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Short report

Multiple independent second-site mutations in two siblings with somatic mosaicism for Wiskott-Aldrich syndrome

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Key words: Wiskott-Aldrich syndrome, somatic mosaicism, somatic reversion, second-site mutation; gene therapy

Short Title: Multiple independent second-site mutations in WAS

Abstract

Wiskott-Aldrich syndrome (WAS) is an X-linked primary immunodeficiency disorder associated with microthrombocytopenia, eczema, autoimmunity and predisposition to malignant lymphoma. Although rare, few cases of somatic mosaicism have been published in WAS patients to date. We here report on two Ukrainian siblings who were referred to us at the age of 3 and 4 years, respectively. Both patients suffered from severe WAS caused by a nonsense mutation in exon 1 of the *WAS* gene. In both siblings, flow cytometric analysis revealed the presence of WASp-positive and WASp-negative cell populations amongst T and B lymphocytes as well as NK cells. In contrast to previously described cases of revertant mosaisicm in WAS, molecular analyses in both children showed that the WASp-positive T cells, B cells, and NK cells carried multiple different second-site mutations, resulting in different missense mutations. To our knowledge, this is the first report describing somatic mosaicism in WAS patients caused by several independent second-site mutations in the *WAS* gene.

Introduction

Wiskott-Aldrich syndrome (WAS) is a complex primary immunodeficiency disorder characterized by microthrombocytopenia, eczema, autoimmune phenomena, and susceptibility to malignant lymphoma. *WAS*, the defective gene in this disorder, encodes for Wiskott-Aldrich syndrome protein (WASp), a key regulator of actin polymerization (1). WASp deficiency causes multiple cellular defects, including defective leukocyte migration, defective T cell proliferation and disturbed formation of the immunological synapse (2, 3).

Several cases of revertant mosaicism have been described for WAS (4-9). The observation that the genetic reversion in a single or few cells can give rise to somatic mosaicism supports the concept that WASp expression confers a selective advantage at least for T lymphocytes and NK cells, which has important implications for the prospect of gene therapy approaches for this disorder (10).

We here report on two 3- and 4-year old siblings suffering from WAS with a novel nonsense mutation in exon 1 of the *WAS* gene, in both of whom somatic mosaicism for WASp was observed. Molecular investigations provided evidence for several independent second-site mutations in both patients. This observation adds to the growing understanding of somatic mosaicism in WAS.

Materials and Methods

Patients and controls

All material from patients and healthy donors was obtained with informed assent/consent in accordance with the Declaration of Helsinki.

Flow cytometry

Immunophenotyping and flow cytometric analysis of WASp expression in peripheral blood mononuclear cells (PBMCs) were performed as described previously (9, 11).

T cell proliferation assays

T cell proliferation assays were performed as described previously with minor modifications (12). In brief, T cell stimulation was performed using anti-CD3 coated 96 well plates with or without additional human IL-2 at a final concentration of 10 IU/ml. Cells were stimulated for 48 hours before pulsing with 1μCi (0.037 MBq) of ^{3}H-Thymidine (Perkin Elmer, Jügesheim, Germany) for 24 hours and subsequent analysis of thymidine incorporation.

Podosome staining

CD14+ cells were labelled with anti-CD14 magnetic beads (Milteny Biotech, Bergisch Gladbach, Germany) and isolated using an AutoMACS device (Milteny Biotech). Podosome analysis was performed as described previously (13).

Molecular analysis

CD3-positive lymphocytes from both patients were sorted into fractions of WASp-positive and WASp-negative cells. Genomic DNA was extracted from both fractions, followed by PCR amplification of all 12 exons of the *WAS* gene including intron-exon boundaries. For exons 1-11, PCR amplification was performed at an annealing temperature of 60°C using the following primer pairs: exon 1 fw: gctcagcctaacgaggagg, exon 1 rev: cgggaaattctaggagaggag, exon 2 fw: ctgcagctcttcctttggg, exon 2 rev: ctgatcaggtctgaggtcttg, exons 3-5 fw: ctcccaaatccagacaccc, exons 3-5 rev: ccctcctcttctctctggg, exon 6 fw: ccagccaatgaaggtgagtc, exon 6 rev: tcaatctacctatccattcaccc, exon 7 fw: tccatgaccatccaacacac, exon 7 rev: cagccatctgcccatctg, exons 8-9 fw: cattcattaattctggcccc, exons 8-9 rev: tgaccaactcctgactgaagc, exon 10 fw: gtcaggagttggtcagtggg, exon 10 rev: ctaaggcctggcactctcc, exon 11 fw: gaaggggactggagtgtgtg, exon 11 rev: acagatggggctgatgtcac. Primers and PCR conditions for amplification of exon 12 have been published previously (14). DNA sequences were analyzed

by using an ABI Prism 3130 DNA Sequencer and the DNA Sequencing Analysis software version 3.4 (Applied Biosystems, Foster City, CA, USA) and Sequencer version 3.4.1 (Gene Codes Corporation, Ann Arbor, USA).

To demonstrate the presence of several independent second-site mutations, exon 1 of the *WAS* gene in the WASp-positive and WASp-negative CD3+ lymphocyte, the WASP-positive CD3-CD56+ NK cell fraction and B cell population (sorted as CD3-CD56-CD19+CD20+ cells) was amplified using the primer pair described above. The product was gel extracted and subcloned into PCR Script cloning vector (Stratagene, Amsterdam, Netherlands). Sequencing of clones was performed using M13 universal primers.

Results

In August 2006, two brothers suffering from Wiskott-Aldrich syndrome were referred to us as potential candidates for a clinical gene therapy study. At this point, the brothers were 4 (patient 1) and 3 years (patient 2) of age, respectively. Patient 1 had eczema from birth and suffered from bloody diarrhea since infancy. At the age of 3 years, the patient had a systemic CMV infection. Patient 2 also suffered from eczema since birth. Due to a marked bleeding diathesis, he had frequent severe epistaxis. Both patients suffered from recurrent upper respiratory tract infections.

We performed T cell proliferation assays in both patients and found decreased proliferative responses upon CD3 stimulation, which could be partially rescued by additional stimulation with hIL-2 (Fig. 1a). Furthermore, we performed podosome analysis in peripheral blood CD14+ monocytes and found complete absence of podosomes in both patients, while a healthy control displayed 65% podosome-positive monocytes (Fig. 1b). Both patients had less than 50,000 platelets/microliter (Table 1). Immunophenotyping revealed decreased numbers of B lymphocytes in both patients as well as inverted CD4-CD8 ratios (Table 1). IgG levels in both patients were within the normal range. Specific antibodies to vaccines were dissociated. Patient 1 mounted a protective response to tetanus (0.11 IU/ml) but not to diptheria (0.08 IU/ml). Patient 2 had 0.15 IU/ml anti-tetanus-IgG and 0.18 IU/ml anti-diptheria IgG.

Flow cytometric analysis of WASp expression in peripheral blood mononuclear cells was performed and revealed the presence of fractions of WASp-positive and WASp-negative cells amongst CD3+ lymphocytes and NK cells in both patients (Fig. 2). In both patients, the majority of CD8+ lymphocytes and NK cells were WASP-positive. A fraction of 31% of CD4+ lymphocytes were positive in the elder brother (patient 1), whereas only a minor fraction of 4% of CD4+ cells were positive in the younger brother (patient 2). A small fraction of B lymphocytes from both patients were also WASp-positive, whereas no WASP-positive cells were identified in monocytes (Fig. 2).

We next performed flow cytometric sorting of WASp-positive and WASp-negative CD3+ lymphocytes from both patients, extracted DNA and sequenced all 12 exons of the WAS gene. The WASp-negative cell fraction harboured a novel mutation in exon 1, leading to a premature stop codon (c.C58T; p.Q20X). In contrast, analysis of the WASp-positive cell fraction showed an ambiguous signal for nucleotides 2 and 3 of the triplet of interest (Fig. 3). Thus, our findings suggested that the WASp+ CD3+ lymphocytes contained cells with different second-site mutations within the same triplet. The chromatograms showed that, for the WASp+ cell fraction, the „dominant" triplet was TAT in patient 1 and TGG in patient 2

while different peaks representing other nucleotides at triplet position 2 and 3 were also apparent.

To confirm the suspected molecular heterogeneity of WASp+ CD3+ cells, we sorted peripheral T cells into WASP-positive and WASP-negative fractions. Exon 1 of the *WAS* gene was PCR amplified and subcloned into a cloning vector prior to sequence analysis. 48 individual clones from patient 1 and 52 clones from patient 2 were analyzed by sequence analysis. In patient 1, we found that 23 out of 48 clones carried the triplet TAT, while 16 clones carried the triplet TGG and 9 clones carried the triplet TAC. For patient 2, 36 out of 52 clones carried the triplet TGG, whereas other clones carried the triplet TAC (13/52), TTG (1/52) or the wildtype sequence CAG (1/52) (Fig. 3). 1 out of 52 clones contained the original mutation TAG, thus representing missorted cells.

In contrast to WASp+ CD3+ cells, the chromatograms for WASp+ NK cells suggested the presence of a predominant TGG triplet for both patients. We found that 19 out of 20 clones from patient 1 contained the TGG sequence, while one clone contained the triplet TAC. For patient 2, 10 clones were analyzed, all of which contained the triplet TGG. Since also a minor B cell population appeared to be reverted in both patients (Fig. 2), we also sorted CD3-CD56-CD19+CD20+ B lymphocytes, subcloned the amplified exon 1 and subjected 123 clones from patient 1 and 114 clones from patient 2 to sequence analysis. 115 out of 123 sequences (93.5%) for patient 1 and 110 out of 114 sequences (96.5%) for patient 2 carried the original mutation, respectively. The second-site mutations in B lymphocytes from patient 1 were identified as CAG (4 out of 123), TGG (3) and TAC (1). For patient 2, 3 clones carried the triplet TAT and 1 clone the triplet TAC, respectively.

At a clinical follow up of the two patients 18 months later, both patients had decreased bleeding and eczema. Interestingly, their thrombocyte counts had risen to normal values. However, immunophenotypic analysis showed that both patients had persistent B cell lymphopenia. The CD4-CD8 ratio remained inverted (Table 1).

Discussion

Wiskott-Aldrich syndrome is a complex, X-linked primary immunodeficiency disorder caused by mutations in the WAS gene (2). More recently, a series of cases of somatic mosaicism have been reported for WAS (4-9). Spontaneous *in vivo* reversion events have also been reported for other primary immunodeficiency disorders such as ADA-SCID (15), X-linked SCID (16), Leukocyte Adhesion Deficiency (LAD) (17) or X-linked immunodeficiency with ectodermal dysplasia due to mutations in NEMO (18). While the majority of cases of somatic mosaicism in WAS is caused by true back reversions, more rare scenarios of a second-site mutations leading to altered but functional gene products has also been observed (5, 6). The study of somatic mosaicsim due to secondary somatic mutations is of considerable interest for the prospect of gene therapy for WAS, as it supports the concept of a potential proliferative advantage of gene-corrected cells at least for T lymphocytes and NK cells (10).

The cases presented here are remarkable in several aspects. Our analyses show that both affected siblings have somatic mosaicism for WAS with the large majority of CD8+ lymphocytes and NK cells expressing WAS protein. Indeed, observations by Wada et al. have demonstrated increasing expansion of WASp-positive lymphocytes over time in a WAS patient with revertant mosaicism (6), in agreement with the proliferative advantage of gene-corrected lymphocytes evident in the Was knockout mouse model (12). Given the high proportion of WASp+ cells in both patients, one may hypothesize that the second-site mutations may have occurred early in life. An alternative explanation may be that the proliferative advantage for WASp-positive lymphoid cells is particularly strong in these patients due to the severity of the null mutation in exon 1.

Sequencing results for the WASp-positive cell fractions suggested the parallel presence of different sequences within the same triplet in which the original mutation occurred, thus representing different second-site mutations. Indeed, we were able to confirm at a molecular level that both siblings carry at least 3 different second-site mutations within their WASp-positive CD3+ lymphocytes. Interestingly, the distribution of the detected second-site mutations is not equal in both siblings. First, the predominant sequence is different for the two patients, with the majority of cells carrying the triplet TAT for patient 1 and the triplet TGG for patient 2. Secondly, some of the observed triplets are either unique to one of the siblings or extremely rarely found in the other sibling, i.e., the triplet TAT was found exclusively in patient 1 and the triplets TTG and the wildtype triplet CAG were found exclusively in patient 2. Nonetheless, in contrast to the variety of second-site mutations found in WASp+ CD3+ cells, almost the entire set of WASp+ NK cells from both patients contained the triplet TGG.

We also found second-site mutations in B cells, consistent with our FACS data which showed that a minor population of B lymphocytes were WASp-positive in both patients.

An open question is whether the altered WAS protein is indeed functional. We have attempted to express the altered WAS protein in a retroviral vector system but could not achieve sufficient protein expression levels. Longitudinal observation of these patients suggests that they may have a clinical benefit from the second-site mutations, with an overall decreased bleeding diathesis and eczema in both patients. Of note, their platelet counts normalized. However, these observations remain anecdotal. In a clinical perspective, it remains difficult to define parameters guiding rational therapeutic decisions in WAS patients showing spontaneous somatic chimerism. Should allogeneic bone marrow transplantation or experimental gene therapy be performed in these patients? In view of the inherent difficulty in judging the consequences of retrovirus-mediated WASp expression, we decided not to offer experimental gene therapy at the present time.

Although there is increasing evidence that the prevalence of spontaneous somatic mosaicism in WAS patients may be higher than previously recognized, we here present, to the best of our knowledge, the first cases of somatic mosaicism caused by multiple second-site mutations in the same patients. The precise mechanism underlying these findings is unclear at present, but it is an intriguing observation that all second-site mutations have occurred within the same triplet. This would suggest that this triplet may be a hotspot for mutational events, a concept which is also supported by the finding that both siblings carry second-site mutations in this triplet. On the other hand, no other mutations within this triplet have been described in WAS patients to date (19) (http://homepage.mac.com/kohsukeimai/wasp/WASPbase.html.). An alternative hypothesis could be environmental influences which the patients may have experienced, such as exposure to increased amounts of radioactivity in many areas in Ukraine following the incident in Chernobyl in 1986. It is tempting to speculate if only such mutational events which confer a proliferative advantage to cells such as true back mutations or second-site mutations leading to an altered but functional protein, may eventually be observed in the patients, whereas many other mutations may remain undetected.

Prospective multicenter longitudinal studies would greatly help to define the clinical spectrum of reversions and their functional and molecular implications – these data are urgently needed to define a practical approach to the clinical management of WAS patients with somatic mosaicism.

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Figure legends

Figure 1.

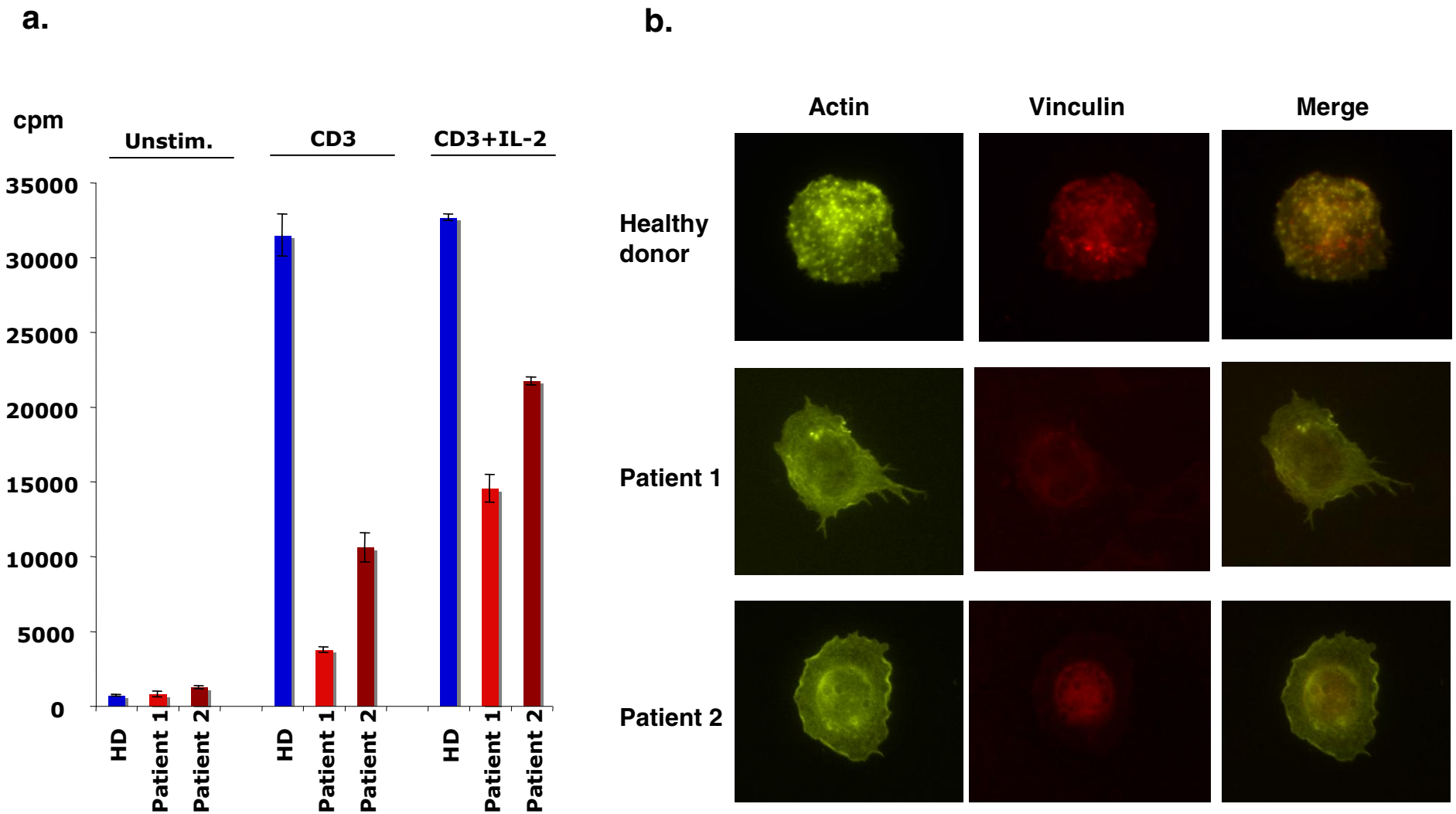
T cell proliferation assay and podosome analysis in CD14+ cells. (a) T cell proliferation upon stimulation with CD3 was decreased in both patients as compared to a healthy donor. This was partially rescued upon co-stimuation with human IL-2. (b) Podosomes were absent in CD14+ cells from both patients, whereas 65% of healthy donor CD14+ cells were podosome-positive.

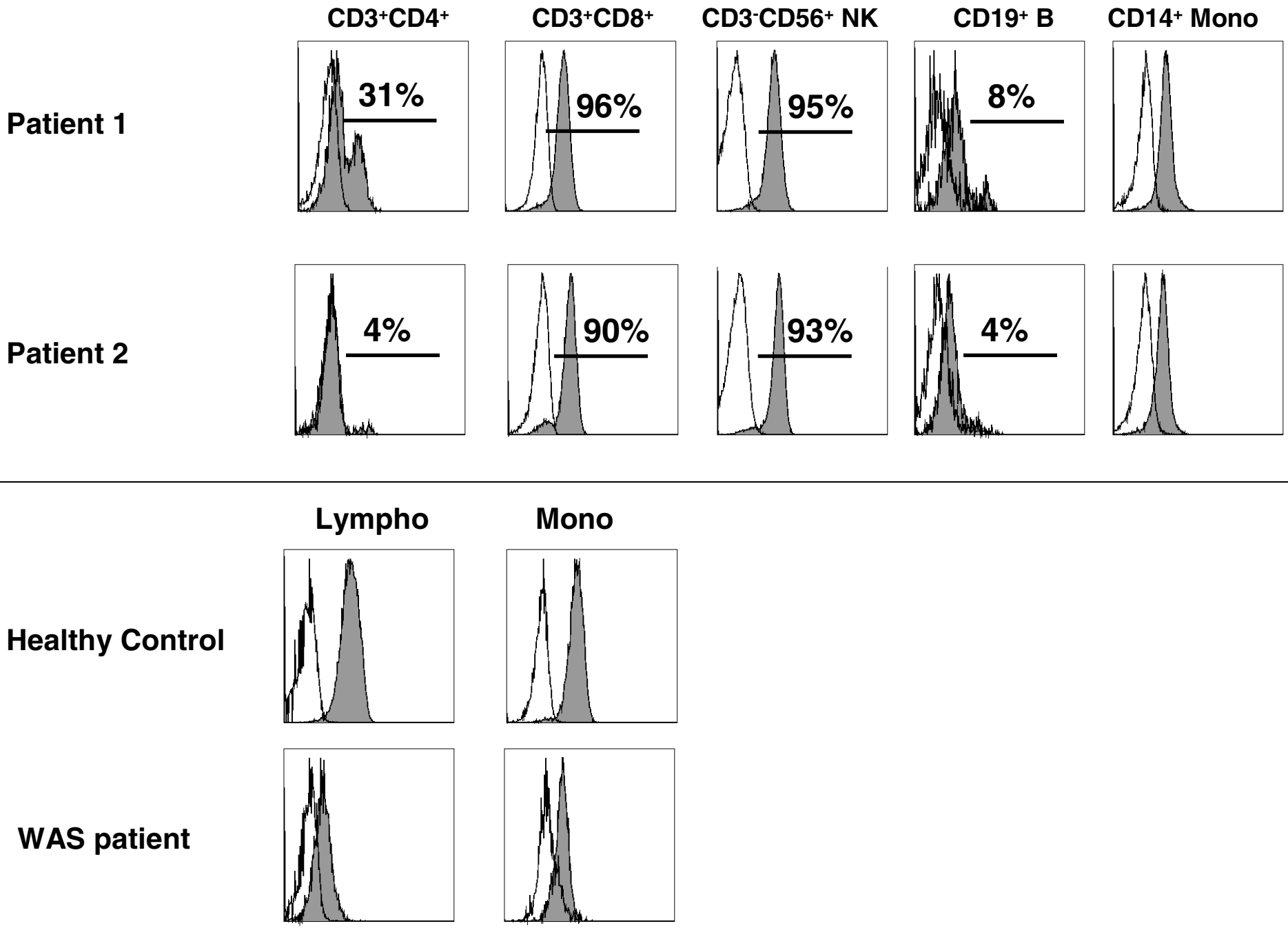
Figure 2.

Intracytoplasmic WASp expression analysis by FACS. Histograms represent staining anti-WASp staining (filled lines) as compared to isotype control (open lines) in different leukocyte subsets as indicated.

Figure 3.

Molecular analysis of second-site mutations in both patients. CD3+ WASp+ cells were FACSsorted and exon 1 of the WASp gene was sequenced, with the chromatogram showing ambiguous signals for nucleotide positions 2 and 3 in the triplet of interest. Subcloning of the DNA and subsequent sequence analysis demonstrated the presence of multiple second-site mutations in both patients.


a.
cpm
Unstim.
CD3
CD3+IL-2
35000
30000
25000
20000
15000
10000
5000
0
HD
Patient 1
Patient 2
HD
Patient 1
Patient 2
HD
Patient 1
Patient 2
b.
Actin
Vinculin
Merge
Healthy donor
Patient 1
Patient 2


CD3+CD4+
CD3+CD8+
CD3-CD56+ NK
CD19+ B
CD14+ Mono
Patient 1
31%
96%
95%
8%
Patient 2
4%
90%
93%
4%
Lympho
Mono
Healthy Control
WAS patient

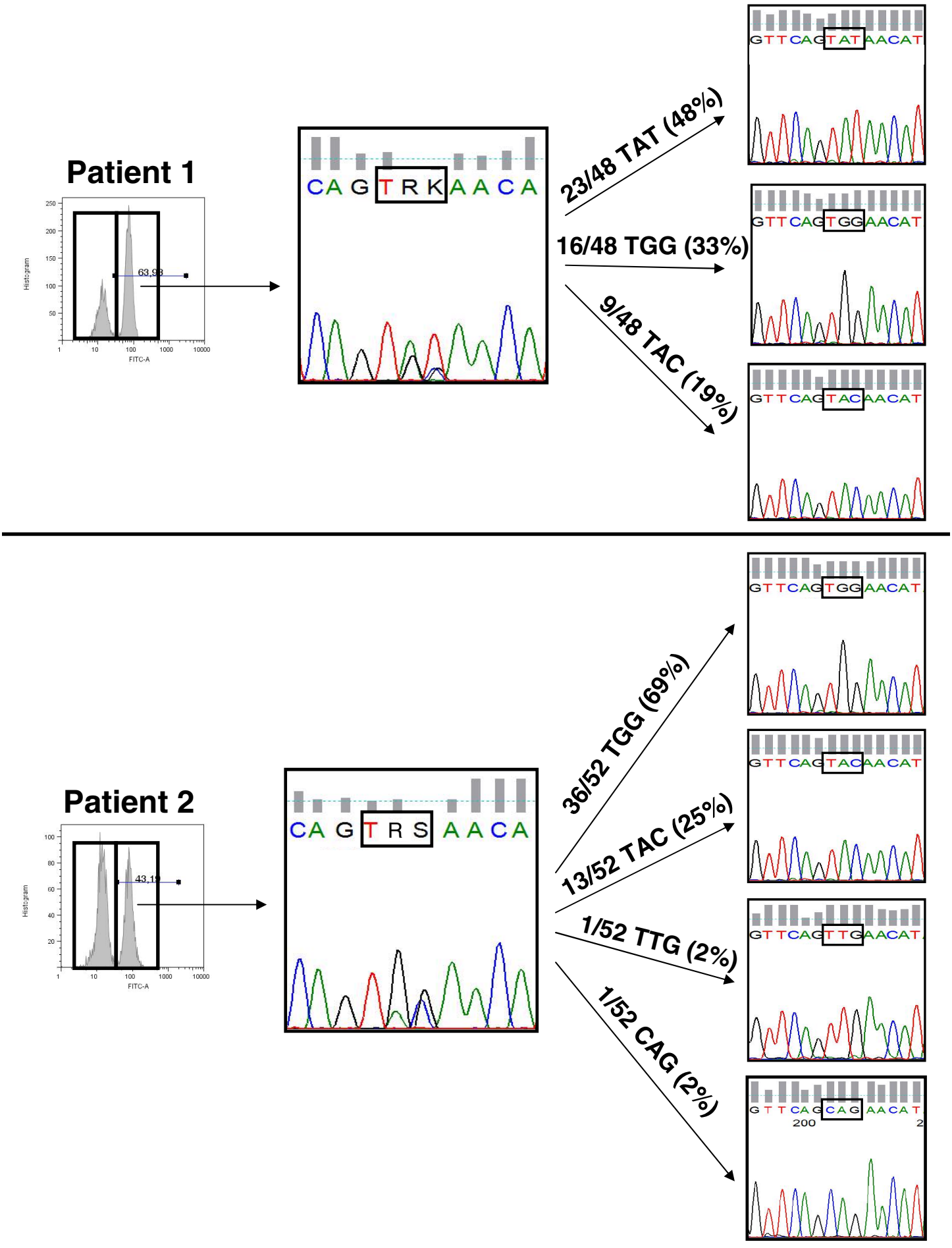

Patient 1
Histogram
63,98
FITC-A
CA G T R K A A C A
23/48 TAT (48%)
16/48 TGG (33%)
9/48 TAC (19%)
GTTCAGTATAACAT
GTTCAGTGGAACAT
GTTCAGTACAACAT
Patient 2
Histogram
43,19
FITC-A
CA G T R S A A C A
36/52 TGG (69%)
13/52 TAC (25%)
1/52 TTG (2%)
1/52 CAG (2%)
GTTCAGTGGAACAT
GTTCAGTACAACAT
GTTCAGTTGAACAT
GTTCAGCAGAACAT
200

Table 1. Blood counts and immunophenotyping in patients 1 and 2.

	Patient 1 Aug 2006	Patient 1 Feb 2008	Patient 2 Aug 2006	Patient 2 Feb 2008	Reference values
Leukocytes [/µl]	5200	10625	7900	7125	4,000-10,000
Thrombocytes [/µl]	45000	260000	20000	280000	150,000-300,000
Lymphocytes [/µl]	3822	3835	1358	3129	1,000-5,300
$CD3^+$ cells [/µl]	2320	1981	727	3835	800-3,500
$CD3^+CD4^+$ cells [/µl]	791	677	308	1228	400-2,100
$CD3^+CD8^+$ cells [/µl]	1333	1348	392	2818	200-1,200
$CD19^+$ cells [/µl]	144	187	91	84	200-600
$CD3^-CD56^+$ cells [/µl]	226	961	456	2141	70-1,200