Role of *miR-15a/miR-16-1* and the *TP53* axis in regulating telomerase expression in chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL), the most prevalent form of leukemia in adults in the western world, has a highly heterogeneous clinical course, and is characterized by genomic instability which gives rise to several chromosomal alterations detectable in more than 80% of CLL cases.[1] While the absence of somatic mutations in the immunoglobulin heavy-chain gene variable region (*IGVH*) and high expression of CD38 and ZAP-70 molecules have been associated with aggressive CLL, the most common chromosomal abnormality, 13q14 deletion (13q14del), has been associated with a more indolent form of the disease.[1,2] The mechanism by which 13q14del contributes to CLL pathogenesis and affects the outcome of patients has not yet been elucidated.

Several studies have focused on the prognostic significance of an interplay between telomeres and telomerase in CLL.[3-6] Telomerase is responsible for the maintenance of telomeres, structures which cap and protect the ends of chromosomes. Telomerase expression, up-regulated in approximately 90% of human cancers, enables continuous and uncontrolled proliferation of the malignant cells driving tumor growth and progression.[7] Catalytic protein with telomere-specific reverse transcriptase activity (TERT) is the rate-limiting component of the telomerase complex, and its expression is correlated with telomerase activity.[8] We and others have shown that levels of TERT and its activity are prognostic markers in CLL.[3-6] Notably, *TERT* levels were found to be lower in 13q14del CLL than in CLL with other chromosomal abnormalities,[4] but mechanistic insight for this difference is still unclear.

The expression of microRNA, small non-coding RNA with regulatory functions, is frequently deregulated in tumors.[9] The microRNA cluster which encodes for *miR-15a* and *miR-16-1* maps within a 30-kilobase region of loss at 13q14.[10] Both these microRNA interact directly with and inhibit the expression of the anti-apoptotic *BCL2* gene, and the loss of the *miR-15a/miR-16-1* cluster due to 13q14 deletion is the main cause of *BCL2* overexpression in CLL.[10] Nonetheless, *miR-15a* and *miR-16-1* also directly target the tumor suppressor *TP53* gene, and their overexpression is associated with repressed TP53 expression at both mRNA and protein levels.[11] Transcription of the *TERT* gene is the key determinant in regulating TERT expression and telomerase activity. TP53 is an important repressor of the promoter of *TERT* gene,[12] and the C-terminus of TP53 interacts with and inhibits telomerase activity.[13]

This study aimed at determining *miR-15a/miR16-1* levels in CLL and correlating them to *TP53* and *TERT* transcripts. Since 13q14del CLL cells would lack *miR-15a/miR-16-1*, and since these two microRNA repress expression of TP53,[11] we hypothesized that the *miRNA/TP53* axis modulates TERT levels, and thus the outcome of patients with 13q14del CLL.

Peripheral blood cells were collected from 155 CLL patients who attended the Hematology Section, Clinical and Experimental Medicine, University of Padua (Italy). This study included 99 cases of CLL with the 13q14 deletion as the sole chromosomal abnormality detected by fluorescence *in situ* hybridization (FISH), and 56 CLL with no chromosomal abnormalities detected by FISH [i.e., 13q14.3, 17p13.1 (*TP53*), and 11q22.3 (*ATM*) deletions, and trisomy 12]. FISH was carried out as previously described.[4] CLL cases with *TP53* mutations were excluded from this study. All samples were collected at the time of diagnosis, and all patients were untreated at the time of sampling. *TERT* transcripts were quantified in all samples by real-time polymerase chain reaction, as previously described.[4] The expression of *TP53* transcripts was quantified in 123 CLL by real-time polymerase chain reaction using a TaqMan Gene Expression Assay-Human TP53:Hs01034249_m1 kit (Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions. The expression of *miR-15a*, *miR-16-1* and *RNU6B*, used as a control to normalize the data, was assessed in available RNA samples from 101 cases of CLL using a standard TaqMan MicroRNA assay kit (Life Technologies), according to the manufacturer's instructions. Statistical analyses were carried out using SAS version 9.1 (SAS Institute, Cary, NC, USA). Time from diagnosis to first treatment was considered as a marker for time to disease progression. Informed consent was obtained according to the Helsinki Declaration and the study was approved by the local Ethics Committee.

As expected, both *miR-15a* and *miR-16-1* levels were lower in 13q14del CLL than in CLL without chromosomal abnormalities [1.97 (1.12-3.34) *versus* 2.86 (1.90-4.66), P=0.009 and 0.15 (0.13-0.31) *versus* 0.25 (0.17-0.37), P=0.001)] (Table 1) and positive correlations were found between *miR-15a* and *miR-16-1* levels (r_s=0.854, P<0.0001). In agreement with the fact that overexpression of *miR-15a* and *miR-16-1* in primary CLL cells is associated with a decrease in *TP53* levels,[11] *TP53* mRNA levels were significantly higher in 13q14del CLL than in CLL with no chromosomal abnormalities [782 (416-1186) *versus* 582 (304-815) copies/10^5 *GAPDH* copies P=0.012] (Table 1). Levels of *miR-15a* and *miR-16-1* were inversely correlated with *TP53* levels in both 13q14del (r_s= -0.775, P<0.0001 and r_s= -0.722, P<0.0001,

Table 1. Clinical and biological characteristics of the patients with chronic lymphocytic leukemia according to 13q14del status.

	13q14del	no FISH abnormalities	*P*-value[a]
Clinical characteristics			
Men/women	46/53	33/23	0.316
Age, mean (IQR)	70 (63-76)	68 (60/75)	0.342
Binet stage A/B/C (%)	85/11/4	79/16/5	0.331
Lymphocytes >10^9/L (%)	42/99	14/56	0.141
Biological characteristics of CLL			
IGVH unmutated (%)	19/88 (21.6)	12/46 (26.1)	0.517
CD38 >30% (%)	9/94 (9.6)	13/50 (26.0)	0.014
miRNA-15a	72/99	29/56	
Median relative levels[b] (IQR)	1.97 (1.12-3.34)	2.86 (1.90-4.66)	0.009
miRNA-16-1	72/99	29/56	
Median relative levels[b] (IQR)	0.15 (0.13-0.31)	0.25 (0.17-0.37)	0.001
TP53	81/99	42/56	
Median relative levels[c] (IQR)	782 (416-1186)	582 (304-815)	0.012
TERT	99/99	56/56	
Median relative levels[d] (IQR)	55 (20-115)	73 (46-198)	0.027

[a]Associations between categorical variables were analyzed by the χ^2 test and the distribution of continuous variables was compared by the Kruskal-Wallis non-parametric test. [b]The relative levels of *miR*-15a and *miR-16-1* were determined from the *miRNA/RNU6B* ratio according to the $2^{-\Delta Ct}$ formula, where $\Delta Ct=(C_{tmiRNA}- C_{tRNU6B})$. [c]*TP53* relative levels were expressed as *TP53* copies/10^5 *GAPDH* copies. [d]*TERT* relative levels are expressed as *TERT* copies/10^6 *GAPDH* copies.

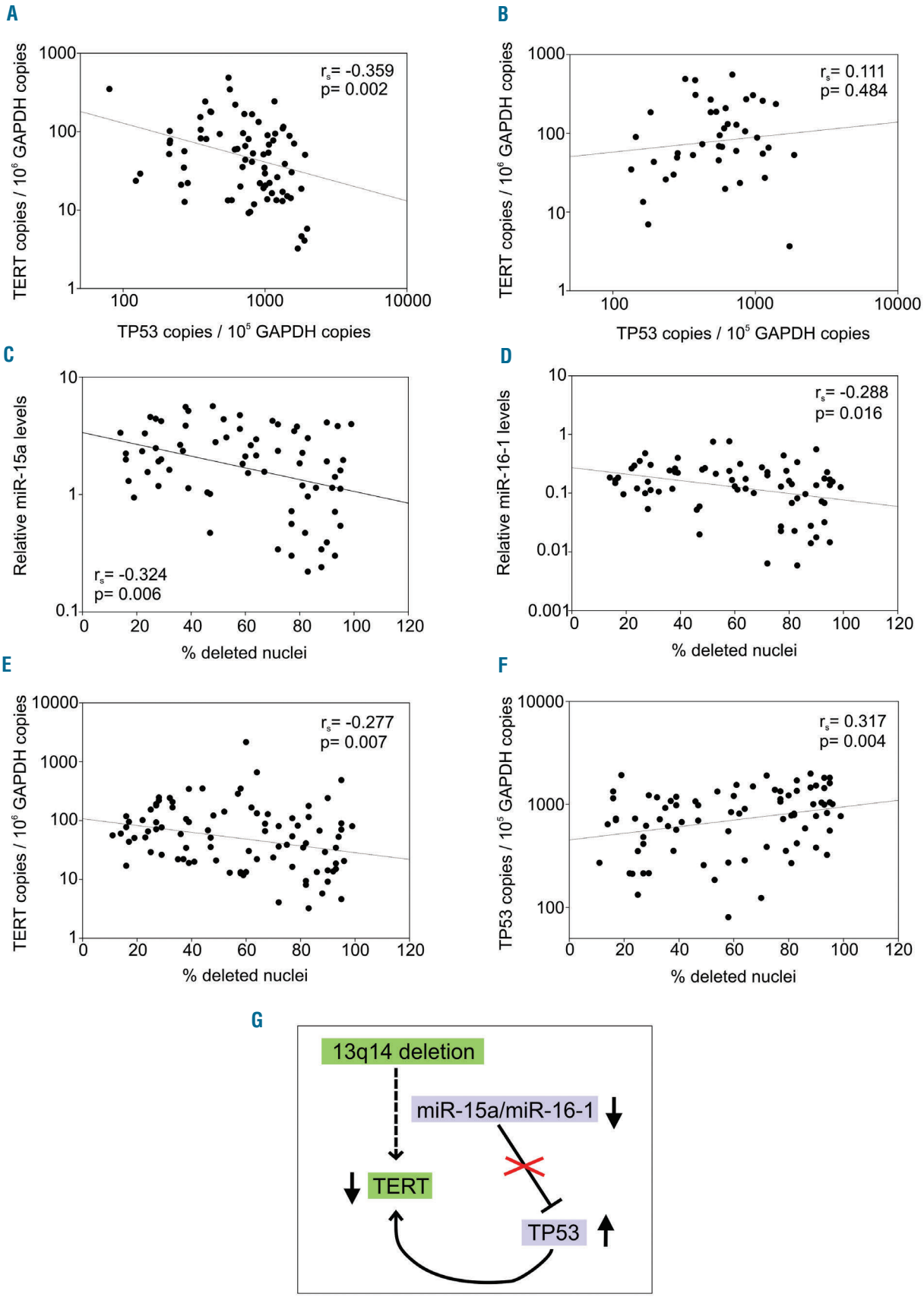

Figure 1. Relationship among *miRNA*, *TP53* and *TERT* levels in 13q14del chronic lymphocytic leukemia. (A, B) Relationship between *TERT* and *TP53* levels in: (A) 13q14del CLL, (B) CLL with no chromosomal abnormalities. (C-F) Relationship between percentage of deleted nuclei and levels of (C) *miR-15a*, (D) *miR-16-1*, (E) *TP53*, (F) *TERT*. (G) Proposed network among *miR-15a/miR-16-1*, *TP53*, and *TERT* expression. Down-regulation of *miR-15a/miR-16-1*, due to 13q14 deletion, leads to increased *TP53* levels which, in turn, down-regulate levels of *TERT*, the catalytic component of the telomerase complex.

Table 2. Time to first treatment and hazard ratio according to *IGVH* status, *TERT* levels, and *TP53* levels showing their effects on disease progression in chronic lymphocytic leukemia with 13q14 deletion.

	TTFT[a] months (95% CI)-	*P*-value log-rank	Hazard ratio (95% CI)[b]	HR *P*-value
***IGVH* status**		< 0.0001		
IGVH mutated	211 (110;-)		1	
IGVH unmutated	32 (6;157)		4.05 (1.90;8.54)	0.0003
***TERT* levels**		0.026		
TERT low[c]	211 (104;-)		1	
TERT high[c]	84 (53;157)		2.24 (1.08;4.67)	0.030
***TP53* levels**		0.038		
TP53 low[c]	96 (32;-)		2.36 (1.02;5.49)	
TP53 high[c]	157 (149;-)		1	0.044
***TERT & TP53* levels**		0.012		
TERT high & *TP53* high	157 (153;-)		0.22 (0.06;0.83)	0.025
TERT low & *TP53* low	- (96;-)		0.27 (0.07;0.99)	0.047
TERT high & *TP53* low	32 (5;-)		1	
TERT low & *TP53* high	221 (104;-)		0.29 (0.11;0.77)	0.013
***TERT, TP53* levels & *IGVH* status**		< 0.0001		
Other; *IGVH* unmutated	157 (1;157)		0.29 (0.12;0.69)	0.006
Other; *IGVH* mutated	221 (104;-)		0.11 (0.03;0.37)	0.0004
TERT high & *TP53* low; *IGVH* unmutated	12 (1;32)		1	
TERT low & *TP53* high; *IGVH* mutated	- (25;-)		0.38 (0.15;0.96)	0.040

For each variable, time to first treatment (TTFT) analysis was estimated using the Kaplan-Meier method and compared with the log-rank test. *TERT* and *TP53* levels were analyzed as dichotomous variables (cut-off: ≤median or >median). Hazard ratios for each category were estimated using univariate Cox proportional hazards models. The independent role of the *TERT/TP53* level profile in predicting TTFT was tested using a Cox proporzional hazard model, and was adjusted for *IGVH* mutational status. [c]*TERT* or *TP53* low: ≤ median level; *TERT* or *TP53* high: > median level. [a]TTFT: time to first treatment. [b]CI: confidence interval.

respectively; *data not shown*) and all CLL (r_s= -0.806, *P*<0.0001 and r_s= -704, *P*<0.0001, respectively; *data not shown*).

13q14del CLL expressed significantly lower levels of *TERT* than CLL with normal cytogenetic profile [55 (20-115) *versus* 73 (46-198) copies/10^6 copies *GAPDH*, *P*=0.027] (Table 1). Of interest, *TP53* levels correlated negatively with *TERT* levels only in 13q14del (r_s= -0.359, *P*=0.002, Figure 1A), but not in CLL without chromosomal abnormalities (r_s= 0.111, *P*=0.484) (Figure 1B). The different percentage of nuclei carrying the 13q14 deletion may explain the variable levels of *miRNA*, *TP53*, and *TERT* observed within the 13q14del CLL; indeed, the percentage of nuclei carrying the 13q14 deletion tended to correlate negatively with *miR-15a*, *miR-16-1* and *TERT* levels (r_s= -0.324, *P*=0.006; r_s= -0.288, *P*=0.016; r_s= -0.277, *P*=0.007, respectively) (Figure 1C-E), and positively with *TP53* levels (r_s=0.317, *P*=0.004) (Figure 1F).

Univariate Cox analyses showed that mutated *IGVH* status and low *TERT* levels (≤ median) were prognostic of better disease outcome, estimated as time from diagnosis to first treatment, in the entire cohort of CLL, with hazard ratios (HR) and 95 confidence intervals (95% CI) of 3.95 (2.11-7.43) *P*<0.001 and 2.19 (1.21-3.97) *P*=0.008, respectively, and also in the subgroup of patients with 13q14del CLL (Table 2). Of interest, cases with high *TP53* expression had a better prognosis in the subgroup of 13q14del CLL (Table 2), but not in that of CLL with normal cytogenetic profile [HR 0.97 (95% CI 0.29-3.28); *P*=0.973]. CD38 (>30%) was not prognostic of disease outcome in 13q14del CLL [HR 2.28 (95% CI 0.86-6.04) *P*=0.107; *data not shown*]. Multivariate Cox analysis showed that within 13q14del CLL, a high *TERT*/low *TP53* level profile defined the subgroup of cases with the worst prognosis (Table 2) and the values of *TERT/TP53* profiles were independent of *IGVH* mutational status (Table 2).

This result, together with the finding that a negative correlation between *TP53* and *TERT* levels can only be observed in 13q14del CLL but not in CLL without chromosomal abnormalities, emphasizes the existence of a network between *miR-15a/miR-16-1*, *TP53* and *TERT* within 13q14del CLL (Figure 1G).

It should be noted that *miRNA* expression also varied in CLL without chromosomal aberrations; therefore, mechanisms other than deletion of the *miRNA* cluster in the 13q14 region probably contribute to regulating their expression. It has recently been demonstrated that *miR-15a/miR16-1* and *TP53* are engaged in a feedback loop in CLL: increased levels of *miR-15a/miR16-1* target and down-regulate *TP53* expression, while TP53 binds to its specific binding sites on chromosome 13 and up-regulates the expression of *miR-15a/miR-16-1* in CLL with a normal cytogenetic profile.[11] As the expression of *TP53* in CLL is influenced by many factors,[14] this variability may influence levels of the *miRNA*, which in turn down-regulate *TP53* expression.

Notably, in this study *TP53* had prognostic value only for 13q14del CLL. It has been advanced that CLL with a high percentage of 13q14 deletion tend to have a worse prognosis.[15] The deletion of *miR-15a* and *miR-16-1* and the consequent lack of inhibition of the anti-apoptotic *BCL2* gene may partially support this trend. However, *miR-15a* and *miR-16-1* also target the tumor suppressor gene *TP53*. Hence, the loss of the *miR-15a/miR-16-1* cluster due to 13q14 deletion not only moves the balance toward higher levels of anti-apoptotic protein, but also toward higher levels of tumor suppressor TP53. With a cut-off of 80% of 13q14 deleted nuclei,[15] we did not find any significant differences in disease outcome (HR 0.597;

95% CI: 0.304-1.173; *P*=0.135). This supports the concept that the effects of deletion, rather than the percentage of deletion *per se*, influence the disease outcome.

An interesting and intriguing result of our study is that levels of *TP53* were inversely correlated with *TERT* levels only in the 13q14del CLL cases. TP53 alone may be inefficient in regulating TERT, since many other factors activate or repress TERT at a transcriptional level.[7] Our findings suggest that TP53 plays an important role in TERT regulation in 13q14del CLL, while in CLL with no or other chromosomal aberrations TERT may be regulated by other factors.

In conclusion, collectively, these findings indicate that, in 13q14del CLL, the *miR15/miR16-1* cluster and *TP53* axis is an important pathway which regulates *TERT* expression, thus influencing disease outcome, and also suggest that analysis of *TERT/TP53* profiles may be useful in refining the prognosis of patients with 13q14del CLL.

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Acknowledgments: the authors would like to thank all the patients who provided samples for the study.

Funding: this study was supported by the Associazione Italiana per la Ricerca sul Cancro (AIRC grant n. 14258) and by Istituto Oncologico Veneto

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doi:10.3324/haematol.2016.157669

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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