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Thrombotic thrombocytopenic purpura (TTP) is a life-threatening thrombotic microangiopathy (TMA) that requires quick diagnosis and urgent treatment[1]

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Prescribing Information: Cablivi (caplacizumab) ▼ 10 mg powder and solvent for solution for injection. Please refer to Summary of Product Characteristics (SPC) before use. Presentation: Each vial of powder contains 10 mg of caplacizumab. Each pre-filled syringe of solvent contains 1 ml of water for injections. **Indication:** Cablivi is indicated for the treatment of adults and adolescents of 12 years of age and older weighing at least 40kg experiencing an episode of acquired thrombotic thrombocytopenic purpura (aTTP), in conjunction with plasma exchange and immunosuppression. **Dosage and Administration:** Treatment with Cablivi should be initiated and supervised by physicians experienced in the management of patients with thrombotic microangiopathies. First dose: Intravenous injection of 10 mg of caplacizumab prior to plasma exchange. Subsequent doses: Daily subcutaneous administration of 10 mg of caplacizumab, into the abdomen, after completion of each plasma exchange for the duration of daily plasma exchange treatment, followed by daily subcutaneous injection of 10 mg of caplacizumab for 30 days after stopping daily plasma exchange treatment. Injections into the area around the navel should be avoided and consecutive injections should not be administered in the same abdominal quadrant. Patients or caregivers may inject the medicinal product after proper training in the subcutaneous injection technique. If at the end of this 30 day period there is evidence of unresolved immunological disease, it is recommended to optimise the immunosupression regimen and continue daily subcutaneous administration of 10 mg of caplacizumab until the signs of underlying immunological disease are resolved (e.g. sustained normalisation of ADAMTS13 activity level). In the clinical development program, caplacizumab has been administered daily for up to 71 days consecutively. Data on retreatment with caplacizumab are available. Missed dose: If a dose of Cablivi is missed, it can be administered within 12 hours. If ≥12 hours have passed since the dose was to have been given, the missed dose should not be administered and the next dose should be administered per the usual dosing schedule. **Special Populations:** Renal impairment: No dose adjustment necessary. Mild-moderate hepatic impairment: No dose adjustment necessary. Elderly: Experience in the elderly is limited, however there is no evidence to suggest that dose adjustment or special precautions are necessary. Paediatric population: The safety and efficacy of caplacizumab in the paediatric population have not been established in clinical trials. The posology of Cablivi in adolescents of 12 years of age and older weighing at least 40kg is the same as in adults. No recommendations can be made on the posology of Cablivi for paediatric patients below 40kg of body weight. **Contraindication:** Hypersensitivity to the active substance or to any of the excipients. **Precautions and Warnings:** Bleeding: Cablivi increases the risk of bleeding. Cases of major bleeding, including life-threatening and fatal bleeding have been reported in patients receiving caplacizumab, mainly in those using concomitant anti-platelet agents or anticoagulants. Caplacizumab should be used with caution in patients with underlying conditions that may predispose them to a higher risk of bleeding. In case of clinically significant bleeding, treatment with Cablivi should be interrupted. If needed, the use of von Willebrand Factor concentrate could be considered to correct haemostasis. Cablivi should only be restarted upon the advice of a physician experienced in the management of thrombotic microangiopathies. If Cablivi is restarted, monitor closely for signs of bleeding. *In the setting of concomitant use of oral anticoagulants, anti-platelet agents, thrombolytic drugs or heparin:* The risk of bleeding is increased with concomitant use of Cablivi with drugs affecting haemostasis and coagulation. Initiation or continuation of treatment with oral anti-coagulants (e.g. vitamin K antagonists or direct oral anticoagulants [DOAC] such as thrombin inhibitors or factor Xa inhibitors), anti-platelet agents, thrombolytic drugs such as urokinase, tissue plasminogen activator (t-PA) (e.g. alteplase) or heparin requires careful consideration and close clinical monitoring. *In patients with coagulopathies (e.g. hemophilia, other coagulation factor deficiencies):* Due to a potential increased risk of bleeding, use of Cablivi in these patients must be accompanied by close clinical monitoring. *In patients undergoing surgery:* If a patient is to undergo elective surgery, an invasive dental procedure or other invasive interventions, the patient must be advised to inform the physician or dentist that they are using caplacizumab, and it recommended to withhold treatment for at least 7 days before the planned intervention. The patient must also notify the physician who supervises the treatment with caplacizumab about the planned procedure. After the risk of surgical bleeding has resolved and caplacizumab is resumed, the patient should be monitored closely for signs of bleeding. If emergency surgery is needed, the use of von Willebrand Factor concentrate is recommended to correct haemostasis. Severe hepatic impairment: No data available in patients with severe acute or chronic hepatic impairment. Use of Cablivi in this population requires a benefit/risk assessment and close clinical monitoring. Traceability: In order to improve the traceability of biological medicinal products, the name and the batch number of the administered product should be clearly recorded. **Pregnancy:** There are no data on the use of caplacizumab in pregnant women. Studies in guinea pigs showed no effect of caplacizumab on the dams or foetuses. As a precautionary measure, it is preferable to avoid the use of Cablivi during pregnancy. **Breastfeeding:** No data available in women. It is unknown whether caplacizumab is excreted in human milk. Therefore, risk to the child cannot be excluded, and decision must be made whether to discontinue breastfeeding or to abstain/discontinue from therapy, considering the benefit of breastfeeding for the child and the benefit of therapy for the women. **Fertility:** The effects of caplacizumab on fertility in humans are unknown. **Interactions:** No data available. **Adverse Reactions:** Very common: Headache, epistaxis, gingival bleeding, urticaria, pyrexia and fatigue. Common: Cerebral infarction, eye haemorrhage, haematoma, dyspnoea, haemoptysis, haematemesis, haematochezia, melaena, haemorrhage (upper gastrointestinal haemorrhoidal, rectal), abdominal wall haematoma, myalgia, haematuria, menorrhagia, vaginal haemorrhage, injection site haemorrhage, injection site pruritus, injection site erythema, injection site reaction and subarachnoid haemorrhage. **List price (UK only):** Cablivi 10mg injection x 1 vial: £4,143; x 7 vials £29,000. **Legal Category:** POM. **Marketing Authorisation Number:** PLGB 04425/0888. **Marketing Authorisation Holder:** Aventis Pharma Limited, 410 Thames Valley Park Drive, Reading Berkshire, RG6 1PT, UK. **Further information is available from:** Sanofi, 410 Thames Valley Park Drive, Reading, Berkshire, RG6 1PT, UK. Or uk-medicalinformation@sanofi.com. **SPC Date:** 09 May 2023 **Date of Preparation:** May 2023

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Loss of *MIR15A* and *MIR16-1* at 13q14 is associated with increased *TP53* mRNA, de-repression of *BCL2* and adverse outcome in chronic lymphocytic leukaemia

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Summary

This study was conducted to investigate the possibility that *TP53* mRNA is variably expressed in chronic lymphocytic leukaemia (CLL) and that under-expression is associated with TP53 dysfunction and adverse outcome. Although *TP53* mRNA levels did indeed vary among the 104 CLL samples examined, this variability resulted primarily from over-expression of *TP53* mRNA in 18 samples, all of which lacked *TP53* deletion/mutation. These patients had higher lymphocyte counts and shorter overall and treatment-free survival times compared to cases with low *TP53* mRNA expression and no *TP53* deletion/mutation. Furthermore, *TP53* mRNA levels did not correlate with levels of TP53 protein or its transcriptional target CDKN1A. We speculated that the adverse outcome associated with *TP53* mRNA over-expression might reflect variation in levels of *MIR15A* and *MIR16-1*, which are encoded on chromosome 13q14 and target *TP53* and some oncogenes including *BCL2*. In keeping with our hypothesis, 13q14 copy number and levels of *MIR15A*/*MIR16-1* correlated positively with one another but negatively with levels of *TP53* mRNA and *BCL2* mRNA. Our findings support a model in which loss of *MIR15A*/*MIR16-1* at chromosome 13q14 results in adverse outcome due to de-repression of oncogenes such as *BCL2*, and up-regulation of *TP53* mRNA as a bystander effect.

Keywords: CLL, 13q14, *MIR15A/16-1*, *TP53*, *BCL2*.

Chronic lymphocytic leukaemia (CLL) is a clonal expansion of antigen-experienced mature B cells with a distinctive immunophenotype. It runs a chronic relapsing course requiring multiple treatment episodes and is notable for its clinical variability (Chiorazzi *et al*, 2005). Among the many prognostic factors described in CLL, the most powerful predictor of adverse outcome is mutation or deletion of *TP53,* which occurs in 5–10% of patients requiring frontline therapy and 40–50% of chemo-refractory patients.

The *TP53* gene is located on chromosome 17p13 and encodes the TP53 tumour suppressor protein. TP53 is activated by and co-ordinates the cellular response to multiple stresses including DNA damage. Once activated, wild-type TP53 protein is stabilized and accumulates in the nucleus as a tetramer transcription factor. Wild-type TP53 tetramers bind to the promotor sequences of multiple target genes to regulate their expression. TP53 inactivation in CLL usually occurs by mutation of one *TP53* allele and deletion of the other and is associated with early disease progression, resistance to chemotherapy and short survival (Döhner *et al*, 2000; Grever *et al*, 2007; Stilgenbauer *et al*, 2008; Zenz *et al*, 2008). These associations reflect the pivotal role of TP53 in regulating important cellular functions including apoptosis, cell cycle arrest, DNA repair and senescence (Meek, 2009). The expression of TP53 protein is tightly regulated through post-translational modifications that influence its interaction with MDM2, an E3 ubiquitin ligase that targets TP53 for proteosomal degradation (Haupt *et al*, 1997; Honda *et al*, 1997). However, recent studies have shown that TP53 protein expression may also be regulated at the mRNA level through the action of microRNAs (miRNAs) (Hermeking, 2012).

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miRNAs comprise a large family of short (20–24 nucleotides) noncoding RNAs that bind to complementary sequences in the 3′ untranslated region (UTR) of mRNAs to reduce stability and block translation of targeted transcripts (Ambros, 2004). In this way, miRNAs play an important role in regulating gene expression. It has been estimated that >60% of protein-encoding genes in the human genome are regulated by miRNAs (Friedman *et al*, 2009). Several miRNAs can directly bind the 3′ UTR of *TP53* mRNA resulting in reduced expression of *TP53* and the induction of phenotypes associated with *TP53* loss (Hermeking, 2012). Among these *TP53*-regulating miRNAs, *MIR15A, MIR16-1* and *MIR125B-1* have been implicated in the pathogenesis of B-cell malignancies. Specifically, *MIR125B-1*, which is encoded at chromosome 11q24, is overexpressed in B-cell precursor acute lymphoblastic leukaemia as a result of the t(11;14)(q24;q32) translocation (Enomoto *et al*, 2011). In contrast, *MIR15A* and *MIR16-1* map to the minimal deletion region (MDR) of chromosome 13q14 that is lost in more than 50% of patients with CLL. Cases of CLL with this deletion have been reported to express reduced levels of *MIR15A* and *MIR16-1* (Palamarchuk *et al*, 2010), and have a favourable outcome (Döhner *et al*, 2000).

We have recently demonstrated that *TP53* deletion/mutation in CLL cells is accompanied by under-expression of mRNAs encoding *TP53* and other genes on chromosome 17p (Lin *et al*, 2013), most likely as a direct result of allelic loss. Since TP53 dysfunction can arise through mechanisms other than *TP53* mutation/deletion (Pettitt *et al*, 2001; Jones *et al*, 2004; Romanov *et al*, 2005; Lin *et al*, 2012), we sought to establish whether *TP53* mRNA is under-expressed in some cases of CLL with wild-type TP53 resulting in adverse outcome due to TP53 dysfunction. To address this question, *TP53* mRNA levels were quantified in a large cohort of unstimulated CLL samples and correlated with other clinical and laboratory variables, including 13q14 deletion and levels of *MIR15A*, *MIR16-1* and *MIR125B*. Although we found wide variation in *TP53* mRNA levels, our findings did not fit with our original hypothesis but instead supported a model in which loss of *MIR15A* and *MIR16-1* at chromosome 13q14 results in increased *TP53* mRNA (but not TP53 protein) and adverse outcome due to de-repression of oncogenes such as *BCL2*.

Materials and methods

CLL samples

This study was approved by the Liverpool Research Ethics Committee (project numbers 01/195, 02/032, 06/Q1505/81 and 06/Q1505/82). All blood samples were obtained with fully informed written consent and had a typical CLL phenotype (CD19+, CD5+, CD23+, weak light-chain-restricted-surface immunoglobulin) with a lymphocyte count of $>30 \times 10^9$/l. Mononuclear cells were prepared from whole blood by centrifugation on Lymphoprep (d = 1·077) and cryopreserved at −196°C in 10% dimethylsulfoxide (DMSO) until used.

Detection of chromosomal abnormalities by FISH

As previously described (Carter *et al*, 2006), interphase fluorescence *in situ* hybridization (FISH) was used to detect recurrent chromosomal abnormalities: deletion of *TP53* and *ATM* and trisomy 12 with Vysis probes specific for 17p13.1, 11q22.3 and the centromeric region of chromosome 12, respectively, and deletion of 13q14.3 with the Vysis probe D13S319. For each case, the average 13q14 copy number was calculated as 2 − [proportion of cells with biallelic deletion × 2] − proportion cells with monoallelic deletion.

Detection of TP53 mutations by denaturing high pressure liquid chromatography (DHPLC)

Genomic DNA was prepared from CLL cells using an AllPrep DNA/RNA mini kit (Qiagen, Crawley, UK) following the manufacturer's instructions (Lin *et al*, 2013). *TP53* exons 4–10 were then amplified by polymerase chain reaction (PCR) from the CLL genomic DNA mixed with a known wild-type DNA control (20%). *TP53* mutations were identified by DHPLC based on the temperature-dependent differences in column-retention time of PCR products generated from homoduplex (wild-type) and heteroduplex (mutated) DNA. All samples were denatured and cooled slowly to room temperature before DHPLC to maximize heteroduplex formation (95°C for 2 min, then decreased by 1°C every 40 s to 45°C for 30 min). Oligonucleotide primers and DHPLC conditions were chosen as previously described (Zenz *et al*, 2008). All samples with heteroduplex formation were sequenced using the Big Dye Terminator Kit and an ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA).

Measurement of TP53 and CDKN1A protein levels by flow cytometry

In vitro un-stimulated CLL cells were fixed, permeabilized and stained with mouse monoclonal antibodies to either TP53 (clone DO-1; Oncogene Research, Nottingham, UK) or CDKN1A (clone EA10; Oncogene Research) in separate tubes followed by a fluorescein isothiocyanate (FITC) -conjugated goat anti-mouse antibody and then a phycoerythrin (PE)-conjugated anti-CD19 (BD Biosciences, San Jose, CA, USA). Intracellular TP53 and CDKN1A protein levels in CD19+ cells were measured by flow cytometry and presented as mean fluorescence intensity (MFI) as previously described (Carter *et al*, 2004).

Measurement of TP53 and BCL2 mRNA by quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA extracted from CLL-cell samples using an RNeasy mini kit (Qiagen) was reverse transcribed using Moloney murine leukaemia virus (M-MLV)reverse transcriptase (Promega, Southampton, UK) and an oligo(dT)$_{15}$ primer

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(Lin *et al*, 2013). In the PCR step, the resulting cDNA was mixed with DyNAmo SYBR Green I qPCR master mix (Finnzymes, Espoo, Finland) and a set of primers for human *TP53* (forward: 5′-CCAGTGGTAATCTACTGGGACG; reverse: 5′-CTGACGCACACCTATTGCAAGC), human *BCL2* (forward: 5′-TGTGGCCTTCTTTGAGTTCG; reverse: 5′-ATT TGTTTGGGGCAGGCATG) or human *ACTB* (forward: 5′- CC TCGCCTTTGCCGATCC; reverse: 5′- GGATCTTCATGAGG TAGTCAGTC). All reactions were performed on a Stratagene Mx3005P QPCR System (Stratagene, Amsterdam, Netherlands) under optimized cycling conditions consisting of a 10-min initial denaturing step at 95°C, followed by 44 cycles of amplification (denaturation at 94°C for 20 s, annealing at 59–61°C for 20 s, extension at 72°C for 30 s, and fluorescence data collection at 78°C or 80°C). Following a final 10-min extension at 72°C, a melting curve was measured from 65 to 98°C. The specificity of each of the PCR products was confirmed as a single band with the expected molecular size on agarose gels and as a narrow peak that appeared in the melting curve when the temperature rose above 78°C. Levels of *TP53* and *BCL2* mRNA are presented relative to that of the control gene *ACTB*.

Quantification of miRNA expression

Total RNAs were extracted with the miRNeasy kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. The quantity and quality of RNAs were checked with Nanodrop spectrophotometer and Agilent 2100 Bioanalyser. cDNAs were prepared in a reverse transcription reaction using the miScript II RT Kit (Qiagen). Levels of *MIR15A*, *MIR16-1* and *MIR125B-1* were then measured by qPCR on a Stratagene Mx3005P QPCR System (Stratagene) using the miScript SYBR Green PCR Kit containing a universal reverse primer and the miRNA-specific miScript Primer Assay kits providing specific forward primers for individual miRNAs (both from Qiagen). These forward primers are *MIR15A* (MS00003178): 5′-UAGCAGCACAUAAUGGUUUGUG; *MIR16-1* (MS00031493): 5′-UAGCAGCACGUAAAUAUU GGCG and *MIR125B-1*(MS00006629): 5′-UCCCUGAGA CCCUAACUUGUGA. The amount of target was normalized to an endogenous reference *RNU6-6P*.

Statistical analysis

Comparisons of clinical and laboratory enumeration data were performed using χ^2 or Fisher's exact tests. Overall survival (OS) was calculated from sample collection to death from any cause. Treatment-free survival (TFS) was timed from sample collection to time of first treatment or death from any cause. Survival curves were constructed using the Kaplan–Meier method and compared using the log-rank test. Pearson's correlation test was performed to examine linear relations between two variables of approximately normal distribution. Spearman's rank correlation test was used to analyse relations between two variables with a skewed distribution of data. The statistical tests were conducted with SSPS version 20 (IBM, Portsmouth, UK). Statistical significance was defined by a 2-sided P value <0·05.

Results

TP53 mRNA is over-expressed in a proportion of CLL samples

We speculated that some cases of CLL with wild-type TP53 might under-express *TP53* mRNA resulting in low TP53 protein levels, TP53 pathway dysfunction and adverse clinical outcome. To test this hypothesis, we first sought to elucidate the quantitative variation in *TP53* mRNA expression between individual cases. To do this, *TP53* mRNA levels were measured by RT-qPCR in un-stimulated primary CLL cells from 104 patients. Levels were found to be low (<0·020) in most of these cases, including all 15 samples with a *TP53* deletion and/or mutation (Fig 1A). However, the distribution of data was clearly skewed, as 18 cases (20% of those without *TP53* deletion/mutation) showed remarkably high levels of *TP53* mRNA (0·028–0·310) (Fig 1A). These 18 patients were regarded as the group with high *TP53* mRNA levels in all subsequent analyses.

Over-expression of TP53 mRNA is associated with clinically aggressive disease but has no impact on TP53 protein levels or transcriptional activity

Our initial hypothesis predicted that patients with wild-type *TP53* and low *TP53* mRNA expression should have an adverse clinical outcome. To test this prediction, OS and TFS were compared between patients with high *TP53* mRNA expression (none of whom had a *TP53* mutation/deletion), those with low *TP53* mRNA expression and no *TP53* mutation/deletion and those with low *TP53* mRNA expression and *TP53* mutation/deletion. As expected, cases with *TP53* deletion/mutation had the shortest OS and TFS (Fig 1B,C). Contrary to expectations, OS and TFS were significantly shorter in patients with high *TP53* mRNA levels compared to those with low *TP53* mRNA levels and no *TP53* mutation/deletion (Fig 1B,C). Importantly, among samples with no *TP53* mutation/deletion, *TP53* mRNA levels did not correlate with levels of TP53 protein (Fig 2A) or its transcriptional target CDKN1A (Fig 2B), although TP53 and CDKN1A protein levels positively correlated with one other as expected (Fig 2C). To exclude the possible confounding effects of *in-vivo* induction of TP53 by DNA-damaging drugs, protein levels were compared between CLL samples from patients who had (n = 18, mean ± SD = 6·09 ± 3·22) or had not (n = 67, mean ± SD = 6·17 ± 2·33) received prior chemotherapy; no difference was found (P = 0·90).

To further characterize CLL cases with high *TP53* mRNA levels, they were compared to cases with low *TP53* mRNA

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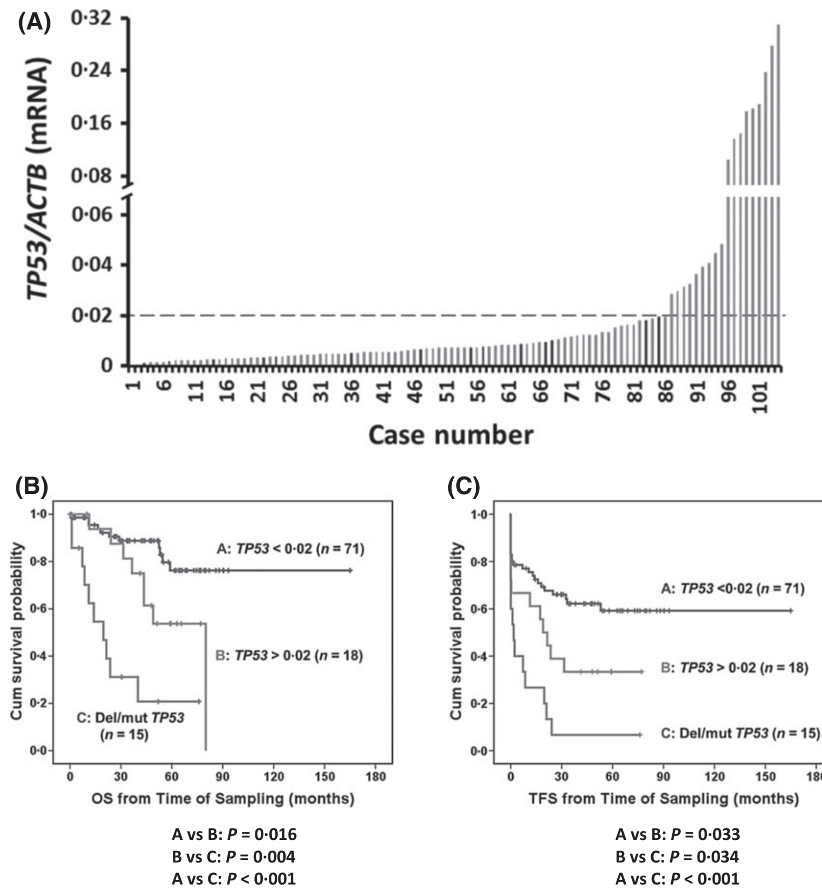

Fig 1. High levels of *TP53* mRNA are associated with adverse clinical outcome among cases of CLL with wild-type *TP53*. (A). Levels of *TP53* mRNA were measured by quantitative reverse transcription polymerase chain reaction in 104 cases, including 14 with *TP53* deletion and mutation and one with sole *TP53* mutation (dark grey bars). The dashed line was used as a cut-off to define high versus low levels of *TP53* mRNA. (B) Kaplan–Meier plots showing overall survival (OS). (C) Kaplan–Meier plots showing treatment-free survival (TFS). Group A = low *TP53* mRNA levels and no *TP53* mutation/deletion, group B = high *TP53* mRNA levels and no *TP53* mutation/deletion; group C = *TP53* deletion/mutation. *P* values were calculated using the Log-rank test.

levels and no *TP53* deletion/mutation for a range of other clinical and laboratory variables. No differences were detected between the two groups at the time of sample collection in terms of age, gender, Binet stage, prior therapy, *IGHV* mutation status, CD38 status, frequency of 11q- and 13q- or follow-up time. However, patients with high *TP53* mRNA levels had a more pronounced lymphocytosis and a borderline increase in the frequency of bi-allelic 13q14 deletion (Table I).

Collectively, these findings indicate that high, rather than low, *TP53* mRNA levels are associated with a more aggressive form of disease amongst CLL patients without *TP53* mutation/deletion, and that this observation cannot be explained by alterations in TP53 protein expression or function.

TP53 mRNA over-expression is associated with low 13q14 copy number and reduced levels of MIR15A and MIR16-1

To elucidate the factor(s) responsible for the adverse outcome associated with high *TP53* mRNA levels, we next sought to establish the mechanisms responsible for this over-expression. We speculated that it might result from a reduction in the expression of *MIR15A* and *MIR16-1* due to deletion of chromosome 13q14. Importantly, 13q14 deletion can affect a variable proportion of one or both alleles (Van Dyke *et al*, 2010; Dal Bo *et al*, 2011). In keeping with observations in mouse models (Klein *et al*, 2010), CLL patients with a bi-allelic deletion of 13q14 have been reported to have a worse outcome compared to patients with a mono-allelic deletion (Pfeifer *et al*, 2007; Chena *et al*, 2008), raising the possibility of a gene dose effect. To test this idea, we calculated the average 13q14 copy number per cell for each CLL sample and related the findings to *TP53* mRNA expression. A significant correlation was found between low 13q14 copy number and high *TP53* mRNA levels in cases without *TP53* deletion/mutation (Fig 3A,B). Given that *MIR15A* and *MIR16-1* are encoded at the MDR at 13q14 and are known to target *TP53* mRNA for degradation, we related *TP53* mRNA levels and 13q14 copy number to *MIR15A* and *MIR16-1* levels using 12 available CLL-cell samples selected

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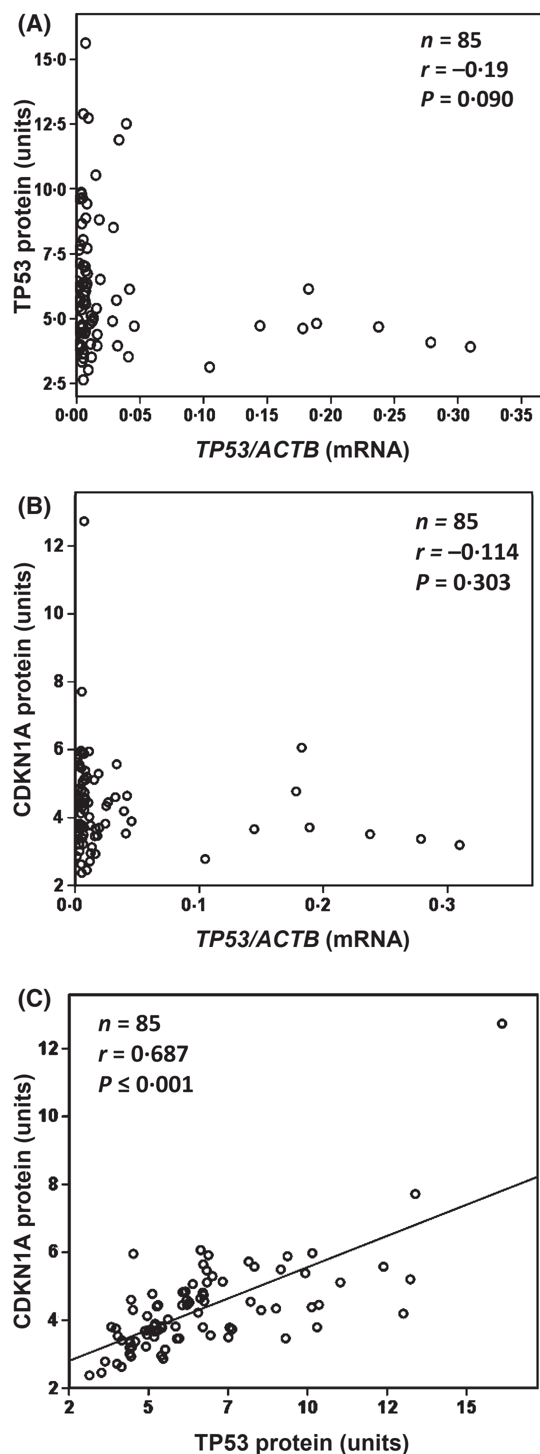

Fig 2. *TP53* mRNA levels do not correlate with TP53 protein expression and transcriptional activity. 85 CLL samples without *TP53* deletion/mutation were analysed for levels of *TP53* mRNA detected by quantitative reverse transcription polymerase chain reaction and levels of TP53 and CDKN1A proteins as measured by flow cytometry. (A) Comparison of *TP53* mRNA versus TP53 protein using Spearman's rank correlation analysis. (B) Comparison of *TP53* mRNA versus CDKN1A protein using Spearman's rank correlation analysis. (C) Comparison of TP53 and CDKN1A proteins using Pearson's linear correlation analysis.

Table I. *TP53* mRNA expression and other clinical and laboratory variables in CLL patients without *TP53* deletion/mutation.

Variables	Low *TP53* mRNA	High *TP53* mRNA	*P*
Age at sampling (years): Mean (95% CI)	65·1 (62·3–67·9)	67·4 (61·5–73·3)	0·457
Gender: Male/Female	50/21	13/5	0·881
Binet stages: (B+C)/A	18/53	6/12	0·556
Treatment: Yes/No	13/58	5/13	0·312
WBC ($\times 10^9$/l): Mean (95% CI)	43·1 (30·6–55·5)	94·2 (45·1–143·3)	0·017
IGHV status: UM/M	27/38 (41·5%)	10/6 (62·5%)	0·132
CD38 expression: +/–	15/38	3/9	>0·999
Del 11q22.3: Yes/No	3/64	1/17	>0·999
Del 13q14			
Mono- or bi-allelic: Yes/No	39/28	13/5	0·279
Bi-allelic: Yes/No	11/56	7/11	0·053
Follow-up months: mean (95% CI)	76 (63–89)	86 (59–114)	0·391

95% CI, 95% confidence interval; WBC, white blood cell count.

to include a broad range of 13q14 copy number. As predicted, a significant positive correlation was observed between 13q14 copy number and levels of *MIR15A* and *MIR16-1* (Fig 4A,B), whereas a negative correlation was observed between levels of *MIR15A* and *MIR16-1* and levels of *TP53* mRNA (Fig 5A,B). In contrast, no correlation was observed between *TP53* mRNA levels and levels of *MIR125B-1* (Fig 5C). The latter is another *TP53*-targeting miRNA, as shown in animals and in human cell lines, but, unlike *MIR15A* and *MIR16-1,* is located on human chromosome 11q24.1 (Le *et al*, 2009; Shaham *et al*, 2012). Together, these observations support a model in which *TP53* mRNA levels in CLL are determined by levels of *MIR15A* and *MIR16-1,* which, in turn, are determined by 13q14 copy number.

Negative regulation of TP53 mRNA by MIR15A/MIR16-1 dominates over positive regulation of MIR15A/MIR16-1 by TP53 protein

In addition to controlling *TP53* mRNA expression, *MIR15A* and *MIR16-1* are also transcriptional targets of TP53 protein. In order to examine the functionality of this latter component of the TP53:*MIR15A/MIR16-1* feedback loop, levels of the two miRNAs were related to levels of TP53 protein and one of its transcriptional targets, CDKN1A, in CLL samples without *TP53* mutation/deletion. No correlations were observed between *MIR15A/MIR16-1* and TP53/CDKN1A protein levels (Figure S1). Furthermore, neither TP53 nor CDKN1A protein levels correlated with 13q14 copy number (Figure S2). These results suggest that 13q14 copy number is more important than TP53 protein levels as a determinant of

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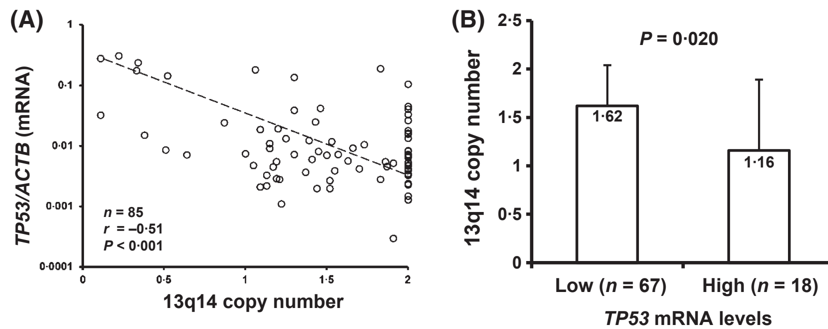

Fig 3. High *TP53* mRNA levels are associated with low 13q14 copy number. *TP53* mRNA levels were compared with 13q14 copy number in 85 CLL samples without *TP53* deletion/mutation. (A) Pearson's linear correlation analysis showing a negative correlation between *TP53* mRNA levels and 13q14 copy number. (B) Comparison of 13q14 copy number in cases with high versus low *TP53* mRNA levels (as defined in Fig 1A) using the student *t* test. The bars show mean values (+standard deviation).

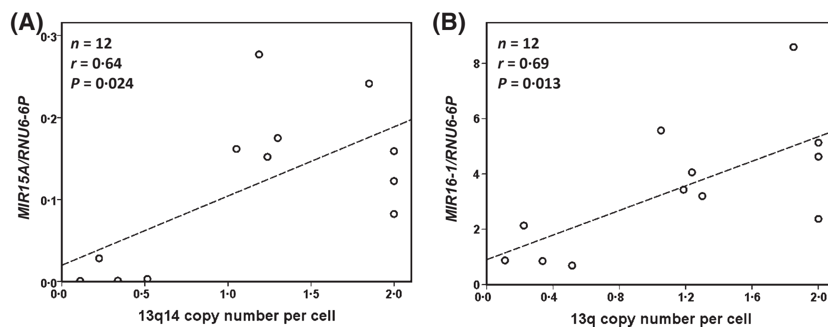

Fig 4. Low 13q14 copy number is associated with low levels of *MIR15A* and *MIR16-1*. Levels of *MIR15A* and *MIR16-1* were measured in 12 CLL samples selected to represent a wide spectrum of 13q14 copy number. (A) Pearson's linear correlation analyses showing positive correlation between 13q14 copy number and *MIR15A* levels. (B) Pearson's linear correlation analyses showing positive correlation between 13q14 copy number and *MIR16-1* levels.

MIR15A and *MIR16-1* levels in CLL cells, and that the negative regulation of *TP53* mRNA by *MIR15A* and *MIR16-1* dominates over the positive regulation of the two miRNAs by TP53 protein.

13q14 and 17p13 deletion have opposing effects on TP53 mRNA levels

We have previously shown that deletion of *TP53* at chromosome 17p13 in CLL cells is associated with reduced expression of *TP53* mRNA (Lin *et al*, 2013). We confirmed this observation in the present study by showing a positive correlation between *TP53* copy number and *TP53* mRNA levels in an extended cohort of 14 cases harbouring a monoallelic *TP53* deletion (Figure S3A). Given the negative correlation between *TP53* mRNA levels and 13q14 copy number (Fig 3), we reasoned that loss of the respective chromosome fragments at 17p13 and 13q14 should have opposing effects on *TP53* mRNA expression. To test this prediction, we divided the 14 cases with 17p13 deletion into those with low *TP53* and high 13q14 copy number (Group 1, predicted to have low *TP53* mRNA levels), those with high *TP53* and low 13q14 copy number (Group 2, predicted to have high *TP53* mRNA levels) and the rest (Group 3, predicted to have intermediate *TP53* mRNA levels). In keeping with our predictions, *TP53* mRNA levels were significantly higher in Group 2 than in groups 1 or 3, although the difference between groups 1 and 3 did not reach statistical significance (Figure S3B). Overall, these data support the notion that *TP53* mRNA levels are governed by the opposing effects of 13q14 and 17p13 loss.

High levels of TP53 mRNA are associated with over-expression of BCL2

Having established that deletion of *MIR15A* and *MIR16-1* at 13q14 is likely to account for the high *TP53* mRNA levels observed in some CLL samples without *TP53* deletion/mutation, we next sought to explain the adverse outcome of these patients. We speculated that the reduced expression of *MIR15A/MIR16-1* resulting from low 13q14 copy number would result in an increase not only in *TP53* mRNA but also in mRNA encoding oncogenes targeted by *MIR15A/MIR16-1*. To test this idea, CLL samples were analysed for

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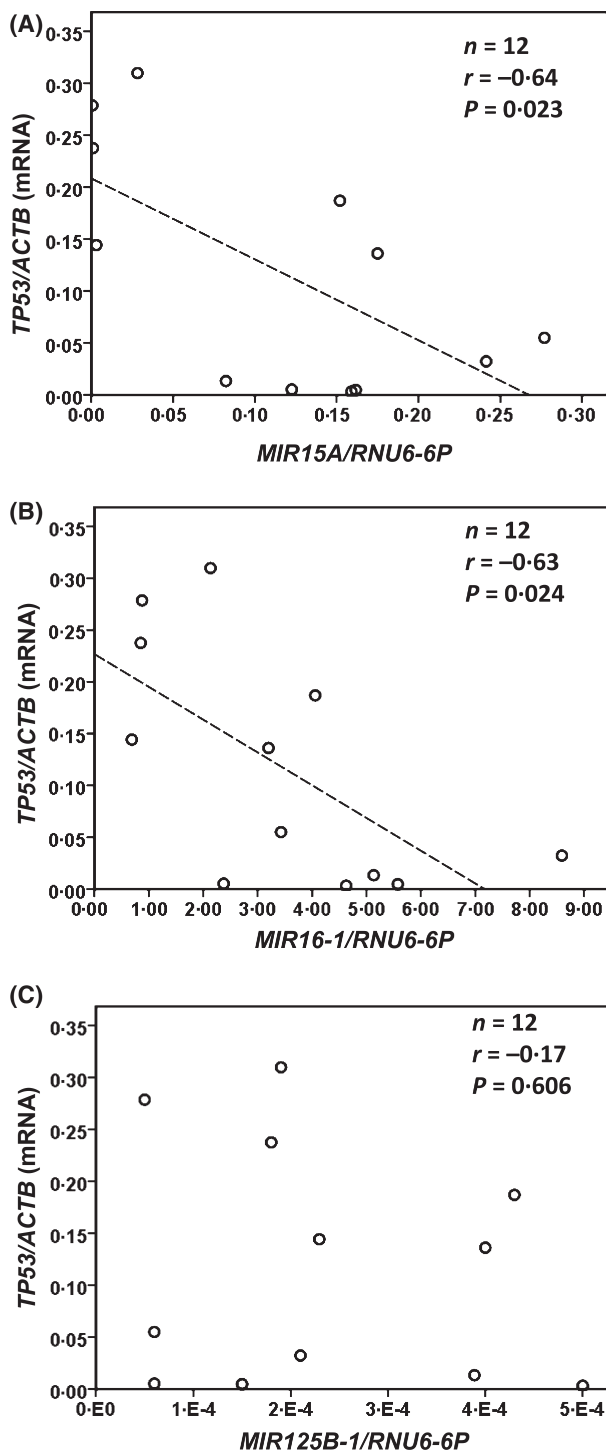

Fig 5. High levels of *TP53* mRNA are associated with low expression of *MIR15A* and *MIR16-1* but not *MIR125B-1*. Levels of *MIR15A*, *MIR16-1* and *MIR125B-1* were measured in the same 12 CLL cases shown in Fig 4 and compared with *TP53* mRNA levels. (A) Pearson's linear correlation analyses showing a negative linear correlation between *TP53* mRNA and *MIR15A*. (B) Pearson's linear correlation analyses showing a negative linear correlation between *TP53* mRNA and *MIR16-1*. (C) Pearson's linear correlation analyses showing no correlation between *TP53* mRNA and *MIR125B-1*.

the expression of one such oncogene, *BCL2*, and the findings related to 13q14 copy number and *TP53* mRNA levels. In keeping with our predictions, *BCL2* mRNA levels correlated negatively with 13q14 copy number and positively with *TP53* mRNA levels (Fig 6). Given the established role of the anti-apoptotic BCL2 protein in CLL biology and its regulation at the mRNA level, our findings may explain why the high levels of *TP53* mRNA are associated with adverse outcome in this disease.

Discussion

Inactivation of *TP53* resulting from gene mutation/deletion is strongly associated with disease progression, drug resistance and short survival in patients with CLL (Döhner *et al*, 2000; Grever *et al*, 2007; Stilgenbauer *et al*, 2008; Zenz *et al*, 2008). We have previously shown that dysfunction of the TP53 pathway can arise in CLL through alternative mechanisms including inactivation of its upstream regulators ATM (Pettitt *et al*, 2001) or ATR (Jones *et al*, 2004), inactivation of the downstream effector CDKN1A (Johnson *et al*, 2009), or suppression by basic fibroblast growth factor (Romanov *et al*, 2005), and have shown that some of these defects may be associated with adverse clinical outcome (Lin *et al*, 2002, 2012). Following our recent observation that *TP53* mRNA levels are reduced in patients with monoallelic *TP53* gene deletion (Lin *et al*, 2013), the present study sought to establish whether *TP53* mRNA levels could be reduced by alternative mechanisms and, if so, whether such under-expression of wild-type *TP53* mRNA might be associated with reduced TP53 protein levels and adverse clinical outcome due to dysfunction of the TP53 pathway.

In keeping with our hypothesis, we found that un-stimulated primary CLL samples exhibited marked variation in *TP53* mRNA levels. However, this variation resulted from marked over-expression of *TP53* mRNA in 20% (18/89) of cases who lacked *TP53* deletion/mutation. On the other hand, all samples with a *TP53* deletion/mutation expressed low levels of *TP53* mRNA, which was negatively correlated with the proportion of cells harbouring a *TP53* deletion. This confirmed our previous finding that *TP53* transcription is down-regulated as a result of monoallelic *TP53* gene deletion (Lin *et al*, 2013).

As expected, *TP53* deletion/mutation was associated with the worst outcome. However, among patients with no such *TP53* defects, those with high levels of *TP53* mRNA had a shorter OS and TFS and a more pronounced lymphocytosis compared to those with low *TP53* mRNA levels. This observation was contrary to expectations and could not be adequately explained by differences in other clinical or laboratory variables. Furthermore, no correlation was observed between *TP53* mRNA levels and TP53 protein expression and function (measured as CDKN1A protein expression), even when the putative TP53-activating effect of prior chemotherapy (Groves *et al*, 2012) was taken into

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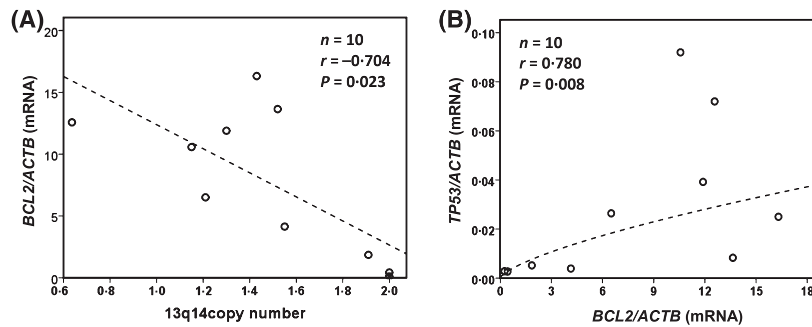

Fig 6. High levels of *TP53* mRNA are associated with over-expression of *BCL2*. 10 CLL samples without *TP53* deletion/mutation were analysed for *BCL2* mRNA levels and the results related to 13q14 copy number and *TP53* mRNA levels. (A) Pearson's correlation analyses showing a negative correlation between *BCL2* mRNA levels and 13q14 copy number. (B) Pearson's correlation analyses showing positive correlation between *BCL2* mRNA levels and *TP53* mRNA levels.

account. This lack of correlation between *TP53* mRNA and TP53 protein levels is likely to reflect the dominant role of post-translational regulation in determining TP53 protein expression (Kruse & Gu, 2009).

Our demonstration amongst CLL patients with no *TP53* mutation/deletion, that *TP53* mRNA levels did not correlate with TP53 protein expression and function and that high, rather than low, *TP53* mRNA levels were associated with adverse outcome, suggests that *TP53* mRNA levels have no effect on clinical outcome but are instead a surrogate for other factor(s) that are co-regulated with *TP53* mRNA and directly influence the clinical course of the disease. In order to elucidate these factors, we sought to explore how *TP53* mRNA is regulated in CLL cells.

A recent study showed that deletion of chromosome 13q14 in CLL cells results in an increase in *TP53* mRNA levels through loss of *TP53* repression by *MIR15A* and *MIR16-1*, which are located in the MDR (Fabbri *et al*, 2011). In agreement with this observation, we found that the average 13q14 copy number per cell correlated positively with levels of *MIR15A* and *MIR16-1* and negatively with levels of *TP53* mRNA. We also found that levels of *MIR15A* and *MIR16-1* correlated negatively with levels of *TP53* mRNA.

In contrast, some of our findings conflicted with those of Fabbri *et al* (2011). In particular, the latter study found that deletion of 13q14 was associated with an increase in TP53 protein levels as well as *TP53* mRNA (Fabbri *et al*, 2011), whereas we did not find any correlation between levels of *TP53* mRNA and protein or between 13q14 copy number and TP53 protein expression. One possible explanation for this discrepancy is that the two studies employed different methods to measure TP53 protein levels. Another explanation may lie in sample selection. Thus, although we excluded prior chemotherapy as a theoretical cause of *in-vivo* TP53 protein up-regulation, it is possible that co-deletion of other genes at 13q14 had a lowering effect on TP53 protein levels in our cohort. For example, *SETDB2*, which is positioned between the MDR and *RB1* at chromosome 13q14.2 and lost in some CLL samples with 13q14 deletion (Chuikov *et al*, 2004), encodes a lysine methyltransferase that can regulate TP53 at the post-translational level through methylation of lysine 372, resulting in protein stabilization (Chuikov *et al*, 2004; Parker *et al*, 2011). Loss of this gene could potentially reduce TP53 protein levels and thereby oppose the up-regulating effect of *MIR15A/MIR-16-1* deletion.

Our observation that high *TP53* mRNA levels are associated with adverse outcome and low 13q14 copy number needs to be reconciled with fact that 13q14 has historically been regarded as a favourable prognostic marker in CLL (Döhner *et al*, 2000). In fact, recent evidence suggests that the situation is considerably more complex than previously thought. Specifically, the clinical consequences of 13q14 deletion appear to depend on the percentage of cells harbouring a deletion, the size of deletion and the overall balance between tumour suppressor and oncogenes loss.

In agreement with our findings, other studies of both animal and human CLL have shown that 13q14 deletion is associated with aggressive disease and poor prognosis if the deletion is biallelic or present in a high percentage of cells (Pfeifer *et al*, 2007; Van Dyke *et al*, 2010; Dal Bo *et al*, 2011). Furthermore, co-deletion of genes outside of the MDR has the potential to affect the clinical impact of 13q14 deletion (Lia *et al*, 2012). Thus, FISH (Van Dyke *et al*, 2010) and single-nucleotide polymorphism array studies (Pfeifer *et al*, 2007; Falandry *et al*, 2010; Ouillette *et al*, 2011) have shown that the size of the 13q14 deletion in CLL is highly variable and that larger deletions are associated with a more aggressive form of the disease (Falandry *et al*, 2010; Dal Bo *et al*, 2011; Ouillette *et al*, 2011). Linking these two concepts together, it is intriguing to note that low 13q14 copy number has been shown to correlate with deletion of *RB1* (Dal Bo *et al*, 2011), a tumour suppressor gene that is more than 2 Mbp centromeric to the MDR.

As an alternative to variable co-deletion of genes outside the MDR, the complex prognostic effect of 13q14 deletion might instead reflect variation in the downstream

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consequences of gene deletion within the MDR. In particular, *MIR15A* and *MIR16-1* are known to target and inhibit multiple genes, including both tumour suppressors and oncogenes (Fabbri *et al*, 2011), with much potential for the balance between tumour suppressor and oncogene repression to vary between individual patients.

With regard to oncogenes, we sought to establish whether the adverse clinical outcome associated with high *TP53* mRNA levels might be explained by de-repression of BCL2 due to deletion of *MIR15A* and *MIR16-1* at 13q14. We focussed our attention on BCL2 as this anti-apoptotic protein is crucial to the survival of CLL cells (Pepper *et al*, 1999; O'Brien *et al*, 2007), is regulated predominantly at the mRNA level (Sanz *et al*, 2004; Otake *et al*, 2007) and is known to be repressed by *MIR-15* and *MIR16-1* (Cimmino *et al*, 2005). By showing that *BCL2* mRNA levels correlated negatively with 13q14 copy number and *MIR-15/MIR16-1* levels and positively with *TP53* mRNA levels, our study provides evidence that the adverse clinical outcome associated with high *TP53* mRNA levels might result at least in part from de-repression of BCL2 due to deletion of *MIR15A* and *MIR16-1* at 13q14.

In summary, our study has shown that high *TP53* mRNA levels are associated with adverse outcome in CLL and supports a model in which loss of *MIR15A* and *MIR16-1* at chromosome 13q14 results in adverse outcome due to de-repression of oncogenes, such as *BCL2*, along with up-regulation of *TP53* mRNA as a 'bystander effect'. At a broader level, our findings illustrate the complex role of 13q14 deletion in determining clinical outcome in CLL and pave the way to future studies to clarify how disease outcome is determined by the balance between tumour suppressor and oncogene deletion or de-repression.

Author contributions

KL and ARP designed research and interpreted data and wrote the manuscript; ARP and NK provided CLL samples and clinical data; KL, MF, YY and GJ performed research; MO collected and managed CLL samples and clinical data; AM and DA contributed to FISH analysis; KL and MF performed statistical analysis.

Competing interests

The authors have no competing interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Pearson's correlation analyses showing lack of correlation between baseline TP53 protein levels and levels of *MIR15A* (A) and *MIR16-1* (B), or between CDKN1A protein expression and levels of *MIR15A* (C) and *MIR16-1* (D) in CLL samples without *TP53* deletion and/or mutation.

Fig S2. Pearson's correlation analyses showing lack of correlation between 13q14 copy number and baseline TP53 (A) or CDKN1A (B) protein levels in CLL samples without *TP53* deletion and/or mutation.

Fig S3. Opposing effects of *TP53* and 13q14 copy number on *TP53* mRNA levels.

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