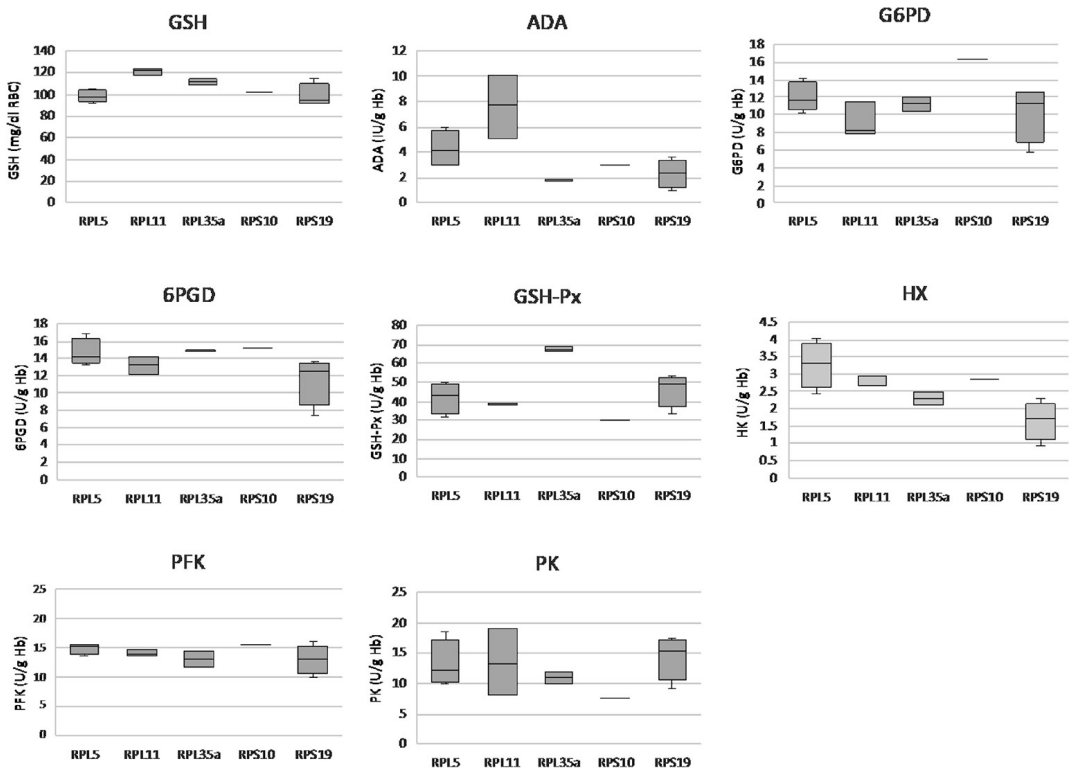


Fig. 2. Association of ribosomal protein (RP) gene mutations and reduced glutathione (GSH) or red blood cell (RBC) enzyme activities in Diamond-Blackfan anemia (DBA) patients. The shaded area indicates the reference value. Reference value: reduced glutathione (GSH), 62.4-98.9 mg/dl RBC; adenosine deaminase (ADA), 0.46-1.50 IU/gHb; glucose-6-phosphate dehydrogenase (G6PD), 6.20-9.70 IU/gHb; 6-phosphogluconate dehydrogenase (6PGD), 7.58-11.3 IU/gHb; glutathione peroxidase (GSH-Px), 30.1-52.7 IU/gHb; hexokinase (HK), 0.84-1.94 IU/gHb; 6-phosphofructokinase (PFK), 10.2-21.0 IU/gHb; pyruvate kinase (PK), 13.7-21.0 IU/gHb.

simultaneous assessment of ADA and GSH may facilitate distinguishing between patients with DBA and unaffected family members, as well as normal subjects.

GSH is synthesized via two enzymatic reactions catalyzed by two enzymes (Wu et al. 2004), GC-S and GSH-S, and undergoes conversion from GSSG to prevent peroxidation of RBC proteins and lipids (Kondoh et al. 2007). GSSG is reduced by GR for reuse. NADPH, a coenzyme of GR, is produced by the reduction of $NADP^+$ by G6PD during the initial stage of the pentose phosphate pathway. To clarify the cause of the elevation of GSH in RBCs of DBA patients, we determined the activities of six enzymes and found that the activities of G6PD, 6PGD, and GPX were elevated. Furthermore, GC-S and GSH-S levels were unchanged, suggesting that GSH was presumably increased by upregulating the reducing reaction of GSSG, but not by promoting the *de novo* synthesis of GSH (Raftos et al. 2010). It seems reasonable that activation of the pentose phosphate pathway is necessary for the reduction of GSSG, as NADPH production is inevitably required. An increase in GSH-Px activity is supported by the upregulation of GSH levels. In the cross-analysis reported in the present study, we noticed that the protein level of G6PD did not increase significantly, suggesting that the discrepancy between activity and protein levels was attributable to the oxidation/reduction states of G6PD (Luzzatto 1967; Taniguchi et al. 2016), the glycosylation states of G6PD (Rao et al. 2015), the post-translational deacetylated state (Wang et al. 2014), and the stimulation caused by the phosphorylation of G6PD (Matte et al. 2020).

Premature death of erythroblasts in DBA is caused by cell cycle arrest in response to p53 activation, which induces the suppression of the glycolytic pathway (Danilova et al. 2011). In the present study, we examined the activity of glycolytic enzymes, HK, PFK, and PK, in RBCs derived from patients with DBA and normal control subjects. The levels of these enzymes were determined by referring to the proteomic data by Pesciotta et al. (2015). The activity and levels of HK enzymes were significantly elevated. On the contrary, two important rate-limiting enzymes of glycolysis, PFK and PK, showed decreased activity and enzyme levels. The high demand for NADPH production through the pentose phosphate pathway necessitates a decrease in the consumption of glucose in the glycolytic pathway. Furthermore, the activation of p53 leads to the suppression of the glycolytic pathway, as previously reported (Bensaad et al. 2006). Although we did not examine the RBC metabolome in DBA, the present data indicated the suppression of glycolysis in the RBCs of DBA subjects.

Several isozymes are involved in the glycolytic pathway. The activity of HK in RBCs is associated with the type 1 isozyme, HK1. Two isoenzymes are known to be expressed at almost the same levels in PFK, namely liver-type and muscle-type isozymes, and they are encoded by different structural genes, namely *PFKM* and *PFKL*. The two types of PK isozymes are expressed in the early stage of erythropoiesis, and the R-type PK encoded by *PKLR* is suggested to be exclusively expressed in mature RBCs. In the proteomic analysis data, the amount of PFKL protein significantly decreased, but the PFKM level remained unchanged. Moreover, the expression level of PKLR protein also decreased, indicating that the expression levels of the two key rate-limiting enzymes of glycolysis were downregulated in RBCs. The decreased expression of PKLR is the most prevalent cause of congenital hemolytic anemia among the defects of the glycolytic pathway. We have previously demonstrated that ineffective erythropoiesis may be partly responsible for the pathogenesis of PK deficiency (Aizawa et al. 2005). Hematologically, RBC aplasia and hemolysis exhibit distinct pathological conditions, and PK activity in patients with DBA may not be as profoundly decreased, as observed in hemolytic anemia associated with PK deficiency (Bianchi et al. 2019). It was also noted that the expression of isoenzyme PFKL was decreased in DBA. PFK deficiency is one of the rare causes of congenital hemolytic anemia owing to the occurrence of glycolytic defects, and the patients analyzed thus far have shown mutations in *PFKM*. The physiological significance of PFKL isozymes in RBCs remains unknown.

We performed the metabolic study to analyze the RBCs derived from patients with DBA to identify a useful biomarker for DBA diagnosis. We confirmed the alteration in RBC enzymatic activity using an increased number of patients and normal subjects. Thus far, no definitive explanation on ADA activity and increase in the GSH content in the RBCs derived from patients with DBA has been reported. Furthermore, the mechanism by which RP gene mutations cause cell death in a particular lineage of hematopoietic cells remains unknown. It is important for us to detect the key metabolic changes in the pathological pathway leading to DBA, and hence, we will continue to comprehensively analyze the metabolome of DBA in future studies.

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Author Contributions

Taiju Utsugisawa, Toshitaka Uchiyama, and Hitoshi Kanno wrote the manuscript. Keiko Shimojima-Yamamoto, Tsutomu Toki, Shouichi Ohga, and Etsuro Ito analyzed the data and edited the manuscript. All authors approved the final version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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