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Erythrocyte glutathione is a novel biomarker of Diamond-Blackfan anemia

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ABSTRACT

Diamond-Blackfan anemia (DBA) is a congenital red cell aplasia with mutations in ribosomal protein (RP) genes. Elevated activity of erythrocyte adenosine deaminase (eADA) has been utilized as a biomarker of DBA. We examined erythrocyte reduced glutathione (GSH) as well as eADA in 22 patients in 18 DBA families, in whom RP gene mutations had been identified. Simultaneous evaluation of both eADA and GSH demonstrated that all examined DBA patients showed elevated values of either eADA or GSH, whereas presence of both eADA and GSH elevation was able to distinguish DBA patients from 34 normal controls and 14 unaffected members of the DBA families. Furthermore, a support vector machines analysis using both eADA and GSH levels yielded a formula to differentiate DBA from both normal controls and non-DBA family members. To confirm the usefulness of the formula, we analyzed additional 7 patients diagnosed by the clinical criteria. Although eADA showed within normal values in 3 patients, all of these patients were diagnosed as 'DBA' by use of the formula. Because extensive analysis of the RP genes failed to detect no causative mutation in approximately 40% of clinically diagnosed DBA patients, GSH may be useful an additional biomarker for diagnosis of DBA.

1. Introduction

Diamond-Blackfan anemia (DBA) is a rare congenital red cell aplasia characterized by growth retardation and congenital malformations [1,2]. The annual incidence is 4.02 cases per million births in Japan [3]. Although the diagnostic and supporting criteria of DBA have been proposed [4], phenotypic heterogeneity often hampers a correct diagnosis. Infants with classical DBA who fulfill the criteria are not difficult to diagnose. On the other hand, non-classical DBA patients over 1 year of age showing mild anemia but no morphological abnormality require genetic study before prolonged corticosteroid therapy [5]. Mutations or large deletions in 13 ribosomal protein (RP) genes are currently thought to be the causative genes of DBA. However, the elaborate screening process of these reported RP genes identifies abnormalities in up to 60% of the patients [6–16]. Elevated activity of erythrocyte adenosine deaminase (eADA) has long been utilized as a biomarker for the differential diagnosis of DBA [17], but 16% of DBA patients are reportedly eADA-negative [18].

We assayed eADA in Japanese DBA subjects and normal controls, and in most cases, heparinized blood samples were dispatched from distant hospitals under refrigerated conditions. To assess sample condition after such transport, we routinely assayed for reduced glutathione (GSH) in blood samples of both patients and controls, because erythrocyte GSH is reportedly decreased during storage [19]. Unexpectedly, we found that GSH concentrations of DBA patients were increased in some cases. To examine whether GSH may be used as another biomarker for DBA, we analyzed GSH as well as eADA in 22 patients of 18 DBA families, in whom we had identified gene mutations in RPS19, RPL5, RPL11, RPS10, RPS17, or RPS35a.

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2. Materials and methods

2.1. Patient samples

Twenty-two DBA patients from 18 families were studied. All clinical samples were obtained with informed consent from 12 pediatric and/or hematology departments throughout Japan. To avoid misinterpretation of biochemical measurements, blood was sampled just before transfusion in regularly transfused DBA patients and after a minimum of a 4-week period following previous red blood cell transfusion. Mutation analyses of the DBA families were performed as previously described by Kuramitsu et al. [20]. and Konno et al. [12]. This study was approved by the Ethics Committee of Tokyo Women's Medical University Graduate School of Medicine.

2.2. Analysis of GSH and eADA activities

GSH was measured by the method described by Beutler et al. [21]. Briefly, 20 μl of heparinized whole blood was lysed with 180 μl of distilled water. The hemolysate was deproteinized with metaphosphoric acid solution, and the resultant supernatant was reacted with 5.5′-dithiobis(2-nitrobenzoic acid) (DTNB) for measurement at 412 nm. The upper cutoff of GSH was 99.8 mg/dl RBC (mean + 2SD). Simultaneously, we measured eADA activity by the standard method [22], using adenosine as a substrate at 265 nm, and defined the eADA level higher than mean + 2SD (1.94 IU/gHb) as elevated.

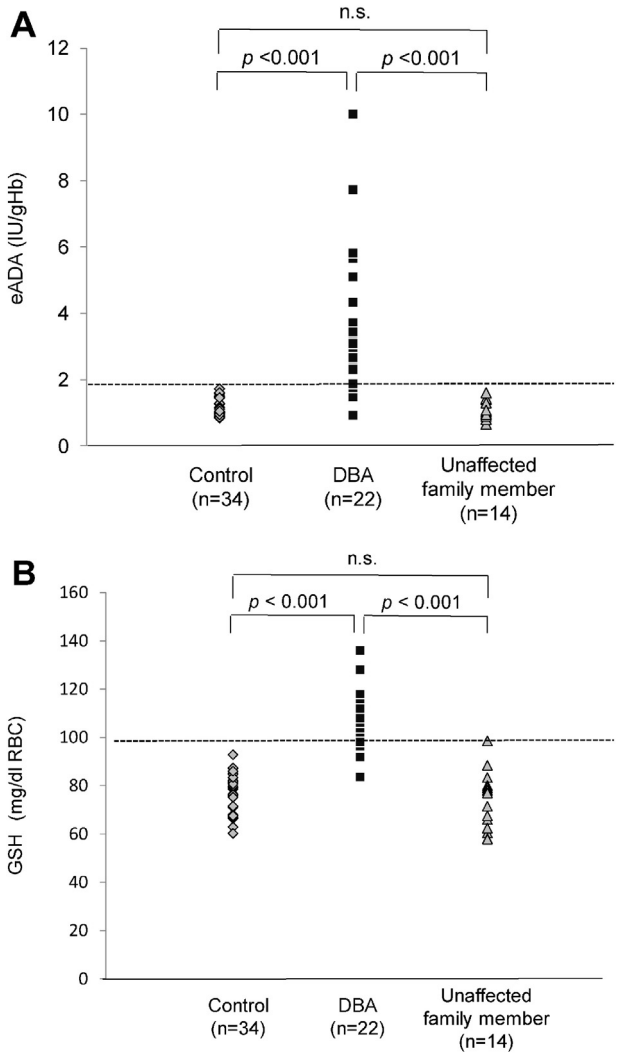

Fig. 1. Comparison of erythrocyte adenosine deaminase activity (eADA, IU/gHb) (A) and reduced glutathione concentration (GSH, mg/dl RBC) (B) of controls, Diamond-Blackfan anemia (DBA) patients with ribosomal protein gene mutations, and unaffected DBA patient family members. The dashed horizontal axis line is the diagnostic upper cutoff of eADA, of 1.94 IU/gHb (mean + 2SD) (A) and GSH of 99.8 mg/dl RBC (mean + 2SD) (B). *p*-values were calculated from Wilcoxon rank-sum test. n.s.,not significant.

Table 1
eADA and GSH of the DBA subjects with RP gene mutations.

DBA	Mutated RP gene	eADA (IU/gHb)	GSH (mg/dl RBC)
1	*RPS19*	4.33[a]	98.3
2		2.64[a]	96.5
3		0.95	114[b]
4		2.35[a]	83.7
5		2.57[a]	92.4
6		3.71[a]	128[b]
7		3.36[a]	136[b]
8		3.44[a]	96.7
9		2.32[a]	94.9
10		0.93	118[b]
11	*RPS17*	5.66[a]	96.3
12	*RPS10*	2.96[a]	103[b]
13	*RPL5*	1.49	110[b]
14		5.82[a]	128[b]
15		5.09[a]	92
16		2.97[a]	106[b]
17		3.09[a]	100[b]
18	*RPL35a*	1.75	108[b]
19		2.67[a]	98.1
20		1.87	114[b]
21	*RPL11*	10.0[a]	118[b]
22		7.73[a]	112[b]

None of the unaffected family members had RP gene mutations. Patients 3 was regularly transfused once a month, patient 4, 5, 7, 9, 12, 16, 17, 18, 19, 20, 21 and 22 were not transfusion dependent, patient 2, 4, 12, 17, 18 and 22 were treated with steroid, 16 and 21 with steroid and cyclosporine, 3, 5, 7, 9, 19 and 20 were not treated.
[a] Higher than diagnostic eADA level upper cutoff of 1.94 IU/gHb (mean + 2SD).
[b] Higher than diagnostic GSH level upper cutoff of 99.8 mg/dl RBC (mean + 2SD).

2.3. Statistical analysis

Statistical analyses were performed using the Wilcoxon rank-sum test. *p* values of <0.05 were considered significant. Support vector machines (SVMs) are supervised learning methods used for classification. They seek the hyperplane that maximizes the margin in the feature space, and are known to have high generalization performance. In the present study, an SVM was applied to divide DBA and non-DBA groups using two variables, eADA and GSH. The SVM used the ksvm function while specifying a linear kernel in the statistical software R.

2.4. Enzymes involved in the pentose phosphate pathway, glutathione biosynthesis, and glutathione metabolism

In order to evaluate whether upregulated biosynthesis or reduction of glutathione is attributable to the elevated GSH in DBA red cells, enzymatic activities of the pentose phosphate pathway (glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD)), glutathione biosynthesis (gamma glutamylcysteine synthetase (GC-S) and glutathione synthetase (GSH-S)), and glutathione metabolism

Table 2
Sensitivity and specificity of eADA and GSH in DBA subjects.

	High eADA[a]	High GSH[b]
Control ($n = 34$)	0/34	0/34
DBA ($n = 22$)	17/22 (77.2%)	13/22 (59.1%)
Unaffected family member ($n = 14$)	0/14	0/14

[a] Higher than diagnostic eADA level upper cutoff of 1.94 IU/gHb (mean + 2SD).
[b] Higher than diagnostic GSH level upper cutoff of 99.8 mg/dl RBC (mean + 2SD).