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

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General discussion

Chapter 6 - General discussion

6.1 Occurrence of circulating clonal T-cells in mycosis fungoides

In the early 1990-ties, several groups applied TCRβ SB techniques to evaluate an extracutaneous spread of malignant T-cells in MF and other CTCL. The majority of these studies detected circulating clonal T-cells only in Sézary syndrome and some cases of advanced MF or pleomorphic CTCL. Therefore, it was postulated that blood involvement is restricted to advanced cutaneous lymphoma and is associated with poorer prognosis, lymph node involvement as well as an enlarged total body tumor burden[1,2,3,4]. In contrast, frequent occurrence of multifocal or diffuse cutaneous MF lesions as well as the recirculational behavior of T-cells[5,6] support the hypothesis of an early occurrence of malignant T-cells in the peripheral blood. As a consequence, a few groups have supposed early hematogeneous involvement in MF[7,8,9,10].

Using a sensitive TCRγ PCR/ HD-TGGE system to investigate a large cohort of well-classified CTCL, we identified circulating clonal T-cells in 45/69 (65%) patients with MF and other CTCL, including high frequencies in MF stage IA (6/13) and IB (15/27)[11]. Other groups, using TCRγ PCR with high-resolution electrophoresis techniques as DGGE[12] and SSCP[13], confirmed the data by showing such T-cell clones in 7/47 (15%) and, respectively, 11/33 (33%) MF stage IA-IIB patients. In a later study, we verified the early occurrence of circulating clonal T-cells by a more objective TCRγ PCR/ FFA assay detecting T-cell clones in peripheral blood in 8/18 (44%) MF stage IA and 19/39 (49%) MF stage IB patients[14].

Data on the analytic sensitivity of SB as well as PCR techniques vary considerably due to the structure of the investigated clonal TCR rearrangement, which influences the enzymatic cleaving in SB and amplification as well as migration pattern in PCR assays. In generally, the analytic sensitivity of TCRγ PCR assays is estimated between 0.5 and 5% clonal T-cells in PBMC/ polyclonal T-cells[10,13,14], TCRβ SB assays reach thresholds of about 2-5%[15]. Despite the comparable analytic sensitivities, PCR approaches have the advantage of an amplification of (all) rearranged TCR genes, which leads to reduction of the non-rearranged background. These technical differences, rather than a pathophysiological event (i.e. transformation of the lymphoma), explain the discrepant outcomes of the studies investigating peripheral blood T-cell clonality in MF. As a consequence, detection of circulating clonal T-cells in MF is probably not related to a poor prognosis (see 6.2).

Although our data indicate an early and frequent occurrence of circulating clonal T-cells in MF, the identity of blood and cutaneous T-cell clone remained controversial. By comparison of gradient gel migration patterns and sequencing of the clonal TCRγ rearrangements, we ensured identity of the circulating with the cutaneous T-cell clone in 14/17 MF stage I patients bearing a peripheral blood T-cell clone[11]. In contrast, Delfau-Larue and colleagues[16], after detecting circulating clonal T-cells in 40/88 patients by TCRγ PCR/ DGGE, 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[12].

Determination of T-cell clonality and of the identity of T-cell clones found in different samples by

DGGE (as well as by TGGE) is very subjective since it depends on the assessment of shape and position of the bands in the gradient gel. FFA, providing peak height ratios and exact size assessment of the (frequently biallelic) clonal TCR rearrangement, objectifies determination of clonality and, thereby, overcomes this problem[17]. Using such a TCRγ PCR/ FFA assay, we identified corresponding T-cell clones in skin and blood of 22/57 MF stage I patients. Peripheral blood T-cell clones, which were not identical with the cutaneous clone, were found in 7/57 patients[14]. Dippel et al.[18], by applying another TCRγ PCR/ FFA assay, were even not able to detect non-identical peripheral blood T-cell clones in their cohort of 19 CTCL patients.

The (objective) FFA data, in connection with our sequencing data[11], 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 corresponding 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. This is in line with the concept of some American colleagues that MF is a lymphoma of the skin-associated lymphoid tissue (SALT): Even when the disease appears confined to the skin clinically, there can be low-level trafficking of tumor cells through the peripheral lymph nodes via the lymphatics and blood vessels[19].

Nevertheless, in a substantial number of MF cases, the peripheral blood T-cell clone differs from the cutaneous clone. Clonal T-cell populations were also detected in peripheral blood of patients suffering from inflammatory dermatoses and autoimmune collagen disorders: In 64% of small plaque parapsoriasis patients[20], in 43% and 37% of cutaneous and systemic lupus erythematosus, respectively, in 75% of generalized morphea, in 75% of localized scleroderma and 60% of CREST syndrome patients[21], and in 34% of systemic sclerosis[22]. Moreover, 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[23,24,25,26].

Using our TCRγ PCR/ FFA assay, we have identified peripheral blood T-cell clones, which were not related to a malignant lymphoproliferation, at a frequency of 12% in MF, 13% in healthy donors, 23% in (non-lymphoma) skin cancer, and 38% in autoimmune collagen disorders. Their occurrence was independent of the donor's age and not related with malignant lymphoproliferation, retroviral infections or idiopathic CD4 lymphopenia. In (non-lymphoma) skin cancer, investigation of paired skin and blood samples clearly excluded linkage of the circulating clones to skin-infiltrating (anti-tumor) T-cells[14].

Surprisingly, clonal rearrangements using the VγII-IV gene segments were much more frequent seen in the control groups of healthy donors, (non-lymphoma) skin cancer and autoimmune collagen disorders than in the MF group[14]. This finding is even more remarkable, since Breit et al.[27], 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, whereas 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[28].

However, the nature of these non-related circulating T-cell clones remains speculative. Therefore, we suggested the term T-cell Expansion of Undetermined Significance (TExUS) to describe this phenomenon in analogy to monoclonal gammopathy of undetermined significance (MGUS)[14]. Although at a rather low frequency, TExUS is even found in (cutaneous) lymphoma patients and should be considered when assessing the T-cell clonality in peripheral blood samples.

6.2 Prognostic relevance of circulating clonal T-cells in mycosis fungoides

Whereas detection of the cutaneous T-cell clone in peripheral blood succeeds in up to 40% of patients with MF stage I[9,10,11], only 14% of MF stage I patients develop a large cell-transformation[29] and long-term survival of early stage MF reaches that of the normal population[30,31]. Therefore, detection of circulating clonal T-cells is unlikely to be a stage independent indicator of a worse prognosis. In contrast, the presence of a peripheral blood T-cell clone as detected by SB analysis has already in 1992 been associated with a rapidly fatal disease in MF patients with lymph node involvement[3]. More recently, the detection of circulating clonal T-cells has been associated with a worse prognosis in MF by Fraser-Andrews et al.[13], using SB and TCRγ PCR/ SSCP in 66 MF patients, and by Beylot-Barry et al.[12], applying TCRγ PCR/ DGGE in 67 cases.

Our group could not confirm such correlation when investigating two different cohorts of MF stage I/II patients by TCRγ PCR/ HD-TGGE (n=67)[32] and, respectively, by TCRγ PCR/ FFA (n=64)[14]. In our first study, peripheral blood T-cell clonality was correlated with the time from first occurrence of circulating clonal T-cells to progression in TNM stage. 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. In the second study, we again monitored MF patients for progression at TNM stage, but applied a more objective TCRγ PCR/ FFA assay. Univariate analysis identified age of >60y and detection of a peripheral blood T-cell clone identical to the cutaneous clone to be of prognostic relevance. Detection of peripheral blood T-cell clonality non-related to the cutaneous clone, 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.

Laetsch et al.[33], reporting that the clinical course of 51 patients with demonstrable blood involvement did not differ from PCR negative cases, support our data. Even in erythrodermic MF and Sézary syndrome, a disease related death rate of 0.01 for the hematological (H) stages H0 and H1, to which our patients would have been assigned, was found by Scarisbrick at al.[34].

The controversial results of the above-cited studies regarding the prognostic relevance of circulating clonal T-cells in MF are caused by the following problems:

1) Cohorts with differently distributed MF stages were analyzed.

In MF, stage T1 is unlikely to progress[30] and stage T3/4 is likely to progress rapidly[35]. Stage T2 patients represent the largest group of MF patients and have the most variable prognosis. Consequently stage T1/2 patients would benefit most of all from additional prognostic markers and, therefore, constituted 94% and 96% of the MF cohort analyzed by us. In contrast, they represented 37% of the CTCL patients in the study of Beylot-Barry et al.[12], and 41% in the analyis of Fraser-Andrews et al.[13], which leads to a predominance of (rapidly progressing) T3/4 patients in both studies.

2) Application of different outcome criteria for the investigation of the prognostic relevance of peripheral blood T-cell clonality, i.e. survival[13], progression at TNM stage[32], correlation to TNM stage[33] and response to treatment[12].

Disease-related survival represents the most relevant outcome criterion in prognostic studies on cancer and was applied by Fraser-Andrews et al.[13]. Since the majority of our MF patients suffered from stage T1/2 disease with rather long or variable survival, we decided to assess progression at TNM stage instead of disease-related survival as the end point of our analysis. Furthermore, we exclusively analyzed blood specimens taken at the initial diagnosis. Response to treatment, as used by Beylot-Barry et al.[12], should rather be avoided because it is not standardized at present and does not represent an independent outcome criterion.

3) Technical issues resulting in subjective determination of T-cell clonality and/or of the identity of T-cell clones found in blood and diagnostic skin sample.

Until now, TCR PCR/ FFA, providing exact fragment lengths and a peak height ratio[17], represents the most objective approach to determine T-cell clonality and to judge identity of T-cell clones found in different samples. Consequently, this approach is used by the new standard protocol Biomed-2[36]. In a recent study[37] we verified our previously published findings on clonally expanded T-cells in CTCL by correlation of the diagnostic sensitivity and specificity of our TCRγ PCR/FFA with the Biomed-2 protocol. Longitudinal investigation by a clone specific PCR detected the clonal T-cells in all blood samples of a MF patient although the TCRγ PCR/ HD-TGGE failed in 3/6 samples[32]. This failure correlated to the lower intensity of the clone-specific PCR products after electrophoresis and indicates variable frequencies of circulating clonal T-cells during the course of the disease.

With respect to these points, the failure to detect circulating clonal T-cells in all MF cases is rather a problem of sensitivity than a prognostic marker and the increasing frequency of PCR positive cases during the course of the disease, might indicate an increase in the number of circulating clonal T-cells in more advanced stages. Quantification of circulating clonal T-cells might help to determine that amount of neoplastic T-cells in the peripheral blood, which has a prognostic significance. Purely qualitative investigation of T-cell clonality in blood samples at the initial diagnosis of MF cannot predict the clinical course of the disease. These findings especially apply for the subgroup of T2 patients, which show the most variable prognosis in MF.

6.3 Correlation between T-cell clonality and genetic aberrations in CTCL

Detection of a T-cell clone by TCR PCR (i.e. a TCR clone) does not unanimously mean malignancy, and the presence of TExUS[14] in about one-eighth of MF patients may be confusing (see 6.1). Moreover, TCR clones have been identified in a substantial portion of blood samples derived from healthy individuals and patients with benign diseases, as well as in skin specimens from benign dermatoses.[20,38,39,40] Besides TCR rearrangements, the detection of clonal chromosomal aberrations (i.e. chromosomal clones) designates a clonal T-cell expansion. Whereas these alterations occur very rarely in blood and normal tissue of healthy adults, a variety of them has been found in the vast majority of CTCL patients[41,42]. Moreover, specific aberrations have been associated with the activity of CTCL: Aberrations of chromosomes 1, 6 and 11, although increasing with activity of the disease, seem to be a hallmark of existing disease, detectable even in remission; aberrations of chromosomes 8 and 17 especially associate with active or progressive disease[43]. Thus, in contrast to TCR clones purely indicating a quantitative change in the TCR repertoire, chromosomal clones, especially with complex somatic alterations, indicate qualitative and functional alteration of the affected cells.

We explored the coexistence of clonal chromosomal abnormalities and TCR clones in a series of 41 samples derived from skin, blood, or lymph nodes of 17 patients suffering from CTCL. Thirty of the 34 specimens (88%), which were successfully studied by both approaches, revealed corresponding results for TCR and chromosomal clonality. In two patients, analysis of micro-dissected cells demonstrated that single neoplastic T-cells bear both, a dominant TCR rearrangement and a complex chromosomal aberration. In the nine patients bearing identical TCR clones in skin and blood, chromosomal analysis confirmed the TCR PCR data by detection of identical chromosomal clones. Analysis of follow-up samples revealed persistence of the initial TCR/chromosomal clone in 79% (11/14) of the patients despite therapy and even if temporary complete clinical remission was achieved.

Of the four divergent specimens, all were derived from peripheral blood and were clonal by TCR PCR analysis only. In two of them, the TCR clone found differed from the primary (cutaneous) TCR clone. This discrepancy may be explained by the occurrence of TExUS[14] or by the evolution of subclones from a dominant clone as suggested by Vega et al.[44]. With regard to the remaining two samples, sensitivity limits of G-banding and MFISH or tumor cell death prior to chromosomal analysis in vitro may play a role, as indicated by the detection of chromosomal clonality in a follow-up sample of one patient.

In our second study on chromosomal aberrations, we were interested in presence and prognostic significance of chromosomal imbalances in CTCL. By comparative genomic hybridization (CGH), chromosomal imbalances were detected in 21 of 32 CTCL patients (66%). Euchromatic loss (dim) was localized most frequently (>16%) at the chromosomal regions 17p (28%), 13q (25%), 6q (19%), and 10q (16%), and gain of chromatin (enh) at 7 (25%), 8q (25%), and 17q (16%). The pattern dim6q–enh7–enh8–dim13 was the most frequent combination. The number of aberrations per tumor sample correlated with clinical tumor stages: from none in stage IA to 8.75 ± 1.8 (mean ± SEM) in stage IVA. Imbalances occurred more frequently in aggressive subtypes (9.33 ± 2.16) than in indolent (2.88 ± 0.8) subtypes. A high number of chromosomal imbalances (≥5) was associated with shorter survival. Gain of chromatin in 8q and loss of 6q and 13q correlated with a significantly shorter

survival, whereas the most frequently observed aberrations (loss in 17p and gain in 7) did not influence the prognosis.

Although CGH does not detect inversions and balanced translocations that are found by G-banding studies, some of our CGH findings confirm reports from earlier conventional cytogenetic studies. The most striking agreement between G-banding studies[45] and CGH studies[46,47] are gains in 8q, and losses of 13q, 10q and 17p.

Gains of chromosome 8q have been reported in a CGH study on B cell lymphomas[48,49], and several oncogenes are located on the long arm of chromosome 8, including MYC, MYC activator oncogene PVT1 (both 8q24), and MOS (8q22). Losses in 13q are a consistent finding of G-banding[45] and of an earlier CGH study[46]. At present, oncogenes in this chromosomal region, as the retinoblastoma oncogene RB-1 (13q14.1–14.2) or the breast cancer type 3 oncogene BRCA3 (13q21), could not be linked to leukemia or lymphoma. In our study, gains of chromosome 8q and losses of chromosome 13q were correlated with a short survival of the patients, indicating that genes located in this region may be pathogenetically relevant.

Aberrations of the chromosome 10q region have, in addition to CTCL[45,50,51], been reported for other hematological malignancies, including acute T-cell leukemia[52], B cell lymphoma[53], and other non-Hodgkin lymphoma.[54] This region encodes tumor suppressor genes such as PTEN (10q23.3), MXI1 (10q25–26), and DMBT1 (10q25–26). Losses of 17p are among the most frequent aberrations in neoplastic disorders of epithelial[55] and mesenchymal[56] origin, and were also reported for leukemia[57]. Well-known tumor suppressor genes such as tumor protein 53 (TP53; 17p13.1) and CT-10 regulator of kinase (CRK; 17p13.1) are encoded in this region. In contrast to other leukemia/lymphoma[58,59] and a CTCL study from Karenko et al.[43], no predictive value of this aberration was found in our study. Although gains of chromosome 7 were rarely described in T-cell lymphoma[60,61], they belong to the most prominent aberrations detected in the present study. This observation may be of particular relevance to T-cell lymphoma, since TCR encoding genes are located on this chromosome (γ: 7p15; β: 7q32–35). Possibly, the gains are a consequence of the naturally high rate of recombination and variation at this locus. In contrast to a study on adult T-cell leukemia/lymphoma[62], gains in chromosome 7 did not correlate with a better survival in our CTCL study.

In conclusion, a consistent pattern of chromosomal alteration in CTCL is still unidentified. But beside chromosomal aberrations that occur in a broad spectrum of malignancies (e.g., dim10q and dim17p), information on more CTCL-specific alterations (dim6q, enh7, enh8q, and dim13q) is emerging. We found alteration of the latter regions in characteristic combination (enh7–dim13q and dim6q–enh7–enh8q–dim13q) in a substantial number (24%) of our CTCL patients. At least in CTCL, complex chromosomal aberrations arise exclusively in clonally expanded T-lymphocytes. Vice versa, the occurrence of TCR clones is strongly associated with the occurrence of complex clonal aberrations of chromosomes. However, even if the lower sensitivity of the chromosomal techniques is taken into account, the occurrence of TExUS has to be considered. Parallel chromosomal analysis confirmed previous TCR data demonstrating an early dissemination of the T-cell clone in both skin and blood. Long-standing persistence of initial T-cell clones indicates that clonal cells were not targeted by current therapies nor were they sensitive to therapy-induced apoptosis as currently suggested[63,64].

In this scenario, it is remarkable, that Vermeer et al.[65] recently made the link between some of the

described chromosomal aberrations and deregulated genes: By high-resolution array-based comparative genomic hybridization and subsequent quantitative PCR on malignant cells from 20 Sézary syndrome patients, they found gain of the proto-oncogene MYC (located on chromosome 8q) in 75%, loss of the MYC antagonists MNT (55%, 17p) and MXI1 (40%, 10q), loss of tumor suppressor gene TP53 (>90%, 17p), gain of STAT3/STAT5 genes (75%, 17q) and IL-2 receptor genes (30%, IL-2RA on 10p).

6.4 Molecular biological findings in the light of the standardized Biomed-2 approach

Recently, several European centers of lymphoma diagnosis and research cooperated in the development of consensus PCR methods for clonality analysis in suspect T- and B-cell proliferations [Biomed-2 Concerted Action]. They have mainly been applied to frozen material of systemic B- and T-cell malignancies. Only limited data exist concerning cutaneous T-cell lymphoma (CTCL) and paraffin-embedded material.

To address the applicability of the Biomed-2 protocol to paraffin-embedded material and to confirm our previous TCR PCR results, we applied the Biomed-2 TCRγ and TCRβ PCR as well as our in-house TCRγ PCR to a collection of 107 archival skin samples (84 CTCL, 3 systemic T-cell lymphomas and 20 controls). In the CTCL samples, the Biomed-2 TCRγ PCR revealed 81%, the in house TCRγ PCR 86%, and the Biomed-2 TCRβ PCR 78% T-cell clonality. In the 20 control samples, the Biomed-2 TCRγ PCR detected a T-cell clone in one sample (cutaneous marginal zone lymphoma, small clonal peak, clonality uncertain), and the Biomed-2 TCRβ PCR in two samples (pseudolymphomas, small clonal peaks, clonality uncertain). By the in-house TCRγ PCR, all of the controls were non-clonal.

These data compare to previous studies on fresh/frozen samples of CTCL[66,67], and to the results of Morgan et al.[68] applying the Biomed-2 assay to both, fresh and paraffin embedded skin sections (without quoting the portions of both materials). The lower integrity of DNA as expected after extraction from paraffin obviously did not reduce the diagnostic sensitivity of the PCR-based clonality analysis. Even the more complex Biomed-2 TCRβ assay revealed comparable frequencies of T-cell clonality. On this condition, it should be emphasized that all samples generated the 300 bp fragment in the Biomed-2 control tube.

A higher complexity of the PCR assay may explain the differences between in-house TCRγ assay and Biomed-2 TCRγ assay: By splitting the investigation of the possible TCRγ rearrangements in three PCR, the in-house assay revealed slightly more clonal rearrangements in CTCL. In fact, the Biomed-2 TCRγ assay failed to detect T-cell clonality in 5 CTCL (MF-patch, n=1; MF-plaque, n=3; lymphomatoid papulosis, n=1) where clonality was shown by the in-house PCR. Conversely, the Biomed-2 TCRγ PCR exclusively showed clonality in one case of MF-patch. Since both approaches revealed almost identical analytical sensitivities of 2.5-10% in formalin-fixed material (depending on the T-cell line), the different outcomes are rather caused by different primer binding positions or primer-primer interactions in the more complex Biomed-2 TCRγ PCR than by different analytical

sensitivities.

Insufficient priming of the TCR gene segments due to germ line configuration, incomplete, deleterious or trans-rearrangements may also explain the general failure to detect clonality in the remaining 11 CTCL cases. Commonly, clonal rearrangements are not detectable using TCRγ PCR in at least 10% of lesional skin samples from CTCL, independently of the PCR and electrophoresis technique applied[69]. Priming at different genes explains the differences between the TCRγ and TCRβ analyses. Combining the Biomed-2 TCRγ and TCRβ methods increased significantly the diagnostic sensitivity. Therefore, the Biomed-2 TCRβ assay (with exception of the TCRβ DJ PCR which did not exclusively indicate a clonal rearrangement) should supplement the TCRγ tests in cases without detectable clonal TCRγ rearrangement.

Complexity of the PCR primers may also result in amplification of pseudoclonal PCR products resulting in a lower diagnostic specificity. If this holds true for the Biomed-2 assays can only be evaluated in larger series of control samples. With respect to the low prevalence of CTCL in routinely investigated skin samples, such a study is also mandatory to estimate the predictive values of the Biomed-2 approach.

In conclusion, our data underline the reliability of the standardized Biomed-2 protocol and show its applicability in the investigation of T-cell clonality of paraffin-embedded skin biopsies of CTCL. The confirmed diagnostic sensitivity and specificity of our in-house TCRγ assay verifies our previously published findings on clonally expanded T-cells in CTCL[11,14,32,70]. Nevertheless, the Biomed-2 TCRγ protocol is nowadays highly recommended for routine analysis of CTCL to permit data comparability and exchange of experience in T-cell lymphoma diagnosis.

However, (appropriate) predictive values have not been estimated for the Biomed-2 protocol. Furthermore, detection of T-cell clonality by PCR assays fails in a substantial portion of CTCL samples, but succeeds in various benign conditions. Thus, accurate integration of clinical, histomorphological, immunohistochemical data still represents the golden diagnostic standard. Demonstration of a T-cell clone only supplements the diagnosis of CTCL.

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