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noted. However, no hypersensitivity to GM-CSF as determined by colony formation assay for BM-MNCs (data not shown) or phosphor-STAT5 staining (data not shown) was observed. DNA sequence for JMML-associated genes, such as *NRAS, KRAS, HRAS, PTPN11*, and *CBL*, was determined, and KRAS G13D mutation was identified (Figure 1B). The mutation was seen exclusively in the hematopoietic cell lineage, and no mutation was seen in the oral mucosa or nail-derived DNA. Granulocytes, monocytes, T cells, and B cells were all positive for KRAS G13D mutation (data not shown). The proportion of mutated cells in each hematopoietic lineage was quantitated by mutation allele-specific quantitative polymerase chain reaction methods, which revealed that mutated allele was almost equally present in granulocytes, T cells, and B cells (Figure 1C). $CD34^+$ hematopoietic stem cells (HSCs) were also positive for KRAS G13D mutation, and 60% of colony-forming units-granulocyte macrophage (CFU-GM) developed from isolated CD34 cells carried the KRAS G13D mutation (data not shown). These observations suggest that the mutation occurred at the HSCs level, and HSC consists of wild-type and mutant HSCs.

NRAS-mutated type IV ALPS was previously characterized by apoptosis resistance of T cells in IL-2 depletion.[3] Then, activated T cells were subjected to an apoptosis assay by FAS stimulation or IL-2 depletion. Remarkable resistance to IL-2 depletion, but not to FAS-dependent apoptosis (Figure 1D-E), was seen. This was in contrast to T cells from FAS-mutated ALPS type 1a, which showed remarkable resistance to FAS-dependent apoptosis and normal apoptosis induction by IL-2 withdrawal (Figure 1D-E). Western blotting analysis of activated T cells or Epstein-Barr virus-transformed B cells showed reduced expression of Bim (Figure 1F).

In case 2, autoimmune phenotype and hepatosplenomegaly were remarkable, as shown in Supplemental data. The patient was initially diagnosed as Evans syndrome based on the presence of hemolytic anemia and autoimmune thrombocytopenia. Double-negative T cells were 1.1% of T-cell receptor-$\alpha\beta$ cells in the peripheral blood, which did not meet with the criteria of ALPS. Although spontaneous colony formation was shown in peripheral blood- and BM-MNCs, and GM-CSF hypersensitivity was demonstrated in BM-MNCs derived $CD34^+$ cell (supplemental Table 2), she showed no massive monocytosis or increased fetal hemoglobin. Thus, the diagnosis was less likely to be ALPS or JMML. DNA sequencing of JMML-related genes, such as *NRAS, KRAS, HRAS, PTPN11*, and *CBL*, identified somatic, but not germline, KRAS G13D mutation (Figure 1B). KRAS G13D mutation was detected in granulocytes and T cells. Mutation was not identified in B cells by conventional DNA sequencing (data not shown). Mutant allele-specific quantitative polymerase chain reaction revealed that mutated allele was almost equally present in granulocytes and T cells, but barely in B cells (Figure 1C). Activated T cells showed resistance to IL-2 depletion but not to FAS-dependent apoptosis (Figure 1D-E).

Both of our cases were characterized by strong autoimmunity, immune cytopenia, and lymphadenopathy or hepatosplenomegaly with partial similarity with ALPS or JMML. However, they did not meet with the well-defined diagnostic criteria of ALPS[2] or JMML.[6] It is interesting that case 2 presented GM-CSF hypersensitivity, which is one of the hallmarks of JMML. Given the strict clinical and laboratory criteria of JMML and ALPS, our 2 cases should be defined as a new disease entity, such as RAS-associated ALPS-like disease (RALD). Recently defined NRAS-mutated ALPS type IV may also be included in a similar disease entity.

There are several cases of JMML reported simultaneously having clinical and laboratory findings compatible with autoimmune disease.[8,9] Autoimmune syndromes are occasionally seen in patients with myelodysplastic syndromes, including chronic myelomonocytic leukemia.[10] These previous findings may suggest a close relationship of autoimmune disease and JMML. Because KRAS G13D has been identified in JMML,[11-13] it is tempting to speculate that KRAS G13D mutation is involved in JMML as well as RALD. In JMML, erythroid cells reportedly carry mutant RAS, whereas B- and T-cell involvement was variable.[13] In both of our cases, myeloid cells and T cells carried mutant RAS, whereas B cells were affected variably. These findings would support a hypothesis that the clinical and hematologic features are related to the differentiation stages of HSCs where RAS mutation is acquired. JMML-like myelomonocytic proliferation may predict an involvement of RAS mutation in myeloid stem/precursor cell level, whereas ALPS-like phenotype may predict that of stem/precursor cells of lymphoid lineage, especially of T cells. Under the light of subtle differences between the 2 cases presented, their hematologic and clinical features may reflect the characteristics of the stem cell level where *KRAS* mutation is acquired. Involvement of the precursors with higher propensity toward lymphoid lineage may lead to autoimmune phenotypes, whereas involvement of those with propensity toward the myeloid lineage may lead to GM-CSF hypersensitivity while still sharing some overlapping autoimmune characteristics.

One may argue from the other viewpoints with regard to the clinicopathologic features of these disorders. First, transformation in fetal HSCs might be obligatory for the development of JMML[14] and, in HSCs later in life, may not have the same consequences. Second, certain KRAS mutations may be more potent than others. Codon 13 mutations are generally less deleterious biochemically than codon 12 substitutions, and patients with JMML with codon 13 mutations have been reported to show spontaneous hematologic improvement.[12,15] Thus, further studies are needed to reveal in-depth clinicopathologic characteristics in this type of lympho-myeloproliferative disorder.

KRAS mutation may initiate the oncogenic pathway as one of the first genetic hits but is insufficient to cause frank malignancy by itself.[16,17] Considering recent findings that additional mutations of the genes involved in DNA repair, cell cycle arrest, and apoptosis are required for full malignant transformation, one can argue that RALD patients will also develop malignancies during the course of the disease. Occasional association of myeloid blast crisis in JMML and that of lymphoid malignancies in ALPS will support this notion. Thus, the 2 patients are now being followed up carefully. It was recently revealed that half of the patients diagnosed with Evans syndrome, an autoimmune disease presenting with hemolytic anemia and thrombocytopenia, met the criteria for ALPS diagnosis.[18,19] In this study, FAS-mediated apoptosis analysis was used for the screening. Considering the cases we presented, it will be intriguing to reevaluate Evans syndrome by IL-2 depletion-dependent apoptosis assay focusing on the overlapping autoimmunity with RALD.

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Authorship

Contribution: Masatoshi Takagi and S.M. designed entire experiments and wrote the manuscript; K.S., N.M., and Mari Takagi treated patients and designed clinical laboratory test; J.P. performed experiments described in Figure 1B-F; K.M., H.M., and S.D. performed colony and mutational analysis; and M.N., T.M., K.K., S.K., Y.K., and A.T. supervised clinical and immunologic experiments or coordinated clinical information.

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X-linked thrombocytopenia (XLT) due to *WAS* mutations: clinical characteristics, long-term outcome, and treatment options

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A large proportion of patients with mutations in the Wiskott-Aldrich syndrome (WAS) protein gene exhibit the milder phenotype termed X-linked thrombocytopenia (XLT). Whereas stem cell transplantation at an early age is the treatment of choice for patients with WAS, therapeutic options for patients with XLT are controversial. In a retrospective multicenter study we defined the clinical phenotype of XLT and determined the probability of severe disease-related complications in patients older than 2 years with documented *WAS* gene mutations and mild-to-moderate eczema or mild, infrequent infections. Enrolled were 173 patients (median age, 11.5 years) from 12 countries spanning 2830 patient-years. Serious bleeding episodes occurred in 13.9%, life-threatening infections in 6.9%, autoimmunity in 12.1%, and malignancy in 5.2% of patients. Overall and event-free survival probabilities were not significantly influenced by the type of mutation or intravenous immunoglobulin or antibiotic prophylaxis. Splenectomy resulted in increased risk of severe infections. This analysis of the clinical outcome and molecular basis of patients with XLT shows excellent long-term survival but also a high probability of severe disease-related complications. These observations will allow better decision making when considering treatment options for individual patients with XLT. (*Blood*. 2010;115(16): 3231-3238)

Introduction

In 1937 Wiskott described a clinical entity characterized by thrombocytopenia, eczema, bloody diarrhea, and recurrent otitis media in male infants. After rediscovery in 1954 by Aldrich as an X-linked recessive disorder, it was designated the Wiskott-Aldrich syndrome (WAS).[1-3] X-linked thrombocytopenia (XLT), sometimes associated with mild eczema and/or infections, was recognized in the 1960s and was suspected to be a variant of WAS.[4-6] This was confirmed when patients with XLT were shown to have mutations in the Wiskott-Aldrich syndrome protein gene (*WAS*).[7-9]

WAS gene mutations result in 3 distinct clinical phenotypes: classic WAS, XLT, and X-linked neutropenia,[10,11] and a strong genotype phenotype correlation has been suggested.[12-15] Mutations completely averting WAS protein (WASP) expression typically lead to the classic phenotype. Missense mutations resulting in expression of defective WASP, often in reduced quantity, most often result in the XLT phenotype, sometimes with only intermittent thrombocytopenia.[16] X-linked neutropenia is caused by gain of function mutations resulting in constitutively activated WASP.[17-19] There are however exceptions to these rules, making it difficult to predict the clinical course of a male infant solely based on the type of *WAS* gene mutation and its effect on WASP expression.

The classic WAS phenotype with microthrombocytopenia, severe eczema, increased susceptibility to pyogenic and opportunistic infections, and increased risk of autoimmune disease and cancer usually leads to death in early childhood or adolescence if left untreated.[10,20,21] Curative treatment by allogeneic hematopoietic stem cell transplantation (HSCT) should be offered to all such patients. The outcome is excellent if performed early in life from a human leukocyte antigen–matched related or unrelated donor.[10,22-24] Hematopoietic stem cell gene therapy might in the future offer an alternative approach in patients lacking a suitable donor.[25-27]

Generally accepted treatment policies do not exist for patients exhibiting the XLT phenotype, in whom HSCT would seem like an excessively risky procedure if they have thrombocytopenia and

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eczema only. Although it has been assumed that patients with XLT have a lower risk of cancer or autoimmunity than patients with WAS, this has never been formally examined. Therefore, the risk–benefit ratio for HSCT is not known in XLT.

In this multicenter study we assessed retrospectively the spectrum of clinical phenotypes, the associated genotypes, and the long-term outcome of the largest cohort of patients with XLT studied so far.

Methods

Data accrual

Questionnaires were sent worldwide to major centers treating patients with primary immunodeficiency diseases (PIDs), asking to enroll their patients with the XLT phenotype and to provide data on the following disease parameters: infections, eczema, thrombocytopenia, bleeding, malignancy, autoimmunity, *WAS* gene mutation, WASP expression, and type and extent of therapy. An alternative possibility was documentation online with the same questionnaire in the European Society for Immunodeficiencies registry (www.esid.org). Patient information was made anonymous by the submitting physician. The study was approved by the ethics committee of the University of Munich, Germany.

Patients

All submitted patient data were evaluated, and patients were included as study patients by consensual decision of a central review board (M.H.A., T.C.B., B.H.B., H.D.O.). To be enrolled into the final study, patients had to fulfill all of the following criteria: (1) confirmed mutation within the *WAS* gene; (2) classified by their treating physician as having XLT; (3) with or without mild-to-moderate eczema or mild, infrequent infections not resulting in sequelae; (4) age older than 2 years; and (5) no severe infection, autoimmunity, or malignancy within the first 2 years of life.

Bleeding events before the age of 2 years were no reason for exclusion from the study. Older than 2 years, severe infections, the development of autoimmunity, or malignancy was recorded and included in the analysis, but it was no reason for exclusion from the study.

If patients underwent allogeneic HSCT, the transplantation was recorded as the last date of follow-up; the resulting events/outcome were not part of this analysis.

Definitions

Life-threatening infections were defined as requiring hospitalization such as sepsis, meningitis, or pneumonia needing oxygen supply or mechanical ventilation. Serious bleeding was defined as a fatal or life-threatening bleeding episode resulting in hospitalization or red blood cell transfusion. Other serious complications were a diagnosis of autoimmunity, malignancy, or death. If a patient experienced more than 1 serious event, only the first event was registered for the analysis of event-free survival. Severity of thrombocytopenia was defined as follows: less than 20.0×10^9/L (20 000/μL) was severe, 20.0 to 50.0×10^9/L (20 000 to 50 000/μL) was moderate, and greater than 50.0×10^9/L (50 000/μL) or cyclic was mild. All patients with normal or reduced levels of WASP detectable by Western blot or fluorescence-activated cell sorting were designated as WASP positive; those with truncated (by Western blot) or undetectable protein were categorized as WASP negative. Intravenous immunoglobulin (IVIG) or antibiotic (AB) prophylaxes were defined as having had IVIG or prophylactic ABs more than once for any period of time.

Mutations are reported according to the current nomenclature of the Human Genome Variation Society (www.hgvs.org).[28]

Statistical analysis

Kaplan-Meier survival estimates and cumulative incidence rates were compared with the use of the log-rank test (Prism; GraphPad Software Inc). Cumulative incidence for different events adjusting for competing risks was estimated with the use of the statistics language R[29] with the cmprsk package that used the method by Gray.[30] Other analyses used the χ^2 or Fisher exact test and were accepted as significantly different at a level of P less than .05.

Results

Study cohort

A total of 69 centers known to treat patients with PID were contacted and 50 responded (72%). Of 213 completed forms, representing 12 countries from 4 continents, 173 (171 male, 2 female) patients from 128 families and 21 centers with a median age of 11.5 years (range, 2.0-74.6 years) fulfilled the inclusion criteria, covering 2830 patient-years. The 2 female patients of our XLT cohort had been reported previously, 1 with a homozygous missense mutation and 1 with a heterozygous missense mutation and skewed X-inactivation in favor of the mutated allele.[31,32]

Mutations in patients with XLT

We identified 62 unique mutations (Table 1), including 3 mutational hotspots, defined as affecting 10 or more nonrelated families with either the identical mutation or a missense mutation affecting the same amino acid. Two hotspots were located in exon 2 affecting either a valine at position 75 (p.Val75Met or p.Val75Leu; 23 patients) or an arginine at position 86 (p.Arg86Gly, p.Arg86Cys, p.Arg86His, or p.Arg86Leu; 33 patients). The third hotspot mutation, located in intron 6 (c.559 + 5G>A) was found in 15 patients. Thus 41% of all patients had a hotspot mutation.

The majority of mutations was located in exon 1 (10% of all patients) and exon 2 (54%). Most mutations were missense (69% of all patients), followed by splice site mutations (19%), deletions (5%), insertions (3%), nonsense mutations (2%), and no-stop mutations (1%; supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). With few exceptions, patients with missense and splice site mutations expressed WASP in reduced quantity or in truncated form (Table 1).

Survival

Without curative treatment classic WAS results in premature death, often during childhood.[21,33] Patients with XLT are expected to have a better prognosis. To verify this perception, we defined the probability of survival in our cohort of patients with XLT.

Overall survival was excellent with 97% (95% confidence interval [95% CI], 95%-100%), 96% (95% CI, 91%-100%), 81% (95% CI, 66%-97%), and 81% (95% CI, 66%-97%) at 15, 30, 45, and 60 years, respectively, and only slightly reduced compared with the survival curve of the normal male German population[34] (Figure 1A). However, survival probability without having experienced a severe disease-related event was less favorable with 74% (95% CI, 65%-82%), 56% (95% CI, 43%-70%), 36% (95% CI, 20%-53%), and 27% (95% CI, 10%-44%) at 15, 30, 45, and 60 years, respectively (Figure 1B).

Thus the excellent survival in patients with XLT is associated with a high rate of severe disease-related events throughout life.

Incidence of severe disease-related events

To better define the nature and occurrence of severe disease-related events, we analyzed the cumulative incidence rate of these events separately.

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Table 1. *WAS* gene mutations in patients with XLT

Exon	Coding DNA mutation	Predicted protein change	Mutation type	Pt*	Fam†	Origin	WASP expression (no. of pt)	Score (no. of pt)
1	c.G5C	p.Ser2Thr	Missense	1	1	Fr	ND	2
1	c.G18A	p.Met6Ile	Missense	2	1	JPN	Reduced (2)	1, 2→5M
1	c.C71T	p.Ser24Phe	Missense	2	2	US (1), JPN (1)	Reduced (1), ND (1)	1, 2→5A
1	c.C79T	p.Leu27Phe	Missense	1	1	US	Reduced	1
1	c.88_90delCAC	p.His30del	Deletion	5	2	UK (4), Ger (1)	Reduced (3), ND (2)	1(4), 2
1	c.G91A	p.Glu31Lys	Missense	1	1	Italy	Absent	2→5A
1	c.T116C	p.Leu39Pro	Missense	6	4	US (3), Italy (2), Ger (1)	Reduced (5), absent (1)	1, 1→5A/M, 2(4)
2	c.C134T	p.Thr45Met	Missense	13	8	JPN (4), US (2), Ger (1), UK (1), Sw (5)	Reduced (6), absent (1), ND (6)	1(6), 1→5A, 2(4), 2→5A /B (2)
2	c.C140A	p.Ala47Asp	Missense	1	1	US	Reduced	2
2	c.A142G	p.Thr48Ala	Missense	1	1	JPN	Reduced	2
2	c.C143T	p.Thr48Ile	Missense	1	1	US	Reduced	1→5M
2	c.C167T	p.Ala56Val	Missense	5	4	US (3), Italy (1), JPN (1)	Reduced (4), ND (1)	1(3), 1→5A, 2
2	c.C172A	p.Pro58Thr	Missense	2	1	US	Normal (2)	1, 2
2	c.C172G	p.Asp58Ala	Missense	1	1	US	Reduced	2→5A/M
2	c.C173G	p.Pro58Arg	Missense	3	1	Italy	Reduced (2), ND (1)	1, 1→5M, 2
2	c.G199A	p.Glu67Lys	Missense	1	1	Fr	Reduced	2
2	c.G223A	p.Val75Met	Missense	22	16	Fr (6), UK (5), US (5), Ger (2), JPN (2), Sp (1), Italy (1)	Normal (1), reduced (10), absent (3), ND (8)	1(6), 1→5A, 2(14), 2→5A
2	c.G223T	p.Val75Leu	Missense	1	1	US	ND	2
2	c.A227C	p.Lys76Thr	Missense	2	2	US	Reduced (1), ND (1)	2(2)
2	c.G229C	p.Asp77His	Missense	1	1	Italy	Reduced	1
2	c.A230G	p.Asp77Gly	Missense	2	1	Italy	Reduced (2)	1, 2
2	c.A239G	p.Gln80Arg	Missense	1	1	Rus	Reduced	2
2	c.248insA	p.Tyr83X	Insertion	1	1	Fr	ND	2
2	c.C256G	p.Arg86Gly	Missense	1	1	US	Reduced	2→5A
2	c.C256T	p.Arg86Cys	Missense	24	18	US (10), Ger (6), JPN (3), UK (3), Italy (1), Sw (1)	Normal (3), reduced (9), ND (12)	1(10), 1→5M, 2(12), 2→5A
2	c.G257A	p.Arg86His	Missense	7	7	JPN (2), Fr (1), Ger (1), Isr (1), Rus (1), US (1)	Reduced (4), absent (1), ND (2)	1→5A, 2(4), 2→5A(2)
2	c.G257T	p.Arg86Leu	Missense	1	1	US	Absent	2
2	c.A263G	p.Tyr88Cys	Missense	1	1	NL	ND	2→5A
2	c.G266A	p.Gly89Asp	Missense	1	1	UK	Normal	1
3	c.A320G	p.Tyr107Cys	Missense	1	1	US	Reduced	2
3	c.326_330insC	p.Thr111HisfsX9	Insertion	1	1	US	Absent	2
3	c.G355A	p.Gly119Arg	Missense	1	1	NL	ND	1
4	c.dup355_361	p.Asp121insGD	Insertion	1	1	JPN	Absent	2
4	c.G399T	p.Glu133Asp	Missense	1	1	US	Reduced	2
5	c.G505T	p.Asn169X	Nonsense	1	1	JPN	Reduced	2→5M
6	c.G538A	p.His180Asn	Missense	1	1	Italy	Reduced	1
7	c.C707G	p.Ala236Gly	Missense	1	1	Italy	Absent	1
7	c.A724T	p.Ser242Cys	Missense	1	1	NL	ND	1
9	c.854_855insG	p.Thr286AspfsX1	Insertion	2	1	UK	Reduced and truncated (1), absent (1)	1(2)
9	c.A919G	p.Met307Val	Missense	1	1	Ger	ND	2
10	c.C961T	p.Arg321X	Nonsense	1	1	JPN	Absent	2→5M
10	c.983_984delC	Multiple products	Deletion	1	1	US	Reduced and truncated	2
10	c.991insA	p.Gly334X	Insertion	1	1	US	Absent	2
10	c.1073_1074delGA	p.Gly358AlafsX135	Deletion	1	1	US	Reduced and truncated	2
10	c.1079delC	p.Pro360HisfsX84	Deletion	2	2	Ger, JPN	Reduced (1), absent (1)	2(2)
10	c.C1090T	p.Arg363X	Nonsense	2	1	Fr	ND (2)	2(2)
11	c.G1430A	p.Arg477Lys	Missense	1	1	Sp	Reduced	2
11	c.T1442A	p.Ile481Asn	Missense	2	1	Italy	Normal (1), reduced (1)	1(2)
12	c.G1453A	p.Asp485Asn	Missense	1	1	US	Reduced	2→5A
12	c.A1454G	p.Asp485Gly	Missense	3	1	Sp	ND (3)	1(3)
12	c.G1508C	p.X503SerextX76	No-stop	2	1	US	Absent (1), ND (1)	2(2)
Int 3	c.360+1G>A	p.Ala92_Asp120del	Splice (donor site)	1	1	JPN	Reduced	2
Int 3	c.361-1G>A	p.fsX201	Splice (acceptor site)	1	1	US	Reduced	2
Int 4	c.[463+1_463+8del; 464-3_464-2insG]	p.fsX178/fsX251	Splice (donor +acceptor site)	1	1	JPN	Reduced	2
Int 6	c.559+5G>A	70% fsX190/30% normal	Splice (donor site)	15	11	US (9), Ger (2), JPN (3), UK (1)	Reduced (12), absent (1), ND (2)	1(6), 1→5M, 2(6), 2→5A(2)
Int 7	c.734+5G>A	ND	Splice (donor site)	4	1	Ger	ND (4)	2 (3), 2→5A
Int 7	c.735-25A>C	ND	Splice (acceptor site)	3	1	UK	Reduced (3)	1(3)
Int 8	c.777+1G>A	p.fsX246	Splice (donor site)	2	2	Australia, US	Absent (1), ND (1)	1, 2
Int 8	c.777+3insT	ND	Splice (donor site)	2	1	Italy	Reduced (2)	1, 2
Int 8	c.778-6G>A	ND	Splice (acceptor site)	1	1	UK	Reduced	1
Int 9	c.(931_932)ins250	ND	Splice site	1	1	JPN	Reduced	1
Int 11	c.(1484_1485)ins118	Normal and abnormal splice products	Splice site	2	1	JPN	Reduced (2)	2→5A(2)

Pt indicates number of patients with the respective mutation; Fam, number of families with the respective mutation; 1→5, WAS score progressing from 1 to 5 because of either A, autoimmunity, or M, malignancy; Fr, France; ND, not done; JPN, Japan; US, United States of America; UK, United Kingdom; Ger, Germany; Sw, Sweden; Sp, Spain; Rus, Russia; Isr, Israel; and NL, The Netherlands.

*There was a total of 173 patients.

†There was a total of 128 families.

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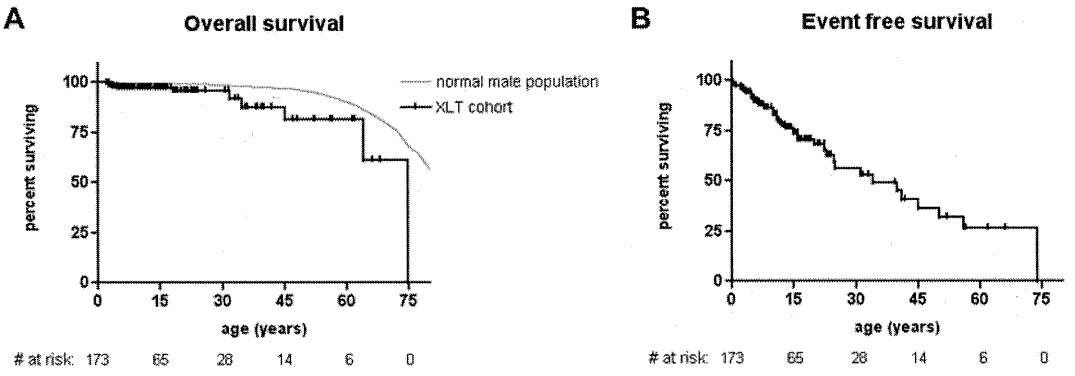

Figure 1. Overall and event-free survival. (A) Kaplan-Meier estimate of overall survival probability of all study patients compared with survival of the normal German male population 2006.[34] (B) Event-free survival probability. Event was defined as a severe or fatal infection, severe or fatal bleeding, autoimmunity, malignancy, or death. Each hash mark on a graph line indicates a censored event; # at risk, number of patients at risk at indicated time point.

Median event-free survival was 10.2 years (range, 0.1-73.9 years). A total of 86 events in 47 patients were reported, some of them occurring in different event categories in the same patient (detailed in Table 2). Cumulative incidences for each event category are detailed separately in Figure 2. If events were analyzed honoring other events as competing, the cumulative incidences were slightly lower because later events in the same patient were ignored (data not shown).

Table 2. Disease-related events

	Total events	Fatal events
Infections*		
Pneumonia	6	0
Bacterial meningitis	4	0
Sepsis	4	2
Gastrointestinal (salmonellosis)	1	1
Orchitis	1	0
Tuberculosis	1	0
No. of events	17†	3‡
No. of patients	12	3
Bleeding§		
ICH‖	18	3
Gastrointestinal	6	1
Ear/nose/throat	4	0
Pulmonary	2	1
Traumatic, not ICH	2	0
Retinal	1	0
No. of events	33	5
No. of patients	24	5
Autoimmunity¶		
Nephropathy	9	0
AIHA	6	0
Vasculitis	3	0
ITP	4	0
Arthritis	3	0
Colitis	1	0
No. of events	26	0
No. of patients	21	0
Malignancy#		
Lymphoma/EBV-LPD	4	1
MDS	1	0
Spinalioma	2	0
Seminoma	1	0
ALL	1	0
Pancreatic cancer	1	1
No. of events	10	2
No. of patients	9	2

ICH indicates intracranial hemorrhage; AIHA, autoimmune hemolytic anemia; ITP, immune thrombocytopenic purpura; ALL, acute lymphoblastic leukemia; EBV-LPD, Epstein-Barr virus–associated lymphoproliferative disease; and MDS, myelodysplastic syndrome.

*Three patients had more than 1 infectious event.

†Eight events were in patients who had undergone a previous splenectomy.

‡Two events were in patients who had undergone a previous splenectomy.

§Four patients had more than 1 bleeding episode.

‖Fifteen were spontaneous, 3 were traumatic.

¶Three patients had more than 1 autoimmune disease.

#One patient had 2 malignancies.

Life-threatening infections occurred at a median age of 24.8 years (range, 2.0-73.9 years), 3 of which were fatal. There was no discernible effect of patient age on the incidence of infectious events (Figure 2A). In contrast, all but 1 serious hemorrhage occurred before the age of 30 years, at a median age of 5.7 years (range, 0.1-74.6 years; Figure 2B). Most serious bleeding events (18 of 33) were intracranial hemorrhages. Five bleeding episodes were fatal at a median age of 4.9 years (range, 2.0-74.6 years). There was no correlation between the recorded platelet counts and the incidence of severe or fatal bleeding, which was 12.5% in mild, 9.7% in moderate, and 18.4% in severe thrombocytopenia ($P = .31$). Autoimmune nephropathy and hemolytic anemia were the most frequent autoimmune manifestations; the former occurring more frequently in Japanese patients than in patients from other countries (5 of 28 vs 4 of 145; $P = .006$). In general, autoimmune diseases were not significantly more frequent in Japanese patients (5 of 28 vs 16 of /145; $P = .34$). Autoimmunity was not restricted to adult patients but occurred at all ages with a median of 12.2 years (range, 4.9-56.0 years; Figure 2C). Malignancies developed at a median age of 34.0 years (range, 7.8-74.0 years; Figure 2D), half (5 of 10) of which were of lymphoid origin. Two patients died of their malignancies, 2 more went on to have HSCT and died of transplantation-related causes and 2 died of other complications.

In conclusion, with the exception of severe bleeding, which seems to be limited to the first 3 decades of life, a relatively high rate of life-threatening or fatal disease-related events was observed in XLT at all ages.

Influence of WAS gene mutation, protein expression, IVIG, or AB prophylaxis on overall and event-free survival

Because some patients with XLT have a largely uneventful course of disease and a normal life expectancy and others have severe or even fatal complications at any age, we asked whether individual *WAS* gene mutations, the presence or absence of WASP, or the prophylaxis with ABs and intravenous immunoglobulin had any influence on outcome.

WASP expression, if assessed, was detectable in 98 patients and absent in 21. Presence or absence of WASP had no influence on overall and event-free survival in patients with the XLT phenotype (Figure 3A). Similarly, there was no significant effect on the incidence of disease-related events (data not shown). The same was true when the influence of IVIG prophylaxis (n = 39) was analyzed in comparison to patients having never received IVIG (n = 134; Figure 3B). AB prophylaxis had no positive influence on

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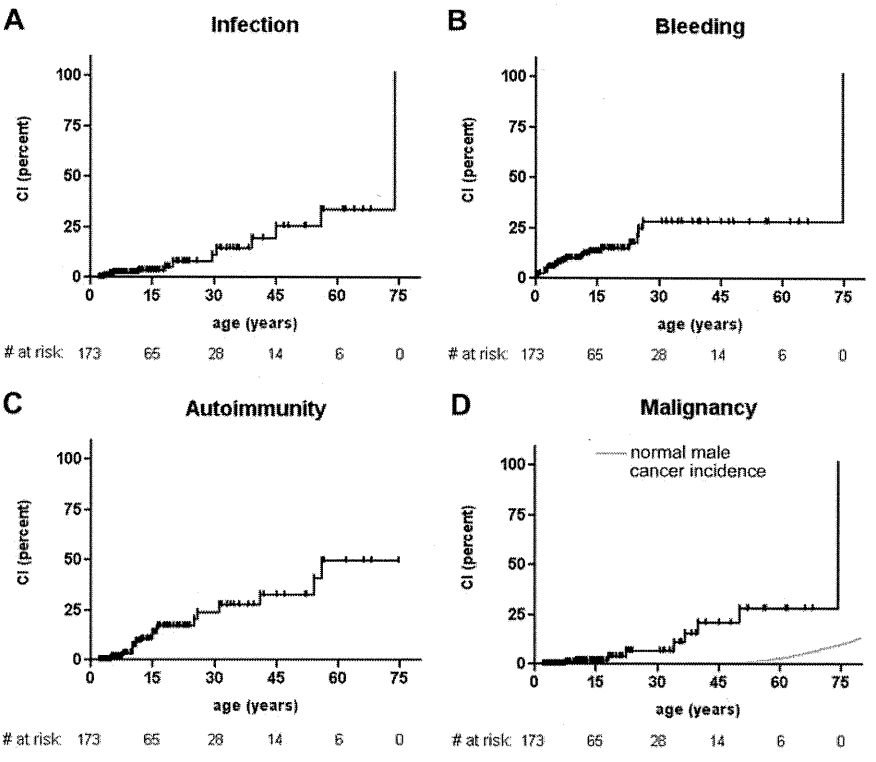

Figure 2. Cumulative incidence rate of severe events. Cumulative incidence of (A) severe or fatal infectious episodes in the study cohort, (B) severe or fatal bleeding episodes, (C) autoimmune disease, and (D) malignancy, compared with cancer incidence in the US male population.[35] Each hash mark on a graph line indicates a censored event, # at risk, number of patients at risk at indicated time point.

outcome (Figure 3C). Patients with hotspot mutations had no different overall and event-free survival and event incidences compared with others (data not shown).

In summary none of the tested outcome variables were of significance in this cohort of patients with XLT selected on the basis of their mild phenotype.

Influence of splenectomy on infections and bleeding episodes

Splenectomy in patients with XLT/WAS usually leads to a sustained increase in platelet counts and is considered an effective measure to control the bleeding predisposition. Therefore, splenectomy has been recommended by some investigators for patients with WAS and patients with XLT.[36,37]

A total of 41 patients (23.7%) underwent splenectomy at a median age of 7.02 years (range, 0.8-43.0 years). The indication for splenectomy was not reported, but 7 of these 41 patients had experienced a severe bleeding episode before splenectomy, and 28 of 41 patients had had severe thrombocytopenia. All 13 patients in whom postsplenectomy platelet counts were available had experienced an increase in platelet numbers, 7 having counts greater than 100.0×10^9/L (100 000/μL). In the 2 patients who experienced a severe bleeding event after splenectomy, platelet counts were not reported. Therefore, it cannot be excluded that these 2 patients may have had low counts despite splenectomy. The overall cumulative incidence rate of serious bleeding events in these patients after splenectomy compared with before splenectomy was reduced although not significantly ($P = .15$). However, there was a significantly higher incidence of severe infectious events after splenectomy than before ($P = .005$). This might possibly be due to negligent AB prophylaxis in some patients. Of the 9 patients who did not receive AB prophylaxis, 3 had a severe (1 fatal) infection up to 53 years after splenectomy. This compared unfavorably, however not statistically significant, to patients who underwent splenectomy with AB prophylaxis in whom only 5 of 32 (1 fatal) had such an event ($P = .34$). Overall survival in patients who underwent splenectomy was not significantly different from patients not undergoing splenectomy (data not shown).

These data indicate that patients with XLT who underwent splenectomy are at significant risk of severe infections and require life-long AB prophylaxis.

Discussion

WAS is a multifaceted disorder with a wide spectrum of disease severity. In contrast to classic WAS, patients with a mild clinical phenotype, termed XLT, require comprehensive assessment in deciding on the strategy to provide optimal treatment. This is true for children who often present with selective microthrombocytopenia and have an uncertain long-term prognosis at a time when they are excellent candidates for allogeneic HSCT.[23,24] Similarly, adult patients with XLT who often are wrongly categorized as having chronic immune thrombocytopenic purpura and who may already have developed complications such as autoimmunity pose unique therapeutic challenges. This retrospective study was designed to better define the type of mutations and the clinical course of patients with XLT and to collect supportive evidence for optimal treatment choices.

The design of such a study requires a stringent definition of inclusion and exclusion criteria. The WAS scoring system has been used successfully in categorizing patients according to their disease severity.[10,11] However, an individual patient is not expected to keep the same score throughout his or her life. Progression from a score of 1 to 4 to a score of 5 by developing cancer or autoimmunity can occur at any age, and patients with classic WAS often present with a relatively mild phenotype during infancy. We, therefore, chose inclusion criteria that best reflect the situation when patients with XLT/WAS present in an immunodeficiency clinic. In addition to the classification as XLT by physicians experienced in treating patients with PIDs, we deliberately chose stringent criteria to prevent the

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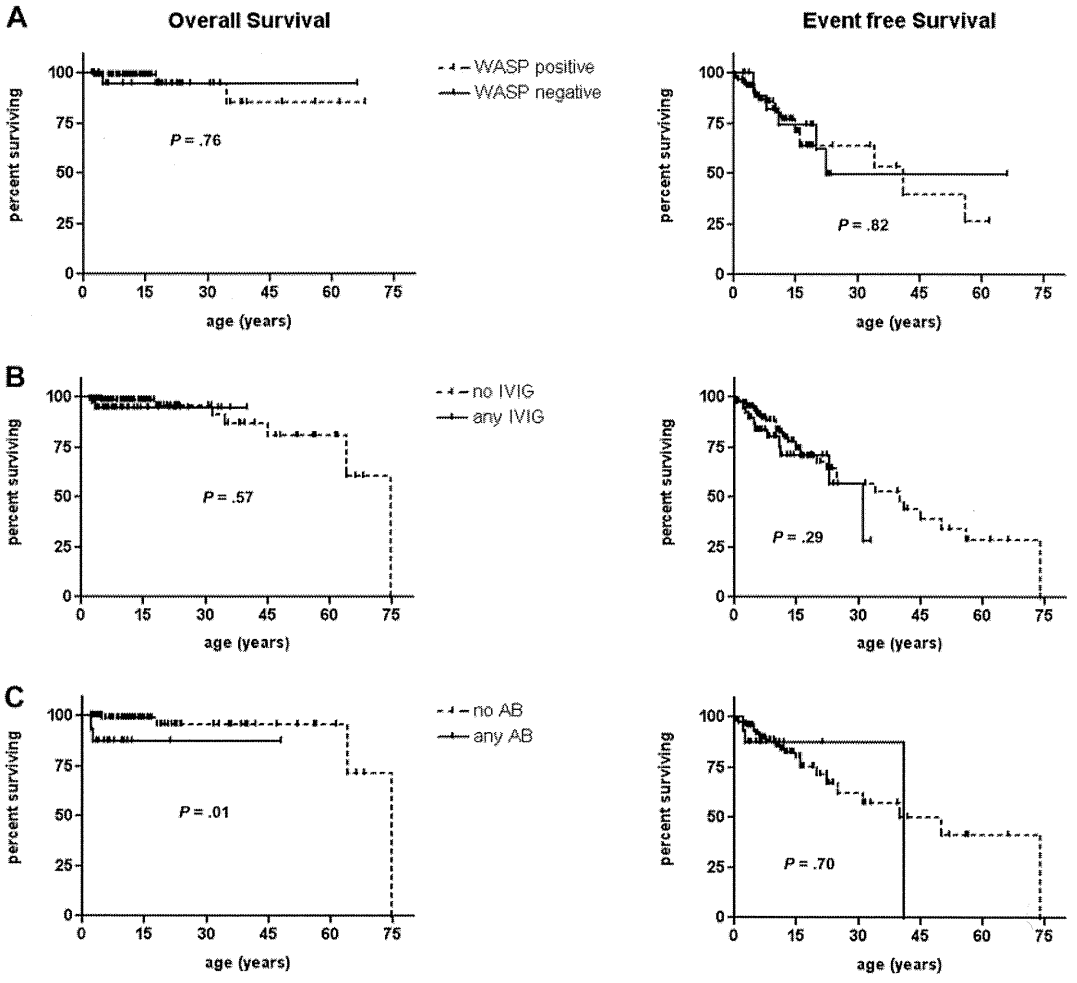

Figure 3. Influence of WASP expression, IVIG, or AB prophylaxis on overall and event-free survival. Kaplan-Meier estimate of overall survival and event-free survival probability of (A) WASP-positive (n = 98, dotted line) and WASP-negative (n = 21, solid line) patients. (B) Patients receiving any IVIG prophylaxis (n = 39, solid line) or no IVIG prophylaxis (n = 134, dotted line) and (C) patients receiving any AB prophylaxis (n = 16, solid line) or no AB prophylaxis (n = 116, dotted line). Patients who underwent splenectomy were excluded from the analysis in panel C. Each hash mark on a graph line indicates a censored event.

inclusion of patients with classic WAS with few disease symptoms as may be the case during the first 2 years of life. One possible drawback of this study could be its retrospective, cross-sectional design. It is probable that some events took place when medical care differed from that of today. Naturally, the study design might encompass a bias by some confounding factors such as patient compliance, physician preference, choice of prophylactic measures, and availability of HSCT. We can also not exclude some selection bias, missing very mild cases that are undiagnosed or misdiagnosed and not referred to an immunology center. But some older patients in this study had lived an uneventful life, before being diagnosed as XLT because their brothers, nephews, or grandsons were discovered to have a *WAS* gene mutation. Of note, the outcome of these older relatives did not differ from that of the rest of the cohort (data not shown). At this time the retrospective study design seems to be the only possible means to assess the clinical characteristics of a large cohort of patients with XLT. Having established this database of patients with XLT, we now have the opportunity to prospectively follow their course of disease.

Only 17.6% of evaluable patients with XLT from this cohort lacked WASP expression. In contrast, the proportion of WASP-negative patients from a multinational cohort of patients with WAS/XLT with known *WAS* mutations was 57% (104 of 184).[15] Some patients may in fact express WASP because the methods used to assess expression, such as Western blot analysis, might not be sensitive enough to detect low protein levels. This possibility is supported by the fact that 10 patients who were WASP negative had mutations (missense and invariant splice site) expected to result in WASP expression. In this selected cohort of patients with XLT, the clinical outcome of patients who did not express WASP was not different from patients who expressed WASP. Similarly, we did not find any beneficial effect of IVIG or AB prophylaxis on overall and event-free survival or on the incidence of life-threatening infectious events. These results have to be interpreted with caution, and a possible beneficial effect of these measures cannot be ruled out because data on AB and IVIG prophylaxis were very heterogeneous about dose and duration of treatment. They might solely reflect the fact that, by definition, most patients with XLT can mount effective antibody responses and therefore do not need IVIG or AB prophylaxis. It is possible that the initiation of these prophylactic measures might have been triggered by slightly more severe disease symptoms.[33]

In this cohort of 173 patients, 108 (62%) had missense mutations in the first 4 *WAS* exons; the remaining 38% (including 11 patients with missense mutations in exons 6-12) were spread over the entire gene, including 19% in noncoding regions. This is in line with previous reports of XLT.[13-15,33] We could not detect any

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influence of the type of mutation on survival or on the incidence of specific disease-related events. A mild phenotype despite a deleterious mutation might be due to other disease-modifying genes, pathogen exposure, or somatic mosaicism caused by in vivo reversion, leading to some WASP expression and thus a milder phenotype. Reversion is an event quite frequent in WAS,[38,39] but it was not specifically analyzed in this cohort.

Forty-one patients (23.7%) had undergone splenectomy, reflecting the acceptance of splenectomy by some health care providers to reduce the risk of bleeding and thus improve quality of life in patients with XLT.[37,40] Interestingly, there was only a nonsignificant reduction of severe bleeding episodes after splenectomy, possibly because of the low overall incidence that decreased with age. However, the incidence of severe infections was significantly increased, especially in patients not receiving AB prophylaxis. These data suggest that, before splenectomy in patients with XLT, one needs to carefully weigh the pros and cons of this procedure. If performed, that is, in patients with recurrent episodes of serious bleeding, the family must understand the risk of infections and be willing to accept the need for AB prophylaxis. In addition, vaccination against pneumococci and meningococci has to be considered, given the fact that most patients with XLT can be effectively immunized.[33] The high incidence of severe infectious complications after splenectomy, including adult patients, highlights the importance of lifelong AB prophylaxis in patients with XLT who have undergone splenectomy.

The excellent overall survival rate that is close to that of the normal male population supports the perception that XLT is a mild, chronic disease and that, as a rule, patients with XLT do not require standard prophylactic interventions. Declining immune function has been observed in XLT, and defective antibody responses may require prophylactic measures such as IVIG in some patients. However, the reduced event-free survival shows substantial risks of severe, life-threatening or potentially debilitating disease-related complications. The cumulative incidence rate analysis of events showed that serious bleeding episodes were generally restricted to the first 30 years of life. In contrast, the risk of developing autoimmune disease, developing malignancy, or having a life-threatening infectious episode was rather constant throughout the patients' lifetime. The prevalence of autoimmunity is 12% in our cohort, suggesting that this complication is less common than in classic WAS whereby it was reported to be as high as 40% to 72%.[20,41,42] Interestingly, we found a significantly higher incidence of autoimmune nephropathy in Japanese patients. Similarly, the prevalence of malignancy was less in our XLT cohort (5%) than in classic WAS (13%).[20,43] Considering the higher mean age of patients with XLT compared with patients with classic WAS who have not received a transplant, these differences are even more significant.

The persistent morbidity associated with XLT might argue for HSCT as a treatment option for these patients. Given the excellent success in young children with classic WAS,[23,24] HSCT might be considered a viable option for patients with XLT if an human leukocyte antigen–identical donor can be identified. However, when discussing HSCT, which requires full conditioning in patients with WAS and patients with XLT, one needs to carefully weigh the advantage of a possible cure against the acute risks and long-term consequences of this procedure, such as risk of secondary malignancy and infertility. Thus, HSCT in XLT has to be decided on an individual patient basis. In our cohort 25 of 173 patients underwent HSCT at a median age of 7.3 years (range, 2.1-38.0 years) and 22 (88%) are alive after a median follow-up of 2.2 years (range, 0.0-12.1 years). Of note, more than half of the patients received their transplant at an age older than 5 years, when matched unrelated transplants in WAS may have a less favorable outcome.[23] Long-term studies of HSCT in patients with XLT, not available at present, are urgently needed.

Because patients with XLT may present to different medical specialists, it seems vital to raise awareness of this probably underdiagnosed or misdiagnosed condition. Although this study showed a high overall survival rate of patients with XLT, it also showed that they are at risk of life-threatening complications. By defining the natural course of XLT and recognizing the life-long medical problems that affect the prognosis and quality of life of these patients, it has become possible to select safe and effective individualized therapies for this unique set of patients with mutations of the *WAS* gene that are generally expected to be less devastating.

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Authorship

Contribution: M.H.A., B.H.B., and H.D.O. designed the study; all authors except P.P. contributed data; M.H.A., T.C.B., P.P., and H.D.O. analyzed the data; and M.H.A., T.CB., B.H.B., and H.D.O. wrote the paper.

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Identification of *TINF2* gene mutations in adult Japanese patients with acquired bone marrow failure syndromes

Mutations of the genes involved in human bone-marrow failure syndromes (BMFS) have been identified in components of the telomerase- and telomere-associated genes, including the *TINF2* gene on chromosome 14q11·2, which encodes the 40 kDa TIN2 component of the telomere-associated shelterin protein complex (Calado & Young, 2008; Savage *et al*, 2008; Walne *et al*, 2008; Walne & Dokal, 2009). Clinically, it is very important to identify patients with pathogenic mutations in the telomere- or telomerase-associated genes, because these patients will probably exhibit refractoriness to conventional immunosuppressive therapy (IST) (Calado & Young, 2008). Several recent studies showed heterozygous *TINF2* mutation in 1–5% of patients with acquired aplastic anaemia (AA) (Walne *et al*, 2008; Du *et al*, 2009). The subjects of these studies were Caucasian, Black and Hispanic. Analysis of the *TINF2* gene among adult Asian populations of AA and myelodysplastic syndrome (MDS), to the best of our knowledge, had never been done. The largest controlled epidemiological study reported that the incidence of AA in the West was 2cases/million/year, but was about two- to three-fold higher in Asia (Issaragrisil *et al*, 2006). Therefore, we carried out an investigation to determine whether mutations in *TINF2* could be found in our cohort of adult Japanese patients with acquired BMFS, and if so, at what frequency. We screened exon 6 of *TINF2*, as it was previously found to be a potential hotspot for disease-associated mutations (Walne *et al*, 2008; Du *et al*, 2009), among 142 Japanese patients who were diagnosed with acquired AA or MDS refractory anaemia between 1993 and 2006 at the Nippon Medical School Hospital. We excluded AA and MDS patients who were found to carry mutations in the telomerase *TERC* or *TERT* gene. We identified two AA patients (1·4%) with *TINF2* heterozygous mutations, which were P283H and n865-866 di-nucleotide CC deletion (Fig 1A). The n865-866 di-nucleotide CC deletion in the *TINF2* gene is a novel mutation that has not been previously identified. These mutations were not found in 300 healthy controls. Because of the lack of biological sample from the relatives of the patients as well as other tissues of the patients, it was not possible to determine whether these were segregational or germline mutations. Using Southern blotting technique, we compared the length of telomeres of mononuclear cells in AA patients who carried the *TINF2* mutations to those of healthy age-matched controls. As shown in Fig 1B, AA patients with *TINF2* mutations (Patients 1 and 2) showed much shorter telomere lengths than those of healthy age-matched controls.

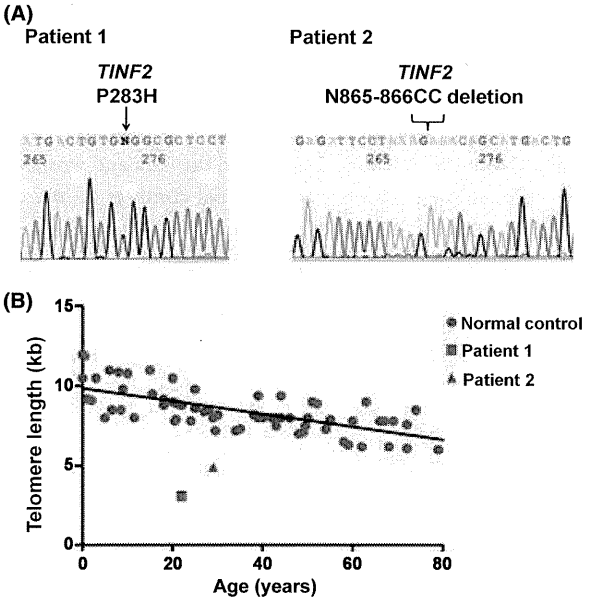

Fig 1. Identification of *TINF2* mutations and telomere length measurements. (A) Gene mutations identified by direct sequencing. In the case of the n865-866 di-nucleotide CC deletion (Patient 2), the *TINF2* PCR product was sub-cloned into pCR2·1-TOPO expression vector and sequenced. (B) telomere lengths of patients with *TINF2* mutations. Patient 1: 3·1 kb, Patient 2: 4·9 kb.

The clinical characteristics of these two patients with *TINF2* mutation are shown in Table I. Both of the patients with *TINF2* mutations were diagnosed with severe AA with no physical features of Dyskeratosis Congenita or its severe variant Hoyeraal-Hreidarsson syndrome (HH) (Walne *et al*, 2008) and showed no clinical response to IST. We attempted treatment of our patients with *TINF2* mutations with metenolone, which is a dihydrotestosterone (DHT)-based anabolic steroid with androgenic properties, but they did not show any favourable clinical responses, unlike a previous report of favourable haematological response in BMFS patients with *TERT* mutations upon androgen treatment (Calado *et al*, 2009). In summary, we report here for the first time *TINF2* natural mutations in 2/142 Japanese patients with acquired BMFS, which is at about the same frequency (1·4%) as reported in patients of other ethnic groups (Caucasian, Black and Hispanic) (Walne *et al*, 2008; Du *et al*, 2009).

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Table I. Clinical characteristics of patients with *TINF2* gene mutations.

Patient	Gene	Location of mutation	Age (years)	Sex	Diagnosis	Family history	Physical anomaly	Neutrophils ($\times 10^9$/l)	Hb (g/l)	Reticulocytes ($\times 10^9$/l)	Platelets ($\times 10^9$/l)	Chromosome abnormality	Shortened telomere	Treatment
1	*TINF2*	P283H	22	M	sAA	–	–	0·4	76	16·8	19	–	+	No response to IST
2	*TINF2*	Del n865-866	29	M	sAA	–	–	0·35	69	15·0	22	–	+	No response to IST

sAA, severe aplastic anaemia; IST, immunosuppressive therapy.

Authorship and disclosures

HY was the principal investigator and takes primary responsibility for the paper. HY, KI, JT, HT and KD recruited the patients. HY, KI, YM and FK performed the laboratory work for this study. HY, HL and KD analyzed the data and wrote the paper. The authors reported no potential conflicts of interest.

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Keywords: *TINF2,* telomere, bone marrow failure, aplastic anaemia, Dyskeratosis congenita.

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Mutation@A Glance: An Integrative Web Application for Analysing Mutations from Human Genetic Diseases

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Abstract

Although mutation analysis serves as a key part in making a definitive diagnosis about a genetic disease, it still remains a time-consuming step to interpret their biological implications through integration of various lines of archived information about genes in question. To expedite this evaluation step of disease-causing genetic variations, here we developed Mutation@A Glance (http://rapid.rcai.riken.jp/mutation/), a highly integrated web-based analysis tool for analysing human disease mutations; it implements a user-friendly graphical interface to visualize about 40 000 known disease-associated mutations and genetic polymorphisms from more than 2600 protein-coding human disease-causing genes. Mutation@A Glance locates already known genetic variation data individually on the nucleotide and the amino acid sequences and makes it possible to cross-reference them with tertiary and/or quaternary protein structures and various functional features associated with specific amino acid residues in the proteins. We showed that the disease-associated missense mutations had a stronger tendency to reside in positions relevant to the structure/function of proteins than neutral genetic variations. From a practical viewpoint, Mutation@A Glance could certainly function as a 'one-stop' analysis platform for newly determined DNA sequences, which enables us to readily identify and evaluate new genetic variations by integrating multiple lines of information about the disease-causing candidate genes.

Key words: genetic disease; mutation; polymorphism; bioinformatics; protein structure

1. Introduction

Genetic diseases are caused by structural changes in genes and/or chromosomes. In the Online Mendelian Inheritance in Man (OMIM, http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim) database, more than 2200 genes are known to have mutations causing genetic diseases.[1] For instance, primary immunodeficiency diseases (PIDs) are caused by congenital defects in genes involved in the development and maintenance of the immune system,[2,3] and they can be diagnosed using mutation analysis that identifies pathogenic mutations in candidate PID genes. This process plays a critical role in improving

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the quality of life for PID patients.[4] In this regard, the recent advances in DNA sequencing technology will extremely expedite this process. Thus, the next bottleneck to be addressed is obviously how to clarify the associations between newly identified patient-specific genetic variations and disease phenotypes, even when familial disease history is absent. To eliminate the bottleneck in mutation analysis, we need a bioinformatics tool that would enable us to readily evaluate the impact of a genetic variation on the structure/function of a gene product at the molecular level. Towards this end, our first step was to develop an integrated 'one-stop' analysis platform where we could cross-reference multiple lines of information regarding known genetic variations, including a huge amount of non-synonymous (ns) single-nucleotide polymorphisms (nsSNPs) in healthy individuals,[5–7] in genes of interest.

Bioinformatics resources and methods played an indispensable role in creating this platform.[8–12] Although a number of databases regarding reported human disease mutations and SNPs have been already constructed,[13–25] these databases were launched as a static archive for genetic variation data, not necessarily an interactive tool for evaluating newly identified sequence variation data. Several computational algorithms for predicting the effects of ns substitutions on a corresponding protein have been developed using evolutionary and protein three-dimensional (3D) structure information.[26–31] However, despite public availability of these software/web servers, there are at least two hurdles, especially for clinical researchers to exploit them for the mutation analysis: (i) since these servers usually require information about the position of the genetic variation occurred in a submitted sequence as a query input, the users have to specify the variation position in the sequence before submitting the query; (ii) since these servers do not necessarily incorporate known disease-associated mutation data into their systems, the users have to manually compare their newly identified genetic variation data from patients with previously reported data. Thus, we thought it was important to integrate predictive bioinformatics tools, such as the one described above, with a comprehensive set of known genetic variation data, to create a 'one-stop' mutation analysis platform.[32]

In this context, here we present Mutation@A Glance (http://rapid.rcai.riken.jp/mutation/), a new web-based integrated bioinformatics tool for analysing mutations from human genetic diseases. The user-friendly graphical interface of Mutation@A Glance makes it possible to allocate known disease-associated mutation data on the nucleotide and amino acid sequences of a gene of interest, and to link these mutation data to the 3D structure of the gene product along with various lines of information about the mutated amino acid residues (e.g. the extent of evolutional sequence conservation, post-translational modifications and molecular interactions). Furthermore, this tool enables users to identify and evaluate newly identified sequence variations in a query DNA sequence from a gene of interest by comparing them with known disease-associated mutation data and using the SIFT program,[26] which is one of the most accurate and widely used program to specifically predict the effects of ns substitutions based on evolutionary information for each residue position.[33] Therefore, Mutation@A Glance surely serves as a 'one-stop' informational platform to identify and evaluate new genetic variations by integrating multiple lines of information about the disease-causing candidate genes.

2. Materials and methods

2.1. *Data resources for disease-associated genes and sequence variations*

Human disease-associated mutation data were obtained from the following three databases: OMIM (http://www.ncbi.nlm.nih.gov/omim/),[1] UniProt (http://www.uniprot.org/)[34] and RAPID (http://rapid.rcai.riken.jp/).[17] Sequence variations that were associated with OMIM in the dbSNP database (Build 130, http://www.ncbi.nlm.nih.gov/projects/SNP/)[18] were considered to be disease-associated mutations and other variations were considered non-disease associated. For the mutation data in the UniProt database, VARIANT features associated with diseases in the human entries were considered. RAPID is a molecular database that we have recently established for reported disease mutation data in genes causing PIDs.[17] The RAPID database is directly connected to our local server and the mutation data (as of August 2009) are retrieved using a Perl script. The human genome sequence (Build 36.3), RefSeq sequences for nucleotides and proteins of human were downloaded from the NCBI ftp site (ftp://ftp.ncbi.nlm.nih.gov/). Information regarding residue-wise functional features (Transmembrane helix, signal peptide, nucleotide binding, disulphide bond, metal binding, active site and post-translational modification site) was extracted from the human entries in the UniProt database. Information regarding the exon–intron structures of each gene was downloaded from the NCBI ftp site.

2.2. *Calculation of sequence conservation in ns substitution sites*

Homologous protein sequences in other organisms to the human proteins encoded by disease-causing genes were identified using the BLAST program[35] against the RefSeq database (6 691 817 amino acid

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sequences) with a cut-off *E*-value of 10^{-4}. If the sequence identity and the coverage between a sequence hit and the human were higher than 40% and 80%, respectively, the sequence was selected as a homologous sequence. When two or more sequences from an organism were found as homologous sequences, the sequence with the highest sequence identity was only considered. The homologous protein sequences from various organisms were aligned using the CLUSTAL W program.[36] A degree of sequence conservation at each amino acid position in the multiple sequence alignment (simply designated as 'residue conservation' in Fig. 1) was defined as the ratio of (the number of the homologous protein sequences which carried an identical amino acid residue to that in the human sequence) to (the number of the aligned homologous protein sequences) at the specified position in the multiple sequence alignment. For example, if Ala appears in an aligned position in the human sequence and the corresponding positions in all of the other homologous sequences are also Ala, the residue conservation in this position is defined as 1.0. The frequency distribution of the residue conservations in disease-associated missense mutation or nsSNP positions for proteins analysed in this study was represented using bins of the interval of 0.2. The value in each bin was normalized by the frequency of the total number of residues in each bin.

2.3. *Protein 3D structure information*

Protein 3D structure data were downloaded from the Protein Data Bank (PDB, http://www.rcsb.org/pdb/).[37] In cases where the 3D structure of a human protein had not yet been determined, we searched the available sequences in the PDB entries for a template structure for homology modelling using the BLAST program as described above. When the alignment of the human protein sequence and a known 3D structure showed >30% identity and >90% coverage, a homology model was built using the MODELLER package.[38] For each target, 20 model structures were generated and their reliabilities were assessed with the Discrete Optimized Protein Energy (DOPE) method.[39] Eventually, the model with the best DOPE score was selected as the final model for each protein. Information about protein quaternary structures was also extracted from the PDB database. Entries from the PDB that contained information about the biological unit structure and entries with polypeptide chains showed >85% identities with a human protein sequence were considered. When a distance of one atom in a residue in a given polypeptide chain was <5.0 Å from that of another residue in the other polypeptide or nucleotide chain, the residue was considered to be located at a molecular interaction interface.

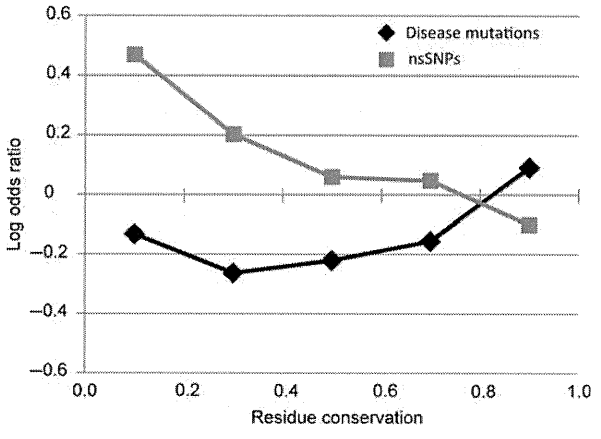

Figure 1. Comparison of frequency distributions of residue conservations in disease-associated missense mutations and nsSNPs. The vertical axis depicts the log-odds ratio of the frequency of ns substitution residue positions (disease-associated mutations or nsSNPs) to those of total number of residues in each residue conservation bin.

2.4. *Solvent accessibility calculations*

The solvent accessibilities of the amino acid residues in a 3D modelled structure were calculated using a modification of the Shrake and Rupley method,[40] with a water molecule represented by a 1.4 Å radius sphere. The solvent accessibility is represented by values ranging from 0 to 1. The residue was considered as an exposed residue on the protein surface, if the solvent accessibility was >0.25 and buried otherwise.

2.5. *Disorder prediction*

We used the DISOPRED2 program[41] to analyse each amino acid sequence of a gene product and predict intrinsically unstructured (disordered) regions in the protein sequence. If the program predicted a region consisting of more than three amino acid residues in a sequence to be 'disordered', we assigned this region as an intrinsically unstructured one.

2.6. *Predicting the effect of ns substitutions on proteins*

The effects of ns substitutions on a given protein were evaluated on a local server using the SIFT program[26] which predicts the effects of missense substitutions on a protein based on evolutionary information from homologous protein sequences.

2.7. *System implementation*

At the server end, a set of common gateway interface programs was written in Perl and is running on an Apache web server. The information regarding the disease-associated genes and the sequence variations

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described above was integrated into a MySQL database implemented in the server. At the client end, JavaScript frameworks such as prototype.js (http://www.prototypejs.org/) and scriptaculous.js (http://script.aculo.us/) were used to make the user interface more interactive. Jmol, a Java applet (http://www.jmol.org/), was implemented for visualizing protein 3D structures in a web browser.

3. Results and discussion

3.1. *Statistics of the sequence variation data on Mutation@A Glance*

From three data resources for human disease mutations, OMIM, UniProt and RAPID, we obtained 25 616 disease-associated mutations and 21 199 nsSNPs in 2656 human genes (Table 1) and integrated into the local database. Functional classification of the proteins encoded by the disease-associated genes showed a wide variety of molecular functions such as metabolic enzymes, protein kinases, transcription factor/regulators and structural proteins (Table 1 and Supplementary Table S1). Because we have been actively analysing mutations found in patients of PIDs with paediatricians in Japan, we constructed RAPID and used it as our original data resource for genetic variations in genes responsible for PIDs.[17] RAPID contains manually curated mutation data from published literature, including nonsense (582 sites in 96 genes), frameshift (851 sites in 101 genes) and insertion/deletion (85 sites in 42 genes) mutations as well as missense mutations (1564 sites in 116 genes) in the protein-coding regions of 155 PID genes (as of August 2009). For non-PID genes, we used two publicly available data sets from UniProt and OMIM. The UniProt database contains only missense mutation data (22 258 entries in 2614 genes). On the other hand, the OMIM database contains a large number of missense mutation (1899 entries in 556 genes) and a relatively small number of the other types of mutations (99 entries in 13 genes). The RAPID and the OMIM databases also contain 699 disease-associated mutation data in intronic regions of 147 genes that cause splice anomaly effects. Thus, the most frequent mutation type in our data sets was missense mutation (89% of the total entry) as reported in the previous study.[13]

3.2. *Evolutionary, structural and functional features of the ns substitution positions*

In general, disease-associated missense mutations tend to occur at evolutionarily conserved positions, because these positions are usually essential for the structure and/or function of a protein.[26,42,43] To verify this using the up-dated data set, we compared the frequency of disease-associated missense mutation sites (19 128 unique positions in 2622 genes) in each residue conservation bin with that of nsSNP sites (20 605 positions in 2494 genes) (Fig. 1). The results indicated that the previously reported tendency was still true for the 2622 genes in our data set; the disease-associated mutation sites were preferably appeared in the highest residue conservation bin, while nsSNP sites showed the opposite trend (Fig. 1). Next, we cross-referenced amino acid positions of the disease-associated missense mutations and nsSNPs to the functional features and 3D structures of the protein data in Mutation@A Glance. We classified these positions in terms of their functional features in a protein (annotated in the UniProt databases; Table 2). More disease-associated missense mutations were found in the positions annotated to have some functional features, except in the 'signal peptides' and 'post-translational modification sites', than nsSNPs. Using a homology modelling technique, we mapped 10 939 out of 19 128

Table 1. Functional classification of disease-associated gene products

Molecular class	No. of genes	No. of mutations[a]	No. of nsSNPs
Enzymes	410	5406 (5003)	2476
Protein kinases	258	1947 (1340)	2452
Transcription factor/regulator	239	2889 (2743)	1502
Structural proteins	132	1588 (1377)	1800
Cell surface receptors	123	1271 (1165)	838
Transport/cargo protein	116	1617 (1411)	1139
DNA/RNA binding proteins	97	429 (369)	580
Integral membrane protein	87	446 (434)	698
Channels	79	958 (948)	639
GTPase/GTPase regulators	71	371 (351)	450
Membrane transport protein	67	755 (751)	523
Immunity proteins	58	496 (183)	423
Extracellular matrix protein	53	886 (884)	939
Proteases	53	345 (284)	368
Cell adhesion molecules	52	390 (363)	428
Others	430	4647 (4091)	3863
Unclassified	331	1175 (987)	2081
Total	2656	25 616 (22 684)	21 199

[a]The numbers in parentheses indicate the number of disease-associated missense mutations.

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Table 2. Structural and functional loci of mutation/nsSNP sites

Property	Disease mutations	nsSNPs
Transmembrane helix	1283	648
Nucleotide binding	670	102
Disulfide bond	385	39
Metal binding site	226	147
Signal peptide	101	262
Post translational modification site	97	104
Binding site[a]	48	12
Active site[a]	25	6

[a]As defined in UniProt database (described in the text).

disease-associated mutation sites (57.2%) to protein 3D structures (Fig. 2). Of these sites, 6616 sites (60.4%) were located in regions buried in protein structures (solvent accessibility <0.25). In the same way, 7106 out of 20 605 nsSNP sites (34.4%) were mapped to 3D structures, and 4258 sites (59.9%) were located on the surfaces of proteins (Fig. 2A). This observation is basically consistent with the previous findings from structural analysis.[44–46] Interestingly, nsSNP sites were located in regions predicted as intrinsically disordered at a three times higher frequency than disease-associated mutation sites (Fig. 2A). This might be ascribed to the observation that conservation in the intrinsically disordered regions is relatively lower than that in ordered regions.[47]

Proteins function with other molecules in molecular networks (e.g. signalling pathways) in many cases. Hence, the effects of mutations on molecular interactions must be intriguing in mutation

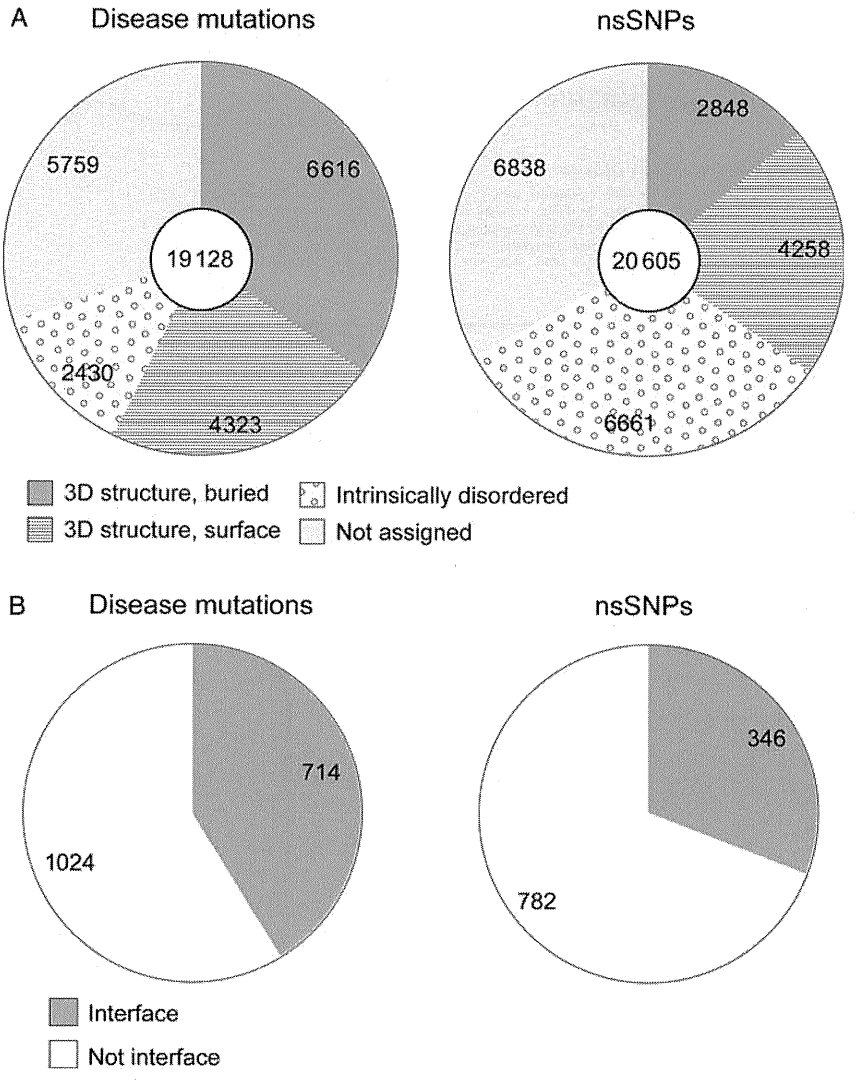

Figure 2. Classification of disease-associated mutations and nsSNPs according to their location on protein 3D structure. (A) The numbers in the pie charts depict those of ns substitution positions. (B) Proportion of ns substitution positions in the disease-associated mutations or nsSNPs that were located on the interface of the experimentally determined quaternary structures.

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analysis.[48] We thus analysed whether or not the missense mutation positions were located in the molecular interaction sites based on the quaternary protein structures available from the PDB. Consequently, 714 out of 1738 disease-associated mutation sites (41.1%) were found to locate at the interfaces of 474 distinct proteins known to be involved in protein complex structures (Fig. 2B; see Section 2.3). In contrast, the same was true for only 346 out of 1128 nsSNP sites (30.7%) in 447 genes. We confirmed that the frequency of disease-associated mutation sites located at the molecular interaction interface was significantly higher than that of nsSNP sites by χ^2 test ($P < 0.01$). These results implicated that ns substitutions at positions involved in the molecular interaction tend to be disease-related as we expected.

3.3. *The user interface for visualizing sequence variations*

Figure 3 shows the front page of the Mutation@A Glance website. It has two types of query forms, for visualizing known disease mutation data (Fig. 3A) and for evaluating novel genetic variations in query DNA sequences (Fig. 3B). For the visualization, a user inputs a given gene symbol of interest in the form. When the user enters some characters in the form, a list of gene names containing the input character string is shown to assist the user input. In addition, a user can also search for the gene name of interest from an entire list of genes available in Mutation@A Glance, which is displayed by clicking 'Select from List' button (Fig. 3A). Just as information for users, the mutation data set used for each gene is noted near the 'Select from List' button. Figure 4 shows

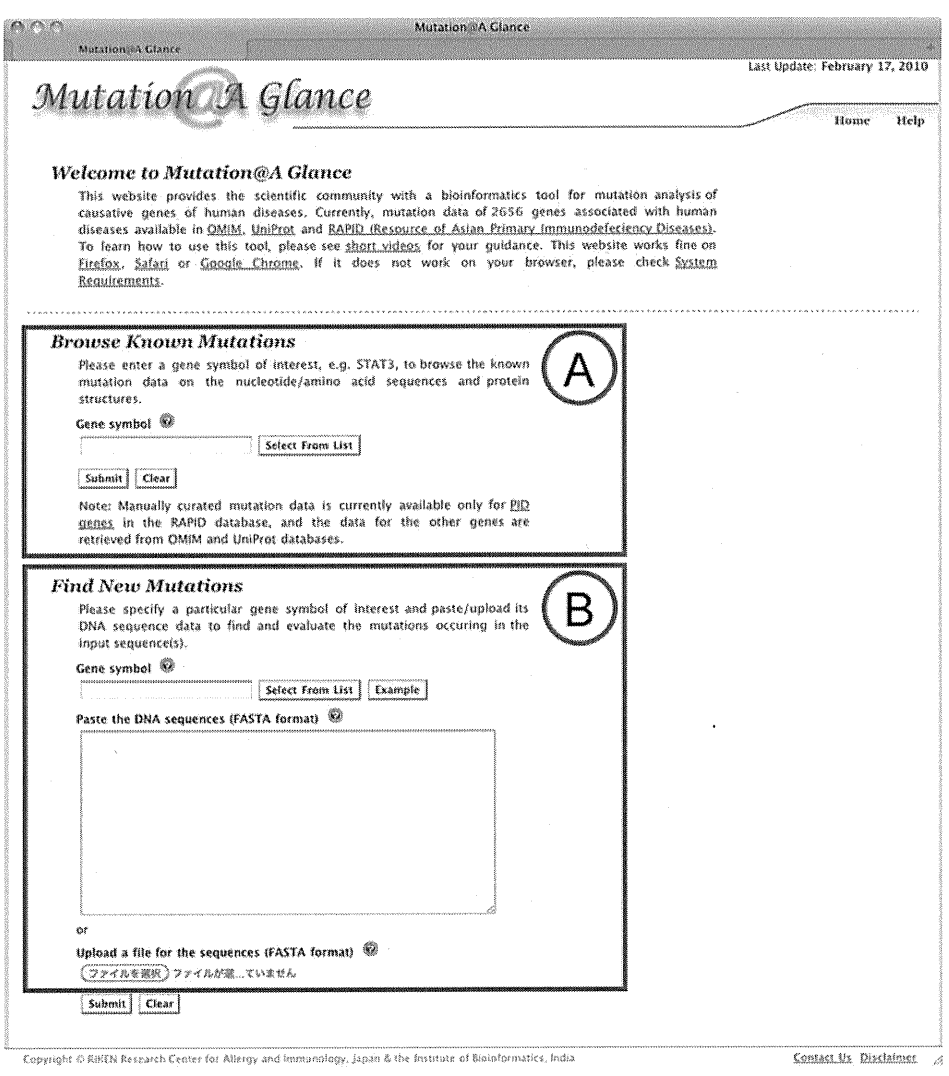

Figure 3. The front page of Mutation@A Glance. There are two types of query interface for (A) browsing known mutation data and (B) evaluating novel sequence variations in DNA sequences of interest. See the main text for details of the mutation data available in Mutation@A Glance.

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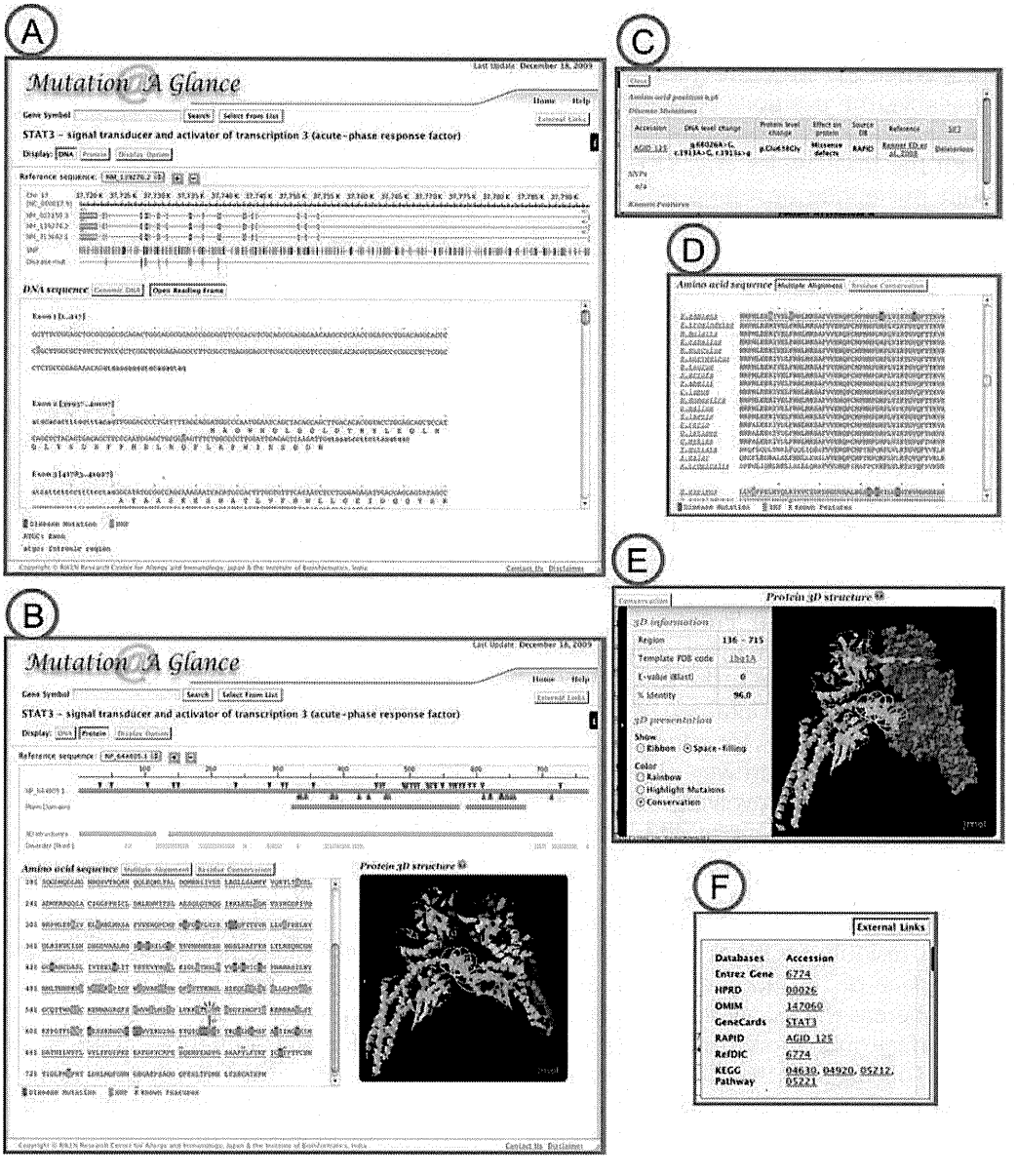

Figure 4. Screenshots of Mutation@A Glance. An example of visualizing mutation data for *STAT3* is shown at the DNA (A) and the protein levels (B). The nucleotide and amino acid positions of disease-associated mutations and SNPs are coloured magenta and green, respectively. At the protein level, various types of information for the mutated amino acid residues can be viewed. (C) The detailed information about the position of nucleotide or amino acid residues selected. (D) A multiple sequence alignment of human and the other organisms STAT3 protein sequences. (E) Detailed information about the 3D structure displayed with Jmol and the representation option menu for 3D structure information. (F) External links to other website for various types of information about the gene, e.g. gene expression and signalling pathway.

sample screenshots for the *STAT3* gene, which is known to be causative to hyper-IgE syndrome (HIES).[49,50] At the DNA level, positions of the disease-associated mutations, including substitution, insertion and deletion, as well as SNPs are shown on a set of exon sequences or genomic DNA sequence with/without the open-reading frame for the gene of interest (Fig. 4A). If two or more alternative transcripts exist in the RefSeq database, the genetic variation data are allocated on the reference sequence that encodes the longest amino acid sequence among the alternative transcripts whereas all the alternative transcripts are indicated in the top panel of the genomic structure. At the protein level, the disease-associated mutation and SNP sites are highlighted in the primary structure of the gene products along with available functional annotation information of the amino acid residues from the UniProt database (e.g. enzymatic active sites and post-translational modification sites, etc.) (Fig. 4B). Information regarding

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