Diagnostic and prognostic markers in tumor stage mycosis fungoides and Sézary syndrome

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Increased expression of *PLS3* correlates with better outcome in Sézary syndrome

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TO THE EDITOR

Patients with Sézary syndrome (SS), a rare erythrodermic and leukemic form of cutaneous T-cell lymphoma, have a poor prognosis with a 5-year overall survival (OS) of 20-42% and a median OS between 2.5 and 5 years.[1-6] Prognostic factors associated with a worse survival reported in SS include advanced age, short duration of skin lesions before diagnosis of SS, previous history of mycosis fungoides (MF), elevated serum lactate dehydrogenase levels, degree of nodal involvement, and factors reflecting blood tumor burden, such as increased leukocyte counts or high Sézary cell counts.[1-8]

However, the results of these studies are not consistent, which may be due to different diagnostic criteria for SS, such as inclusion of patients without a T-cell clone in the peripheral blood, and analysis of mixed populations of patients with SS and erythrodermic MF.

Recently, we investigated the diagnostic significance of a large number of immunophenotypic and molecular biomarkers for SS in a group of patients with SS that fulfilled the diagnostic criteria of the World Health Organization - European Organization for Research and Treatment of Cancer classification.[9;10] None of these patients had a history of MF. Molecular biomarkers diagnostic for SS were copy number alterations in *MYC* (gain) and/ or *MNT* (loss); increased expression of *DNM3*, *TWIST1*, *EPHA4*, and *PLS3* and decreased expression of *STAT4*.

We investigated the prognostic significance of these molecular biomarkers and previously reported clinical prognostic markers using the same cohort of SS patients. Between September 2009 and October 2013, 64 SS patients from six European Organization for Research and Treatment of Cancer centers, including Helsinki, Finland; London, United Kingdom; Leiden, The Netherlands; Mannheim, Germany; Turin, Italy; and Paris, France were included and followed up until January 30, 2015. At the inclusion of the study, clinical variables (sex, age at diagnosis, duration of skin lesions before diagnosis of SS, lymph node involvement, leukocyte count, absolute CD4 count, and Sézary cell count) were recorded, and peripheral blood samples were collected for copy number variation and gene expression quantitative PCR analysis, as described previously.[9] Lymph node involvement was defined by presence of enlarged lymph nodes of 1.5 cm or larger in the longest transverse diameter on computed tomography scan or histologically confirmed lymph node involvement.

Aberrant gene expression in the SS samples was compared with samples from patients with erythrodermic inflammatory dermatoses (EID) and healthy control samples. Receiver operating characteristic curve analysis was used to determine fixed cut-off thresholds for each individual gene expression quantitative PCR assay with a specificity of 100% and an accuracy above 0.80. An one-tailed Mann-Whitney test was applied to test for significant differential expression between the SS and EID samples. *P*-values below 0.05 were regarded as statistically significant. The results of aberrant expression of the *DNM3*, *TWIST1*, *EPHA4*, *PLS3*, and *STAT4* genes were included in the statistical analysis. A more detailed Methods section including these thresholds is included in the **Supplementary Materials** and **Supplementary Table S1**.

Survival was calculated with the Kaplan-Meier method from the date of diagnosis

until the patient's death or date of last follow-up. The median follow-up time after diagnosis was 45 months (range = 1-129 months). Twenty-seven patients died during follow-up, including 21 SS-related deaths. The disease-specific survival (DSS) after 1, 2, 3 and 5 years was 89%, 82%, 76% and 59%, respectively, and OS was 86%, 79%, 72% and 49%, respectively.

Univariate analysis of parameters with possible prognostic significance for DSS and OS was performed using Cox proportional hazards regression analysis, and parameters that were significant at the 0.1 level were included in a multivariate analysis model. *P*-values below 0.05 were regarded as statistically significant.

Both in univariate and multivariate analyses, up-regulation of *PLS3* was associated with a significantly better outcome for DSS and OS (multivariate $P = 0.006$ and $P = 0.002$, respectively). Patients with up-regulation of *PLS3* had a median survival of 71 months (range = 9-129 months), compared with only 33 months (range = 1-72 months) in SS patients with normal expression of *PLS3* (**Figure 1**).

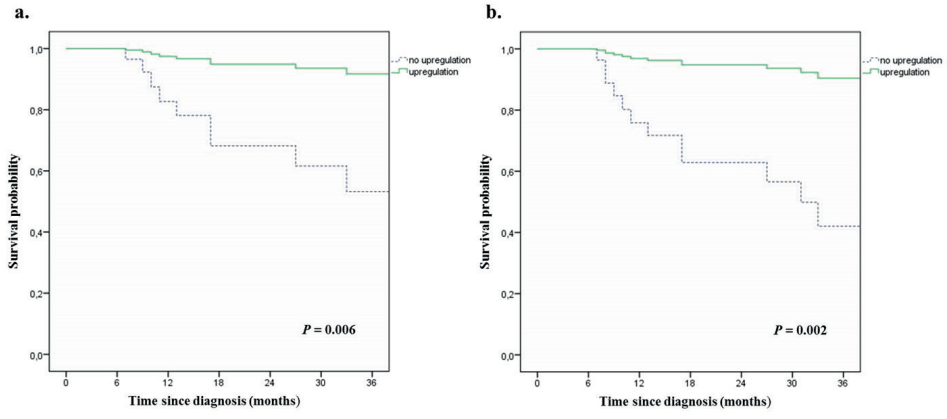

Figure 1. Disease-specific survival (a) and overall survival curve (b) according the groups with and without up-regulation of *PLS3.*

Up-regulation of *DNM3* and *TWIST1* were associated with a better OS in univariate analysis ($P = 0.008$ and $P = 0.043$, respectively) but not in multivariate analysis ($P = 0.658$ and $P = 0.342$, respectively). Gain of *MYC*, loss of *MNT*, up-regulation of *EPHA4* and down-regulation of *STAT4* showed no association with DSS and OS (**Table 1**).

Of the clinical parameters, both univariate and multivariate analyses showed that leukocyte count was a significant prognostic factor for DSS and OS (multivariate $P = 0.005$ and $P = 0.005$, respectively), whereas sex, age, duration of skin lesions before diagnosis, lymph node involvement, absolute CD4 count and Sézary cell count were not (**Table 1**).

PLS3 (T-plastin) is an actin-binding protein that is expressed in all normal cells of solid tissues that have a replicative role, but it is normally not expressed in T cells.[11] Expression of *PLS3* has been described as a specific marker of Sézary cells.[12;13] Studies

Table 1. Results of univariate and multivariate analyses for variables at Sézary syndrome diagnosis. Parameters significant at the 0.1 level were included in multivariate analysis.

Variables	Median survival	Univariate analysis DSS	
	(months)	HR (95% CI)	*P*-value
Gain in copy number of *MYC*			0.843
Yes, n=21	72 (1-129)	0.90 (0.32-2.54)	
No, n=31	49 (7-86)	1	
Loss in copy number of *MNT*			0.355
Yes, n=35	68 (1-129)	0.61 (0.22-1.74)	
No, n=17	49 (7-80)	1	
Up-regulation of *DNM3*			0.337
Yes, n=36	71 (9-129)	0.56 (0.17-1.83)	
No, n=13	33 (1-72)	1	
Up-regulation of *TWIST1*			0.197
Yes, n=32	71 (1-129)	0.48 (0.16-1.46)	
No, n=17	31 (7-80)	1	
Up-regulation of *EPHA4*			0.312
Yes, n=32	49 (7-129)	1.95 (0.53-7.12)	
No, n=17	68 (1-80)	1	
Up-regulation of *PLS3*			0.027
Yes, n=32	71 (9-129)	0.29 (0.10-0.87)	
No, n=17	33 (1-72)	1	
Down-regulation of *STAT4*			0.356
Yes, n=44	68 (1-129)	#	
No, n=5			
Sex			
Female, n=25	49 (1-129)	1.21 (0.51-2.85)	
Male, n=39	68 (1-115)	1	
Age at SS diagnosis in years, n=64		1.01 (0.96-1.05)	0.827
Duration skin lesions, n=60		0.98 (0.96-1.00)	0.075
Lymph node involvement			0.356
Yes, n=18	49 (1-74)	1.82 (0.51-6.53)	
No, n=23	Not reached	1	
Leukocyte count, n=61		1.04 (1.01-1.06)	0.007
Absolute CD4 count, n=59		1	0.146
Sézary cell count, n=47		1	0.123

CI, confidence interval; DSS, disease-specific survival; HR, hazard ratio; OS, overall survival; SS, Sézary syndrome. # means no statistics can be computed.

Multivariate analysis DSS		Univariate analysis OS		Multivariate analysis OS	
HR (95% CI)	***P*-value**	**HR (95% CI)**	***P*-value**	**HR (95% CI)**	***P*-value**
			0.366		
		0.66 (0.26-1.63)			
		1			
			0.388		
		0.68 (0.28-1.65)			
		1			
			0.008		0.658
		0.29 (0.12-0.72)		0.74 (0.20-2.75)	
		1		1	
			0.043		0.342
		0.39 (0.16-0.97)		0.57 (0.18-1.81)	
		1		1	
			0.311		
		1.70 (0.61-4.74)			
		1			
	0.006		0.001		0.002
0.14 (0.03-0.56)		0.19 (0.07-0.49)		0.12 (0.03-0.46)	
1		1		1	
			0.250		
		3.28 (0.43-24.77)			
		0.92 (0.43-1.98)			
		1			
		1.00 (0.97-1.04)	0.850		
0.99 (0.97-1.02)	0.447	0.99 (0.97-1.00)	0.152		
			0.420		
		1.55 (0.53-4.52)			
		1			
1.06 (1.02-1.10)	0.005	1.03 (1.01-1.06)	0.011	1.05 (1.01-1.08)	0.005
		1	0.265		
		1	0.204		

investigating the mechanism underlying dysregulation of *PLS3* expression in SS cells found no evidence for *PLS3* mutations within coding or promoter regions but showed significant hypomethylation of CpG dinucleotides 95-99 within the *PLS3* CpG island, which was restricted to the *PLS3*+ cells.[13] Reanalysis of recently published DNA methylation profiles from nine patients with SS and four healthy control subjects included in this study confirmed this correlation between DNA methylation and *PLS3* expression (data not shown).[14] A recent study found that constitutive *PLS3* expression was associated with apoptotic resistance to etoposide and suggested a role for cell survival in SS.[15] How T-plastin expression is linked to a better outcome in patients with SS is not known and should be the subject of further study.

Although for a disease as rare as SS the number of included patients is relatively high, a limitation of this study is a small sample size, which yielded wide confidence intervals, and these observations should be confirmed in an independent study.

In conclusion, we show that up-regulation of *PLS3* is associated with a favorable disease outcome in patients with SS and that increased leukocyte count is a significant adverse prognostic factor for survival.

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SUPPLEMENTARY MATERIAL

METHODS

Peripheral blood mononuclear cells were isolated from peripheral blood from 59 patients with Sézary syndrome (SS), 19 patients with erythrodermic inflammatory dermatoses (EID) and 4 healthy controls (HC) and enriched for CD4+ T helper cells, by depletion of non-CD4+ T cells, using the CD4+ T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) for copy number variation (CNV) and gene expression (GE) assays, as described previously.[1]

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). RNA was isolated using the RNeasy Mini Kit (Qiagen), which included on-column DNase digestion. RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA) using random priming. In order to quantify copy number changes accurately, quantitative PCR assays with FAM labelled hydrolysis MGB probes (Life Technologies, Carlsbad, CA) were developed for target genes *JUNB*, *TWIST1*, *MYC*, *MNT* and reference genes *ABT1*, *ARG2* and *DNM3*. For gene expression analysis, quantitative PCR assays with FAM labelled hydrolysis MGB probes (Life Technologies) were developed for target genes *PLS3*, *DNM3*, *CDO1*, *TRAIL*, *CD1D*, *GATA3*, *MYC*, *JUNB*, *TWIST1*, *EPHA4*, *STAT4* and reference genes *ARF5*, *ERCC3* and *TMEM87A*. Optimisation of both copy number as gene expression assays and sample analysis were performed as described previously.[1]

To compare aberrant gene expression in SS samples, relative to EID and HC samples, receiver operating characteristic curve analysis was used to determine fixed cut-off thresholds for each individual gene expression quantitative PCR assay with a specificity of 100% and an accuracy above 0.80. An one-tailed Mann-Whitney test was applied to test for significant differential expression between the SS and EID samples. *P*-values below 0.05 were regarded as statistically significant. The results of aberrant expression of genes *DNM3*, *TWIST1*, *EPHA4*, *PLS3* and *STAT4* were included in the statistical analysis (**Supplementary Table S1**).

All statistical calculations were performed using IBM SPSS Statistics 20.0. Survival was calculated from the date of diagnosis of SS until the patient's death or date of last follow-up. The Kaplan-Meier method was used to estimate survival curves and comparison between curves was performed using the log-rank test. Median follow-up was calculated

Supplementary Table S1. This table shows the thresholds determined by receiver operating characteristic curve analysis with an accuracy above 0.8 and specificity of 100% for the genes *DNM3*, *TWIST1*, *EPHA4*, *PLS3* and *STAT4.*

Gene	Accuracy (>0.8)	Std. error	Threshold	Sensitivity at 100% specificity
DNM3	0.919	0.032	8.826	75%
TWIST1	0.86	0.041	13.7865	69%
EPHA4	0.88	0.039	2.0035	66%
PLS3	0.842	0.045	31.602	66%
STAT4	0.99 (= 1-0.01)	0.008	0.24611	91%

using the reverse Kaplan-Meier method.

Univariate analysis of parameters with possible prognostic significance for disease-specific survival and overall survival was performed using Cox proportional hazards regression analysis. Parameters that were analyzed for their prognostic significance were gain in copy number of *MYC* (yes vs no), loss in copy number of *MNT* (yes vs no), up-regulation of *DNM3* (yes vs no), up-regulation of *TWIST1* (yes vs no), up-regulation of *EPHA4* (yes vs no), up-regulation of *PLS3* (yes vs no), down-regulation of *STAT4* (yes vs no), sex (male vs female), age at diagnosis (continuous variable), duration of skin lesions before diagnosis SS (continuous variable), lymph node involvement (yes vs no), leukocyte count (continuous variable), absolute CD4 count (continuous variable) and Sézary cell count (continuous variable). The parameters that were significant at the 0.1 level were included in a multivariate analysis model. *P*-values below 0.05 were regarded as statistically significant.

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