Nutlin-3 Downregulates the Expression of the Oncogene \textit{TCL1} in Primary B Chronic Lymphocytic Leukemic Cells

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Abstract

\textbf{Purpose:} The oncogene \textit{TCL1} plays a key role in the development of B chronic lymphocytic leukemia (B-CLL), but it is not known whether TCL1 could be modulated by therapeutic approaches.

\textbf{Experimental Design:} B-CLL patient samples (\(n = 35\)) and B leukemic cell lines (EHEB, JVM2, JVM3, MEC1, MEC2, and BJAB) with different p53 status were exposed to Nutlin-3, a small-molecule inhibitor of the p53–MDM2 interaction. Modulations of the steady-state mRNA levels of TCL1 were analyzed by quantitative real-time PCR and Western blotting in both primary B-CLL samples and leukemic cell lines. In addition, transfection experiments with either p53 siRNA or with a TCL1 expression plasmid were carried out in the EHEB B-CLL cell line.

\textbf{Results:} Upon \textit{ex vivo} treatment with Nutlin-3, TCL1 was significantly \((P < 0.05)\) decreased in 23 of 28 B-CLL p53\textsuperscript{wild-type}. The functionality of the p53 pathway in the same leukemic cell samples was underscored by the concomitant ability of Nutlin-3 to significantly \((P < 0.05)\) upregulate the p53 target gene \textit{MDM2} in the p53\textsuperscript{wild-type} leukemic cells. The dependence of TCL1 downregulation by a functional p53 pathway was confirmed in a panel of B lymphoblastoid cell lines and by p53 knockdown experiments with p53 siRNA. The importance of TCL1 in promoting leukemic cell survival was underscored in transfection experiments, in which TCL1 overexpression significantly counteracted the Nutlin-3–mediated induction of apoptosis in EHEB.

\textbf{Conclusions:} Our data indicate that the Nutlin-3 downregulates TCL1 mRNA and protein, which likely represents an important molecular determinant in the proapoptotic activity of Nutlin-3. \textit{Clin Cancer Res}; 17(17); 5649–55. \textcopyright 2011 ACR.

Introduction

The T-cell leukemia/lymphoma 1 (\textit{TCL1}) oncogene was discovered as a target of chromosomal translocations and inversions at 14q31.2 in T-cell prolymphocytic leukemias (1). The B-cell transforming potential of TCL1 has been subsequently shown in IgVH/E\textit{m}-TCL1 transgenic mice, which exhibit emergence of clonal CD5\textsuperscript{+}/IgM\textsuperscript{+} cells expansion resembling the course and phenotype of IgVH-unmutated human B chronic lymphocytic leukemia (B-CLL; refs. 2–4). It has been previously described that TCL1 shows a differential and regulated expression pattern in B-CLL (5). Different groups of investigators have observed association of high protein levels of TCL1 with features of aggressive disease in B-CLL (6, 7). These results indicate that deregulation of TCL1 is critically important in the pathogenesis of the aggressive form of B-CLL (3). Although the mechanism(s) through which \textit{TCL1} mediates its oncogenic effects is incompletely understood, it has been proposed that the pro-oncogenic role of \textit{TCL1} could be mediated by hyperactivation of the Akt pathway (8–10).

Nutlin-3 is a small-molecule inhibitor of the p53–MDM2 interaction, which leads to increased levels of p53 protein and, subsequently, promotion of induction of cell-cycle arrest and apoptosis in a variety of tumor cells (11). The use of Nutlin-3 has been suggested for innovative therapeutic purposes (11). Interestingly, unlike solid tumors, mutations and/or deletions of p53 in hematologic malignancies have been detected in less than 20% of patients at diagnosis, mostly in patients with 17p monosomy, which is often associated with \textit{TP53} mutation of the...
second allele (12). Of note, recent studies have shown that Nutlin-3, used alone or in combination with chemotherapeutic drugs, effectively increases the degree of apoptosis in different hematopoietic malignancies (13–15), including B-CLL (16–19). In spite of these studies, the potential mechanisms of action of Nutlin-3 are still not completely understood. In particular, both transcriptional-dependent and transcriptional-independent mechanisms have been proposed to explain the cytotoxic activity of Nutlin-3 in primary B-CLL patient samples and in a panel of B lymphoblastoid cell lines.

Materials and Methods

Primary B-CLL patient cells and continuous cell lines

For experiments with primary cells, peripheral blood samples were collected in heparin-coated tubes from 35 B-CLL patients following informed consent, in accordance with the Declaration of Helsinki and with approval obtained from the Institutional Review Board of the University-Hospital of Udine (Udine, Italy). The diagnosis of B-CLL was made by peripheral blood morphology and immunophenotyping. Rai stage and history of relapse. Peripheral blood mononuclear cells were characterized for ZAP70 mutational status, and chromosomal abnormalities by interphase FISH, as previously described (17). All patients had untreated CLL without history of relapse. Peripheral blood mononuclear cells were isolated and cultured as described in Supplementary Methods.

The p53-deleted/mutated (MEC1, MEC2, and BJAB) B leukemic cell lines were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and cultured as described in Supplementary Methods.

The oncogene TCL1 has been shown to play a key role in promoting leukemic transformation in B chronic lymphocytic leukemia (B-CLL), but whether TCL1 can be modulated by pharmacologic treatments is currently unknown. By analyzing the effect of Nutlin-3, a nongenotoxic activator of the p53 pathway, on primary B-CLL patient cells and in a panel of B leukemic cell lines with different p53 status, the authors showed for the first time that Nutlin-3 induces the downregulation of endogenous TCL1 in a p53-dependent manner. Given the lack of toxic effects of Nutlin-3 in initial human testing, the results of this study provide insight for further clinical evaluation of Nutlin-3 in B-CLL.

Translational Relevance

The oncogene TCL1 has been shown to play a key role in promoting leukemic transformation in B chronic lymphocytic leukemia (B-CLL), but whether TCL1 can be modulated by pharmacologic treatments is currently unknown. By analyzing the effect of Nutlin-3, a nongenotoxic activator of the p53 pathway, on primary B-CLL patient cells and in a panel of B leukemic cell lines with different p53 status, the authors showed for the first time that Nutlin-3 induces the downregulation of endogenous TCL1 in a p53-dependent manner. Given the lack of toxic effects of Nutlin-3 in initial human testing, the results of this study provide insight for further clinical evaluation of Nutlin-3 in B-CLL.

Culture treatments and assessment of cell viability and apoptosis

Both primary B-CLL cells and lymphoblastoid cell lines were seeded at a density of 1 × 10^6 cells/mL before treatment with Nutlin-3 (10 μmol/L; Cayman Chemical). Cell viability was examined at 24 hours after treatment by Trypan blue dye exclusion (25) and confirmed by MTT colorimetric assay (Roche Diagnostics Corporation). The induction of apoptosis was quantified by Annexin V-fluorescein isothiocyanate/propidium iodide staining (Immuno) followed by flow cytometric analysis, as previously detailed (26).

RNA analyses

Total RNA was extracted from cells by using a Qiagen RNeasy Plus mini kit (Qiagen) according to the supplier’s instructions and as previously described (27). For detailed description of procedures, refer to Supplementary Methods.

Western blot analyses

Cells were lysed in radioimmunoprecipitation assay buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.1% SDS, 1% Nonidet P-40, 0.25% sodium deoxycholate), supplemented with protease inhibitors (Roche), and processed as previously described (28). Equal amounts of protein for each sample were migrated in acrylamide gels and blotted onto nitrocellulose filters. The following monoclonal antibodies were used in our experiments: anti-ZAP70 (Millipore), anti-MDM2 (Santa Cruz Biotechnology), anti-TCL1 (Cell Signaling Technology), and anti-tubulin (Sigma-Aldrich). After incubation with peroxidase-conjugated anti-mouse IgG, specific reactions were revealed with the enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

Transfection experiments

EHEB cells (7 × 10^6) were resuspended into 0.1 mL Nucleofector solution of human nucleofector kit V (Amaxa). Two micrograms of plasmid DNA [green fluorescent protein (GFP) construct] were mixed with the 0.1 mL cell suspension, transferred into a 2.0-mm electroporation cuvette, and nucleofected using an Amaxa Nucleofector II apparatus (program U-013), following the manufacturer’s guidelines. Transfection efficiency was estimated in each experiment by scoring the number of GFP-positive cells by flow cytometric analysis. In other experiments, cells were transfected with the mammalian vector pcDNA3TCL1fl expressing the full-length TCL1 (29), or with the control empty plasmid pcDNA3. For siRNA transfection description, refer to Supplementary Methods.

Statistical analysis

Data are shown as mean ± SD or as median and interquartile range, according to the distribution. The results were evaluated by using ANOVA with subsequent comparisons by Student’s t test and with the
Mann–Whitney rank-sum test. Statistical significance was defined as $P < 0.05$.

Results

Differential baseline levels of TCL1 in primary B-CLL samples and B lymphoblastoid cell lines with ZAP70$^\text{high}$ versus ZAP70$^\text{low/absent}$

B-CLL patient samples enrolled in this study ($n = 35$) were characterized for ZAP70 expression levels, chromosomal aberrations, and presence of TP53 deletion and/or mutations, as summarized in Table 1. Baseline levels of TCL1 mRNA evaluated by real-time PCR (Fig. 1A) were significantly ($P < 0.01$) higher in those patient samples with higher ZAP70 expression, evaluated by Western blot analysis as exemplified in Figure 1B. Similar findings were observed when TCL1 mRNA was analyzed in a panel of B lymphoblastoid cell lines with different ZAP70 levels (Fig. 1B). Also in this case, EHEB, MEC1, and MEC2 cell lines, characterized by ZAP70$^\text{high}$ expression, showed significantly ($P < 0.05$) higher levels of TCL1 mRNA than BJAB, JVM2, and JVM3 cell lines, characterized by ZAP70$^\text{low/absent}$ expression (Fig. 1A and B).

### Table 1. Clinical and laboratory features of untreated CLL patients

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Abbreviations: mut, mutated; wt, wild-type; NA, not available.

$^a$FISH defects were found using a B-CLL FISH panel.

$^b$ZAP70 expression was determined by Western blot analysis.
The nongenotoxic activation of the p53 pathway downregulates TCL1 in p53wild-type but not in p53mutated primary B-CLL cells and B lymphoblastoid cell lines

Among the 35 B-CLL patients involved in our study, 12 showed a 17p− deletion at FISH analysis (patients 1, 2, 6, 11, 22, 23, 28, 29, 30, 31, 33, 34 of Table 1). Six patients with 17p− deletion also presented a mutation in the remaining TP53 allele, consisting mainly of amino acid substitutions (patients 28, 29, 30, 33, 34) or deletion (patient 31) in exons involved in DNA-binding function. An additional patient showed TP53 mutation resulting in an amino acid substitution in the absence of 17p− deletion (patient 32 of Table 1). These parameters are relevant taking into consideration that the mechanism of action of Nutlin-3 requires an intact p53 pathway for optimal activity (11).

After treatment with Nutlin-3 for 24 hours, a significant (P < 0.05) downmodulation of TCL1 mRNA with respect to untreated samples was observed in 22 of 28 B-CLL with p53wild-type, including 6 samples carrying 17p− and only in 1 of 7 B-CLL with p53mutated. On the other hand, TCL1 mRNA levels were completely unaffected in 5 B-CLL samples carrying 17p− and p53mutated and in 1 p53mutated B-CLL without 17p− (Fig. 2A). Consistently, Nutlin-3 downregulated TCL1 mRNA levels also in p53wild-type but not in

Figure 1. Correlation between ZAP70 expression and high baseline levels of TCL1 mRNA in B-CLL cells. Baseline TCL1 expression was comparatively analyzed by quantitative real-time PCR in primary B-CLL patient samples (Table 1) and in B-CLL lines (EHEB, MEC1, MEC2, JVM2, JVM3, and BJAB). A, levels of TCL1 mRNA were analyzed in relationship to the ZAP70 (low vs. high) expression. B, ZAP70 protein levels, analyzed by Western blot analysis, are shown for representative B-CLL patient (Pt.) samples and for the B-CLL cell lines. Tubulin staining is shown as the loading control.

Figure 2. Transcriptional downmodulation of TCL1 by Nutlin-3 in p53wild-type B-CLL cells. B-CLL leukemic patient samples (Table 1) and cell lines (EHEB, MEC1, MEC2, JVM2, JVM3, and BJAB) were either left untreated or exposed to Nutlin-3 (10 μmol/L). After 24 hours of treatment, mRNA levels of TCL1 and MDM2 (analyzed as the internal control, for comparison) were assessed by quantitative real-time PCR. Results were expressed as fold of either TCL1 (A) or MDM2 (B) modulation in Nutlin-3–treated cultures with respect to the control untreated cultures, which were set to 1 (white bars). TCL1 and MDM2 mRNA modulation was compared between the p53wild-type (p53wt) and the p53mutated (p53mut) leukemic patient cell samples or cell lines. In A and B, data are expressed as means ± SD. *, P < 0.05. In C, TCL1 and MDM2 protein modulation by Nutlin-3, analyzed by Western blot analysis, are shown for representative B-CLL patient (Pt.) samples and for the B-CLL cell lines after 24 hours of treatment. Tubulin staining is shown as the loading control.
p53mutated B lymphoblastoid cell lines (Fig. 2A). The functionality of the p53 pathway in these leukemic cell samples was underscored by the concomitant ability of Nutlin-3 to significantly \( P < 0.05 \) upregulate the steady-state mRNA levels of the p53 target gene MDM2 (11) in p53wild-type but not in the p53mutated cell lines (Fig. 2B). The opposite modulation of TCL1 and MDM2 in p53wild-type, but not in p53mutated, leukemic cells in response to Nutlin-3 treatment was also confirmed at protein levels (Fig. 2C).

**The knockdown of p53 counteracts the Nutlin-3 downregulation of TCL1**

The data illustrated earlier suggested, but did not prove, that the Nutlin-3–mediated downregulation of TCL1 requires a functional p53 pathway. In this respect, a direct role for p53 in regulating/repressing the TCL1 levels was further suggested by the observation that computational analysis (as predicted by SABiosciences' Text Mining Application and the UCSC Genome Browser) on TCL1 promoter showed the presence of some p53 consensus DNA-binding sites (Fig. 3A). To clarify the p53 dependence of the observed gene modulations, we analyzed the effect of Nutlin-3 in EHEB cells transiently transfected with p53 siRNA (Fig. 3B). Because in EHEB cells, transfection efficiency is approximately 30% to 40%, p53 is not completely silenced but yet significantly knocked down, as evaluated by quantitative real-time PCR assay (data not shown). The ability of Nutlin-3 to decrease TCL1 RNA levels was significantly \( P < 0.05 \) attenuated with respect to cells transfected with control scramble siRNA (Fig. 3B).

**TCL1 overexpression counteracts the Nutlin-3–induced apoptosis in EHEB cells**

Considering previous studies suggesting that TCL1 expression has been linked to an aggressive behavior of B-CLL (5, 6), in the last group of experiments we sought to investigate the role played by TCL1 in modulating the response to Nutlin-3. For this purpose, upon transfection of the p53wild-type EHEB cell line with the TCL1-expressing plasmid (Fig. 4A), we could observe that the overexpression of TCL1 efficiently \( P < 0.05 \) counteracted the cytotoxicity induced by Nutlin-3, as indicated by the recovery of the cell viability and by the reduction of the percentage of apoptotic cells (Fig. 4B). These data support the idea for a critical role of TCL1 in modulating the antileukemic activity of Nutlin-3.

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**Figure 3.** Role of p53 in TCL1 transcriptional downmodulation by Nutlin-3.

A, regulatory p53 transcription factor binding sites in the TCL1 gene sequence. B, TCL1 mRNA modulation by Nutlin-3 was analyzed after 24 hours of treatment in EHEB cells either not transfected (Not transf.) or transiently transfected with p53 siRNA. Data are reported as means ± SD of results from at least 3 experiments, each carried out in triplicate. *P < 0.05.

**Figure 4.** TCL1 overexpression counteracts Nutlin-3 cytotoxicity. EHEB cells were transiently transfected with pTCL1-expressing vector or with control empty vector (A). EHEB cell culture, either left not transfected or transfected as indicated, were exposed to Nutlin-3 (10 μmol/L) and analyzed for apoptosis induction (B), after 48 hours of treatment. Data are reported as means ± SD of results from at least 3 experiments, each carried out in triplicate. *P < 0.05.
Discussion

Previous studies have shown that the pattern of response to B-cell receptor (BCR) engagement in B-CLL is highly correlated with cellular levels of the lymphoid oncogene TCL1 and with the formation of activation complexes at the BCR that include TCL1, Akt, and membrane-proximal tyrosine kinases such as ZAP70. The CLL cases with high TCL1 also showed more aggressive growth features in vivo, including advanced clinical stage, higher white blood cell counts, shorter lymphocyte doubling time, and poor response to all therapy types, with TCL1 levels as an independent predictor of outcome in multivariate models (5, 6).

Although little doubt exists on the link between Nutlin-3–mediated transcriptional activity of p53 and cell-cycle arrest mediated by p21, some recent studies have provided evidence for a transcription-independent induction of apoptosis by Nutlin-3 (20–22, 30, 31). In particular, these studies have shown that the transcription-independent mitochondrial p53 program plays an important role in Nutlin-3–induced p53-mediated tumor cell apoptosis. In fact, aside from nuclear stabilization, Nutlin-3a causes cytoplasmic p53 accumulation and translocation to mitochondria (20, 30). However, previous studies from our group have shown that the ability of Nutlin-3 to induce a characteristic gene expression signature directly correlates with the cytotoxic activity of Nutlin-3 (24). Starting from these bases, in this study, we have shown for the first time that Nutlin-3 downregulated the oncogene TCL1 in the majority of primary B-CLL samples carrying wild-type p53 (22 of 28), including 6 samples with the unfavorable chromosomal abnormality 17p–, whereas B-CLL samples carrying mutated p53 did not show any decrease of TCL1. Nutlin-3 significantly downregulated TCL1 mRNA levels also in p53Wild-type but not in p53Mutated B lymphoblastoid cell lines. The p53 dependence of TCL1 downregulation was shown by experiments carried out with siRNA specific for p53. In fact, knockdown of p53 counteracted the ability of Nutlin-3 to downregulate TCL1 in leukemic cells. Although relatively little is known about the regulation of TCL1 expression, and previous data have shown that TCL1 can be regulated by selective miRNA (32), 2 potential p53 binding sites are present in the TCL1 promoter, suggesting that p53 might directly induce the transcriptional repression of TCL1.

Another important conclusion of our study is that TCL1 overexpression counteracts Nutlin-3–mediated apoptosis in lymphoblastoid B cells, substantiating an important role of TCL1 in promoting leukemogenesis. Thus, although it has been argued that transcriptional-independent mechanisms play a key role in mediating the cytotoxic activity of p53 (20–22, 30, 31), perhaps due to the presence of negative feedback loops (33), our data show for the first time that the transcriptional downregulation of the oncogene TCL1 likely represents an important mechanism of action by which Nutlin-3 promotes apoptosis in leukemic cells.

In conclusion, we have shown for the first time that activation of p53 represses the transcription of TCL1 in primary B-CLL cells and p53Wild-type lymphoblastoid cells and we propose that such downregulation is an important mediator of Nutlin-3 cytokotoxic activity. Therapeutic strategies able to downregulate TCL1 should be further explored to improve the antileukemic activity of Nutlin-3.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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p53-Dependent Downregulation of TCL1


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Rebecca Voltan, Maria Grazia di Iasio, Raffaella Bosco, et al.


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