

Real-time Quantitative Polymerase Chain Reaction (RQ-PCR) using the LightCycler: A Rapid, High-throughput Method for Detecting and Quantifying Fusion Transcripts in Childhood Leukaemias for Disease Stratification and Prognostication

LH Loh,¹MRCPC, SP Chen,²MSc, TC Quah,³M Med (Paed), AEJ Yeoh,⁴M Med (Paed), H Ariffin,⁵M Med (Paed), MRCP

Introduction

Cancer is a genetic disease. Its uncontrolled growth is driven by underlying genetic mutations that prevent differentiation and cause relentless cell divisions and apoptotic death. The genetic basis for leukaemogenesis is well elucidated and specific mutations can be found in up to 50% of patients.

As drivers of leukaemogenesis, oncogene fusions are the most accurate diagnostic markers and predictors of treatment outcome. It is, hence, crucial in modern leukaemia therapy to detect these fusion transcripts so that the correct treatment intensity can be applied to improve outcome and decrease side effects. Conventional cytogenetics, the gold standard, is laborious, laboratory-dependent and completely misses submicroscopic translocations like t(12;21)/TEL-AML1.¹

Reverse-transcription polymerase chain reaction (RT-PCR) can detect these oncogene fusions but is also time-consuming and requires post-PCR handling, greatly increasing the risk of PCR-product contamination.² Moreover, it is a qualitative assay and cannot quantify transcripts for monitoring disease post-therapy.

Early response to therapy as determined by Ig/TCR rearrangements, is one of the most important prognostic markers of ALL therapy.³⁻⁶ Unfortunately, they require high levels of diagnostic blasts to identify a leukaemia-associated marker and are susceptible to clonal evolution. We postulate that fusion transcripts are not constrained by these parameters and can complement Ig/TCR for disease stratification.⁷

Real-time quantitative PCR (RQ-PCR) is a new molecular platform that allows specific quantitation of gene copy numbers. It is rapid, has high-throughput and does not require post-PCR handling.^{7,8}

Here, we studied the feasibility of using RQ-PCR to detect and quantify fusion transcripts in childhood leukaemia for diagnosis and prognostication.

Materials and Methods

RNA extraction and Reverse Transcription

Bone marrow (BM) aspirates were collected in EDTA, isolated by density gradient centrifugation and total RNA extracted using TRIzol reagent. cDNA was synthesized with random hexamers using the Omniscript Reverse Transcriptase kit. Primers and probes were obtained from published data.^{9,10}

Qualitative Detection of Fusion Transcripts

Multiplex RT-PCR setup. This was designed to screen patients for the commonly reported splice variants of TEL-AML1, E2A-PBX1,

MLL-AF4 and BCR-ABL p210 transcripts. RQ-PCR was performed on a LightCycler Instrument using Taqman probes.

Positive, negative and no-template controls were included in each run by replacing the template DNA with positive cell lines for the specific fusion transcripts, total RNA from HL60 cell line or PCR-grade water, respectively. Positive multiplex panels underwent split-out PCR with individual primers to confirm the specific splice variant.

Quantitative Monitoring of Fusion Transcripts

Standard curves were prepared using 10-fold dilutions of plasmids containing the specific fusion transcripts cloned into pCR® II-TOPO vector. GUS was selected as the housekeeping gene to compensate for variations in sample volume, target recovery during RNA extraction and inhibitors.

Results

Qualitative Screening

A total of 107 diagnostic or relapse BMs were screened for the common fusion transcripts in childhood leukaemia. Twenty-eight (26%) patients tested positive for fusion transcripts (Table I). RQ-PCR results correlated perfectly with conventional RT-PCR and even detected a case originally missed by the latter.

Quantitative Studies

Quantitative assays were performed on 41 BM samples from 12 patients (Table II), including 10 diagnostic, 2 relapse and 29 follow-

TABLE I: RESULTS OF QUALITATIVE SCREEN FOR ONCOGENE FUSIONS

Diagnosis	Fusion transcript	Positive cases n = 28	Percentage affected	Published data ¹ (%)
ALL n = 86	TEL-AML1	12	14	25
	MLL-AF4	3	4	2
	BCR-ABLp190	5	6	5
	BCR-ABLp210	*	1	0.5
	E2A-PBX1	0	0	5
	Total	21	24	25-50
AML n = 18	CBFβ-MYH11	1	6	10
	AML1-ETO	3	17	14
	Total	4	22	20-40
CML n = 3	BCR-ABLp210	3	100	>95
Overall n = 107		28	26	

*biphenotypic leukaemia

¹ Registrar

² Laboratory Officer

³ Associate Professor and Senior Consultant

⁴ Assistant Professor and Consultant

Division of Paediatric Haematology-Oncology, The Children's Medical Institute, National University Hospital, National University of Singapore, Singapore

⁵ Associate Professor and Consultant

Department of Paediatrics, University of Malaya, Malaysia

Address of Correspondence: Dr Loh Lih Hwa, Division of Paediatric Haematology-Oncology, The Children's Medical Institute, National University Hospital, National University of Singapore, 5 Lower Kent Ridge Road, Singapore 119074.

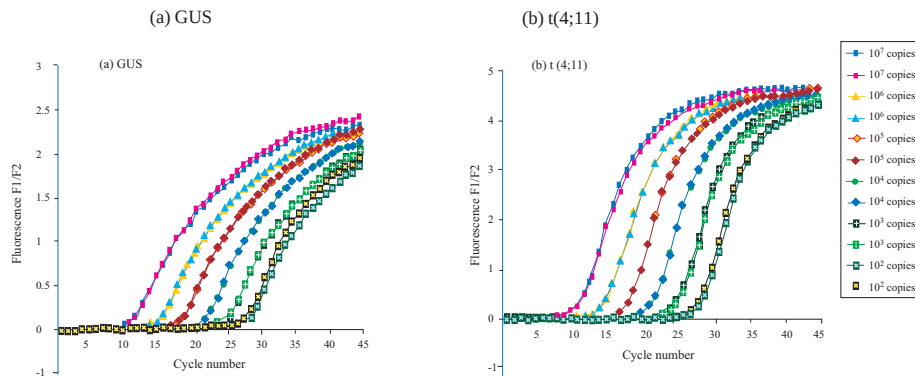


Fig. 1. Representative graphs showing increase in fluorescence during amplification of sequences from 10^2 – 10^7 copy numbers of (a) GUS and (b) t(4;11) plasmid DNA.

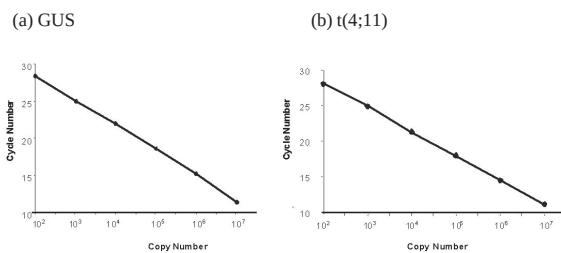


Fig. 2. Representative graph showing linear relationships between crossing point and starting copy number of cDNA standards. 10^2 – 10^7 copies of GUS plasmid cDNA were amplified for 40 cycles. The slopes were -3.424 cycles/log decade. The slope of the standard curve was converted to amplification efficiency E by the following algorithm: $E = 10^{-1/\text{slope}}$ (E should not defer by more than 1.5–2.2). The regression coefficients were -1.00 for all runs.

up samples. The median duration of follow-up was 65 days (range 1–12 months).

Standard Curves⁷

Regression coefficients were -1.00 (ideal -1.00) for all runs. The mean $\pm$ SD slope was -3.41 ± 0.15 (ideal -3.3) with mean $\pm$ SD intercept of 36.01 ± 2.15 . For the GUS standard curves, slope was -3.44 ± 0.02 and intercept 35.64 ± 0.27 (Figs. 1 & 2).

Sensitivity

The detection limit of each assay was 10–50 fusion transcript copies.

Reliability of Assay

Intra-assay variability analysis was determined using ten-fold

serial dilutions of a positive control sample (cell line cDNA) in triplicates from 100% to 0.01% of template concentration. Inter-assay variability was studied by repeating the analysis of the same samples in 3 consecutive assays. The inter- and intra-assay comparisons, expressed as crossing thresholds (CTs), showed good reproducibility with less than 0.93 cycle difference, reflecting a coefficient of variation of less than 3%.

Fusion Transcript Quantitation in ALL (Table III)

Four (67%) of six *TEL-AML1* patients showed complete transcript clearance by day 33 while 2 (33%) had low-level disease (normalised ratio 8×10^{-5}).^{11–13} These correlated perfectly with the Ig/TCR method of minimal residual disease (MRD) detection:¹⁴ the 2 patients with low-level disease had MRD of 2×10^{-4} and 7×10^{-4} , respectively.

Similarly, both patients with *MLL-AF4* transcripts demonstrated clearance by day 33, although 1 had intermediate-level disease (3×10^{-3}) by Ig/TCR MRD levels.

For the patient with *BCR-ABLp190*-positive ALL, this transcript persisted at day 33 and even 6 months later. There was, however, a 2-log reduction of MRD from 63,510 to 636.8 copies/uL cDNA (normalised ratio 6.57 to 0.04) by day 33. Using Ig/TCR markers, she had intermediate MRD at day 33 and clearance of disease at 5 months. This suggests that fusion transcripts are potentially more sensitive than Ig/TCR rearrangements for MRD detection.

Fusion Transcript Quantitation in AML

Neither AML patient showed transcript clearance after the first course of chemotherapy. The *CBF β -MYH11* transcripts demonstrated a 4-log reduction between diagnosis and treatment cessation¹⁵. The *AML1-ETO* transcripts showed a 3-log reduction after 2 courses of chemotherapy.¹⁶

TABLE II: QUANTITATION STUDIES COMPARING ONCOGENE FUSION TRANSCRIPTS AND IG/TCR MARKERS

Patient	Diagnosis (FT)	Fusion transcript at diagnosis	FT/Gus after induction tx	FT/ Gus after induction tx	MRD level (Ig/TCR)	Clinical progress No.
1	B-ALL	<i>TEL-AML1</i>	1.53	0.00000	Not done	CR* at 7.5 months
2	B-ALL	<i>TEL-AML1</i>	2.12	0.00000	$<5 \times 10^{-5}$	CR at 5.83 month
3	B-ALL	<i>TEL-AML1</i>	1.26	0.00000	$<1 \times 10^{-4}$	CR at 3.77 months
4	B-ALL	<i>TEL-AML1</i>	2.78	0.00008	2×10^{-4}	CR at 3.27 month
5	B-ALL	<i>TEL-AML1</i>	6.91	0.00008	7×10^{-4}	CR at 7 months
6	B-ALL	<i>TEL-AML1</i>	1.55	0.00000	$<2 \times 10^{-5}$	CR at 2.1 months
7	B-ALL	<i>MLL-AF4</i>	4.12	0.00000	3×10^{-3}	CR at 6.43 months
8	B-ALL	<i>MLL-AF4</i>	1.16	0.00000	$<5 \times 10^{-5}$	CR at 4.07 months
9	B-ALL	<i>BCR/ABLp190</i>	6.57	0.25763	8×10^{-4}	CR at 8.03 months
10	AML-M4	<i>CBFβ-MYH11</i>	2.13	0.00177	Not done	CR at 8.47 months
11	AML-M2	<i>AML1-ETO</i>	3.34	0.00076	Not done	CR at 3.73 months
12	CML	<i>BCR/ABLp210</i>	NA**	NA**	NA**	Deceased

* CR clinical remission

** The CML patient's BM samples were timed differently. Please refer to Figure 3.

TABLE III: SUMMARY OF SENSITIVITY OF VARIOUS FUSION TRANSCRIPTS USED FOR MRD QUANTITATION

Fusion transcript	No. of patients	At diagnosis/relapse		At Day 33 (end of induction remission treatment)*	
		Transcript Copies/ uL cDNA Median (range)	Normalised with GUS Median (range)	Transcript copies/ uL cDNA Median (range)	Normalised with GUS Median (range)
<i>TEL-AML1</i>	6	91225 (38720-217100)	1.84 (1.26 - 6.91)	0.60 (0.00 - 3.96)	0.00004 (0.00000-0.00012)
<i>MLL-AF4</i>	2	42255 (40690-43820)	2.64 (1.12- 4.12)	0.00	0.00000
<i>BCR-ABLp190</i>	1	63510	6.57	4153.00	0.25763
<i>CBFβ-MYH11</i>	1	34710	2.13	67.43	0.00177
<i>AML1-ETO</i>	1	366300	3.34	105.30	0.00076

* In AML patients, a repeat BM was performed before commencing on the second course of chemotherapy rather than on day 33.

† Data for the CML patient is presented separately.

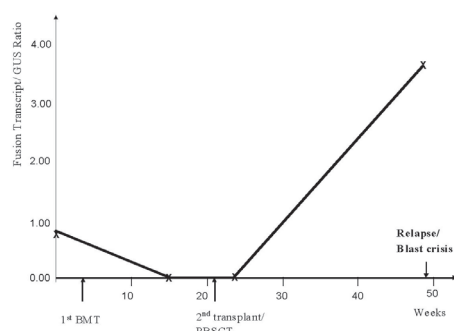


Fig. 3.

BCR-ABL Levels and Clinical Progression¹⁷

Transcript levels of the CML patient correlated well with his clinical course. There was complete clearance after his first BMT, low transcripts (7×10^{-3}) after his second transplant which increased to 3.57 at relapse (Fig. 3).

Correlation with Cytogenetics

Our results correlated with conventional karyotyping where available. *TEL-AML1* was missed entirely by the latter.

Discussion

As the local cure rates for childhood ALL and AML improve to 75% and 50%, respectively, it is clear that leukaemia is a highly heterogeneous disease. Fusion transcripts are extremely useful for disease stratification as they accurately identify different risk groups. For example, *TEL-AML1* patients have 90% EFS, while *BCR-ABL* and *MLL-AF4* patients have less than 20% EFS. Similarly, for AML, *AML1-ETO* and *CBFβ-MYH11* have excellent outcome (70% EFS) on our therapy. It is, hence, crucial to incorporate the detection of oncogene fusion screening for modern management of leukaemia.

We have demonstrated that RQ-PCR is superior to conventional karyotyping and RT-PCR as a screening tool for fusion transcripts in childhood leukaemia.¹⁸⁻²¹ Conventional karyotyping, only successful in 60% to 80% of cases, is highly laboratory-dependent and completely misses submicroscopic fusions like the common *TEL-AML1* fusion gene (14% of ALL cases here).

RQ-PCR has a much faster turnaround time (7h versus 13h versus 1 week) and a higher maximal throughput (29 versus 6 versus 4 screens per technician day) compared to RT-PCR and cytogenetics, respectively. This is crucial for large studies like our Malaysia-Singapore ALL 2003 study, where timely delivery of results is vital.

RQ-PCR permits accurate quantitation of PCR products during the exponential phase of the amplification process, in contrast to end-point quantitation in classical PCR, making it a highly valuable

quantitative tool.⁷ The intra- and inter-assay reproducibility is excellent in our study.

Conventional microscopy is very insensitive: $<5 \times 10^{-2}$ (5 blasts in 100 nucleated cells) is considered complete remission. We have previously demonstrated that IgH MRD markers increase sensitivity of detection to 5×10^{-5} , a 3-log improvement compared to conventional microscopy. Moreover, IgH-based MRD levels at 1 month of therapy was highly correlated with outcome.²²

To determine if fusion transcripts have comparable MRD quantitative capabilities as IgH/TCR markers, we compared MRD determination using both techniques for 8 patients with oncogene fusion transcripts. Both methods have comparable sensitivities with complete concordance in 6 cases. In one case of *MLL-AF4*, fusion transcripts were undetectable despite intermediate-level disease by IgH, while the reverse was true for a case of *BCR-ABL*-positive ALL, where fusion transcripts showed persistent disease²³ whilst IgH was negative.

In this study, 24% of ALL cases carried fusion transcripts while 97% carried IgH/TCR gene rearrangements. In AML and CML, it was informative in 22% and 100% of cases, respectively, compared to 0% in IgH/TCR. Fusion transcripts, thus, play a bigger role in the monitoring of these conditions as Ig/TCR is not useful. *BCR-ABLp210* has been used to monitor response to BMT. Patients who are persistently PCR-negative have a low risk of relapse. Conversely, return of PCR positivity often heralds relapse.¹⁷

IgH/TCR methodology requires high blast counts at diagnosis to identify a marker, making it unsuitable for previously treated patients. Fusion transcripts are present in large numbers at diagnosis and have high sensitivities of 10-50 copies. They should, thus, be detectable 1-2 weeks after commencing therapy. It is a robust marker as it does not undergo evolution with treatment, in contrast to Ig/TCR gene rearrangements. Fusion transcript quantitation is, thus, highly complementary to IgH/TCR MRD risk stratification. This will be explored in our current Malaysia-Singapore ALL 2003 trial.

Conclusion

We conclude that RQ-PCR detection and quantitation of fusion transcripts is a new and important tool in the armamentarium of the paediatric oncologist. It supersedes conventional RT-PCR and karyotyping in qualitative assays and complements current methods of MRD detection using Ig/TCR gene rearrangements. In AML and CML, fusion transcript quantitation is the MRD marker of choice. In addition, it is a highly efficient tool with a fast turnaround time and increased throughput.

REFERENCES

1. Raimondi SC. Cytogenetics of acute leukaemias. In: Pui CH, editor. Childhood Leukaemias. Cambridge, United Kingdom: Cambridge University Press, 1999:168-

- 86.
2. Cross NCP. Annotation: Quantitative PCR techniques and applications. *Br J Haematol* 1995; 89:693-7.
3. Neale GAM, Campana D, Pui CH. Guest Commentary: Minimal residual disease detection in acute lymphoblastic leukaemia: real improvement with the real-time quantitative PCR method? *J Pediatr Hematol Oncol* 2003; 25:100-1.
4. Pui CH, Campana D. New definition of remission in childhood acute lymphoblastic leukaemia. *Leukaemia* 2000; 14:783-5.
5. van Dongen JJ, Seriu T, Panzer-Grumayer ER, Biondi A, Pongers-Willems MJ, Corral L, et al. Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. *Lancet* 1998; 352:1731-8.
6. Cave H, van der Werff ten Bosch J, Suciu S, Guidal C, Waterkeyn C, Otten J, et al. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia. European Organization for Research and Treatment of Cancer—Childhood Leukemia Cooperative Group. *N Engl J Med* 1998; 339:591-8.
7. van der Velden VH, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, van Dongen JJ. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukaemia* 2003; 17:1013-34.
8. Cazzaniga G, Rossi V, Biondi A. Monitoring minimal residual disease using chromosomal translocations in childhood ALL. *Best Practice Research Clinical Haematology* 2002; 15:21-35.
9. Krauter J, Hoellge W, Wattjes MP, Nagel S, Heidenreich O, Bunjes D, et al. Detection and quantification of CBFB/MYH11 fusion transcripts in patients with inv(16)-positive acute myeloblastic leukaemia by real-time RT-PCR. *Genes Chromosomes Cancer* 2001; 30:342-8.
10. Wattjes MP, Krauter J, Nagel S, Heidenreich O, Ganser A, Heil G. Comparison of nested competitive RT-PCR and real-time RT-PCR for the detection and quantitation of AML1/MTG8 fusion transcripts t(8;21) positive acute myelogenous leukaemia. *Leukaemia* 2000; 14:329-35.
11. Seeger K, Kreuzer KA, Lass U, Taube T, Buchwald D, Eckert C, et al. Molecular quantification of response to therapy and remission status in TEL-AML1-positive childhood ALL by real-time reverse transcription polymerase chain reaction. *Cancer Res* 2001; 61:2517-22.
12. Bolufer P, Barragan E, Verdeguez A, Cervera J, Fernandez JM, Moreno I, et al. Rapid quantitative detection of TEL-AML1 fusion transcripts in pediatric acute lymphoblastic leukaemia by real-time reverse transcription polymerase chain reaction using fluorescently labeled probes. *Haematologica* 2002; 87:23-32.
13. Pallisaard N, Clausen N, Schroder H, Hokland P. Rapid and sensitive minimal residual disease detection in acute leukaemia by quantitative real-time RT-PCR exemplified by t(12;21) TEL-AML1 fusion transcript. *Genes Chromosomes Cancer* 1999; 26:355-65.
14. de Haas V, Breunis WB, Dee R, Verhagen OJ, Kroes W, van Wering ER, et al. The TEL-AML1 real-time quantitative polymerase chain reaction (PCR) might replace the antigen receptor-based genomic PCR in clinical minimal residual disease studies in children with acute lymphoblastic leukaemia. *Br J Haematol* 2002; 116:87-93.
15. Guerrasio A, Pilatino C, De Micheli D, Cilloni D, Serra A, Gottardi E, et al. Assessment of minimal residual disease (MRD) in CBF α -MYH11-positive acute myeloid leukaemias by qualitative and quantitative RT-PCR amplification of fusion transcripts. *Leukaemia* 2002; 16:1176-81.
16. Barragan E, Bolufer P, Moreno I, Martin G, Nomdedeu J, Brunet S, et al. Quantitative detection of AML1-ETO rearrangement by real-time RT-PCR using fluorescently labeled probes. *Leuk Lymphoma* 2001; 42:747-56.
17. Hochhaus A. Minimal residual disease in chronic myeloid leukaemia patients. *Best Practice Research Clinical Haematology* 2002; 15:159-78.
18. Pallisaard N, Hokland P, Riishoj DC, Pedersen B, Jorgensen P. Multiplex reverse transcription-polymerase chain reaction for simultaneous screening of 29 translocations and chromosomal aberrations in acute leukaemia. *Blood* 1998; 92:574-88.
19. Elia L, Mancini M, Moleti L, Meloni G, Buffolino S, Krampera M, et al. A multiplex reverse transcription-polymerase chain reaction strategy for the diagnostic molecular screening of chimeric genes: a clinical evaluation on 170 patients with acute lymphoblastic leukaemia. *Haematologica* 2003; 88:275-9.
20. Kwan E, Norris MD, Zhu L, Ferrara D, Marshall GM, Haber M. Simultaneous detection and quantification of minimal residual disease in childhood acute lymphoblastic leukaemia using real-time polymerase chain reaction. *Br J Haematol* 2000; 109:430-4.
21. Pine SR, Moy FH, Wiemels JL, Gill RK, Levendoglu-Tugal O, Ozkaynak MF, et al. Real-time quantitative PCR: standardized detection of minimal residual disease in pediatric acute lymphoblastic leukaemia. *J Pediatr Hematol Oncol* 2003; 25: 103-8.
22. Kham SKY, Zhou JB, Tan PL, Quah TC, Yeoh AEJ. The kinetics of early response to therapy as determined by PCR-based minimal residual disease (MRD) is highly predictive of outcome in childhood acute lymphoblastic leukaemia. National Healthcare Group 1st Annual Scientific Congress; 2002 Aug 17-18; Singapore, 1992.
23. Radich JP. Molecular measurement of minimal residual disease in Philadelphia-positive acute lymphoblastic leukaemia. *Best Practice Research Clinical Haematology* 2002; 15:91-103.

Young Investigator's Award Finalist

Angiomyogenesis using Human Myoblast Carrying Human VEGF₁₆₅ for Injured Heart

L Ye,¹MD, KhH Haider,²BSc, MPharm, PhD, SJ Jiang,³MD, MSc, LH Ling,⁴MBBS, FRCP, FAMS, MI Niagara,⁵BSc (Appl Sci), AJ Rufaihah,⁵BSc (Appl Sci), PK Law,⁶PhD, EKW Sim,⁷MBBS, FRCS

Introduction

Therapeutic angiogenesis using vascular endothelial growth factor (VEGF) for the treatment of ischaemic heart disease has been studied in animal models and clinical trials.^{1,2} The common modes of VEGF delivery include direct introduction of angiogenic protein and injection of plasmid or recombinant viral vectors.³ Recent studies have suggested therapeutic angiogenesis through cell-mediated gene transfer.⁴ The transplantation of genetically modified cells serves as a reservoir to produce biologically active angiogenic factors in a localised and sustained pattern.

Skeletal myoblast, the precursors of skeletal muscle fibres, can be induced to proliferate, propagate and differentiate *in vitro*. Recently, the promising results for improved heart function using myoblast transplantation have generated considerable interest amongst researchers.⁵ Myoblast transplantation improves both *in vivo* and *ex vivo* indices of global ventricular dysfunction and prevents deleterious remodelling to achieve global myocardial function after myocardial infarction. Considering skeletal myoblast is a good carrier for gene therapy, the genetically engineered skeletal myoblast has been studied for cardiac repair.⁴

¹ PhD Student, Department of Cardiothoracic Surgery, National University Hospital, Singapore

² Research Scientist, National University Medical Institute, National University of Singapore

³ Research Officer, National Heart Center, Singapore

⁴ Associate Professor, Department of Cardiology, National University of Singapore, Singapore

⁵ Master Student, Department of Cardiothoracic Surgery, National University Hospital, Singapore

⁶ Founder Chairman, Cell Transplantation Inc, Singapore

⁷ Associate Professor and Senior Consultant, Department of Cardiothoracic Surgery, National University Hospital, Singapore

Address of Correspondence: Dr L Ye, Department of Cardiothoracic Surgery, National University Hospital, 5 Lower Kent Ridge Road, Singapore 119074.

In the present study, we assessed the efficacy of genetically engineered human skeletal myoblast carrying human VEGF₁₆₅ (HSM-VEGF₁₆₅) in a rodent heart model of cryo-injury. We hypothesised that this combined strategy could achieve concurrent angiogenesis and myogenesis.

Materials and Methods

Cell Culture

The purity of myoblast was assessed by desmin immunostaining. Human umbilical vein endothelial cell line (HUVEC), HEK-293 cells and FLYA-4 cell lines were cultured in respective basal medium supplemented 10% FBS.

Propagation of Adenovirus Vector Carrying hVEGF₁₆₅ and Transduction of Myoblast

Adenovirus vector carrying hVEGF₁₆₅ (Ad-hVEGF₁₆₅) was propagated using HEK-293 cells. At 70% confluence, the cells were infected with adenoviral vector carrying VEGF₁₆₅ (Ad-VEGF₁₆₅). At the stipulated time, the supernatant from HEK-293 cells was removed, centrifuged and used for transduction of myoblast.

Myoblasts were propagated in laminin coated tissue culture flasks. The cells were exposed to virus medium for 8 hours followed by refresh Super medium. The transduction procedure was repeated thrice with 24 hours' interval each time.

Characterisation of Ad-VEGF₁₆₅ Transduced Myoblast In vitro Immunohistochemical Staining of Myoblast

Myoblasts transduced with Ad-hVEGF₁₆₅ (HSM-VEGF₁₆₅) were grown on glass slides and fixed with -20°C cold methanol. After blocking with 1% BSA and incubating with 3% H₂O₂, cells were washed with PBS and incubated with 1:500 dilution of anti-VEGF₁₆₅ overnight followed by 1: 200 goat anti-rabbit IgG conjugated with HRP. The VEGF-positive cells were observed and visualised with DAB visualisation system.

Enzyme-linked Immunosorbent Assay for hVEGF₁₆₅

The detection of hVEGF₁₆₅ secreted from myoblast transduced with Ad-VEGF₁₆₅ (M-VEGF₁₆₅) was carried out using hVEGF₁₆₅ Sandwich ELISA kit (Chemicon Inter., USA) according to the manufacturer's instructions.

RT-PCR Analysis for hVEGF₁₆₅ Gene Expression in Transduced Human Skeletal Myoblast

The total RNA was isolated by using Total RNA Isolation Kit (PURESCRIPT USA). The RT-PCR analysis of isolated mRNA was done using QIAGEN One-step RT-PCR Kit (QIAGEN). The RT-PCR primers of hVEGF₁₆₅ 5'→3' (5' ATGAACCTTCTG CTGTCTTGGGTG 3') and 3'→5' (5' TCACCGCTCGGCTTG TCACA 3') and human GAPDH were 5'→3' (5' GTGGAGGAGT GGG TGTCGCTG 3') and 3'→5' (5' CGGATTTGGTCGTATT GGGCG 3') were used for amplification.

Thymidine [³H] Incorporation Assay

HUVEC (1x10⁵ cell / well) were seeded in 6-well plates in triplicate for each sample. After 12 hours' culture with DMEM containing 2% FBS, the cells were cultured with conditioned medium from various samples. Each well was supplemented with 2 μCi/ ml Thymidine [³H] (ICN Biomedical Inc., USA). The cells were harvested after 96 hours for radioactivity count.

Rat Heart Model of Cryo-injury

After anaesthesia and intubation, rat heart was exposed through a 1.5-cm left lateral thoracotomy. Cryo-injury was induced on the free wall of the left ventricular wall using a liquid nitrogen cooled 8-mm diameter stainless probe. Cryo-injury was induced by 3 freezing cycles of 10 sec each. All animals received humane care in compliance with the institutional guidelines for animal research at NUS. A total of 21 rats were randomised into basal medium group

(n = 7), HSM group (n = 7) and HSM-VEGF₁₆₅ group (n = 7).

Myoblast Transplantation

The cells were labelled with retroviral lac-z reporter gene. Eight days after cryo-injury, 0.25 mL of medium containing 3 x 10⁶ myoblast or without cells was transplanted into rat cryo-injured heart using 27G needles. Three days before cell transplantation and until 6 weeks post-transplantation, a daily dose of 5 mg/kg/day cyclosporine was administered.

Rat Heart Function

Echocardiographic assessment of rat heart was carried out using 12 mHz probe positioned on the left anterior side of the chest. The heart was imaged in 2D mode at minimum depth settings to measure ejection factor (EF) and fractional shortening (FS).

Tissue RT-PCR Analysis of Rat Heart Post-myoblast Transplantation

Rat heart tissue was collected for RT-PCR analysis. The isolation of total RNA and RT-PCR analysis was performed as described earlier. The same primers were used for hVEGF₁₆₅. The primers for rat GAPDH were (forward) 5'-TTCTTGTGCAGTGCCAGCCT CGTC-3', (reverse) 5'-TAGGAACACGGAAGGCCATGCCAG-3'. The comparison of signals was performed using Microimage Analysis Software (Olympus, Japan).

Immunostaining of Rat Tissue for von Willebrand Factor VIII Expression

Tissue sections of rat heart were fixed in 100% acetone for 30 seconds. After blocking with 1% BSA, 1:2000 dilutions of rabbit anti-von Willebrand Factor VIII antibody was added. After 24 hours incubation at room temperature, the antigen was visualised using goat anti-mouse IgG conjugated with HRP and developed by DAB system.

Statistical Analysis

Statistical analysis was performed using SPSS (version 11.0). All data were presented as mean ± standard error mean (SEM) and analysed by analysis of variance (ANOVA). *P* < 0.05 was considered statistically significant.

Results

The purity of myoblast was >98% as assessed by desmin expression (Fig. 1a). The transduction efficiency of retroviral vector carrying lac-z reporter gene with myoblast was 75-80% (Fig. 1b). A high transduction efficiency (>98%) was achieved for VEGF₁₆₅ by a triple transduction procedure at a ratio of 1:1000PFU myoblast to Ad-VEGF₁₆₅ vector (Fig. 2a). ELISA revealed that the HSM-VEGF₁₆₅ secreted VEGF₁₆₅ up to 30 days *in vitro* with peak level at day 7 post-transduction. The peak expression level was 37 ± 3 ng/mL which was significantly higher than non-transduced myoblast (0.2 ± 0.04 ng/mL, *p* = 0.000). RT-PCR analysis of HSM-VEGF₁₆₅ was consistent with ELISA data and further confirmed high expression level of hVEGF₁₆₅ (Fig. 2b) Thymidine incorporation assay revealed that HUVEC cultured with conditioned medium from HSM-VEGF₁₆₅ incorporated significantly high level of Thymidine than any other group (*p* = 0.000).

β-gal staining revealed that myoblast successfully survived in rat heart (Fig. 3). Immunostaining for von Willebrand Factor VIII expression revealed that blood vessel density in HSM-VEGF₁₆₅ group at low power field (x200) was 17.5 ± 1.05. It was significantly higher than DMEM group 3.6 ± 0.7 (*p* = 0.000), and myoblast group 5.44 ± 0.82 (*p* = 0.000) (Fig. 4).

Echocardiograph demonstrated that EF and FS in myoblast group (93.6 ± 1.52%; *p* = 0.019, 76.0 ± 4.95%; *p* = 0.032) and HSM-VEGF₁₆₅ group (94.4 ± 2.22%; *p* = 0.012, 77.26 ± 3.25; *p* = 0.021) was significantly higher than DMEM injected group (82.7 ± 5.43%; 54.8 ± 6.44%) (Fig. 5).



Fig. 1a.

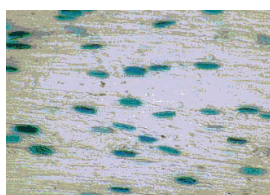


Fig. 1b.

Fig. 1. Characterisation of myoblast *in vitro*. (a) Myoblast immunostained for desmin expression. (magnification 100x) (b) Myoblast transduced with retroviral vector carrying lac-z reporter gene. (magnification 400x)

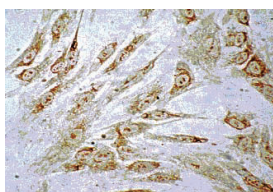


Fig. 2a.



Fig. 2b.

Fig. 2. *In vitro* characterisation of human skeletal myoblast carrying human VEGF₁₆₅. (a) Immunostaining of HSM-VEGF₁₆₅ for VEGF₁₆₅ expression. (magnification 400x) (b) RT-PCR analysis of HSM-VEGF₁₆₅ (samples 1 and 2 = myoblast, samples 3 and 4 = null-ad transduced myoblast, samples 5-12 = VEGF₁₆₅ transduced myoblast at 1, 8, 18 and 30 days post transduction).

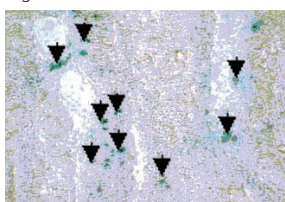


Fig. 3. Lac-z staining of myoblast transplanted rat heart (a) Myoblast located in cardiomyocytes. (magnification 200x) (Black arrow shows the presence of lac-z carrying myoblast)

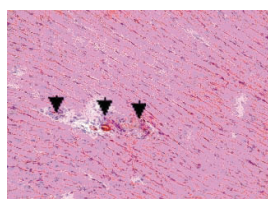


Fig. 4a.

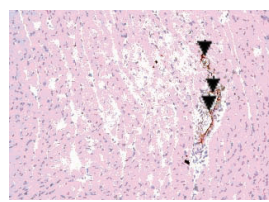


Fig. 4b.

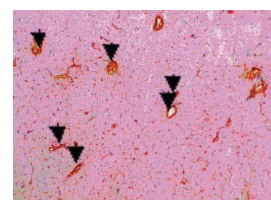


Fig. 4c.

Fig. 4. Rat cardiac tissue immunostaining vWF-VIII expression. The number of the blood vessels in HSM-VEGF₁₆₅ transplanted rat heart (c) was significantly higher than DMEM injected rat hearts (a) and myoblasts transplanted rat hearts (b). (magnification 200x) (Black arrow shows the blood vessel)

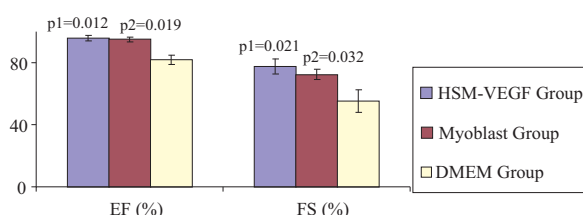


Fig. 5. Rat heart function post-myoblast transplantation. Ejection fraction (EF) in myoblast and HSM-VEGF₁₆₅ groups was significantly higher than DMEM-injected control group animals. Fractional shortening (FS) of myoblast and HSM-VEGF₁₆₅ transplanted animals was significantly higher than DMEM-injected control group (p1: HSM-VEGF₁₆₅ group vs DMEM group, p2: myoblast group vs DMEM group).

Discussion

The present study demonstrated that transplantation of human skeletal myoblasts carrying human VEGF₁₆₅ gene efficiently improved heart function in a rat heart model of myocardial infarction. The genetically engineered myoblast efficiently achieved therapeutic angiogenesis.

The efficiency of therapeutic angiogenesis using VEGF is related to its dose and delivery strategy. It has been shown that low dose for longer term administration has shown better prognosis with less side effects as compared to higher dose administration.^{6,7} Apart from the dose, route of administration significantly influences the result of angiogenesis.³ Thus, a regional and sustained level for a sustained term delivery of VEGF is necessary to achieve therapeutic angiogenesis. In the present study, we choose myoblast as a carrier of hVEGF₁₆₅ for therapeutic angiogenesis in cardiac repair. HSM-VEGF₁₆₅ efficiently secreted hVEGF₁₆₅ up to 30 days *in vitro* and *in vivo*. The transplanted HSM-VEGF₁₆₅ served as a reservoir to secrete biologically active hVEGF₁₆₅ in a localised pattern.

Myoblast was transplanted into centre and peri-infarction as a localised therapy. No angioma formation was observed at 6 weeks post transplantation. Tissue RT-PCR analysis revealed that HSM-

VEGF₁₆₅ worked as an expression vehicle and expressed hVEGF₁₆₅ in a regulated manner. We hypothesised that this novel strategy could deliver VEGF₁₆₅ in a site specific and sustained pattern.

We concluded that transplantation of human skeletal myoblast carrying human VEGF₁₆₅ gene resulted in a regional, sustained and transient expression of VEGF₁₆₅ for myocardial repair. This strategy leads to increased vascular density and improved heart function. This clinical related strategy is promising for patients suffering from myocardial infarction.

Sources of Support Funding

National Medical Research Council Grant No. R-176-000-042-213.

REFERENCES

- Rosengart TK, Lee LY, Patel SR, Sanborn TA, Parikh M, Bergman GW, et al. Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease. *Circulation* 1999; 100:468-74.
- Moore MA, Hattori K, Heissig B, Shieh JH, Dias S, Crystal RG, et al. Mobilization of endothelial and hematopoietic stem and progenitor cells by adenovector-mediated elevation of serum levels of SDF-1, VEGF, and Ang-1. *Ann N Y Acad Sci* 2001; 938:36-45.
- Kornowski R, Fuchs S, Leon MB, Epstein SE. Delivery strategies to achieve therapeutic myocardial angiogenesis. *Circulation* 2000; 101:454-8.
- Suzuki K, Murtuza B, Smolenski RT, Sammut IA, Suzuki N, Kaneda Y, et al. Cell transplantation for the treatment of acute myocardial infarction using vascular endothelial growth factor-expressing skeletal myoblast. *Circulation* 2001; 104[suppl1]:I207-I212.
- Pouzet B, Vilquin JT, Hagege A, Scorcin M, Messas E, Fiszman M, et al. Factors effecting functional outcome of autologous skeletal myoblast transplantation. *Ann Thorac Surg* 2001; 71:844-51.
- Lopez JJ, Laham RJ, Stamler A, Pearlman JD, Bunting S, Kaplan A, et al. VEGF administration in chronic myocardial ischemia in pigs. *Cardiovasc Res* 1998; 40:272-81.
- Lazarous DF, Shou M, Scheinowitz M, Hodge E, Thirumurti V, Kitsiou AN, et al. Comparative effects of basic fibroblast growth factor and vascular endothelial growth factor on coronary collateral development and the arterial response to injury. *Circulation* 1996; 96:1074-82.