

Spectral karyotyping demonstrates genetically unstable skin-homing T lymphocytes in cutaneous T-cell lymphoma

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Abstract: We initially established cell lines from skin biopsies from four patients (MF8, MF18, MF19 and MF31) in early stages of cutaneous T-cell lymphoma (CTCL) in 1999. After 3 weeks of culture, skin-homing T lymphocytes were stimulated with phytohaemagglutinin. Metaphase spreads were analysed using spectral karyotyping (SKY), a molecular cytogenetic technique. MF18 and MF19 had predominantly normal karyotypes. MF8 had recurrent numerical aberrations resulting in two T lymphocyte clones: one with trisomy 21 (12/20 cells) and the other with monosomy chromosome 22 (3/20 cells). MF8 also exhibited a clonal deletion, del(5)(p15.1), as well as multiple non-clonal structural aberrations. MF31 had a clonal deletion, del(17)(p12) and other non-clonal deletions involving chromosomes 2, 5, 10, 11. MF18 had a single abnormal cell that contained two reciprocal translocations t(1;2)(q32;p21) and t(4;10)(p15.2;q24). In 2001, three of the original patients had new skin biopsies taken and cell

lines were established. SKY analysis revealed the continued presence of a T-cell clone in MF8 with trisomy 21 (4/20 cells). Additionally, a new clone was seen with a del(18)(p11.2) (17/20 cells). MF31 had only one aberrant cell with a del(17)(p12). MF18 had a clonal deletion, [del(1)(p36.1) in 3/20 cells] and non-clonal aberrations involving chromosomes 3, 4, 5, 6, 12, 13, 17 and 18. Thus, three of four patients continued to show numerous numerical and structural aberrations, both clonal and non-clonal, with only MF8 having a recurring T lymphocyte clone (+21). Our findings demonstrate high genetic instability among skin-homing T lymphocytes even in early stages of CTCL. We did not see genetic instability or evidence of clones in cell lines from a patient with atopic dermatitis and one with psoriasis.

Key words: cutaneous T-cell lymphoma – cytogenetic analysis – genomic instability – mycosis fungoides – spectral karyotyping

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Introduction

Cutaneous T-cell lymphoma (CTCL) is a rare skin disorder characterized by a lymphocytic skin inflammation with pleomorphic T lymphocytes forming epidermal Pautrier's microabscesses (1,2). Several studies have described karyotypic changes of peripheral blood lymphocytes from patients with CTCL; however, to date no consistent pattern of chromosomal aberrations has been observed in blood and skin-homing T lymphocytes (3–8). Patients with advanced disease have numerous structural and numerical chromosomal aberrations (4–11). Recent studies have revealed recurrent losses of chromosome regions 1p, 6q, 9p, 10q, 17p and 19p (8,9). Other investigators have observed losses of 10q and 13q(5) or 1p loss and 17q gain (10).

The consensus today is that neither recurrent pattern of chromosomal changes nor specific reciprocal translocations is associated with CTCL, but the variety and complexity of changes observed indicate a general genomic instability of the T lymphocytes involved. We have previously termed these cells 'geno-traumatic' T lymphocytes (12) and considered such cells to be in malignant transformation (13).

Cancer is the result of multiple genetic changes (14). The goal of our study was to determine if skin-homing lymphocytes grown in vitro from patients with early-stage CTCL are associated with recurrent chromosomal aberrations, which correlate with malignant transformation of T lymphocytes. Previous chromosome analyses of CTCL patients have primarily used conventional banding methods with few reports utilizing molecular cytogenetic methods

(6,9,10). We have used spectral karyotyping (SKY), a method that allows one to visualize all human chromosomes within a single metaphase in different colours (15). SKY has proven instrumental in revealing hidden rearrangements in cells derived from haematological malignancies as well as defining complex karyotypes, and refining breakpoints in solid tumors (16,17).

Patients and methods

We established cell lines from four patients (designated MF8, MF18, MF19 and MF31) with early stages of CTCL after obtaining informed consent (Table 1). Classification was made according to the Scandinavian Mycosis Fungoides Group (18). The first cell lines were established in 1999. In 2001 we obtained new skin biopsies from the same patients and established cell lines, except for one patient who declined to participate as she suffered from severe osteoarthritis. Patients indicated as being in remission had a skin biopsy taken from an area of previous disease.

A cell line was established from a 24-year-old woman suffering from atopic dermatitis and one from a 19-year-old woman with psoriasis vulgaris. Both were included as 'control cell lines' from non-malignant inflammatory skin diseases.

Establishment of cell lines was as previously described (19). Skin tissue was placed in sterile medium and within 1 h transferred into a Nunc 50 ml culture flask containing 10 ml of prewarmed (37°C) RPMI-1640 with 10% human AB serum, antibiotics, interleukin 2 (1000, units/ml; Chiron, Amsterdam, the Netherlands) and IL-4 250 units/ml (Schering-Plough, Kenilworth, NJ, USA). Cell numbers were counted on a weekly basis using a Coulter Counter ZM (Beckman, Fullerton, CA, USA). After approximately 3 weeks of culture, cells were phytohaemagglutinin (PHA)-stimulated for 3 days (1 µg/ml) and then treated with colchicine (0.4 µ/ml) for 1 h to arrest cellular proliferation. They were then spun gently down and resuspended and stored in ethanol. The study was approved by the Ethical Committee

of Aarhus County. Patient information was not disclosed to HPN (first author) until the SKY analysis was finished.

Spectral karyotyping analysis

In 1999, cell pellets (samples were coded) from four CTCL patients were shipped on dry ice to HPN and upon receipt, the cells were resuspended in fixative [methanol:acetic acid, 3:1 (v/v)]. Cell suspensions were dropped onto slides in a Thermanon to control for humidity and temperature. The slides were then aged in a 40°C drying oven for approximately 1 week. The preparation of SKY probes, slide pretreatment, slide denaturation, detection and imaging have been described previously, and protocols are available at <http://www.riedlab.nci.nih.gov/protocols> (20).

Two years later (2001) cell pellets from three of the four original patients were received and treated in the same manner as described previously. Fifteen to twenty metaphase cells were analysed for each patient using SKYview v1.6TM (Applied Spectral Imaging, Carlsbad, CA, USA).

Results

The patients initially received topical nitrogen mustard in combination with topical steroids. In 2001 MF8 was treated with a low dose of retinoid in order to control her disease, which was clinically active. MF18 and MF19 had gone into clinical remission and did not receive treatment. The biopsies were taken from areas of skin with previous plaques. MF31 was still displaying active disease, but being treated with only topical steroids as he is physically too weak for further nitrogen mustard therapy. Thus the initial topical nitrogen mustard therapy seems not likely to have induced the karyotypic changes.

The karyotypes of all four patients are presented in Table 2 using chromosome nomenclature rules from ISCN (21). A structural aberration is considered clonal if two or more cells contain the same aberration. With respect to numerical aberrations, gains of chromosomes are considered clonal if two or more cells contain the same extra

Table 1. Clinical information about the patients and controls who provided skin biopsies for the establishment of cell lines

Cell line code	Age (years)	Sex	CTCL stage	Duration of disease	First skin biopsy	Second skin biopsy	Clinical status 2002
MF8	84	Female	MF st. I parapsoriasis	14	1999	2001	Stable but in treatment
MF18	80	Male	Erythroderma	7	1999	2001	In remission
MF19	78	Female	MF st. I parapsoriasis	5	1999	Not done	In remission
MF31	87	Male	MF st. II	2	1999	2001	Almost in remission
Control 1	19	Female	Psoriasis	5–6	2005	Not done	Active psoriasis
Control 2	24	Female	Atopic dermatitis	Lifelong	2005	Not done	Active eczema

The control persons had active skin disease when biopsied. They had no immediate treatment prior to their biopsies.

chromosome, and losses are classified as clonal if they are observed in three or more cells. The karyotypes for all patient samples have been entered into the NCI and NCBI SKY/CGH interactive online database; <http://www.ncbi.nlm.nih.gov/sky/skyweb.cgi> (submitter: padilla-nash). In this database, the patient's karyotypes are depicted as coloured human ideogram, called a SKYGRAM. Each SKYGRAM presents the complement of normal and abnormal chromosomes with the position of their breakpoints linked to the DNA sequence database.

The control cell lines showed no gains of chromosomes or consistent loss of chromosomal material and no evidence of clonal T cells. They were not different from what is seen in metaphase spreads of normal individuals (Table 2).

Patient MF8

The SKY analysis of MF8 in 1999 revealed both non-clonal numerical losses and gains (losses of chromosomes 6, 11, 13, 15, 16, 17, 19 and 20; gains of chromosomes 4, 6, 7, 9, 13, 14, 15, 16, 19 and 20), and multiple structural aberrations. In addition, two clones were detected, one containing trisomy 21 (12/20 cells) (see Fig. 1). The patient did not have Down syndrome. Additionally, loss of chromosome 22 was found in three of 20 cells. In 2001, four cells from MF8 had the karyotype 47,XX,+21. Additionally, a new clone had developed containing the deletion, del(18)(p11.2) in 17 of 20 cells.

Patient MF18

Spectral karyotyping analysis of patient MF18 in 1999 revealed only two abnormal cells, one with monosomy of

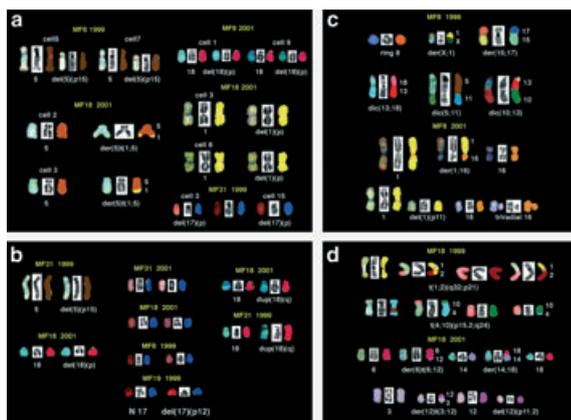


Figure 1. Summary of structural aberrations resolved by spectral karyotyping (SKY) in MF patients. (a) All the clonal structural aberrations found in the MF8, MF18 and MF31 using SKY. (b) Non-clonal but recurrent structural aberrations found by SKY in all four patients. (c) Summary of non-clonal chromosome exchanges found in MF8 in 1999 and those observed in 2001. (d) Non-clonal reciprocal translocations found in MF18 in 1999 and some of the non-clonal structural aberrations found in 2001.

chromosome 2, the other cell contained two reciprocal translocations involving chromosomes 1 and 2, and 4 and 10, respectively, as shown in Table 2 and Fig. 1c. In 2001, 11 cells were observed with two recurrent aberrations, del(1)(p36.1) and der(5)t(1;5)(p36.1;q33), as well as non-clonal deletions and translocations involving chromosomes 3, 4, 6, 12, 14, 17 and 18.

Patient MF19

Three cells were seen in patient MF19 revealing a non-clonal unbalanced translocation, and two deletions, including the del(17)(p12). No sample was analysed in 2001.

Patient MF31

In 1999, six cells were found by SKY to have non-clonal deletions involving chromosomes 2, 5, 10, 11 and 17. The del(17)(p12) was clonal. Interestingly, seven of 20 cells displayed a propensity for satellite association between the acrocentric chromosomes 15 and 22. In 2001, only one cell was found with del(17)(p12).

A pictorial summary of both the clonal and non-clonal rearrangements revealed by SKY analysis is shown in Fig. 1a,b. The non-clonal structural aberrations seen in MF8 and MF18 are displayed in (c) and (d). As many

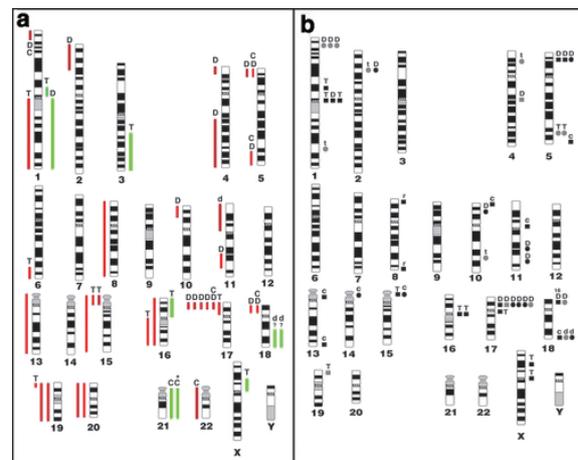


Figure 2. Summary of chromosome losses, gains and breakpoints in MF patients. Both figures present a human ideogram with a summary of cytogenetic analysis of four MF patients using SKY (spectral karyotyping). (a) Both structural and numerical aberrations result in loss and gains of entire chromosomes or chromosome regions. The aberrations which are clonal are identified by the **C**. Gains are shown as a green bar to the right of the chromosomes and losses are shown as a red bar to the left. **D** refers to regions lost by deletions; **d** refers to regions lost or gained via duplications; **T** refers to regions lost or gained as a result of translocations. *Gain of chromosome 21 found in MF8 in 2001. (b) The chromosome breakpoints determined by SKY analysis for the four patients are shown, using the same key as described in (a). Each individual is indicated by a different symbol. MF8 is depicted as a black square, MF19 as a grey square, MF18 as a grey circle and MF31 as a black circle.

Table 2. Summary of SKY karyotypes for MF patients for 1999 and 2001

Patient code	Karyotypes for 1999	Karyotypes for 2001 and 2005 (controls)
MF8	46,XX (1) ^a 47,XX,+21(5) 46,XX,-20,+21 (1) 45,XX,-19,-20,+21 (1) 46,XX,-17,+21 (1) 56,XX,+4,+4,dic(5;11)(q35;q11)+6,+7, +9,-11,+13,+14,-15,+16,del(17)(p12), +19,+20, +21 (1) 46,XX,tas(10;13)(pter;qter),+21 (1) 48,XX,+der(1;X)(p10;q10)del(1)(p13) del(X)(p21) (1) 41,XX,del(5)(p15.1),-6,-13, dic(13;18)(p12;q23),der(15;17) (q10;q10), -16,-17, -22,+21 (1) 44,XX,del(5)(p15.1),-19,-22 (1) 43,XX,r(8)(p23q24.3),-15,-16,-22 (1)	45,XX,-19 (1) 47,XX,+21 (4) 46,XX,del(18)(p11.2) (13) 48,XX,+1,del(1)(q11),+del(16)(p12) ^b , del(18)(p11.2) (1) 45,XX, der(1;16)(q10;p10),-16,del(18)(p11.2)(1) 47,XX, pulv(1p) ^c ,del(18)(p11.2), +22, (1) 40,XX,-3,-6,-7,-17,del(18)(p11.2),-20 (1)
MF18	46,XY (8) 45,XY,-2 (1) 46,XY,t(1;2)(q32;p21),t(4;10) (p15.2;q24) (1)	46,XY (10) 46,XY,del(1)(p36.1) (2) 44,XY,-21 (1) 46,XY,der(5)t(1;5)(p36.1;q33) (1) 45,XY,del(1)(p36.1?),der(5)t(1;5) (p36.1;q33),-11 (1) 46,XY,dup(18) (q23q?) (1) 47,XY,der(6)t(6;12)(q25;?),+13 (1) 45,XY,der(14)t(14;18)(p11.2;q11.2),-18, (1) 47,XY,del(12)(p11.2),+der(12)t(3;12)(q21;q12) (1) 46,XY,del(4)(p16),inv(13)(q32q14) (1) 46,XY,del(17)(p12),del(18)(p11.2) (1)
MF19	46,XX (16) 47,XX,+17 (1) 46,XX,del(4)(q22) (1) 46,XX,del(17)(p12) (1) 46,XX,der(19)t(8;19)(?;p13.3) (1)	NA
MF31	46,XY (11) 45,XY,-11 (1) 45,XY,-20 (1) 42,XY,-18,19,-22 (1) 40,XY,-3,-4,-8,-9,-12,-16,-16 (1) 45,XY,dup(18)(q23q?),-19, (1) 42,XY,-1,del(2)(p721),del(5)(p15.1),-8, -20 (1) 46,XY,del(17)(p12) (2) 46,XY,der(14;15)(q10;q10) (1) 46,XY,del(10)(p13),del(11)(q21q23) (1)	46,XY (19) 46,XY,del(17)(p12) (1)
Control 1		46,XX (15) 46,XX,chtb(6qter),+ace(6) (1) 45,XX,-13,+ace(20) (1) 44,XX,-4,-13 (1) 41,XX,-6,del(8)(q11.2),-17,-18,-22 (1) 37,XX,-3,-4,-7,-9,-10,-13,-14,-15,-22 (1)
Control 2		46,XX (15) 45,X-X (1) 45,XX,-17 (1) 44,XX,-4,-14 (1) 44,XX,-16,-17 (1) 44,XX,-10,-15 (1)

^aTri-radial arrangement.^bPulvarized p arm of chromosome 1.^cNumbers in parentheses indicate number of cells containing aberration.

telomere regions of chromosomes are G-light (unbanded), SKY is especially useful for detecting unbalanced translocation such as the der(5)t(1;5)(p36.1;q33) seen in MF18. The chromosome regions gained and/or lost as a consequence of numerical aberrations and/or structural rearrangements have been displayed as coloured bars alongside an ideogram of human chromosomes as presented in Fig. 2. In (a) the clonal and non-clonal gains are shown as green bars, and losses of chromosomal regions as red. In Fig. 2b the breakpoints of all structural aberrations found in the CTCL patients are shown. Although many of the breakpoints are non-clonal with respect to each individual patient, the structural aberrations found by SKY yielded recurrent breakpoints and imbalances of chromosome regions among the four patients. These included 5pter->5p15.1, 17pter->17p12 and 18pter->18p11.2.

Discussion

This cytogenetic analysis as revealed by SKY shows that T-skin-homing lymphocytes from patients with CTCL have a heterogeneous pattern of chromosomal aberrations in early-stage CTCL. Second, chromosomal aberrations are observed in patients with early stages of CTCL even though the disease has lasted for approximately 20 years (MF8; Table 1). Third, in one patient (MF8) we observed a recurring clone (47,XX,+21) first in 1999 and then in 2001. Two other clones were also found in MF8, one in 1999 containing loss of chromosome 22 (3/20 cells), and in 2001 a new clone with a del(18)(p11.2) (17/20 cells). Fourth, within the individual patients, few recurrent structural aberrations were retained over 2 years. Trisomy 21 has been shown as a recurrent numerical aberration in three patients with Sézary syndrome (22). Interestingly, in one patient, in our study, MF31, we found one cell with der(14;15)(q10;q10). This translocation has been reported as a recurrent aberration in two MF/SS patients (22). Although three deletions, del(5)(p15.1), del(17)(p12) and del(18)(p11.2) were non-clonal for MF8, MF18 and MF19, they were recurrent for two, four and two patients, respectively, as shown in Table 2. Only del(17)(p12) was found in all four patients and was present in both 1999 and 2001 for patient MF31, reinforcing the hypothesis that this region may contain genes (e.g. *TP53*) relevant to tumor progression in CTCL. Lastly, our study demonstrates that treatment followed by remission can lead to the disappearance of diseased cells (MF31; Tables 1 and 2). We cultured our cells in vitro for approximately 3 weeks using the T-cell growth factors IL-2 and IL-4 where approximately nine to 12 cell doublings took place to get an outgrowth of cells. We have observed a similar outgrowth of cells from non-malignant skin diseases as atopic dermatitis and psoriasis (23). When studying cell lines from atopic dermatitis and psoriasis we did

not find either evidence of genetic instability or presence of abnormal clones (Table 2).

The high degree of variability of the chromosomal changes in the CTCL patients as revealed by SKY is indicative of a general genetic instability within the T lymphocytes. Previous studies have documented chromosomal changes in freshly isolated and PHA-stimulated blood lymphocytes (3–8,24). However, our results demonstrate the utility of using molecular cytogenetic methods such as SKY to reveal recurrent aberrations such as del(5)(p15), del(17)(p12), del(18)(p11.3) and dup(18)(q23) during progression of MF not previously described by other investigators in skin-homing T cells which may prove of value in further defining the progression of CTCL.

The conclusions from our observations are that we are able to confirm previous findings of karyotypic abnormalities in CTCL and that these changes do not follow a consistent pattern. More importantly, different clones in the same patient are often present, and chromosomal changes are occurring in very early stages of CTCL. This finding substantiates our proposal that the common denominator for T lymphocytes in CTCL is the presence of an inherent genetic instability in these cells possibly linked to an inhibition of DNA repair mechanisms (13). Therefore, future studies should focus on genes and mechanisms of importance for DNA repair.

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