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ORIGINAL ARTICLE

High-resolution chromatin immunoprecipitation (ChIP) sequencing reveals novel binding targets and prognostic role for SOX11 in mantle cell lymphoma

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Sex determining region Y-box 11 (SOX11) expression is specific for mantle cell lymphoma (MCL) as compared with other non-Hodgkin's lymphomas. However, the function and direct-binding targets of SOX11 in MCL are largely unknown. We used high-resolution chromatin immunoprecipitation sequencing to identify the direct target genes of SOX11 in a genome-wide, unbiased manner and elucidate its functional significance. Pathway analysis identified WNT, PKA and TGF-beta signaling pathways as significantly enriched by SOX11-target genes. Quantitative chromatin immunoprecipitation sequencing and promoter reporter assays confirmed that SOX11 directly binds to individual genes and modulates their transcription activities in these pathways in MCL. Functional studies using RNA interference demonstrate that SOX11 directly regulates WNT in MCL. We analyzed SOX11 expression in three independent well-annotated tissue microarrays from the University of Wisconsin (UW), Karolinska Institute and British Columbia Cancer Agency. Our findings suggest that high SOX11 expression is associated with improved survival in a subset of MCL patients, particularly those treated with intensive chemotherapy. Transcriptional regulation of WNT and other biological pathways affected by SOX11-target genes may help explain the impact of SOX11 expression on patient outcomes.

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INTRODUCTION

Mantle cell lymphoma (MCL) represents ~6% of all non-Hodgkin's lymphoma, which is the commonest hematological malignancy worldwide. Despite advances in chemotherapy and immunotherapy, the majority of MCL patients relapses and dies of their disease, and advances in prognostication and therapeutics are urgently needed.[1] The pathogenesis of MCL is characterized by cell cycle dysregulation and CCND1 (Cyclin D1) overexpression. However, CCND1 overexpression is not universal in this disease and mouse models overexpressing CCND1 do not recapitulate the phenotype of the disease,[2] indicating that additional genetic lesions are therefore necessary for lymphomagenesis and in need of characterization.

Sex determining region Y-box 11 (SOX11) immunohistochemical expression is present in the majority (78–93%) of MCL patients and is specific for MCL as compared with other non-Hodgkin's lymphomas.[3–5] SOX11 belongs to the SOXC family of high-mobility-group (HMG) transcription factors, which consists of SOX4, SOX11 and SOX12.[6] HMG transcription factors bind to, and facilitate conformational changes in DNA, that allow binding of other transcription factors to regulate downstream gene expression. SOXC transcription factors have overlapping roles in nervous system development and it is likely that these factors may have a role in B-cell development as well, as SOX11-null mice do not form spleens and SOX4-deficient mice do not form B lymphocytes.[6,7] Although SOX11 expression has been demonstrated in the majority of patients with MCL, data on its functional role in MCL pathogenesis are lacking. Gene expression arrays have been used to compare the transcriptional profiles of SOX11-transfected MCL cell lines to infer the genes regulated by SOX11.[8] However, this approach is limited by two aspects: (a) gene expression changes by SOX11 modulation may be indirect and not reflective of the direct-binding targets of this gene; (b) expression microarrays are limited by vendor-specific variation

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in coverage of human genome depending on array design. Next-generation sequencing of chromatin immunoprecipition (ChIP) products can identify direct-binding targets in an unbiased and genome-wide manner. We, therefore, performed ChIP-seq (chromatin immunoprecipition coupled with massive parallel sequencing) using the MCL cell line Granta-519 as a model to identify SOX11 direct-binding targets, which were then validated in MCL patients and cell lines. Based on our cell line data, we hypothesized that SOX11 negatively regulates cell proliferation *in vivo* and its expression may be associated with differences in chemotherapy sensitivity and survival in MCL. Prior studies examining the impact of SOX11 expression on patient outcomes have been hampered by small numbers and treatment heterogeneity. We, therefore, performed a meta-analysis of three well-annotated cohorts of patient samples to examine the association of SOX11 with patient outcomes and the impact of treatment on this association.

RESULTS

High-resolution ChIP sequencing identifies SOX11-binding targets in critical cellular pathways regulating proliferation in MCL cell lines and patients

Genomic DNA from the Granta-519 MCL cell line was immunoprecipitated by SOX11 antibody and IgG and genomic enrichment sites were sequenced by Illumina Hiseq2000 system (Illumina, San Diego, CA, USA). Using stringent statistical criteria (*P*-value less than 0.01, fold change more than 1.5) we identified significant enrichment of ChIP-seq peaks (Supplementary Tables S1 and S2) corresponding to 1912 unique genes common in both replicates (Supplementary Table S3). Figure 1a showed the genomic distribution of genomic DNA fragments directly bound by SOX11. The most common motifs enriched in the SOX11-binding targets contained the core (T/A) TTGT sequence (Figure 1b) bound by highly related members of the SOX family of transcription factors.[9,10] Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of SOX11-binding targets revealed the most significant network to be 'pathways in cancer' ($P < 10^{-12}$) which were enriched in WNT and transforming growth factor, beta (TGF-beta) pathway genes (Figure 1c, Supplementary Figure S1A). We validated direct binding of SOX11 to SMAD3, transforming growth factor, beta receptor 1 (TGFBR1), WNT4, nemo-like kinase (NLK) and protein kinase A catalytic unit alpha (PRKACA) from these pathways as well as known SOX11 targets SET domain and mariner transposase fusion gene (SETMAR), DBN1 (drebrin 1) and HIG2 (hypoxia-Inducible Protein 2)[8] by quantitative PCR of ChIP products from Granta-519, Z138 and JEKO-1 (Figure 1d, Supplementary Figures S1B and C).

SOX11 directly binds to and transcriptionally regulates genes in the WNT signaling pathway in MCL

Pathway analysis of ChIP-seq data identified several SOX11-binding targets in the WNT pathway (Figure 2a, marked in red). Among these SOX11-binding targets in the WNT pathway, NLK and SMAD3 are of particular interest for their roles in regulating the WNT pathway. NLK, a SOX11 direct-binding gene, is a negative regulator of WNT signaling pathway and has been shown to

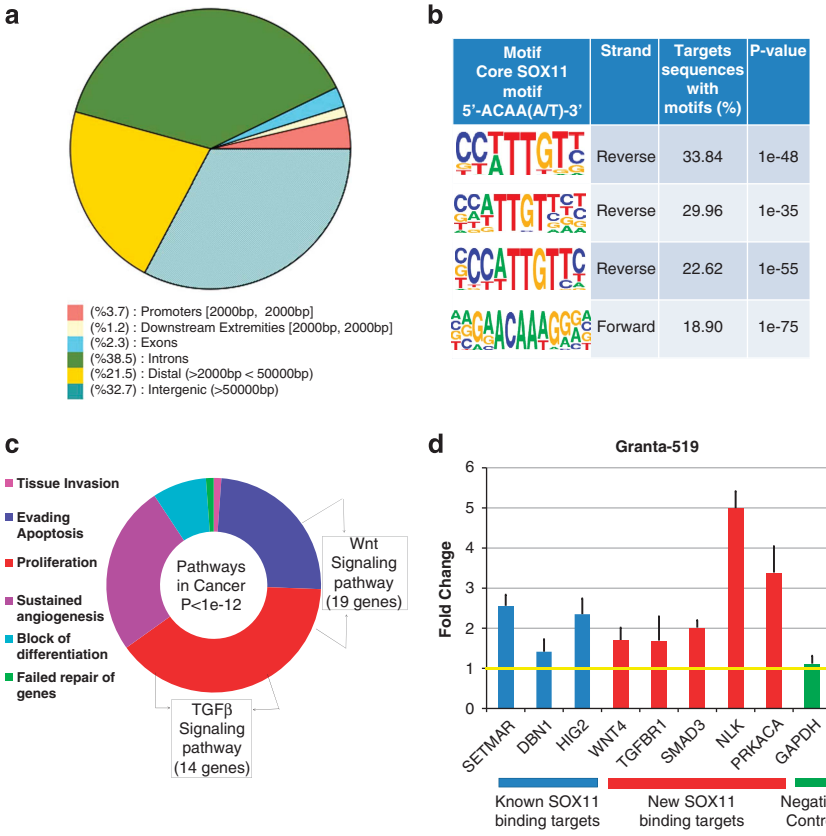


Figure 1. High-resolution ChIP sequencing identifies SOX11-binding targets in critical cellular pathways regulating proliferation in MCL cell lines and patients. (**a**) Genomic distribution of SOX11 direct-binding peaks identified by ChIP-seq. (**b**) Analysis of motifs enriched in SOX11 ChIP-seq. (**c**) Critical intracellular processes within the most significant pathway enriched by SOX11-target genes are indicated in the pie chart. WNT (19 SOX11-binding targets) and TGF-beta signaling (14 SOX11-binding targets) occur within this pathway and independently in the list of top 10 pathways. (**d**) Single locus validation of ChIP by PCR showing fold enrichment of target genes on the Y axis and individual genes on X axis. GAPDH was used as a negative control and SETMAR, DBN1 and HIG2, known SOX11 targets, were used as a positive control.

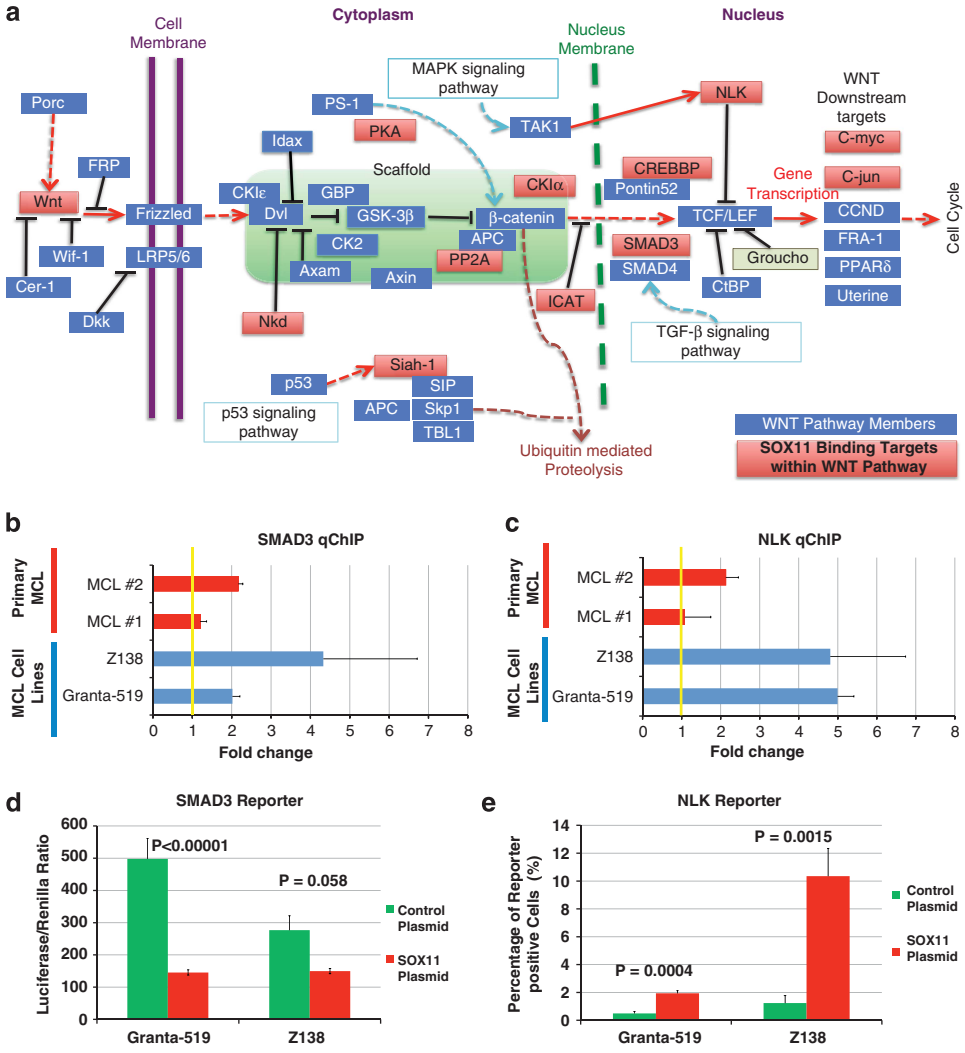


Figure 2. SOX11 directly binds to and transcriptionally regulates genes in the WNT signaling pathway in MCL. (**a**) SOX11-binding targets predicted by ChIP-seq in the canonical WNT pathway are indicated in red. (**b**, **c**) Quantitative PCR of ChIP products from MCL cell lines and primary MCL samples showing fold enrichment of SMAD3 (**b**) and NLK (**c**) with anti-SOX11 antibody compared with IgG control. Error bars represent s.d. (**d**) SMAD3 promoter reporter assay showing repression of SMAD3 promoter activity after SOX11 overexpression in Granta-519 cells. Control or SOX11 plasmid along with SMAD3p-Luc and *Renilla* plasmid were co-transfect into Granta-519 cells. SOX11 binding to the promoter of SMAD3p-Luc repressed the expression of firefly luciferase reporter gene. SMAD3 reporter activity was represented by luciferase to *Renilla* ratio. This is a representative view from three individual experiments. Error bars represent s.d. (**e**) NLK promoter reporter assay showing specific increase of NLK activity after SOX11 expression in Granta-519 cell line. NLK promoter region containing SOX11 consensus-binding sequence upstream of the transcription starting site was cloned before the mCherry reporter gene. Control or SOX11 plasmid along with NLK promoter reporter construct were electroporated into Granta-519 cells. SOX11 binding to the promoter region of NLK reporter construct specifically modulates the expression of mCherry reporter gene. Percentage of mCherry-positive cells was analyzed using FACS. NLK reporter activity is represented by the percentage of reporter gene-positive cells (mCherry-positive) in total viable cells. The bar chart shows the average of three independent experiments. Error bars represent s.d.

downregulate the β-catenin/T-cell factor/lymphoid enhancer factor-1 (TCF/LEF) transcription activity via phosphorylation of TCF4 to prevent DNA binding of the β-catenin/TCF4 complexes.[11] SMAD3 is a key mediator between the TGFβ pathway and the WNT pathway[12] and has an important role in activating WNT/ β-catenin pathway by protecting β-catenin from ubiquitin–proteasome-dependent degradation and mediating its nuclear translocation.[13] We validated the enrichment of SMAD3 and NLK (Figures 2b and c) in MCL cell lines and patients by ChIP coupled with quantitative PCR. ChIP coupled with quantitative PCR shows significant enrichment of SOX11 binding compared to IgG control in both primary MCL samples and MCL cell lines (Figures 2b and c). Additional ChIP-coupled with quantitative PCR validation of SOX11-binding targets of WNT pathway members, WNT4 and PRKACA, is also included in Supplementary Figures S2A and B. We further studied the effects of SOX11 binding to its target genes via reporter assays using promoter constructs of SMAD3 and NLK (Figures 2d and e), which contain a SOX11 consensus-binding sequence upstream of the transcription starting site. SOX11 binding to the reporter constructs modulates the transcription of the reporter genes (firefly luciferase in SMAD3 reporter construct and mCherry in NLK reporter construct) and thus the protein level of the reporter genes. The reporter assays showed that overexpression of SOX11 specifically increases NLK

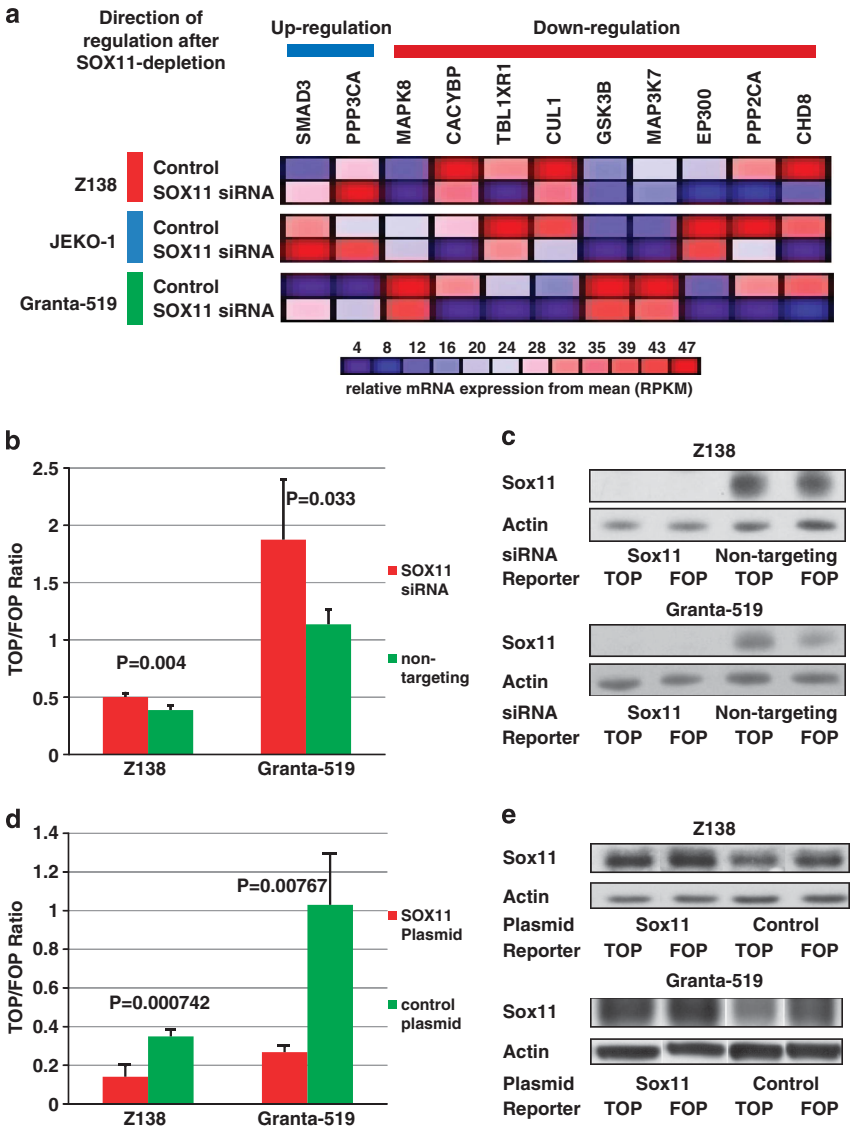


Figure 3. SOX11 functionally represses WNT/β-catenin activity in MCL. (**a**) Heatmap of the mRNA expression values of 11 genes enriched in the WNT pathway using gene-set enrichment analysis after SOX11 depletion by siRNA treatment in Granta-519, Z138 and JEKO-1 cells. (**b**) WNT/β-catenin signaling is assayed by the ratio of TOPFLASH:FOPFLASH reporter activity (Y axis) in Z138 and Granta-519 cells after SOX11 depletion by siRNA for SOX11. One representative experiment is shown out of three. Error bars represent s.d. (**c**) SOX11 protein levels are shown following transfection of siRNA in Z138 and Granta-519 cells compared with control non-targeting siRNA. (**d**) WNT/β-catenin signaling is assayed by the ratio of TOPFLASH:FOPFLASH reporter activity (Y axis) in Z138 and Granta-519 cells after SOX11 overexpression. One representative experiment is shown out of three. Error bars represent s.d. (**e**) SOX11 protein levels following transfection of SOX11 expression plasmid compared with empty vector control.

promoter activity by 6–12 times, while decreasing SMAD3 promoter activity by 1.8–3.5 times as compared with control plasmid in two MCL cell lines, Z138 and Granta-519.

Taken together, our data support direct binding and transcriptional regulation of WNT pathway members by SOX11 and demonstrated that SOX11 can exert both transcriptional activation and transcriptional repression functions in a gene-specific manner.

SOX11 functionally represses WNT/β-catenin activity in MCL cell lines

Next, we studied SOX11-mediated regulation of gene transcription by examining genes differentially expressed following SOX11 depletion in three MCL cell lines, Granta-519, Z138 and JEKO-1, using RNA-sequencing. Poly-A containing mRNA from both control (untransfected) cells and SOX11-depleted cells (48 h after small interference RNA (siRNA) transfection) were used for cDNA library construction. Comparing the differences in expression between SOX11-depleted and control samples on a gene-by-gene basis and then ranking them on the basis of a one-sample *t*-test of the paired, we obtained 2799 differentially expressed genes (Supplementary Table S4). Eleven genes in the WNT pathway were differentially expressed across all three cell lines after SOX11 depletion (Figure 3a). Downregulation of negative regulators of the WNT signaling pathway, such as CHD8 (chromodomain helicase DNA-binding protein 8),[14] GSK3B (glycogen synthase kinase-3 beta),[15] CUL1 (Cullin-1)[16] and CACYBP (calcyclin-binding protein),[17] were seen simultaneously with upregulation of SMAD3, which has been shown to stabilize and to assist in β-catenin nuclear translocation[13] after SOX11 depletion. These expression changes suggest that SOX11 represses WNT signaling.

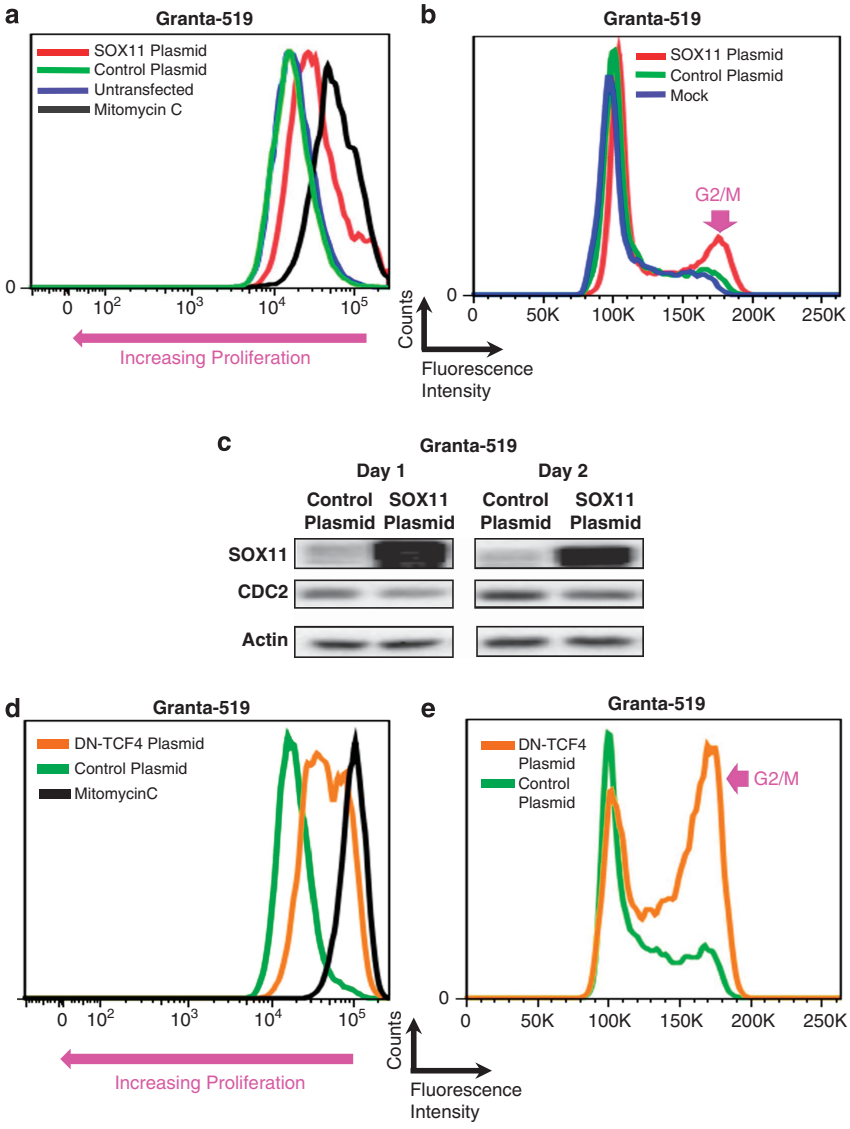


Figure 4. SOX11 overexpression induced G_2/M accumulation and repressed cell proliferation in MCL. (**a**) Effect of SOX11 overexpression on cell division in Granta-519 cells is assayed using CellTrace™ staining. FACS tracing of cell division in SOX11 overexpressing cells (red) is shown with fluorescence intensity on the X axis and number of events on the Y axis. Mitomycin C-treated (black) and tracings from untransfected cells (blue) and an empty vector (green/negative control) are shown for comparison. This is a representative view from three individual experiments. (**b**) Effect of SOX11 overexpression on cell cycle distribution in Granta-519 cells. FACS tracing of cell cycle distribution in Granta-519 cells following SOX11 overexpression (red) as compared with mock control cells (blue) and an empty vector (green/negative control) shows an increase in cells in the G_2/M phase. Representative view from three individual experiments is shown here. (**c**) Western blot showing protein expression of CDC2 and SOX11 24 and 48 h after transfection with SOX11 plasmid, as compared with control (empty vector) plasmid. Actin is shown as loading control. (**d**) Effect of DN-TCF4 overexpression on cell division in Granta-519 cells is assayed using CellTrace™ staining. FACS tracing of cell division in DN-TCF4 overexpressing cells (orange) is shown with fluorescence intensity on the X axis and number of events on the Y axis. Mitomycin C-treated (black) and tracings from an empty vector (green/negative control) are shown for comparison. This is a representative view from two individual experiments. (**e**) Effect of DN-TCF4 overexpression on cell cycle distribution in Granta-519 cells. FACS tracing of cell cycle distribution in Granta-519 cells following DN-TCF4 overexpression (orange) as compared with an empty vector (green/negative control) shows an increase in cells in the G_2/M phase. Representative view from two individual experiments is shown here.

To confirm the functional impacts of SOX11 on WNT signaling, we depleted SOX11 levels by siRNA and directly examined TCF/LEF activity using a reporter assay in MCL cell lines Granta-519 and Z138. Treatment of cells with anti-SOX11 siRNA resulted in significant increase ($P < 0.04$) in WNT activity (Figure 3b) compared with control non-silencing siRNA. Conversely, overexpression of SOX11 (Figure 3d) resulted in decreased WNT signaling ($P < 0.008$). Reduction of SOX11 and overexpression of SOX11 at the same time point (24 h for both SOX11 depletion and Sox11 overexpression) were confirmed at the protein level by western blotting (Figures 3c and e). Taken together, these observations confirm that SOX11 represses the WNT pathway in MCL.

SOX11 overexpression induced G_2/M accumulation and repression of cell proliferation in MCL

Previous studies have shown constitutive activation of the WNT pathway in primary MCL and identify the WNT pathway as important targets for regulating cell proliferation in MCL cell lines *in vitro*.[18,19] To test whether SOX11-mediated WNT pathway

repression can functionally repress cell proliferation, we transfected SOX11 using a CMV-driven SOX11-IRES-eGFP-expressing plasmid into Granta-519 cells and measured proliferation by tracing cell division in living cells using the Celltrace Violet Cell Proliferation Assay 5 days after plasmid transfection. Transfected cells (eGFP-positive fraction) showed significant proliferative arrest (Figure 4a) similar to Mitomycin-treated cells (black). Cell cycle analysis of SOX11 transfected cells showed an increased proportion of cells in the G_2/M phase (in red Figure 4b) as compared with eGFP (negative control plasmid) transfected cells. Three additional cell lines, Z138, HBL2 and JVM2, with varying levels of intrinsic SOX11 expression were similarly treated and showed proliferation arrest and an increase in G_2/M independent of p53 status (Supplementary Figures S3A–G).

The cell cycle progression from G_2 to M phase requires the activation of CDC2.[20–22] To investigate mechanisms involved in the regulation of G_2/M arrest by SOX11, we overexpressed SOX11 in Granta-519 and Z138 cells and tested the protein level of CDC2 by western blotting. As expected, our result showed that CDC2 protein level reduced as early as 24 h after SOX11 overexpression as compared with control plasmid in Granta-519 and Z138 cells (Figure 4c; Supplementary Figure S4).

We further confirmed that direct inhibition of WNT pathway downstream effector TCF/LEF activity via a dominant negative TCF4 (DN-TCF4) construct[23] can recapitulate the functional phenotypes of proliferation repression and G_2/M arrest induced by SOX11-forced expression. Using Granta-519 (Figures 4d and e) and Z138 (Supplementary Figures S5A and B) cell lines, we showed that DN-TCF4-transfected cells (orange) exhibited significant proliferative arrest (Figure 4d, Supplementary Figure S5A) compared with control plasmid-transfected cells (green). Cell cycle analysis of DN-TCF4-transfected cells (orange) showed a significantly increased proportion of cells in the G_2/M phase on day 2 as compared with eGFP (negative control plasmid) transfected cells (green) (Figure 4d, Supplementary Figure S5B).

SOX11 expression is associated with improved overall survival in MCL patients treated with R-Hyper-CVAD

We hypothesized that the repression of proliferation and cell cycle inhibition by SOX11 may confer differential sensitivity to chemotherapy used in the clinic to treat MCL patients. To test this hypothesis, we examined the association between SOX11 and patient outcomes in three independent cohorts of patients from three different countries with well-annotated treatment data. Tissue microarrays (TMA) of 131 patient tumor samples were stained for SOX11 and scored by two independent pathologists. Representative TMA sections staining for SOX11 are shown in Figure 5a. The association between SOX11 expression and overall survival was examined via Cox proportional hazard models separately for each cohort and treatment type (CHOP versus R-Hyper-CVAD) (Figures 5b and c, Supplementary Table S5). There was a statistically significant improvement in overall survival associated with higher SOX11 expression in patients treated with R-Hyper-CVAD[24] but not with CHOP (combined cohort, SOX11 low expression hazard ratio 6.96, $P < 0.0004$) (Figures 5b and c, Supplementary Table S5). Kaplan–Meier plots for Karolinska cohort and UW cohort stratified by treatments further showed a longer overall survival time in patients with high SOX11 expression receiving R-Hyper-CVAD (Figures 5d and e). This association retained significance following multivariate analysis incorporating MIPI and Ki-67 (adjusted SOX11 low expression hazard ratio 8.02, $P = 0.002$) (Supplementary Table S6). P53 overexpression is associated with mutation and loss of function in MCL, and can independently predict poor prognosis.[25] Furthermore, SOX4 has been shown to act as a tumor suppressor in non-small cell lung carcinoma by stabilizing p53 protein from Mdm2-mediated p53 ubiquitination and degradation.[26] We controlled for the impact of p53 expression in combined UW and Karolinska cohorts, and still found a statistically significant ($P = 0.004$) association between SOX11 expression and improved survival in R-Hyper-CVAD-treated patients (Supplementary Table S7). Our cell line data also showed inhibition of cell proliferation in both p53 wild type and p53-mutant cell lines (Figures 4a, Supplementary Figures S3A, C and G), suggesting that inhibition of MCL proliferation by SOX11 is independent of p53 status. A previous study by Wiestner *et al.*[27] showed MCL tumors having a higher level of cyclin D1 mRNA expression preferentially expressed the shorter cyclin D1a isoform and were associated with inferior survival in patients. Our observation of the treatment-specific prognostic value of SOX11 protein expression in patients receiving R-Hyper-CVAD appears to be independent from the level of cyclin D1. For this, we interrogated the relationship between SOX11 and CCND1 RNA and protein expression levels using gene expression array datasets and immunohistochemistry of primary tissue microarrays. SOX11 and cyclin D1 RNA expression had a Spearman rank-order correlation coefficient: $r = 0.41$ $P = 0.008$ for UNMC dataset (Iqbal, J. [UNMC] unpublished data) but differed from the NCI dataset (Wiestner, A. *et al.* [NCI] unpublished data) ($r = 0.32$, $P = 0.15$ for NCI dataset). Furthermore, the cyclin D1 IHC AQUA scores did not show any correlation to the SOX11 IHC AQUA scores (linear regression and correlation model; $R^2 = 0.05$, $P = 0.1$; TMA data from UW).

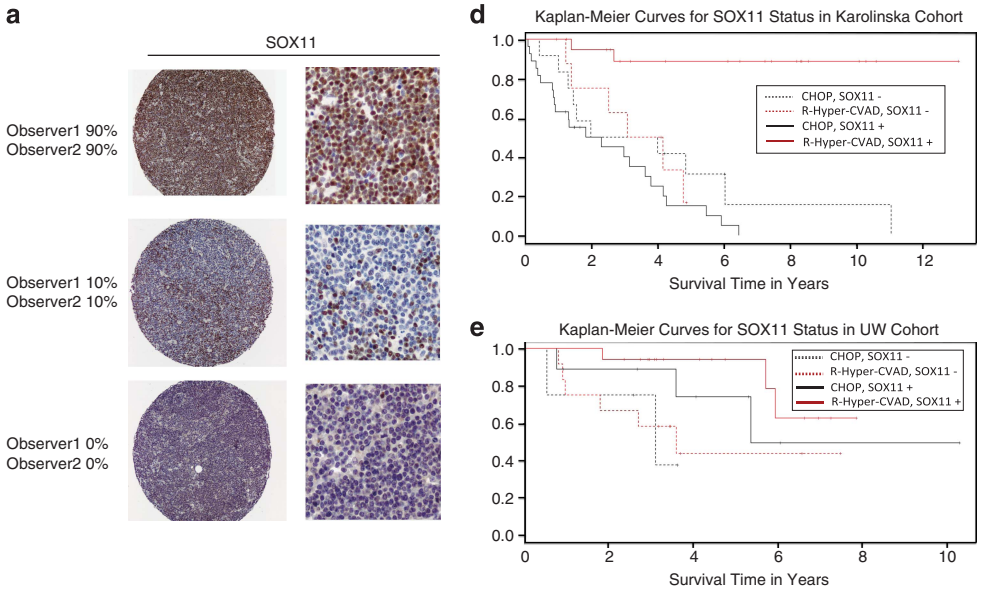
DISCUSSION

Our ChIP-seq results described the target genes directly bound to by the transcription factor SOX11 in MCL. On the basis of pathway analysis of the SOX11-binding targets, we predicted that functional networks associated with SOX11-binding targets could repress proliferation. We specifically demonstrated that the WNT signaling pathway, which controls expression of pro-proliferative genes such as Myc, is functionally repressed by SOX11.

WNT signaling pathway activation results in de-repression of the transcriptional activity of downstream effectors TCF/LEF (T-cell factor/lymphoid enhancer factor), which subsequently regulate target genes in diverse biological contexts including stem cell maintenance, embryonic patterning and tissue homeostasis.[28] WNT signaling must be tightly controlled as elevated β-catenin and TCF/LEF activity leads to cancer in many tissues.[29–32] Nuclear β-catenin expression and constitutively active WNT signaling have been previously demonstrated in MCL[18] and inhibition of WNT signaling by a depletion of WNT pathway intermediate DVL-2 by RNA interference or inhibitor of β-catenin reduces cell proliferation in MCL cell lines.[18] More recently, β-catenin-independent activation of TCF/LEF activities has also been demonstrated in MCL.[33]

The SOX transcription factors were first shown to physically interact and antagonize β-catenin activity in *Xenopus* embryos.[34] The interaction between SOXC transcription factors and WNT signaling has been described in colon carcinoma and other malignancies.[35,36] The SOX11/WT1 complex has been shown to regulate WNT4 in embryonic kidney cells.[37] NLK, a SOX11 direct-binding gene, is a negative regulator of the WNT signaling pathway and has been shown to downregulate the β-catenin/T-cell factor/Lymphoid enhancer factor-1 (TCF/LEF) transcription activity via phosphorylation of TCF4 to prevent DNA binding of the β-catenin/TCF4 complexes.[11] Our functional data showing direct repression of WNT signaling represent one pathway through which SOX11 can exert its anti-proliferative effect in MCL. In addition to WNT, other SOX11-regulated pathways, such as TGF-beta, may also contribute to its anti-proliferative effect (Figure 1c). Previous studies[38] using promoter array-based ChIP-chip have focused on plasmacytic differentiation as one aspect of its function.

SOX11 expression is present in pre-malignant lymph nodes examined in serial biopsies from MCL patients, suggesting that the



b

Cohort (Treatment)	HR (SOX11 low vs. SOX11 high)	SE	p-value	N
Karolinska (R-Hyper-CVAD)	9.97	2.28	0.005	29
UW (R-Hyper-CVAD)	4.53	2.28	0.07	17
Combined (R-Hyper-CVAD)	6.96	1.8	1e-04	46
Karolinska (CHOP)	0.53	1.49	0.12	40
UW (CHOP)	3.32	2.77	0.24	13
BCCA (CHOP)	1.25	1.52	0.61	32
Combined (CHOP)	0.89	1.32	0.65	85

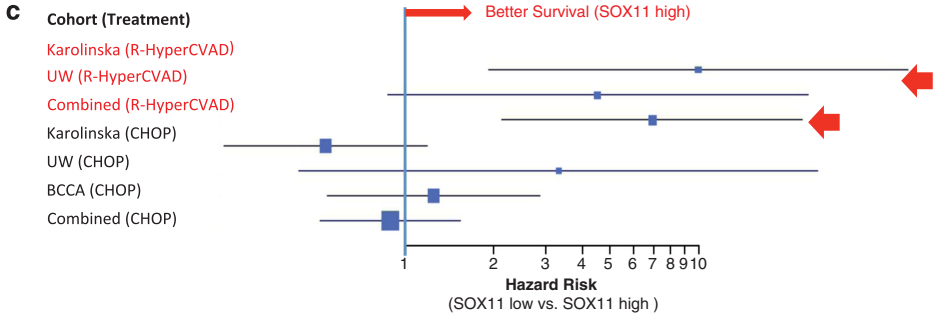


Figure 5. SOX11 expression is associated with improved overall survival in R-Hyper-CVAD-treated MCL patients. (**a**) Representative tissue microarray images from primary MCL patients with negative, low and high SOX11 expression scored by two independent pathologists. (**b**) Hazard Ratio of SOX11 expression and overall survival by treatment cohort. (**c**) Forest plot of the hazard ratio of SOX11 expression and overall survival by cohort. Cohorts marked in red arrows were statistically significant ($P < 0.05$) and thus had 95% confidence intervals that excluded a ratio of 1.0. (**d**, **e**). Kaplan–Meier plot for Karolinska cohorts (**d**) and UW cohorts (**e**) stratified by treatment.

expression of this protein is an early event in the malignant transformation of lymphocytes in MCL.[39] It belongs to the SOXC family of high-mobility-group transcription factor. High-mobility-group transcription factors are epigenetic regulators, facilitating conformational changes in DNA structure that allow binding of other transcription factors. It is interesting to note that the expression of SOX11 is itself controlled by epigenetic regulation. We have found SOX11 expression to be associated with promoter hypomethylation in both MCL cell lines and patient samples.[40] SOX11 expression in MCL has also been associated with activating histone marks (H3K9/14Ac and H3K4me3).[41]

Previous reports suggest that lack of SOX11 is associated with an indolent disease course in MCL,[3] where patients usually present with non-nodal disease and peripheral blood involvement and can be observed without therapy for months or years. These findings were contrary to a well-annotated independent Swedish study (which is part of our meta-analysis) where SOX11 was expressed in 50% of patients with indolent disease.[42] Additionally, analysis of an independent dataset from UW as part of our meta-analysis similarly showed that SOX11 was expressed in a majority of patients with indolent disease (10/11 patients were SOX11-positive). The majority of patients with indolent disease in these two cohorts had nodal involvement. Our meta-analysis of three independent cohorts of samples demonstrates a strong association between SOX11 and survival in R-Hyper-CVAD-treated patients. Intensive induction therapeutic approaches like R-Hyper-CVAD[24] and the Nordic[43] regimens have been associated with improved survival in MCL, but can cause considerable morbidity from myelosuppression, infection and other chemotherapy toxicities, particularly in older patients, which constitute the

bulk of the MCL population. Pre-selection of patients for intensive therapy is usually based on clinical parameters such as age and performance status, which can be interpreted subjectively. Our results present a unique opportunity for a bio-assay (SOX11 expression) as a way of pre-selecting patients at higher likelihood for better survival with these intensive chemotherapy regimens in MCL. The association of SOX11 expression and improved survival has also been seen in ovarian epithelial tumors.[44] Our results may be relevant beyond MCL to the other SOX11-expressing solid tumors, such as ovarian tumors,[45] medulloblastoma[46] and gliomas.[47]

SOX11 is associated with better survival in two of three cohorts in our meta-analysis, suggesting a strong treatment effect. The British Columbia Cancer Agency cohort,[48] which did not show this association, has more heterogeneity in treatments, as patients were collected over several years and also has fewer patients treated with intensive therapy.

In summary, we show that SOX11 directly binds to genes in critical intracellular pathways controlling cell cycle and proliferation in MCL. Further functional dissection using mouse models may help us understand the role of SOX11 in MCL pathogenesis. A subset of the SOX11-binding targets genes may identify new pathways for therapeutic intervention for the treatment of this challenging disease. Our results showing improved survival in SOX11 overexpressing MCL patients treated with intensive chemotherapy lay the foundation for examining SOX11 expression in prospective clinical trials, to ultimately support its routine incorporation as a bio-assay for pre-selecting patients more likely to benefit from intensive approaches in MCL.

MATERIALS AND METHODS

Patient samples for ChIP-seq

De-identified fresh frozen samples from MCL patient tumors were obtained from the tumor bank of the Hackensack University Medical Center, after IRB permission (IRB#: 07-11-437X) and in keeping with Helsinki protocols. A minimum of 2×10^7 cells were recovered after thawing each sample for ChIP and PCR.

Cell culture and transient transfection

MCL cell lines Granta-519, HBL2, JVM2, JEKO-1 and Z138 were grown in a humidified incubator at 37 °C and 5% CO_2 with Roswell Park Memorial Institute 1640 medium (Cellgro, Herndon, VA, USA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA, USA), 100 units/ml of penicillin G and 100 μg/ml of streptomycin (Cellgro). Transient transfection was done using the Amaxa 2B system (Kit T, program G016, Lonza, Basel, Switzerland).

Plasmid and siRNA constructs

SOX11 overexpression plasmid (Ex-M0425-M60), control plasmid (Ex-eGFP-M02) and NLK promoter reporter construct (CS-HPRM24544-PM02) were purchased from Genecopoeia (Genecopoeia, Rockville, MD, USA). WNT reporter constructs TOPFLASH (wild type, Addgene plasmid 12456) and FOPFLASH (mutated, Addgene plasmid 12457) were purchased from Addgene (Cambridge, MA, USA). SMAD3 reporter construct (SMAD3p-Luc) containing SOX11 consensus sequence was kindly gifted by Dr Thomas J Kelly.[49] DN-TCF4 was a kind gift by Dr Stuart A Aaronson[23] and was subsequently cloned into an expression vector containing IRES-eGFP (Ex-M60) for overexpression experiment. SOX11 siRNA sequences were listed in Supplementary Table 8.

ChIP, ChIP-seq and ChIP-PCR

A total of 10^8 or $2–3 \times 10^7$ of cells from MCL cell lines or MCL patients, respectively, were 1% formaldehyde-fixed, lysed and sonicated for 6×30 s in a Bioruptor sonicator (Diagenode, Denville, NJ, USA). Supernatants were precleared with protein A/G agarose beads (Roche, Indianapolis, IN, USA). Chromatin fragments were immune-precipitated by using an anti-SOX11 affinity-purified rabbit polyclonal antibody (HPA000536, Sigma-Aldrich, St Louis, MO, USA) or normal rabbit polyclonal antibody (Cell Signaling, Danvers, MA, USA) and purified using QIAquick PCR purification columns (Qiagen, Hilden, Germany) followed by 40 cycles of PCR amplification. Primers used for PCR are listed in Supplementary Table S8. A total of 10 ng of amplified DNA was used to prepare sequencing libraries using the Illumina ChIP-Seq HT Sequencing Library preparation protocol. Sequencing was performed using Illumina HiSeq2000 system (Illumina) with each library sequenced in a 50 bases single-read run. Reads were aligned to the *Homo sapiens* genome (Hg18) using Bowtie 9. Reads with more than four matches were excluded. Peak calling was performed by using the ChIPSeeqer[50] with *P*-value less than 0.01, fold change greater than 1.5 between peaks enriched by SOX11 antibody minus IgG control. Chip-seq was performed in two replicates and the genes in common between the two were used for further network and ontogeny analysis.

Gene network, gene ontology and motif analyses

A publicly available Gene Set Analysis Toolkit v2 (http://bioinfo.vanderbilt.edu/webgestalt/) was used to carry out network composition analyses. The genes in common from two ChIP-seq replicates were used for the Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis. A hypergeometric test was used to determine the pathways enriched in SOX11-target genes. The top 10 pathways with the most significant *P*-values were selected (Supplementary Figure S1A). Adjusted *P*-values were calculated based on the multiple test adjustment method proposed by Benjamini and Hochberg. Homer (Hypergeometric Optimization of Motif EnRichment) was used for motif discovery.[51]

RNA-sequencing and RNA-sequencing data analysis

Total RNA was isolated using PureLink RNA mini kit (Life Technologies, Carlsbad, CA, USA). In total, 100 ng of total RNA were used to construct cDNA libraries using TruSeq RNA sample preparation kit (Illumina) following the manufacturer's instructions. Sequencing was done on a HiSeq 2000 system using 100 bases, single-end read sequencing. All samples obtained at least 18 000 000 raw reads with higher than 88% of the clusters passing Illumina purity filter. Sequence reads were aligned to the human reference sequence (GRCh37/Hg19) using gsnap.[52] Aligned reads were subsequently analyzed using the RNA-Seq workflow module in Partek Genomics Suite 6.6 (Partek Inc., St Louis, MO, USA; http://www.partek.com/). All genes and transcripts have been normalized and assigned a relative coverage rate as measured in RPKM units ('reads per kilobase per million mapped reads') as described by Mortazavi *et al.*[53]

Statistical method for RNA-seq data analysis

Expression levels (X) in SOX11-depleted and control samples within each MCL cell line were compared on a gene-by-gene basis by computing the relative change in cell line expression (defined as: $D = X_{KO} - X_{control}/X_{CONTROL}$) and then ranked on the basis of a one-sample *t*-test. False discovery rate[54] was also computed in order to assess the statistical significance of genes in the context of multiple testing and to quantify the expected proportion of false positives.

Cell proliferation and cell cycle analysis

Cell proliferation was studied based on dye dilution using CellTrace Violet Cell Proliferation Kit (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instruction. In total, 2×10^6 cells/ml of MCL cells were labeled with CellTrace Violet at a final concentration of 4 μM in phosphate-buffered saline. Labeled Cells were then transfected with plasmids and cultured for 72 to 120 h. Cell cycle analysis was performed by staining MCL cells with 4 μg/ml of Hoechst 33342 (Life Technologies) in RPMI medium plus 10% fetal bovine serum at a density of 2×10^6 cells/ml for 45 to 60 min at 37 °C in the dark. A total of 2.5 ug/ml of propidium iodide was used for dead cell discrimination. FACS was performed using BD LSR II (Becton Dickinson, Mountain View, CA, USA) and data were analyzed with FlowJo (Tree Star Inc., Ashland, OR, USA). Transfected cells were gated based on the intensity of eGFP expression.

Reporter assays

WNT pathway activity was assayed with TOPFLASH/FOPFLASH constructs. SMAD3 activity is assayed with SMAD3p-Luc.[49] Reporter constructs were co-transfected with either SOX11 plasmid or siRNA (WNT reporter assay only) and *Renilla* luciferase (pGL4.74, Promega, Madison, WI, USA).

Luciferase activity was assessed using Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instruction on AutoLumat LB 953 Multi-Tube Luminometer and normalized to activity of *Renilla l*uciferase. WNT activity was expressed as the ratio of TOPFLASH/FOPFLASH. Experiments were performed in duplicate and repeated as least twice for each cell line. NLK reporter activity (CS-HPRM24544-PM02, Genecopoeia) was studied by con-transfection with SOX11 plasmid and analyzed by FACS. Results were represented as percentage of reporter positive cells averaged from three independent experiments.

Immuno-blotting analysis

Immuno-blotting was performed following standard procedures by using primary antibodies specific for SOX11 antibody (HPA000536), CDC2 (Cat# 9112, Cell signaling technology, Danvers, MA, USA) and actin antibody (C-11, HRP, Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

Immunohistochemistry

Immunohistochemistry was performed as previously described,[54,55] Anti-SOX11 antibody (HPA000536, Sigma-Aldrich) was used at 1:400 dilutions. Two independent pathologists scored protein at $\times 20$ objective magnification. SOX11 expression was identified as strong nuclear staining in lymphoid cells and quantified in 5% increments by visual estimation. Ki-67 IHC was performed using Ki-67 antibody (Clone SP6, Biocare Medical, Concord, CA, USA). The Ki-67 index was defined as the percentage of Ki-67-positive tumor cells in representative areas of lymphoma evaluated by one observer (DTY) counting 100 cells in two representative high power fields at $\times 400$ magnification for each core and then averaged between the triplicate cores for each case.

Patient cohort analysis

We estimated a Cox proportional hazards model for each cohort and treatment type (CHOP versus R-Hyper-CVAD) to examine the association between SOX11 and patient survival (defined as the time to death or last follow-up from the initiation of therapy) in three independent cohorts of patients. SOX11 was categorized using an *a priori* cut-point of 0.54 for each model. An exhaustive evaluation of all potential cut-points was performed for each cohort in order to validate the initial *a priori* selection. Cut-points in the range of 45–60% were similar with respect to the resulting association between the categorized SOX11 and overall survival. Furthermore, treating SOX11 as a continuous covariate resulted in similar findings within each cohort and treatment type. Multivariable cohort-stratified Cox PH models were estimated using the combined data set in order to examine the effect of SOX11 on survival after adjustment for other clinically relevant covariates (MIPI, ki67 and p53 status).

Forest plots using the rmeta package (version 2.14, R) were created in order to display cohort and treatment-specific hazard ratios along with corresponding 95% confidence intervals. The effect of SOX11 was estimated using cohort-stratified models.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)