Clinical significance of T-cell clonality in mycosis fungoides and other cutaneous T-cell lymphomas

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Prognostic relevance of circulating clonal T-cells in CTCL

Chapter 3 - Prognostic relevance of circulating clonal T-cells in CTCL

3.1

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3.1 Peripheral blood T-cell clonality in mycosis fungoides – An independent prognostic marker?

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J Invest Dermatol. 2000; 115: 504-5

To the Editor.

Mycosis fungoides (MF) represents the prototype of cutaneous T-cell lymphoma which is defined as clonal expansion of skin-homing T-lymphocytes.[1] By PCR analysis up to 90% of MF skin biopsy samples can be demonstrated to carry clonal T-cells.[2] More recently, clonal T-cells corresponding to the cutaneous T-cell clone have also been detected in the peripheral blood of up to 40% of patients with early stage MF and of nearly all patients with advanced MF.[3,4,5] These data were controversially discussed to reflect either an enlarged total body tumor burden and a poor prognosis or the physiological and stage independent recirculation of the neoplastic cells via lymph nodes and peripheral blood to the skin.

The lack of data regarding the prognostic significance of circulating clonal T-cells in MF has recently been filled by the publication of Fraser-Andrews et al.[6] in this journal. After confirming the occurrence of circulating clonal T-cells corresponding to the cutaneous tumor clone in all MF stages, the authors correlated the results of the TCR gene analysis with the clinical outcome in 48 patients (24 T1/2, 24 T3) using proportional hazard analysis. Thereby, analysis of peripheral blood clonality by PCR was found to provide independent prognostic information on multivariate analysis.

Unfortunately, no data were given regarding the survival or time to progression in stage of the reported MF patients with or without peripheral blood T-cell clonality. Survival analysis was performed from the date of diagnosis but not from the date of first occurrence of circulating clonal T-cells. Furthermore, peripheral blood T-cell clonality is found in up to 40% of early stage MF but only 14% of these cases develop a large cell-transformation[7] and long-term survival of early stage MF reaches that of the normal population.[8,9] Therefore, the detection of circulating clonal T-cells is not likely to represent a stage independent indicator of a worse prognosis. Accordingly, Laetsch et al.[10] reported in this journal that the clinical course of patients with demonstrable blood involvement did not differ from PCR-negative cases.

We, herewith, report on 67 stage I-II MF patients with PCR detectable circulating clonal T-cells in 58% of the cases (table 1). For the investigation, a TCRγ PCR with subsequent heteroduplex-loaded temperature gradient gel electrophoresis was applied.[5] Since PCR assessment of peripheral blood T-cell clonality is applied routinely for a few years only, peripheral blood T-cell clonality was correlated with the time from first occurrence of circulating clonal T-cells to progression in stage instead of to patient death (table 2). By comparing the frequency of PCR-positive cases within the progressing and non-progressing group, significant differences between both groups were found neither after 2 months nor after 1, 2, 3 and 4 years of observation. Longitudinal investigation by a clone specific PCR detected the clonal T-cells in all blood samples of a MF patient although the TCRγ PCR/ heteroduplex-loaded temperature gradient gel electrophoresis failed in 3/6 samples. Failure of routine PCR correlated to the intensity of the bands indicating variable frequencies of circulating clonal T-cells during the course of the disease.

In conclusion, our data on 67 stage I-II MF patients do not support the findings of Fraser-Andrews et al.[6]. The occurrence of circulating clonal T-cells from the beginning of the disease reflects more likely a recirculation via lymph nodes and peripheral blood to the skin which is typical for skin-homing T-cells[11] and their neoplastic counterparts. The failure to detect circulating clonal T-cells in all MF cases is rather a problem of sensitivity than a prognostic marker and the increase in the

frequency of PCR-positive cases, with the course of the disease, might indicate an increase in the number of circulating clonal T-cells in more advanced stages. Accordingly, a somewhat lower sensitivity of the method of Fraser-Andrews et al.[6] might be assumed, which enables detection of peripheral blood T-cell clonality in advancing cases only. Quantification of circulating clonal T-cells might help to determine the amount of neoplastic T-cells in the peripheral blood which has a prognostic significance.

MF Stage	**Total**	**Progression in stage**	**T-cell clonality peripheral blood**		**T-cell clonality skin**	
			mc[a]	**pc[b]**	**mc[a]**	**pc[b]**
T1N0M0	18	1	11	7	12	6
T2N0M0	41	8	25	16	31	10
T2N1M0	4	0	2	2	2	2
T3N0M0	4	2	1	3	3	1
Total	67	11	39	28	48	19

Table 1. MF-patients, stage, and T-cell clonality. a, monoclonal; b, polyclonal

Observation (months)	**Stage progression**	**T-cell clonality peripheral blood**			**Observation[c] (months)**	**Significance[d] (p)**
		total	**mc[a]**	**pc[b]**		
>1	Y	11	8	3	3-60 (41)	
	N	56	31	25	2-62 (25)	0.286
>12	Y	8	7	1	29-60 (42)	
	N	43	24	19	12-62 (29)	0.092
>24	Y	8	7	1	29-60 (42)	
	N	29	17	12	24-62 (37)	0.130
>36	Y	6	5	1	41-60 (48)	
	N	18	10	8	36-62 (41)	0.224
>48	Y	3	3	0	53-60 (55)	
	N	6	4	2	48-62 (53)	0.257

Table 2. Clinical outcome (progression in stage) of the MF-patients. a, monoclonal; b, polyclonal ; c, observation/ time to stage progression; d, significance as determined by chisquare test.

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3.2 Peripheral Blood T-cell Clonality in mycosis Fungoides and Non-Lymphoma Controls.

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Diagn Mol Pathol. 2003; 12: 142-50

Abstract.

In mycosis fungoides (MF), T-cell clonality is reported in about 90% of skin and 40% of blood samples. However, identity of blood and cutaneous T-cell clone and prognostic relevance of blood T-cell clonality remain controversial. By PCR/fluorescence fragment analysis with estimation of clonal fragment lengths and relative peak heights, we objectively identified T-cell clonality unrelated to malignant lymphoproliferation in healthy donors (5/38), autoimmune dermatoses (3/8) and non-lymphoma skin cancer (9/39). This T-cell expansion of undetermined significance (TEXUS) was also found in 8/64 MF patients. Dissemination of neoplastic cells into blood, as identified by identical clonal fragment lengths in blood and skin, was detected in 23/64 MF patients. When monitoring for progression at TNM stage for a mean of 45.7 months, univariate analysis identified age of >60y and detection of a related blood T-cell clone to be of prognostic relevance, whereas, detection of TEXUS, sex, TNM stage at initial diagnosis, and detection of a cutaneous T-cell clone were irrelevant. Although multivariate analysis was not possible, further stratification clearly indicated an age of >60y to be the predominating prognostic factor. In conclusion, investigation of T-cell clonality in skin and blood samples at the initial diagnosis cannot predict the clinical course of MF and the occurrence of TEXUS should be considered when assessing blood T-cell clonality.

Introduction.

Mycosis fungoides (MF) represents the prototype of cutaneous T-cell lymphomas (CTCL) which are defined as a neoplastic proliferation of skin-homing T-lymphocytes[1]. By PCR amplification of T-cell receptor (TCR) rearrangements and subsequent high-resolution electrophoresis, a single expanded (predominant) T-cell clone can be demonstrated in skin biopsy samples of up to 90% of the MF cases[2,3,4] and detection of a predominant T-cell clone at the initial diagnosis has been found to be an independent negative predictive marker of treatment response in MF[5]. Since, with respect to the current pathogenetic model of lymphoproliferative diseases, the T-cell clone detected by PCR is generally assumed to represent the malignant T-cells, PCR methods have been applied for the detection of clonal, i.e. neoplastic T-cells in extracutaneous sites of MF patients.

Regarding the peripheral blood, a predominant T-cell expansion has been found in 46-57% of the investigated MF cases[6,7,8]. However, the identity of the circulating T-cell clone to the cutaneous tumor and the relevance of its detection to the prognosis of MF patients remain controversial.

Clonal T-cell expansions have also been found within subsets of peripheral blood T-lymphocytes in non-malignant conditions, e.g. in Vβ subsets derived from peripheral T-cells of healthy individuals[9,10,11,12,13,14] and patients suffering from autoimmune diseases[15,16,17]. Here, the accumulation of expanded T-cell clones was found to be age-dependent and started earlier, as well as being more pronounced in the CD8 positive fraction than in CD4 positive cells[14]. With regard to MF, Delfau-Larue and colleagues[18], after detecting circulating clonal T-cells in 40/88 patients by TCRγ-PCR and denaturing gradient gel electrophoresis, reported 29/40 peripheral blood T-cell clones to differ from the cutaneous tumor. In another study, which detected clonal T-cells in the peripheral blood of 33/67 MF patients by means of the same approach, T-cell clones were distinct from the skin tumor in 16/33 cases[19]. In contrast, and with the use of TCRγ-PCR with temperature gradient gel electrophoresis, we detected circulating T-cell clones in 26/45 MF patients and skin and blood clones differed in only one case. Out of five cases, in which the clonal TCRγ junctional region had been sequenced, four revealed identical sequences in both compartments[7]. Dippel et al.[20], by applying TCRγ-PCR in combination with fluorescence fragment analysis (FFA), were even not able to detect non-identical peripheral blood T-cell clones in their cohort of 19 CTCL patients.

Already in 1992, the presence of a peripheral blood T-cell clone as detected by southern blot analysis has been associated with a rapidly fatal disease in MF patients with lymph node involvement[21]. More recently, the detection of circulating clonal T-cells has been associated with a worse prognosis in MF by Fraser-Andrews et al.[6], using southern blot and TCRγ-PCR with subsequent single strand conformational polymorphism analysis in 66 MF patients, and by Beylot-Barry et al.[19], applying TCRγ-PCR and denaturing gradient gel electrophoresis in 67 cases. However, our group[22] could not confirm such correlation when investigating samples of 67 MF patients with TCRγ-PCR and temperature gradient gel electrophoresis. Accordingly, Laetsch et al.[23], after analyzing 51 CTCL patients with TCRγ-PCR and denaturing gradient gel electrophoresis, reported that the clinical course of patients with demonstrable blood involvement did not differ from PCR-negative cases.

Controversies in the above-cited studies are caused by the following facts: Determination of T-cell clonality by assessing the migration pattern in gradient gels is very subjective. Estimation of the

identity of T-cell clones found in different samples by comparing the position of clonal band(s) in such gels is even more prone to errors. Different outcome criteria for the investigation of the prognostic relevance of peripheral blood T-cell clonality were chosen, i.e. survival[6], progression at TNM stage[22], correlation to TNM stage[23] and response to treatment[19]. Cohorts with differently distributed MF stages were analyzed: Unlike stage T1, which is unlikely to progress[24], and stage 3/4, which is likely to progress rapidly, stage T2 MF has the most variable prognosis and, therefore, would benefit most of all from additional prognostic markers.

In order to solve the controversies, a technique is required which allows for an objective determination of T-cell clonality, as well as for a simple and objective comparison of T-cell clones detected in different samples. We are reporting here on the first results of an extended study that has utilized FFA to objectively compare composition and length of the clonal TCRγ junctional region in blood and skin samples taken from 72 MF patients at the time of initial diagnosis, and from 85 controls derived from healthy volunteers and patients suffering from autoimmune dermatoses (AID) or non-lymphoma skin cancer (NLSC). Progression at TNM stage was chosen to address the prognostic relevance of peripheral blood T-cell clonality in our T2 predominated MF cohort.

Methods.

MF patients. From all consecutive patients diagnosed with MF between January 1995 and June 2000 at the Department of Dermatology and Allergy of the Charité Berlin, blood and skin samples were prospectively obtained at the time of initial diagnosis and after written informed consent. Thirty-five patients were female and 37 male, the median age at diagnosis was calculated at 64y (females 68; males 61; 27---85y). The diagnosis of MF was based according to the EORTC classification for primary cutaneous lymphomas[1] and patients were staged in accordance with the TNM system[25] as T1N0M0 (n=20), T2N0M0 (n=44), T2N1M0 (n=4), T3N0M0 (n=4). After initial diagnosis, all patients received standard therapies (PUVA, PUVA+IFNα, local irradiation), were monitored for progression at TNM stage for a mean of 45.7 months (7---81 months) and grouped as progressive and non-progressive.

Controls. Blood samples from 85 individuals served as controls. They were grouped as healthy/young (n=18; female/male=9/9; age ≤60y, mean age 36y), healthy/old (n=20; female/male=16/4; age >60y, mean age 87y), NLSC (n=39; female/male=15/24, age >60, mean age 71y), and AID (n=8; female/male=7/1; age >60, mean age 70y). With the exception of type 2 diabetes and senile dementia, individuals in the healthy groups had no signs of ongoing disease after clinical investigation and medical history. All patients in the NLSC group presented with either untreated basal or squamous cell carcinoma or untreated melanoma stage IA-IIC in accordance with the American Joint Committee on Cancer[26]. In these patients, corresponding skin samples were available for investigation. Patients of the AID group suffered from ongoing lupus erythematodes, sclerodermia or lichen sclerosus et atrophicus.

The cell lines JM (rearranged Vγ8 and Vγ11) and PEER (rearranged Vγ9), as well as peripheral blood of patient Ra suffering from γδ+ T-cell acute lymphatic leukemia (Vγ10 positive), served as positive controls.

Sample preparation and TCRγ-PCR. Peripheral blood mononuclear cells (PBMC) were prepared from 10 mL of heparinized blood by density gradient centrifugation through Ficoll-HyPaque (Pharmacia, Freiburg, Germany). Genomic DNA was prepared from about $1x10^6$ cells by a standard procedure using proteinase K digestion. For preparation of genomic DNA from the paraffin embedded skin specimens, the paraffin of 10 sections per sample (10 µm each) was dissolved with xylene. After centrifugation, the pellet was washed with ethanol and also digested by proteinase K.

To amplify the TCRγ rearrangements, 2 consecutive PCR were performed as described previously[7]: PCR-g1 using the consensus primers VG1 (5'CTACATCCACTGGTACCT) and JG1/2 (5'CAACAAGTGTTGTTCCAC, 5' labeled with 5-carboxy-fluorescein), annealing at the Vγ1-8 and Jγ1/2 segments, respectively, and PCR-g2 with primers for the Vγ9, Vγ10, Vγ11 and the Jγ1/2 segments (VG9: 5'ATTGGTATCGAGAGAGAC, VG10/11: 5'CACTGGTACKKGCAGAAAC, JG1/2).

Determination of T-cell clonality. Labeled PCR products were subjected to FFA on the ABI 310 PRISM CE sequencer (PE Applied Biosystems, Weiterstadt, Germany). Before separation, 12 µl deionized formamide and 0.5 µl Genescan 500^{TM} ROX internal lane standard (PE Applied Biosystems, Weiterstadt, Germany) were added to 1µl of the PCR amplificate, the mixture was denatured at 90°C for 2 minutes and chilled on ice. Each run was performed at 60°C and 15 kV with a 5s injection and 36 min separation time in a 47 cm POP 6-filled capillary (PE Applied Biosystems, Weiterstadt, Germany).

Runs were analyzed using the GeneScan software (PE Applied Biosystems, Weiterstadt, Germany). To determine the clonality of a PCR product, peak-height ratios (PHR) were calculated as h1/h2, whereby, h1 represents the peak height of the largest peak above the fragment profile, and h2 represents the mean height of the two peaks surrounding the largest peak[27]. FFA analysis was assessed clonal in the case of 1 to 2 peaks yielding a PHR >2 in the fragment profiles of PCR-g1 and/or -g2, questionable clonal in the case of 1 to 2 peaks with 1.5<PHR≤2, oligoclonal in the case of a non-Gaussian distribution of the peaks, and non-clonal in the case of a Gaussian distribution of the peaks. Fragment lengths corresponding to the predominating peaks (clonal fragment lengths) were recorded for the comparison between blood and skin samples, as well as for control of the FFA validity.

Statistical analysis. FFA outcome in the different groups was investigated by Fisher's exact test. Kaplan-Meier curves were used to examine the prognostic relevance of age, sex, peripheral blood T-cell clonality, and skin T-cell clonality. The time since initial diagnosis was defined as a time scale. Differences between the curves (exposure to prognostic factor or no exposure to prognostic factor) were assessed using the log rank test. As our statistical power was limited, no multivariate analysis could be performed.

Results.

FFA succeeded in blood specimens of 64 MF patients and in all control blood samples. Specimens were investigated twice and, in the case of predominating peaks, fragment lengths were used to

control FFA reproducibility. Peaks with PHR higher than 1.5 were always reproducible in our series (data not shown). Application of a PHR higher than 2 as criterion for clonality led to an analytic sensitivity of the approach of 1-3%, when dilutions of Jurkat or Molt-16 cells in polyclonal PBMC were used[27]. Fragment lengths of clonal skin and blood specimens were compared in MF and NLSC patients. Peripheral blood T-cell clones were classified as being identical to the cutaneous clone in case of one/ two peak(s) matching the clonal fragment length(s) of the skin sample and showing PHR higher than 1.5. Non-related peripheral blood T-cell clonality was assumed in case of different clonal fragment lengths in blood and skin. T-cell clonality, detected in the remaining control samples (AID and healthy volunteers), was generally classified as non-related (to malignant lymphoproliferation).

Peripheral blood T-cell clonality in MF and controls. Non-related peripheral blood T-cell clonality was detectable in 25/149 samples (17%). Its frequency was higher at >60y of age (22/109 [20%] vs. 3/40 [8%]), but statistical significance of this difference was not found (p=0.084). Concerning the distinct groups analysed (table 1), the lowest frequencies were observed in healthy volunteers and MF patients (5/38 [13%] and 8/64 [12%], respectively). Analysis of the NLSC and the AID groups identified considerably more peripheral blood samples as being clonal (38% and 23%, respectively). However, statistical analysis did not reveal significant differences between the distinct groups and healthy volunteers (MF, p=1; NLSC, p=0.38; AID, p=0.13) Interestingly, even the subgroups of healthy volunteers (≤60y and >60y of age) and the different stages of MF (T1N0M0, T2N0M0, T2N1M0, T3N0M0) were not found to bear significantly different frequencies of non-related peripheral blood T-cell clonality.

Questionable clonal fragment profiles (PHR 1.5-2) were identified in 7 out of 85 control specimens and in none of the MF samples, whereas, oligoclonal profiles were detected in 8 out of 85 controls and in one out of 64 MF blood samples. All patients revealing oligoclonal FFA outcomes were older than 60y.

Group	FFA outcome blood samples[a]									
	n	monoclonal[b]		monoclonal-x[c]		oligoclonal		Polyclonal		
Healthy (total)	38	nd		5	(13)	3	(8)	30	(79)	
Healthy (≤ 60y)	*18*	*nd*		*2*	*(11)*	*0*		*16*	*(89)*	
Healthy (> 60y)	*20*	*nd*		*3*	*(15)*	*3*	*(15)*	*14*	*(70)*	
Autoimmunity	8	nd		3	(38)	1	(12)	4	(50)	
Neoplasia	39	0	(0)	9	(23)	4	(10)	26	(67)	
MF	64	23	(36)	8	(12)	1	(2)	32	(50)	
T1N0M0	*18*	*6*	*(33)*	*3*	*(17)*	*0*		*9*	*(50)*	
T2N0M0	*39*	*16*	*(41)*	*4*	*(10)*	*1*	*(3)*	*18*	*(46)*	
T2N1M0	*4*			*1*	*(25)*	*0*		*3*	*(75)*	
T3N0M0	*3*	*1*	*(33)*	*0*		*0*		*2*	*(67)*	

Table 1. T-cell clonality of peripheral blood samples. [a] percentages in brackets; [b] not determined (nd) in healthy volunteers and autoimmune dermatoses; [c] peripheral blood T-cell clone not identical with skin T-cell clone.

Correlation of T-cell clones in corresponding skin and blood specimens. In MF and NLSC, T-cell clonality in blood and skin could be correlated (table 2). In MF patients, 47 (73%) skin samples were clonal, 16 (25%) non-clonal, and one (2%) oligoclonal. Blood T-cell clones related to the cutaneous clone were found in 23 out of 64 MF patients (36%). Significant differences regarding the TNM stage were not observed, but stages T2N1M0 and T3N0M0 included only 4 and 3 patients, respectively.

Out of the 41 remaining MF patients, 20 revealed clonal fragment profiles exclusively in the skin (including one case with oligoclonal blood sample), 13 were non-clonal in skin and blood (including one case with oligoclonal skin sample), 4 showed different T-cell clones in skin and blood, and 4 were found clonal exclusively in blood.

Of the NLSC patients, 4 (10%) skin samples were clonal and 2 (5%) questionably clonal, but none of these T-cell clones was identified in the corresponding blood sample. The remaining 33 specimens were either non-clonal (n=22, 57%) or not amplifiable by PCR-g1/2 despite successful DNA extraction as proved by amplification of the β globin gene (n=11, 28%).

		FFA outcome[a]					
	skin	monoclonal				non-clonal[b]	
	blood	monoclonal	monoclonal-x[c]	non-clonal[b]		monoclonal-x[c]	non-clonal[b]
Group	n						
MF	64	23 (36)	4 (6)	20 (32)		4 (6)	13 (20)
T1N0M0	*18*	*6*	*2*	*6*		*1*	*3*
T2N0M0	*39*	*16*	*2*	*10*		*2*	*9*
T2N1M0	*4*	*-*	*-*	*2*		*1*	*1*
T3N0M0	*3*	*1*	*-*	*2*		*-*	*-*
NLSC[d]	39	0 (0)	2 (5)	4 (10)		7 (18)	26 (67)

Table 2. T-cell clonality of corresponding skin and peripheral blood samples. [a] percentages in brackets; [b] includes questionable monoclonal, oligoclonal, polyclonal; [c] peripheral blood T-cell clone not related to skin T-cell clone; [d] T-cell receptor genes could not be amplified in 11/39 skin samples despite sufficient DNA quality.

Vγ repertoire in peripheral blood T-cell clones. To identify putative differences between related and non-related peripheral blood T-cell clonality, the distribution of clonal fragment profiles with regard to the Vγ families was analyzed (table 3). Surprisingly, differences were not observed when stratifying for related and non-related peripheral blood T-cell clonality, but when differentiating between MF and control samples. In the controls, PCR-g1 amplifying the VγI rearrangements and PCR-g2 amplifying the VγII-IV rearrangements, revealed clonal fragment profiles in a comparable portion (53% vs. 88%), whereas, in MF patients, remarkably more clonal fragment profiles were found after PCR-g1 (87% vs. 16%).

Group		Detection of T-cell monoclonality (%) in	
	n	VγI[a]	VγII-IV[a]
Controls	17	53	88
Healthy	*5*	*40*	*100*
Autoimmunity	*3*	*67*	*100*
Neoplasia	*9*	*56*	*78*
MF	31	87	16
MF related	*23*	*91*	*13*
MF non-related	*8*	*75*	*25*

Table 3. Vγ repertoire of peripheral blood T-cell clones. [a] VγI is represented by PCR-g1, VγII-IV by PCR-g2, a FFA peak may be detected in both PCR (biallelic rearrangement).

Factors predicting progression at TNM stage. Out of the 64 MF patients with successful PCR analysis, TNM progression occurred in 12 patients: 3 patients with initial T1N0M0 disease (towards T2N0M0, n=2; T3N0M0, n=1), 8 with initial T2N0M0 (T3N0M0, n=4; T4N0M0, n=2; T2N3M0, n=1; T3N3M1, n=1), and one with initial T3N0M0 (T3N3M0).

In univariate Kaplan-Meier analysis, two factors were significantly associated with progression at TNM stage: age of >60y (p=0.01), and detection of a peripheral blood T-cell clone related to the cutaneous clone (p=0.007). Concerning the stage T2 patients, 8/43 advanced in TNM stage. Again, age of older than 60y (p=0.01), and detection of related peripheral blood clonality (p=0.01) were significantly associated with progression at TNM stage in univariate Kaplan-Meier analysis. In both groups, MF and MF T2, no significant association with TNM progression was found for sex, TNM stage at initial diagnosis, detection of a cutaneous T-cell clone, and detection of peripheral blood T-cell clonality not related to the skin.

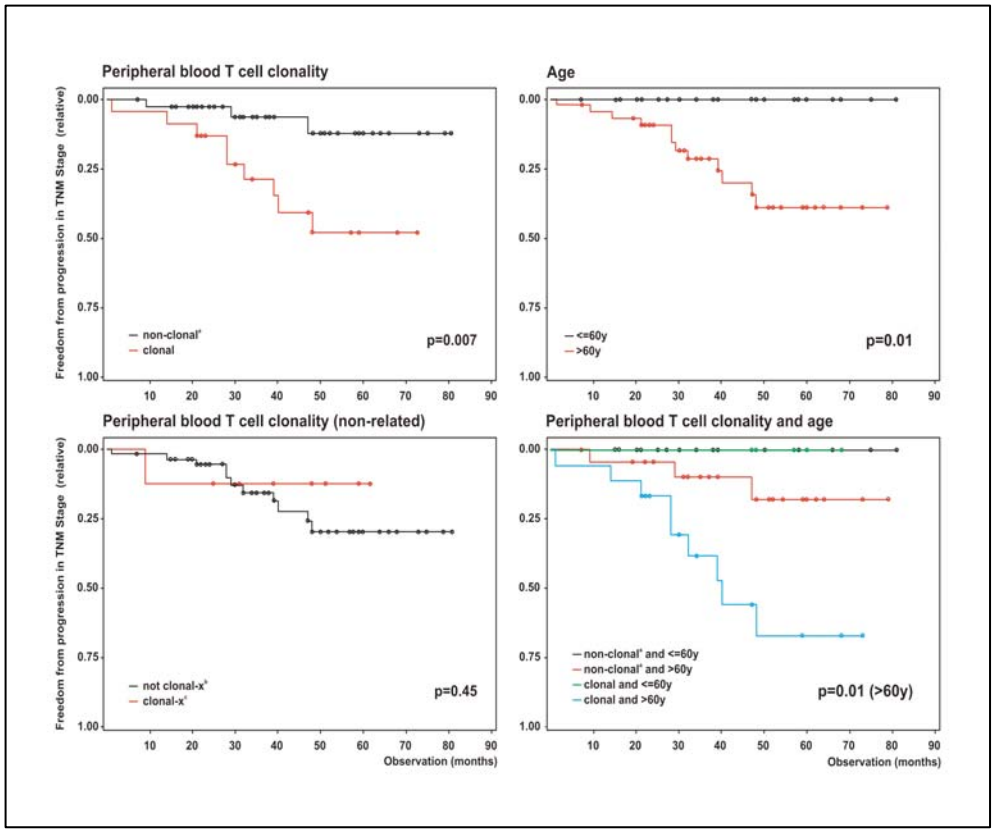

Figure 1. Factors predicting progression at TNM stage in the MF group. Univariate Kaplan-Meier analysis revealed age of >60y (p=0.01), and detection of a peripheral blood T-cell clone related to the cutaneous clone (p=0.007), but not detection of a non-related peripheral blood T-cell clone (TEXUS, p=0.45) to be of prognostic relevance. When stratifying for age and peripheral blood T-cell clonality, all patients at the age of ≤60y did not progress at TNM stage independently from the state of peripheral blood T-cell clonality, whereas, patients at the age of >60y were at significantly higher risk of TNM progression when peripheral blood T-cell clonality was detected (p=0.01). [a] includes non-clonal, oligoclonal, questionable clonal and non-related clonal; [b] includes non-clonal, oligoclonal, questionable clonal and clonal; [c] includes non-related clonal.

Since the proportion of patients with stage progression was only 19% in the MF and the MF T2 group, multivariate analysis of the prognostic relevance of age and peripheral blood T-cell clonality related to the skin was not possible. However, when stratifying for age and peripheral blood T-cell clonality, all patients at the age of ≤60y did not progress at TNM stage independent of the state of peripheral blood T-cell clonality, whereas, in the group of >60y of age, patients with peripheral blood T-cell clonality were at a significantly higher risk of TNM stage progression (p=0.01; figure 1).

Discussion.

PCR of TCRγ rearrangements has become a standard procedure for the detection of predominant T-cell clones and an essential tool in the diagnosis of T-cell lymphoma, such as CTCL. However, the limited heterogeneity of TCRγ-PCR products requires high-resolution electrophoresis for the differentiation between non-clonal and clonal samples. So far, gradient gels, which separate PCR products according to their length and base sequence, were most frequently applied[3,7,8]. However, determination of T-cell clonality and of the identity of T-cell clones found in different samples by this technique is very subjective since it depends on the assessment of shape and position of the bands in the gradient gel.

FFA, which was applied in the present study, overcomes this problem. It allows for an objective determination of clonality by calculating the peak height ratio[27]. Each T-cell clone detected by FFA constitutes at least 1-3% of the T-cells present in the investigated sample. Moreover, FFA provides a one base pair exact size assessment of each DNA amplicon which defines a detected T-cell clone by means of its fragment length. This definition is even more specific in (frequently observed) cases of biallelic TCRγ rearrangement. Determination of clonal fragment lengths enables the comparison of T-cell clones found in various samples of a given patient without sequencing, whereas, the possibility of false-positive results, i.e. the detection of pseudoclonality defined as varying clonal fragment lengths in repeated analyses of a given sample[20,28] is substantially reduced.

However, even objectively assessed T-cell clonality does not equate with malignancy. Regarding peripheral blood of healthy individuals, the occurrence of clonal T-cell expansions in distinct Vβ subsets is known to be a common feature in persons at >65y of age, to be age-dependent and to start earlier, as well as being more pronounced in the CD8 positive fraction than in CD4 positive cells [10,12,14,29]. But data regarding frequency and nature of T-cell clones predominating the entire peripheral blood T-cell population are rare.

In the present study, we have identified peripheral blood T-cell clones, which were not related to a malignant lymphoproliferation, in 25/149 specimens at a frequency of 12% in MF, 13% in healthy donors, 23% in NLSC, and 38% in AID. Since their detection was independent of the donor's age, an age-related accumulation of antigen-driven clones, as suspected for the T-cell expansions detected in Vβ subsets[14] remains unlikely or at least cannot be demonstrated for the T-cell clone predominant in the blood. Malignant lymphoproliferation or the occurrence of reactive peripheral blood T-cell clones, i.e. in the case of retroviral infections or idiopathic CD4 lymphopenia, were also excluded in the control samples.

In AID, the predominant peripheral blood T-cell clones may be disease related, since T-cell clonality of Vβ subsets has been associated with disease activity in particular in systemic lupus erythematodes, and some of the T-cell clones identified in peripheral blood were also found in pleural or pericardial effusions of patients with lupus serositis[16,30,31]. In the NLSC group, investigation of paired skin and blood samples clearly excluded linkage of the circulating clones to tumor infiltrating T-cells in all 39 cases analyzed. Moreover, circulating tumor specific T-cells are expected to represent less than 0.1% of peripheral blood lymphocytes[32] and, therefore, would not have been detected by FFA. More likely, the identified T-cell clones may be the sign of a restricted immune repertoire responsible for cancer development or of an immune suppression caused by the malignancy.

Surprisingly, the Vγ usage in the clonal rearrangements differed completely in our groups. Whereas, at least one TCRγ allele of the predominant T-cell clone rearranges VγII-IV in healthy controls and AID patients, the usage of VγII-IV was slightly reduced in the NLSC patients and decreased dramatically in MF patients. In the latter group, VγI rearrangements predominated as observed for the peripheral blood T-cell clones related to the cutaneous clone. This finding is even more remarkable, since Breit et al.[33], analyzing the Vγ repertoire of TCRγδ positive T-cells, found a predominance of VγI in thymocytes and acute T-cell leukemia, whereas, peripheral blood lymphocytes of healthy donors rearranged VγII in the vast majority. Thus, VγII rearranging T-lymphocytes may represent cells of normal physiology, and the decrease of VγII rearranging cells in the NLSC patients may indicate an immune suppression, as discussed above. In contrast, VγI rearranging clonal T-cells may bear the potential of malignant transformation. In this scenario, the detection of VγI rearranging non-related T-cell clones in MF patients supports the concept of genetically instable (genotraumatic) T-lymphocytes as MF precursors, which accumulate genetic alterations until one of the clones finally undergoes malignant transformation[34].

In summary, the nature of the non-related circulating T-cell clones remains speculative. Functional characterization and investigation of follow-up samples are required to exclude clinically inapparent benign or malignant T-cell expansions and to identify the (patho)physiology behind this clonal T-cell expansion. So far, we suggest the term T-cell Expansion of Undetermined Significance (TEXUS) to describe this phenomenon in analogy to monoclonal gammopathy of undetermined significance. Although at a rather low frequency, TEXUS is even found in lymphoma patients and should be considered when assessing the T-cell clonality in peripheral blood samples.

By means of objective determination of clonal fragment lengths, peripheral blood T-cell clones related to the cutaneous clone were identified in 36% of our MF cases. These findings confirm previous data from gradient gels showing such T-cell clones in 23/55 (42%), 11/55 (20%), and 20/40 stage IA-IIB patients[6,7,19], but contradict another study which found related peripheral blood T-cell clones in only 6/69 (9%) of the cases suffering from MF stage IA-IIB[18]. Most probably, these differences are due to methodological issues, i.e. assessment of the identity of clonal rearrangements in a gradient gel.

Nevertheless, the data indicate an early dissemination of the neoplastic T-cell clone into both compartments, skin and blood, as expected from the clinical impression that skin lesions occur multi-focally already at early stages of MF. It remains to be discussed whether the detection of related peripheral blood T-cell clones should be termed systemic disease. In our opinion, it is rather a sign of

the physiological recirculation of skin-homing T-cells and the term systemic disease should be reserved for cases in which the neoplastic cells have lost their affinity to the skin.

By monitoring our MF patients for progression at TNM stage, univariate analysis identified age of >60y and detection of a related peripheral blood T-cell clone to be of prognostic relevance, whereas, detection of non-related peripheral blood T-cell clonality, sex, TNM stage at initial diagnosis, and detection of a cutaneous T-cell clone were irrelevant. Although multivariate analysis was not possible in our cohort, further stratification clearly indicated an age of >60y to be the predominating prognostic factor, since all patients at the age of ≤60y did not progress at TNM stage, irrespective of the state of peripheral blood T-cell clonality. I.e., investigation of T-cell clonality in skin and blood samples at the initial diagnosis of MF cannot predict the clinical course of the disease. These findings also apply for the subgroup of T2 patients, which show the most variable prognosis in MF.

Our data are supported by our previous findings[22], and by the results of Scarisbrick at al.[35] showing a CTCL related death rate of 0.01 for the hematological (H) stages H0 and H1, to which our patients would have been assigned, but appear to contradict earlier data from Fraser-Andrews et al.[6] and Beylot-Barry et al.[19]. These discrepancies are most probably due to imbalances in the TNM stage distribution of the investigated patients and to the application of different outcome criteria. Whereas the portion of stage IA-IIA patients, which show survival rates comparable to the normal population[24], was 37% and 41% in the cited studies[19,6], these patients constituted 96% in our investigation. Consequently, we decided to assess the prognostically relevant progression at TNM stage instead of disease-related survival as the end point of our analysis.

In conclusion, we report here on an extended study that has utilized FFA for objective characterization of T-cell clonality in skin and blood of 149 individuals. The occurrence of predominant T-cell clones in peripheral blood was demonstrated in one-sixth of donors not suffering from malignant or reactive lymphoproliferation. These so-called TEXUS is even found in lymphoma patients and should be considered when assessing the T-cell clonality in peripheral blood. As indicated by demonstration of T-cell clonality, dissemination of the neoplastic cells into skin and blood occurs in a significant percentage of early stage MF, but remains without prognostic relevance in these cases constituting the majority of all MF patients.

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