Clinicopathological Characterization and Genomic Aberrations in Subcutaneous Panniculitis-Like T-Cell Lymphoma

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Subcutaneous panniculitis-like T-cell lymphomas (SPTLs) represent a rare, difficult-to-diagnose, and poorly characterized subtype of cutaneous T-cell lymphomas (CTCLs) affecting younger people more than the other CTCL forms. We performed a thorough clinical, immunohistological, and molecular analysis of nine Finnish SPTL patients. Specifically, we performed single-cell comparative genomic hybridization (CGH) from laser microdissected, morphologically malignant SPTL cells, as well as loss of heterozygosity (LOH) and fluorescence *in situ* hybridization (FISH) analysis for the *NAV3* (neuron navigator 3) gene. CGH revealed large numbers of DNA copy number changes, the most common of which were losses of chromosomes 1pter, 2pter, 10qter, 11qter, 12qter, 16, 19, 20, and 22 and gains of chromosomes 2q and 4q. Some of the DNA copy number aberrations in SPTL, such as loss of 10q, 17p, and chromosome 19, overlap with those characteristic of common forms of CTCL (mycosis fungoides (MF) and Sezary syndrome (SS)), whereas 5q and 13q gains characterize SPTL. Allelic *NAV3* aberrations (LOH or deletion by FISH), previously found in MF and SS, were identified in 44% of the SPTL samples. This study demonstrates that SPTL is also moleculocytogenetically a uniform entity of CTCL and supports the current World Health Organization–European Organization for Research and Treatment of Cancer (WHO–EORTC) classification defining SPTL as a subgroup of its own.

Journal of Investigative Dermatology (2008) **128,** 2304–2309; doi:10.1038/jid.2008.6; published online 13 March 2008

INTRODUCTION

Subcutaneous panniculitis-like T-cell lymphomas (SPTLs) are a rare and poorly characterized subgroup of cutaneous T-cell lymphomas (CTCLs). World Health Organization (WHO) classification identifies SPTL as a separate entity of T-cell lymphoma with an aggressive clinical behavior (Jaffe *et al.*, 2001), and the latest WHO–European Organization for Research and Treatment of Cancer (WHO–EORTC) classification separated for the first time two different subgroups within SPTL (Willemze *et al.*, 2005). Of these, only SPTL with α/β T-cell phenotype (SPTL-AB), presenting with cells usually expressing CD8, and restricted to the subcutaneous tissue should be considered as SPTL. In the γ/δ T-cell phenotype SPTL (SPTL-GD), the infiltrating malignant cells usually are CD8− and CD56+. They may show (epi)dermal involvement and should be classified among the cutaneous γ/δ T-cell lymphomas (Willemze *et al.*, 2005; Willemze *et al.*, 2008).

To date, there are only a few publications on SPTL, describing mainly the clinicopathological characteristics of sporadic cases (Burg *et al.*, 1991; Salhany *et al.*, 1998; Weenig *et al.*, 2001; Santucci *et al.*, 2003; Hoque *et al.*, 2003; Massone *et al.*, 2004, 2006; Takeshita *et al.*, 2004, Ghobrial *et al.*, 2005). A very recent, as yet unpublished, large clinicopathologic study of European patients has been completed (Willemze *et al.*, 2008). However, nothing is known about the molecular mechanisms leading to SPTL. Thus, we undertook a thorough clinical, immunohistological, and molecular analysis of Finnish SPTL-AB patients and present the results herein. Further usage of SPTL in this paper presents SPTL-AB.

Clinically, SPTL presents with solitary or multiple subcutaneous nodules and plaques, predominantly affecting the lower extremities and trunk. Initial systemic symptoms such as fever, fatigue, and weight loss are frequent, and hemophagocytic syndrome may be present (Gonzalez *et al.*, 1991), indicating a rapidly progressive course and worse prognosis (Marzano *et al.*, 2000; Willemze *et al.*, 2008). Histologically, SPTL is characterized by subcutaneous infiltrates of pleomorphic, small- to medium-sized T lymphocytes

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Abbreviations: CGH, comparative genomic hybridization; CTCL, cutaneous T-cell lymphoma; FISH, fluorescence in situ *hybridization; LOH, loss of heterozygosity; MF, mycosis fungoides; SPTL, subcutaneous panniculitis-like T-cell lymphoma; SS, Sezary syndrome*

Received 29 June 2007; revised 7 October 2007; accepted 12 November 2007; published online 13 March 2008

rimming the individual fat cells, whereas the epidermis and dermis are typically uninvolved. Additionally, necrosis, karyorrhexis, leukocytoclasia, and cytophagocytosis are often present. The immunophenotype is usually CD3+, CD4−, CD8+, CD56−, and cytotoxic proteins are frequently expressed. The disease response to therapy is usually favorable, with a 5-year survival of more than 80% (Massone *et al.*, 2004; Willemze *et al.*, 2008) and without hemophagocytic syndrome it is even 91% (Willemze *et al.*, 2008).

RESULTS

Clinical and immunohistological features of SPTL

The patients were relatively young (13–59 years of age, mean age 26 years; Table 1) when presenting with the first symptoms compared with patients presenting with the more common variants of CTCL, and there was a male predominance. The presenting symptoms were typically fever of 38–39 °C lasting for a few weeks and followed by the gradual development of violaceous, subcutaneous plaques or nodules anywhere on the body (Figure 1a). Before the diagnosis of SPTL, most patients had undergone wide examinations to reveal the cause of the fever, and the only abnormal laboratory values common for these patients were as follows: slight leukopenia, moderately elevated erythrocyte sedimentation rate and C-reactive protein, and elevated lactate dehydrogenase (Table 1). In whole-body computed tomography and bone marrow biopsy, extracutaneous lymphoma could not be verified, although it was suspected in two cases (cases 2 and 3). The delay from the appearance of the first symptoms to the definite SPTL diagnosis varied from 3 weeks to 10 years. For three cases (cases 1, 2, and 4), the delay was 10 years, which further underlines the diagnostic challenges. Cases 7–9 were diagnosed recently, and an accurate diagnosis was achieved within a few months, due to experience gained from this study. Three patients had a history of autoimmune disease, one of idiopathic thrombocytopenic purpura (ITP) (case 1), one of systemic lupus erythematosus (SLE) (case 6), and one of psoriatic arthritis (case 7). Additionally, SLE was suspected in three other cases (cases 1, 2, and 7) because the skin lesions resembled lupus skin lesions, but the American College of Rheumatology (ACR) criteria for SLE (Tan *et al.*, 1982) were not fulfilled. Case 7 had been treated with antirheumatic drug leflunomide for psoriatic arthritis before the onset of SPTL. None of the patients fulfilled the hemophagocytic syndrome criteria, although hemophagocytic syndrome was suspected in two cases (cases 3 and 6).

Histologically, all skin biopsies exhibited an infiltrate of small- to medium-sized pleomorphic cells with mitoses in the deeper dermis and fat tissue. Lobular panniculitis and rimming (atypical lymphocytes surrounding the fat cells in a lace-like manner; Figure 1b) were present for all nine cases. Additionally, erythrophagocytosis, karyorrhexis, and angiocentricity were seen in the lymphoid infiltrate. The immunophenotypes of the malignant cell infiltrate were CD3+, CD4−, CD8+ (Figure 1c), CD56−, and GranzB+ in all nine cases, whereas T cell-restricted intracellular antigen (TIA1) was expressed in six cases. Active cell proliferation was observed in all nine samples as measured by the nuclear Ki-67 antigen expression (MIB-1 immunostaining). All cases were positive for TCR α/β A chain (Figure 1d). No difference in the clinical course was found in relation to these markers.

Common DNA copy number changes characteristic to SPTL, as defined with CGH

To ensure the best possible representativeness of the sample material, we chose to collect the morphologically malignant cells by single-cell laser microdissection. The pure malignant cell population thus obtained showed several previously unreported DNA copy number abnormalities by comparative genomic hybridization (CGH) (Figure 2). The overall CGH profiles were consistent between individual patient samples, and all samples showed chromosomal imbalances by CGH. The number of chromosomal imbalances in case 3 was less than in all other cases. The most common CGH findings were DNA copy number losses in chromosomes 1p (6/6 cases), 2p (5/6), 2q (4/6), 5p (5/6), 7p (4/6), 9q (5/6), 10q (5/6), 11q (5/6), 12q (5/6), 16 (5/6), 17q (4/6), 19 (4/6), 20 (6/6), 22 (6/6), and gains in chromosomes 2q (5/6), 4q (5/6), 5q (4/6), 6q (4/6), and 13q (4/6) (Figure 2).

NAV3 deletion is found in SPTL by fluorescence *in situ* hybridization and loss of heterozygosity analysis

Deletion of *NAV3* (neuron navigator 3) gene, previously found to be associated with mycosis fungoides (MF) and Sezary syndrome (SS) (Karenko *et al.*, 2005), by fluorescence *in situ* hybridization (FISH) on touch preparates made from fresh frozen tissue skin biopsy samples, was demonstrable in 34, 52, and 8% of the cells of the three patient samples studied (cases 2, 3, and 8, respectively). Loss of heterozygosity (LOH) results for the *NAV3* region in the skin samples could be obtained from four patients. Three patients (cases 2, 3, and 5) showed LOH at D12S326, the closest marker relative to *NAV3*, and in two of these (cases 2 and 3), marker D12S1684 was shown to be involved as well. Case 6 was uninformative for D12S326 but showed LOH at D12S1684.

DISCUSSION

This study reveals DNA copy number changes common to SPTL. Previously, it has proven to be difficult to obtain any molecular cytogenetic data on SPTL, because the number of malignant cells circulating in the blood, if any, is scarce, and the malignant cells in the subcutaneous tissue are surrounded by fat cells, reactive T cells, and other normal cells of the skin. By microdissecting the morphologically malignant cells from the biopsy sections of SPTL lesions, isolating the genomic DNA thereof, and amplifying the obtained DNA with a reliable whole-genome amplification method, it was possible to assess the DNA copy number changes occurring in the malignant cell population of SPTL.

CGH revealed DNA copy number aberrations in the majority of the chromosomes. The large amount of aberrations is due to the relatively pure malignant cell population that was studied. The DNA copy number changes were very uniform between individual patients with a large number of changes occurring in the vast majority of cases (Figure 2).

Table 1. Clinical presentation, treatment and outcome of the patients

Case	Sex and age (years) at time of first symptoms	First clinical presentation and abnormal laboratory parameters	Initial therapy	Staging (CT or BM)	Therapy after SPTL diagnosis	Outcome
1	F, 18	Recurrent fever and subcutaneous nodules in extremities and face, loss of weight; SR 27, CRP 14, WBC $3.2 \times 10^9\,l^{-1}$, LD 1,206 U l^{-1}	Prednisone→ remission for 5 years; azathioprine, hydroxychloroquine	CT: negative	Cytostatics (M-BAIOD/ BACOD 6 cycles)	CR for 7 years
2	M, 16	Recurrent fever, 2 years later subcutaneous nodules on trunk and later on extremities; SR 19, CRP 31, WBC $1.9 \times 10^9\,l^{-1}$, LD 1,125 U l^{-1}	Hydroxychloroquine	CT: negative BM: negative	Cytostatics (CHOP 6 cycles)	CR for 4 years
3	M, 13	Fever for 4 weeks and subcutaneous nodules on face, trunk, and extremities, fatigue, dry cough; SR 16, CRP 35, WBC $3.1 \times 10^9\,l^{-1}$, ALAT 123, ASAT 125, prealbumin 43 mg l^{-1}, LD 1,581 U l^{-1}	Antimicrobials	CT: liver involvement suspected, pleural effusion and slight ascites (HPS criteria not fulfilled) BM: negative	Prednisone+ cyclophosphamide, CHOP-E[1], 3 cycles→ PR in skin, sepsis; relapse 1 month later, melphalan+ autologous stem cells twice[2]	Died of *Pseudomonas* sepsis, complicating autologous stem cell transplants, 6 months after first symptoms of SPTL
4	M, 18	Fever and subcutaneous plaques, patches and nodules on trunk and extremities, during relapse weight loss; LD 2,084 U l^{-1}, WBC $2.5 \times 10^9\,l^{-1}$	Prednisone→ remission for 10 years	CT: negative BM: negative	Cytostatics (M-BACOD 6 cycles)	CR for 6 years
5	M, 23	Recurrent fever and tender subcutaneous violaceous nodules on face, trunk, arms, and legs, weight loss; WBC $2.8 \times 10^9\,l^{-1}$, SR 35, ASAT 338, ALAT 417, AFOS 1,180, LD 4,320 U l^{-1}	Prednisone→no remission	CT: ND BM: infiltration of histiocytes with erythrophagocytosis in spleen→ splenectomy	Cytostatics (Bleo-CHOP 3 cycles)	CR for 18 years
6	F, 48	Recurrent fever episodes, subcutaneous nodules; WBC $1.7 \times 10^9\,l^{-1}$, platelets $105 \times 10^9\,l^{-1}$, S-ferritin 4,800 μg l^{-1}, ASAT 254, ALAT 122, AFOS 830, LD 1,063 U l^{-1}; SLE diagnosis 19 years earlier with complications (hepatitis, AIHA, thrombocytopenia)	Prednisone→no remission Cyclosporine, azathioprine, methylprednisolone Symptoms were considered SLE related	CT: negative BM: negative	Cytostatics (CHOP 7 cycles); radiotherapy for local recurrence	CR for 5 years
7	F, 59	Subcutaneous violaceous nodules on forehead and arms; no B-symptoms; LD 239 U l^{-1}		CT: negative BM: ND	Electron beam→ partial response Prednisone 60 mg for 2 weeks, reduction to 10 mg within 3 months→CR	CR; follow-up time <2 years
8	M, 27	Subcutaneous, firm nodules on top of the head; no B-symptoms		CT: negative BM: negative	Prednisone 60 mg→ CR, relapse within 1 month→ prednisone 80 mg for 2 weeks, reduction to 5 mg within 5 months→CR	CR; follow-up time <2 years
9	F, 15	Subcutaneous, tender nodules on upper and lower extremities and abdomen; no B-symptoms; LD 242 U l^{-1}		CT: ND BM: ND	Prednisone 40 mg for 2 weeks, end of the treatment within 6 weeks→CR	CR; follow-up time <2 years

AFOS, alkaline phosphatase; AIHA, autoimmune hemolytic anemia; ALAT, alanine amino transferase; ASAT, aspartate aminotransferase; BM, bone marrow; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; CR, complete remission; CRP, C-reactive protein; CT, computed tomography; F, female; HPS, hemophagocytic syndrome; LD, lactate dehydrogenase; M, male; M-BACOD, methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, dexamethasone; M-BAIOD, methotrexate, bleomycin, doxorubicin, ifosfamide, vincristine, dexamethasone; ND, not determined; PR, partial response; SR, sedimentation rate; WBC, white blood cell.
[1]CHOP-E=cyclophosphamide, doxorubicin, vincristine, prednisone, etoposide, combined with G-CSF as described in the Nordic Lymphoma Group phase II study of peripheral T-cell lymphomas (NLG-T-01) (Sendoxan, Adriamycin, VP16, Oncovin).
[2]Autologous stem cells second time after busulfan, thiotepa, etoposide, and ARA-C.

In previous CGH studies on other CTCL forms, performed on non-microdissected heterogeneous cell populations, the CGH changes identified have not been that uniform, although they were common to the majority of cases studied (Karenko *et al.*, 1999; Mao *et al.*, 2002; Fischer *et al.*, 2004).

In this SPTL patient series, the number of losses in DNA copy number exceeded the number of gains. This has also been the trend in some previous CGH studies of the most common CTCL subgroups, for example, MF and SS (Karenko *et al.*, 1999). DNA copy number gains were detected in large areas in the q-arms of chromosomes 2, 4, 5, and 13 (Figure 2). Previous CGH studies on MF and SS have demonstrated gains in chromosomes 2q (Mao *et al.*, 2002; Karenko *et al.*, 1999) and 4q (Mao *et al.*, 2002; Hahtola *et al.*, 2006). By gene expression analysis of these common CTCL subtypes, chromosome arm 4q is also among the areas with the highest frequency of upregulated genes (Hahtola *et al.*, 2006). Losses were identified in the terminal parts of many chromosomes, as well as covering the whole small group F and G chromosomes. The most common areas of DNA copy number losses were 1qter, 2qter, 10qter, 11qter, 12qter, 17p, 19, 20, and 22. These findings also share a great deal of similarities when compared to the CGH findings published for the most common CTCL subgroups. Chromosomal losses of 1p, 17p, 10q, and 19 were the most common CGH changes in a British study of 34 CTCL patients (Mao *et al.*, 2002), and other groups have demonstrated losses of 10q and 17p to be the key aberrations in CTCL (Karenko *et al.*, 1999; Fischer *et al.*, 2004). On the other hand, losses of chromosomes 5q and 13q occur in the most common forms of CTCL (Karenko *et al.*, 1999; Fischer *et al.*, 2004), but in SPTL these areas showed DNA copy number gains by CGH. Our current finding of losses in chromosome 12q, in most cases of SPTL, is in concordance with the recent identification of both DNA copy number loss and a downward gene expression bias in chromosome 12q (Hahtola *et al.*, 2006). We also recently reported on the recurrent CTCL-associated gene aberration, a putative, previously unknown, non-classical (that is,

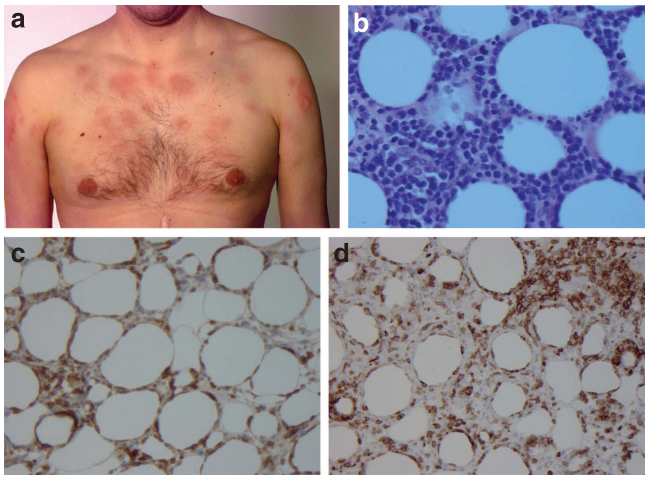

Figure 1. Clinical and immunohistological characteristics of SPTL. (**a**) SPTL presents clinically with subcutaneous, usually multiple reddish nodules on the trunk and extremities. (**b**) Histologically, SPTL is characterized by atypical lymphocytes rimming the subcutaneous adipocytes. Immunohistologically, SPTL is characterized by (**c**) CD8 positive and (**d**) TCR α/β infiltrating lymphocytes.

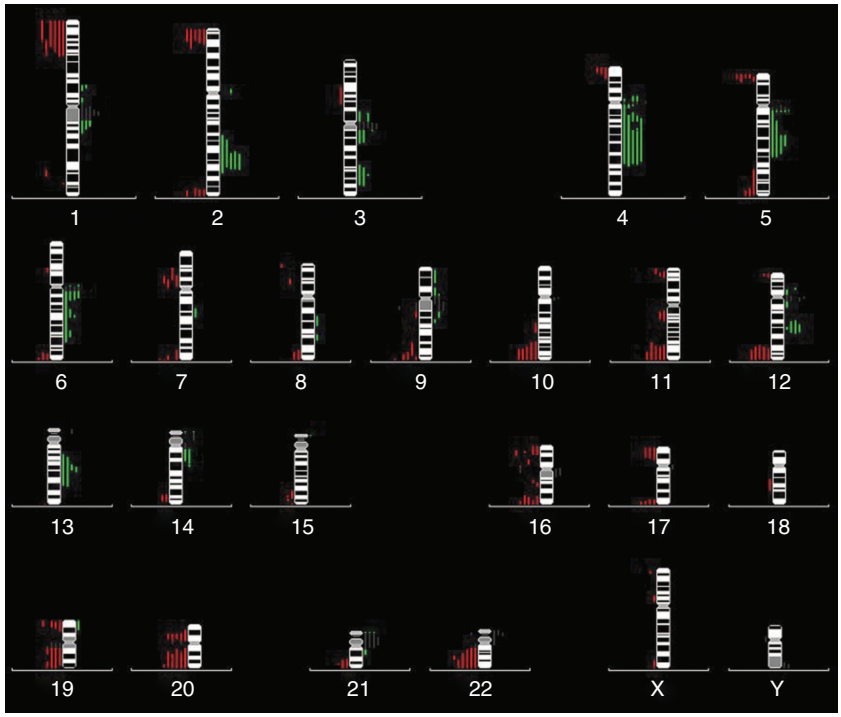

Figure 2. DNA copy number changes of true malignant cells of seven patients with SPTL. The most common CGH findings were DNA copy number losses in chromosomes 1p (6/6 cases), 2p (5/6), 5p (5/6), 9q (5/6), 10q (5/6), 11q (5/6), 12q (5/6), 16 (5/6), 20 (6/6), 22 (6/6), and gains in chromosomes 2q (5/6) and 4q (5/6).

haploinsufficient) tumor suppressor gene *NAV3*, located in the chromosomal area 12q21, to be deleted or translocated in all stages of CTCL (Karenko *et al.*, 2005). *NAV3* was also deleted in the four SPTL patients studied, as detected by both FISH and LOH analyses, and thus the *NAV3* gene seems to be important for several CTCL subtypes, namely MF, SS, and SPTL. Taken together, in addition to *NAV3* deletion, chromosome regions 10q, 17p, and 19 are likely to harbor genes common for the molecular pathogenesis of several subtypes of CTCL (MF, SS, and SPTL), whereas 5q and 13q gains characterize SPTL.

It may be speculated that aberrations in chromosome arms, common to both SPTL and MF/SS, may not influence the prognosis, as the prognosis of SPTL is favorable and that of SS less favorable. It has previously been observed that 17p loss and 7 gain do not influence the prognosis of CTCL (MF and SS) patients (Fischer *et al.*, 2004). Instead, 13q loss has previously been found to associate with shorter survival among MF and SS patients (Fischer *et al.*, 2004) and, interestingly, SPTL patients did not show that abnormality, but instead, frequently have 13q gain.

Our findings also provide previously unknown information on the usage of systemic steroids as a first-line therapy for SPTL. Although it took almost 10 years to reach the definite SPTL diagnosis for three of our first cases, they were all treated with oral steroids for unclassified panniculitis, and long-lasting remissions, even for 10 years, were achieved. Moreover, three of the more recent patients (cases 7–9) have responded completely to long-term systemic steroid therapy. However, the factors determining response to sole high-dose systemic corticosteroid therapy and the requirement of multiagent chemotherapy need to be defined in a larger prospective study.

Autoimmune phenomena occur frequently among SPTL patients (Willemze *et al.*, 2008). Three of our cases had an autoimmune disease, and two other cases presented with laboratory abnormalities suggestive of an autoimmune disorder, for example, antinuclear autoantibodies, a positive Coombs test, but the criteria for any specific autoimmune disease were not fulfilled. It is noteworthy that the autoimmune phenomena (either a confirmed autoimmune disease or sole laboratory abnormalities) of four of our cases (cases 1, 2, 4, and 6) completely disappeared after the successful treatment of SPTL with multiagent chemotherapy. This observation supports a link between the autoimmune disorder and the development of SPTL. However, such association still needs to be confirmed in a multinational prospective study.

Taken together, our findings demonstrate the clinical and immunohistological characteristics of SPTL with the α/β T-cell phenotype. In addition, CGH revealed previously unidentified DNA copy number abnormalities, some of which were common with other CTCL subtypes, but a great number were also SPTL specific, namely gains of 5q and 13q. Thus, the WHO–EORTC classification of SPTL as being a separate CTCL subgroup is also confirmed at a molecular cytogenetic level. Moreover, demonstration of a NAV3 gene deletion by FISH was found to characterize SPTL apart from the more common CTCL subtypes MF and SS.

MATERIALS AND METHODS

Patient samples

Nine patients diagnosed with SPTL were included in the study (Table 1). All patients were treated at the Helsinki University Central Hospital between 1987 and 2007, and the study was approved by the ethical review boards of Helsinki and Uusimaa Hospital District and of the Skin and Allergy Hospital, Helsinki University Central Hospital. The additional skin and blood samples obtained for this study were obtained with the patient's (or parents' in the case of children) informed consent.

Sections cut from the paraffin-embedded biopsies were immunostained for membrane antigens (manufacturer and dilutions are given in parentheses) CD3 (Novocastra, New Castle, UK; 1:100), CD4 (Novocastra; 1:150), CD5 (Novocastra; 1:25), CD7 (Novocastra; 1:100), CD8 (Novocastra; 1:25), CD30 (Dako, Glostrup, Denmark; 1:25), CD56 (Zymed, South San Francisco, CA; 1:50), Granzyme B (Monosan, Uden, The Netherlands; 1:100), TIA1 (Biocare, Birmingham, UK; 1:200), Ki-67 (MIB-I antibody, recognizes proliferating cells at all stages of the cell cycle but not cells in G_0 phase, Dako; 1:50), and TCR α/β (GeneTex, TX; 1:100) according to the manufacturers' instructions and visualized with DakoEnvision (Dako).

DNA extraction

Sections of 5 μm were cut from formalin-fixed paraffin-embedded tissue samples ($n=6$) using a microtome and mounted onto a 1.35-μm-thin polyethylene membrane (PALM Microlaser Technologies, Bernried, Germany) attached to a glass slide. Tissue sections were then deparaffinized and stained with hematoxylin (Stoecklein *et al.*, 2002) and hematoxylin–eosin for morphological control (standard protocol). Single lymphocytes or areas of malignant lymphocytes rimming the fat lobules covering altogether 50,000 μm^2 were laser capture microdissected using the PALM Laser-Microbeam System (PALM Microlaser Technologies). Thereafter, proteinase K digestion was performed and the DNA was amplified with single-cell CGH, as previously described (Klein *et al.*, 1999; Stoecklein *et al.*, 2002). The success of the amplification was PCR-tested with microsatellite markers D5S500 and D17S1161, as previously described (Stoecklein *et al.*, 2002). Isolation of genomic DNA was successful in all seven cases. The amplified DNA of the microdissected cells was then used for CGH and LOH analyses, as described below.

Comparative genomic hybridization

CGH was performed as previously described (Klein *et al.*, 1999; Stoecklein *et al.*, 2002). Briefly, microdissected and proteinase K-digested DNA was digested with *Mse*I restriction enzyme (New England BioLabs, Ipswich, MA), adaptors were ligated to the 5′ overhangs, and DNA fragments were amplified by PCR. The amplified DNA was then labeled with digoxigenin-dUTP (Roche, Mannheim, Germany) and similarly processed aliquots of reference DNA obtained from peripheral blood mononuclear cells of healthy volunteers with biotin-dUTP (Roche). The labeled probes were hybridized on normal male metaphase slides for 2–3 nights. After post-hybridization washes, metaphases were viewed under a fluorescence microscope and three-color digital images were captured using an epifluorescence microscope (Axioplan imagining 2; Carl Zeiss AG, Oberkochen, Germany) equipped with a CCD camera. Images were analyzed with ISIS digital image analysis system (MetaSystems GmbH, Altlussheim, Germany) using statistical limits for

green to red ratios to determine DNA copy number gains and losses. For each case, 8–12 metaphases were included in the analysis. As an internal control, normal male and female DNA was cohybridized and only differences in sex chromosomes were identified.

Locus-specific FISH for NAV3

Interphase FISH was performed on frozen tissue samples of four patients (cases 2, 3, 7, and 9, of whom enough material was available) to identify deletion of *NAV3* gene by using *NAV3*-specific bacterial artificial chromosomes (BACs), as previously described (Karenko *et al.*, 2005). Briefly, digoxigenin-labeled BACs, 136F16 and 36P3, spanning the frequently deleted area of *NAV3* gene were cohybridized with biotin-labeled chromosome 12 centromere on touch preparates made of frozen tissue samples. After post-hybridization staining with anti-digoxigenin rhodamine and avidin-FITC (both from Invitrogen, Carlsbad, CA), the interphase nuclei were viewed under a fluorescence microscopy and signals were counted from the BACs and centromeres in at least 50 non-overlapping nuclei of each case. A nucleus with an equal number of fluorescence signals from the centromere probe and the BAC probes was considered normal, and a deletion was recorded if the centromere probe gave a higher number of signals than the BAC probes. A cutoff level for at least 10% of abnormal nuclei was introduced based on our previous expertise on lymphoma and non-lymphoma skin lesions (Karenko *et al.*, 2005).

NAV3 LOH analysis

For *NAV3* LOH analysis, we chose three microsatellite markers spanning the *NAV3* gene locus at 12q21.2–q21.31 and surrounding the gene from both directions (physical distances between loci in mega-bases according to http://www.ensembl.org are given in parentheses): pter D12S1684-(0.8 Mb)-D12S326-(0.2 Mb)-*NAV3*-(3.8 Mb)-D12S1708 qter. The PCR amplification primers were D12S1684F 5′-cctgcatgcctcagttatga-3′, D12S1684R 5′-aacaagccata ccagtcagg-3′, D12S326F 5′-accaggctcccctaaaagtg-3′, D12S326R 5′-agaatgaccagacccacagg-3′, D12S1708F 5′-gggaacttatgtcaaggctag ga-3′, D12S1708R 5′-gatctagtgctcaagaggttttcaa-3′. The forward primers were fluorescently labeled with 6-carboxyfluorescein, the PCR fragments were run on the ABI3730 sequencer/genotyper, and the results analyzed using GeneMapper v3 software (Applied Biosystems Europe/Applera Finland, Espoo, Finland). A sample was scored as showing LOH if one of the alleles in the tumor sample had decreased 40% or more compared to its matching normal.

CONFLICT OF INTEREST

Dr Karenko and Dr Ranki are inventors in a Finnish patent (PCT/FI2006/113666) that has been assigned to Dermagene Oy.

ACKNOWLEDGMENTS

We thank Kaija Järvinen and Saila Saarinen for skillful technical assistance and Pauliina Luimula for skillful performance of the TCR α/β stainings.

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