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Enhanced Detection of Chromosomal Abnormalities in Chronic Lymphocytic Leukemia by Conventional Cytogenetics Using CpG Oligonucleotide in Combination with Pokeweed Mitogen and Phorbol Myristate Acetate

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Abstract

Reproducible cytogenetic analysis in CLL has been limited by the inability to obtain reliable metaphase cells for analysis. CpG oligonucleotide and cytokine stimulation have been shown to improve metaphase analysis of CLL cytogenetic abnormalities, but is limited by variability in the cytokine receptor levels, stability and biological activity of the cytokine in culture conditions and high costs associated with these reagents. We report here use of a novel, stable CpG, GNKG168 along with pokeweed mitogen (PWM) and phorbol 12-myristate 13-acetate (PMA) for conventional cytogenetic assessment in CLL. We demonstrate that the combined use of GNKG168+PWM/PMA increased the sensitivity of detection of chromosomal abnormalities compared to PWM/PMA (n=207, odds ratio=2.2, p=0.0002) and GNKG168 (n=219, odds ratio=1.5, p=0.0452). Further, a significant increase in sensitivity to detect complexity ≥ 3 with GNKG168+PWM/PMA compared to GNKG168 alone (odds ratio 8.0, p=0.0022) or PWM/PMA alone (odds ratio 9.6, p=0.0007) was observed. The trend toward detection of higher complexity was significantly greater with GNKG168+PWM/PMA compared to GNKG168 alone (p=0.0412). The increased sensitivity was mainly attributed to the addition of PWM/PMA with GNKG168 because GNKG168 alone showed no difference in sensitivity for detection of complex abnormalities (p=0.17). Comparison of fluorescence *in situ* hybridization (FISH) results with karyotypic results showed a high degree of consistency, although some complex karyotypes were present in cases with no adverse FISH abnormality. These studies provide evidence for potential

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The authors have no conflict of interest.

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use of GNKG168 in combination with PWM and PMA in karyotypic analysis of CLL patient samples to better identify chromosomal abnormalities for risk stratification.

INTRODUCTION

Chronic lymphocytic leukemia is the most common form of adult B cell leukemia. At least 7000 patients are diagnosed and 4500 die due to CLL each year in the US. The disease has a variable clinical course. While many patients do not require treatment for years and have survival equal to age matched controls, others exhibit aggressive disease and have a poor prognosis. (1) Although treatment outcome has improved in recent years with alkylator-based therapies, purine analogs and rituximab, all patients eventually relapse and become refractory to fludarabine and other chemoimmuno therapeutics. Staging systems by Binet et al. (2) and Rai et al. (3) have been used for many years to aid in disease prognosis. However, because of the variable course of the disease, much research has recently focused on the elucidation of new prognostic factors predicting rapid disease progression and therapy response. Concurrent with the advances in combination therapeutics, several high risk genetic features including the IgV_H mutational status (4-5), select interphase cytogenetic abnormalities including del(17)(p13.1) and del(11)(q22.3) and the presence of non silent *TP53* mutations have been linked to early disease progression and inferior survival in CLL (6). Recently, several of these chromosomal aberrations have been identified as important prognostic indicators for predicting disease progression and therapy response (6-13).

The majority of the leukemic B cells isolated from CLL patients are in the G0/G1 phase of the cell cycle due to progressive accumulation of slowly proliferating cells (14). They exhibit a poor mitotic index when cultured in-vitro (15-18), limiting their usefulness for detection of aberrant metaphase karyotypes. Due to the limited proliferation of CLL B cells in-vitro, until recently use of conventional metaphase cytogenetics has played a minor prognostic role in CLL. Inability of traditional mitogens to effectively promote CLL cells to divide was a major impediment to this approach. Hence, interphase cytogenetics, i.e., fluorescence *in situ* hybridization (FISH), has been applied to clinical use in CLL, with an abnormality detected in 75-80% of cases (6,12,19-21). While FISH allows detection of genetic abnormalities in non-dividing cells, it detects only abnormalities of the probes applied; it does not detect complexity, which has been shown to correlate with outcome (11,16-18), and it does not permit identification of new or specific abnormalities. In order to overcome the limitations associated with the intrinsic defective proliferation that hinders metaphase analysis in CLL, stimulation of CLL cells with traditional B-cell mitogens, such as pokeweed mitogen (PWM), phorbol 12-myristate 13-acetate (PMA), [also designated 12-O-tetradecanoyl-phorbol-13- acetate (TPA)], or lipopolysaccharide (LPS) has been attempted with varying results. These in-vitro stimulatory methods enhance the yield of abnormal metaphases, but result in detection of abnormal metaphases in only 40%-50% of cases (15,17,22-23). To improve the efficiency of the detection of abnormal clones in CLL, the combination of an immunostimulatory synthetic oligodeoxynucleotide (ODN) containing unmethylated CpG motifs (CpG) along with cytokines such as interleukin-2 (IL2), IL12 or IL15 has been considered for karyotyping (24-29). Recently, the ODN, CpG-DSP30 was shown to induce cell division, thereby allowing metaphase cytogenetic detection of chromosomal aberrations in 80% of CLL patients (26). Additionally, CpG-DSP30 stimulation along with IL2 has been utilized to promote efficient metaphase analysis (24-29). Limitation of these assays is associated with variability in the cytokine receptor levels, stability and variability in biological activity of the cytokine in culture conditions and high costs. These limitations prompted us to explore more reliable, reproducible and cost effective methodologies for detection of chromosomal abnormalities in CLL.

Towards this goal, we assessed a novel stable CpG along with PWM and PMA for conventional cytogenetic assessment in CLL. We have recently developed a novel CpG ODN (GNKG168) with potent stimulatory properties in CLL B cells, resulting in induction of robust activation leading to S phase entry, similar to other CpGs (24-25,29). We report here comparative evaluation of GNKG168 with or without PWM/PMA, two mitogens that were routinely used in our laboratory at the time these experiments were initiated. We also performed a comparative analysis of enhanced efficacy and detection of complexity using GNKG168 in combination with PWM/PMA and with either of these mitogens alone. The chromosome banding analysis results were also compared with the FISH results. Additionally, the effectiveness of the addition of IL-2/IL-15 to GNKG168 was compared with that of PWM/PMA for detection of abnormalities.

MATERIALS AND METHODS

Patients and Samples

All patients enrolled in this study had immunophenotypically defined B-cell CLL as outlined by National Cancer Institute-Working Group 1996 criteria (30). Peripheral blood or bone marrow was obtained from patients after written informed consent in accordance with the Declaration of Helsinki and under a protocol approved by the institutional review board of The Ohio State University (Columbus, OH). Samples were obtained from patients with CLL between the period of 10/1/07 and 10/22/08. A total of 338 unselected cases, 174 bone marrow and 164 peripheral blood samples, referred for routine analysis of CLL at The Ohio State University, either at diagnosis or during follow-up were included. The median age of the patients was 60.7 years (range 29.7 - 86.8 yrs) and the male: female ratio was 2.16:1.00. Outcome data relative to specific cytogenetic abnormalities noted will be reported separately.

Conventional Cytogenetic Analyses

Cells from peripheral blood or bone marrow (2.0×10^6 cells/ml) were incubated in RPMI 1640 medium (Fischer Scientific, Houston, TX) with 2% L-Glutamine (Gibco Invitrogen, Carlsbad, CA), supplemented with 20% fetal bovine serum (Hyclone Laboratories, Logan TX), and 2% penicillin and streptomycin (Gibco, Invitrogen, Carlsbad, CA). The mitogens used included: pokeweed mitogen (PWM, final concentration = 10 µg/mL, Sigma Aldrich, St. Louis, MO) and phorbol 12-myristate 13-acetate (PMA, final concentration = 40 ng/mL, Sigma Aldrich) [PWM/PMA]. This was the standard for the laboratory prior to these studies. GNKG168 (20 µg/mL; SBI Biotech Co., Ltd, Tokyo, Japan) + PWM/PMA (above concentrations), and GNKG168 only (20 µg/mL) were also used in these experiments. The mitogens were added to the cultures, and the cells were cultured for 72 (303 samples) or 96 (34 samples) hours at 37° C in an atmosphere of 5% CO₂, with the longer culture time for samples obtained on Thursdays, as the laboratory is closed on Sunday. One sample with abnormal results was inadvertently cultured for only 24 hours. Previous studies showed that the longer culture time did not affect results (data not shown). Harvest was by standard laboratory procedures. All samples were G-banded using trypsin and were stained with Wright stain according to standard laboratory procedures. Studies using these mitogen combinations on normal samples resulted in normal results in all cases (data not shown). Culturing and analyses were done in a single laboratory in an attempt to add precision to the experiment. Each CLL sample was analyzed under all culture conditions applied as detailed below, unless there were insufficient cells. Analysis of the same number of cells per culture was done whenever possible, for a total of at least 20 metaphases. For each culture, the first analyzable cells observed were studied. A minimum of 20 cells was analyzed for a sample to be considered to have normal chromosomes. A clone was defined as two cells with the same chromosomal gain(s) or structural abnormality(ies) or three cells with loss of the same

chromosome(s) (31). All mitogen combinations were compared as follows: PWM/PMA vs GNKG168+PWM/PMA (118 samples); three independent stimulatory treatment groups including 1) PWM/PMA, 2) GNKG168+PWM/PMA and 3) GNKG168 only (89 samples); and GNKG168+PWM/PMA vs GNKG168 only (131 samples). In addition, GNKG168+PWM/PMA was compared with stimulation with GNKG168+IL2 (25 U/ml) +IL15 (10 ng/ml; both from Sigma Aldrich, St. Louis, MO; 41 additional samples).

Chromosomal aberrations

Karyotypes were described according to ISCN 2009 (31). When a particular aberration was observed in only one metaphase, it was considered as clonal when confirmed in at least one metaphase in a parallel culture (or two metaphases in case of loss of a whole chromosome) or in a significant proportion of interphase nuclei by interphase FISH. Failure was defined as less than 10 normal metaphases in the culture under consideration, whereas success was defined as 10 or more normal metaphases or the presence of a clonal abnormality. At least 20 metaphases combining all culture conditions were required for inclusion of a normal case.

Fluorescence *in situ* hybridization (FISH)

FISH was done on the PWM/PMA only cultures for the first 207 samples. For the last 131 samples, FISH was done on cells from the GNKG168+PWM/PMA cultures. Comparison of FISH results on unstimulated samples and GNKG168+PWM/PMA stimulated samples showed no difference in detection of any abnormalities, although the stimulated samples sometimes had a higher frequency of an aberration (data not shown). FISH probes for the standard CLL FISH panel (Abbott Molecular, Des Plaines, IL) were used, D13S319 (13q14.3), D12Z3 (chromosome 12 centromere), *ATM* (11q22.3) and *TP53* (17p13.1). Hybridization was according to the manufacturer's directions. Two hundred cells/probe were analyzed, 100 by each of two independent observers. FISH control values were calculated using the Beta-inverse function. Each case was compared for consistency of FISH results with conventional karyotyping results.

Statistical analysis

The primary focus was on the sensitivity to detect abnormal clones across the culture conditions. Because each patient's cells were analyzed under two or three medium conditions and the outcomes of multiple medium conditions were correlated, a mixed effect logistic regression model was used to account for the resulting dependencies. Under this model, hypothesis tests focused on the difference in odds (odds ratios) of detecting abnormalities across the culture conditions. We used medium condition as the fixed factor and patient as the random factor to model their effect on the log odds. P-values were generated by this model (32). SAS software (SAS Institute, Inc., Cary, NC) was used for all statistical analyses.

RESULTS

Samples from a total of 338 cases were cultured with various stimulatory agents, as detailed in the methods. Of these, 242 (71.6%) cases had abnormal cytogenetic results in one or more cultures, and 96 (28.4%) had normal results in all cultures. Quality, including banding level (400-450), of the preparations varied among samples, but with no differences among the different mitogen combinations (data not shown). Among the 242 abnormal cases, the previously reported abnormalities of trisomy 12, deletions of 13q, 11q and 17p were common. The only other recurring abnormalities noted were translocations with *IGH* at 14q32. However, most karyotypes were unbalanced, with balanced karyotypes in only ten of the 242 abnormal cases (4.1%). Of the 338 cases, 65 patients had sequential (two to six)

samples. All repeat samples had related abnormal clones, sometimes with additional or fewer abnormalities, or had normal results (data not shown), indicating the veracity of the abnormal clones. Nonclonal cells are frequent in CLL, and they were found in all of the culture conditions and in both normal and abnormal cases. They were most frequent in the PWM/PMA cultures (6.2% of cells), followed by the GNKG168+PWM/PMA cultures (4.4%) and least frequent in the GNKG168 only cultures (2.6%). Nonclonal cells were present more often in cases with normal results (70.8% of normal cases) than in those with abnormal results (45.9% of abnormal cases). The significance of nonclonal cells is not known. No recurrent nonclonal abnormalities were observed, excepting gains and losses of the X chromosome and losses of the Y chromosome.

Combined use of GNKG168+PWM/PMA enhances the sensitivity of detection of chromosomal abnormalities compared to PWM/PMA or GNKG168 alone

We first compared the probability of detecting abnormal cells in the combined use of GNKG168+PWM/PMA with that in PWM/PMA alone. This comparison showed that abnormal clones were detected in the cultures with the addition of GNKG168 more frequently (67.2% vs. 51.7%, Table 1). GNKG168+PWM/PMA resulted in significantly greater sensitivity in the detection of chromosomal abnormalities compared to PWM/PMA alone (odds ratio=2.2, $p=0.0002$, Table 2). In addition, sensitivity of detection of abnormal clones was significantly improved when the GNKG168+PWM/PMA cultures were compared with the GNKG168 alone cultures (67.2% vs. 58.5%, odds ratio 1.5, $p=0.0452$, Tables 1 & 2). When GNKG168 alone was compared with PWM/PMA alone, however, there was no significant difference in the detection of abnormal clones, although there was a suggestion that GNKG168 alone might be more sensitive in detection of an abnormal clone (58.5% and 51.7%, respectively, Table 1; odds ratio 1.4, $p=0.1218$, Table 2),

Combination of GNKG168 with PWM/PMA increases the sensitivity of detection of complex abnormalities compared to either mitogen alone

Since complexity is a significant clinical prognostic factor in CLL (11,17-18,33), the impact of different mitogens in detecting complex karyotypes was investigated. Given that a case had a complex karyotype in at least one culture, the probabilities of detecting complex (≥ 3 unrelated abnormalities) abnormal clones were 97.5%, 82.9% and 80.3% in GNKG168+PWM/PMA, GNKG168, and PWM/PMA cultures, respectively (Table 1). GNKG168+PWM/PMA as a mitogen was 9.6 times more likely to reveal a complex karyotype than PWM/PMA alone ($p=0.0007$, Table 2). Moreover, using GNKG168+PWM/PMA was 8 times more likely to result in detection of a complex karyotype compared to using GNKG168 alone ($p=0.0022$, Table 2). Linear trend analysis revealed that as the complexity increased, the probability of detecting the abnormal clone increased with GNKG168+PWM/PMA (Table 3, $p=0.0008$). This greater sensitivity seemed mainly due to the PWM/PMA, because GNKG168 alone showed no difference in sensitivity as complexity increased ($p=0.1734$) and the difference of the trends between GNKG168+PWM/PMA and GNKG168 alone in detecting the abnormal clone as complexity increased was significant ($p=0.0412$). Furthermore, PWM/PMA showed significantly increased sensitivity in detection of abnormal clones as the complexity increased ($p=0.0287$).

In addition to evaluating detection of complexity overall, each case was examined for complexity within each culture method, and the degree of complexity between culture methods was compared (Figure 1). Comparison of GNKG168+PWM/PMA cultures with PWM/PMA cultures (205 samples, data not available for two cases), showed a difference in complexity in 75 samples. In 59 (79%) samples the GNKG168+PWM/PMA culture resulted in more complex karyotypes, and in 16 (21%) samples the PWM/PMA only culture resulted in more complex karyotypes. Comparison of the GNKG168+PWM/PMA with the

GNKG168 only cultures (214 samples, data not available for five samples) showed differing complexity in 58 samples. Thirty-eight (66%) samples were more complex in the GNKG168+PWM/PMA cultures, and 20 (34%) samples were more complex in the GNKG168 only culture. Comparison of the PWM/PMA only and GNKG168 only cultures (83 samples) showed a difference in complexity in 32 samples. In 24 (75%) cases, the GNKG168 only culture resulted in a higher degree of complexity, and in eight (25%) cases the PWM/PMA only culture was more complex. Thus in individual cases, GNKG168+PWM/PMA cultures had higher complexity than either mitogen alone, and GNKG168 only resulted in higher complexity than PWM/PMA only.

GNKG168 in combination with PWM/PMA or IL-2/IL15 yields comparable results in detection of simple or complex karyotypes

Recently, CpG in combination with IL2 or IL15 has been found to be effective in detecting chromosomal abnormalities (24-27,29). We compared GNKG168+PWM/PMA to GNKG168+IL2+IL15 in 41 additional CLL samples to determine if there were any differences in these two combinations. There were 26 abnormal cases in GNKG168+PWM/PMA cultures and 26 abnormal cases in GNKG168+IL2/IL15 cultures, with 13 cases with complexity ≥ 3 in both culture conditions.

In conclusion, the mitogen combination GNKG168+PWM/PMA gave the highest frequency of detection of cytogenetic abnormalities and was more effective than either mitogen alone in detecting complex abnormal clones. The mitogens could be categorized in terms of finding clonal abnormalities (best to worst) as: GNKG168+PWM/PMA (67.2% abnormal) > GNKG168 only (58.5% abnormal) > PWM/PMA (51.7% abnormal).

Comparison of FISH results with conventional karyotyping results

Of the 242 cases with abnormal cytogenetics, FISH and the karyotypic results were completely consistent in 151 cases (62.4%; Table 4). FISH was not done in two cases. In 79 cases (32.6%), abnormalities were detected by FISH that were not seen in the karyotypes; however, 69 (28.5%) of these were deletions of 13q, which are known to be cryptic frequently (34). As 13 cases (5.4%) had more than one FISH abnormality, 25 other abnormalities were seen in the FISH analyses and not in the banded karyotypes; 12 deletions of *ATM*, eight deletions of *TP53*, three trisomy D12Z3 and two losses of D12Z3. There were 16 metaphase cytogenetic abnormalities involving the chromosome bands represented by the FISH probes that were not detected by FISH, nine deletions of 17p13.1 (*TP53*), three deletions of 11q22.3 (*ATM*), three trisomy 12 (D12Z3), and one 13q14 (D13S319) deletion. Of the 96 cases with normal banded metaphase cytogenetics, 41 (44.6%) were also normal using the above FISH panel. Fifty-five cases (57.3%) had FISH abnormalities, 51 of which had deletions of 13q (D13S319, 53.1% of cytogenetically normal cases). Only seven other abnormalities were detected by FISH in the cytogenetically normal cases, two deletions of *ATM*, two deletions of *TP53*, and three cases with three copies of D12Z3. The three copies of D12Z3 and deletions of *TP53* were in low frequencies of cells (*TP53* in 10.5% and 13.5%, and D12Z3 $\times 3$ in 1.0%, 1.5% and 1.5%). Deletions of *ATM* were in high frequencies of cells (80.5% and 64%). Only three cases had more than one FISH abnormality, two had loss of D13S319 and of *TP53*, and one had loss of D13S319 and gain of D12Z3. Thus, no complexity ≥ 3 was detected by FISH in the cytogenetically normal cases.

Complexity of three or greater also was compared with deletion of *ATM* and/or of *TP53* by FISH, both of which are considered poor prognostic indicators (6-8,10,13). Of the 242 cases with abnormal cytogenetics, 12 had a complexity of ≥ 3 and did not have loss of either *ATM* or of *TP53* by FISH. An additional nine cases had complexity ≥ 3 and had gain of the chromosome 12 centromere by FISH. In 20 of these cases, the abnormal clone was detected

in the GNKG168+PWM/PMA culture. The one exception was a GNKG168+PWM/PMA culture failure. These cases would not be recognized as having a poor prognosis by FISH alone, whereas the banded metaphase analyses identified them as cytogenetically poor prognosis cases. Thus, both FISH and metaphase cytogenetics detect abnormalities not detected by the other method, and both should be used to accurately classify patients for risk stratification.

DISCUSSION

Cytogenetic aberrations are considered major prognostic indicators for predicting response to treatment and the survival of CLL patients. The heterogenous biology of the CLL cell, as well as the poor in-vitro mitotic index of B cells obtained from CLL patients pose a great challenge for obtaining reproducible and reliable abnormal metaphases. Attempts to improve culture conditions to increase the cycling status of the CLL cells for efficient cytogenetic analysis have been investigated. Several B cell mitogens/stimulatory agents including PMA (TPA), PWM and LPS have been applied, and used as a standard method in many cytogenetic laboratories. While each of these mitogens has its own advantages and disadvantages, recently, use of novel immunostimulatory agents including CpG oligodeoxynucleotides, CD40 ligand and cytokines such as IL2, and IL15 were evaluated (24-27,29,35-36). CD40 ligand-induced cell cycle stimulation has been shown to result in the detection of abnormalities in 89% of cases compared with 22% after stimulation with conventional B-cell mitogens such as PMA, LPS, and PWM (35). However, this technique is labor-intensive and cost prohibitive in standard cytogenetic laboratories. Further, it is dependent on the levels of surface CD40 expression and hence is less applicable for routine practice. Bacterial DNA and synthetic single-stranded oligodeoxynucleotides containing unmethylated CpG motifs (CpG-ODN) stimulate cellular immune responses by Toll-like receptor 9 mediated mechanisms (37). There is evidence that internalization of the nucleotide is required for activity (38). Combination of an immunostimulatory CpG ODN with IL2 has identified chromosomal aberrations in 77-82% of cases (12,26-27), a percentage that is almost equivalent to interphase FISH. The CpG ODN GNKG168 resulted in a slightly lower frequency of abnormal karyotypes (70.1%, combined abnormality frequency in GNKG168 cultures with or without PWM/PMA). This lower abnormality rate with GNKG168 may be due to patient differences, as some of the patients reported in this study had been heavily treated and were in pathological remission; additionally, FISH results in the cases with normal cytogenetics identified only seven abnormalities other than deletions of D13S319, five of which were in low percentages of cells. A recent multi-center study identified use of CpG-DSP30 ODN in combination with IL2 to result in enhanced detection of cytogenetic abnormalities compared with PMA (28). Interestingly, in this study, as in our study, neither conventional cytogenetics nor FISH detected all aberrations, demonstrating the complementary nature of these techniques and need for application of each of these techniques. While combination of CpG ODNs along with cytokines such as IL2, IL12 or IL15 has been utilized for karyotyping, limitation of these assays associated with patient variability in the cytokine receptor levels, stability and variability in biological activity of the cytokine in culture conditions, as well as the high costs of cytokines, warrant more reliable, reproducible and cost effective methodologies for detection of chromosomal abnormalities in CLL.

The studies described here validate the use of CpG ODNs along with a PWM and PMA combination in routine cytogenetic analysis. We recently developed the novel GNKG168 that is in a phase 1 clinical trial for treatment of CLL. Induction of activation antigens such as CD40, CD8, HLA-DR and associated induction of DNA synthesis upon application of the ODN to CLL cells prompted us to explore the use of this CpG ODN for mitotic stimulation for cytogenetic analysis. While this CpG ODN by itself was not significantly different from

PWM/PMA (odds ratio=1.4; $p=0.1218$), combined use of GNKG168+PWM/PMA resulted in significantly greater sensitivity in the detection of chromosomal abnormalities compared to PWM/PMA alone ($n=207$, odds ratio=2.2, $p=0.0002$). Moreover, the combination of GNKG168+PWM/PMA showed significantly greater sensitivity compared to GNKG168 alone in detecting abnormal cells as the complexity increased ($p=0.0412$ for linear trend). This greater sensitivity seemed mainly due to the PWM/PMA, because GNKG168 alone showed no difference in sensitivity as complexity increased; and PWM/PMA showed significantly increased sensitivity in detecting abnormal clones as complexity increased ($p=0.00287$). Since complexity is a prognostic factor in CLL (8,13-15), this is a significant finding. Furthermore, use of this mitogen combination resulted in identification of cases with complex karyotypes that did not have a poor prognosis FISH abnormality, resulting in better patient risk stratification. Our analysis of limited sample size revealed lack of a significant difference when GNKG168+PWM/PMA treatment was compared to CpG-NO658+IL2/IL15 in detection of either simple or complex karyotypes. The superiority of GNKG168+PWM/PMA could be attributed to enhanced mitogenic response in the presence of these two B cell mitogens on the CLL cells. These studies provide evidence for potential use of GNKG168 in combination with PWM and PMA in karyotype analysis of CLL patient samples.

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Figure 1.

The percentage of cases with the same or different complexity after culture with the different mitogens. Each column represents comparison of two different culture conditions. The frequencies with higher complexities are indicated.

Table 1

Frequency of detection of cytogenetic abnormalities in different culture conditions

Medium	Sensitivity to Detect Abnormality (Standard Error)	No. of Samples Assessed
GNKKG168+PWM /PMA	67.2% ($\pm 2.5\%$)	338
GNKKG168	58.5% ($\pm 3.3\%$)	219
PWM/PMA	51.7% ($\pm 3.4\%$)	207
Sensitivity^a to Detect Complexity ≥ 3		
GNKKG168+PWM /PMA	97.5% ($\pm 1.6\%$)	120
GNKKG168	82.9% ($\pm 4.4\%$)	70
PWM/PMA	80.3% ($\pm 4.9\%$)	76

^a Sensitivity to detect complexity was calculated as the ability to detect an abnormal clone in a particular culture when seen in at least one culture.

Table 2

Estimated odds ratios in detecting abnormal cells across three culture conditions based on mixed-effects logistic regression model:

Comparisons	Odds ratio (95% CI)	p-value
Detection of Abnormality		
GNKG168 + PWM/PMA vs PWM/PMA	2.2 (1.4, 3.3)	0.0002
GNKG168 + PWM/PMA vs GNKG168	1.5 (1.0, 2.3)	0.0452
GNKG168 vs PWM/PMA	1.4 (0.9, 2.3)	0.1218
Detection of Complexity ≥ 3		
GNKG168 + PWM/PMA vs PWM/PMA	9.6 (2.7, 34.9)	0.0007
GNKG168 + PWM/PMA vs GNKG168	8.0 (2.1, 29.9)	0.0022

Table 3

Probability of detecting abnormal clones increases with GNKG168+PWM/PMA as complexity increases

Comparisons	Estimate	95% Confidence Intervals		p-value
Linear trend for GNKG168+PWM/PMA	13.6	3.0	62.1	0.0008
Linear trend for GNKG168	2.1	0.7	5.8	0.1734
Linear trend for PWM/PMA	3.0	1.1	7.9	0.0287
GNKG168+PWM/PMA vs GNKG168	6.6	1.1	40.5	0.0412
GNKG168+PWM/PMA vs PWM/PMA	4.6	0.78	27.1	0.1098

With GNKG168+PWM/PMA or PWM/PMA, the probability of detecting abnormal cells increased as cell complexity increased ($p=0.0008$ and 0.0287 , respectively). No evidence suggested that the probability had a similar trend with GNKG168 alone ($p=0.1734$). The trend/slope difference between GNKG168+PWM/PMA and GNKG168 is 6.6 ($p=0.0412$).

Table 4

Discrepancies between FISH and Metaphase Cytogenetics

13F	11F	17F	13F 11F	13F 17F	13F 12F	13F 17F 12F	12F 12F	12F LOOS	13F 11C	13F 17C	13C	11C	12C	17C	13F 11F 12C	11F 17C
ABNORMAL CYTOGENETICS																
51	3	4	7	3	1	1	1	2	1	2	1	2	1	6	1	1
NORMAL CYTOGENETICS																
48	2			2	1		2									

F = Seen by FISH, not seen by conventional cytogenetics, C = Seen by conventional cytogenetics, but not by FISH. Abnormalities by conventional cytogenetics were any that included the chromosomal band of the probe. For example, 13F indicates a deletion of D13S319 by FISH, which was not observed by conventional cytogenetics and was seen in 51 cases with abnormal cytogenetics and in 48 cases with normal cytogenetics. 13 = D13S319 (FISH), chromosome band 13q14 (conventional cytogenetics), 11 = ATM (FISH), band 11q22.3 (conventional cytogenetics), 12 = D12Z3 (FISH), trisomy 12 (conventional cytogenetics), 17 = TP53 (FISH), band 17p13.1 (conventional cytogenetics).