



Chapter 8

Survivin expression in follicular lymphoma and transformed follicular lymphoma: a target for therapy?

Mariëlle J Wondergem¹, Martine ED Chamuleau¹, Laura M Moesbergen²,
Nathalie J Hijmering², Joost J Oudejans³, Sonja Zweegman¹, Josée M Zijlstra¹,
Chris JLM Meijer², Saskia AGM Cillessen²

1) Department of Hematology VU University Medical Center, Amsterdam
2) Department of Pathology VU University Medical Center, Amsterdam
3) Department of Pathology, Diakonessenhuis, Utrecht, The Netherlands.

Transformed lymphoma (TFL) is a high-grade aggressive non-Hodgkin lymphoma (mostly diffuse large B cell lymphoma, DLBCL) diagnosed in a patient with an underlying follicular lymphoma (FL). Transformation occurs in around 2-3% of FL patients per year and results in an inferior survival due to 30-40% of primary refractoriness to currently available treatment, consisting of rituximab in combination with chemo- and/or radiotherapy (1,2). In order to improve outcome, alternative treatments using novel targets are urgently needed. Survivin, a member of the family of inhibitor of apoptosis proteins, has a role in regulation of mitosis and inhibition of apoptosis and might be such a target (3). Firstly, it is overexpressed in many tumors including B-cell non-Hodgkin lymphoma and known to be associated with resistance to therapy and poor outcome (4,5,6). Furthermore, survivin expression is more elevated in aggressive lymphomas as compared to indolent lymphomas (7). Knockdown of survivin was found to inhibit cell growth and to restore apoptosis, resulting in a decreased survival in B-cell non-Hodgkin lymphoma cell lines and in animal models of aggressive B-cell non-Hodgkin lymphomas (8,9). Moreover, YM155 (sepantronium bromide), a small molecule inhibitor of survivin has shown activity in patients with refractory DLBCL and demonstrated synergistic activity with chemotherapy (10,11). Expression of survivin and the effect of its inhibition in TFL is unknown. Therefore, we investigated survivin expression in TFL patient samples and cell lines and examined the effect of inhibition of survivin by YM155.

We assessed survivin mRNA expression by MLPA (for material and methods: see supplemental data) in isolated lymphoma cells of seven patients with TFL, twelve patients with FL without the development of transformation (median FU time 64 months, range 2-125 months) and eight patients with FL patients that developed transformation later on during the disease course (median time between sample and TFL, 17 (range 7-84) months). Additionally, mRNA expression of survivin was examined in 10 non-transformed GCB-DLBCL and 10 non-transformed ABC-DLBCL patient samples. DLBCL were defined as GCB-like and ABC-like DLBCL by immunohistochemistry using the algorithms adopted from Choi et al. and Hans et al. (12).

Survivin expression in TFL patient samples was significantly higher than in FL samples of patients who never transformed (Figure 1A, p=0.0013). The mRNA survivin expression was equal in TFL and in both types of DLBCL (P=0.74).

When comparing survivin expression in FL patients that never transformed to survivin expression in FL patients that developed transformation during their disease course, expression was not significantly different (Figure 1A, P=0.18). However, this might be

caused by the long time interval between FL sample and diagnosis of transformation, as we found that survivin mRNA expression increased closer to diagnosis of transformation in the FL that developed TFL (Figure 1B). That this increase in survivin mRNA expression is of biological importance is supported by the fact that we did not observe a relation between follow up time and survivin expression in the FL that never transformed (Figure 1C). These data suggest that upregulation of survivin has a role in the process of transformation, for instance by favoring aberrant progression of transformed cells through mitosis bypassing apoptotic checkpoints, enabling accumulation of additional mutations and subsequent transformation (4,13). Whether upregulation of survivin expression in a FL will be able to predict transformation needs to be prospectively validated in more patients.

In order to unravel the functional role of survivin in more detail, additional functional analyses were performed in two TFL cell lines: WSU-DLCL2 and RL. Furthermore, survivin mRNA expression was examined in five DLBCL cell lines (SUDHL4, SUDHL5, HT, OCI-LY3, OCI-LY10). Expression in the TFL and DLBCL cell lines was similar to expression in the patient samples and higher when compared to tonsil B-cells from a healthy donor (Figure 1D). Using western blot analysis we could confirm the mRNA expression levels with actual protein expression levels of survivin in the cell lines (Figure 1E).

In non-malignant cells expression of survivin is cell cycle-dependent: In the G₁ phase, survivin is repressed whereas in the G₂/M phase it is highly expressed (3). However, in malignant cells its expression is independent of the cell cycle (14). This was similar in our TFL cell lines: with 17.9% and 10.4% of the cells in the G₂/M phase upregulation of survivin was evidently not caused by a G₂/M arrest. Possible factors that might upregulate survivin expression in malignant cells independent from the cell cycle might be loss of P53 and NF-κB activation, events known to occur frequently in TFL (13). Small molecule compound YM155 can block survivin expression via inhibition of the survivin promotor. To determine the cytotoxic effect of YM155 on TFL cells, cultured lymphoma cells from 3 TFL patients were exposed to increasing concentrations of YM155 for 24 hours. Treatment with YM155 demonstrated moderate levels of cell death (25-50%) in all TFL patient samples tested (Figure 2A).

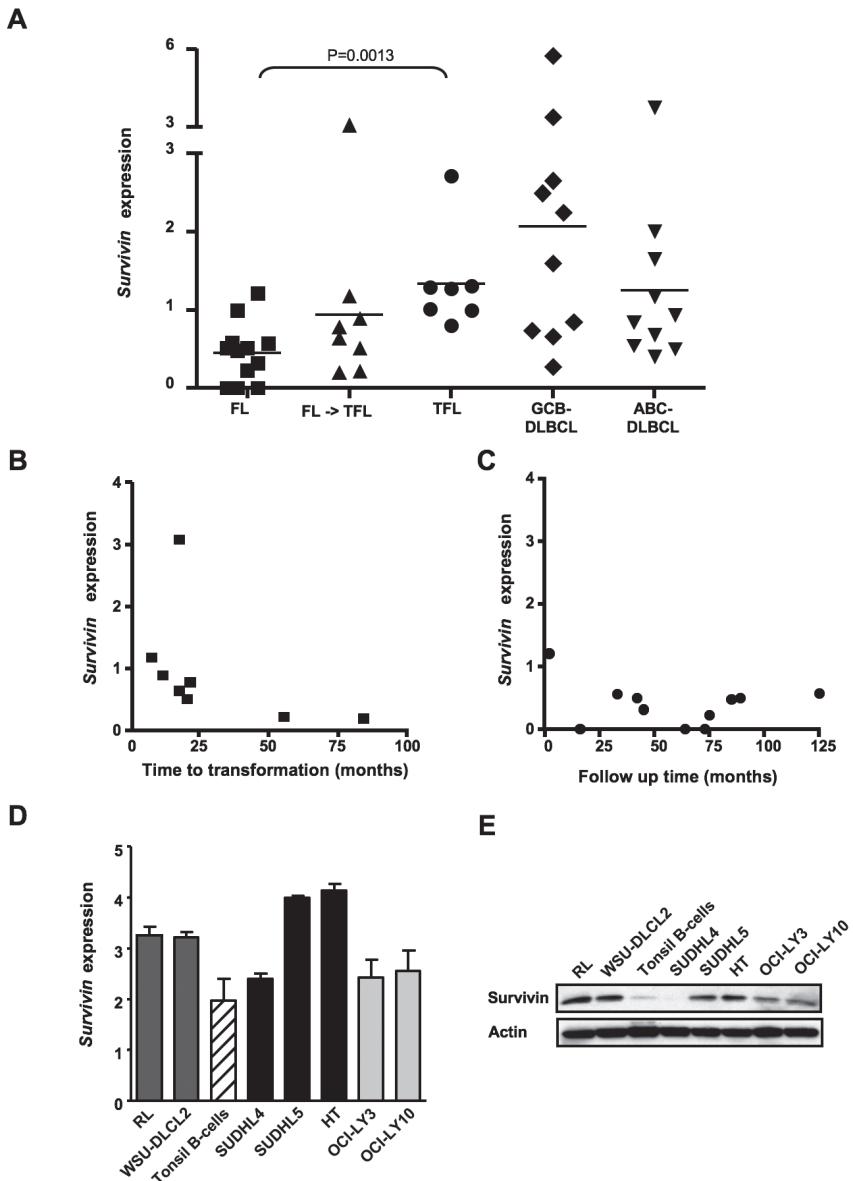


Figure 1. Upregulated survivin expression in TFL patient samples and TFL cell lines.

Figure 1: Survivin expression is depicted in relation to the expression of the housekeeping gene β -glucuronidase (A) Survivin mRNA expression in samples from patients with TFL, from patients with FL (grade 1 and 2), from patients with FL that developed transformation during the disease course (FL->TFL) and from patients with GCB- and ABC-DLBCL. (B) Relation between survivin mRNA and the time from sample to diagnosis of transformation expression in FL patients who developed transformation. (C) No relation between survivin mRNA expression and follow up time in FL patients who never transform. (D) Expression of survivin mRNA in TFL cell lines. (E) Protein expression of survivin in TFL cell lines.

The effect of YM155 on apoptosis sensitivity was further investigated in TFL cell lines. Both TFL cell lines were highly sensitive to the survivin antagonist (Figure 2B). Mean LD₅₀ values for TFL cell lines were 1.0nM for WSU-DLCL2 and 6.46nM for RL. SUDHL4 DLBCL cells with low survivin expression showed less sensitivity to YM155 (LD₅₀=41.0nM). Compared to patient samples, the TFL cell lines were more sensitive to YM155. These findings might be explained by the notion that cell lines are more proliferative compared to primary material. Moreover, Purroy et al demonstrated that the microenvironment of the tumor cells contributes to sensitivity to YM155 by increasing the proliferation state of the tumor cells (14). The relative low levels of sensitivity of the patient samples to YM155 are in agreement with other studies and might be overcome by continuous infusion with higher doses of YM155 as shown by *in vitro* and *in vivo* studies (8,9,14).

In order to investigate whether YM155 would be superior or additive to current treatments for TFL such as chemotherapy and rituximab, sensitivity to YM155 was compared with current therapies and was found to be higher than sensitivity to etoposide in both TFL cell lines (67.6% more apoptosis with YM155 for RL, P<0.001; 82.6% for WSU-DLCL2, P=0.0016). Accordingly, levels of YM155-induced cell death of RL cells were higher compared to rituximab-induced cell death (24.4%, P=0.0016). In contrast, no difference in cell death of WSU-DLCL2 cells was found after YM155 and rituximab exposure (Figure 2C). Furthermore, we investigated if survivin antagonist YM155 could sensitize TFL cells to etoposide or rituximab. Therefore, WSU-DLCL2 and RL cells were treated with YM155 in combination with etoposide or rituximab for 24 hours. No significant additional increase in apoptosis was detected by combining

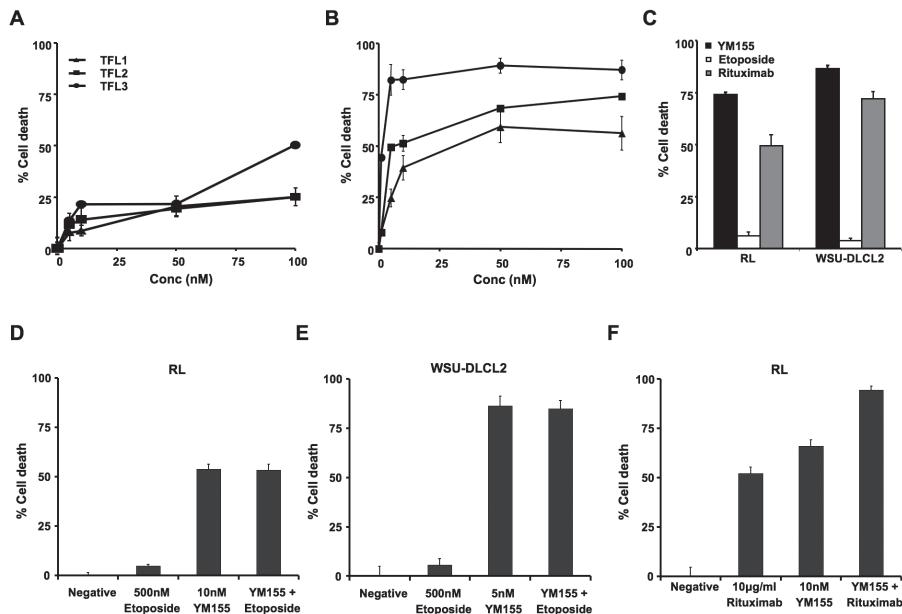


Figure 2. The effect of YM155 as single agent and in combination with etoposide and rituximab in TFL cells.

Figure 2 (A) Dose-response curves of TFL patient samples after 24 hours of treatment with YM155. (B) Dose-response curves of TFL cell lines RL (■) and WSU-DLCL2 (●) and DLBCL cell line SUDHL4 (◆) with YM155. (C) Detection of cell death in TFL cell lines treated with 10nM YM155 (■), 500nM etoposide (□) or 10 μ g/ml rituximab (▨) for 24 hours. (D+E) RL and WSU-DLCL2 cells were incubated with 10nM YM155 antagonist either with or without 500nM etoposide. (F) RL cells was incubated with 10nM YM155 antagonist either with or without 10 μ g/ml rituximab.

YM155 with etoposide in both cell lines (Figure 2D+E). In contrast, the combination of YM155 with rituximab showed an increase in cell death compared with either agent alone. (Figure 2F) This observation is in agreement with a recent clinical study in DLBCL (9). Subsequently, apoptosis regulating mechanisms by which YM155 induces cytotoxicity in TFL tumor cells were examined. We first investigated the effect of YM155 on survivin expression in TFL. Significant downregulation in protein expression levels of survivin was detected after incubation with YM155 in WSU-DLCL2 and RL using western blot analysis (Figure 3A) These findings are consistent with results in leukemia cells (15). Treatment with YM155 showed a dual effect: a decrease in the number of cells in S- and G₂/M phase was seen, in agreement with a study on the effect of YM155 in chronic lymphocytic leukemia, indicating decreased proliferation (Figure 3E) (14). Additionally a clear increase in the subG₁ population was observed consistent with an increase in apoptotic cells.

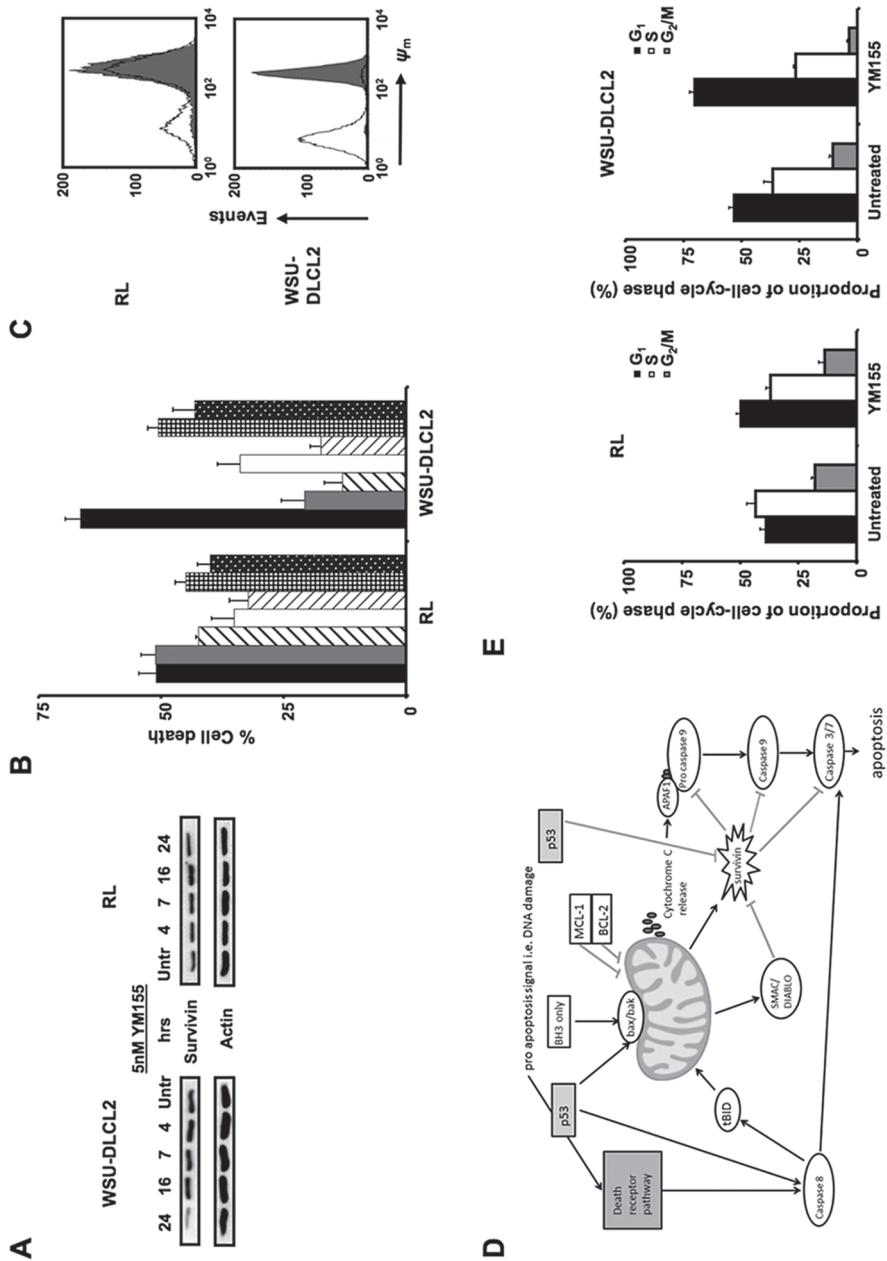


Figure 3. Apoptosis regulating mechanisms by which survivin antagonist YM155 induces cell death

Figure 3 (A) Protein expression of survivin from a time-course treatment of TFL cells with 10nM YM155 using western blot analysis. (B) Cell death detected in TFL cell lines after treatment with 10nM YM155 in combination with increasing concentrations of the pancaspase inhibitor z-VAD-fmk, or the caspase-8 inhibitor LEHD-fmk, the caspase-9 inhibitor IBD, respectively for 16 hours. 10nM YM155 (■), YM155 + 10μM zVAD-FMK (▨), YM155

+ 25 μ M z-VAD-fmk (□), YM155 + 10 μ M LETD-FMK (□), YM155 + 25 μ M LETD-FMK (▨), YM155 + 10 μ M LEHD-FMK (checkered), and YM155 + 25 μ M LEHD-FMK (dots). (C) TFL cell lines were treated with or without 10nM YM155 for 24 hours and stained with TMRE. $\Delta\Psi$ was determined as the % unstained cells in YM155 treated samples – the % unstained cells in untreated samples. (D) Schematic overview of intrinsic and extrinsic apoptosis pathway, incorporating the anti-apoptotic function of survivin. Inhibition of survivin by YM155 will lead to restoration of the caspase-9 mediated intrinsic apoptosis pathway and reactivation of effector caspases 3 and 7. (E) Cell cycle analysis of TFL cells after treatment with 10nM YM155.

Addition of pancaspase inhibitor z-VAD-fmk inhibited YM155 induced killing, indicating that the induced cell death is caspase-dependent (Figure 3B). Moreover, treatment with YM155 resulted in clearly detectable loss of mitochondrial membrane potential (Ψ_m) (Figure 3C) and experiments with a specific caspase-8 inhibitor LETD-fmk and caspase-9 inhibitor LEHD-fmk demonstrated that the observed cell death was more dependent on caspase-8 than caspase 9 in TFL cell lines (Figure 3B). These findings suggest that YM155-induced apoptosis in TFL involves activation of both the intrinsic and the extrinsic apoptosis pathway (Figure 3D) which is in agreement with a report in leukemia cells (15).

In conclusion, survivin expression in TFL is higher than in FL and comparable to DLBCL. Survivin expression levels in FL patients that develop TFL seem to increase closer to the diagnosis of transformation. However, survivin expression levels do not increase over time in samples from FL patients that never transform, suggesting a role for survivin in the process of transformation. Our preclinical data show that inhibition of survivin by YM155 induces apoptosis in TFL patient samples and TFL cell lines. Whether upregulation of survivin expression in FL is a predictor of transformation deserves prospective clinical evaluation.

References

1. Villa D, Crump M, Panzarella T et al. Autologous and allogeneic stem-cell transplantation for transformed follicular lymphoma: a report of the Canadian blood and marrow transplant group. *J Clin Oncol.* 2013;31(9):1164-1171
2. Eide MB, Lauritzsen GF, Kvalheim C et al. High dose chemotherapy with autologous stem cell support for patients with histologically transformed B-cell non Hodgkin Lymphomas. A Norwegian multi center phase II study. *Br J Haematol.* 2011;152(5):600-610
3. Mita AC, Mita MM, Nawrocki ST, Giles FJ. Survivin: Key regulator of mitosis and apoptosis and novel target for cancer therapeutics. *Clin Cancer Res.* 2008;14(16):5000-5005.
4. Li, F., Ambrosini, G., Chu, E. Y., et al. Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* 1998; 396:580-584
5. Markovic O, Marisavljevic D, Cemerikic-Martinovic V et al. Survivin expression in patients with newly diagnosed nodal diffuse large B cell lymphoma (DLBCL). *Med Oncol.* 2012;29(5):3515-3521.
6. Adida C, Haioun C, Gaulard P, et al. Prognostic significance of survivin expression in diffuse large B-cell lymphoma. *Blood.* 2000;96(5):1921-1925.
7. Tracey L, Pérez-Rosado A, Artiga MJ, et al. Expression of the NF-kappaB targets BCL2 and BIRC5/Survivin characterizes small B-cell and aggressive B-cell lymphomas, respectively. *J Pathol.* 2005;206(2):123-134.
8. Jacquemin G, Granci V, Gallouet AS et al. Quercetin-mediated Mcl-1 and survivin downregulation restores TRAIL-induce apoptosis in non-Hodgkin's lymphoma B cells. *Haematologica* 2012;97(1):38-46
9. Kita A, Mitsuoka K, Kaneko N, et al. Sepantonium Bromide (YM155) enhances response of human B-cell non-Hodgkin lymphoma to rituximab. *J Pharmacol Exp Ther.* 2012;343(1):178-183.
10. Tolcher AW, Mita A, Lewis LD, et al. Phase I and pharmacokinetic study of YM155, a small-molecule inhibitor of survivin. *J Clin Oncol.* 2008;26(32):5198-5203.
11. Kaneko N, Mitsuoka K, Amino N, et al. Combination of YM155, a survivin suppressant, with bendamustine and rituxima: a new combination therapy to treat relapsed/refractory diffuse large B-cell lymphoma. *Clin Cancer Res.* 2014;20(7):1814-1822.
12. Choi WW, Weisenburger DD, Greiner TC et al. A new immunostain algorithm classifies diffuse large B-cell lymphoma into molecular subtypes with high accuracy. *Clin Cancer Res.* 2009;15(17):5494-5502.
13. Pasqualucci L, Khiabanian H, Fangazio M, et al. Genetics of follicular lymphoma transformation. *Cell Rep.* 2014;6(1):130-140.
14. Purroy N, Abrisqueta P, Carabia J, et al. Targeting the proliferative and chemo resistant compartment in chronic lymphocytic leukemia by inhibiting survivin protein. *Leukemia.* 2014;28(10):1993-2004
15. Feng W, Yoshida A, Ueda T. YM155 induces caspase-8 dependent apoptosis through downregulation of survivin and Mcl-1 in human leukemia cells. *2013;435(1):52-57.*

SUPPLEMENTARY INFORMATION

Supplementary Methods

Lymphoma patient samples and cell lines

Cells from seven patients with TFL, twelve FL patients (grade 1 and 2) that did not develop transformation and eight FL patients that developed transformation later on during the disease course and twenty DLBCL patients, which were classified according to the World Health Organization (WHO) at the Comprehensive Cancer Center of Amsterdam, were included in this study.¹ All protocols for obtaining and studying human tissues and patients' data were approved within the local ethical procedures of the institutional ethical review board at the VU University Medical Center and complied with the Code for Proper Secondary Use of Human Tissue in The Netherlands (www.federa.org). Informed consent was provided according to the Declaration of Helsinki.

Lymphoma suspensions were isolated from tissue biopsies by a combination of mechanical dissociation and collagenase/DNase digestion, and frozen until further testing.² Cells were thawed 1 hour prior to experimental testing and cultured in Iscove's modified Dulbecco's medium (BioWhittaker, Lonza, Belgium) supplemented with 40% fetal calf serum, 100 IU penicillin/100µg/ml streptomycin (1% P/S) at 37°C with 5% CO₂ in a humidified atmosphere.

TFL cells were isolated and selected similar as DLBCL patient cells, as described previously.² FL tumor cells were FACS-sorted from tissue biopsies based on phenotype obtained by immunohistochemistry using antibodies against the cell surface markers including CD20, CD10, CD5 (all BD biosciences, San Jose, CA, USA).

The following cell lines were used in this study; TFL cell lines: RL and WSU-DLCL2 and DLBCL cell lines: SUDHL4, SUDHL5, HT, OCI-LY3 and OCI-LY10. WSU-DLCL2, SUDHL4 and HT were cultured in RPMI 1640 medium (BioWhittaker) containing 10% fetal calf serum and 1% P/S. RL cells were cultured in RPMI 1640 medium (BioWhittaker) containing 20% fetal calf serum and 1% P/S. SUDHL4 and OCI-LY10 were cultured in IMDM medium (BioWhittaker) containing 10% fetal calf serum and 1% P/S.

RT-MLPA analysis

Total RNA was isolated from primary FL and TFL cells and TFL and DLBCL cell lines. Reverse Transcriptase-Multiplex Ligation-dependent Probe Amplification (RT-MLPA) was performed on total RNA as described previously.^{3,4} For detailed description of

the design of the probes, www.mrc-holland.com. Survivin expression data were analyzed using GeneMapper (Applied Biosystems, Warrington, UK) and Coffalyser 7 (MRC-Holland, Amsterdam, the Netherlands) software. The β -glucuronidase (GUS-B) housekeeping gene was used as internal reference.

Assessment of cell death

Primary TFL cells and TFL cell lines were incubated with increasing concentrations of YM155 (Selleckchem.com, USA) for 24 hours. TFL cell lines were pre-incubated with increasing concentrations of the caspase-8 inhibitor LEHD-FMK, the caspase-9 inhibitor LEHD-FMK and pancaspase inhibitor z-VAD-fmk (Enzo life sciences, Lausen, Switzerland) 1 hour prior to treatment. Etoposide (Sigma, St Louis, MO, USA) was used to assess sensitivity to chemotherapy-induced apoptosis as it activates the intrinsic apoptosis pathway and therefore it is representative of the many chemotherapeutic drugs used in the treatment of TFL.^{5,6,7} TFL cell lines were incubated with 500nM etoposide for 24 hours. Sensitivity to rituximab-induced cell death was determined by incubation of TFL cell lines with 10 μ g/ml rituximab (Roche, Basel, Switzerland) for 15 minutes at room temperature. Subsequently normal human serum was added as source of complement and cells were incubated at 37°C for 24 hours.

Cell death of primary cells and TFL cell lines was determined as described previously.² Briefly, cell death was detected using a standard number of fluorescent beads (Fluorospheres, Becton Dickinson, San Jose, CA, USA) in combination with 7-amino-actinomycin D (7AAD, ViaProbe, BD Pharmingen, Erembodegem, Belgium) to determine the number of viable (7AAD-negative) cells in each individual sample. Fluorescence was detected by the FACSCalibur flow cytometer and analyzed using CellQuest software (both Becton Dickinson). All tests were performed in triplicate.

Measurement of $\Delta\Psi_m$

Depolarization of the mitochondrial membrane (Ψ_m) was detected using the fluorescent probe tetramethylrhodamine ethyl ester perchlorate (TMRE, Invitrogen, Eugene, OR, USA) which accumulates in mitochondria. Briefly, cells (10⁶ cells/ml) were incubated with 25nM TMRE at 37°C for 15 minutes in the dark and analyzed using FACS analysis. Mitochondrial membrane depolarization was observed as a shift to the left in the emission spectra. $\Delta\Psi_m$ was determined as the percentage unstained cells in YM155 treated samples minus the percentage unstained cells in untreated samples.

Cell cycle analysis

DAPI staining was used to measure each phase of the cell cycle according to DNA content of TFL cells. Cells were fixated in 1% paraformaldehyde for 15 minutes. Subsequently, cells were washed and incubated with DAPI according to the procedure as described previously.⁸ TFL cells were analyzed with a Partec PAS II mercury lamp-based flow cytometer (Partec Instruments), using trout erythrocytes as external control cells.

Western blotting

Western blot analysis was performed as described previously.⁹ Survivin expression was detected using an antibody against survivin (Cell Signaling Technology, Boston, MA, USA). The protein was visualized with the enhanced chemiluminescence technique (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis

Comparisons of differences in continuous variables between two groups were performed using the Mann-Whitney U test. *P*-values of <0.05 were considered significant.

References:

1. Swerdlow SH, Campo E, Harris NL, et al. WHO classification of Tumours of Haematopoietic and Lymphoid Tissues, Lyon: IARC Press; 2008.
2. Cillessen SA, Meijer CJ, Ossenkoppele GJ, et al. Human soluble TRAIL/Apo2L induces apoptosis in a subpopulation of chemotherapy refractory nodal diffuse large B-cell lymphomas, determined by a highly sensitive *in vitro* apoptosis assay. *Br J Haematol.* 2006;134(3):283-293.
3. Cillessen SA, Hess CJ, Hooijberg E, et al. Inhibition of the intrinsic apoptosis pathway downstream of caspase-9 activation is a cause for chemotherapy resistance in diffuse large B-cell lymphoma. *Clin Cancer Res.* 2007;13(23):7012-7021.
4. Hess CJ, Denkers F, Ossenkoppele GJ, et al. Gene expression profiling of minimal residual disease in acute myeloid leukaemia by novel multiplex-PCR-based method. *Leukemia.* 2004;18(12):1981-1988.
5. Robertson JD, Gogvadze V, Zhivotovsky B, Orrenius S. Distinct pathways for stimulation of cytochrome c release by etoposide. *J Biol Chem.* 2000;275:32438-32443.
6. Schwartz PS, Waxman DJ. Cyclophosphamide induces Caspase 9-dependent apoptosis in 9L tumor cells. *Mol Pharmacol.* 2001;60:1268-1279.
7. Gamen S, Anel A, Pérez-Galán P, et al. Doxorubicin treatment activates a Z-VAD-sensitive caspase, which causes deltaprim loss, caspase-9 activity, and apoptosis in Jurkat cells. *Exp Cell Res.* 2000;258:223-235.
8. Bergers E, van Diest PJ, Baak JP. Tumour heterogeneity of DNA cell cycle variables in breast cancer measured by flow cytometry. *J Clin Pathol.* 1996;49:931-937.
9. Dukers DF, Oudejans JJ, Vos W, ten Berge RL, Meijer CJ. Apoptosis in B-cell lymphomas and reactive lymphoid tissues always involves activation of caspase 3 as determined by a new *in situ* detection method. *J Pathol.* 2002;196(3):307-315.