A study on complex karyotypes in leukemia by routine G-banding and whole chromosome painting: A report on four Filipino cases

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ABSTRACT

Cytogenetic or chromosome abnormalities seen in leukemia patients at diagnosis are used to stratify patients into various risk groups and also serve as predictors of disease progression and response to therapy. Routine cytogenetic analysis using G-banding remains the standard procedure in establishing normal as well as abnormal karyotypes. But this method has its own resolution issue so describing multiple chromosome aberrations in complex karyotypes is challenging. This paper aims to document complex karyotypes (CK) in Filipino leukemia patients seen at diagnosis and at the same time compare the strengths and limitations of routine karyotyping and whole chromosome painting (WCP) technology (either SKY or M-FISH platforms). Follow-up cytogenetic analyses and clinical correlation of results were not done on these cases. There are four adult leukemia cases presented: two acute, one chronic, and one unclassified that were found with complex karyotypes using routine cytogenetic banding technique (GTG banding) and subsequently verified either by SKY or M-FISH. Case # 1 was a 39-year old male diagnosed with AML whose initial karyotype was 46,XY,t(3;20)(q13;q13.2). This finding was confirmed in SKY with an additional t(9;22)(q34;q11.2) aberration. Case #2 was a 37-year old male with a complex karyotype, 48,XY,ins(1)(q21q24q31), inv(1)(p2?1p3?6.1),+8,+8. SKY confirmed the presence of two extra copies of chromosome 8, but not the insertion and inversion in chromosome 1 pair. Case # 3 was a 87-year old female initially diagnosed with anemia but was found to carry a complex karyotype 45,XX,t(2;4)(q11.2;q35),del(5)(p11), add(17)(p13),+mar. SKY confirmed the abnormalities in chromosomes 2,4,5 and 17 and trisomy 8. The composition of the marker chromosome was revealed coming from chromosomes 2,4 and 14; and the additional material on 17p was a segment of chromosome 5. Nine minute chromosomes or acentric fragments not earlier reported by G-banding were also observed. Lastly, case #4 was a 41-year old male with chronic myelogenous leukemia (CML) presenting an atypical karyotype 47,XY,+8,t(12;17)(p13;q23). M-FISH confirmed all these abnormalities. Complex karyotypes presented in this paper demonstrated the advantages and limitations of both technologies. Identification of aberrant chromosomes by G-banding provided a good take off point in identifying directly the aberrant genomic segments and

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indirectly the genes lying in these segments that may have been disrupted and altered and are believed to contribute to the malignancy. Whole chromosome paint technology using either SKY or M-FISH was very useful in describing marker chromosomes and acentric fragments, as well as in verifying segments involved in translocation. However, it failed to recognize structural rearrangements within the same chromosome pair. The tandem of routine G-banding technique and SKY paint proved to be an accurate and powerful approach in identifying chromosome abnormalities especially in complex karyotypes.

INTRODUCTION

Leukemia is a group of disease that affects the blood forming cells and is characterized by abundance of white blood cells in the body. This hematological malignancy is classified either as acute or chronic based on the course of the disease and either lymphoid or myeloid as to cellular origin (Chennamadhavuni, Lyengar, and Shimanovsky 2022). Certain types of leukemia are associated with specific chromosome abnormalities providing evidence that specific mutated genes are responsible for the neoplastic transformation of cells (Brunning 2003). Several leukemias are defined by reciprocal translocations: t(9;22)(q34;q11.2) for chronic myelogenous leukemia (CML), t(8:21)(q22'q22) for acute myelogenous leukemia (AML), and t(15;17)(q22;q21) for acute promyelocytic leukemia (APL). These translocations result in gene fusions (BCR-ABL; AML ETO; and PML-RARA respectively) creating aberrant proteins that are responsible for the disease. Initial cytogenetic findings play an important role in the evaluation of hematologic malignancies and particularly in acute leukemias as these are used to stratify patients into favorable, intermediate, and unfavorable genetic risks groups (Brunning 2003).

A complex karyotype (CK) is defined as the presence of three or more chromosome abnormalities in a patient's genome (Mrozek 2008, Gohring et al. 2010, Jarosova et al. 2019). In AML, CK accounts for 10-14% of cases and up to 23% among older AML patients and this group presents adverse genetic risk based on the recommendations of the European Leukemia Net (ELN) and the UK National Cancer Research Institute Adult Leukemia study group (MRC for Medical Research Council) (Mrozek 2008, Mrozek et al. 2019).

G-banding or G-bands by Trypsin Using Giemsa (GTG) staining method is used routinely in most laboratories for detecting chromosome abnormalities. This differential staining method produces banding patterns on the chromosomes with an average band resolution of 400-650 bands per haploid set. Cytogenetic analysis or karyotyping is done by examining these banded chromosomes under the microscope for abnormalities. The resolution limit of karyotyping by G-banding is between 5-10 megabases which means it cannot detect chromosome aberrations lower than this limit (Roone, ed. 2001. More often, when a cell presents a complex karyotype, routine G-banding is no longer able to pinpoint exact breakpoints and reunion in multiple chromosomes (He et al. 2022). This problem is addressed by whole chromosome paint technology which uses combinatorial labelling of five fluorophores and consequently assign specific spectral signature (unique color) for each chromosome number. This technology called Multicolor FISH is now commercially available using either of the two systems: Spectral Karyotyping or SKY and Multiplex-Fluorescence in situ hybridization or M-FISH. Multicolor FISH allows a fast and precise assessment of acquired numerical and large structural chromosomal changes associated with malignant diseases including hematological disorders (O'Connor 2008). The limit of resolution of SKY/M-FISH in detecting chromosome rearrangement is between 500 and 2000 kilobases (Liehr et al. 2004).

MATERIALS AND METHODS

Patients included in the study

The four patients included in this paper were part of the sample population in the research project entitled, "Cytogenetic, Immunophenotypic and Molecular Genetic Characterization of Adult Acute Leukemia". Bone marrow samples were drawn from patients prior to treatment. The project was approved by the Ethics Review Board of St. Luke's Medical Center and informed consent was obtained from each patient.

Cell culture, chromosome preparation and G-banding analysis

Four patients (Cases 1 to 4) referred upon their doctor's request for cytogenetic analysis were included in the study. From each patient, a minimum of 2 mL of bone marrow aspirate was collected in a green top sodium heparin tube. Routine cytogenetic preparation following the modified protocol of Verma and Babu (1995) was carried out. Briefly, about 0.8 to 1 mL of bone marrow cells were cultured in a sterile T25 culture flask containing 10 mL of complete RPMI 1640 media with glutamine (20% fetal bovine serum (FBS), and 1% antibiotic (PenStrep). Two to three culture flasks were set-up for each patient. Initial cell density per flask was adjusted not to exceed 1x 10^6/mL. The cultures were incubated at 37^0C with 5% CO_2 for 48 hours and each flask was exposed to colcemid (0.2 ug/mL) 30 minutes prior to harvest to arrest cells at metaphase. The cell suspensions were centrifuged and the supernatant removed until a cell pellet was collected. Cells were treated with hypotonic solution, a prewarmed 0.075M KCl, and consequently fixed and washed several times using Carnoy's fixative (3:1 methanol and acetic acid) until a clean white precipitate was obtained. The final cell pellet was resuspended in an appropriate volume of Carnoy's fixative and from this a small amount was dropped onto precleaned slides. The slides were placed in a 37ºC oven for one day before staining. Slides were stained following the standard GTG technique. G-bands are produced when chromosomes are pre-treated with trypsin (an enzyme that digests proteins) prior to Giemsa staining (Verma and Babu 1995). There is an established normal banding pattern for each chromosome number; based on these patterns structural and numerical abnormalities are identified (Bickmore 2001, McGowan-Jordan, Simmons and Schmid, eds 2016). For each case, a minimum of 25 metaphase cells were captured and screened using an Axioimager microscope (Zeiss) and analyzed using the Cytovision software (Applied Imaging). The software analyzed each chromosome based on its size, centromeric location, and banding pattern. The final image of each metaphase spread was an orderly arrangement of paired chromosomes from numbers 1-22 and XY following the International System of Human Cytogenetics Nomenclature 2016 (McGowan-Jordan, Simmons, and Schmid, eds 2016). To establish a complex karyotype, a minimum of two cells carrying the same aberrations must be seen. These complex karyotypes based on G-banding were verified by whole chromosome painting using either SKY or M-FISH platform.

Multicolor FISH assays: SKY and M-FISH

The spectral karyotyping (SKY) of Cases 1, 2, and 3 was done in the laboratory of the Applied Spectral Imaging (Israel). That of Case # 4 was analyzed using multiplex fluorescence in situ-hybridization (M-FISH) at the Molecular Cytogenetics laboratory of SLMC. SKY and M-FISH are whole paint karyotyping technologies that use a combinatorial labelled

chromosome-specific fluorochromes (fluorescent dyes) to differentiate and classify nonhomologous chromosomes (Imataka and Arisaka 2012). The suppliers' protocols were followed in the conduct of SKY and M-FISH. These essentially consisted of the following steps: (1) slide preparation (same as in G-banding), (2) slide enzyme (pepsin) pretreatment, (3) denaturation of the chromosomes on the slide and the probes, (4) hybridization of the probe to the target (chromosomes), and (5) post hybridization washing. SKY images were captured using a CCD (charge-coupled device) and image analysis was carried out by an interferometer attached to an epifluorescence microscope (GenAsis Spectral Imaging System from Applied Spectral Imaging). In M-FISH, each homologous pair of chromosomes was identified based on the fluorochrome's presence or absence when visualized with specific filters. A dedicated software measured this defined emission spectra. The final outputs were computer generated fluorescence images where each chromosome pair was painted with a distinct color.

RESULTS AND DISCUSSION

Case # 1

This was a 39-year old male who presented with spontaneous appearance of hematoma in his trunk and extremities. The condition was also accompanied by pallor, fatigue, and cough. Initial CBC showed platelet count of about 19,500 µL. He was initially diagnosed with acute myeloid leukemia (AML). Chromosomal analysis of the G-banded lymphocytes chromosomes revealed an abnormal karyotype bearing a reciprocal translocation between 3 and 20 as shown in Figure 1. The karyotype was described as 46,XY,t(3,20)(q13;q13.2).

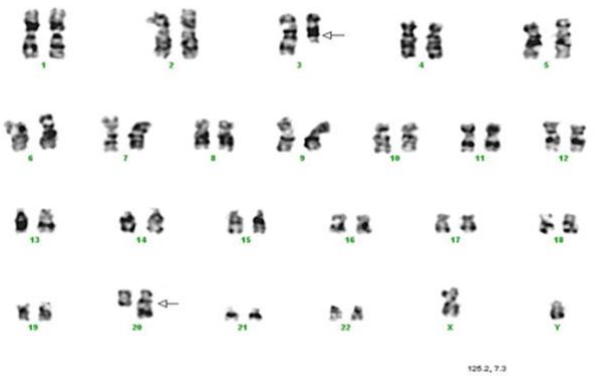

Figure 1: Case # 1. A G-banded karyogram of a 39-year old male with AML. The image shows a reciprocal translocation between chromosome 3 and 20 (arrows). The karyotype is described as 46,XY,t(3;20)(q13; q13.2).

Figure 2 shows the spectral karyotype of Case #1 confirming the reciprocal translocation between chromosomes 3 and 20 earlier seen in G-banding. However, a second reciprocal translocation between chromosomes 9 and 22 was observed. This added aberration, t(9;22)(q34;q11) makes this karyotype a complex one, with a total of four abnormal chromosomes. The t(9;22) translocation could be a cryptic (submicroscopic) abnormality, one that could not be detected by routine G-banding (Soliman et al. 2018). Even with the SKY image, the translocated segment of chromosome 9 was seen as a very thin white band at the end of the abnormal chromosome 22q (right) while the segment of chromosome 22 (pink) that translocated to the q arm of chromosome 9 (right) was wider. This small abnormal chromosome 22 is popularly referred to as the "Philadelphia chromosome" and is considered the hallmark of CML. The t(9;22)(q34;q11.2) is typically seen in 90-95% chronic myelogenous leukemia patients and in 20 to 30% of adult cases of ALL (Faramarz 2013, Liu-Dumlao et al. 2012). Such a translocation creates the BCR-ABL gene fusion that encodes an overexpressed tyrosine kinase (ABL) believed to be responsible for the active proliferation of lymphocytes (Pane et al. 2002).

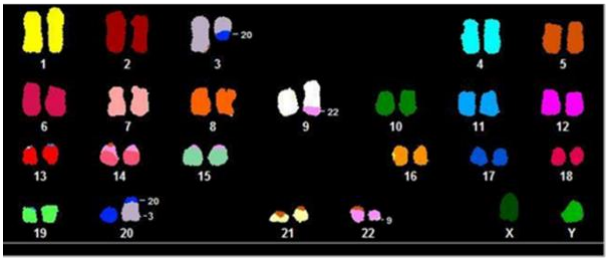

Figure 2: Case # 1. This spectral karyotyping (SKY) image shows a complex karyotype with the presence 4 aberrant chromosomes resulting from two translocations: t(3;20)(q13;q13.2) and t(9;22)(q34;q11.2). The second translocation was not earlier seen in G-banding.

Case # 2

The patient was a 37-year old male diagnosed with acute lymphocytic leukemia (ALL). At diagnosis, his karyotype was described as complex because of the presence of 3 abnormalities: 2 structural and one numerical. Figure 3 presents an insertion in the long (q) arm of one copy of chromosome 1 and an inversion in the short arm (p) in the other chromosome 1. In addition, 2 extra copies of chromosome 8 were seen. For the insertion, the long arm segment between bands 1q24 and 1q31 have inserted into the long arm of the same chromosome at band 1q21. For the paracentric inversion (non-involvement of centromere), breakage and reunion could have occurred at bands 1p2?1 and 1p3?6.1 The spectral karyotype image (Figure 4) was not able to clearly support the structural rearrangements in both copies of chromosome 1 as these were painted in solid yellow color. This is one limitation of SKY/M-FISH which cannot detect structural chromosome abnormalities like duplication, inversion, and insertion especially if these occur within the same chromosome (one color). Question marks (?) are placed before the band numbers to indicate uncertainty of the breakpoints because such chromosomes are not long enough to exhibit thinner bands due condensed morphology (McGowan-Jordan, Simmons, and Schmid, eds 2016).

Case # 3

The patient was an 87-year old female who was initially admitted for fever and chills in a provincial hospital after wound infection. The initial clinical impression was leukemia and a complete blood count was done and revealed the presence of anemia. Since people with leukemia are more likely to develop anemia, a cytogenetic study was requested. Analysis of chromosomes prepared from her cultured peripheral blood lymphocytes revealed a complex karyotype. Figure 5 shows three structurally abnormal chromosomes: 2, 4, and 17, with monosomy 5 and 20. Of the 25 cells analyzed, the modal chromosome number was established between 45 and 47. Modal chromosome number is defined as the most common number of chromosomes in the majority of metaphase cells analyzed and may be expressed as a range between two numbers (McGowan-Jordan, Simmons, and Schmid eds 2016). The following clonal abnormalities were observed from among the cells studied: (a) an abnormal long chromosome 4 resulting from breakage and fusion of the long (q) arms of chromosomes 2 and 4, (b) an abnormal chromosome 5 with a deleted short (p) arm, (c) one

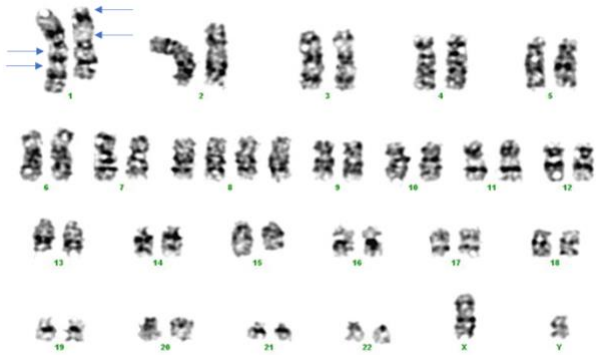

Figure 3: Case #2. A complex karyotype with three abnormalities; 2 structural and one numerical. There are 48 chromosomes. Both copies of chromosome 1 are abnormal. Chromosome 1 (L) has an insertion; the long arm segment between bands 1q24 and 1q31 has been inserted into the long arm at band 1q21. The other chromosome 1 (R) carries a paracentric inversion, where breakage and reunion occurred probably at band1p2?1 and 1p3?6.1. Chromosome 8 is present in 4 copies.

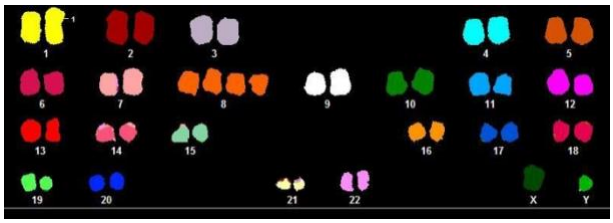

Figure 4: Case # 2. A SKY image showing both copies of chromosome 1 in solid yellow color which makes intrachromosomal abnormalities such as insertion and inversion difficult to show. The assumption of insertion is indicated on chromosome 1(R) as it is longer than the other allele. Four copies of chromosome 8 are also shown confirming earlier result seen in GTG banding.

aberrant chromosome 17 with an additional material at its p-arm, (d) monosomy 20, and (e) a marker chromosome. Arrows in Figure 5 point to structurally abnormal chromosomes.

The SKY image presented interesting findings. The marker chromosome in the G-banded karyotype was composed of segments from chromosomes 2, 4, and 14. A marker chromosome (mar) is a structurally abnormal chromosome in which no part can be identified by routine G-banding (McGowan-Jordan, Simmons, and Schmid eds 2016). The identity of this aberrant chromosome can only be established using whole chromosome paint technique (SKY or M-FISH). The additional material at the end of the p arm of chromosome 17 turned out to be the short arm of chromosome 5; this was earlier not identified by G-banding. An additional copy of chromosome 8 plus nine minute (min) chromosomes from chromosome 8 were also seen in SKY but not in G-banding. Minute chromosomes are cytogenetic markers of genomic amplification and are clonally present in cancer (Fan et al. 2011). The nonreciprocal translocation (one way transfer) between chromosomes 2 and 4 was verified.

Case # 4

The patient was a 41-year old male initially diagnosed with chronic myelogenous leukemia (CML). Figure 7 reveals a translocation between chromosomes 12 and 17 and trisomy 8. The complex karyotype revealed a male with 47 chromosomes and carrying a reciprocal translocation between chromosomes 12 and 17 and three copies of chromosome # 8. The absence of the typical t(9;22)(q34;q11.2) in cells analyzed, qualified this case as an atypical CML (aCML), a subset of myelodysplastic syndrome/myeloproliferative diseases (MDS/MPN) (Drozd-

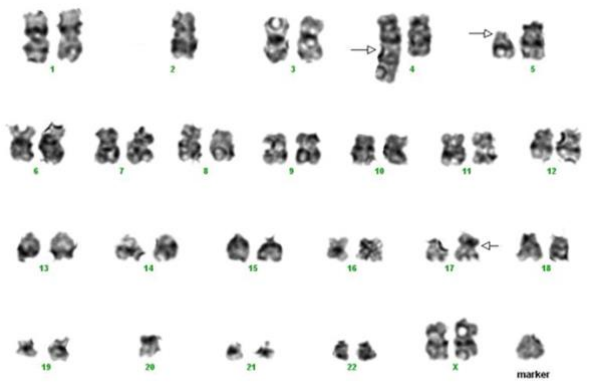

Figure 5: Patient #3. A metaphase cell from an 87-year old female presenting a complex karyotype with a modal chromosome number of 45-47. The figure shows structural aberrations (translocations) and aneuploidy (monosomy). Note the presence of a marker chromosome. Arrows point to segments of chromosomes where breakages and reunion occurred.

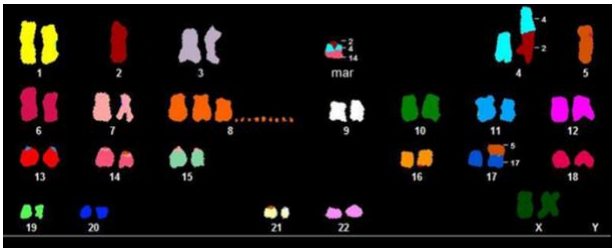

Figure 6: Patient #3. Chromosome analysis using SkyPaint® showing structural abnormalities in chromosomes 2,4, 5,17, trisomy 8, 9 minute chromosomes and a marker chromosome made up of segments from chromosomes, 2,4 and 14. The aberrant chromosome 5 is not shown in this image.

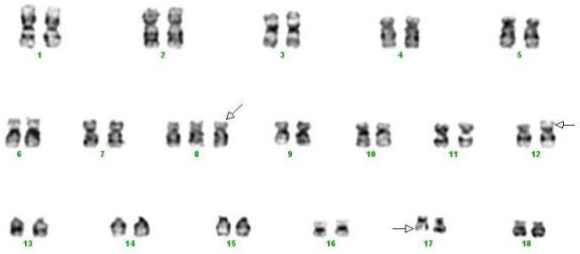

Figure 7: Patient # 4. The complex karyotype revealed a male with 47 chromosomes and carrying a reciprocal translocation between chromosomes 12 and 17; and trisomy 8. This is a case of an atypical CML.

Sokolowska et al. 2018, Belkhair et al. 2019). At present, the diagnosis of aCML is made in accordance with the WHO guidelines (2016). The criteria have become more precise since 2009; still, the absence of BCR-ABL2 remains a criterion. Published studies claim that these aCML cases have a high rate of transformation to AML (Faramarz ed 2013). Figure 8 is an M-FISH karyotype of one metaphase spread from Case #4. The presence of the t(12;17) and trisomy 8 were confirmed.

DISCUSSION

In chromosome analysis, the most essential material is the metaphase cell and adequate number of these cells is needed to establish the clonal origin (derived from a single parent) of any abnormality. The accurate delineation of breakpoints in structural chromosome aberrations such as translocations is largely dependent on banding resolution, which is the measure of the number bands that are visible (McGowan-Jordan, Simmons, and Schmid (eds) 2016). G-bands are seen as a series of monochromatic bands (black, white, and gray) along the vertical stretch of each chromosome. The longer the vertical

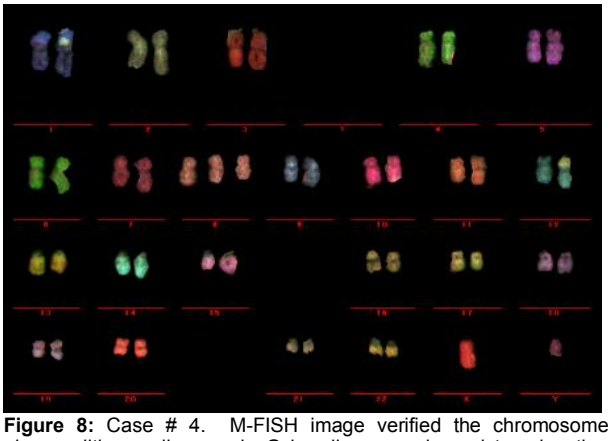

Figure 8: Case # 4. M-FISH image verified the chromosome abnormalities earlier see in G banding: a reciprocal translocation between the short arm of chromosome 12 and the long arm of chromosome 17 and trisomy 8.

vertical stretch of each chromosome. The longer the chromosomes the more bands are seen. Highly condensed chromosomes (shorter and more compact) have low level resolution and are seen with fewer bands (approximately 300 bands per haploid set). Chromatin condensation causes several bands to lie very close to each other and are seen as one thick band (McGowan-Jordan, Simmons, and Schmid eds 2016). To describe where breakage and reunion occurs is difficult in these condensed chromosomes, and this difficulty is even heightened in complex karyotypes. Chromosomes of cases presented in this paper are largely condensed thus the exact breakpoints at the band level are difficult to pin point. When uncertain, the symbol (?) is placed before the arm or band (p or q) (McGowan-Jordan, Simmons, and Schmid eds 2016).

Leukemia patients with complex karyotypes (CKs) at diagnosis are classified under a separate category of hematologic malignancies. Complex karyotypes are associated with poor prognosis with many altered genes that may play important roles in the disease development. Studies have shown that the appearance of complex karyotypes increases with age and the treatment outcomes of patients with this genetic make-up are very poor (Mrozek 2008). Unfortunately, patient follow-ups were not covered in this study.

WCP which includes SKY or M-FISH addresses the limitations of cytogenetic analysis. SKY and M-FISH allow the painting of the entire chromosome complement in a single hybridization by labelling each chromosome with a different fluorophore (a chemical compound that can absorb and emit light). The resulting image presents a simultaneous visualization of all the human chromosomes (pairs of # 1-22, X and Y) in 24 different colors, thus chromosome abnormalities are easily identified (Imataka and Arisaka, 2012). But like any other technologies, SKY and M-FISH also have limitations; their inability to discriminate intrachromosomal rearrangements such as duplication, inversion, and insertion (Knutsen 2017). This was demonstrated by Case # 2 in this paper. Moreover, the exact assignment of breakpoint and reunion is also not easy with SKY or M-FISH because they paint each chromosome homolog with a solid color (no bands). G-banding, on the other hand, with the black, white, and gray band pattern along the chromosome length, provides a good estimate where the breakage occurred. An accurate description of where breakage and reunion occurred in aberrant chromosomes depends on the band resolution of the metaphase spread.

Two patients (# 3 and 4) presented in this paper reported extra copies of chromosome 8. The clinical impact of trisomy 8 on cancer progression and treatment response have been reported in a number of studies (Seghezzi et al. 1996, Bakshi et al. 2012, Ashangari and Tumula 2018). These studies looked into the possible roles of the following genes mapped on chromosome 8: c-myc, c-mos, MOZ, and ETO in leukemogenesis (Bakshi et al. 2012, Moosavi et al. 2019). Abnormalities of chromosome 5 are commonly reported in MDS and AML with deletion 5q as the more frequent occurrence. Monosomy 7 is not so common. Both abnormalities indicate poor prognosis (Galvan et al. 2010). Case # 3 presented with monosomy 5 (Figure 5) and an added material in the short arm of #17. The SKY image (Figure 6) confirmed one whole copy of chromosome 5 and identified the extra material in 17p as a segment of chromosome 5 (could be from the other missing #5). Interestingly, P53, a tumor suppressor gene (TSG) that is often mutated in cancer is mapped at 17p. An unbalanced translocation involving chromosome 5q and 17p resulting in the deletion of *P53* is a recurrent aberration in MDS/AML (Warnstorf et al. 2021). In addition, Case number 3 presented with 9 min/ace, which when carrying oncogenes, can effectively increase the copy number of these genes. More so, some studies have shown that min are able to enhance and maintain tumor heterogeneity and make cancer cells resistant to targeted therapy (Fan et al. 2011, Nathanson et al. 2014, Xu et al. 2019).

An adequate number of quality metaphase cells and a good band resolution are key factors in describing accurate chromosome aberrations present in complex karyotypes. This paper described the limitations and advantages of each technology as well as the usefulness of their use in tandem in describing the various chromosome abnormalities in leukemia. Amidst the rapid development of molecular technologies, cytogenetic analysis remains a vital laboratory technique in the diagnosis and prognosis of human diseases, particularly cancer.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Enriquez ML and Caguioa PB conceptualized the project; Abad CS, Arnante M, Enriquez MD did the laboratory work and cytogenetic analysis. All the authors contributed in the drafting and revising of the manuscript, and gave final approval of the version to be published. All figures in this manuscript are original.

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