Blood Flow Cytometry in Sézary Syndrome: New Insights on Prognostic Relevance and Immunophenotypic Changes During Follow-up.

Novelli M, Fava P, Sarda C, Ponti R, Osella-Abate S, Savoia P, Bergallo M, Lisa F, Fierro MT, Quaglino P.
Blood Flow Cytometry in Sezary Syndrome: New Insights on Prognostic Relevance and Immunophenotypic Changes During Follow-up.
AMERICAN JOURNAL OF CLINICAL PATHOLOGY (2015) 143
DOI: 10.1309/AJCP1NA3YCHCDEIG.

Blood Flow Cytometry in Sézary Syndrome
New Insights on Prognostic Relevance and Immunophenotypic Changes During Follow-up

Mauro Novelli[1], Paolo Fava[1], Cristina Sarda[1], Renata Ponti[1], Simona Osella-Abate[1], Paola Savoia[1], Massimiliano Bergallo[2], Francesco Lisa[1], Maria Teresa Fierro[1] and Pietro Quaglino[1]

Author Affiliations
[1]From the Dermatologic Clinic, Department of Medical Sciences, and
[2]Department of Public Health and Pediatrics, University of Torino, Torino, Italy.

Address reprint requests to Dr Fierro: Dermatologic Clinic, Dept of Medical Sciences, University of Torino, via Cherasco 23, 10126, Torino, Italy; mariateresa.fierro@unito.it.

Abstract
Objectives: Sézary syndrome (SS) is characterized by erythroderma, generalized lymphadenopathy, and the presence of circulating atypical lymphocytes, which are difficult to identify by morphologic data.
Methods: We revised our series of 107 patients in an attempt to better define the phenotypic aberrancies in blood at diagnosis and the immunophenotypic stability over time detected by flow cytometry. Polymerase chain reaction assay was also used to study CD26/dipeptidyl peptidase IV (DPPIV) gene methylation.
Results: The most common aberrancies were represented by the lack of CD26 (96/107) or CD38 (101/107) expression and the presence of a "dim" CD3, CD4, or CD2 population. There was a high variability in CD7 expression. In total, 31% of the patients had phenotypical heterogeneity in CD26 and CD7 expression at diagnosis. The phenotype was stable over time in 73 of 95 patients with available follow-up data, while 22 of 95 patients developed changes in CD26, CD7, or CD2 expression. CD4+CD26− SS showed hypermethylation of the CpG islands for the promoter region of CD26/DPPIV. Multivariate analysis showed that CD26 expression is a favorable prognostic factor (hazard ratio, 2.94; P = .045).
Conclusions: We confirm the relevance of CD26 negativity in SS diagnosis and monitoring. Nevertheless, the presence of rare CD26+ cases suggests that a multiparameter flow cytometry approach should be used. Changes in methylation profile could account for phenotypical heterogeneity.

Key Words: Sézary syndrome, CTCL, CD26, Flow cytometry, Hypermethylation, Immunophenotypic changes

Sézary syndrome (SS), characterized by erythroderma, generalized adenopathies, and peripheral blood involvement, is considered the leukemic variant within the spectrum of cutaneous T-cell lymphomas (CTCLs).1 Blood involvement is a constitutive SS feature and is included in the diagnostic criteria of the International Society for Cutaneous Lymphomas (ISCL) and the European Organization of Research and Treatment of Cancer (EORTC).2,3 The correct morphologic identification of Sézary cells (SCs) can be difficult and presents a high interobserver variability, making SS a suitable model for a flow cytometric study, which is more sensitive and accurate for both diagnostic purposes and tumor burden monitoring during follow-up.4–7 Circulating SCs display a postthymic T (CD3+, CD5+, CD28+, TCRαβ+) helper (CD4+CD8−), "central memory" (CD45RO+, CCR7+, CD27+)8,9 phenotype, with a peculiar skin-homing tendency due to the presence of the cutaneous lymphocyte antigen10 and chemokine receptors CCR4 and CCR10.11,12 SS is regarded as a Th2-dominant disease or, in a subgroup of patients, as a Treg disorder,13,14 opposite to the Th1 pattern of early mycosis fungoides (MF). The most frequent aberrancies are represented by the lack of CD26,15–20 CD7,21–23 and CD24 on their surface or by phenotypically aberrant populations that express a different amount of cell surface proteins than do normal T-cell populations ("dim" or "bright").4,24 Although flow cytometric analysis of the T-cell receptor (TCR)–Vb chain variable region can also be used to demonstrate clonality, this method is time-consuming and can identify only around 70% of the cases.25,26 Tumor burden is a major prognostic factor1,27,28 in patients with SS and can be monitored by flow

cytometry. The reliability of this method is based on the assumption that the phenotype remains stable over time.

Few studies in the literature discuss the phenotype heterogeneity at diagnosis and its stability during followup,6,23 and most group SS and MF in all disease stages as the same disease, despite the possibility of their arising from different functional T-cell subsets.

This article reports our retrospective review of serial peripheral blood lymphocyte (PBL) immunophenotyping of 107 patients with SS at diagnosis and during followup aimed at the identification of the most useful diagnostic markers. We also investigated the possibility of a phenotypical heterogeneity of the neoplastic cells at diagnosis or an instability during follow-up. As CD26 surface negativity is the most frequent abnormality in SS, we also decided to investigate CD26 gene silencing by means of hypermethylation of its promoter; this is a well-known feature of neoplastic cells and plays an important role in normal cell differentiation and development. Indeed, DNA methylation is an essential mechanism for normal cellular development, imprinting, X-chromosome inactivation, and maintenance of tissue specificity: hypermethylation represses transcription, while hypomethylation can lead to increased transcription levels.

Materials and Methods

Patients

Flow cytometry was used to screen blood samples from 805 primary cutaneous or secondary T-cell lymphomas from October 1985 to December 2012 at the Dermatologic Clinic of Turin University. Initial diagnosis was established according to ISCL/EORTC criteria,1,2 using a combination of clinical, histologic, and immunohistochemical findings. All patients had a skin examination, morphologic analysis of blood smears, flow cytometric immunophenotyping, and TCR gene rearrangement analysis performed by polymerase chain reaction (PCR) or GeneScan electrophoresis analysis (Applied Biosystems, Weiterstadt, Germany).29,30 We found the presence of phenotypically atypical circulating T lymphocytes in 168 (20.9%) of 805 cases. Among these, the most frequent disease was SS (107/168 [63.7%]) followed by MF (38/168 [22.6%]), other peripheral T-cell lymphomas (11/168 [6.5%]), and T-cell prolymphocytic leukemia (12/168 [7.1%]). Molecular analysis was done on archival samples in cases diagnosed before 1995. The study was performed according to the principles of good clinical practice and the Declaration of Helsinki principles. All patients gave their written informed consent for diagnostic procedures; ethical approval for this study was obtained from the ethical committee of the "Azienda Ospedaliera Città della Salute e della Scienza di Torino."

Flow Cytometry

Our archives contain 2,434 flow cytometry standard (FCS) files collected since 1985 using FACSCan (software, CONSORT 30 and FACScan Research), FACSCalibur (software, CellQuest), and, since 2009, FACSCanto II cytometer (BD Biosciences, San Jose, CA), equipped with three lasers: blue (488 nm), red (633 nm), and violet (405 nm). Repeated immunophenotypic studies (minimum of three blood samples) during follow-up were available in 95 of 107 patients with SS. Currently, PBLs are analyzed according to their immunofluorescence reactivity using a standard red blood cell lysis method: 20,000 peripheral lymphocytes included in a forward angle light scatter vs right-angle light scatter and CD45 gate are acquired and analyzed by FACSDiva 6.1.2 software (BD Biosciences). Eight-color immunofluorescence analyses are performed simultaneously using antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein–Cy5.5, (PerCP-Cy5.5), phycoerythtin-Cy7 (PE-Cy7), allophycocyanin (APC), allophycocyanin-H7 (APC-H7), Horizon V450 (V450), and Horizon V500 (V500).

Where it was deemed necessary to recheck carefully the phenotype of older cases, Flow Explorer software (freeware, Ron Hoebe compilation, Academic Medical Center, Department of Cell Biology and Histology, University of Amsterdam, The Netherlands) was used to locate FCS files in the archives.

Listmode files were retrospectively analyzed based on ad hoc templates created on FACSDiva 6.1.2 software (BD Biosciences). The old two-color CONSORT 30 FCS files (ie, CD4/CD7 and CD4/CD26) were reviewed by Infinicyt software (Cytognos SL, Salamanca, Spain), on the basis of a CD4 common parameter to obtain the information that was not available at the time of the acquisition, as a CD4/CD7/CD26 new combination using "merge and recalculation" steps.

The fluorochrome-conjugated monoclonal antibodies used against T-cell antigens included anti-CD2 FITC, PE, or PE-Cy7 (clone S5.2); anti-CD3 FITC, PE, or PerCP-Cy5.5 (clone SK7); anti-CD4 FITC, PE, or V450 (clone SK3); anti-CD5 FITC or APC (clone L17F12); anti-CD7 FITC or PE (clone 4H9) or anti-CD7 PerCP-Cy5.5 (clone M-T701); anti-CD8 PE or APC-Cy7 (clone SK1); anti-CD26 PE (clone L272); anti-CD27 FITC or APC (clone M-T271); anti-CD28 PE (clone CD28.2); anti-CD38 PE or PE-Cy7 (clone HB-7); anti CD45RA FITC (clone 4KB5); anti-CD45RO PE (clone UCHL1); anti-CD45 PerCP or V500 (clone HI30); and CD62L APC (clone DREG-56), all from BD Biosciences, as well as anti-CD26 APC (clone CLB-22C3) from Caltag Laboratories (Burlingame, CA). The negative staining threshold was established by the addition of an isotype-matched control tube. The analysis of the TCR-Vb repertoire was done using a commercially available kit (IOTest Beta Mark TCR-Vβ Repertoire Kit; Beckman Coulter, Miami, FL) designed to quantitate 24 different TCR-Vβ specificities covering approximately 70% of the normal human TCR-Vβ repertoire.

Over the past 2 years, the panel was enhanced by the addition of anti-KIR3DL2/CD158k PE (clone AZ158k), kindly provided by Innate-Pharma (Marseille, France), as well as anti-PD1/CD279 PerCP-Cy5.5 (clone EH12.1) and anti-NKP46/CD335 PE (clone 9E2/Nkp46), both from BD Biosciences.

Neoplastic T-cell populations were identified on the basis of patterns of aberrant antigen expression with respect to the normal internal phenotypic populations according to definitions by Vaughan et al7 and Jamal et al.31 A change in immunophenotype over time was defined as a gain or loss of an antigen on atypical cells stable in at least two consecutive samples. A modification of antigen intensity was accepted only when there was a log difference of at least 0.5. To identify any shifts in relative fluorescence over time caused by changes in reagents or instruments, we compared levels of antigen expression with normal internal cell populations.

Molecular Analysis

TCR-γ Gene Rearrangement Analysis

PCR was routinely used to detect the presence of a clonal TCR rearrangement. PCR with primers directed against the TCR-γ gene was performed as previously described.28 TCRγ-GR was studied on serial blood determinations by GeneScan capillary electrophoresis analysis, as previously described, in 24 of 107 patients.30

DNA Methylation PCR Array for the CD26/Dipeptidyl Peptidase IV Gene

The Methyl-Profiler qPCR Primer Assay for Human DPP4 (CpG Island 03780): MePH03780-2 (Qiagen, Valencia, CA) uses the MethylScreen technology (Orion Genomics, St Louis, MO). This method is based on the detection of the leftover input DNA after cleavage with a methylation-sensitive and/or a methylation-dependent restriction enzyme. These enzymes will digest unmethylated and methylated DNA, respectively. The remaining DNA is quantified after digestion by real-time PCR in each individual enzyme reaction, using primers that flank a promoter (gene) region of interest. The relative fractions of hypermethylated, intermediately methylated, and unmethylated DNA are subsequently determined by comparing the amount in each digest with that of a no-enzyme added reaction.

Three digestion reactions were prepared, three with each of the enzymes and one not digested reaction (Mo, Ms, Md, and Msd), according to the Qiagen instructions. The SYBR Green quantitative PCR array was performed as follows: 95°C for 10 minutes, three cycles at 99°C for 30 seconds and 72°C for 1 minute, and 40 cycles at 97°C for 15 seconds and 72°C for 1 minute. SYBR Green fluorescence from each well during the annealing step of each cycle was detected and recorded. EpiTect Methyl PCR Array Ct analysis was performed on an Excel-based data template, available at www.sabiosciences.com/ dna_methylation_data_analysis.php.

The minimum level of hypermethylation considered positive can be set at 10% to 20%. However, this is dependent on the ratio of target vs nontarget cells present in the sample (ie, normal cells mixed with cancerous cells). To overcome this threshold problem, we checked methylation on 98%/99.5% CD4+ purified cells.

Statistical Analyses

Statistical analyses were performed using the STATA 12.0 statistical software (StataCorp LP, College Station, TX). Data that were significant according to the Kolmogorov-Smirnov tests were considered to have a non-Gaussian distribution and were described by median and range. The cell percentages were compared

between groups using a one-way nonparametric analysis of variance (Kruskal-Wallis) test, followed by Dunn's multiple-comparison post hoc test for multiple groups. Data were considered significant when $P < .05$. Overall survival (OS) was calculated from the date of SS diagnosis to the date of death or last checkup for all patients. In the univariate analyses, product limit estimates were derived using the Kaplan-Meier method, and statistical comparisons were carried out by the log-rank Mantel-Cox test. Univariate/multivariate analyses were carried out to evaluate the influence that different variables had on OS. In the univariate/multivariate analysis, sex, B1/B2 blood stage, the presence of dim marker expression on atypical cells, loss of T-cell markers, and CD7 and CD26 expression (positive/mixed vs negative) were dichotomized; age at diagnosis, number of atypical cells, percentage of atypical cells, and CD4/CD8 ratio were continuous.

Results

Patient Characteristics

A total of 107 patients with SS (58 males and 49 females, median age 69.5 years at diagnosis) were studied with a median follow-up of 24.9 months (range, 1.2–128.9 months). At diagnosis, all patients had pruriginous erythroderma (two of 107 also displayed skin nodules), diffuse superficial adenopathies, atypical circulating SCs (>1,000 cells/μL), and circulating T-cell clones detected by TCR-g chain analysis with the use of PCR. An identical clone was evidenced in all patients tested (n = 50), both in blood and skin, by GeneScan analysis. Twelve patients had a history of MF and 20 a long-standing erythroderma that had been misdiagnosed as reactive dermatitis.

The median WBC count at diagnosis was 11,220/μL (range, 3,270–61,670/μL). The median percentage and absolute lymphocyte count were 39% (range, 10%–95.1%) and 4,314/μL (range 1,002–53,036/μL), respectively.

Immunophenotypic Findings: Heterogeneity at Diagnosis

The most commonly observed lymphocyte immunophenotypic pattern in patients with SS was CD3+, CD5+CD4+, CD8−, CD27+, CD28+, and CD45RO+, with a consequent increase in the CD4/CD8 ratio (median, 11.19; range, 0.95–990). The most common aberrancy at diagnosis was a lack of CD26 expression Figure 1A, Figure 1B, and Figure 1C (central dot plots) and was observed in 96 (89.7%) of 107 cases. While 11 patients (10.3%) showed a "mixed" phenotype with a variable percentage of atypical CD26+ SCs Figure 1D, Figure 1E, and Figure 1F (central dot plots), this was predominant in only three cases (Figure 1E). In 84 of 107 cases, clonality was investigated by means of anti-TCR antibodies: in 50 (40%) cases, a clonal population was positively identified. The CD4+CD26− subset in the 96 CD26− patients with SS (median, 66; range, 16–97.50) matched with the atypical population detected by the analysis of the TCR-Vb chain variable region and/or by T-marker dim expression (linear regression analysis: $R2 = 0.77$). A high variability in CD7 expression was documented: 51 (47.7%) patients were CD7− (Figure 1A,D, dot plots on the right), 26 (24.3%) were CD7+ (Figure 1C,F, dot plots on the right), and 30 (28%) displayed the simultaneous presence of CD7+ and CD7− SCs (Figure 1B,E, dot plots on the right). A total of 28 (26.2%) of 107 cases had a loss of T-cell markers other than CD26 and CD7. The most frequent was a CD2 loss in 23 of 28 cases, with four complete Figure 2B (central dot plot) and 19 partial Figure 2A (central dot plot), followed by a CD4 loss in five of 107 cases (complete in two and partial in three). One case showed a coexistent CD2 and CD4 loss, and another had a CD45RO− CD45RA− phenotype. None of the 107 patients with SS showed either a CD3 or CD5 loss.

Either a "dim" or, more rarely, a "bright" population was found in 103 (96.3%) of 107 cases. The most frequently expressed "dim" markers were CD3 (82/107 [76.6%]), CD4 (52/107 [48.6%]), CD2 (47/107 [43.9%]), and CD7 (21/107 cases, with 20% considering only the positive and mixed cases). Several patients had more than one "dim" population (Figure 2A, central dot plot); the most common was CD2dimCD3dim (31 cases [29.0%]; 21/31 were CD2dimCD3dimCD4dim).

CD38 expression was significantly lower (median, 17.5%; range, 1%–94%) than in healthy participants (median, 65%; range, 20%–88%) in all but six patients. The CD4+CD38− population (median, 70.0%; range, 5%–97.90%) matched with the CD4+CD26− one (linear correlation = 0.86 in CD26− patients with SS) (Figures 2A and 2B, dot plot on the right).

Beginning in 2010, CD279/PD1, KIR3DL2/CD158k, and NKP46/CD335 were added to our panel and investigated in 17 of 107 patients. CD279/PD1 was constantly detected on SCs Figure 3A and Figure 3B (second dot plot from right), whereas CD158k was expressed in only 40% of patients (Figure 3B, second dot plot from left), and NKP46 was negative (Figures 3A and 3B, first dot plot from right) in all but one of the patients.

Both subsets within the "CD26-mixed" cases belonged to the same TCR-Vβ clone, as demonstrated by flow cytometry (Figures 1D, 1E, and 1F, central dot plots) and GeneScan analysis Figure 4, which clearly identified the presence of a single neoplastic clone. It was possible to repeat the GeneScan analysis during follow-up in four of 11 patients, confirming the stability of the clone.

No relevant phenotypical differences were found in the 12 patients with a prior MF diagnosis with respect to SS right from the beginning: two of 12 showed immunophenotypic heterogeneity at diagnosis with a CD26+ subset, one was CD7+, two were CD7 mixed, and nine were CD7−.

Immunophenotypic Stability During Follow-up

A stable phenotype over time was documented in 73 (76.8%) of 95 patients with SS with serial determinations (minimum of three samples), in contrast to an unstable one observed in 22 (23.2%) of 95. Phenotypic changes concerned a single antigen in 17 cases and two simultaneous antigens in five cases. In detail, 11 (12.1%) patients acquired a CD4+CD26+ subset variable from 2% to 86%, and two lost the small CD4+CD26+ fraction that had been detected at diagnosis. Figure 5 shows the variations over time of the CD4+CD26± subpopulations according to treatment in a patient; interestingly, Gene Scan analyses performed during time showed no molecular changes despite the phenotypical switch observed. Positive or negative changes in CD7 expression were observed in nine (8.5%) patients (four cases from negative to mixed or positive and five cases from mixed to negative or positive). Five cases had changes in CD2 expression (four from negative to positive and one from positive to negative). No variations in fluorescence intensity were observed during follow-up, and the "dim" or "bright" CD3 or CD4 populations detected at diagnosis remained stable over time. In 11 of 22 cases, the onset of a new population was preceded by a treatment-induced, almost complete disappearance of circulating SCs (alemtuzumab in seven patients and fludarabine in four patients).

CD26/Dipeptidyl Peptidase IV Gene Hypermethylation

The DNA methylation profile of the CpG islands for the promoter region of CD26/dipeptidyl peptidase IV (DPPIV) on PBLs was examined in 10 patients with SS and five healthy donors. Since the results were ambiguous, we decided to repeat the experiment on CD4+ purified lymphocytes (five patients with SS and two healthy donors) and report the data as follows: all SS cases with a CD26− phenotype (CD4+CD26− population >85%) were hypermethylated, whereas a lower level of hypermethylation was observed (82% hypermethylated and 18% unmethylated) in an SS case with a consistent CD4+CD26+ percentage (24%). All normal samples were unmethylated Table 1.

Univariate/Multivariate Analyses of Prognostic Factors

Five- and 10-year OS was 31.6% and 15.4%, respectively, and 68 patients died during follow-up. The following parameters detected at diagnosis were considered for univariate survival analysis: sex, age, percentage and absolute number of circulating SCs, CD4/CD8 ratio, B1/B2 blood stage, previous MF diagnosis, marker loss (CD7, CD26, and others), and the presence of "dim" populations Table 2. The parameters related to tumor burden (ie, percentage and absolute SC number) were both identified as having a statistically significant association with poor survival, whereas sex and age, previous MF diagnosis, and phenotypic aberrancies related to CD7 Figure 6A and CD2 (data not shown) expression at diagnosis had no impact on survival. Interestingly, patients characterized by the presence of a variable proportion of CD26+ atypical cells at diagnosis showed a statistically significant higher OS (P = .050) Figure 6B. CD26 expression maintained a significant role (hazard ratio [HR], 2.94; P = .045) in the Cox multivariate model when corrected for main EORTC prognostic factors: B1/B2 blood stage (HR, 2.14; P = .013) and CD4/CD8 ratio (HR, 1.00; P = .031) (Table 2).

Discussion

CTCLs are a clinically heterogeneous group derived from skin-homing T lymphocytes with different biological profiles and clinical course.32,33 Since a large series of patients with SS with a median follow-up of 24.9 months was available to us, we were able to carry out a retrospective immunophenotypic study to evaluate the efficacy that flow cytometry has in the quantification of atypical circulating SCs. This method was applied to define the most frequent aberrancies that had a diagnostic impact and to investigate the phenotype stability over time.

The most frequent aberrancies observed in more than 90% of patients were a defective CD26 expression or a modification of antigen expression intensity (dim or bright), whereas CD7 expression always varied greatly. Since 1987, our group15,34 has focused on the CD26 molecule, underlying its importance in SS diagnosis and suggesting that a cutoff of 30% of the CD4+CD26− population provides a reliable tool when differentiating SS from inflammatory erythroderma.13 In our experience, the PE-CD26 clone L272 (BD Biosciences) is the most reliable of the antibodies available, in agreement with the Euroflow Consortium.35 The right choice of antibody is critical to avoid misinterpretations.5 The use of an antibody that underestimates the CD26+ population could lead to a consequent increase of the CD4+CD26− subset in both normal and reactive skin.

CD26/DPPIV, considered a T-cell activation antigen, is a characteristic transmembrane glycoprotein with multiple biological functions—it acts as a serine protease, receptor, costimulator, and adhesion molecule, and it induces apoptosis. Herein we discuss the role that the CpG hypermethylation of the CD26 gene promoter region plays in the loss of the CD26 surface marker, the same as that observed in adult T-cell leukemia (ATL).36 Tsuji et al36 demonstrated that CD26− ATL cells had faintly detected transcripts of the gene that were aberrantly methylated. CpG methylation of various genes may be one of the most important molecular events in the late stages of leukemogenesis, and an analogous mechanism could be hypothesized also for other T-cell marker loss (eg, CD7).37 The survival advantage observed in CD26+ or mixed SS cases, reported here for the first time, to our knowledge, might well be attributed to the tumorigenicity of CD26 loss. Since the SS cohort at our disposal was larger than the one we analyzed in 1998, we were able to confirm that all parameters related to tumor burden are significantly associated with poor survival, but not the prognostic value of CD7 negativity, in agreement with the findings from Vonderheid et al.23

Considering the large panel of antibodies used, we would like to stress the importance of CD38 negativity in SS, something rarely described in the literature.4 CD38 is a glycoprotein involved in cellular adhesion, signal transduction, and intracellular calcium regulation, and its loss leads to a reduction in immune functions. Nowadays, it is considered an important prognostic factor used to stratify patients with B-cell chronic lymphocytic leukemia.38 In our experience, the evaluation of a CD4+CD38− population is a sensitive and reliable marker and can provide an additional parameter when defining the atypical population.

Another interesting observation that emerged from our retrospective analysis is that CD3 or CD5 loss is an extremely rare phenomenon in patients with SS39: in our experience (data not shown), a CD3 loss can be found only in primary cutaneous CD30+ or follicular T-cell lymphomas. A “dim” T-cell marker expression in the atypical population represents the most frequent alteration in SS, as reported by other authors.4–6 In our opinion, the finding of a dim population, especially when coupled with the CD26 and/or TCR-Vb analysis, represents a good tool for a reliable tumor burden assessment; nevertheless, the correct choice of the fluorochrome is important for a good resolution of the “dim” population.35 Some molecules have recently been proposed as positive markers for SS. PD-1 plays a role in the maintenance of self-tolerance and prevention of autoimmunity, and its major goal is the inhibition of T-cell function, including proliferation and cytokine production. The present study confirms that PD-140,41 is widely expressed in SCs. It is not suitable for diagnostic purposes, being also expressed in activated T and B cells, but its expression in SCs can be relevant from a functional point of view, defining the suppressor nature of these cells and representing a potential target for therapy. Although our experience is somewhat limited, our findings on CD158k-positive SCs are in disagreement with those of Poszepczynska-Guigné et al42 and Bahler et al,43 who reported a very high percentage of CD158k-positive SCs (ie, from 82%–97%). Indeed, we observed CD158k expression in only 40% of cases. However, this bias may well be due to the different techniques employed: the PE-conjugated AZ158k Ab could be less sensitive than the CD158k/KIR3DL2 mAb

plus PE-coupled goat anti–mouse secondary mAb used by Poszepczynska-Guigné et al42 and the Q66 Ab used by Bahler et al.43

The natural cytotoxicity receptor NKP46 was identified as the activating receptor that is mainly expressed by natural killer lymphocytes but also by the circulating SCs. For this reason, Bensussan et al44 have recently proposed it as an additional marker on the circulating malignant T lymphocytes of patients with SS, in whom it functions as an inhibitory coreceptor that is capable of interfering with the processes governing their CD3-dependent proliferation. Again, our results differ from those of Bensussan et al,44 showing a negativity of NKP46 in all but one patient with SS.

Phenotypical heterogeneity has already been described6,16,22 in a small series of patients with SS, and our finding confirms this observation on a wide series, in which a heterogeneous phenotype was detected in 32% of the study population. Notably, the review of our cases shows that in approximately 15% of cases, a subset of CD26+ cells can be present at diagnosis or arise during the follow-up. Moreover, we carried out evaluations to determine whether the positive and negative subsets for the same antigen belong to the same neoplastic clone at a molecular level. To this aim, two different approaches were used—the cytometric detection of TCR-Vb regions and TCR GeneScan analysis—and a single clone was observed by both techniques. This strengthens the hypothesis that the neoplastic population possesses a plasticity that allows for heterogeneity at a phenotypical level.

A stable phenotype over time was documented in most patients (76.8%), including those with an aberrant mixed phenotype, in agreement with Washington et al,24 who stated that immunophenotypic aberrancies were maintained over the course of disease in 95% of the 44 CTCLs analyzed despite varying treatments. The observation of an onset of new phenotypic subsets over time in a minority of our patients could be explained by the presence of several small undetectable populations since diagnosis: a different subset belonging to the minimal residual disease could proliferate after an effective treatment such as alemtuzumab.45 However, on the basis of our findings, this phenomenon does not seem to bear prognostic relevance, since alemtuzumab was used in 21 (67.7%) patients in our series who achieved an almost complete response (data not shown) without any evidence of phenotypical changes.

In conclusion, we confirm the relevance that CD26 negativity has for a diagnostic purpose in SS. Nevertheless, due to the finding of stable phenotypical changes sometimes involving CD26, multiparameter flow cytometry is recommended. This should combine the assessment of routinely used T-cell markers and the cytometric TCR-Vβ analysis should the routine panel produce ambiguous results. In detail, we believe that the first-step panel for SS diagnosis should include the evaluation of CD26, CD27, CD28, CD38, CD45RO, and CD45RA, in addition to T-cell markers (CD2, CD4, CD7, CD8) and CD3 and CD45 as backbone markers, as suggested by the Euroflow Consortium.35 As already suggested by other groups,6,46 multiparameter flow cytometry may also help the clinician in monitoring tumor burden in response to therapy, specifically alemtuzumab. At our institution, after evaluating the CD52 expression in basal conditions, this approach is used to guide alemtuzumab dose frequency to keep the side effects of this therapy to a minimum.

Address reprint requests to Dr Fierro: Dermatologic Clinic, Dept of Medical Sciences, University of Torino, via Cherasco 23, 10126, Torino, Italy; mariateresa.fierro@unito.it.

This research was supported in part by grants from University of Turin (ex 60%).

Acknowledgments: We thank Alessandra Stacchini, Flow Cytometry Unit, Diagnostic Laboratory Department, Citta' della Salute e della Scienza, Turin, for critical review of the manuscript and Barbara Wade for linguistic advice.

References

1. Olsen EA, Rook AH, Zic J, et al. Sézary syndrome: immunopathogenesis, literature review of therapeutic options, and recommendations for therapy by the United States Cutaneous Lymphoma Consortium (USCLC).J Am Acad Dermatol. 2011;64:352-404.

2. Vonderheid EC, Bernengo MG, Burg G, et al. ISCL Update on erythrodermic cutaneous T-cell lymphoma: report of the International Society for Cutaneous Lymphomas. J Am Acad Dermatol. 2002;46:95-106.
3. Olsen E, Vonderheid E, Pimpinelli N, et al; ISCL/EORTC. Revisions to the staging and classification of mycosis fungoides and Sézary syndrome. Blood. 2007;110:1713-1722.
4. Lima M, Almeida J, dos Anjos Teixeira M, et al. Utility of flow cytometry immunophenotyping and DNA ploidy studies for diagnosis and characterization of blood involvement in CD4+ Sézary's syndrome. Haematologica. 2003;88:874-887.
5. Klemke CD, Brade J, Weckesser S, et al. The diagnosis of Sézary syndrome on peripheral blood by flow cytometry requires the use of multiple markers. Br J Dermatol. 2008;159:871-880
6. Hristov AC, Vonderheid EC, Borowitz MJ. Simplified flow cytometric assessment in mycosis fungoides and Sézary syndrome. Am J Clin Pathol. 2011;136:944-953.
7. Vaughan J, Harrington AM, Hari PN, et al. Immunophenotypic stability of Sézary cells by flow cytometry: usefulness of flow cytometry in assessing response to and guiding alemtuzumab therapy. Am J Clin Pathol. 2012;137:403-411.
8. Fierro MT, Novelli M, Quaglino P, et al. Heterogeneity of circulating CD4+ memory T-cell subsets in erythrodermic patients: CD27 analysis can help to distinguish cutaneous T-cell lymphomas from inflammatory erythroderma. Dermatology. 2008;216:213-221.
9. Campbell JJ, Clark RA, Watanabe R, et al. Sézary syndrome and mycosis fungoides arise from distinct T-cell subsets: a biologic rationale for their distinct clinical behaviors. Blood. 2010;116:767-771.
10. Picker LJ, Terstappen LW, Rott LS, et al. Differential expression of homing-associated adhesion molecules by T cell subsets in man. J Immunol. 1990;145:3247-3255.
11. Notohamiprodjo M, Segerer S, Huss R, et al. CCR10 is expressed in cutaneous T-cell lymphoma. Int J Cancer.
2005;115;641-647.
12. Sokolowska-Wojdylo M, Wenzel J, Gaffal E, et al. Circulating clonal CLA(+) and CD4(+) T cells in Sézary syndrome express the skin-homing chemokine receptors CCR4 and CCR10 as well as the lymph node–homing chemokine receptor CCR7. Br J Dermatol. 2005;152:258-264.
13. Heid JB, Schmidt A, Oberle N, et al. FOXP3+CD25– tumor cells with regulatory function in Sézary syndrome.
J Invest Dermatol. 2009;129:2875-2885.
14. Krejsgaard T, Gjerdrum LM, Ralfkiaer E, et al. Malignant Tregs express low molecular splice forms of FOXP3 in Sézary syndrome. Leukemia. 2008;22:2230-2239.
15. Bernengo MG, Novelli M, Quaglino P, et al. The relevance of The CD4+ CD26– subset in the identification of
circulating A, Sézary cells. Br J Dermatol. 2001;144:125-135.
16. Jones D, Dang NH, Duvic M, et al. Absence of CD26 expression is a useful marker for diagnosis of T-cell lymphoma in peripheral blood. Am J Clin Pathol. 2001;115:885-892.
17. Introcaso CE, Hess SD, Kamoun M, et al. Association of change in clinical status and change in the percentage of the CD4+CD26– lymphocyte population in patients with Sézary syndrome. J Am Acad Dermatol. 2005;53:428-434.
18. Sokolowska-Wojdylo M, Wenzel J, Gaffal E, et al. Absence of CD26 expression on skin-homing CLA+ CD4+ T
lymphocytes in peripheral blood is a highly sensitive marker for early diagnosis and therapeutic monitoring of patients with Sézary syndrome. Clin Exp Dermatol. 2005;30:702-706.
19. Kelemen K, Guitart J, Kuzel TM, et al. The usefulness of CD26 in flow cytometric analysis of peripheral blood in Sézary syndrome. Am J Clin Pathol. 2008;129:146-156.
20. Steinhoff M, Schöpp S, Assaf C, et al. Prevalence of genetically defined tumor cells in CD7 as well as CD26
positive and negative circulating T-cell subsets in Sézary syndrome. Leuk Res. 2009;33:88-99.
21. Wood GS, Hong SR, Sasaki DT, et al. Leu-8/CD7 antigen expression by CD3+ T cells: comparative analysis of skin and blood in mycosis fungoides/Sézary syndrome relative to normal blood values. J Am Acad Dermatol.
1990;22:602-607.

22. Ginaldi L, Matutes E, Farahat N, et al. Differential expression of CD3 and CD7 in T-cell malignancies: a quantitative study by flow cytometry. Br J Haematol. 1996;93:921-927.
23. Vonderheid EC, Bigler RD, Kotecha A, et al. Variable CD7 expression on T cells in the leukemic phase of cutaneous T cell lymphoma (Sézary syndrome). J Invest Dermatol. 2001;117:654-662.
24. Washington LT, Huh YO, Powers LC, et al. A stable aberrant immunophenotype characterizes nearly all cases of cutaneous T-cell lymphoma in blood and can be used to monitor response to therapy. BMC Clin Pathol. 2002;2:5.
25. Feng B, Jorgensen JL, Jones D, et al. Flow cytometric detection of peripheral blood involvement by mycosis
fungoides and Sézary syndrome using T-cell receptor Vbeta chain antibodies and its application in blood staging. Mod Pathol. 2010;23:284-295.
26. Vonderheid EC, Boselli CM, Conroy M, et al. Evidence for restricted Vbeta usage in the leukemic phase of cutaneous T cell lymphoma. J Invest Dermatol. 2005;124:651-661.
27. Vidulich KA, Talpur R, Bassett RL, et al. Overall survival in erythrodermic cutaneous T-cell lymphoma: an analysis of prognostic factors in a cohort of patients with erythrodermic cutaneous T-cell lymphoma. Int J Dermatol. 2009;48:243- 252.
28. Olsen EA, Whittaker S, Kim YH, et al; International Society for Cutaneous Lymphomas; United States Cutaneous Lymphoma Consortium; Cutaneous Lymphoma Task Force of the European Organisation for Research and Treatment of Cancer. Clinical end points and response criteria in mycosis fungoides and Sézary syndrome: a consensus statement of the International Society for Cutaneous Lymphomas, the United States Cutaneous Lymphoma Consortium, and the Cutaneous Lymphoma Task Force of the European Organisation for Research and Treatment of Cancer. J Clin Oncol. 2011;29:2598-2607.
29. Ponti R, Fierro MT, Quaglino P, et al. TCRgamma-chain gene rearrangement by PCR-based GeneScan: diagnostic accuracy improvement and clonal heterogeneity analysis in multiple cutaneous T-cell lymphoma samples. J Invest Dermatol. 2008;128:1030-1038.
30. Fierro MT, Ponti R, Titli S, et al. TCRgamma-chain gene rearrangement by GeneScan: incidence and significance of clonal heterogeneity in Sézary syndrome. J Invest Dermatol. 2010;130:2312-2319.
31. Jamal S, Picker LJ, Aquino DB, et al. Immunophenotypic analysis of peripheral T-cell neoplasms: a multiparameter flow cytometric approach. Am J Clin Pathol. 2001;116:512-526.
32. Willemze R, Kerl H, Sterry W, et al. EORTC classification for primary cutaneous lymphomas: a proposal from the Cutaneous Lymphoma Study Group of the European Organization for Research and Treatment of Cancer. Blood. 1997;90:354-371.
33. Wong HK, Mishra A, Hake T, et al. Evolving insights in the pathogenesis and therapy of cutaneous T-cell lymphoma (mycosis fungoides and Sézary syndrome). Br J Haematol. 2011;155:150-166.
34. Bernengo MG, Meregalli M, Novelli M, et al. Relevance of MoAb BT5.9 and DP IV activity for the diagnosis of Sézary syndrome. J Invest Dermatol. 1987;89:329.
35. van Dongen JJ, Lhermitte L, Böttcher S, et al. EuroFlow antibody panels for standardized n-dimensional flow cytometric immunophenotyping of normal, reactive and malignant leukocytes. Leukemia. 2012;26:1908-1975.
36. Tsuji T, Sugahara K, Tsuruda K, et al. Clinical and oncologic implications in epigenetic down-regulation of CD26/ dipeptidyl peptidase IV in adult T-cell leukemia cells. Int J Hematol. 2004;80:254-260
37. Rogers SL, Zhao Y, Jiang X, et al. Expression of the leukemic prognostic marker CD7 is linked to epigenetic modifications in chronic myeloid leukemia. Mol Cancer. 2010;9:41.
38. Malavasi F, Deaglio S, Damle R, et al. CD38 and chronic lymphocytic leukemia: a decade later. Blood. 2011;118:3470-3478.
39. Sano S, Matsui Y, Itami S, et al. Immunological study on CD3 defective cutaneous T cell lymphoma cells from a patient with Sézary syndrome. Clin Exp Immunol. 1998;113:190-197.
40. Samimi S, Benoit B, Evans K, et al. Increased programmed death-1 expression on CD4+ T cells in cutaneous T-cell lymphoma: implications for immune suppression. Arch Dermatol. 2010;146:1382-1388.
41. Cetinözman F, Jansen PM, Vermeer MH, et al. Differential expression of programmed death-1 (PD-1) in Sézary syndrome and mycosis fungoides. Arch Dermatol. 2012;148:1379-1385.

42. Poszepczynska-Guigné E, Schiavon V, D'Incan M, et al. CD158k/KIR3DL2 is a new phenotypic marker of Sézary cells: relevance for the diagnosis and follow-up of Sézary syndrome. J Invest Dermatol. 2004;122:820-823
43 Bahler DW, Hartung L, Hill S, et al. CD158k/KIR3DL2 is a useful marker for identifying neoplastic T-cells in Sézary syndrome by flow cytometry. Cytometry B Clin Cytom. 2008;74:156-162.
44. Bensussan A, Remtoula N, Sivori S, et al. Expression and function of the natural cytotoxicity receptor NKp46 on circulating malignant CD4+ T lymphocytes of Sézary syndrome patients. J Invest Dermatol. 2011;131:969-976.
45. Bernengo MG, Quaglino P, Comessatti A, et al. Low-dose intermittent alemtuzumab in the treatment of Sézary syndrome: clinical and immunologic findings in 14 patients. Haematologica. 2007;92:784-794.
46. Horna P, Deaver DM, Qin D, et al. Quantitative flow cytometric identification of aberrant T cell clusters inerythrodermic cutaneous T cell lymphoma. Implications for staging and prognosis. J Clin Pathol. 2014;67:431-436.

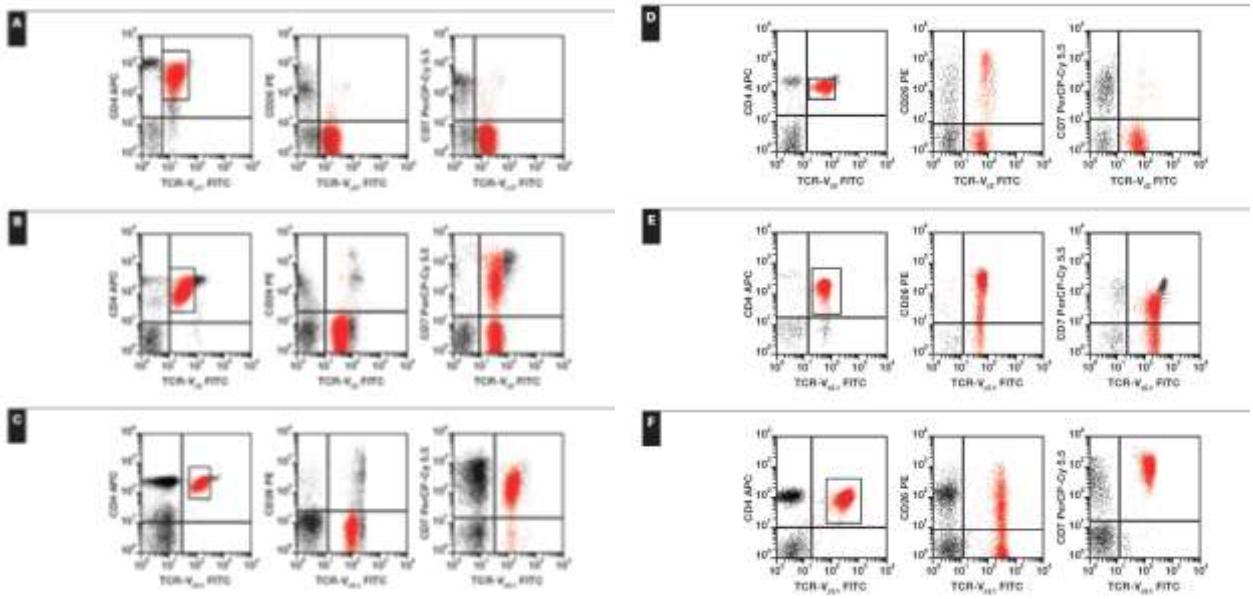

Figure 1. Six different Sézary syndrome cases in which the atypical Sézary cells population (in red) is identified at baseline by CD4+ T-cell receptor (TCR)–Vb positivity (A–F). Different CD26 expression: dot plots show CD26 negativity (A–C) and a CD26 mixed pattern with different amount of CD26 (D–F). CD7 expression in dot plots is negative (A, D), mixed (B, E), and positive (C, F).

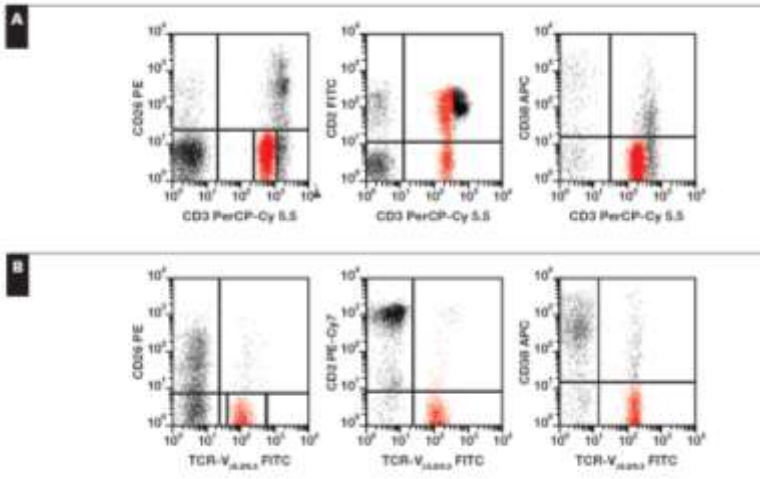

Figure 2. Aberrancies in CD2 and CD38 expression in two different Sézary syndrome (SS) cases. A, Patient with SS with CD3+dim CD26− phenotype, aberrant mixed CD2− and CD2+dim populations, and CD38 negativity. B, Patient with SS with T-cell receptor (TCR)–Vb 5.2/5.3+ CD26− phenotype, CD2 negativity, and CD38 negativity.

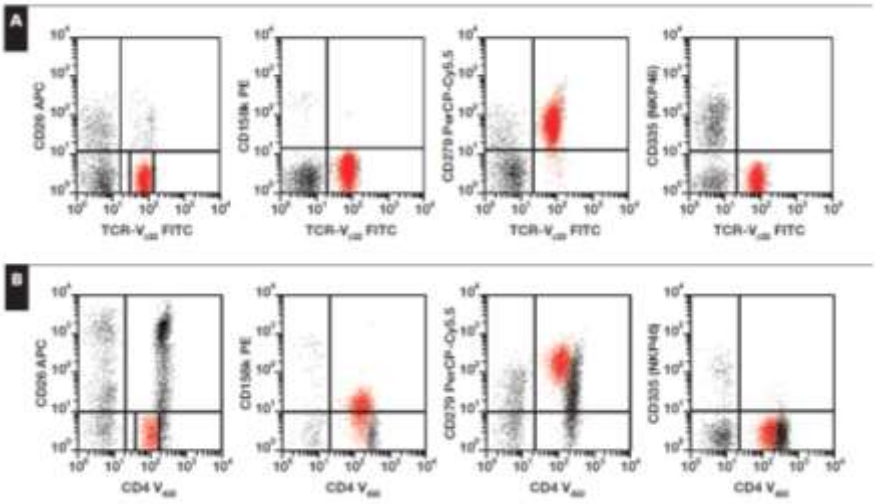

Figure 3. A, Patient with Sézary syndrome (SS) patient with high tumor burden: immunophenotype T-cell receptor (TCR)–Vb22+, CD26−, CD158k−, CD279+, NKP46− (red). B, Patient with SS with low tumor burden: immunophenotype CD4+dim, CD26−, CD158k+dim, CD279+, NKP46− (red). Residual normal CD4+ lymphocytes are CD26+, CD158k−, CD279±, NKP46− (black).

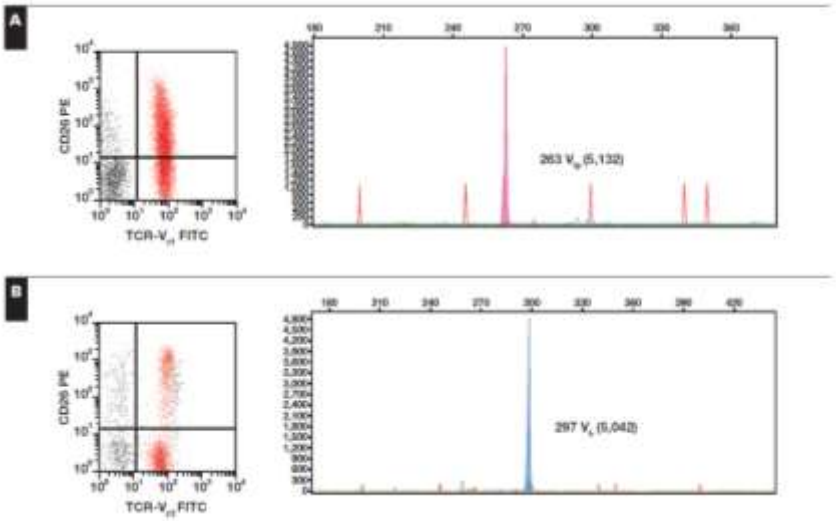

Figure 4. GeneScan identifies a single pathologic clone (filled peak), whereas cytometric analysis shows a continuous spectrum of CD26 expression from positive to negative (case A) and the coexistence of two CD26+ and CD26− subsets (case B), both expressing a single T-cell receptor (TCR)–Vb chain in the same peripheral blood lymphocyte sample.

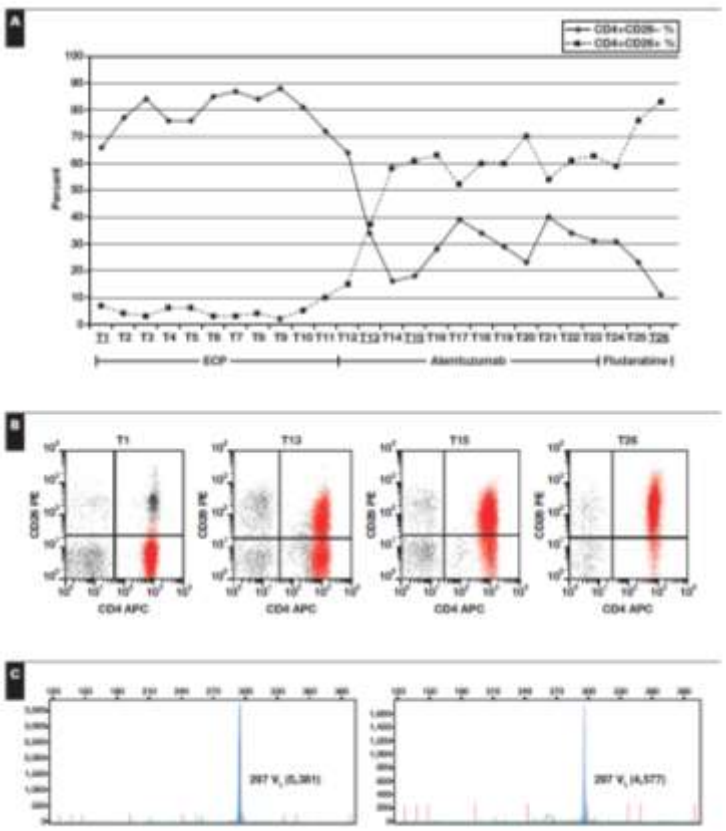

Figure 5. Phenotypical switch from CD4+CD26− to CD4+CD26+ in a patient with Sézary syndrome. A, Fluctuation of the two subsets during follow-up according to treatment (extracorporeal photopheresis, ECP; alemtuzumab; fludarabine). B, Dot plots at time T1, T13, T15, and T26 show the progressive increase of the CD4+CD26+ population. C, GeneScan analysis of a peripheral blood lymphocyte before (left) and after (right) the phenotypical switch show an identical clone at the molecular level despite the phenotypical diversity.

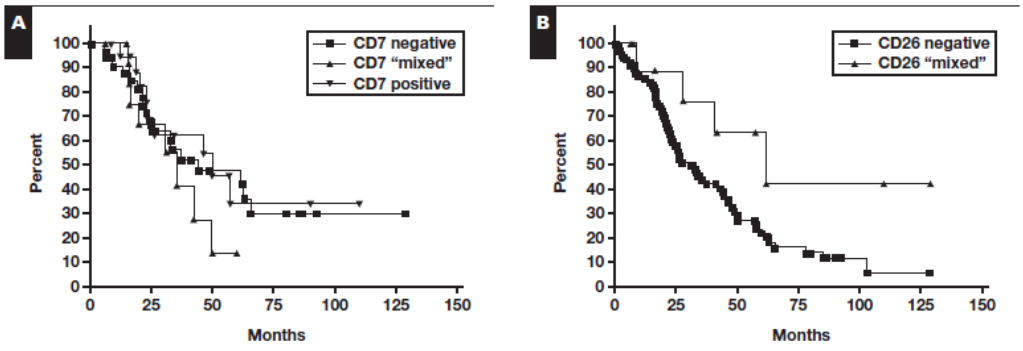

Figure 6. Survival according to CD7 (A; P = .4717) and CD26 (B; P = .0469) expression.

	Sezary cells	CD4+ lymph.	CD4+CD26- lymph.	Hypermethylated	Unmethylated
B.D. (SS CD4dim)	95	96	96	95.1	4.9
B.C. (SS CD4dim)	82	91	82	97.5	2.5
M.E. (SS TCR Vβ 13.2)	67	79	76	89.13	10.87
LR.G. (SS TCR Vβ 13.1)	91	95	93	98.7	0.83
D.A. (SS TCR Vβ 5.1)	91	93	15	6.67	93.33
Healthy donor 1	0	58	10	1.46	98.54
Healthy donor 2	0	60	6	0.07	99.93

Table 1: CD26 DNA methylation profile on CD4 purified lymphocytes from 5 SS cases and 2 healthy donors. All data are expressed as percentage values.

Characteristic	HR	**HR (95% CI)**	**P Value**
Gender	.88	(.542774 1.418286)	0.593
age at diagnosis	.99	(.9793775 1.018717)	0.909
B1/B2 blood stage	2.39	(1.3335 4.272529)	0.003
% of atypical cells	1.02	(1.010752 1.031901)	0.000
number of atypical cells	1. 00	(1.000007 1.000049)	0.008
CD4/CD8 ratio	1.00	(1.000867 1.00464)	0.004
“dim” marker expression	2.59	(.3586435 18.78389)	0.345
Marker loss (CD2, CD4)	1.60	(.955714 2.680926)	0.074
CD7 expression	.89	(.551187 1.444637)	0.643
CD26 expression	2.70	(.9745076 7.474271)	0.050
Gender	.86	(.5234651 1.427505)	0.569
age at diagnosis	1.00	(.9850816 1.023853)	0.665
B1/B2 blood stage	2.14	(1.176012 3.904511)	0.013
CD4/CD8 ratio	1.00	(1.00021 1.004365)	0.031
CD26 expression	2.94	(1.025964 8.423034)	0.045

Table 2: Summary of a Univariate Analysis to Evaluate an Association With OS and a Multivariate Analysis to Identify Independent Predictors of OS
Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival..