The utility of mRNA analysis in defining SOX11 expression levels in mantle cell lymphoma and reactive lymph nodes

Mantle cell lymphoma (MCL) is a rare, but distinctive, B cell derived non-Hodgkin lymphoma (NHL) subtype that constitutes approximately 8% of all NHL.[369] The higher prevalence of MCL in individuals of advanced age and the frequent relapses and resistance to therapy makes this disease difficult to cure.[2,3] Interestingly, there are subsets of MCL which display a more indolent clinical course with slow progression and longer survival, despite not requiring immediate treatment.[4-6] The molecular hallmark of MCL is the t(11;14)(q13;q32) cyclin D1 translocation, by which the *CCND1* gene is juxtaposed to the immunoglobulin heavy chain enhancer resulting in aberrant cyclin D1 expression.[1] Rare cases of MCL lack cyclin D1 expression.[1,7] Such cases constitute a diagnostic challenge.

Most MCL, including t(11;14) negative cases, express the neural transcription factor SRY (sex-determining region Y)-box 11 (*SOX11*).[8,9] Detection of SOX11 by immunohistochemistry (IHC) is therefore a diagnostic marker for cyclin D1 negative MCL,[8,9] and SOX11 is also expressed in 93-95% of cyclin D1 positive cases.[6,8,10] It has also been suggested that SOX11 expression could serve as a surrogate marker for biological behavior, including disease aggressiveness.[5,6,11,12] The value of SOX11 IHC as a prognostic marker in MCL is, however, partly conflicting. One explanation for this could be the use of insufficiently specific antibodies,[13] but recently a highly specific monoclonal antibody, MRQ-58, has become available[13,14] that could overcome this problem. Another explanation for conflicting results is the lack of defined cut-off levels of SOX11 expression.[5,6,10,15] This is reflected by the different immunohistochemical cut-offs used in recent studies.[6,10,13,15] In order to investigate if there is a natural cut-off for SOX11 expression we have carried out an extensive analysis of SOX11 mRNA expression by quantitative PCR (qPCR) and by IHC in MCL (n=102) and reactive lymph nodes (n=16) (ethical approval granted from The Regional Central Ethical Review Board of the Karolinska Institutet, and performed in accordance with the Declaration of Helsinki protocol). The patient samples were derived from a well-defined population based cohort of MCL (a subset of which has been previously evaluated for SOX11 by IHC[6]). As part of this study we investigated the performance of the MRQ-58 antibody (MRQ-58, Cell Marque; cat. no. 382M) compared to the polyclonal SOX11 antibody HPA000536 (Atlas Antibodies). Several cases that were negative with the

Table 1A. Clinical and pathological features of investigated cases.

Clinical and pathological features of MCL	The 10% MCL cases with lowest SOX11 mRNA expression (n=10)	The 90% MCL cases with highest SOX11 mRNA expression (n=92)	*P* (Fisher's exact test)
Median age in years (range)	67.0 (58.0-87.2)	69.7 (32.1-91.7)	
Male, (%)	5/10 (50.0)	46/92 (69.6)	n.s.
Age>65 years, (%)	6/10 (60.0)	59/92 (64.1)	n.s.
Tumor cells by flow cytometry median (range)	57.5 (11-87)	72.0 (17-94)	n.s.
B-symptoms, (%)	2/10 (20.0)	23/87 (26.4)	n.s.
>4 nodal sites, (%)	2/9 (22.2)	59/87 (67.8)	0.0107
Splenomegaly, (%)	3/9 (33.3)	40/83 (48.2)	n.s.
Ann Arbor stage IV	9/10 (90.0)	73/88 (83.0)	n.s.
Lymphocytosis >5*10(9)/L, (%)	6/10 (60.0)	14/86 (16.3)	0.0049
High serum LDH*, (%)	5/10 (50.0)	33/81 (40.7)	n.s.
MIPI high risk, (%)	6/8 (75.0)	28/70 (40.0)	n.s.
Ki-67 high ≥ 30%, (%)	3/9 (33.3)	41/89 (46.1)	n.s.
Blastoid variant, (%)	2/10 (20.0)	13/88 (14.8)	n.s.
P53 positivity > 20%, (%)	4/9 (44.4)	13/85 (15.3)	n.s.
$SOX11^{+}$ by IHC, (%)	2/10 (20.0)	92/92 (100)	n.s.
RFI SOX11, median (range)	8.8 (0.01-76)	544.3 (92.0 - 7419.41)	

**Elevated LDH as defined by Karolinska University Hospital Laboratory.*

Table 1B. Clinical and pathological features of reactive lymph nodes.

Clinical and pathological features of reactive lymph nodes	All (n=16
Median age in years (range)	46,2 (13.2-71.4)
Male. (%)	9/16 (56.2)
Follicular hyperplasia, (%)	10/16 (62.5)
B lymphocytes by flow cytometry, % of total, median (range)	30.5 (14-49)
At least 2 SOX11 positive cell nuclei by immunohistochemistry (%)	6/16 (37.5)
RFI SOX11, median (range)	4.87 (1.26-58.35)

HPA000536 antibody became positive when using the MRQ-58 antibody, and the results of IHC by MRQ-58 are more in line with the mRNA expression (*Online Supplementary Table S1*). Furthermore, we correlated SOX11 levels to clinical parameters in order to address the potential clinical significance of low *versus* high levels of SOX11 mRNA expression.

MCL cases were selected based on availability of frozen tissue (lymph node biopsies n=80, tonsils n=6, bone marrow cells n=4, spleens n=3, peripheral blood cells n=7, pleural cells n=1 and gastrointestinal biopsy n=1). The tumor cell content is given in Table 1. The diagnosis was established according to WHO criteria[1] and all cases but one were cyclin D1 positive by IHC and/or FISH. The single cyclin D1 negative case was $CD5^+$, $CD23^-$, $CD200^-$ and $SOX11^+$ by IHC (MRQ-58) and had a Ki-67 index of 32%. All IHC stainings were performed on full tissue sections.

The qPCR method, using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen), has recently been described.[14] Primers for SOX11 were: forward 5' -CATGTAGACTAATGCAGCCATTGG-3', reverse 5' -CACGGAGCACGTGTCAATTG-3' and for β-actin: forward 5'-AAAGACCTGTACGCCAACACA-3', and reverse: 5' -AGTACTTGCGCTCAGGAGGA-3. Samples were run in triplicates and the relative amount of SOX11 (Ct) was calculated from the expression of the housekeeping gene, β-actin, for respective sample, and then related to the relative expression of SOX11 in a reactive lymph node (ΔCt). Data quantification and analysis were conducted in the CFX manager (BioRad).

Among the MCL cases the relative fold increase (RFI) values ranged from 0.01-7419.41. There was no correlation between tumor cell content and SOX11 RFI values (P=0.73) (*Online Supplementary Figure S1*). The clinical and pathological characteristics of the 10 % of cases with lowest SOX11 mRNA expression (SOX11 low) as compared to the other MCL cases are presented in Table 1. Nodal disease was present in 22% of SOX11 low cases and in 68% of SOX11 high cases (P=0.01). Furthermore, the SOX11 low cases were associated with lymphocytosis in 60% of cases while this feature was only present in 16% of SOX11 high cases (P=0.005). Thus our findings are in line with previous data suggesting that low SOX11

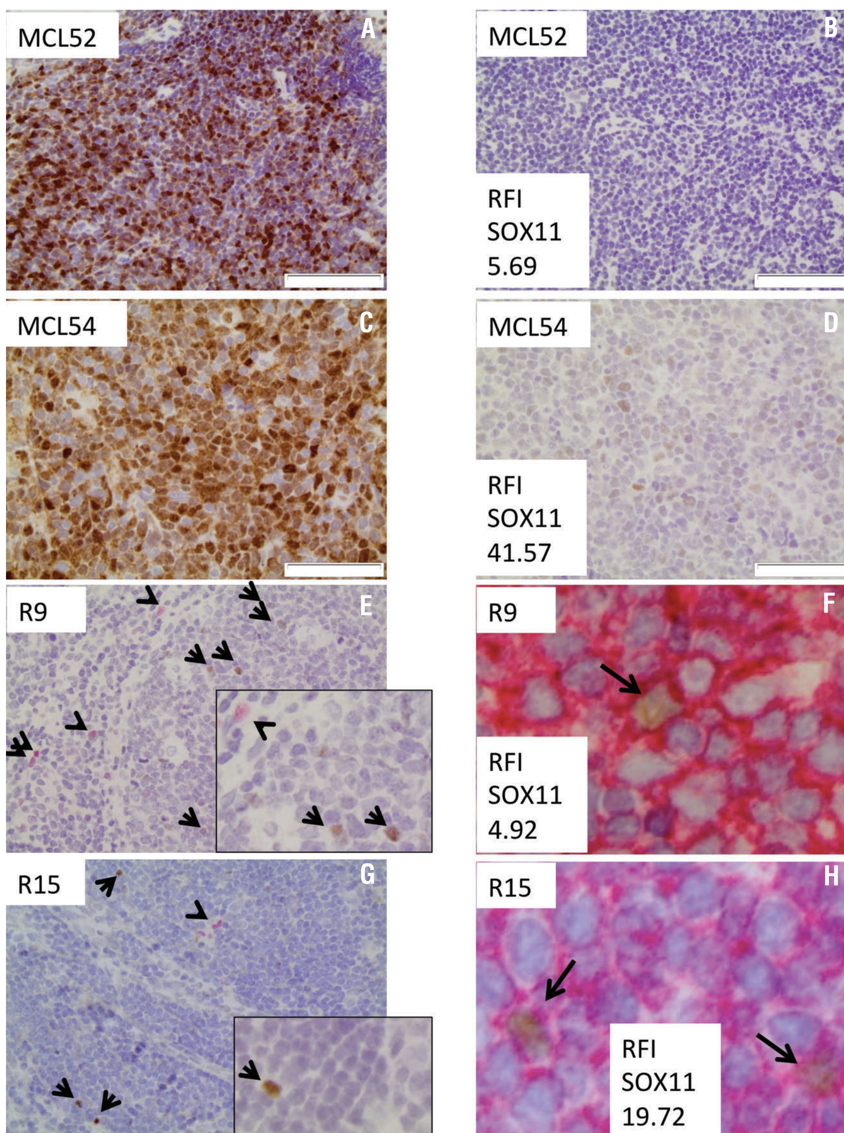

Figure 1. SOX11 immunohistochemistry in MCL with low SOX11 mRNA expression and in reactive lymph nodes. (A and B): MCL (case 52) stained for cyclin D1 (A). The SOX11 RFI value was 5.69 and the SOX11 staining (B) is negative. (C and D). MCL (case 54, blastoid MCL) stained for cyclin D1 (C). The SOX11 RFI value was 41.57 and SOX11 staining (D) is very weak. (E and F). Reactive lymph node (sample 9) had a SOX11 RFI value of 4.92. The tissue was stained for cyclin D1 in red (arrowheads) and SOX11 in brown (arrows) (E). $SOX11^+$ cells are sparse and negative for cyclin D1 and double staining with CD20 (F) demonstrates that the $SOX11^+$ cells are B cells. (G and H). Reactive lymph node (sample 15) had a SOX11 RFI value of 19.72. Double staining for cyclin D1 in red (arrowheads) and SOX11 in brown (arrows) demonstrates a few $SOX11^+$, cyclin $D1^-$ cells (G) which by double staining for CD20 (H) were confirmed to be B-cells. Original magnification x 40.

expression is often associated with non-nodal leukemic disease.[12,16] Analysis of overall survival (OS) in 73 patients who had not received autologous stem cell transplantation (ASCT) showed that the quartile of patients with the lowest SOX11 mRNA levels had significantly shorter OS (*Online Supplementary Figure S2*), supporting previous studies where SOX11 has been evaluated by IHC.[6,10] It is important to note that since the studied cohort was selected based on the availability of frozen material in a non-homogenously treated patient group, our current results should be interpreted with some caution. Out of the 10 cases in the SOX mRNA low group, 8 were SOX11⁻ by IHC using the MRQ-58 antibody as exemplified for MCL 52 in Figure 1A,B. However, two cases were clearly SOX11⁺ by IHC, the weakest expression among these cases (MCL 54) is shown in Figure 1C,D. All of the 92 cases with high SOX11 mRNA were SOX11⁺ by IHC.

We further investigated whether the SOX11 mRNA expression in non-malignant lymph nodes could serve as

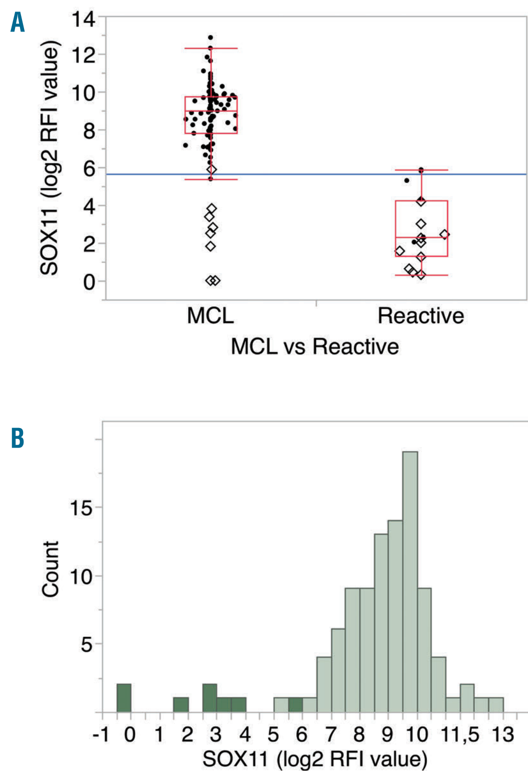

Figure 2. Analysis of SOX11 expression by quantitative PCR. (A). Samples are grouped into MCL and reactive lymph nodes. Diamonds mark cases that are SOX11- by IHC. The two lowest cases with RFI 0.98 and RFI 0.01 are grouped together for clarity. The log2 SOX11 RFI values for these cases were -0.02 and -6.64, respectively. The mRNA expression levels were significantly different between the entire group of MCL and all reactive lymph nodes (P<0.0001) but not between the MCL SOX11⁻ by IHC and reactive lymph nodes (Wilcoxon). The blue line represents the mean of the means of the log2 SOX11 RFI of MCL and reactive lymph nodes, 5.7. (B). Distribution of the SOX11 mRNA expression. All 102 MCL cases are included in this frequency plot. The 8 cases that did not express SOX11 by IHC are shown in dark grey. The two lowest cases with RFI 0.98 and RFI 0.01 are grouped together for clarity. The log2 SOX11 RFI values for these cases were -0.02 and -6.64, respectively. The mRNA expression in the 94 cases that were SOX11⁺ by IHC is normally distributed and there is no natural cut-off to define cases that weakly express mRNA.

a relevant detection cut-off for defining SOX11 positive MCL. To that end, 16 consecutive lymph nodes, removed for diagnostic purposes on the suspicion of malignancy but with no evidence of malignant disease or clonal lymphoid populations, were used. The SOX11 RFI values in these lymph nodes ranged from 1.26-58.35 which was significantly lower than in the whole MCL group (P<0.0001) (Figure 2A), but not significantly different from the SOX11 low MCL cases [(P=0.24 (Wilcoxon)]. SOX11 IHC of the reactive lymph nodes demonstrated a few scattered SOX11⁺ cells in 6/16 cases. These cells, which were mostly located in mantle zones, were morphologically similar to small lymphocytes and were CD20⁺ but cyclin D1⁻, as exemplified by reactive lymph node R9 (Figure 1E, F). In one case of reactive hyperplasia (R15) 1/67 follicles contained scattered SOX11⁺, cyclin D1- cells (Figure 1G, H). Our qPCR data are in line with a previous report on low level of SOX11 mRNA expression in non-malignant B-lymphocytes by gene expression profiling[17] (*Online Supplementary Information*). In addition, our findings suggest that SOX11 protein expressing CD20⁺ cells might infrequently be detected during immune responses in reactive lymph nodes. Therefore it is important to score SOX11 by IHC only in the tumor areas of MCL.

The SOX11 mRNA levels for all MCL and reactive cases are presented in Figure 2A (log2 transformed RFI values for the clarity of presentation), which also indicates which cases were SOX11⁻ by IHC. The blue line in the figure represents the mean of the means of SOX11 levels in MCL and reactive lymph nodes, RFI 5.7. Only two out of 16 non-malignant lymph node biopsies had a value above 5.7, while 94/102 (92.2%) of MCL had SOX11 mRNA levels above 5.7. Seven of the eight MCL cases that were SOX11⁻ by IHC had RFI levels below 5.7 and 93/94 (98.9%) of MCL cases that were immunohistochemically SOX11⁺ had mRNA values above 5.7. These results suggest that there is a good correlation between SOX11 expression at the mRNA and protein level (using MRQ-58 antibody) as recently shown by ourselves[14] and Soldini *et al.*[13] Using the cut-off value of 5.7 resulted in a discordant classification of only 2/102 MCL by these two methods in our study. Importantly, the SOX11 mRNA levels as measured by qPCR were normally distributed among the MCL cases that were SOX11⁺ by IHC (Figure 2B). Thus among the SOX11 protein positive cases, the mRNA analysis could not identify a natural cut-off that would identify cases with low expression. Our results therefore suggest that cyclin D1⁺ MCL can be reliably defined as SOX11 positive or negative either by qPCR analysis or by scoring IHC staining for SOX11 in the tumor areas with the MRQ-58 antibody. Based on our results, we propose that MCL with weak and variable IHC SOX11 expression should be considered SOX11⁺ and cases with no SOX11 positivity in tumor cell areas should be defined as SOX11⁻. The currently used gold standard, immunohistochemical analysis of SOX11 by the MRQ-58 antibody, seems to be justified when compared to qPCR methodology.

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