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Differential gene expression in non-malignant tumour microenvironment is associated with outcome in follicular lymphoma patients treated with rituximab and CHOP

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Summary

Rituximab in combination with chemotherapy (immunochemotherapy) is one of the most effective treatments available for follicular lymphoma (FL). This study aimed to determine whether differences in gene expression in FL tissue correlate with outcome in response to rituximab and CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy (R–CHOP). We divided 24 patients into long- [time to treatment failure (TTF) >35 months] and short-term (TTF <23 months) responders, and analysed the gene expression profiles of lymphoma tissue using oligonucleotide microarrays. We used a supervised learning technique to identify genes correlating with outcome, and confirmed the expression of selected genes with quantitative polymerase chain reaction (qPCR) and immunohistochemistry. Among the transcripts with a high correlation between microarray and qPCR analyses, we identified *EPHA1*, a tyrosine kinase involved in transepithelial migration, *SMAD1*, a transcription factor and a mediator of bone morphogenetic protein and transforming growth factor-β signalling, and *MARCO*, a scavenger receptor on macrophages. According to Kaplan–Meier estimates, high *EPHA1*, and low *SMAD1* and *MARCO* expression were associated with better progression-free survival (PFS). Immunohistochemistry showed that EphA1 was primarily localised in granulocytes. In addition, both EphA1 and Smad1 were expressed in vascular endothelia. However, no difference in vasculature was detected between long- and short-term responders. In a validation set of 40 patients, a trend towards a better PFS was observed among patients with high EphA1 expression. We conclude that gene expression in non-malignant cells contributes to clinical outcome in R–CHOP-treated FL patients.

Keywords: follicular lymphoma, rituximab and CHOP, microarray, treatment outcome.

Follicular lymphoma (FL) is the second most frequent subtype of all non-Hodgkin lymphomas (NHL). It is an indolent and chemotherapy-sensitive disease, which is, however, considered rarely curable because of its continuous relapse pattern. As no chemotherapy regimen has been shown to prolong survival, no globally established standard treatment for FL exists. In recent years, chemotherapy regimens have been explored in combination with rituximab (Mabthera, Rituxan), a chimeric (mouse–human) IgG1 monoclonal antibody directed against the B-cell antigen CD20. Rituximab kills lymphoma cells by activating the complement system, via antibody-dependent cellular cytotoxicity (ADCC), and directly by inducing apoptosis (Harjunpaa *et al*, 2000; Maloney *et al*, 2002; Cartron *et al*, 2004). Emphasising the importance of ADCC, Fcγ R polymorphism has been shown to correlate with the effect of rituximab treatment (Cartron *et al*, 2002). The concurrent administration of rituximab with different chemotherapies has resulted in higher response rates and longer progression-free and overall survival compared with chemotherapy alone (Forstpointner *et al*, 2004; Hiddemann *et al*, 2005; Marcus

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et al, 2005). Despite these advances, responses to treatments are heterogeneous and the treatment outcome is often unpredictable. Furthermore, treatments are toxic and costly. These aspects raise the need to identify more accurately patients, who will benefit from immunochemotherapy.

Recently, a specific Follicular Lymphoma International Prognostic Index (FLIPI) has been proposed (Solal-Celigny *et al*, 2004). In estimating the prognosis of FL, FLIPI appears to be more discriminatory than the International Prognostic Index for aggressive NHL (The International Non-Hodgkin's Lymphoma Prognostic Factor Project, 1993). It is likely that FLIPI will be a helpful tool in selecting the most appropriate treatment for individual FL patients. However, five clinical characteristics of FLIPI (age, tumour stage, nodal stage, haemoglobin level and serum lactate dehydrogenase concentration) do not provide information on the molecular features of the FL. It also remains to be shown how the FLIPI model can be applied to predict the outcome in response to immunochemotherapy.

The advent of microarray analysis has provided an opportunity to assess gene expression profiles and their value in predicting outcome of NHL, including FL (Alizadeh *et al*, 2000; Rosenwald *et al*, 2002; Shipp *et al*, 2002; Bohen *et al*, 2003; Dave *et al*, 2004; Bjorck *et al*, 2005). Thus far, however, it has not been addressed, whether gene expression in B-cell lymphomas could be used to predict a patient's outcome in response to a combination of rituximab and chemotherapy. In the present study, we have searched for biological factors that could explain the significant differences in the clinical outcome of FL patients treated concurrently with rituximab and CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy. Oligonucleotide-based microarray technique was used to identify genes that could be used to separate patients into long-term and short-term responders. Differential gene expression was verified by quantitative polymerase chain reaction (qPCR), and at the protein level.

Materials and methods

Patients and samples

A total of 64 follicular NHL (FL) patients treated with a combination of rituximab and CHOP chemotherapy between 1999 and 2003 at the Helsinki University Central Hospital (HUCH) were included in this study. Twenty-four patients were selected for the training group on the basis of the availability of freshly frozen lymph node tissue containing enough material for mRNA analyses. Paraffin-embedded tissue for immunohistochemistry was available for 62 patients. All tissue samples were taken before patients were treated with rituximab. Lymphoma classifications, including histopathology and immunophenotyping, were performed at the Department of Pathology at HUCH Laboratory Diagnostics according to the World Health Organization classification (Jaffe *et al*, 2001). Treatment records of all patients were reviewed to confirm the appropriate treatment protocols and to document clinical characteristics, prognostic factors and long-term follow up. The study was approved by the ethical committee of the HUCH.

Gene expression profiling and data analyses

RNA isolation, microarray and statistical methods are described in detail in Appendix S1. Briefly, total RNA was isolated from tumour samples according to established procedures, and examined for gene expression with the use of Agilent Human 1A oligo microarray (Agilent Technologies, Palo Alto, CA, USA) containing 18 716 features. Before supervised learning classification, all samples were sorted into long-term responders [time to treatment failure (TTF) >36 months; $n = 11$] and short-term responders (TTF <23 months; $n = 13$). In order to find differentially expressed genes and to separate the samples into different groups based on clinical information, we used a weighted voting algorithm (WVA) (Golub *et al*, 1999; Alizadeh *et al*, 2000; Shipp *et al*, 2002). In parallel, we used a two-tailed *t*-test to find differentially expressed genes with a confidence level of 0·05. Subsequently, the genes were sorted using the obtained *P*-values.

Quantitative real-time PCR

RNA was available from 22 patients. Expression of mRNA for six genes identified by microarray and two endogenous control genes, *TBP* and *GAPDH* was measured by real-time reverse transcription PCR (RT-PCR) with the TaqMan methodology using a Gene Amp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The detailed description of the method is available in *Appendix S1*. All assays were performed in duplicate.

Immunohistochemistry

Immunohistochemistry for EphA1, Smad1 (Santa Crutz) and CD31 (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) was performed on 22 formalin-fixed, paraffin-embedded FL sections from tissue microarrays (TMA). The sections were treated in an autoclave in 0·01 mol/l sodium citrate (pH 5·0) for 2 min and washed with phosphate-buffered saline. Subsequent staining for EphA1, Smad1 and CD31 were performed using antibody dilutions 1:200, 1:100 and 1:200 respectively. Immunohistochemistry was completed using the Vectastain ABC kit reagents (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. The immunoreactions were visualised with 3-amino-9-ethylcarbazole, and the slides were counterstained with haematoxylin.

Initially, EphA1 and Smad1 stains were evaluated by semiquantitative grading (low, intermediate or high expression). As this method showed poor reproducibility, we also counted the most positive cells, which were granulocytes from EphA1 stains. As tissue arrays included two to three replicates from each lymphoma tissue, the results of continuous scaling

were averaged. CD31-positive vessels were also counted from the total area of tissue arrays.

Statistical analyses

Chi-squared test and Mann–Whitney tests were used to compare the baseline characteristics and the expression of mRNAs or the EphA1 protein or vessels in lymphomas from patients with short or long time with TTF. To evaluate the correlation between data from microarray and qPCR, the Spearman correlation coefficient (r_s) was calculated. Cox univariate analysis was used to test the prognostic impact of the identified genes on TTF. Survival rates were estimated by the Kaplan–Meier method and the differences were compared by the log-rank test. TTF was determined as a period between the first day of therapy and the date of the documented progression or lack of response, first relapse or death for any reason. Time to progression (progression-free survival, PFS) was determined as an interval between the first day of therapy and the date of relapse. These analyses were carried out using the Statistical Package for the Social Sciences (SPSS) software for Macintosh (SPSS, Inc., Chicago, IL, USA). Probability values below 0·05 were considered statistically significant. All *P*-values were two-tailed.

Results

Patient characteristics

In this pilot study, we searched for molecular factors correlating with outcome in response to rituximab and CHOP chemotherapy. Twenty-four samples were available for the screening of gene expression profiles. The baseline characteristics of these patients, which formed the test group, are shown in Table I. The groups were well balanced for age, grade, disease status and FLIPI. All patients received rituximab for the first time. Rituximab was administered four times at 375 mg/m^2 along with three to seven cycles of CHOP chemotherapy (cyclophosphamide 750 mg/m^2, doxorubicin 50 mg/m^2, vincristine 1·4 mg/m^2 on day 1 and prednisone 100 mg on days 1–5). The overall response rate [complete response (CR) + partial response (PR)] in this study population was 88% (21/24).

Based on the clinical outcome patients were divided into groups of responders (CR and PR, $n = 21$) and non-responders (SD, $n = 3$). PFS varied significantly among the responding patients (median 40 months, range 4–66 months). Half of the patients ($n = 11$) had significantly longer PFS (35–66 months), when compared with the other half ($n = 10$, PFS 4–23 months). On this basis, the screening population was divided into 'long-term responders' (TTF > 36 months) and 'short-term responders', which included the non-responders (TTF < 23 months, $n = 13$). In addition to test group, 40 samples were available for validation with immunohistochemistry. The clinical characteristics of these patients are shown in Table II.

Table I. Characteristics of the screening population.

Characteristic	All patients ($n = 24$) (%)	Short-term responders ($n = 13$)	Long-term responders ($n = 11$)	*P*-value
Gender				
Female	15 (62·5)	6	9	NS
Male	9 (37·5)	7	2	
Age (years)				
<60	16 (66·7)	9	7	NS
≥60	8 (33·3)	4	4	
Disease status				
Primary	17 (70·8)	8	9	NS
Relapse	7 (29·2)	5	2	
1. Relapse	4	3	1	
2. Relapse	3	2	1	
FL grade				
I	10 (41·7)	7	3	NS
II	6 (25·0)	1	5	
III	2 (8·3)	1	1	
Not known	6 (25·0)	4	2	
FLIPI score				
0–2	14 (58·3)	6	8	NS
3–5	10 (41·7)	7	3	
Response				
Complete	16 (66·7)	6	10	0·059
Partial	5 (20·8)	4	1	
Stable disease	3 (12·5)	3	0	

FLIPI, Follicular Lymphoma International Prognostic Index; NS, not significant.

Identification of genes differentially expressed between long- and short-term responders

Hierarchical clustering was performed for 24 samples to get an overview and group the patients according to differences in gene expression. The expression patterns were relatively homogeneous and the clusters that arose did not associate with clinical parameters (data not shown). Subsequently, we conducted a supervised learning classification and sorted genes by their degree of correlation with the favourable *versus* unfavourable distinction. Two separate comparisons were performed: response *versus* no response and long response *versus* short response. In both settings, WVA and *t*-test were used to identify the genes whose expression varied most significantly between the groups, and chose only the genes identified by both methods for further analysis (Table III).

Validation of microarray data by qPCR

To validate the results, qPCR was applied on six differentially expressed genes, as well as on two housekeeping genes (*GAPDH* and *TBP*) as a control. The selection of genes to be validated was performed randomly from the group of distinctive genes with interesting biological function and prognostic impact according to univariate analysis (Table III). Instead of

Table II. Characteristics of the validation group.

Characteristic	All patients ($n = 40$) (%)	EphA1 low ($n = 14$)	EphA1 high ($n = 26$)	*P*-value
Gender				
Female	27 (67·5)	9	18	NS
Male	13 (32·5)	5	8	
Age (years)				
<60	29 (72·5)	9	20	NS
≥60	11 (27·5)	5	6	
Disease status				
Primary	12 (30·0)	4	8	NS
Relapse	28 (70·0)	10	18	
FL grade				
I	12 (30·0)	4	8	NS
II	6 (15·0)	1	5	
III	3 (7·5)	1	2	
Not known	19 (47·5)	8	11	
FLIPI score				
0–2	28 (70·0)	10	18	NS
3–5	9 (22·5)	3	6	
Not known	3 (7·5)	1	2	

FLIPI, Follicular Lymphoma International Prognostic Index; NS, not significant; EphA1 low, the lowest tertile; EphA1 high, two upper tertiles.

directly choosing the numerically best genes, we used random selection, as the small size of training set could cause at least minor distortions leading to inaccuracies of *P*-values. mRNA for qPCR was available from 22 FLs. In general, when compared with microarray analyses, the differences in mRNA levels were more pronounced when measured by qPCR. Nevertheless, qPCR results were in agreement with the microarray data. The best correlation between microarray and qPCR data was observed for *SMAD1* ($r_s = 0{\cdot}665$, $P = 0{\cdot}003$) and *EPHA1* ($r_s = 0{\cdot}688$, $P = 0{\cdot}002$). The correlation coefficient for *MARCO* was 0·529 ($P = 0{\cdot}020$), for *RRAD* 0·509 ($P = 0{\cdot}026$) and for *MXI1* 0·476 ($P = 0{\cdot}038$). Differential expression of *CUL4B* mRNA could not be verified by qPCR.

Evaluation of PFS according to qPCR data

Among the transcripts with the best correlation between microarray and qPCR analyses, *MARCO* was overexpressed in non-responders, as well as short-term responders. Likewise, *SMAD1* was overexpressed in short-term responders, whereas *EPHA1* was downregulated (Fig 1). As these genes are likely to contribute to lymphoma cell growth, they were selected for further analyses.

At the median follow up of 49 months (3–66 months), 21 of the 24 patients were alive (87·5%) and 10 in remission (41·7%). The median TTFs for patients with low *SMAD1* ($P = 0{\cdot}019$) or *MARCO* ($P = 0{\cdot}018$) qPCR levels were significantly better than the ones with high levels (Fig 2A and B). In addition, a trend towards better TTF was observed among the patients with high *EPHA1* pPCR levels ($P = 0{\cdot}077$) (Fig 2C). In comparison, the clinically based FLIPI could separate the high-risk patients from the low and intermediate risk groups, but presumably because of the small number of patients, the difference was not statistically significant ($P = 0{\cdot}467$; Fig 2D). Together, the results suggest that the expression of these genes in lymphoma tissue correlates with the outcome in response to rituximab and CHOP chemotherapy in FL.

Table III. A partial list of genes differentially expressed between short- and long-term responders, and their prognostic impact on time to treatment failure.

Gene	*t*-test (*P*)	Cox univariate analysis (*P*)
Proliferation/cell cycle regulation		
STAG1	0·002502	0·072
CD30	0·004042	0·001
KITLG	0·004564	0·019
AZGP1	0·008374	0·208
FGF18	0·008597	0·040
IHPK2	0·009781	0·005
RBL1	0·010187	0·157
CD69	0·022971	0·003
CUL4B	0·047001	0·044
Differentiation		
TNFSF11	0·003466	0·008
ZNF1/SZF1	0·029707	0·004
Signal transduction		
SOSC1	0·005218	0·003
SP110/IFI41	0·006568	0·004
CXCR4	0·008012	0·006
SHC3	0·015706	0·009
SMAD1	0·02414	0·002
PIK3C2G	0·031392	0·003
GNG11	0·000153	0·009
PLD1	0·007591	0·023
MXI1	0·009706	0·004
Transcription		
FOXJ2	0·000583	0·022
CALCOCO1	0·009545	0·002
NFYB	0·010742	0·002
PRDM1	0·017607	0·088
Regulation of immune response and inflammation		
CCRL1	0·004277	0·419
NFKBIZ/MAIL	0·006568	0·336
FCGR1A	0·015554	0·050
MARCO	0·034342	0·069
IFITM3	0·009698	0·085
Transendothelial migration		
EPHA1	0·037338	0·012
Tumour suppression		
CLCA1	0·002537	0·007
Apoptosis		
AATK	0·002221	0·309

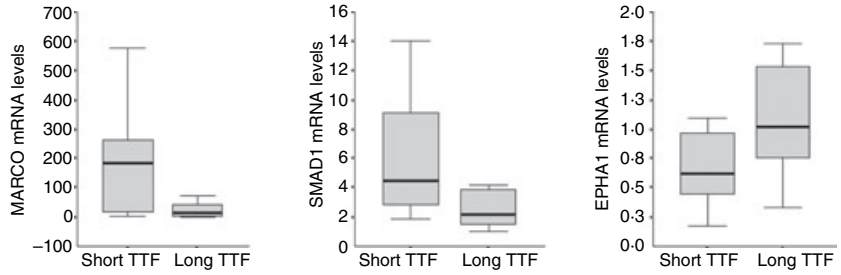

Fig 1. Quantitative polymerase chain reaction analysis of *MARCO*, *SMAD1* and *EPHA1* genes in long-term and short-term responders. Box blots represent the expression values of the percentiles 25% and 75% for each tumour. The extremes of vertical lines represent maximum and minimun expression values.

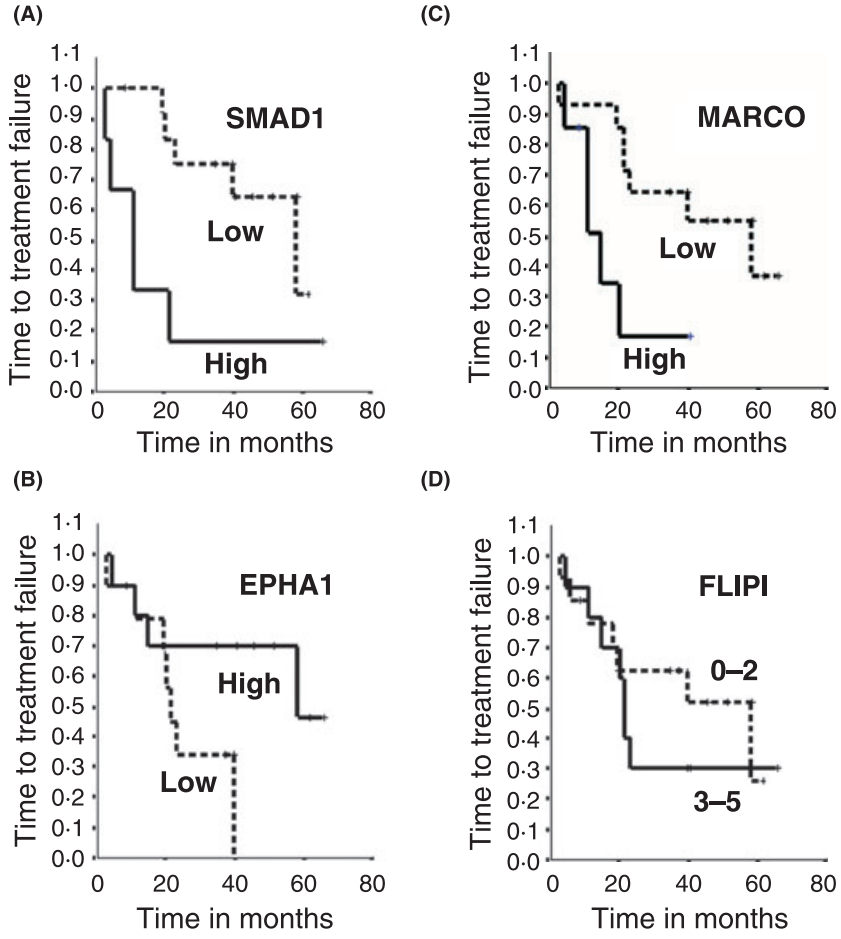

Fig 2. The outcome of follicular lymphoma patients ($n = 24$) treated with rituximab and CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy. mRNA levels were analysed by quantitative polymerase chain reaction (qPCR). (A) Patients according to low ($n = 13$) and high ($n = 6$) *SMAD* mRNA levels [median time to treatment failure (TTF) 58 vs. 11 months; $P = 0{\cdot}019$]. (B) Patients according to low ($n = 14$) and high ($n = 7$) *MARCO* mRNA levels (median TTF 58 vs. 15 months; $P = 0{\cdot}018$). (C) Patients according to low ($n = 10$) and high ($n = 10$) *EPHA1* mRNA levels (median TTF 58 vs. 21 months; $P = 0{\cdot}077$). (D) Patients according to Follicular Lymphoma International Prognostic Index (FLIPI) scores 0–2 ($n = 14$) and FLIPI scores 3–5 ($n = 10$) ($P = 0{\cdot}467$).

EphA1 and Smad detection by immunohistochemistry

In order to extend the gene expression-based analyses to protein and cellular localisation level, we performed immunohistochemical stainings on paraffin-embedded lymphoma tissue obtained from 22 patients (Fig 3). EphA1 and Smad1 proteins were chosen for further analyses, because previous reports have demonstrated an active role for them in cellular signalling in lymphocytes and FL (Husson *et al*, 2002; Munoz *et al*, 2004; Aasheim *et al*, 2005), and appro-

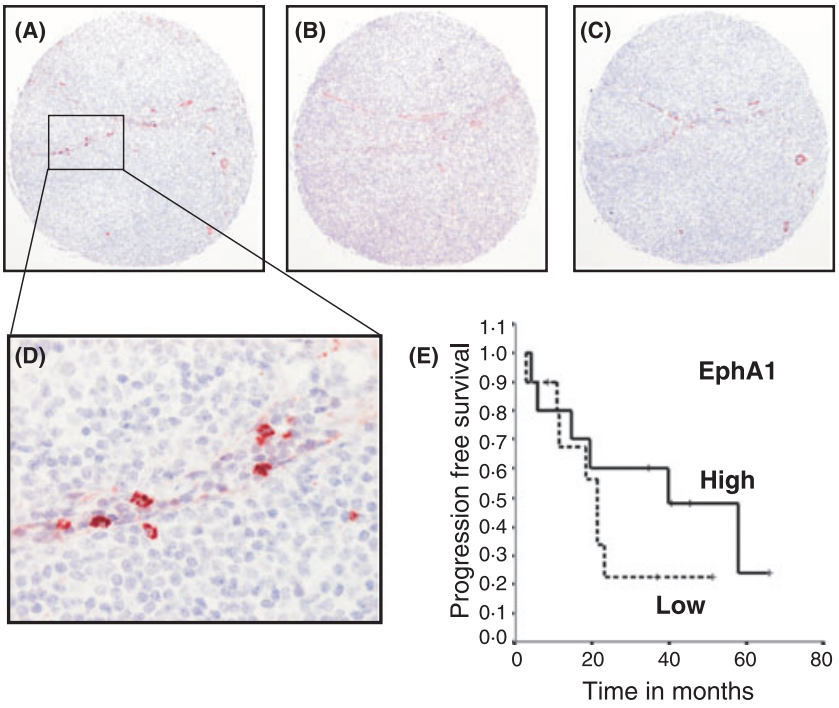

Fig 3. Immunohistochemical staining of follicular lymphoma tissue for EphA1, Smad and CD31. (A) Representative example of EphA1 immunoreactivity, which was detected close to vascular endothelia, but not in lymphoma cells. In (D), a magnification of panel (A) is shown. (B) Smad1 was expressed in lymphoma cells and vascular endothelia. (C) CD31 was expressed only in vascular structures. (E) Progression-free survival according to EphA1-high ($n = 10$) and -low ($n = 10$) immunoreactivity ($P = 0{\cdot}259$). Original magnification ×100 (A–C), ×630 (D).

priate antibodies for immunohistochemistry were commercially available.

The immunohistochemical analysis of EphA1 in lymphoma tissue showed wide variation, from complete absence to intense reactivity. Interestingly, however, the immunoreactivity was not observed in malignant lymphocytes but in the surrounding microenvironment, including vascular endothelia and especially granulocytes close to high endothelial venules (Fig 3A and D). With respect to intracellular compartmentalisation, EphA1 immunoreactivity was localised to the cell surface and cytoplasm.

Similarly to EphA1, Smad1 immunoreactivity showed wide variation, from completely absent to high positivity (Fig 3B). Although the expression of Smad1 was generally more diffuse than EphA1, and observed also in malignant lymphocytes, the most prominent expression appeared to localise to vascular structures.

Overall, differences in protein expression observed immunohistochemically were less apparent than the differences in mRNA levels. The semiquantitative grading of protein expressions failed to correlate reliably with mRNA data. In contrast, a 'hot spot' approach, in which the number of strongly stained granulocytes in the whole sample was measured, showed a good correlation between mRNA and protein expression ($r_s = 0{\cdot}628$, $P = 0{\cdot}003$). Furthermore, EphA1-positive granulocyte counts were higher among long-term responders than short-term responders (mean 14 vs. 8 per TMA core, $P = 0{\cdot}039$). Although we could not identify a cut-off level for EphA1 positivity, which separated this small patient population into two groups with significant differences in PFS, we observed a trend towards a better outcome among the patients having high EphA1 protein expression in their lymphoma tissue (58 vs. 21 months, $P = 0{\cdot}259$) (Fig 3E). Taken together, the data showed that, although the prognostic significance of EphA1 expression was more prominent at the mRNA level, the differences could also be seen at the protein level. Moreover, the prognostic value of EphA1 appeared to be localised primarily to granulocytes and, to a lesser extent, to endothelial cells.

To evaluate the vasculature of tumour tissue in more detail, CD31 staining was performed (Fig 3C). When the vessel density was evaluated, no significant differences in vasculature were found between long- and short-term responders (mean number of CD31-positive vessels 13·9 vs. 13·2 per TMA core, $P = 0{\cdot}74$). Interestingly, however, *SMAD1* but not *EPHA1* mRNA expression correlated with *CD31* mRNA levels ($r_s = 0{\cdot}588$, $P = 0{\cdot}003$). Furthermore, PFS was significantly better among patients having low *CD31* mRNA levels (median not reached *versus* 21 months, $P = 0{\cdot}036$). Together, the data suggest that *SMAD1* and *CD31* expression in FL tissue reflected a molecular signature in the vessels, which was associated with outcome in response to R–CHOP therapy.

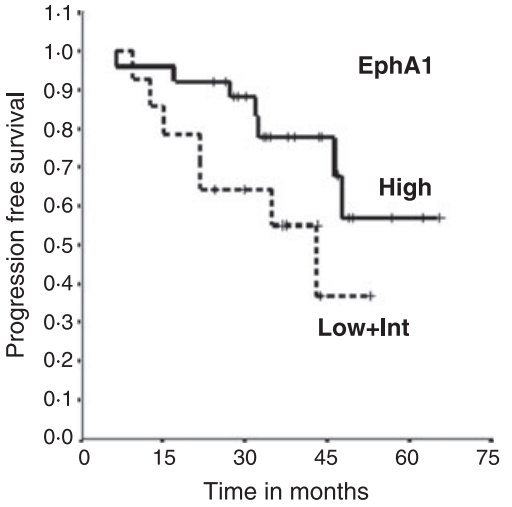

Fig 4. Progression-free survival of the validation group according the EphA1-high ($n = 26$) and -low/intermediate ($n = 14$) immunoreactivity ($P = 0{\cdot}07$).

Prognostic impact of EphA1 protein expression in a validation group

After identifying *EPHA1* as a marker associated with outcome in FL, we analysed the prognostic value of EphA1 protein in a validation group of 40 R–CHOP-treated patients. The characteristics of these patients were well balanced for grade, FLIPI and disease status (Table II). The patients with high EphA1 expression were somewhat younger than the ones in EphA1-low group but the difference was not statistically significant. After a median follow up of 44 months, 39 of 40 patients were alive (97·5%) and 26 (65%) in remission. Although no cut-off level for a significant difference between EphA1-low and -high groups could be determined, we observed a trend towards a better outcome among patients with high EphA1 protein expression (two upper tertiles *versus* the lowest tertile) in their lymphoma tissue ($P = 0{\cdot}07$). Of the 26 patients within the EphA1-high group, 19 (73%) were in remission (median PFS not reached). In comparison, seven (50%) of the remaining 14 patients in the EphA1-low group were in remission (median PFS 43 months) (Fig 4). The association of EphA1 expression with PFS in the validation group confirmed the reproducibility of the primary data.

Discussion

The aim of our study was to identify genes, whose expression in lymphoma tissue correlates with outcome of FL patients treated with rituximab and CHOP chemotherapy. Our results suggest that certain genes have predictive power in this experimental setting and that, by using oligonucleotide microarrays, patients who would benefit from immunochemotherapy could be identified. The data further show that differential expression and prognostic impact of a set of selected genes, such as *SMAD1*, *MARCO* and *EPHA1*, were verified by qPCR. Finally, immunohistochemical staining demonstrated that the prognostic influence of Smad1 and EphA1 was associated with their expression in non-malignant cells.

As the first approach, we used hierarchical clustering to distinguish subgroups that differed with respect to their gene expression profiles. The samples showed relative homogeneity, even though some clusters were found. In line with previous microarray studies of chemotherapy-treated FL patients, the clusters did not correlate with the clinical outcome (Glas *et al*, 2005). This suggests that additional factors may account for responses to immunochemotherapy. When gene expression data were combined with the clinical information, a correlation with outcome was found, and we could identify genes that predicted the outcome of 24 FL patients. The data showed that long- and short-term responders could be separated by analysing the expression of a few genes. The differences in gene expression may be partially influenced by the coincidence that the group of short-term responders contained more relapsed patients than long-term responders. However, as shown in Table I, the association was not statistically significant.

The genes implicated in the long- *versus* short-term outcome distinction included those of multiple regulators for cell growth and apoptosis. Of the selected three representatives, Marco is a scavenger receptor on macrophages, whose expression is rapidly induced in response to inflammatory stimuli (Elomaa *et al*, 1995; van der Laan *et al*, 1999). Its expression in the tumour tissue may reflect the lymph node response to tumour cells, as Marco is thought not only to play an important role in the immune responses by mediating binding and phagocytosis of bacteria, but also to be involved in tumour antigen stimulated phagocytosis by dendritic cells (Grolleau *et al*, 2003).

Smad1 is a transcription factor that mediates gene expression in response to bone morphogenetic protein (BMP) and transforming growth factor-β signalling. In many cell types, including B lymphocyte lineage cells, Smad1 can serve as a mediator of growth arrest and apoptosis (Ishisaki *et al*, 1999; Yamato *et al*, 2001; Munoz *et al*, 2004). Our finding that Smad1 expression is primarily localised on vascular endothelia supports the use of whole tumour tissue instead of isolated tumour cells. It also suggests that endothelial cells are part of the pathophysiology of FL. However, as the expression of *SMAD1* and *CD31*, but not the amount of CD31-positive vessels, are associated with outcome, it is likely that the molecular signature in vessels rather than vessel density has an impact in immunochemotherapy.

Of particular interest is EphA1, a receptor tyrosine kinase, which has been shown to be involved in trans-endothelial migration (Sharfe *et al*, 2002, 2003; Aasheim *et al*, 2005). It is known to be expressed on $CD4^+$ T lymphocytes but not on B lymphocytes (Aasheim *et al*, 2005). However, our immunohistochemical stains showed that, in FL tissue, the most prominent expression of EphA1 was detected in the vascula-

ture and in granulocytes located close to high endothelial venules. To our knowledge, there is no previous data implicating the role of granulocytes in the prognosis of FL. However, it is tempting to speculate that the ability or sensitivity of granulocytes to invade malignant lymph nodes is important for rituximab-mediated cell killing. Invasion and activation of granulocytes could also indicate a pre-existing immune response against malignant lymphocytes, which is further enhanced by rituximab. This is supported by mouse studies showing that granulocytes contributed to the biological antitumour activity of rituximab (Hernandez-Ilizaliturri *et al*, 2003). The finding that granulocyte colony-stimulating factor and granulocyte–macrophage colony-stimulating factor enhanced rituximab activity is also in agreement with the hypothesis (Hernandez-Ilizaliturri *et al*, 2005).

Gene expression profiling provides valuable molecular information on lymphomas, and can be used to screen for new biomarkers. However, it cannot be incorporated into routine diagnostics and clinical evaluation of patients. A better approach would be to use less laborious and costly methods, such as flow cytometry, immunohistochemistry or qPCR, of which the latter two approaches have been utilised in recent diffuse large B-cell lymphoma (DLBCL) studies (Hans *et al*, 2004; Lossos *et al*, 2004; Berglund *et al*, 2005). In the present study, we used both qPCR and immunohistochemistry to validate the microarray data, and demonstrated that mRNA analyses can also be extended to protein studies. According to our findings, high EphA1 expression and low Smad1 expression in non-malignant cells of the FL tissue were associated with a more favourable TTF. The prognostic impact of EphA1 was associated with its expression in granulocytes, whereas the impact of Smad1 was related to its localisation in vessels. It will be interesting to determine whether the same genes associate with outcome in response to R–CHOP in DLBCL, in which the tumour microenvironment is less prominent.

The initial semiquantitative grading of both Smad1 and EphA1 protein expression showed poor reproducibility. This may be due to inaccurate scaling and subjective nature of estimating the immunoreactivity. However, a hot spot method with continuous scaling resulted in reproducible EphA1 counts, which correlated well with microarray data. This, together with the reproducibility of the prognostic impact of EphA1 expression in an independent validation set, encourages us to believe that the microarray-based results and prognostic evaluations can be extended to the protein level. Although EphA1 correlated positively with TTF both in the test and validation sets, we could not determine a prognostically significant cut-off level for the number of EphA1-positive cells. It is thus plausible that the possibilities to quantify EphA1 protein expression by immunohistochemistry remain limited, and that it would be valuable to apply other more accurate methods, such as flow cytometry, for quantification in future studies.

In conclusion, we have identified genes that may help to identify those FL patients likely to benefit from currently available rituximab and chemotherapy combinations. Clearly, our study population was small and the results need additional validation with more accurate methods. A longer follow up will also show whether this approach has a predictive impact for overall survival. Nevertheless, our study applying the gene expression-based outcome predictors for FL patients treated with immunochemotherapy is novel, and illustrates the importance of molecular information for both the optimal use of existing combination therapies and the development of novel treatments for FL.

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Supplementary material

The following supplementary material is available for this article online:

Appendix S1. Materials and methods.

The material is available as part of the online article from http://www.blackwell-synergy.com.