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Expression level of DEK in chronic lymphocytic leukemia is regulated by fludarabine and Nutlin-3 depending on p53 status

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Human oncogene DEK has been shown to be upregulated in a number of neoplasms. The purpose of this study was to investigate DEK expression level in chronic lymphocytic leukemia (CLL), analyze the correlation between DEK expression and CLL prognostic markers, and characterize the role of DEK in the response to either chemotherapeutic drugs or nongenotoxic activators of the p53 pathway. DEK mRNA was evaluated by real-time quantitative reverse transcriptase-polymerase chain reaction (qPCR), and primary CLL samples were treated in vitro with either fludarabine or Nutlin-3 to explore the interaction of p53 status and DEK mRNA expression. The median expression levels of DEK mRNA were 6.792×10^{-2} (1.438×10^{-2}–3.201×10^{-1}) in 65 patients with CLL. A marked increase of DEK mRNA expression was observed in the CLL patients with unmutated immunoglobulin heavy chain variable (IGHV) gene ($p = 0.025$), CD38-positive ($p = 0.047$), del(17p13) ($p = 0.006$). Both fludarabine and Nutlin-3 significantly downregulated DEK in the primary CLL cells which were with normal function of p53, or without deletion or mutation of p53 ($p = 0.042$, $p = 0.038$; $p = 0.021$, $p = 0.017$; $p = 0.037$, $p = 0.017$). However, the downregulation of DEK was not observed in the primary CLL cells which were with dysfunction of p53, or with deletion or mutation of p53 ($p = 0.834$, $p = 0.477$; $p = 0.111$, $p = 0.378$; $p = 0.263$, $p = 0.378$). These data show that DEK might be applied for the assessment of prognosis in patients with CLL, and fludarabine and Nutlin-3 regulate DEK expression depended on p53 status.

Introduction

Human oncogene DEK located on chromosome 6p22-23.[1,2] It is a 375 amino acid (43 kDa) abundant nuclear protein with important functions in the architectural regulation of chromatin assembly.[3,4] DEK was initially identified as a fusion protein with CAN nucleoporin in a subtype of acute myeloid leukemia (AML) involving the t(6;9) translocation.[5] Subsequent studies have repeatedly identified DEK as a frequently overexpressed gene independent of the t(6;9) translocation in a number of neoplasms including melanoma, hepatocellular carcinoma, glioblastoma, retinoblastoma, uterine cervical cancers, ovarian cancers, and bladder cancer.[1,6-12] Furthermore, autoantibodies to DEK have been detected in juvenile rheumatoid arthritis, systemic lupus erythematosus and sarcoidosis.[13,14] Though precise cellular function of DEK remains unclear, several studies have implicated DEK in a variety of cellular processes, such as DNA replication, splice site recognition, and gene transcription, as well as in the control of cell viability, differentiation, and cell-to-cell signaling.[15-23] The mechanisms through DEK mediates its oncogenic effects are only partially understood. However, it has been proposed that the oncogenic role of DEK is mediated by its ability to destabilize p53 protein and to inhibit p53 activity and p53-mediated apoptosis.[10,24-26] DEK can cooperate with the oncogenes E6 and E7 to overcome senescence,[27] and promote epithelial transformation in vitro and in vivo when overexpressed.[28,29]

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the Western world, but less frequent in Eastern countries. The clinical course of CLL is highly variable. One third of CLL patients require therapy as soon as they are diagnosed, one third survive for many years without therapy, and one third have disease progression over the years and require treatment at some point.[30] Previous study reported upregulation of DEK in a subset of CLL with del(11q23) and advanced clinical stage.[31]

In this study, we detected DEK expression by real-time quantitative reverse transcriptase-polymerase chain reaction (qPCR) in 65 Chinese patients with CLL to investigate the DEK expression level in CLL, and analyze the correlation between DEK expression and CLL prognostic markers such as clinical stage, immunoglobulin heavy-chain variable region (IGHV) mutational status, ZAP-70, CD38, and chromosomal abnormalities. Furthermore, primary CLL samples were treated in vitro with

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either fludarabine, which represented the treatment of choice for CLL, or Nutlin-3, which showed promising cytotoxic activity against CLL,[32-36] to explore the role of DEK in the response to either chemotherapeutic drugs or nongenotoxic activators of the p53 pathway.

Results

Clinical characteristics of CLL patients. The characteristics of 65 CLL patients are summarized in **Table 1**. Forty-three patients were male and 22 were female (male: female, 2.0), and the median age was 60 y (rang: 44–84). According to the Binet staging system,[37] 29 (44.6%) patients were in stage A, 10 (15.4%) in stage B, and 26 (40.0%) in stage C.

DEK mRNA expression in 65 CLL patients and the correlations between DEK expression and prognostic factors. The median expression levels of DEK mRNA were 6.792×10^{-2} (1.438×10^{-2}-3.201×10^{-1}) in 65 patients with CLL. The correlations between DEK expression and prognostic factors were shown in **Table 2**. A marked increase of DEK mRNA expression was observed in CLL patients with unmutated IGHV ($p = 0.025$), CD38-positive ($p = 0.047$), del(17p13) ($p = 0.006$).

The clinical characteristics of the CLL patients with cell culture. For preparation of primary cell cultures, CLL cells were from 22 untreated CLL patients. The clinical and biological characteristics of these patients are detailed in **Table 3**. Four cases showed p53 mutations paired to del(17p13), one cases showed del(17p13) in the absence of p53 mutations. The function of p53/p21 was detected by flow cytometry both at the beginning of isolation and after 24 h fludarabine treatment. The level of p53 protein was not increased after treatment with fludarabine in five patients. The level of p21 protein of eight patients showed no increase after fludarabine-treatment, implying p53 dysfunction. In the rest cases, the p21 protein was increased after fludarabine-treated, suggesting p53 normal function.[38]

Fludarabine and Nutlin-3 regulate DEK expression depended on p53 status. To assay the interaction of p53 status and DEK mRNA expression, we detected the mRNA expression in fludarabine-treated (n = 22) or Nutlin-3-treated (n = 11) CLL cells by qPCR. The primary CLL cells with normal p53 function, without deletion or mutation of p53, the level of DEK expression was significantly decreased after 24 h treatment with fludarabine and Nutlin-3 compared with the cells in medium only ($p = 0.042$, $p = 0.038$; $p = 0.021$, $p = 0.017$; $p = 0.037$, $p = 0.017$) (**Fig. 1**). However, the DEK was not downregulated in the primary CLL cells with dysfunction of p53, with deletion or mutation of p53 ($p = 0.834$, $p = 0.477$; $p = 0.111$, $p = 0.378$; $p = 0.263$, $p = 0.378$) (**Fig. 1**). These results indicated that fludarabine and Nutlin-3 regulated DEK expression depended on p53 status, but certainly not consistently.

Discussion

The chromatin architectural factor DEK is an oncogene located on the region of 6p22–23.[1,2] It was discovered by the identification of the t(6;9) in a subset of patients with AML, and was

Table 1. Clinical and biological characteristics of 65 patients with chronic lymphocytic leukemia

Characteristics	Value (%)
Gender	
Male	43 (66.2)
Female	22 (33.8)
Age (years)	
≥ 60	33 (50.8)
< 60	32 (49.2)
Binet stages	
Binet A	30 (46.2)
Binet B or C	35 (53.8)
LDH (n = 45)	
≥ 250U/L	12 (26.7)
< 250U/L	33 (73.3)
β2-MG (n = 41)	
≥ 3 mg/L	25 (61.0)
< 3 mg/L	16 (39.0)
CD38 (n = 63)	
> 30%	22 (34.9)
≤ 30%	41 (65.1)
ZAP-70 (n = 63)	
> 20%	14 (22.2)
≤ 20%	49 (77.8)
Del(13q14) (n = 53)	
Positive	19 (35.8)
Negative	34 (64.2)
Trisomy 12 (n = 50)	
Positive	15 (30.0)
Negative	35 (70.0)
Del(17p13) (n = 54)	
Positive	9 (16.7)
Negative	45 (83.3)
IgH rearrangements (n = 47)	
Positive	11 (23.4)
Negative	36 (76.6)
Del(11q22.3) (n = 57)	
Positive	9 (15.8)
Negative	48 (84.2)
IGHV mutational status (n = 59)	
Mutated	44 (74.6)
Unmutated	15 (25.4)
p53 mutations (n = 58)	
Mutated	11 (19.0)
Unmutated	47 (81.0)

Abbreviations: β2-MG, β 2-microglobulin; IGHV, heavy chain variable region; LDH, lactate dehydrogenase.

Table 2. The differences of DEK mRNA expression level between various groups of patients

Clinical features	DEK [*M (P5-P95)*]	p value
Age (years)		0.773
≥ 60	7.815×10^{-2} (1.712×10^{-2}-3.828×10^{-2})	
< 60	8.390×10^{-2} (2.238×10^{-2}-5.455×10^{-1})	
Binet stages		0.612
A	7.757×10^{-2} (2.903×10^{-2}-2.364×10^{-2})	
B+C	8.407×10^{-2} (1.841×10^{-2}-6.077×10^{-1})	
LDH (n = 45)		0.115
≥ 250U/L	9.121×10^{-2} (1.438×10^{-2}-2.535×10^{-1})	
< 250U/L	6.255×10^{-2} (2.20×10^{-2}-4.989×10^{-1})	
β2-MG (n = 41)		0.33
≥ 3 mg/L	9.016×10^{-2} (1.771×10^{-2}-4.029×10^{-1})	
< 3 mg/L	6.987×10^{-2} (2.281×10^{-2}-5.885×10^{-1})	
CD38 (n = 63)		0.047
≤ 30%	9.333×10^{-2} (2.210×10^{-2}-6.029×10^{-1})	
> 30%	7.563×10^{-2} (1.835×10^{-2}-3.713×10^{-1})	
ZAP-70 (n = 63)		0.552
≤ 20%	8.098×10^{-2} (1.738×10^{-2}-4.531×10^{-1})	
> 20%	8.205×10^{-2} (2.247×10^{-2}-5.407×10^{-1})	
Del(13q14) (n = 53)		0.159
Positive	6.562×10^{-2} (1.745×10^{-2}-3.861×10^{-1})	
Negative	9.190×10^{-2} (2.247×10^{-2}-6.364×10^{-1})	
Trisomy 12 (n = 50)		0.546
Positive	7.962×10^{-2} (3.443×10^{-2}-5.407×10^{-1})	
Negative	8.520×10^{-2} (2.128×10^{-2}-6.364×10^{-1})	
Del(17p13) (n = 54)		0.006
Positive	1.374×10^{-1} (2.256×10^{-2}-3.201×10^{-1})	
Negative	7.405×10^{-2} (1.919×10^{-2}-5.885×10^{-1})	
IgH rearrangements (n = 47)		0.883
Positive	7.778×10^{-2} (3.126×10^{-2}-1.103×10^{-1})	
Negative	8.935×10^{-2} (2.241×10^{-2}-6.449×10^{-1})	
Del(11q22.3) (n = 57)		0.498
Positive	1.070×10^{-1} (2.256×10^{-2}-3.861×10^{-1})	
Negative	7.899×10^{-2} (1.607×10^{-2}-7.126×10^{-1})	
IGHV mutation status (n = 59)		0.025
Mutated	1.115×10^{-2} (1.724×10^{-2}-2.264×10^{-1})	
Unmutated	6.976×10^{-2} (2.386×10^{-2}-6.021×10^{-1})	
p53 mutations (n = 58)		0.655
Mutated	7.513×10^{-2} (1.438×10^{-2}-1.142×10^{-1})	
Unmutated	8.55×10^{-2} (2.350×10^{-2}-4.406×10^{-1})	

Abbreviations: β2-MG, β 2-microglobulin; IGHV, heavy chain variable region; LDH, lactate dehydrogenase.

named based on the initials of the patient DEK.[5] The observation that this chromosomal change was associated with an accelerated tumor onset and poor prognosis prompted a series of studies that ultimately support a causative role of DEK in tumor development.[3,4] DEK has been shown to be upregulated in AML,[5,39,40] retinoblastoma,[2,41,42] glioblastoma,[6] hepatocellular carcinoma,[7] melanoma,[9] and in an increasing list of other tumor types.[1,8,10] However, the mechanisms leading to this preferential accumulation of DEK in cancer cells are not completely understood.

With the increasing list of tumor types, high expression of DEK raises the exciting possibility of using DEK as a tumor marker. The finding that DEK expression levels can distinguish benign nevi from malignant melanomas is a prime example of a clinically relevant setting in which DEK may prove to be highly useful.[10] Moreover, as DEK may be present at higher levels in immature cells than in their differentiated counterparts,[43] it could also aid in gauging the differentiation potential of tumor cells. In fact, t(6;9) translocation has been suggested to be considered in AML prognostic stratification.[44] In this study, the correlations between DEK expression and prognostic factors of CLL were analyzed. A marked increase of DEK mRNA expression was observed in CLL patients with unmutated IGHV, CD38-positive, and del(17p13). This is the first study to correlate DEK expression levels in CLL with prognostic factors to understand the role of DEK upregulation in CLL progression. Our results suggest that the expression level of DEK might be applied for the assessment of prognosis in patients with CLL.

The crosstalk between DEK, p53 and the apoptotic machinery deserves attention, as this information may guide the development of improved therapies. In response to a variety of stimuli, such as cellular stress induced by chemotherapeutic drugs, the p53-MDM2 interaction is disrupted and p53 rapidly accumulates within the cells.[32] Alternatively, p53 can accumulate in response to selective small-molecule inhibitors of the p53-MDM2 interaction, which binds MDM2 in the p53 binding pocket with high selectivity and can release p53 from negative control leading to effective stabilization of p53 and activation of the p53 pathway.[36] In this study, we have shown that both chemotherapeutic drugs (fludarabine) and nongenotoxic activators of the p53 pathway (Nutlin-3) significantly downregulated DEK in the primary CLL cells with normal p53 function, or without deletion or mutation of p53. However, the DEK was not downregulated in the primary CLL cells with p53 dysfunction, or with deletion or mutation of p53. Although these data clearly indicate that a p53 status is necessary to observe the DEK downregulation in response to either fludarabine or Nutlin-3 in CLL cells, the exact role of p53 in DEK regulation remains to be determined.

In conclusion, we provide evidence that increased expression of DEK correlates with IGHV mutational status, CD38-positive and del(17p13), and DEK can therefore be considered as potential prognostic factor. As the result that fludarabine and Nutlin-3 regulate DEK expression depended on p53 status, therapeutic strategies able to downregulate DEK expression should be further explored to improve the antileukemic activity of both conventional and novel antileukemic drugs.

Table 3. Clinical and biological characteristics in 22 CLL patients with cell culture

No.	Gender	Age (years)	Binet stages	IGHV mutation status	Fludarabine-treated		p53 mutation status	p53 deletion	ATM deletion
					p53	p21			
1	Female	78	A	Mutated	Yes	Yes	Wild type	No	No
2	Female	57	B	Unmutated	No	No	Mutated	Yes	Yes
3	Female	62	C	Mutated	Yes	Yes	Wild type	No	No
4	Male	83	B	Mutated	Yes	No	Mutated	Yes	No
5	Female	62	C	Mutated	Yes	No	Wild type	No	No
6	Female	52	C	Unmutated	Yes	Yes	Wild type	No	Yes
7	Male	48	C	Unmutated	No	No	Mutated	Yes	No
8	Male	58	C	Unmutated	No	Yes	Wild type	No	No
9	Male	50	A	Mutated	Yes	Yes	Wild type	No	No
10	Female	54	B	Mutated	Yes	Yes	Wild type	No	No
11	Male	80	C	Unmutated	Yes	Yes	Wild type	No	No
12	Female	70	B	Unmutated	No	No	Wild type	No	No
13	Female	67	A	Mutated	Yes	Yes	Wild type	No	No
14	Female	71	A	Unmutated	Yes	Yes	Wild type	No	No
15	Male	52	A	Unmutated	Yes	Yes	Wild type	No	No
16	Male	55	A	Mutated	Yes	Yes	Wild type	No	Yes
17	Female	61	C	Mutated	No	No	Wild type	No	No
18	Male	68	A	Mutated	Yes	No	Mutated	No	No
19	Male	53	C	Mutated	Yes	Yes	Wild type	No	No
20	Male	49	C	Unmutated	Yes	No	Mutated	Yes	No
21	Female	64	A	Unmutated	Yes	Yes	Wild type	Yes	No
22	Female	57	A	Mutated	Yes	Yes	Wild type	No	Yes

"Yes" stands for raise up or positive; "No" stands for no change or negative.

Materials and Methods

Patients. Our study population consisted of 65 consecutive patients with newly diagnosed and untreated CLL between December 2004 and January 2011. All patients provided their informed consent and the research project was approved by the University and Institutional Review Boards. The diagnosis was based on the revised NCI criteria.[45] The staging of CLL was performed according to the Binet stage system.[37] Data collected at diagnosis included: age, gender, Binet stages, β2-microglobulin (β2-MG) and lactate dehydrogenase (LDH). A range of other prognostic markers was also analyzed for the majority of patients: IGHV and p53 mutational status, CD38 and ZAP-70 expression and cytogenetics by florescence in situ hybridization (FISH).

Detection of CD38 and ZAP-70 by flow cytometry. Flow cytometric analysis of CD38 and ZAP-70 was performed as previously described.[46] Cut-off points of 30% and 20% were used to define positivity for CD38 and ZAP-70, respectively.

IGHV mutational status analysis. IGHV mutational status was detected by IGHV gene primer and IGH Somatic Hypermutation Assay for Gel Detection kit (InVivoScribe Company). The multiplex PCR products of IGHV were detected by direct sequencing as previously described.[47] A germline homology of 98% was used as the cut-off between IGHV mutated and unmutated cases.

p53 mutational status analysis. p53 mutational status was studied by PCR and direct sequencing. We used the same primers as those mentioned in the previous study.[48] PCR products of p53 were purified by standard methods (Invitrogen) and directly sequenced using the ABI3730XL 96-capillary DNA Analyzer (Applied Biosystems).

Detection of molecular cytogenetic aberrations by FISH. FISH analysis was performed on the sample for conventional cytogenetic studies. In order to detect prognostically relevant anomalies of chromosomal regions 6q, 11q, 13q, 14q, 17p and chromosome 12, the following fluorescent-labeled probes were used in interphase cytogenetic analyses: LSI MYB (6q23), LSI ATM (11q22), LSI D13S319 (13q14), LSI IGHC/IGHV (14q32), LSI p53 (17p13) and CEP12 (centromere 12) (all probes were purchased from Vysis, Downers Grove, IL, USA). FISH was performed as described.[49] The cut-off levels for positive values (mean of normal control ± 3 SD), determined from samples of 8 cytogenetically normal persons, was 7.5%, 7.7%, 10.3%, 8.9%, 5.2% and 3.0% for del(6q23), del(11q22), del(13q14), 14q32 translocation, del(17p13) and trisomy 12, respectively.

The protocol of primary cell culture. For preparation of primary cell cultures, CLL cells from 26 untreated patients were

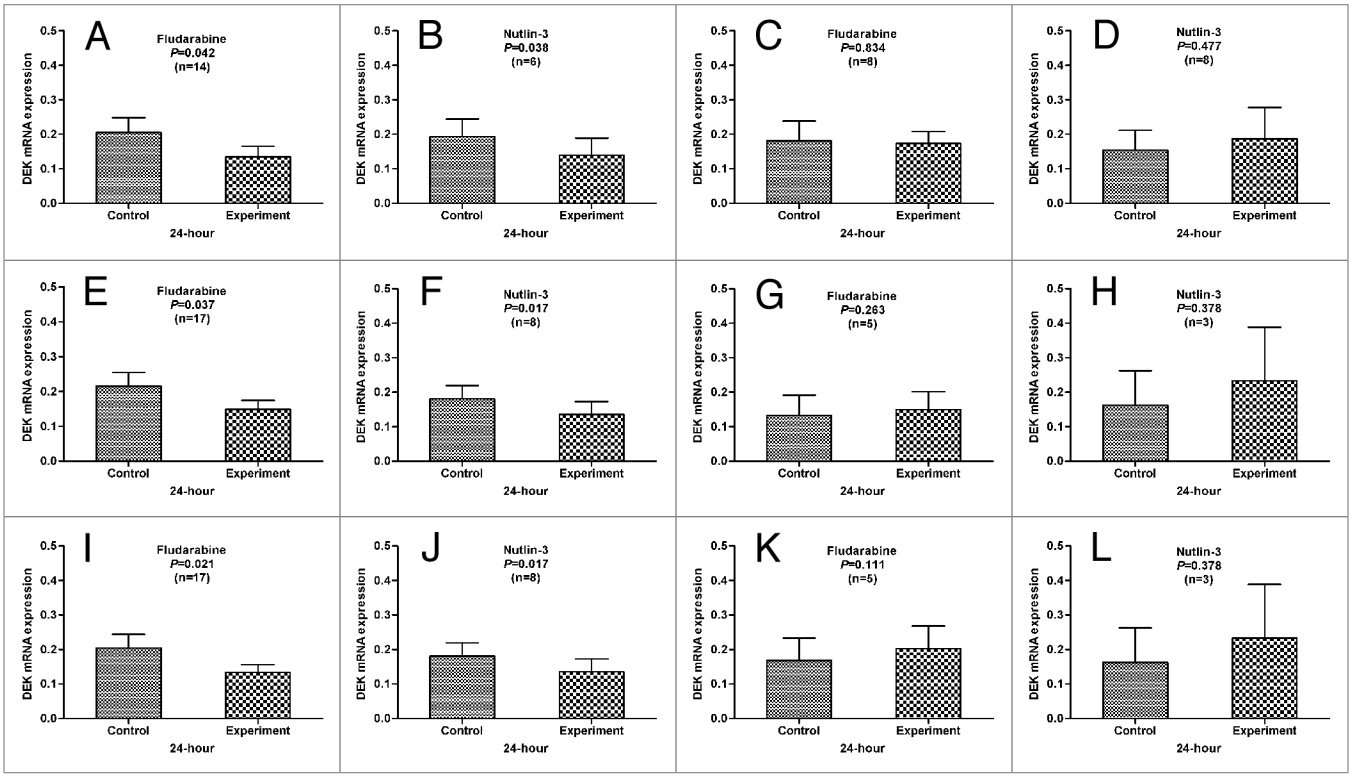

Figure 1. The correlation between the p53 status and the level of DEK expression. In the primary CLL cells with normal p53 function (**A and B**), or without deletion (**E and F**) or mutation (**I and J**) of p53, DEK expression was significantly decreased after 24 h treatment with fludarabine or Nutin-3 compared with the cells in medium only. However, this downregulation of DEK expression was not observed in the primary CLL cells with p53 dysfunction (**C and D**), or deletion (**G and H**) or mutation (**K and L**) of p53.

Table 4. The sequences of qRT-PCR primers of DEK and β-actin

Primer		Sequence	Length of product
DEK	forward	5'-TCC AAA GCC TTC TGG CAA ACC ATT-3'	214 bp
	reverse	5'-TGG TGG CTC CTC TTC ACT TTC TTT A-3'	
β-actin	forward	5'-AGC GAG CAT CCC CCA AAG TT-3'	285 bp
	reverse	5'-GGG CAC GAA GGC TCA TCA TT-3'	

isolated from heparinized venous blood by density gradient centrifugation. The isolated cells were predominantly CLL B cells (> 90% $CD5^+CD19^+$), as assessed by flow cytometry (FACScan, Becton Dickinson). Freshly isolated CLL cells were seeded in 6-well plates (5-10 × 10^6 cells/well), treated by 3.5 μmol/L fludarabine (Sigma) or 10 μmol/L Nutlin-3 (Sigma) or not, and cultured in RPMI-1640 medium supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO_2 at 37°C for 24 h.

Detection of the p53/p21 function by flow cytometry. The function of p53/p21 gene was detected by flow cytometry. Cells were harvested after in vitro culture 24 h with 3.5 μmol/L fludarabine (Sigma) treatment. 5 × 10^6 cells were fixed in 2% paraformaldehyde, -4°C 30 min, washed with PBS, and overnight in 80% ethanol at -20°C. Fixed cells were washed with PBS and cell membrane ruptured with cell permebilization kit (BD Biosciences FIX&PERM) at room temperature for 30 min. CLL cells were labeled with CD19-allophycocyanin away from light for 15 min, and then washed with PBS. Fixed cells were stained with p53-phycoerythrin antibody (BD Biosciences) and p21-fluorescein isothiocyanate (Calbiochem) or the corresponding isotype controls. After incubation at ambient temperature for 15 min away from light, cells were detected on the FACSCalibur and data were analyzed using the CellQuest Pro software.[38]

qPCR analysis for DEK. DEK mRNA expression was investigated by qPCR. Total RNA was isolated from peripheral blood mononuclear cells or culture cells, which had > 90% $CD5^+CD19+$ cells measured by flow cytometry. RNA (1μg) was reverse transcribed using random hexamers, and then amplification was performed with fluorescent dye SYBR Green I, PCR Master Mix and primers (**Table 4**). The β-actin was used as internal reference. Cycle conditions for DEK and β-actin were 1 cycle for 5 min at 95°C, 35 cycles for 10 sec at 95°C, 30 sec at 62°C, 30 sec at 72°C, and finally, 1 cycle for 10 min at 72°C. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passes the fixed threshold, and each sample was normalized based on its endogenous β-actin RNA content. Sequences of amplified products were verified by DNA sequencing. Each sample was replicated for two times.

Statistical analysis. All statistical analyses were performed using the SPSS program for Windows (version 17.0). ΔCt was calculated by subtracting the Ct of β-actin from the Ct of DEK. The relative quantitative value of DEK mRNA was calculated by the equation $2^{-\Delta Ct}$. The difference of DEK mRNA expression between groups with different prognostic factors was described using the Mann-Whitney U test. Differences of gene expression levels between primary CLL cells treated with or without fludarabine and Nutlin-3 were analyzed by matched-pairs t test. For all tests, a p value of 0.05 was considered significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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