Primary Cutaneous T-Cell Lymphomas Show a Deletion or Translocation Affecting NAV3, the Human UNC-53 Homologue

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Abstract

Multicolor fluorescent in situ hybridization (FISH) was used to identify acquired chromosomal aberrations in 12 patients with mycosis fungoides or Sézary syndrome, the most common forms of primary cutaneous T-cell lymphoma (CTCL). The most frequently affected chromosome was 12, which showed clonal deletions or translocations with a break point in 12q21 or 12q22 in five of seven consecutive Sézary syndrome patients and a clonal monosomy in the sixth patient. The break point of a balanced translocation t(12;18)(q21;q21.2), mapped in the minimal common region of two deletions, fine mapped to 12q2. By locus-specific FISH, the translocation disrupted one gene, NAV3 (POMFIL1), a human homologue of unc-53 in Caenorhabditis elegans. A missense mutation in the remaining NAV3 allele was found in one of six cases with a deletion or translocation. With locus-specific FISH, NAV3 deletions were found in the skin lesions of four of eight (50%) patients with early mycosis fungoides (stages IA-IIA) and in the skin or lymph node of 11 of 13 (85%) patients with advanced mycosis fungoides or Sézary syndrome. Preliminary functional studies with lentiviral small interfering RNA-based NAV3 silencing in Jurkat cells and in primary lymphocytes showed enhanced interleukin 2 expression (but not CD25 expression). Thus, NAV3 may contribute to the growth, differentiation, and apoptosis of CTCL cells as well as to the skewing from Th1-type to Th2-type phenotype during disease progression. NAV3, a novel putative haploinsufficient tumor suppressor gene, is disrupted in most cases of the commonest types of CTCL and may thus provide a new diagnostic tool.

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Introduction

Cutaneous T-cell lymphomas (CTCL) represent a heterogeneous group of non-Hodgkin’s lymphomas, where the neoplastic cell is a mature CD4+ T lymphocyte in most subtypes (1). The most common subtypes of CTCL are mycosis fungoides and the leukemic Sézary syndrome, both of which are steadily increasing in incidence (2, 3). Earlier, a number of recurrent, acquired chromosomal alterations and the genes involved in them have been identified in many hematopoietic malignancies (4) and in non-Hodgkin’s lymphomas other than CTCL. These genetic lesions have typically been either activation of proto-oncogenes as a result of chromosome translocation or disruption of tumor suppressor genes (5). However, no specific chromosomal aberration common to a majority of CTCL cases has been described thus far.

A large variety of chromosomal aberrations, both numerical and structural, have been detected in CTCL (6–9). Most of these abnormalities have been nonclonal in the early phases of the disease. Clonal cytogenetic changes have been shown to precede the histologically identifiable malignancy (6, 7, 10, 11), but CTCL studies have been hampered by the presence of numerous reactive T-cells in the skin lesions of CTCL and the difficulty of propagating the truly malignant cells in vitro. In a previous multicolor fluorescent in situ hybridization (FISH) study, only two recurrent unbalanced translocations, der(1)(1;10)(p2;q2) and der(14)(14;15)(q;q?), were reported in 2 of 17 patients with Sézary syndrome (9). Chromosome 12 aberrations were common, as two of six patients with a chromosomal clone showed a structural and three patients a numerical aberration of chromosome 12 (9). Previous G-banding studies have shown chromosome 12 abnormalities with a notable frequency (9).

The aim of our study was to identify recurrent chromosomal changes, and genes involved therein, in CTCL by using molecular cytogenetic tools. First, multicolor FISH showed that the chromosome most often affected in a series of seven patients with Sézary syndrome was chromosome 12, with recurrent break points in 12q21 or 12q22. We fine-mapped the break points of overlapping deletions and by observing a translocation in the minimal common region of the deletions, were able to identify a putative target gene NAV3, either deleted or disrupted by the translocation. Finally, we showed NAV3 deletion in the majority of 21 randomly selected patients representing different stages of CTCL.

Materials and Methods

Patient samples. Peripheral blood samples of seven consecutively diagnosed patients with Sézary syndrome and five randomly selected patients with mycosis fungoides (Table 1) were studied with multicolor FISH. Touch preparations of snap-frozen skin or lymph node biopsies from eight of these patients (1–3, 8–12), skin samples from one other Sézary syndrome patient (case 13), and 12 further randomly selected mycosis fungoides patients (cases 14-25) were available for locus-specific FISH. Altogether, 8 of the 25 patients had early-stage mycosis fungoides (stages...
Six of the 20 snap-frozen skin samples (stored in liquid nitrogen) dated back from years 1987 to 1994. The study was approved by the Ethical Review Boards of Helsinki University Hospital and the Medical University of Gdansk.

**Cell lines and reference samples, cell viability, and apoptosis analyses.** For immunofluorescence and Western blot analyses, two human neural cell lines and peripheral blood lymphocytes derived from healthy voluntary subjects, and touch preparations of frozen skin biopsies from five voluntary patients with inflammatory skin diseases, were used. The neural cell lines were the neural crest–derived tumor cell line, Paju, which undergoes spontaneous neural differentiation in vitro (13), and SH-SY5Y neuroblastoma cell line (American Type Culture Collection, Manassas, VA). For the reverse transcription-PCR (RT-PCR) assays, human fetal liver cDNA library (Clontech, Palo Alto, CA) and the human astrocyte-derived cell line, CCF-STTG1 (a gift from professor Jorma Isola, University of Tampere, Tampere, Finland), served as a reference (14). Also, a skin lesion biopsy of an additional CTCL patient with translocation t(2;5)(p23;q21) was studied (data not shown). For lentiviral short hairpin RNA studies, the Jurkat E6-1 cell line and fresh peripheral blood lymphocytes from healthy donors were used. Parallel cultures of nonstimulated and phytohemagglutinin (PHA)-stimulated (KaryoMAX; Life Technologies, Gaithersburg, MD) cells were used. Viable cells were identified with trypan blue and apoptotic cells with the TUNEL method (ApopTaq Peroxidase in situ; Chemicon International, Temecula, CA).

**Table 1. Aberrations of chromosome 12 and NAV3 gene aberrations in 25 patients and disease characteristics**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis/stage</th>
<th>Treatment</th>
<th>Disease outcome</th>
<th>Peripheral blood clonal chromosome 12 abnormalities by multicolor FISH/G-banding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SS</td>
<td>PUVA; EB; Ch</td>
<td>DOD</td>
<td>10/10</td>
</tr>
<tr>
<td>2</td>
<td>SS</td>
<td>EB; Ch</td>
<td>DOD</td>
<td>20/22</td>
</tr>
<tr>
<td>3</td>
<td>SS*</td>
<td>PUVA</td>
<td>DOD</td>
<td>11/11</td>
</tr>
<tr>
<td>4</td>
<td>SS</td>
<td>PUVA; EB; Ch; R</td>
<td>DOD</td>
<td>30/39</td>
</tr>
<tr>
<td>5</td>
<td>SS</td>
<td>PUVA; Ch</td>
<td>DOD</td>
<td>16/24</td>
</tr>
<tr>
<td>6</td>
<td>SS</td>
<td>PUVA; Ch</td>
<td>DOD</td>
<td>3/45</td>
</tr>
<tr>
<td>7</td>
<td>SS</td>
<td>CP</td>
<td>REM</td>
<td>5/24</td>
</tr>
<tr>
<td>8</td>
<td>MF/IA</td>
<td>PUVA; EB</td>
<td>AR</td>
<td>2/70</td>
</tr>
<tr>
<td>9</td>
<td>MF/IIA</td>
<td>EB</td>
<td>AR</td>
<td>2/25</td>
</tr>
<tr>
<td>10</td>
<td>MF/IIIB</td>
<td>PUVA; EB; I; Ch</td>
<td>AR</td>
<td>0/54</td>
</tr>
<tr>
<td>11</td>
<td>MF/IIIB</td>
<td>PUVA; EB;I;Ch</td>
<td>AR</td>
<td>0/43</td>
</tr>
<tr>
<td>12</td>
<td>MF/IIIB</td>
<td>PUVA; EB; Ch</td>
<td>DOD</td>
<td>7/50</td>
</tr>
<tr>
<td>13</td>
<td>SS</td>
<td>UVA; Ch</td>
<td>DOD</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>MF/IB</td>
<td>PUVA</td>
<td>AR</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>MF/IB</td>
<td>PUVA</td>
<td>AR</td>
<td>G³</td>
</tr>
<tr>
<td>16</td>
<td>MF/IB*</td>
<td>EB</td>
<td>REM</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>MF/IB</td>
<td></td>
<td>REM</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>MF/IB*</td>
<td>PUVA</td>
<td>Other**</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>MF/IB**</td>
<td>PUVA; EB; Ch</td>
<td>AR</td>
<td></td>
</tr>
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<td>PUVA; EB</td>
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<td></td>
</tr>
<tr>
<td>21</td>
<td>MF/IIIB*</td>
<td>EB</td>
<td>DOD</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>MF/IIIB*</td>
<td>I; R</td>
<td>DOD</td>
<td></td>
</tr>
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<td>23</td>
<td>MF/III*</td>
<td>Ch</td>
<td>DOD</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>MF/IVA</td>
<td>PUVA; EB; Ch</td>
<td>DOD</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>MF/IVA</td>
<td>PUVA</td>
<td>DOD</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: MF, mycosis fungoides; SS, Sézary syndrome; PUVA, psoralen + UVA irradiation; EB, electron beam; I, IFN; Ch, chemotherapy; CP, chlorambucil + prednison; R, retinoids; DOD, died of disease; REM, clinical remission; AR, alive, relapsing disease; pbl, peripheral blood lymphocytes; In, lymph node; sk, skin.

*Breakpoints specified by G-banding and locus-specific FISH; clones defined as in (refs. 7, 12).
†The comparative genomic hybridization karyotype of cases 1 and 3 has been published elsewhere (13).
‡Metaphases.
¶Preceded by mycosis fungoides.
||Analyzed with G-banding only.

*Biopsies of skin lesion obtained 5 to 15 years earlier and stored in liquid nitrogen.

**Died of another cause than CTCL.
††CD30 positive.


Table 1. Aberrations of chromosome 12 and NAV3 gene aberrations in 25 patients and disease characteristics (Cont’d)

<table>
<thead>
<tr>
<th>Abnormality [no. of cells with abnormal chromosome 12/total cells]</th>
<th>Chromosome 12 by comparative genomic hybridization</th>
<th>Percentage of cells with NAV3 aberration by FISH</th>
<th>Material</th>
<th>Deletion</th>
<th>Translocation</th>
</tr>
</thead>
<tbody>
<tr>
<td>der(12)(q15q15)(q21q24)</td>
<td>dim(12)(q15q23)/pbl</td>
<td>pbl(^1), sk</td>
<td>sk</td>
<td>68%</td>
<td></td>
</tr>
<tr>
<td>t(12;18)(q24;p11.3)</td>
<td>t(12;18)(q24;p11.3)</td>
<td>sk</td>
<td>44%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>del(12)(q12p12) (^*)</td>
<td>del(12)(q12p12) (^*)</td>
<td>sk</td>
<td>48%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t(12;21)(q21;q21)</td>
<td>t(12;21)(q21;q21)</td>
<td>sk</td>
<td>sk</td>
<td>32%</td>
<td></td>
</tr>
<tr>
<td>der(18)(12;18)(?-?)(12;12)(?-?)(12;22)(?-?)</td>
<td>der(18)(12;18)(?-?)(12;12)(?-?)(12;22)(?-?)</td>
<td>sk</td>
<td>sk</td>
<td>44%</td>
<td></td>
</tr>
<tr>
<td>der(4)(t;4)(12)(q31;?);der(12)(t;10)(12)(?-?)(21;3or22)</td>
<td>der(4)(t;4)(12)(q31;?);der(12)(t;10)(12)(?-?)(21;3or22)</td>
<td>sk</td>
<td>sk</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>Nonclonal aberrations of chromosome 12 [-12, 2/3]</td>
<td>enh(12)(q14q21.1)/pbl</td>
<td>enh(12)(q14q21.1)/pbl</td>
<td>sk</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>-12 [5/5]</td>
<td></td>
<td></td>
<td>sk</td>
<td>8%</td>
<td></td>
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<tr>
<td>Nonclonal aberrations of chromosome 12 [0/2]</td>
<td></td>
<td></td>
<td>sk</td>
<td>3%</td>
<td></td>
</tr>
<tr>
<td>Nonclonal aberrations of chromosome 12 [0/2]</td>
<td></td>
<td></td>
<td>sk</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td>Nonclonal aberrations of chromosome 12 [0/0]</td>
<td></td>
<td></td>
<td>sk</td>
<td>32%</td>
<td></td>
</tr>
<tr>
<td>Nonclonal aberrations of chromosome 12 [1/7]</td>
<td></td>
<td></td>
<td>sk</td>
<td>22%</td>
<td></td>
</tr>
<tr>
<td>Nonclonal aberrations of chromosome 12 [1/7]</td>
<td></td>
<td></td>
<td>sk</td>
<td>58%</td>
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</tr>
<tr>
<td>Nonclonal aberrations of chromosome 12 [1/7]</td>
<td></td>
<td></td>
<td>sk</td>
<td>4%</td>
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</tr>
<tr>
<td>Nonclonal aberrations of chromosome 12 [1/7]</td>
<td></td>
<td></td>
<td>sk</td>
<td>38%</td>
<td></td>
</tr>
<tr>
<td>derivative(12)(q21q23) ([4/4])</td>
<td>?dim(12)(q15q21)/sk</td>
<td>?dim(12)(q15q21)/sk</td>
<td>sk</td>
<td>44%</td>
<td></td>
</tr>
<tr>
<td>derivative(12)(q21q23) ([4/4])</td>
<td>dim(12)(q15q21)/sk</td>
<td>dim(12)(q15q21)/sk</td>
<td>sk</td>
<td>28%</td>
<td></td>
</tr>
</tbody>
</table>

epifluorescence microscope (Axioplan imagining 2, with a charged coupled device camera; Zeiss, Germany) and analyzed with a multicolor FISH program module in infrared screening and inspection solutions image analysis system (MetaSystems GmbH).

**Locus-specific fluorescent in situ hybridization.** Chromosomes 12 and 18 were further studied with locus-specific probes in cases from which enough cell material was available (cases 1, 2, and 3). The region 12q14 to 12q21 was studied with 15 overlapping or contiguous yeast artificial chromosome (YAC) probes of contig 12.4, the regions 12q12 to 12q24 with 10 other YACs (Fondation Jean Dausset, France), and 12q21 and 12q24 further with one bacterial artificial chromosome (BAC) and three P1-derived artificial chromosome (PAC) probes, respectively (Research Genetics, Inc., Huntsville, AL). Chromosomal regions 18p11.2 to 18p11.3, 18q12.3, and 18q21 were studied with altogether 24 BACs and 4 YACs (Supplementary Data). All probes were selected using National Center for Biotechnology Information (NCBI) databases (MapViewer program). The probe identities were confirmed using PCR with locus-specific primers according to NCBI databases. The YAC, BAC, and PAC DNAs were isolated using routine techniques. The chromosomes were identified with centromere-specific probes of chromosomes 12 (p122H8) and 18 (p18R). All probes were labeled with nick translation and dual-color hybridizations were done (Supplementary Data) as previously described (7, 8, 17).

**Fluorescent in situ hybridization on interphase nuclei.** Touch preparations of available snap-frozen skin or lymph node biopsies from 21 patients with mycosis fungoides or Sézary syndrome, and of nine reference skin samples (see above; lupus erythematosus discoides or eczema), were hybridized with two-color interphase fluorescence in situ hybridization (FISH) as described earlier (11) with the following modification: Digoxigenin-labeled BACs 136F16 and 36P3 were cohybridized together with a centromere-specific probe labeled with biotin. The translocation was detected with digoxigenin-labeled BACs 136F16 and 36P3 with biotin-labeled BACs 786A1 and 49K17. At least 50 interphase nuclei were analyzed for each patient. 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 from the centromere than from the BAC areas. In a translocation, the distance of green and red signals is altered. The analyses were done blinded to the diagnosis or sample identity. The highest percentage of cells with aberrant signal patterns observed in reference samples was considered as cutoff level.

**Comparative genomic hybridization.** Comparative genomic hybridization was done as described previously (8).

**Sequencing and denaturing high-performance liquid chromatography.** All exons and one intron region (intron 20) of the NAV3 gene in blood cell-derived DNA of two patients (cases 1 and 3) were amplified with primers specific for each exon or the intron and sequenced with ABI PRISM 310 sequencer. The primer sequences and PCR conditions are available on request. All exons of cases 2, 4, 5, 6, and 13 were studied with denaturing high-performance liquid chromatography (DHPLC) as described before (18). Exons showing abnormalities were sequenced. The mutation and polymorphisms were sequenced in reverse direction, too. To study the frequency of sequence variations in the normal population, exon 37 and intron 35 in the DNA samples of 50 healthy volunteers and all exons from one healthy control sample were amplified and sequenced.

**Immunofluorescence.** Immunofluorescence analysis, imaging, and analyses were done as described earlier (11). For the demonstration of NAV3 protein, a polyclonal rabbit antibody, produced against a 19-mer synthetic peptide (residues 212-230, exon 10 of NAV3; ref. 14), was used on cytoospin preparations of the neural tumor cell line, Paju (13), normal lymphocytes, and touch preparations from frozen skin biopsies of six CTCL.
patients and of five reference patients with inflammatory skin diseases. For cultured and lentivirally infected cells, the following additional antibodies were used: monoclonal mouse anti–interleukin 2 (IL-2; R&D Systems Europe, Ltd., Abingdon, United Kingdom), anti–CD25 (DAKO Cytomation, Glostrup, Denmark), anti–green fluorescent protein (GFP; Molecular Probes, Leiden, the Netherlands), and polyclonal goat anti–IL-4 (Santa Cruz, Santa Cruz, CA), and rabbit anti-GFP (Molecular Probes) antibodies. Secondary antibodies were used as described previously (Supplementary Data; ref. 11). Double stainings with anti-GFP antibodies and other antibodies were done. In all analyses, 50 to 100 cells were examined, as previously described (11), with a computer-connected UV microscope (Olympus BX50, Tokyo, Japan) equipped with a charged coupled device camera.

NAV3 expression by reverse transcription-PCR and Western blot. The expression of NAV3 mRNA was studied by RT-PCR in fresh and PHA-stimulated (3d) normal blood lymphocytes (Life Technologies Invitrogen, Rockville, MD), in the skin lesion biopsies of case 15 and in human fetal liver cDNA library (Clontech). The human astrocyte-derived cell line, CCF-STTG1 (see above), served as a reference (14). For performance, see Supplementary Data.

For Western blot, aliquots of two neural cell lines (Paju and SH-SYSY) and both fresh and PHA-stimulated normal lymphocytes were used. The cells were suspended in 2× SDS-PAGE sample buffer (100 μL/106 cells), boiled for 10 minutes, sheared by repeated passage through a 20-gauge needle, and centrifuged to remove the insoluble material. After resolving in 12% SDS-PAGE Standards Broad Range (Bio-Rad Laboratories), the proteins were transferred to Trans-Blot Transfer Medium nitrocellulose membranes (Bio-Rad Laboratories), probed with anti-NAV3 rabbit antisemur (1:500; Vector Laboratories, Inc., Burlingame, CA) and peroxidase substrate kit DAB (Vector Laboratories). Flow cytometry analyses. Expression of CD4, CD25, and IL-2 on NAV3-silenced (see below) and nonsilenced Jurkat E6-1 cells and peripheral blood lymphocytes was investigated with fluorescence-activated cell sorter (FACSCalibur; BD Biosciences, San Jose, CA). As primary antibodies, monoclonal mouse anti-CD4, anti-CD25, or isotype control antibodies (DAKO Cytomation, Glostrup, Denmark), and as secondary antibody polyclonal antimouse IgG conjugated with phycoerythrin (Jackson Immunoresearch, West Grove, PA), were used. Intracellular IL-2 detection was done on fixed and permeabilized (0.1% saponin in buffer) cells by indirect staining with monoclonal mouse anti–IL-2 (R&D Systems Europe) and phycoerythrin-conjugated polyclonal antibodies. Before staining, the cells were incubated 4 to 6 hours with 1 μg/mL GolgiPlug (PharMingen, San Diego, CA) to inhibit cytokine secretion. Gating was done on forward and side-angle scatter characteristics and GFP expression of the cells.

NAV3 gene silencing with small interfering RNA-expressing lentivirus constructs. Several DNA sequences encoding small interfering RNA (siRNA) precursors based on the NAV3 sequence were cloned in the lentiviral vector pLL3.7 for expression under the U6 promoter, including GFP expression (from a separate pol III promoter).9 Their inhibitory potential was tested by cotransfection into HeLa cells with renilla luciferase control plasmid (pRL-null; Promega, Madison, WI) and a β-actin promoter-driven firefly luciferase expression vector (psiRNA-luc) into which a relevant fragment of NAV3 cDNA had been inserted between the luciferase open reading frame and the polyadenylation signal (psiRNA-luc NAV3; psiRNA-luc is an unpublished vector provided by Tiina Tissari, IMT, Tampere, Finland). Two days after transfection, firefly/renilla luciferase ratios were compared in cells transfected with the parental pLL3.7 or its siRNA-encoding derivatives with psiRNA-luc or psiRNA-luc NAV3. The most potent and specific inhibition was observed with a pLL3.7-derivative carrying a 23-nucleotide sequence from the NAV3 exon 19 (bp 4,623-4,645), which was named pLL3.7siRNA4. This derivative was used for production of infectious short hairpin RNA–expressing lentiviruses as follows: The pLL3.7 or pLL3.7-siRNA4 constructs were transfected into 293T cells together with pDELTA-8.9 (19) and pSVSγ (envelope); cells were gently washed 12 hours after transfection; and the supernatant was collected 48 hours later, filtered, and pelleted. For infection, a total of 106 Jurkat E6-1 cells were infected with 40 μL of concentrated virus at 37°C for 2 hours, and the number of infected cells (GFP-positive) were estimated by fluorescence-activated cell sorting (FACS). The NAV3 expression of FACS-sorted (BD FACSaria; BD Biosciences) GFP-positive cells (pLL3.7 or pLL3.7siRNA4) was studied with quantitative RT-PCR by Light Cycler device (Roche Diagnostics, Mannheim, Germany) according to previously published guidelines (20). For primer and cycling conditions, see Supplementary Data.

Interleukin-2 analysis of culture supernatants. IL-2 concentration was analyzed with Quantikine II-2 ELISA kit (Quantikine Immunoassay, R&D) according to the instructions of the manufacturer.

Results

Aberrations of chromosome 12 are frequently found in cutaneous T-cell lymphoma patients. The most often affected chromosome in the peripheral blood clones observed by multicolor FISH or spectral karyotyping was chromosome 12. Five of seven consecutive patients with Sézary syndrome showed a clonal structural aberration of chromosome 12 and one (case 7) showed a nonclonal deletion of 12q with a clonal monosomy of chromosome 12 (Fig. 1; Table 1; Supplementary Data). Five of the six mycosis fungoides patients studied showed nonclonal deletions of chromosome 12 (Table 1). All structural clonal aberrations of chromosome 12 involved bands q21 or q22. Structural aberrations of chromosome 17 were also detected in five Sézary syndrome patients, but these aberrations could involve either p or q (Table 1). Three cases (cases 1, 3, and 4) showed a translocation with chromosome 18 in multicolor FISH. One had a balanced translocation with 18q (case 3) and another showed a translocation with 18p with loss of much of the 12q-arm (case 1; Fig. 1; Table 1; Supplementary Data). Speciation of the break point in chromosome 12. The aberrations of cases 1, 2, and 3 were further studied with locus-specific FISH. Cases 1 and 2 showed large deletions of chromosome 12, del(12)(q15q15)(q21.1q24), and del(12)(q1q2q1), respectively (Fig. 2). The balanced translocation of case 3 was within the minimal common region of deletions in cases 1 and 2, and divided the signal of YAC 855F7 between chromosomes 12 and 18 (Fig. 2), enabling us to fine map the gene affected. The

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9 For details, see http://csbi.mit.edu/rnl/-vector.
YAC 855F7 is part of the YAC contig WC12.4 (NCBI) and spans the region between markers CHLC.GATA65A12 and WI-6487. Four overlapping BAC probes, RP11-781A6, RP11-494K17, RP11-136F16, RP11-36P3, each with a marker represented in the YAC 855F7 by PCR analysis (SHGC-155034, G62498, SHGC-79622, D12S2006, respectively), were further used. Signal division in FISH analyses indicated that the translocation break point lies within BAC probes RP11-494K17 and 136F16 (Fig. 2), which both contain parts of the NAV3 gene (genomic contig NT_019546) disrupted by the translocation (Fig. 3). No other mapped genes or expressed sequence tags were located in the translocation break point.

The break point of 18q involved in the balanced translocation of case 3 splits YAC 852H2 (located between markers AFM357TD5 and WI-6487). Four overlapping BAC probes, RP11-781A6, RP11-494K17, RP11-136F16, and RP11-36P3, each with a marker represented in the YAC 855F7 by PCR analysis (SHGC-155034, G62498, SHGC-79622, D12S2006, respectively), were further used. Signal division in FISH analyses indicated that the translocation break point lies within BAC probes RP11-494K17 and 136F16 (Fig. 2), which both contain parts of the NAV3 gene (genomic contig NT_019546) disrupted by the translocation (Fig. 3). No other mapped genes or expressed sequence tags were located in the translocation break point.

The break point of 18q involved in the balanced translocation of case 3 splits YAC 852H2 (located between markers AFM357TD5 and AFM191XC9P) into two parts, one giving a signal in 18q and the other in 12q. All BACs located in 18q proximal to 450M22 remain in 18q, whereas BACs and YACs below the break point distally move to a new location.

**Figure 2.** Locus-specific hybridizations of blood lymphocyte metaphases revealed the extension of the deletions in 12q in two Sézary syndrome patients and the break point of the reciprocal translocation t(12;18)(q21.1;q21.2) of the third Sézary syndrome patient in the minimal common region in 12q21.1 of the two deletions specified by the division of two BAC probe signals between chromosomes 12q and 18q. A, schematic representation of the deletions and the translocation break point in 12q. Parts of the chromosomes studied are shown as vertical columns. Fill-in symbols representing the hybridization results are explained in lower right. B, BAC 494K17 (green) originates in the normal 12, and contains part of NAV3 gene. BAC 450M22 (bright red) originates in the normal 18 (E). Translocation chromosome 12 (C) and translocation chromosome 18 (D) show parts of both BACs. Chromosome 12 centromere (wine red) and chromosome 18 centromere (green). F, combined colors.
chromosome 12q in the translocation (Fig. 2; Supplementary Data). Although most of the material lost from the aberrant 12q in case 1 was totally deleted (for comparative genomic hybridization, see ref. 8), a small part of 12q24 was translocated to 18p (PAC 144J4; Fig. 2) and to the region of BAC 683L23, the latter partly translocated to 12q24. Other more proximal BACs studied in 18p remained in their respective locations.

**NAV3 deletion/translocation is found in interphase cells of skin lesions of cutaneous T-cell lymphoma patients.** The translocation observed in blood lymphocytes of one Sézary syndrome patient (case 3) was also observed in the locus-specific FISH to his lesional skin touch preparation (Table 1; Fig. 4). Deletions of the NAV3 gene were observed in solid tissue samples from the three other Sézary syndrome patients studied (case 1, lymph node; cases 2 and 13, skin) and in the lesional skin from 11 of 17 (65%) patients with various stages of mycosis fungoides (Table 1; Fig. 4).

Altogether, NAV3 deletions were found in the skin lesions from four of eight (50%) patients with early mycosis fungoides (stages IA-IIA), and a deletion or a translocation was observed in 11 of 13 (85%) patients with advanced mycosis fungoides or Sézary syndrome with locus-specific FISH (Table 1; Fig. 4). The deletion was equally well found in touch preparations from archival liquid nitrogen–stored skin samples as well as in more recent samples. There was no consistent association between the NAV3 deletion and the type of previous therapy (Table 1). All patients with NAV3 deletion or translocation had a frequently relapsing disease, despite therapy, or had died of CTCL. Of the six patients not showing NAV3 deletion in their skin lesions, four had an early stage disease. Two of them had received psoralen plus UVA irradiation or electron beam therapy and one was untreated (case 17).

**Demonstration of NAV3 mutation in the microscopically intact allele.** Of the blood lymphocyte DNA from the seven cases, six with a cytogenetic aberration of 12q, studied with sequencing or DHPLC, only one showed a missense mutation. Case 1 had a point mutation G→A in exon 37 (cDNA nucleotides 10106643; NM_019403), resulting in an amino acid change E2200K. Several single-nucleotide polymorphisms or intronic deletions were detected. Seven polymorphic variations have been recorded in NAV3 coding region (NT_019546) and two of these changes (4509G→A and 4830C→T; NM_019403) were observed in cases 1 and 3. Altogether, the NAV3 gene region, spanning ~381 kb of chromosomal sequence, contains 849 polymorphic sites. Thus, point missense mutations in CTCL blood samples were not common.

**NAV3 expression in cell lines and primary cells.** With RT-PCR, NAV3 mRNA could be detected in polyclonally activated T lymphocytes, as well as in human fetal liver cells and astrocytes (Supplementary Data). With immunofluorescence and Western blot assays, using the polyclonal antibody (14), NAV3 protein was

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Figure 3. DNA represented in BACs 786A1, 494K17, 136F16, and 36P3 together comprise the NAV3 gene. Hybridization of BACs RP11-781A6, RP11-494K17, RP11-136F16, RP11-36P3 (AC073552.1, AC022885.5, AC073571.14, and AC073608.19, respectively), together spanning the whole NAV3 gene, indicated the translocation break point as division of BAC probes RP11-494K17 and 136F16 between chromosomes 12q and 18q. The whole BAC 781A6 remained in chromosome 12 and the whole BAC 36P3 was translocated to chromosome 18q. Fill-in symbols of bars indicating BACs and their parts remaining in chromosome 12 or translocated to chromosome 18q are explained in lower left.

Figure 4. Deletion of NAV3 was shown in skin or lymph node tissues of patients representing different stages of CTCL. Two adjacent BACs in NAV3 region, 136F16 and 36P3, were labeled with digoxigenin and detected with antidigoxigenin rhodamine (red). Centromere of chromosome 12 was labeled with biotin and detected with avidin–FITC. In cells with a deletion, the number of red signals is less than the number of centromeres. A and B, cases 9 and 1 (skin and lymph node). Normal cells show two red and two green signals. C and D, case 1 (lymph node) and control (eczema skin lesion). Bar, 10 μm. E, percentages of abnormal cells in individual patient and control samples studied as above. The highest control aberration percentage (10%) defining the cutoff level between normal and abnormal is shown as a white horizontal line.
expressed by cell lines of neural origin and by polyclonally activated T-lymphocytes, but not by resting normal human lymphocytes (Fig. 5). In frozen skin touch preparations, the proportion of NAV3-expressing lymphocytes was lower in six CTCL patients with NAV3 deletion (median 18%, range 4-46%) when compared with five samples from reference inflammatory skin disorders (median 44%, range 20-52%). The difference was not statistically significant with Mann-Whitney U-test.

The effect of NAV3 silencing in lymphoid cells enhances interleukin-2 production. The lentiviral infection efficacy in Jurkat cells was 40% as indicated by the GFP reporter gene. The lentiviral silencing lowered the relative expression of NAV3 by 77% as measured by Light Cycler (Fig. 6A). NAV3 transcriptional silencing did not effect the viability of the infected Jurkat cells or primary lymphocytes, but a slight growth advantage, of 8% in average, in the NAV3-silenced cells was observed compared with those infected with the empty vector. The rate of apoptotic cell death was somewhat increased in the NAV3-silenced Jurkat cell cultures (5-10% TUNEL-positive nuclei compared with 1%, respectively). Neither did the silencing of NAV3 effect CD4 or CD25 expression as detected by FACS or immunofluorescence analyses. By FACS analysis, NAV3 silencing increased the proportion of IL-2+/GFP+ Jurkat cells from 12% to 35% (Fig. 6B). The finding was confirmed by double immunofluorescence in both unstimulated and PHA-stimulated Jurkat cells: Unstimulated Jurkat cells with no NAV3 silencing (pLL3.7 or native Jurkat) showed <10% clearly IL-2–positive cells and a very weak IL-2 expression in the majority of the cells. In contrast, 70% of the siRNA4-producing Jurkat cells were positive for IL-2. Upon PHA stimulation, 70% to 90% of Jurkat cells infected with the empty vector (pLL3.7, GFP-positive) showed weak IL-2 positivity, and 96% to 100% of the GFP-positive pLL3.7siRNA4 Jurkat cells showed IL-2 positivity (Supplementary Data). Similar findings were observed in primary lymphocytes. The result was confirmed with IL-2 ELISA of culture supernatants showing ~40% higher concentration of IL-2 in NAV3-silenced (pLL3.7-siRNA4) primary lymphocyte cultures compared with corresponding empty vector-infected lymphocyte cultures (74 pg/mL compared with 12 pg/mL, or 549 pg/mL compared with 369 pg/mL, respectively, at various time points of culture). No comparative significant effect on IL-4 expression was observed (data not shown).

Discussion

Our aim was to identify recurrent chromosomal alterations associated with the development or progression of CTCL by molecular cytogenetic techniques. First, we found that a deletion or translocation of the q-arm of chromosome 12 was the most common recurrent change detected by multicolor FISH in the blood lymphocytes in six of seven (86%) consecutively presenting Sézary syndrome patients. Three of them, studied with locus-specific FISH, showed weak IL-2 positivity, and 96% to 100% of the GFP-positive pLL3.7siRNA4 Jurkat cells showed IL-2 positivity (Supplementary Data). Similar findings were observed in primary lymphocytes. The result was confirmed with IL-2 ELISA of culture supernatants showing ~40% higher concentration of IL-2 in NAV3-silenced (pLL3.7-siRNA4) primary lymphocyte cultures compared with corresponding empty vector-infected lymphocyte cultures (74 pg/mL compared with 12 pg/mL, or 549 pg/mL compared with 369 pg/mL, respectively, at various time points of culture). No comparative significant effect on IL-4 expression was observed (data not shown).
Previous cytogenetic studies have suggested that aberrations of 12q are among the most common alterations in CTCL (6, 9), but the reported frequencies of chromosomal abnormalities have been influenced by the detection methods used (9). Only techniques such as multicolor FISH or spectral karyotyping, which enable the identification of the rearranged chromosome parts and reveal the composition of aberrations (designated only as markers in G-banding; ref. 12), made the present findings possible. In a review of 274 karyotypes (most of them G-banded; ref. 9), the most commonly observed aberrations, those of 1p, occurred in 11% of cases, whereas structural aberration of 12q were found in 7% of the CTCL cases. Previously, we detected nonclonal aberrations of 12q in the blood of 8 of 10 mycosis fungoides patients (data not shown) and a clonal aberration in only one patient (7). However, when the skin lesions of five of the first mentioned cases (cases 8, 15, 16, 20, and 21) were studied with locus-specific FISH in this study, four of them showed a deletion of NAV3. One (case 15) showed later a clonal deletion in 12q in blood G-banding. The fifth patient with no NAV3 deletion (case 16) has remained in remission for over 10 years now (10).

Our finding that the aberration type in 12q was deletion strongly suggests that the region harbors a tumor suppressor gene. The two Sézary syndrome patients studied, with long deletions proximally and distally in 12q, showed the minimal common region in 12q21 covered by a seven-YAC-long contig, with approximate size of 6 Mb. This region may well contain tens or hundreds of genes. By serendipity, a third Sézary syndrome patient showed a balanced translocation with break point right in the middle of the minimal region of deletion. Reciprocal translocations, even from one donor chromosome to several recipient chromosomes, have often pinpointed the location of target tumor suppressor genes, as was the case for example for the retinoblastoma gene (21). The mapping of translocation break point in the above-mentioned Sézary syndrome patient showed that the translocation disrupted a gene for the human homologue of unc-53, the NAV3 (also named POMFIL1; refs. 14, 22).

The function of NAV3 in human lymphoid cells has not been known previously and NAV3 was thus an unexpected target of the recurrent aberration associated with CTCL. Association of the reduced or absent expression of NAV3/POMFIL1 has been reported in neuroblastoma cell lines (14). The NAV3 gene is large, spanning around 400 kb of genomic sequence, and has only recently been cloned, although not in full length (14, 22). NAV3 is one of the three human homologues of unc-53, a gene involved in axonal elongation in Caenorhabditis elegans (22–24). NAV3 consists of 40 exons and is expressed in brain, placenta, and colon. NAV3 has apparently arisen through duplication of NAV1 and NAV2 (HELADI, RAINBI). In particular, NAV3 shows a complexity of splicing events (14, 22). All three NAV proteins have an AAA domain characteristic of ATPases, and ATP/GTP binding sites (P-loops). NAV3 shows a large number of phosphorylation sites, a leucine zipper domain, coiled-coil domain, potential SH3-binding sites (14), as well as calponin-like (CH) domains (22), suggesting that NAV3 may be involved in cellular signaling (25). Mouse NAV3/POMFIL1 was recently shown to locate in nuclear pore complexes (14), which may indicate a function in nucleocytoplasmic transport regulation, cell cycle regulation, and kinetochore formation (26). Like NAV2, NAV3 also shows the properties of a helicase and exonuclease as predicted by its protein sequence (27). Helicases have a role in the maintenance of the stability of chromosomes, and their deficiency, like that of BLM and WRN, could cause a hyperrecombination phenotype, with deletion mutants and possibly also loss of heterozygosity and increase in sister chromatid exchanges, observed in CTCL, too (28–30). Thus, a defective NAV3 might, with other possible defects, contribute to the genomic instability observed in CTCL (31).

In classic tumor suppressor genes, inactivation of the remaining allele of the gene, either through mutation or by epigenetic events (such as promoter hypermethylation), is often found. Of the six studied patients with a deletion or translocation in NAV3, one had a missense mutation showing that both alleles were aberrant. The functional consequence of the mutation is difficult to predict. Whether NAV3 is hypermethylated in CTCL needs to be studied. Another possibility is that the loss of one copy of the gene causes a functional dose effect as is the case with the more recently described nonclassic haploinsufficient tumor suppressor genes (32–34).

The deletion of NAV3 seems to be a relatively early event during the pathogenesis of CTCL because it is detectable with locus-specific FISH in the skin of half of the patients with early mycosis fungoides (stages IA-IIB) compared with 85% of cases with a later stage CTCL. In previous studies, genetic aberrations of some known tumor suppressor genes, like PTEN, p15, p16, and p53, or overexpression of the latter, have been observed, but each with lower frequencies than deletions of NAV3, especially at early stages of the disease (29, 35–38).
To understand the functional consequences of NAV3 deficiency, we infected lymphoid cell cultures with a NAV3 expression-inhibiting siRNA construct (designed against exon 19 of NAV3). Interestingly, NAV3 silencing increased the IL-2 expression in Jurkat cells, as well as in primary lymphocytes stimulated with PHA, as shown by double immunofluorescence (IL-2/GFP), FACS analysis, and by secreted IL-2 levels. IL-2 is known to promote growth, differentiation, and/or apoptosis of lymphoid cells (39). We did not find a comparative effect on IL-4 expression, the other cytokine relevant in Sézary syndrome. Unexpectedly, no up-regulation of CD25 (IL-2Rα) was found.

This preliminary finding of NAV3 functional properties in lymphocytes would explain earlier observations that the malignant cells in mycosis fungoides preferentially express Th1 cytokines, like IL-2 and IFN-γ, and along with disease progression a skewing toward a type 2 cytokine profile (IL-4) occurs (40, 41). Also, IL-2 has been shown to play a critical role in the polarization of naïve CD4 T cells toward the Th2 phenotype by stabilizing the accessibility of the IL-4 gene (42), and, thus, an enhanced expression of IL-2 because early mycosis fungoides (as a consequence of NAV3 gene deletion) might explain the Th2 skewing in Sézary syndrome. That we did not observe a concomitant increase in IL-2Rα expression would also fit earlier observations showing that only a minority of mycosis fungoides tumors do express CD25, the expression being dependent on tissue site (1, 43). Also, a slightly reduced CD25 mRNA expression has been found in Sézary syndrome patient cells following IL-2 induction (44).

Recently, a loss of IL-2–inducible Stat5-dependent gene expression has been observed in Sézary syndrome patients, and the T cells of patients showed a marked inability to express transcription-competent full-length Stat5 protein in the nucleus even after potent activation (e.g., IL-2 treatment) but rather a dominance of the truncated Stat5 protein, (44). The Stat5 gene is not known to be aberrant, but a constitutive activation of both Stat3 and Stat5 have been observed in Sézary syndrome (45, 46). The IL-2–induced proliferative signals to T cells are mediated by two IL-2R–coupled pathways, one involving activation of Stat5 (46). The up-regulation of CD25 in response to IL-2 also requires functionally active Stat5 (47). Interestingly, the NAV homologue UNC-53 interacts with SEM-5, the nematode homologue of human GRB2, an intermediate in, e.g., proliferative cell signaling in T lymphocytes (24, 48, 49). Thus, we may hypothesize that the IL-2 proliferative signaling in CTCL cells is aberrantly regulated by some NAV3 interactome-associated, as yet undefined mechanism. Our observations of the functional consequences of NAV3 silencing would thus provide some gene level explanation for the previous observations of signaling defects in CTCL cells. NAV3 may well be haploinsufficient, because unc53H2, the mammalian NAV2 homologue, shows gene dosage effects for development and behavior in mice (34).

Also, these preliminary results give a hint toward the signaling pathways that should be explored in more detail in future experiments.

The deletion of 12q and the target gene, NAV3, is the first chromosomal/gene aberration found to be associated with the majority of the most common forms of CTCL. We believe that the demonstration of NAV3 deletion/translocation with, e.g., FISH in fresh or fixed tissue samples will provide a new diagnostic aid, facilitating the early diagnosis of mycosis fungoides as well as the follow-up of a residual disease. Namely, the diagnosis of mycosis fungoides is often notoriously difficult in the early stages when histologic features are nonspecific. The only molecular marker currently in use, and with relatively high specificity, is the demonstration of T-cell clonality by T-cell receptor (TCR) gene (50, 51). The chromosomal clones are at least as sensitive and specific as TCR-rearranged clones (52), and NAV3-deleted clones would now provide a new marker for 50% of the early cases of mycosis fungoides and for 85% of the more advanced cases. It is obvious that also other aberrations are required to explain the complex pathogenesis of CTCL, and various subgroups of CTCL are expected to be revealed through the identification of these additional aberrations.

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References


Correction: NAV3 Gene Deletion/Translocation in CTCL

In the article on NAV3 gene deletion/translocation in CTCL in the September 15, 2005 issue of *Cancer Research* (1), inactivation of the NAV3/POMFIL1 gene on chromosome 12 by deletion or translocation was found to be associated with and suggested to be causative for cutaneous T-cell lymphoma. A putative tumor suppressive role of NAV3/POMFIL1 was formerly also suggested by Coy et al. in 2002 (2). Coy et al. described a translocation event affecting the chromosomal regions 1p and 12q which resulted in an inactivation of NAV3/POMFIL1 (2).


Primary Cutaneous T-Cell Lymphomas Show a Deletion or Translocation Affecting NAV3, the Human UNC-53 Homologue

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