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Immunostimulation by cytosine-phosphate-guanine oligodeoxynucleotides in combination with IL-2 can improve the success rate of karyotype analysis in chronic lymphocytic leukaemia

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ABSTRACT

Purpose: To assess whether immunostimulatory cytosine-phosphate-guanine oligodeoxynucleotides (CpG-ODN) combined with interleukin-2 (IL-2) improves the number of mitotic metaphases and the detection rate of chromosomal abnormalities in chronic lymphocytic leukaemia (CLL).

Materials and methods: Bone marrow specimens were collected from 36 patients with CLL. CLL cells were cultured with CpG-ODN type DSP30 plus IL-2 for 72 h, following which R-banding analysis was conducted. Conventional culture without the immunostimulant served as the control group. The incidence of genetic abnormalities was measured by fluorescence *in situ* hybridisation (FISH) using a panel of five specific probes: D13S25 (13q14.3), *RB1* (13q14), *P*53 (17p13), *ATM* (11q22.3) and CSP12 (trisomy 12, +12).

Results: In the control group, chromosome analysis achieved a success rate of only 22.2, and 11.1% of abnormal karyotypes were detected. After immunostimulation with DSP30 plus IL-2, chromosome analysis achieved a success rate of up to 91.6, and 41.6% of abnormal karyotypes were detected. FISH analysis detected 77.7% of abnormalities. FISH combined with CpG-ODN DSP30 plus IL-2 improved the detection rate of chromosomal abnormalities in CLL to 83.3%.

Conclusion: CpG-ODN DSP30 combined with IL-2 is effective in improving the detection rate of chromosomal abnormalities in CLL cells. This combination with FISH analysis is conducive to increasing the detection rate of genetic abnormalities in CLL.

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Introduction

Chronic lymphocytic leukaemia (CLL) is an abnormal clonal expansion of mature B lymphocytes and it is characterised by the aggregation of lymphocytes in the peripheral blood, bone marrow, spleen and lymph nodes. CLL has a highly heterogeneous clinical course and requires parameters to stratify patients into prognostic subgroups to receive various treatment ranging from 'watch and wait' to stem cell transplantation. Different parameters such as Rai and Binet stage, lymphocyte doubling time, β-2 microglobulin, the mutational status of the V_H genes, molecular markers such as ZAP-70 and CD38, and genetic abnormalities determined by fluorescence *in situ* hybridisation (FISH) have been used to predict survival in clinical practice.[1,2]

Many studies showed that the detection of genetic abnormalities has great value for predicting prognosis and selecting therapeutic regimes.[3,4] FISH is a rapid and sensitive technique in analysing genetic abnormalities in CLL, but new structural rearrangements, such as translocations or inversions, are not visible by FISH with a defined probe set, which underscores the importance for karyotype analysis. However, CLL originates from end-stage mature lymphocytes, and the mitotic index of CLL cells is substantially low, making it difficult to obtain metaphase tumor cells for chromosome banding analysis even in the presence of B-cell mitogens such as lipopolysaccharide and pokeweed mitogen.[5,6] Previous studies had added T-cell mitogens, such as phytohemagglutinin and 12-O-tetradecanolyphorpol-13-acetate [7–9] and was also unsatisfied with the results of mostly normal karyotypes.

Therefore, it is necessary to find a novel cytogenetic approach that can stimulate a sufficient number of high-quality metaphases for karyotype analysis in the clinics. It has been reported that unmethylated cytosine-phosphate-guanine oligodeoxynucleotide (CpG-ODN) DSP30 combined with interleukin-2 (IL-2) can effectively stimulate mitotic metaphases of CLL cells, allowing for detecting 83% of chromosomal abnormalities in CLL patients.[10] In the present study, we cultured CLL cells from 36 CLL patients with CpG-ODN DSP30 plus

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IL-2 and then investigated the cytogenetic characteristics of CLL. The results were compared with data obtained by panel FISH.

Materials and methods

Subjects

Bone marrow specimens were extracted from 36 patients with CLL who attended our hospital between March 2014 and August 2015. There were 24 men and 12 women, with a median age of 65 years (range, 43–100 years). Thirty-one cases were preliminarily diagnosed and five were re-examined. All patients underwent routine peripheral blood test, bone marrow cell morphology analysis and multi-parameter flow cytometry immunophenotyping; CLL diagnosis and staging were in accordance with the Guidelines of National Cancer Institute-sponsored Working Group on CLL.[11] The study was approved by the Ethics Committee of Fujian Medical University Union Hospital, and bone marrow samples were collected after obtaining written informed consent from each patient.

CLL cell culture

Peripheral blood or bone marrow specimens (6–8 mL) were extracted using a heparin-wetted syringe and the number of nucleated cells was counted. Nucleated cells (1×10^7) were seeded into 5 mL of Roswell Park Memorial Institute 1640 medium containing 16% fetal bovine serum, followed by addition of CpG-ODN DSP30 (1 μM; Sangon, Shanghai, China, http://www.life-biotech.com/) and IL-2 (200 U/mL; PeproTech, USA). A blank control was prepared. Cells were cultured at 37 °C under 5% CO_2 for 66–72 h and then harvested for conventional chromosome preparation as follows: Colcemid was added 1h before harvesting, and then centrifuge the cell suspensioned at 1000 rpm for 10 min and aspirate off the supernatant. The cells were then treated with 8ml prewarmed hypotonic salt solution (0.075 M KCl) and incubated 37 °C for 30 min; finally, the cells were permanently fixed using 8ml Carnoy's solution (3:1 Methanol: Glacial acetic acid). Adjusted cell concentration and dropped 3 drops of cell suspension onto the slide after 3 changes of fixative.[12] Meanwhile, 4×10^7 cells were taken to prepare specimens using the direct method for FISH analysis. The sequence of CpG-ODN DSP30 was TCG TCG CTG TCT CCG CTT CTT CTT GCC; the skeleton was a stable form with phosphorothioate modification and the product was purified by high-performance liquid chromatography.[13]

Karyotype analysis

Conventional karyotype analysis was performed using the R-banding technique.[14] Slides were spread with cell suspension for each patient and placed in the Earle's solution for 100 min, which was placed in the waterbath at 87.5 °C. The slides were stained with 50 mL 10% Giemsa stain solution in a Coplin jar for 1 hour, were rinsed in tap water and were air dried; then were assessed the metaphases under a microscope. Karyotypic abnormalities were described in detail in accordance with the International System for Human Cytogenetic Nomenclature (2013).[15]

FISH analysis

CLL detection kit was provided by GP Medical Technologies (Beijing, China, http://www.gpmedical.com.cn/), with a probe panel consisting of CSP12 (red), D13S25 (13q14.3, red), *RB1* (13q14, green), *ATM* (11q22.3, red) and *P*53 (17p13.1, green). Bone marrow cell suspension was dropped on glass slides. The slides were then dried at 60 °C in a slide warmer for 30 min, buffered in 2× SSC (saline sodium citrate buffer: 0.3M NaCl, 0.03 M sodium citrate) at 37 °C for 30 min, and dehydrated in gradient ethanol solutions (70, 85, and 100%). A work solution was prepared with a probe: hybridisation buffer ratio of 2:8, and 10 μL of work solution was added to the hybridisation zone on the slides. The slides were covered with a coverslip and the gap was sealed using a mounting medium. The samples were placed in a hybridisation oven for denaturation at 72 °C for 7 min and hybridisation at 42 °C overnight. After hybridisation, the mounting medium and coverslip were opened, and the samples were washed with 2× SSC at 46 °C for 10 min, followed by 2× SSC/0.1% NP-40 at room temperature for 5 min and 70% ethanol for 3 min. The samples were air-dried in the dark and each was added with 10 μL of 4′,6-diamidino-2-phenylindole (DAPI) counterstain, covered with a coverslip, and stained for 5 min. Bone marrow specimens from normal donor served as controls. The percentage of fluorescence-positive cells ≥ the mean ± 3 standard deviations for controls was used as a positive criterion. The threshold for CSP12 trisomy was 7.7%; the threshold for D13S25 deletion was 7.2%; the threshold for *RBl* deletion was 6.8%; the threshold for *ATM* deletion was 6.9%; and the threshold for *P*53 deletion was 7.25%. Fluorescent hybridisation signals were counted in 300 cells underneath a fluorescence microscope (Olympus BX51, Tokyo, Japan) using an oil immersion objective and filter sets for DAPI, FITC and RHOD. Raw fluorescence images were analysed using IMSTAR FISH 2.1 (GP Medical Technologies).

Statistical analysis

The detection rate of karyotypic abnormalities was compared between groups using χ^2 test. All analyses were performed using SPSS l8.0 Statistics (SPSS Inc., Chicago, IL, USA).

Results

Conventional karyotype analysis of CLL cells in two groups

In the conventional group, chromosome analysis achieved a success rate of 22.2% (8/36, >10 metaphases

analysed) and 11.1% (4/36) of abnormal karyotypes were detected. After stimulation by CpG-ODN DSP30 plus IL-2, chromosome analysis achieved a success rate of up to 91.6% (33/36) and 41.6% (15/36) of abnormal karyotypes were detected, including one chromosomal abnormality in nine cases each, two chromosomal abnormalities in two cases each, and complex karyotype in four cases. All abnormalities detected in the conventional group were also found in the CpG-ODN DSP30+IL-2 group; however, only four of 12 abnormalities in the CpG-ODN DSP30+IL-2 group were found in the conventional culture group.

In the CpG-ODN DSP30+IL-2 group, 39 abnormalities of 31 types were detected in 36 patients. Of these, 15 (38.4%) were numerical abnormalities: For chromosome gain, +12 or +12 combined other abnormalities were most common (10.2%); moreover, +9 was detected in one case and marker in two cases. For chromosome loss, –15, –16, –17 and –Y were detected in one case each, and –6 and –21 were detected in two cases each. Additionally, there were 24 (61.5%) structural abnormalities: 13q– was most frequent and found in six cases (15.4%); 6q– and 11q– in two cases each (5.1%); and 3q–, 9q+, 14q+, and 17q+ in one case each. Five cases had balanced translocation, namely t(12;17)(q23;p11), t(17;18)(p11;p11), t(11;11)(q24;q25), t(1;12;13;2)(p13;q22;q11;q11), and t(7;14)(q32;q11) in one case each. Unbalanced translocation was found in one case, der(2)t(2;2)(p15;q13). Other chromosomal rearrangements included chromosomal inversion in one case, isochromosome in one case, chromosomal insertion in one case and partial chromosome duplication in one case (Table 1).

Panel FISH

Chromosomal abnormalities were detected in 28 (77.7%) of 36 patients; the detection rates of trisomy 12, D13S25 deletion, *RB1* deletion, *ATM* deletion and *P*53 deletion were 16.7, 63.9, 55.6, 13.9 and 11.1%, respectively. Significant differences were found in the detection rate of abnormalities between FISH, CpG-ODN DSP30+IL-2, and conventional groups: the FISH group was higher than the CpG-ODN DSP30+IL-2 group ($P = 0.004$), while the CpG-ODN DSP30+IL-2 group was higher than the conventional group ($P = 0.003$) (Table 2). Two of eight FISH-negative patients demonstrated abnormalities in the CpG-ODN DSP30+IL-2 group, namely dup(12)(q12q15) and –Y. FISH detected abnormalities in 12 of 18 patients with a normal karyotype in the CpG-ODN DSP30+IL-2 group and 11 of them showed D13S25 deletion. Only

Table 1. Comparison of the karyotype analysis and FISH results from 36 patients with CLL.

Case	+12	D13S25	*RBI*	*ATM*	*P*53	1UM DSP30+IL-2	Conventional group
1	–	–	+	–	–	46,XY[20]	No metaphase
2,6,10	–	+	+	–	–	46,XY[20]	No metaphase
3	–	+	+	+	+	46,XY[10]	No metaphase
4	–	+	–	–	–	46,XY,del(11)(q21) [6]/46,XY[34]	No metaphase
5	–	+	+	+	–	46,XY,del(11)(q21q25)[2]/46,XY,add(17)(q25)[7]/46,XY[11]	46,XY,del(11)(q21q25) [5]/46,XY,add(17)(q25)[6]/46,XY[9]
7	+	+	+	–	+	45,XX,der(2)t(2;2)(p15;q13),–6,del(6)(q13q23),+12,t(12;17)(q23;p11),del(13)(q21q32), t(17;18)(p11;p11),–21[6]/46,XX[4]	No metaphase
8	+	+	+	+	–	46,XY,t(11;11)(q24;q25) [10]/46,XY[5]	No metaphase
9	–	–	–	–	–	46,XX,dup(12)(q12q15) [10]	No metaphase
11	–	–	–	–	–	46,XY[20]	46,XY[20]
12	+	–	–	–	–	47,XY,+12[10]	No metaphase
13	–	+	+	–	–	46,XY[10]	No metaphase
14	–	+	+	–	–	46,XY[10]	46,XY[10]
15	–	+	+	–	–	46,XX,del(6)(q22q23),inv(12)(q15q23), del(13)(q12q22) [20]	No metaphase
16	+	–	–	–	–	47,XY,+12[10]	No metaphase
17	–	–	–	–	+	38–42,XX,t(1;12;13;2)(p13;q22;q11;q11),ins(2;12)(p11;q23q24),del(3)(p22),–15,–16,–17,i(20q–),–21,+mar[CP18]/46,XX[2]	38–42,XX,t(1;12;13;2)(p13;q22;q11;q11),ins(2;12)(p11;q23q24),del(3)(p22),–15,–16,–17,i(20q–),–21,+mar[CP18]/46,XX[2]
18	–	–	–	–	–	46,XX[10]	No metaphase
19	–	+	+	–	–	46,XX[10]	No metaphase
20	–	–	–	–	–	45,X,–Y[19]/46,XY[1]	45,X,–Y[10]
21,24,27	–	–	–	–	–	46,XY[10]	No metaphase
22	–	+	+	–	–	47,XY,–6,+9,add(9)(q22),del(13)(q12q22),add(14)(q32),+mar[20]	No metaphase
23	–	+	–	–	–	46,XX[20]	46,XX[20]
25	–	+	+	–	–	46,XX,t(7;14)(q32;q11), del(13)(q12q14) [20]	46,XX,t(7;14)(q32;q11), del(13)(q12q14) [20]
26	–	+	+	–	–	46,XY[12]	No metaphase
28	–	+	+	+	–	No metaphase	No metaphase
29	+	+	–	–	–	No metaphase	No metaphase
30	–	+	+	–	+	No metaphase	No metaphase
31	+	–	–	–	–	47,XY,+12[5]/46,XY[5]	No metaphase
32	–	+	–	–	–	46,XX[20]	No metaphase
33	–	–	–	–	–	46,XY[20]	No metaphase
34	–	+	+	–	–	46,XY,del(13)(q12q22)[6]/46,XY[5]	No metaphase
35	–	+	+	–	–	46,XY,del(13)(q12q22)[14]/46,XY[6]	No metaphase
36	–	+	+	–	–	46,XY[20]	46,XY[20]

Table 2. Comparison of detected abnormal chromosomes in the FISH, DSP30+IL-2 and conventional groups.

		Abnormal metaphases		
	N	*n*	%	*P*
FISH group	36	28	77.7**	<0.0001
DSP30+IL-2 group	36	15	41.6*	
Conventional unstimulated group	36	4	11.1	

*DSP30+IL-2 vs. conventional group, $P = 0.003$ (OR = 5.7, 95% CI 1.7–19.6); **FISH vs. DSP30+IL-2 group, $P = 0.004$ (OR = 4.9, 95% CI 1.8–13.7)

six of 20 patients with *Rbl* deletion in FISH showed the abnormality in the CpG-ODN DSP30+IL-2 group. Four of six cases with +12 in FISH were detected in the CpG-ODN DSP30+IL-2 group. Three of five cases with *ATM* deletion in FISH were found in the CpG-ODN DSP30+IL-2 group. Two of four cases with *P*53 deletion in FISH were found in the CpG-ODN DSP30+IL-2 group. Karyotype analysis and FISH detection showed advantages and disadvantages. Combination of these two approaches improved the detection rate of cytogenetic abnormalities in CLL to 83.3% (see Table 3).

Discussion

The use of immunostimulatory CpG-ODN DSP30 has been reported to significantly improve the mitotic index and detection rate of cytogenetic abnormalities in CLL.[16,17] Studies [18,19] have shown that the key for synthetic CpG-ODN DSP30 to stimulate B cell expansion *in vitro* lies in the presence of unmethylated or low-methylated CpG motif. The structure of the CpG motif is characterised by a 6-base nucleotide sequence (5′-PuPuCGPyPy′-3′), wherein p represents the phosphodiester bond. Decker and Peschel [20] have found that CpG-ODN DSP30 can induce the expansion of B-CLL leukaemic cells, upregulate the expression of costimulatory molecules, and increase the expression of potential target antigens. IL-2 receptor existing on the B cell surface can promote activated B cells *in vitro* to expand and produce antibodies. A study [21] has reported that IL-2 can stimulate CLL cell mitosis and improve the detection rate of chromosomal abnormalities. Thus, the combined use of CpG-ODN DSP30 plus IL-2 can stimulate the metaphases of leukaemia cells, allowing for the detection of chromosomal abnormalities.[22–24]

Fifteen of 36 CLL patients (41.6%) showed karyotype abnormalities in the CpG-ODN DSP30+IL-2 group, a detection rate significantly higher than that obtained in the unstimulated conventional group (11.1%). Our result is similar to the reports of Jenderny et al. [25] although markedly lower than that reported by Haferlach et al. [10] who found chromosomal abnormalities in 415 of 506 (83%) patients with CLL after CpG-ODN DSP30+IL-2 stimulation of CLL cells. The discrepancy in the results may be caused by the procedure, in which we used whole-blood stimulation instead of using lymphocyte isolation solution to isolate tumor cells before stimulation, and the analytic skills of the researchers. Therefore, more in-depth studies need to be conducted in the future. In this study, four patients with abnormalities in the CpG-ODN DSP30+IL-2 group were also detected in the conventional group. This revealed that these abnormal karyotypes were induced by tumor cells, other than the immunostimulant. Of the chromosomal abnormalities detected, +12 was the most common numerical abnormality, in agreement with the literature.[25] The most frequent structural abnormality was 13q–, consistent with the report of Haferlach et al. [10] In summary, CpG-ODN DSP30+IL-2 stimulation can significantly improve the detection rate of abnormal karyotypes in patients with CLL. It is therefore expected to replace other polyclonal cell immunostimulants and to optimise the cell culture system for CLL.

FISH can significantly improve the detection rate of genetic abnormalities in patients with CLL. In the present study, up to 77.7% of abnormalities were detected by FISH. Therefore, it is necessary to perform FISH simultaneously in patients with normal karyotypes. FISH-negative patients still demonstrated karyotype abnormalities after CpG-ODN DSP30+IL-2 stimulation. This is because one probe can only detect one type of abnormalities in FISH. We can only select appropriate probes according to known abnormalities, making it impossible to analyse all chromosomes using FISH. In particular, FISH probes cannot identify new structural rearrangements (translocations and inversions) or complex karyotype abnormalities. In the present study, 15 numerical abnormalities were detected in the CpG-ODN DSP30+IL-2 group; of these, only 4 cases of +12 and one case of –17 were detected in FISH, whereas the remaining 10 were undetectable. Meanwhile, there were 24 structural abnormalities; of these, 10 involving segmental deletions on chromosomes 11, 13, and 17 were detected in FISH, whereas the remaining 14 involving chromosomes 2, 3, 6, 9, 12, 14, 18 and 20 were undetectable.

Therefore, FISH cannot replace the role of cytogenetic analysis in predicting the prognosis of CLL. The combination of karyotype analysis and FISH after *in vitro* culture of CLL cells with CpG-ODN DSP30 can complement each other, facilitating the detection of cytogenetic abnormalities in CLL more comprehensively and sensitively. This work represents an advance in biomedical science because it shows that FISH combined with CpG-ODN DSP30 plus IL-2 can greatly improve the detection rate of chromosomal abnormalities in CLL.

Informed consent

The study was approved by the Ethics Committee of Fujian Medical University Union Hospital, and bone marrow samples were collected after obtaining written informed consent from each patient.

Table 3. Summary.

What is known about this subject
• There hasn't an effective culture system that can stimulate a sufficiently number of high-quality metaphases for karyotype analysis in CLL
What this paper adds
• DSP30 plus IL-2 improve the success rate and detection of abnormal karyotypes in chromosome analysis in CLL
• FISH combined with CpG-ODN DSP30 plus IL-2 is most effective in improvement of detection rate of chromosomal abnormalities in CLL

Disclosure statement

The authors declare that they have no conflicts of interest regarding this work.

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