Published in final edited form as:

Cancer Genet Cytogenet. 2010 December ; 203(2): 134–140. doi:10.1016/j.cancergencyto.2010.07.128.

Stimulation of Chronic Lymphocytic Leukemia (CLL) Cells with CpG Oligodeoxynucleotide (ODN) Gives Consistent Karyotypic Results among Laboratories: a CLL Research Consortium (CRC)^h Study

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Abstract

Cytogenetic abnormalities in CLL are important prognostic indicators. Historically, only interphase cytogenetics was clinically useful in CLL because traditional mitogens are not effective mitotic stimulants. Recently, CpG-oligodeoxynucleotide (ODN) stimulation has shown effectiveness in CLL. The CLL Research Consortium (CRC) tested the effectiveness and reproducibility of CpG-ODN stimulation to detect chromosomally abnormal clones by five laboratories. More clonal abnormalities were observed after culture of CLL cells with CpG-ODN than with pokeweed mitogen (PWM)+12-O-tetradecanoyl-phorbol-13-acetate (TPA). All clonal abnormalities in PWM+TPA cultures were observed in CpG-ODN cultures, whereas CpG-ODN identified some clones not found by PWM+TPA. CpG-ODN stimulation of one normal control and 12 CLL samples showed that excepting clones of del(13q) in low frequencies and one translocation, results in all five laboratories were consistent, and all abnormalities were concordant with FISH. Thus, abnormal clones in CLL are more readily detected with CpG-ODN stimulation than with traditional B-cell mitogens. After CpG-ODN stimulation, abnormalities were reproducible among cytogenetic laboratories. CpG-ODN did not appear to induce aberrations in cell culture and enhanced detection of abnormalities and complexity in CLL. Since karyotypic

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complexity is prognostic and is not detectable by standard FISH analyses, stimulation with CpG-ODN is useful to identify this additional prognostic factor in CLL.

Introduction

Assessment of cytogenetic aberrations contributes to the diagnosis, risk stratification, prognosis and biology of many leukemias and lymphomas [1]. Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in the western world. Cytogenetic aberrations are important prognostic indicators in this disease; however, the application of metaphase cytogenetics to diagnosis, risk stratification, prognosis and biology in CLL has been limited. In place of metaphase cytogenetics, which describes the entire genome, albeit at a relatively low level of sensitivity, fluorescence *in situ* hybridization (FISH) has been widely accepted and applied in CLL [2–7]. However, FISH is limited to detection of only those regions of the genome for which the FISH probes have been designed. It does not detect any other aberrations, and importantly, it does not detect karyotypic complexity. Karyotypic complexity, defined as three or more unrelated aberrations, is an adverse prognostic factor in CLL [8–11].

CLL is a clonal disease of B-lymphocytes. CLL cells are arrested at the G₀G₁ phase of the cell cycle and do not divide spontaneously; they primarily accumulate as a result of lack of apoptosis, rather than accelerated cell division. CLL cells will respond somewhat to several traditional B-cell mitogens, such as pokeweed mitogen (PWM), 12-O-tetradecanoylphorbol-13-acetate (TPA), and lipopolysaccharide, but at a low level, with detection of abnormal clones at best in only 40–50% of cases [10,12–14]. Consequently, attempts to use other cell cycle stimulants have been applied to CLL, some with success, including CD40-ligand and CpG-oligodeoxynucleotide (ODN). CD40-ligand has been used with some success, but it is difficult to use in a clinical setting [15–16]. CpG-ODN have been used with more success. They are synthetic or bacterial short single strands of DNA, usually 19–25 base (mer), in which the CpG motifs are not methylated. They enter the B-cells and stimulate response to cytokines through toll-like receptor-9 mediation [17–20]. Stimulation of CLL cells with CpG-DSP30 plus interleukin 2 (IL2) has resulted in detection of abnormal karyotypes in up to 80% of cases [21–25]. The abnormalities detected were consistent with the FISH results in these cases, suggesting that the CpG-ODN did not induce abnormalities *in vitro*. However, the question of reproducibility among different laboratories with CpG-ODN stimulation of CLL cells has not been addressed.

The CLL Research Consortium (CRC) studies the biology and treatment of CLL. It consists of several different member institutions in multiple geographic locations all dedicated to the intense study of CLL, five with participating cytogenetic laboratories. The consortium collects and validates detailed clinical data on participating CLL patients from each site. Metaphase cytogenetic and FISH data are routinely collected from the five sites with participating cytogeneticists. Stimulation of CLL cells for cytogenetic analysis was not uniform, with some of the laboratories employing CpG-ODN mitogen, and some not. We asked (1) whether CpG-ODN would enhance the detection of abnormal cytogenetic clones, (2) whether CpG-ODN might induce cytogenetic abnormalities in CLL cells *in vitro* and (3) whether use of CpG-ODN stimulation for karyotypic analysis of CLL gives reproducible cytogenetic results in independent laboratories, thereby ensuring that pooling data from CRC laboratories is acceptable for clinical research collaborations.

Methods

These studies were approved by the Institutional Review Board at each participating site. Informed consent was obtained in accordance with the Declaration of Helsinki. Initially, one laboratory (OSU) compared the CpG-ODN, CpG-685 (also known as GNKG168) (20 µg/ml, Gingko Biologicals, Japan) stimulation of CLL cells with the traditional previously tested mitogens PWM (10µg/mL, Sigma Aldrich, St. Louis, MO) plus TPA (40ng/ml, Sigma Aldrich) in the same samples. Two-hundred twenty-nine peripheral blood or bone marrow samples were compared. Culturing and analyses were as below for the reproducibility experiments (manuscript in preparation). Secondly, to test reproducibility of CpG-ODN stimulation of CLL cells among the laboratories, fresh peripheral blood samples from one normal control (blinded sample) and 12 CLL patients were collected at the University of California at San Diego and sent directly to the local laboratory and by overnight delivery to the other four participating laboratories.. These were sent as five different experiments with 2 or 3 samples per experiment. Four laboratories stimulated with CpG-DSP30 (10 ug/ml) + IL2 (100 U/ml, PeproTech Inc) +IL15 (10ng/ml, PeproTech Inc), and one with CpG-685 + PWM/TPA as above. Cultures were for 3 days, and then subsequent harvest, banding and analyses were by the standard procedures of each laboratory. Analysis consisted of at least 20 cells per culture in each laboratory whenever possible. If a single abnormal (nonclonal) cell was detected, an additional 10 cells (>30 cells) were analyzed. Analysis was done at each laboratory blind to the results of the other laboratories. Karyograms were prepared, and each laboratory submitted its ISCN (2009) [26] interpretation for comparisons. Subsequently, at least two representative karyograms of each abnormal clone were sent to three of the participating cytogeneticists (PDC, DVD & NAH) for review, and the three reached agreement on the interpretation of results. FISH was not required; however, two of the laboratories (OSU, CLL FISH panel –Abbott Molecular, Des Plaines IL; Mayo - homebrew) did FISH on all of the samples.

Results

When the same blood samples were stimulated with PWM/TPA and with CpG-ODN in a single laboratory, the frequency of detection of abnormal clones was higher after CpG-ODN stimulation (147/229; 64%) than with PWM/TPA (110/229; 48%) ($p = 0.005$). Furthermore, every clonal abnormality observed on slide preparations from the PWM/TPA cultures was also observed on slide preparations from the CpG ODN-stimulated cultures. However, some abnormalities observed in the CpG-ODN cultures were not seen in the PWM/TPA cultures (manuscript in preparation).

Using CpG-ODN stimulation on 12 CLL samples and a normal control, all five laboratories obtained results from 11 of the samples (Tables 1 & 2). Two laboratories failed to obtain results for Pt #1, and one laboratory failed to obtain results for Pt #2. These culture failures are likely due to lack of experience with using CpG-ODN stimulation in cell culture in these two laboratories. All laboratories reported the normal control as normal. Five CLL samples (Patients #1, 3, 5, 7, & 9) were judged to be normal, one initially reported as normal by all laboratories with successful analyses (Patient #1, Table 1). Two patients had small clones with gain or loss of a sex chromosome with otherwise normal karyotypes, detected by one (Patient #3) or two (Patient #5) laboratories. As sex chromosome abnormalities are not thought to be clinically significant in the context of CLL [27–28], patients #3 and #5 with only these abnormalities were recorded as normal. Minor clones with del(13q) as the only abnormality were found in three patients (#2, #10, #12), and were confirmed by FISH in all three cases. Patient #2 had a monosomy X clone (detected by two laboratories), a trisomy X clone (one laboratory), and a del(13q) clone (two laboratories). A del(13q) was found by two laboratories for Patient #12 and by one laboratory for Patient #10. A different

abnormality was also reported by one laboratory for Patient #10. The latter was not confirmed by review. Prior to review, three additional cases were reported as having an abnormal clone by one laboratory (Patients #9 and #11) or different abnormal clones by two laboratories (Patient #7) (Table 1). An abnormal clone was confirmed by review only in Patient #11: a t(12;15)(q11;q26) in 2 of 30 cells.

An abnormal clone was detected by all five laboratories in patients #4, #6 & #8 (Tables 1 & 2). In each case, all five laboratories described abnormalities of the same chromosomes, although their initial ISCN descriptions of these abnormalities varied. Prior to review of patient #4, all five laboratories described a t(2;14)(q21-32;q32),der(5)t(5;11)(p13-15;q11-13),-11,del(13)(q12-14q22),+mar; four laboratories described an add(11)(q23-25), four reported a t(4;17)(q31;q11.2-21), and two described an add(8)(q22-23). Other abnormalities of chromosomes 4, 13 and 14 and various markers were reported by some of the laboratories.

For patient #6, who had a very complex karyotype, all five laboratories described loss of the Y chromosome, and abnormal chromosomes 5, 6, 9, 10, 11, 13, and 17. Other than the ISCN for del(17)(p11.2), each laboratory used different nomenclature to describe the various abnormalities.

For Patient #8, three laboratories reported a t(2;13)(p21-23;q13-22), whereas the other two described an abnormal 2 and an abnormal 13, so again each laboratory identified the same abnormalities but employed varying nomenclatures. Likewise, three of the laboratories described a del(11)(q21q23), one described a different chromosome 11 abnormality, and one laboratory did not observe an abnormal chromosome 11.

To determine whether the abnormalities with different interpretations reported truly were different, the case with t(12;15) (Patient #11) and the three complex abnormal cases (Patients # 4, 6, and 8) were reviewed by three of the cytogeneticists (PDC, DVD, NAH). All three agreed regarding the t(12;15) in patient #11. In the other three cases (Patients # 4, 6 & 8), it was apparent that all laboratories had detected the same abnormalities, but due to complexity and banding quality, had submitted different ISCN details (see Consensus Interpretations, Table 3). The consensus ISCN for Pt #8 was 46,XX,t(2;13)(p21;q14),del(11)(q21q23), and this abnormal karyotype was present in the analyses by all five laboratories.

The karyotype of patient #4 was complex with several related subclones (Figure 1 & Table 3). The consensus ISCN description was: 46,XY,t(2;14)(q21;q32),der(5)t(5;11)(p15.3;q13.1),-11,add(11)(q22.3),del(13)(q12q22),+mar/46,sl,t(4;17)(q31.1;q11.2)/46,sdl1,inv(8)(q22.3q24.3),-21,+r/46,sl,t(4;13)(q11.2;q14). The stemline and sideline 1 were seen in all five laboratories, the inv(8) in three laboratories, and the t(4;13)(q11.2;q14) in two laboratories. Because the latter was seen in only two cells in each of the two laboratories, its absence in the analyses by the other laboratories was not considered significant. FISH with the commonly used CLL FISH panel (probes for detection of a deletion of 13q, trisomy 12, deletion 11q and deletion of 17p) detected only a deletion 13q in this patient (Table 1).

Although each laboratory submitted a different ISCN description for the complex karyotype of patient #6 (Table 3), all five did identify abnormalities of the same chromosomes. Upon review, it was evident that the karyotypes were the same in all five laboratories even though the ISCN descriptions differed. The consensus ISCN for this case was: 45,X,-Y,add(13)(q12),del(17)(p11.2)/45,sl,-5,del(6)(p21.1),t(9;19)(q34;p13.1),der(10)t(6;10)(p21.1;q22),der(11)t(5;11)(p12;q13),+der(?)t(?)5(?)q13).

In all 13 cases studied, the FISH results were consistent between the two laboratories that performed FISH. Nine cases had a deletion of 13q by FISH (Table 1). In two of those cases (Patients #4 and #6), all laboratories detected a cytogenetically visible 13q deletion and in one case (Patient #8), all laboratories reported a breakpoint in band13q14. In three cases with a 13q deletion observed by FISH, the deletion was not detected on the banded chromosome analyses; this deletion is known to be frequently cytogenetically cryptic [29]. Interphase FISH analysis also identified an 11q deletion in one case, and a 17p deletion in another, both of which were also detected cytogenetically by all five laboratories. Thus, karyotypic analysis using CpG-ODN detected the same abnormalities as FISH, with the exception of the 13q deletion that was occasionally detected with a higher sensitivity using FISH.

Nonclonal abnormalities (Table 2) were seen in all except one specimen, including the normal control. The exception was patient #4, for whom all analyzed cells represented the complex abnormal clone described above. Between two and ten nonclonal cells per patient were identified collectively by the five laboratories among the 92 to 137 cells analyzed per case. Among a total of 1506 cells analyzed by the five laboratories, 73 (4.8%) nonclonal abnormal cells were identified (Table 2), and no specific recurring pattern for the non clonal cells was detected.

Discussion

The CRC has a large database of CLL cases, including the karyotypic results of a significant proportion of these cases. However, methods of stimulating the CLL cells varied among the CRC cytogenetic laboratories. To improve the quality of the database, as well as to detect a higher frequency of abnormalities, a better and consistent stimulation method was desired. Use of CpG-ODN for stimulation of CLL had not been used routinely by all of the CRC participants. One participant laboratory first demonstrated that stimulation of CLL with CpG-ODN improved the detection rate of abnormal clones (64% vs 48% of cultures), consistent with previous studies [21–25,30]. This analysis also showed that abnormalities detected in the PWM/TPA stimulated cultures were also found in the CpG-ODN treated cultures.

Therefore, we performed a study to determine whether all of the CRC cytogenetic laboratories could use CpG-ODN to detect cytogenetic aberrations successfully and consistently. Overall, the samples were cultured successfully by the five laboratories, indicating that different laboratories can successfully use CpG-ODN to induce divisions in CLL cells. The five CRC laboratories detected the same genetic abnormalities, indicating that the results are highly reproducible among different laboratories. Thus, stimulation of CLL cells with CpG-ODN to detect cytogenetic defects is both reliable and reproducible.

Another goal of these studies was to determine whether CpG-ODN stimulation induces clonal abnormalities *in vivo*. With the exception of del(13q) and sex chromosome loss or gain, the five CRC laboratories detected the same abnormal clones. In each case where only one or two of the laboratories detected a del(13q), the abnormality was confirmed by FISH analysis. Deletions of 13q are known to be frequently cryptic; and their detection can be very difficult, especially as the band level of cytogenetic preparations in malignancies is often not sufficiently high to detect such aberrations [29]. Small clones of sex chromosome gain or loss most likely did not reflect the leukemic clone, and their detection is not considered clinically significant [27–28]. One reciprocal translocation was found in two cells by one laboratory and not detected by any others. This translocation may represent a small CLL clone or subclone, but at a sufficiently low frequency such that it was not detected by the other laboratories. Alternatively, it may have been present in only the

portion of the sample received by that laboratory, or the CpG-ODN could have induced this one abnormality. In the cases with other clonal abnormalities, the five laboratories detected the same abnormalities. FISH confirmed deletion of 11q and 17p in the cases with these cytogenetic abnormalities. Thus if CpG-ODN induces cytogenetically abnormal clones in vitro, the same abnormalities would have to have been induced in independent 3-day cell cultures in different laboratories, which seems exceedingly unlikely. Other than 13q deletion, the abnormal clones were represented by 38% to 100% of analyzed cells in each case, and by multiple independent CRC laboratories. Thus, we conclude that it is highly unlikely that the CpG ODN induced the observed cytogenetically abnormal clones.

A third goal of the study was to increase the frequency of detection of clinically relevant cytogenetic abnormalities in CLL. Few studies have done a direct comparison of CLL abnormality detection rates in the same samples after different types of stimulation. Use of unstimulated cultures is largely unsuccessful in CLL, and the use of traditional B-cell mitogens such as PWM, TPA and lipopolysaccharides results in detection of abnormalities in only 40–50% of CLL cases [10,12–14]. Application of CpG-ODN greatly increases the detection of abnormal cases when compared with stimulation with PWM/TPA (64% vs 48% in this study). Similar results were obtained in a multi-institutional study comparing CpGDSP30-ODN+IL2 and TPA stimulation, in which 51% of cases had abnormalities after stimulation with CpGDSP30 + IL-2 and only 38% had abnormalities after stimulation with TPA [25], similar to our findings.

A complex karyotype is associated with an adverse outcome in CLL [8–11]. One case (Patient #4) in our series had a complex karyotype, yet the standard FISH CLL panel detected only a deletion of 13q, which is associated with a good prognosis [3,7,8]. If only the standard CLL FISH panel had been done on this patient, he would have been classified as genetically good risk, whereas the metaphase analysis showed a poor risk complex karyotype. This illustrates the significance of metaphase analysis using appropriate methods for detection of aberrations for the correct risk stratification of CLL patients.

One caveat concerning complex karyotypes is that the ISCN descriptions differed among the laboratories. Since complexity per se, as opposed to the specific abnormalities present, is an important prognostic factor in CLL, different interpretations would still result in appropriate patient risk determination.

This is the first demonstration of the reproducibility of stimulation of CLL cells with CpG-ODN to detect cytogenetic abnormalities among different laboratories. Differences among the laboratories were minor (mainly variable ISCN descriptions of the same abnormalities), confirming reproducibility of this test. The abnormal clones were detected consistently and reproducibly. CpG-ODN stimulation of CLL cells is the most effective known method to detect cytogenetically abnormal clones, including those with complex karyotypes, which are clinically significant for patient risk stratification. Additional research will be useful to demonstrate whether this prognostic factor (complex karyotype) is independent of other known prognostic factors, improve CLL patient risk stratification and lead to more informed treatment decisions.

Acknowledgments

Funded by CLL Research Consortium P01 CA081534-07A1

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Figure 1.

Four karyograms of Patient #4, from four different laboratories. A. Clone 1; B. Clone 2; C. Clone 3; D. Clone 4. See Table 3 for ISCN descriptions.

Table 1

Results from the five laboratories prior to review.

Lab	1	2	3	4	5	FISH
Control	Normal	Normal	Normal	Normal	Normal	Normal
Pt 1	Normal	Normal	Normal	Failure	Failure	del(13q)
Pt 2	Sole del(13q) & sole -X	Sole -X & sole +X	Sole del(13q)	Normal	Failure	del(13q)
Pt 3	Normal	Sole +X	Normal	Normal	Normal	del(13q)
Pt 4	Abnormal	Abnormal	Abnormal	Abnormal	Abnormal	del(13q) & IGH rearrangement
Pt 5	Sole -Y	Sole -Y	Normal	Normal	Normal	Normal
Pt 6	Abnormal	Abnormal	Abnormal	Abnormal	Abnormal	del(13q) & del(17p)
Pt 7	Normal	Normal	del(6q),del(11q)	add(5q)	Normal	del(13q)
Pt 8	Abnormal	Abnormal	Abnormal	Abnormal	Abnormal	del(13q) & del(11q)
Pt 9	Normal	Normal	add(4p)	Normal	Normal	Normal
Pt 10	Normal	Sole del(13q)	Normal	del(11q)	Normal	del(13q)
Pt 11	t(12;15)	Normal	Normal	Normal	Normal	Normal
Pt 12	Normal	Sole del(13q)	Normal	Sole del(13q)	Normal	del(13q)

All del(13q) confirmed with FISH

del(11q) not confirmed with FISH

Sole indicates the only abnormality detected in that clone.

Table 2

Results from the five laboratories after review.

Patient	Number of Laboratories with the following results					#Nonclonal cells
	Normal	Abnormal	Failure	# Labs With nonclonal cells		
Control	5			1		3
1	3		2	3		10
2	2	2 del(13q)[4]	1	4		6
3	5			3		8
4		5 Complex				
5	5			2		4
6		5 Complex		3		5
7	5			4		9
8		5 Abnormal		5		8
9	5			3		9
10	4	1' del(13q)[5]		3		3*
11	4	1 t(12;15)[2]		2		2
12	3	2' del(13q)[5]		3		6

' del(13q) confirmed with FISH

* 1 nonclonal cell had del(13)

Table 3

Consensus ISCN of Abnormal Karyotypes in CRC CpG ODN Trial

Patient	
Pt #4	46,XY,t(2;14)(q21;q32),der(5)t(5;11)(p15.3;q13.1), -11,add(11)(q22.3),del(13)(q12q22),+mar Seen in 5 of 5 laboratories
	46,sl,t(4;17)(q31.1;q11.2) Seen in 5 of 5 laboratories
	46,sdl,inv(8)(q22.3q24.3), -21,+r Seen in 3 of 5 laboratories
	46,sdl,t(4;13)(q11.2;q14) Seen in 2 of 5 laboratories
Pt #6	45,X, -Y,add(13)(q12),del(17)(p11.2) Seen in 3 of 5 laboratories
	45,sl, -5,del(6)(p21.1),t(9;19)(q34;p13.1),der(10)t(6;10)(p21.1;q22), der(11)t(5;11)(p12;q13)+der(?)t(?;5)(?:q13) Seen in 5 of 5 laboratories
Pt #8	46,XX,t(2;13)(p21;q14),del(11)(q21q23) Seen in 5 of 5 laboratories