Original Article

Dedicated Cytogenetics Factor is Critical for Improving Karyotyping Results for Childhood Leukaemias – Experience in the National University Hospital, Singapore 1989-2006

JL Heng,[1,2] YC Chen,[3 MSc], TC Quah,[1,2 MBBS, MMed], TC Liu,[3 MBBS, MMed, MRCP], AEJ Yeoh,[1,2 MBBS, MMed]

Abstract

Introduction: Childhood leukaemia accounts for more than 40% of new childhood cancer cases. Karyotyping of cytogenetic abnormalities in such cases continues to provide critical prognostic information which allows the delivery of an appropriate intensity of treatment. Unfortunately, karyotyping of childhood leukaemia is difficult, laborious and often unsuccessful. Banding resolution tends to be poor unlike routine antenatal cytogenetics. The aim of the study is to highlight the benefit of dedicated cytogenetics in improving karyotyping results. Materials and Methods: We analysed the impact of setting up a team of cytogeneticists in the National University Hospital (NUH) on the success of karyotyping, evaluating cytogenetic data collected from 1989 to 2006. From 1989 to 2006, 4789 cases have been processed. Among them, 369 newly diagnosed and relapsed childhood acute leukaemia cases [281 acute lymphoblastic leukaemia (ALL) and 88 acute myeloid leukaemia (AML)] have been diagnosed at NUH. A dedicated cytogenetics laboratory with clearly defined standard operating procedures and quality control was set up in 2002. It used the established recommendation of a complete analysis of at least 20 metaphases per analysis. Results: Overall, the frequency of successful karyotyping was significantly higher (P = 0.002) at 90.7% (185/204) from 2002-2006 compared to 79.4% (131/165) from 1989-2001. For ALL cases, the success rate improved from 77.6% (97/125) in 1989 to 2001 to 89.1% (139/156) in the 2002 to 2006 cohort. For AML, the success rate also was significantly improved (P = 0.04) from 85% (34/40) to 95.8% (46/48). Significantly, this high rate of success is still maintained despite a yearly increase in volume. Conclusion: The establishment of a dedicated cytogenetics service leads to an improvement in results.

Ann Acad Med Singapore 2010;39:102-6

Key words: Childhood leukaemia, Dedicated cytogenetics service, Successful karyotyping

Introduction

Acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML) make up the bulk of childhood leukaemia cases. With risk stratified therapy being one of the cornerstones of the treatment of childhood leukaemia, it is important to determine the prognostic factors on which risk stratified therapy depends. Chromosomal aberrations – numerical and structural – are especially important, as they are prognostic determinants in the outcome of therapy, since they can be used in determining prognosis and predicting the response to certain therapeutic strategies.[1-4] Conventional cytogenetics is the gold standard used in the karyotyping of cytogenetic abnormalities, which currently cannot be replaced by current molecular cytogenetics methods in clinical work, as its high investigative resolution is able to provide an overview of any observable abnormalities at a relatively low cost.[5] In contrast, many molecular cytogenetics methods such as fluorescence in-situ hybridisation (FISH) can only reveal specific known abnormalities and are less cost-efficient than conventional cytogenetics.

Unfortunately, karyotyping of childhood leukaemia is difficult, laborious and often unsuccessful in many laboratories. Many factors contribute to the poor morphology of chromosomes in karyotyping, especially in the karyotyping of leukaemia cases. External factors include the culture medium used, the protocol used in chromosome bandings, sample drawing procedure, and travel time. Internal factors include the poor inherent viability of leukaemia samples – in acute lymphoblastic and myeloid leukaemia samples, leukaemia cells have

[1] Viva-University Children's Cancer Centre, University Children's Medical Institute, National University Hospital, National University Health System, Singapore
[2] Division of Paediatric Haematology-Oncology, Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore, National University of Singapore, Singapore
[3] Department of Haematology-Oncology, National University Cancer Institute, National University Hospital, National University Hospital, National University Health System, Singapore
Address for Correspondence: Dr Allen Eng Juh Yeoh, Department of Paediatrics, Division of Haematology and Oncology, Yong Loo Lin School of Medicine, National University Health System, c/o Level 4, Main Building, National University Hospital, 5, Lower Kent Ridge Road, Singapore 119074.
Email: allen_yeoh@nuhs.edu.sg

much lower viability and tend to die much more quickly than normal. Banding resolution tends to be poor unlike routine antenatal cytogenetics. Thus, it is also important for results from karyotype analysis to be produced in time to allow early intervention in therapy. In addition, there were questions as to whether results from sending samples to external cytogenetics laboratories were comparable to results from in-house cytogenetics analysis. The aim of this paper is to assess the impact of a dedicated team of cytogeneticists on diagnostic results.

Patients and Methods

The intake of patients for this study started in 1989 when the authors' hospital set up a cytogenetics section in the haematology laboratory. From 2002 onwards, a dedicated cytogenetics team was started in the hospital and all samples were processed and analysed in-house. Before 2002, most of the samples were sent to external laboratories for analysis, and the rest of the samples were processed by the hospital's only cytogeneticist. As the dedicated cytogenetics team was in place from 2002 onwards, results will be stratified into 2 categories for comparison: 1989 to 2001 and 2002 to 2006.

Of 4749 cases screened from 1989 to 2006, only ALL and AML cases with a cytogenetic analysis made at the point of diagnosis or at the point of relapse were selected. This amounted to 369 patients aged 0 to 18 years old who had de novo or relapsed ALL and AML. Relapse cases are classified on the basis of a previous exposure to chemotherapy or radiotherapy and a history of leukaemia. Of the 369 cases, 324 are de novo cases (246 ALL and 78 AML) and 45 are relapse cases (35 ALL and 10 AML). Among the 45 relapse cases, 44 are primary relapses, and 1 had two relapses.

A stringent criterion of success is defined according to the established recommendation of a complete analysis of at least 20 metaphases.[6] A success rate is therefore defined as the total number of successes as a percentage of the total number of cases.

The final report of chromosome analyses is made available to the clinician within a certain number of calendar days of specimen receipt. This length of time taken is defined as the turn-around time. A "good" turn-around time expectation was established by the laboratory director to be 21 calendar days or less.

Before the dedicated cytogenetics section was set up, there was no fixed protocol followed by the hospital's only cytogeneticist. From 2002 onwards, standard operating procedures were drawn up by the laboratory director, following closely checklists by the College of American Pathologists, and laboratory protocol modified for best results.

Cytogenetics

One to two mL of bone marrow is aspirated aseptically from the posterior superior iliac spine of the patient and placed in 5 mL of transport media, and mixed well to prevent clotting. The sample is transported to the cytogenetics laboratory at room temperature without delay. Approximately 0.3 mL is removed from the 5 mL sample and run through the cell counter to determine the number of total nucleated cells. A final cell concentration of 1-2 x 10^6 cells per mL is ideal for setting up a 10 mL culture. If the cell count is too high, the sample can be diluted with culture media; otherwise, the sample is centrifuged at 1000 rpm for 10 minutes, and cultures set up with the buffy coat obtained. The centrifugation removes a significant fraction of red blood cells and concentrates the number of cells in the sample. The cell concentration is adjusted accordingly to set up an appropriate number of cultures. If there is a sufficient amount of sample, direct harvest, 24-hour and 48-hour stimulated cultures are set up. Otherwise, if the volume of the sample is only enough for 2 cultures, direct harvest and 24-hour cultures are set up. All cultures are set up in a 37°C, 5% CO_2 incubator.

Synchronisation

Upon reaching the ideal concentration or after the incubation period, cultures are blocked with fluorodeoxyuridine and uridine for 17 hours. The block is released with bromodeoxyuridine, a thymidine analogue. Cells are harvested after 7 hours of release, with colcemid added in the last half an hour. Sychronisation of cell cultures allows increased chromosome length and an improved quality of metaphase spread.

Harvesting

After half an hour of incubation with colcemid at 37°C, the culture is removed and poured into test tubes, which are centrifuged at 1000 rpm for 10 minutes. The supernatant is removed with a pipette, leaving 0.5 mL above the cell pellet. Cells are then re-suspended by mixing gently with a vortex mixer, and 8 to 10 mL of pre-warmed potassium chloride solution is added in drop-wise while mixing. The potassium chloride solution allows for hypotonic treatment of the cells, to swell the cells for better chromosome spread and to lyse the unneeded erythrocytes. The suspension is then left for 15 minutes in a 37°C water bath.

The suspension is then centrifuged at 1000 rpm for another 10 minutes. Again, the supernatant is removed, leaving 0.5 mL above the cell pellet, and the cells re-suspended using a vortex mixer. Once the suspension is completely mixed, 10 mL of freshly prepared 4:1 methanol and glacial acetic acid fixative is added drop-wise, and mixed gently. The suspension is centrifuged again as previously, and

the supernatant is then decanted. This "washing" of the cell pellet is repeated twice with 3:1 methanol and glacial acetic acid fixative. The supernatant is removed, and 4 mL of fresh fixative is added after the final wash.

Slide-making

A small volume of fixative, depending on the size of the cell pellet (1 to 2 mL is usually used), is then added to form a slightly turbid suspension. Two to 3 drops of the suspension are dropped onto a clean glass slide held at an angle of 15°, and allowed to dry. The resulting slide is examined using a phase-contrast microscope under low power objective, to check for metaphase spreads. The concentration of the cell suspension is adjusted appropriately based on microscope observations for subsequent slide making. All the slides are dried overnight at 68°C.

G-banding

G-banding is done according to a modified protocol described by Seabright M.[7] After overnight drying, the slides are removed, and placed into a 6% hydrogen peroxide solution for 4 to 5 minutes. They are rinsed in a pH 7.3 buffer solution twice. The slides are then placed one at a time into a 0.005% Gibco trypsin solution and given a gentle shake. They are then rinsed twice in a pH 6.8 buffer solution, and then placed in Leishman stain for 2 to 3 minutes. Subsequently, they are rinsed in a pH 6.8 buffer solution, and washed with distilled water. They are dried and mounted, ready for karyotype analysis. A trial slide is made, and the banding examined under a light microscope. If optimal staining is not achieved, the trypsinisation time is varied accordingly.

Karyotype Analysis

Karyotype analysis was done in accordance with the International System for Human Cytogenetic Nomenclature (ISCN).[8] Strict laboratory protocol was followed to maintain high culture success rates, and rigorous screening for abnormalities was carried out. Full analysis was carried out as far as possible on all cultures, with a normal successful result being a complete analysis and an observation of a minimum of 20 metaphases, which is the established recommendation. A sub-optimal result is regarded as an observation of between 5 to 19 metaphases, with 2 metaphases displaying identical abnormalities. Beyond that, the culture would be regarded as a culture failure if only 4 or fewer metaphases were observed. Nevertheless, in most of the sub-optimal cultures, a complete karyotype in 2 or more metaphases was achieved; the karyotype could be analysed. However, there were a few sub-optimal results where the chromosome morphology was so poor that they will be excluded from the analysis of results.

Results

For the 369 ALL and AML cases, there was a success rate of 90.7% (185/204) from 2002 to 2006, as compared to a success rate of 79.4% (131/165) from 1989 to 2001, showing an increase of 11.3% ($P = 0.002$). For the 281 ALL cases, there was a success rate of 89.1% (139/156) from 2002 to 2006, as compared to 77.6% (97/125) from 1989 to 2001, showing an increase of 11.5% ($P = 0.008$). For the 88 AML cases, there was a success rate of 95.8% (46/48) from 2002 to 2006, as compared to 85.0% (34/40) from 1989 to 2001, showing an increase of 10.8% ($P = 0.04$). Overall, for the 4749 cases handled by the cytogenetics laboratory from the period 1989 to 2006, there was a success rate of 94.9% (2807/2957) from 2002 to 2006, as compared to 88.8% (1592/1792) from 1989 to 2001. These results are illustrated in Table 1.

The yearly percentage of cases with a turn-around time (TAT) from sample date to report date is represented graphically in Figures 1 and 2. The average TAT from 1989 to 2001 was 21.6 days, with 72.9% of cases having a TAT of 21 days or less, as compared to 15.6 days from 2002 to 2006, with 85.0% of cases having a TAT of 21 days or less.

Discussion

Conventional cytogenetics has long been regarded as the gold standard for chromosome analysis when it comes to diagnosing childhood leukaemia, despite the advent of molecular cytogenetics.[4, 9-11] Conventional cytogenetics allow the survey of the whole genome of visible abnormalities of chromosome number and structure. However, the turn-

Table 1. Overview of Success Rates

Cases	1989-2001	2002-2006	Total
Overall No.	1792	2957	4749
Successful No.	1592	2807	4399
Successful %	88.8%	94.9%	92.6%
Overall No. (ALL+AML)	165	204	369
Successful No. (ALL+AML)	131	185	316
Successful % (ALL +AML)	79.4%	90.7%	85.6%
Overall No. (ALL)	125	156	281
Successful No. (ALL)	97	139	236
Successful % (ALL)	77.6%	89.1%	84.0%
Overall No. (AML)	40	48	88
Successful No. (AML)	34	46	80
Successful % (AML)	85.0%	95.8%	90.9%

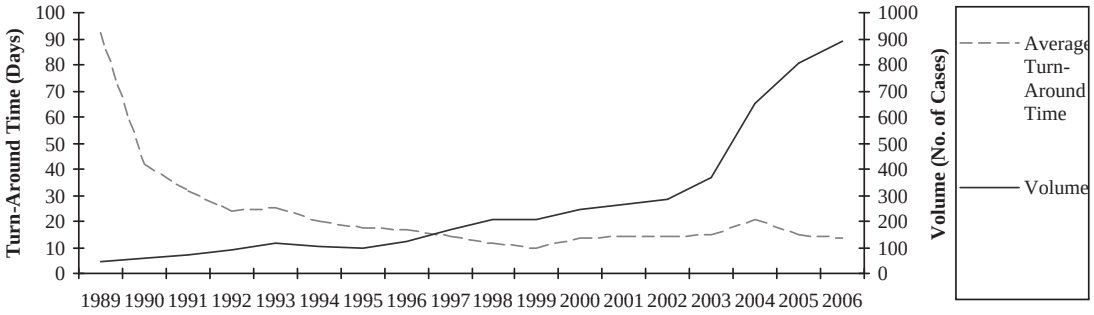

Fig. 1. Graphical representation of comparison of overall number of cases processed in cytogenetics laboratory per year vs. average turn-around time.

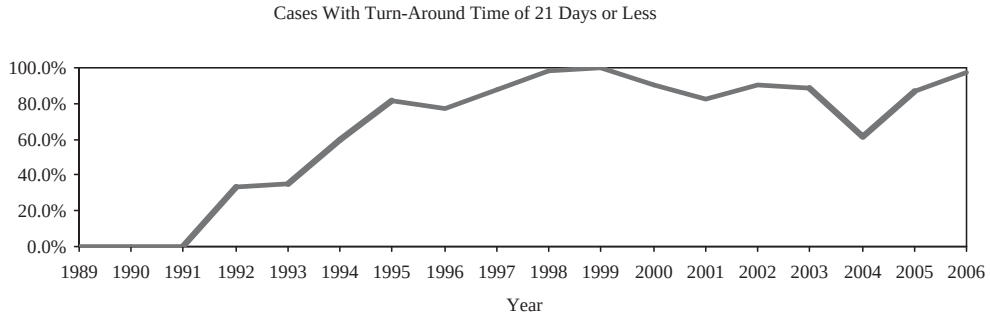

Fig. 2. Graphical representation of percentage of cases with turn-around time of 21 days or less by year.

around time of 21 days in conventional cytogenetics does not allow risk-adapted therapy to proceed immediately. In addition, conventional cytogenetics tests are prone to failure if not carried out properly, resulting in the lack of clinical markers for a proper prognosis. These potential problems are addressed by advanced complementary studies for specific abnormalities, otherwise known as molecular cytogenetics. The latter expands our capabilities by making more accurate and rapid cytogenetic diagnoses, both for constitutional abnormalities and acquired chromosome aberrations in cancer cells, some of which are otherwise not detected by conventional cytogenetics.[12-14] If there are adequate resources and facilities, conventional cytogenetics should be coupled with complementary tests such as FISH and DNA flow cytometry to improve the accuracy of results.

Since molecular cytogenetics allows the detection of numerous clinically relevant abnormalities, which are beyond the resolution of conventional cytogenetics, plans to introduce complementary tests such as FISH are underway at the laboratory. A new automated machine has been purchased, which allows karyotyping to proceed at a quicker pace, and has FISH capabilities. Validation of FISH probes is in progress, and plans are to incorporate FISH procedures into the laboratory by 2010. This includes probe sets for chromosome 21, AML1, MLL, TEL-AML1, trisomies 4, 10 and 17. In addition, real-time polymerase chain reaction (PCR) tests have been established to screen for common oncogene fusion transcripts in paediatric ALL (Table 2), which will be carried out by molecular biologists at Molecular Diagnostic Centre, NUH. Nevertheless, the conventional cytogenetic approach, coupled with strict laboratory protocols to encourage proper karyotype analysis, will detect most cases of aneuploidy, mosaicism, and syndromes that result from chromosome aberrations.[15]

The dedicated cytogenetics laboratory was set up in 2002, as results produced from external laboratories were sub-optimal. Previously, processing methods such as culture and collection procedures were not standardised, and different analysis methods were occasionally used. This led to large

Table 2. List of Common Oncogene Fusion Transcripts Screened in: (A) Childhood Acute Lymphoblastic Leukaemia and (B) Childhood Acute Myeloid Leukaemia

(A)

Chromosomal Alteration	Fusion Gene
t(12;21)(p13;q22)	*TEL-AML1*
t(1;19)(q23;p13)	*E2A-PBX1*
t(4;11)(q21;q23)	*AF4-MLL*
t(9;22)(q34;q11)	*BCR-ABL*
t(1;1)(p34)	*SIL-TAL1*

(B)

Chromosomal Alteration	Fusion Gene
t(9;22)(q34;q11)	*BCR-ABL*
inv(16)(p13q22)	*CBFβ-MYH11*
t(8;21)(q22;q22)	*ETO-AML1*
t(15;17)(q21;q22)	*PML-RARα*

variability in karyotyping measures, leading to sub-optimal results. When the dedicated cytogenetics laboratory was set up, with standards fixed for subsequent cytogenetics testing, there was a marked improvement in the success rates of karyotyping of acute leukaemia cases. By keeping processing and analysis in-house, the level of control over quality is greatly enhanced. As a result, in both ALL and AML, success rates were up by more than 10% each.

Critically, despite the sharp increase in cases handled by the cytogenetics laboratory from year 2002 onwards, success rates for the duration of 2002 to 2006 were still an improvement over success rates for the years of 1989 to 2001, when one would have normally expected success rates to decline due to an increased caseload. One of the reasons for the higher caseload was due to the formation of the Malaysia-Singapore ALL 2003 Study, which led to more samples being sent from all over the region to the authors' hospital for cytogenetics testing. This was done in a co-operative setting where greater quality control was assured and experience was shared.

It is also important that the turn-around time is improved as well. The lower the turn-around time, the earlier the results are delivered to the clinician, which would allow the earliest possible intervention in therapy.

The marked improvement in results was due to the adoption of a strict laboratory protocol for cytogenetics, and the refinement of techniques. The cytogenetics team first adopted a protocol that would meet the accreditation standard of the College of American Pathologists,[6] currently the international gold standard for a cytogenetics laboratory. Culturing, synchronisation and harvesting procedures were experimented with, and different types of culture media were used to see which results worked best for them. The protocol was then modified to integrate the procedures with the best metaphase spreads. To further improve results, the cytogenetics team used a trial slide before slide-making and G-banding, so as to make adjustments to the procedures based on results with the trial slide, which would net them higher quality metaphase spreads.

However, the success of a cytogenetics laboratory does not depend solely on its success rates of karyotyping. Part of its success depends on a successful working relationship with the clinician. The clinician provides important background information that allows the cytogeneticist to render a more precise diagnosis. The close working relationship between the clinicians and cytogeneticists at the authors' hospital has contributed to the improved results.

From the significant jump in success rates and turn-around time, it can be inferred that a dedicated cytogenetics laboratory would confer an important benefit on the management of leukaemia. With the karyotype, an important prognostic determinant in the outcome of therapy in the treatment of childhood leukaemia, any measures taken to improve karyotyping results would be invaluable. As our results have shown, dedicated in-house cytogenetics testing would improve the quality of karyotyping results and turn-around time, and in turn, the quality of patient management.

In light of this, the establishment of dedicated, national centres for cytogenetics is important for the modern management of childhood leukaemia, to aim for the best patient care possible at a minimal cost of therapy.

Acknowledgements: The authors wish to thank the cytogenetics team from the National University Hospital for providing cytogenetic data.

REFERENCES

1. Grimwade D, Walker H, Oliver E, Wheatley K, Harrison C, Harrison G, et al. The importance of diagnostic cytogenetics on outcome in AML: Analysis of 1612 patients entered into the MRC AML10 trial. Blood 1998;92:2322-33.
2. Slovak ML, Kopecky KJ, Cassileth PA, Harrington DH, Theil KS, Mohamed A, et al. Karyotypic analysis predicts outcome of preremission and postremission therapy in adult acute myeloid leukemia: A Southwest Oncology Group/Eastern Cooperative Oncology Group study. Blood 2000;96:4075-83.
3. Byrd JC, Mrozek K, Dodge RK, Carroll AJ, Edwards CG, Arthur DC, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). Blood 2002;100:4325-36.
4. Moorman AV, Harrison CJ, Buck GA. Karyotype is an independent prognostic factor in adult acute lymphoblastic leukemia (ALL): analysis of cytogenetic data treated on the Medical Research Council (MRC) UKALLXII/Eastern Cooperative Oncology Group (ECOG) 2993 trial. Blood 2007;109:3189-97.
5. Frohling S, Skelin S, Liebisch C, Scholl C, Schlenk RF, Dohner H, et al. Comparison of cytogenetic and molecular cytogenetic detection of chromosome abnormalities in 240 consecutive adult patients with acute myeloid leukemia. J Clin Oncol 2002;20:2480-85.
6. College of American Pathologists. Laboratory Accreditation Program – Cytogenetics Checklist. Available at: http://www.cap.org/apps/docs/laboratory_accreditation/checklists/cytogenetics_december2006.pdf. Accessed 25 July 2007.
7. Seabright M. A rapid banding technique for chromosomes. Lancet 1971;21:971-2.
8. Shaffer LG, Tommerup N, editors. ISCN (2005): An International System for Human Cytogenetic Nomenclature. Basel, Switerland: S. Karger, 2005.
9. Teixeira MR. Combined classical and molecular cytogenetic analysis of cancer. Eur J Cancer 2002;38:1580-4.
10. Mrozek K, Prior TW, Edwards C, Marcucci G, Carroll AJ, Snyder PJ, et al. Comparison of cytogenetic and molecular genetic detection of t(8;21) and inv(16) in a prospective series of adults with de novo acute myeloid leukemia: a Cancer and Leukemia Group B Study. J Clin Oncol 2001;19:2482-92.
11. Romeo M, Chauffaille Mde L, Silva MR, Bahia DM, Kerbauy J. Comparison of cytogenetics with FISH in 40 myelodysplastic patients. Leuk Res 2002;26:993-6.
12. Cherry AM, Brockman SR, Paternoster SF, Hicks GA, Neuberg D, Higgins RR, et al. Comparison of interphase FISH and metaphase cytogenetics to study myelodysplastic syndrome: an Eastern Cooperative Oncology Group (ECOG) study. Leuk Res 2003;27:1085-90.
13. Teixeira MR, Micci F, Dietrich CU, Heim S. Detailed genomewide screening for inter- and intrachromosomal abnormalities by sequential G-banding and RxFISH color banding of the same metaphase cells. Canc Gene Cyto 2000;119:94-101.
14. Shali W, Hélias C, Fohrer C. Cytogenetic studies of a series of 43 consecutive secondary myelodysplastic syndromes/acute myeloid leukemias: conventional cytogenetics, FISH and multiplex FISH. Canc Gene Cyto 2006;168:133-45.
15. Dave BJ, Sanger WG. Role of cytogenetics and molecular cytogenetics in the diagnosis of genetic imbalances. Semin Pediatr Neurol 2007;14:2-6.