

## NF- $\kappa$ B Plays a Protective role in Nitric Oxide-induced Neuronal Apoptosis

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### Introduction

The physiological signalling molecule, nitric oxide (NO), plays an important role in numerous functions in cells. However, excessive excitatory neurotransmission can result in its over-production, and accumulating evidence suggests a role of NO in neuronal apoptosis, which contributes to many neurological disorders, including stroke, seizure and Alzheimer's disease.

NF- $\kappa$ B is a ubiquitous transcription factor that participates in many neuronal processes, including synaptic transmission, neural development and neurodegenerative diseases. Recent data revealed that NF- $\kappa$ B can have anti-apoptotic or pro-apoptotic roles under different circumstances. In NO-induced neuronal apoptosis, NF- $\kappa$ B was activated, however, its significance was still unclear. Here, we demonstrated that NF- $\kappa$ B plays a protective role against NO-induced apoptosis in human neuroblastoma cells. The protective mechanism may involve either a delay or inhibition of cytochrome C release from the mitochondria into the cytosol.

### Materials and Methods

**Cell culture:** Human SHEP-1 neuroblastoma cells were maintained in Dulbecco's modified Eagles's medium containing 10% fetal calf serum, 100 units/mL penicillin, and 100 ug/mL streptomycin. LipofectAMINE was used in all transfections, employing the protocol provided by Life Technologies, Inc. Transformed clones were selected and maintained with 500 ug/mL G418.

**Cell death assay:** Different concentrations of the NO donor, sodium nitroprusside (SNP) was added to the medium for 16 to 24 hours. Cell death was measured by crystal violet staining.

**Preparation of cytosolic and mitochondrial proteins:** Wildtype and stably transformed SHEP-1 cells were harvested and washed in ice-cold phosphate-buffered saline. To isolate cytosolic proteins, cells were homogenised in a dounce homogeniser in cold buffer-1 (10mM Tris-HCl pH=7.5, 0.3M sucrose, complete protease inhibitor cocktail). After centrifugation at 10,000xg for 60 minutes at 4°C, the supernatant was collected and designated as the cytosolic protein fraction. The cell pellet was resuspended in cold Buffer-2 (10 mM Tris-HCl pH = 7.5, 1% Triton X-100, 150 mM NaCl, complete protease inhibitor cocktail) and sonicated using a Ultra sonicator. Following centrifugation at 10,000xg for 30 minutes at 4°C, the supernatant was collected and designated as the mitochondrial protein fraction.

**Western blot:** Twenty micrograms of cytosol and mitochondria proteins were electrophoresed in 14% polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblotting was performed with antibodies diluted in phosphate-buffered saline (PBS) containing 0.2% Tween 20 and 5% bovine serum albumin (BSA). After several washing steps, the membrane was probed with a horseradish peroxidase-conjugated secondary antiserum. This was followed by further washing and chemiluminescence detection of antibody binding.

**Immunohistochemistry:** SHEP-1 cells were seeded on chamber slides and treated with insulin growth factor- I (IGF-1) for 1 to 6 hours. This was followed by fixation of the cells with 1%

paraformaldehyde and blocking with 5% BSA. Fixed cells were incubated with an antibody against NF- $\kappa$ B (P65) for 1 hour. After several washes with PBS, cells were incubated with FITC conjugated secondary antibody. The staining results were visualised using a fluorescence microscope.

### Results

*NF- $\kappa$ B is activated by NO and plays a protective role in neuronal apoptosis induced by NO*

The generation of NO in cells can be mimicked with chemical donors of NO. The NO donor, SNP, releases NO intracellularly, which induces apoptosis of neuroblastoma cells partly via oxidative stress. Our previous work has demonstrated that 0.5-2 mM SNP can effectively kill neuroblastoma cells after 8 hours of treatment. The transcription factor NF- $\kappa$ B is activated prior to cell death, at 1 to 4 hours post treatment. To investigate the significance of NF- $\kappa$ B activation, we generated SHEP-1 cell lines expressing I- $\kappa$ B $\alpha$ M, a dominant negative mutant of I- $\kappa$ B $\alpha$ . I- $\kappa$ B $\alpha$ M prevents the release of NF- $\kappa$ B from its associated complex in the cytoplasm and therefore inhibits NF- $\kappa$ B translocation to the nucleus. With NF- $\kappa$ B activation blocked, I- $\kappa$ B $\alpha$ M cells show markedly increased sensitivity to apoptosis induced by SNP. Similar results were obtained with another I- $\kappa$ B $\alpha$ M expressing clone. Therefore, our data indicates that NO stimulated NF- $\kappa$ B activation protects SHEP-1 cells from apoptosis.

*NF- $\kappa$ B may help to stabilise the mitochondria*

NO has been demonstrated to induce cytochrome C release, which in turn trigger a series of events that ultimately leads to apoptosis. To analyse the mechanism by which NF- $\kappa$ B protects SHEP-1 cells against NO-induced apoptosis, we measured the release of cytochrome C from the mitochondria. Cytosolic proteins were isolated from wildtype and I- $\kappa$ B $\alpha$ M cells treated with 1mM SNP. Western blot was performed using a cytochrome C specific antibody. Interestingly, the results reveal cytochrome C to be clearly present in the cytosol of I- $\kappa$ B $\alpha$ M cells, but in significantly lesser amounts in wildtype cells. This suggests NF- $\kappa$ B activation in wildtype cells can prevent cytochrome C release from mitochondria. Since cytochrome C release plays an important role in the execution phase of apoptosis, NF- $\kappa$ B may, via blocking cytochrome C release, protects SHEP-1 cells from cell death.

*NF- $\kappa$ B is required for neuronal protection by insulin-like growth factor I*

To translate our results into therapeutic application, a search was done for factors with the potential of activating NF- $\kappa$ B and at the same time, protect neurons from apoptosis. IGF-1 has been shown to be capable of causing NF- $\kappa$ B activation, and to protect against cell death induced by various apoptotic stimuli. To determine whether IGF-1 can activate NF- $\kappa$ B and protect SHEP-1 cells from NO-induced apoptosis, NF- $\kappa$ B activation was measured by nuclear translocation of its p65 subunit. Consistent with our hypothesis, IGF-1 prevented cell death caused by NO, and we observed p65 immunofluorescence in the nuclei of IGF-1 treated SHEP-1 cells. In contrast, IGF-1 loses its protective effects in I- $\kappa$ B $\alpha$ M cells where the

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activity of NF- $\kappa$ B was blocked. These results demonstrated that IGF-1 can protect neurons from NO-induced apoptosis and this neuroprotection is dependent on NF- $\kappa$ B activation.

#### Conclusion

Our study demonstrated a central role of NF- $\kappa$ B in protecting neurons against the oxidative stress initiated by NO. NF- $\kappa$ B is also

required for the neuroprotection mediated by IGF-1 application. The mechanism probably involves NF- $\kappa$ B activation of a signalling pathway that stabilises the mitochondria, and thus prevents cytochrome C release into the cytosol. The results we obtain will be useful in helping to design a therapeutic strategy for various neurological disorders in the future.

## Best Oral Presentation Award Finalist – Medicine/Paediatrics

# Molecular Monitoring of Minimal Residual Disease in Childhood Acute Lymphoblastic Leukaemia Using Antigen Receptor Gene Rearrangements is Highly Feasible for Disease Stratification and Prognostication

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#### Introduction

Acute lymphoblastic leukaemia (ALL) is the most common form of childhood malignancy in Singapore. Despite cure rates of over 60%, relapse remains the most important cause of death. Treatment intensification reduces the risk of relapse but increases toxicity. Further improvement in cure can only be achieved through careful tailoring of the intensity of treatment to fit the patient's risk of relapse in order to achieve the optimal risk-benefit ratio.

To accomplish this, we need to accurately predict each patient's risk of relapse because the best chance for a cure is to deliver the correct intensity of therapy at initial diagnosis. Unfortunately, conventional risk stratification is based on presenting leukocyte count and age, which are imperfect predictors of outcome, giving risk-stratified therapy a low success rate. The patient's early response to therapy is the best indicator of treatment outcome as it reflects the intrinsic sensitivity of leukaemic blasts to therapy.<sup>1-3</sup> Conventional microscopy is subjective, with a low detection sensitivity of only 5 leukaemic cells in a background of 100 normal cells, below which patients are deemed to be in complete clinical remission. Leukaemic blasts below that limit are termed 'minimal residual disease' (MRD).<sup>2,4</sup> Using new molecular techniques, we are able to detect and accurately quantitate MRD levels as low as 10<sup>-5</sup>, a 3-log improvement over conventional morphology.<sup>1,3,5,6</sup> This provides an unprecedented ability to closely track a patient's response to therapy.

We proved previously that early response to therapy, as determined by MRD at 1 month of therapy, is a statistically significant predictive factor (Fig. 1), using Southern blotting and immunoglobulin heavy chain (IgH) rearrangements.<sup>7</sup> ALL arises from clones of a single lymphoblast, thus we utilised the unique junctional regions resulting from random insertion and deletion of nucleotides.<sup>8,9</sup> Southern blotting is hampered by its labour-intensiveness, and we were able to find an MRD marker for only 60% of precursor-B ALL patients, severely limiting its widespread use.

Timeliness in delivery of a patient's risk status for treatment modification is necessary, and the ability to use it on the majority of patients will determine the viability of MRD as an important prognostic factor. This study reports our turnover time for MRD reports and their applicability. The new real-time quantitative polymerase chain reaction (RQ-PCR) technology measures the product formed as the reaction progresses, removing laborious post-PCR Southern blotting. This reduces the risk of contamination and eliminates the use of radioactive reagents, making it a more rapid, more accurate and safer platform.<sup>9</sup> To increase the number of informative MRD markers obtained, we investigated the feasibility of using other antigen receptors like immunoglobulin kappa deleting element (IgKde), T-cell receptor (TCR) gamma and delta gene rearrangements as targets for MRD monitoring.

#### Materials and Methods

##### Patients and Cell Samples

All newly diagnosed patients (n = 48) enrolled in the Malaysia-Singapore (MA-SPORE) ALL-2002 clinical trial and 6 relapse patients were included in this study (Table I). Week 5 samples were available from 34 patients; week 12 samples were available from 16 patients.

##### Detection of Gene Rearrangements

PCR amplification for IgH, IgK and TCR was carried out using primers and conditions as described previously.<sup>10</sup> Eleven separate PCRs and heteroduplex analysis were performed for each patient. The PCR products were electrophoresed on a 6% non-denaturing polyacrylamide gel. Distinct bands were excised, precipitated, sequenced and compared against human germline sequences (<http://www.ncbi.nlm.gov/BLAST/>).

##### RQ-PCR

Patient-specific primers were designed from the unique junctional regions and paired with a germline Taqman reverse probe.<sup>11-13</sup>

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TABLE I: PATIENT CHARACTERISTICS

|                  |                                                     |
|------------------|-----------------------------------------------------|
| Total number     | 54                                                  |
| De novo ALL      | 48                                                  |
| Relapse ALL      | 6                                                   |
| Precursor-B ALL  | 51                                                  |
| T-lineage ALL    | 3                                                   |
| Mean age (range) | 6 years 1 month<br>(7 months to 14 years 10 months) |

TABLE II: FREQUENCY OF GENE REARRANGEMENTS

|                  | IgH             | TCRG          | TCRD            | IgK             |
|------------------|-----------------|---------------|-----------------|-----------------|
| Pre-B ALL (n=51) | 89.8% (n=46)    | 77.5% (n=39)  | 63.2% (n=32)    | 61.2% (n=31)    |
| Oligoclonal      | 63.3%           | 53.1%         | ND <sup>b</sup> | 40%             |
| T-cell ALL (n=3) | ND <sup>a</sup> | 66.7% (n=2)   | 100% (n=3)      | ND <sup>a</sup> |
| Overall          | 89.8% (46/51)   | 75.9% (41/54) | 64.8% (35/54)   | 61.2% (31/51)   |

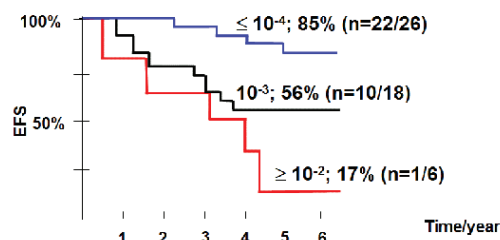
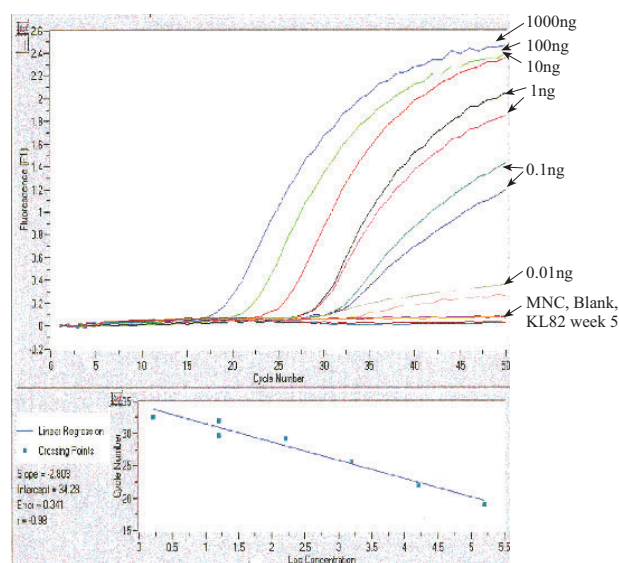
<sup>a</sup> ND, not done, as cross-lineage gene rearrangements are rare in IgH and IgK.<sup>9,15</sup><sup>b</sup> Monoclonality analysis not done as only one panel screened.Fig. 1. MRD levels at 1 month of induction therapy is highly predictive of eventual outcome ( $p < 0.05$ ).<sup>7</sup>

Fig. 2. A typical standard curve and week 5 sample of patient KL82. Standard curves were constructed using 10-fold dilutions of the patient's diagnostic marrow into mononuclear cells.

Diagnostic DNA samples were serially diluted 10-fold in pooled normal MNC to simulate the conditions of a remission marrow and standard curves constructed (Fig. 2). Water and MNC were used as negative controls, and the quality and quantity of the patients' DNA normalised using the albumin gene.<sup>12,13</sup>

#### End-point Measurements

Reports for provisional and final risk group stratifications must be ready by weeks 10 and 14 respectively for marrows done on weeks

5 and 12 to enable changes in treatment protocol. Our stratification method requires at least one marker of sensitivity of  $10^{-4}$ .

## Results

### Gene Rearrangements

IgH gene rearrangements have the highest frequency but suffer from the highest rate of oligoclonality, while IgK rearrangements have the most monoclones (Table II). All patients had at least one gene rearrangement using our panel of markers, with 94.4% (51/54) exhibiting 2 or more different gene rearrangements.

As at least one marker of sensitivity  $10^{-4}$  is required, 53 of the 54 patients (98.1%; 47/48 newly diagnosed, 6/6 relapse) can be stratified according to our protocol. The levels of sensitivity and reproducibility achieved by markers from different gene rearrangements (chi-square test) showed no significant difference ( $p > 0.05$ ). MRD reports were delivered in time for review of treatment for all patients who completed week 5 and week 12 therapy.

### Risk Group Stratification

MA-SPORE ALL-2002 protocol stratifies patients at week 5 and week 12 (Fig. 3). Of the 34 remission marrows obtained at week 5, 18 (52.9%) were classified as standard risk, 12 (35.3%) as intermediate risk and 4 (11.8%) as high risk. All 4 high-risk patients were stratified thus because of the presence of high-risk oncogene fusions. At week 12, no additional patients were upgraded to a higher risk group.

To study the concordance between MRD levels determined by 2 different markers, 32 marrows from 16 patients were compared. 90.6% (13/16) were stratified into the same risk group, with only 3 discordants. Patient KL32 was initially stratified as intermediate risk based on the IgH marker which failed to reach a sensitivity of  $10^{-4}$ , but his TCRG marker showed clearance below  $10^{-4}$ , indicating standard risk. For patient KL76, the MRD between the two markers

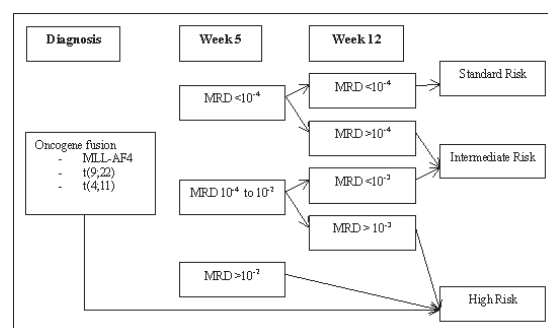


Fig. 3. Stratification of patients under MA-SPORE ALL-2003.

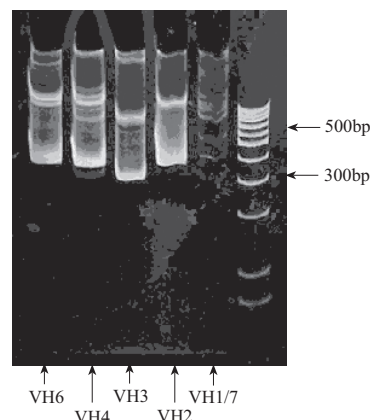


Fig. 4. Oligoclonal IgH gene rearrangements for patient D212.

TABLE III: SENSITIVITY OF THE DIFFERENT TYPES OF GENE REARRANGEMENTS USING RQ-PCR

| Type of gene rearrangement | Limit of detection | Maximal sensitivity <sup>a</sup> |
|----------------------------|--------------------|----------------------------------|
| IgH (n = 29)               | <10 <sup>-4</sup>  | 89.7% (26/29)                    |
| TCRG (n = 10)              | <10 <sup>-4</sup>  | 80% (8/10)                       |
| TCRD (n = 6)               | <10 <sup>-4</sup>  | 100%                             |
| IgK (n = 3)                | <10 <sup>-4</sup>  | 66.7% (2/3)                      |

<sup>a</sup> The maximal level of sensitivity was defined as the maximal dilution with at least 1 replicate amplifying.<sup>17</sup> When non-specific amplification was observed in MNC, only amplification at least 3 cycles from that of MNC was considered.

differed by one-log, which is within the standard error for MRD,<sup>14</sup> but this difference resulted in different risk group classifications. Finally, clonal evolution caused the IgH markers of patient D212 to give false-negative results.

### Discussion

We previously reported that MRD using a single IgH marker is an exceptionally powerful predictor of clinical outcome ( $p = 0.03$ ).<sup>7</sup> However, only 60% of patients could be stratified, limiting its applicability. By expanding our screening to include IgK and TCR gene rearrangements, we were able to substantially increase the percentage of patients for whom an informative marker could be obtained, from 60% to 98% ( $p < 0.05$ ), including previously excluded T-cell ALL patients. The distribution of risk groups was similar to our previous study ( $p > 0.05$ ).

This is an ongoing study where our protocol is to screen for a marker from IgH first, followed by TCR and IgK. Hence, of the 48 markers used for stratification, 60% were from IgH, compared with 20% or less for the other gene rearrangements (Table III). However, the highest rate of oligoclonality was also observed in IgH (63.3% vs 53.1% vs 40% for IgH, TCRG and IgK, respectively). Oligoclonal gene rearrangements are more unstable and prone to secondary rearrangement, possibly leading to false-negative MRD results.<sup>12,15</sup> This was illustrated by patient D212, who exhibited IgH oligoclonality (Fig. 4), and clonal evolution occurred where 2 IgH markers were lost by week 5, giving a false-negative MRD level. Confirmation by a TCRG marker, however, detected MRD at intermediate risk level.

The AIEOP-BFM-ALL 2000 study requires 2 markers of sensitivity at least 10<sup>-4</sup> for risk group stratification to avoid inaccurate results due to clonal evolution. Unfortunately, this stringent requirement makes it possible for the group to deliver results on time for only 67% of patients. Our study differs from the AIEOP-BFM group in that we require a minimum of 1 stable marker for MRD monitoring.

It has been widely reported that the stability of MRD targets is inversely proportional to oligoclonality, particularly for IgH.<sup>16</sup> As we continue to screen for markers, we will prospectively explore if 1 marker is comparable to a combination of 2 markers as long as care is taken to avoid clonal evolution. If we can prove the validity of this simplified method, which is applicable to 98% of patients compared to only 67% under the AIEOP-BFM protocol, it will greatly improve the widespread application of our stratification protocol.

By using RQ-PCR, we were able to reach very high levels of sensitivity of 10<sup>-4</sup> or more for 98% of patients, a considerable improvement over conventional techniques. As there is no significant difference between the different antigen receptor markers in terms of limit of detection and reproducibility, IgH, IgK $\delta$ , TCR gamma and delta gene rearrangements are all suitable targets for molecular monitoring of MRD.

### Conclusion

Conventional risk stratification uses presenting leukocyte count and age which are imperfect risk factors, limiting its usefulness. We showed that molecular monitoring of MRD in childhood ALL is

feasible and can be applied to 98% of patients in a timely manner. With its high predictive value, MRD measurement could replace conventional risk stratification methods. Using RQ-PCR, precise quantitative monitoring of MRD with antigen receptor gene rearrangements is both sensitive (up to 3-log improvement) and highly reproducible. We have shown that in 98% of childhood ALL patients, this method can be used to tailor therapy to each child, enabling a reduction in both cost of therapy and treatment-related toxicity while maintaining an optimal anti-leukaemic effect.

### REFERENCES

1. Rubnitz JE, Pui CH. Recent advances in the treatment and understanding of childhood acute lymphoblastic leukaemia. *Cancer Treat Rev* 2003; 20:31-44.
2. Scrideli CA, Queiroz RG, Bernardes JE, Valera ET, Tone LG. PCR detection of clonal IgH and TCR gene rearrangements at the end of induction as a non-remission criterion in children with ALL: comparison with standard morphologic analysis and risk group classification. *Med Pediatr Oncol* 2003; 41:10-6.
3. Van Dongen JJM, Seriu T, Panzer-Grumayer ER, Biondi A, Pongers-Willemsse MJ, Corral L, et al. Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. *Lancet* 1998; 352:1731-8.
4. 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 leukemia. *J Pediatr Hematol Oncol* 2003; 25:103-8.
5. Cavé H, Bosch JW, Suciu S, Guidal C, Waterkeyn C, Otten J, et al. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia. *N Engl J Med* 1998; 339:591-8.
6. Neale GAM, Campana D, Pui CH. Minimal residual disease detection in acute lymphoblastic leukemia: real improvement with the real-time quantitative PCR method? *J Pediatr Hematol Oncol* 2003; 25:100-2.
7. 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 1<sup>st</sup> Annual Scientific Congress; 2002 Aug 17-18; Singapore.
8. Cazzaniga G, d'Aniello E, Corral L, Biondi A. Results of minimal residual disease (MRD) evaluation and MRD-based treatment stratification in childhood ALL. *Best Pract Res Clin Hematol* 2002; 15:623-38.
9. Szczepański T, Flohr T, van der Velden VHJ, Bartram CR, van Dongen JJM. Molecular monitoring of residual disease using antigen receptor genes in childhood acute lymphoblastic leukaemia. *Best Pract Res Clin Hematol* 2002; 15:37-57.
10. Pongers-Willemsse MK, Seriu T, Stolz F, d'Aniello E, Gameiro P, Pisa P, et al. Primers and protocols for standardized detection of minimal residual disease in acute lymphoblastic leukemia using immunoglobulin and T cell receptor gene rearrangements and TAL1 deletions as PCR targets. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. *Leukemia* 1999; 13:110-8.
11. Verhagen O, Willemsse M, Breunis W, Jacobs D, Joosten S, van Wering E, et al. Application of germline IgH probes in real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia. *Leukemia* 2000; 14:1426-35.
12. Van der Welden VHJ, Willemsse MJ, van der Schoot CE, van Wering ER, van Dongen JJM. Immunoglobulin kappa deleting element rearrangements in precursor-B-acute lymphoblastic leukemia are stable targets for detection of minimal residual disease by real-time quantitative PCR. *Leukemia* 2002; 16:928-36.
13. Van der Velden VHJ, Wijkhuijs JM, Jacobs DCH, van Wering ER, van Dongen JJM. T cell receptor gamma gene rearrangements as targets for detection of minimal residual disease in acute lymphoblastic leukemia by real-time quantitative PCR analysis. *Leukemia* 2002; 16:1372-80.
14. Van der Velden VHJ, Hochhaus A, Cazzaniga G, Szczepański T, Gabert J, van Dongen JJM. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. *Leukemia* 2003; 17:1013-34.
15. Szczepański T, Beishuizen A, Pongers-Willemsse MJ, Hählen K, van Wering ER, Wijkhuijs AJM, et al. Cross-lineage T cell receptor gene rearrangements occur in more than ninety percent of childhood precursor-B acute lymphoblastic leukemias: alternative PCR targets for detection of minimal residual disease. *Leukemia* 1999; 13:196-205.
16. Szczepański T, Willemsse MJ, Brinkhof B, van Wering ER, van der Burg M, van Dongen JJM. Comparative analysis of Ig and TCR gene rearrangements at diagnosis and at relapse of childhood precursor-B-ALL provides improved strategies for selection of stable PCR targets for monitoring of minimal residual disease. *Blood* 2002; 99:2315-23.

# Clinical Features and Predictors for Mortality in a Designated National SARS ICU in Singapore

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## Introduction

Severe acute respiratory syndrome (SARS) is a new disease attributed to a novel coronavirus (SARS-CoV).<sup>1,2</sup> It began in November 2002 in Guangdong Province in southern China.<sup>3</sup> The World Health Organisation (WHO) issued a global alert on this contagious disease on 12 March 2003. In Singapore, the first index case was hospitalised on 1 March 2003, which subsequently led to an outbreak of 206 probable SARS patients.<sup>4</sup>

On 22 March 2003, the Ministry of Health (Singapore) designated Tan Tock Seng Hospital (TTSH) as the only hospital for isolation and management of all SARS patients.<sup>5</sup> In other countries, e.g., Hong Kong and Canada, SARS patients were managed in several hospitals.<sup>6-9</sup> In TTSH, we have the unique opportunity of reviewing the clinical features and outcomes of critically ill probable SARS patients in a national designated SARS intensive care unit (ICU).

## Materials and Methods

From 1 March to 9 June 2003, 206 probable SARS patients were diagnosed in Singapore. Of the 7 probable SARS patients that were not managed in TTSH, 2 ICU patients were too ill for transfer, 2 were diagnosed by autopsy in out-of-hospital deaths, 1 died in the general ward in another hospital and 2 were discharged from a third hospital. Forty-six (23.1%) of 199 patients treated in TTSH required ICU admission. We retrospectively reviewed all the 46 probable SARS patients admitted to TTSH's ICU during this study period.

Probable SARS patients were identified using WHO's criteria current at that time.<sup>10</sup> Patients were referred to the ICU if they developed progressive respiratory failure or required at least 50% O<sub>2</sub> in inspired air to maintain SpO<sub>2</sub> ≥ 92%. Patients were intubated if they were deteriorating clinically and/or could not maintain SpO<sub>2</sub> ≥ 90% while breathing 100% O<sub>2</sub> via a face mask.

## Statistical Analysis

Survival time was defined as the interval between onset of illness and death or censoring as of 9 June 2003. The following risk factors for mortality were studied: sex, age, race, co-morbidities, PaO<sub>2</sub>/FiO<sub>2</sub> ratio on admission to ICU, lowest PaO<sub>2</sub>/FiO<sub>2</sub> ratio, Acute Physiology and Chronic Health Evaluation (APACHE) II score, highest serum lactate dehydrogenase (LDH), lowest serum sodium, lowest serum potassium, lowest absolute lymphocyte count (ALC), highest total white count (TWC), highest prothrombin time (PT) and highest activated partial thromboplastin time (PTT).

It was observed that three-quarters of ICU deaths occurred on day 6 or later of ICU admission. Therefore, the relevant laboratory measurements within the first 5 days of admission to ICU were used in the analysis for prognostic factors. Univariate Cox regression models were fitted for each of the variables.

Starting from the most significant, variables that were significant in the univariate analysis were included in the multivariate model in

turn. The log-likelihood ratio test was used to test whether inclusion of a new covariate helped improve the fit of the model. The adjusted hazard ratios and their confidence intervals were provided for the final model.

The Nelson-Aalen cumulative hazard plot (on the log scale) was used to graphically examine the proportional hazard assumption. The Schoenfeld test was used to formally ascertain if the assumption of proportional hazards was violated for the final model. Data analysis was done in Stata (V6.0) and level of significance was set at 5%.

## Results

The demographic and clinical characteristics of 46 critically ill probable SARS patients are shown in Table I. All the patients presented with fever; the other common symptoms were shortness of breath (89.1%) and cough (80.4%) (Table II). The list of co-morbidities is tabulated in Table III. The laboratory values within the first 5 days of ICU admission are shown in Table IV. Significant morbidity (Table V) and in-ICU of 52.2% (24/46) were observed.

The survival experience of the entire SARS ICU cohort is shown in Figure 1. Table VI highlights the univariate predictors for mortality. The hazard ratio was 2.6 (95% CI: 1.1 to 6.2) among male as compared to female ( $p = 0.027$ ). The hazard decreased by 0.98 (95% CI: 0.97 to 0.99) for every unit increase in PaO<sub>2</sub>/FiO<sub>2</sub> ratio ( $p = 0.012$ ). Lowest PaO<sub>2</sub>/FiO<sub>2</sub> ratio, APACHE II score, presence of congestive heart failure (CCF), cerebrovascular accident (CVA) and peptic ulcer were also found to be statistically significant.

In the multivariate analysis, only sex, APACHE II score and presence of CCF were found to be independently related to mortality (Table VII). Likelihood ratio tests indicated that none of the other predictors that were significant at the univariate analysis were significant, after adjustment for these covariates. The hazard ratio was 3.2 (95% CI: 1.2 to 8.4) among male as compared to female, after adjusting for APACHE II score and presence of CCF. The hazard

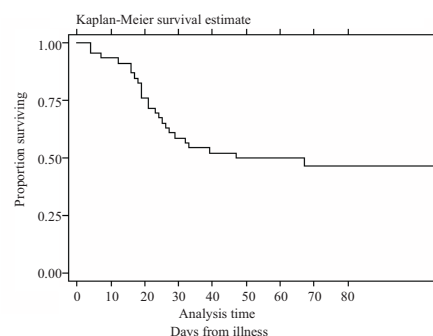


Fig. 1. Kaplan-Meier survival experience.

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TABLE I: DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF ICU PATIENTS (n = 46)

| Variables                                                         |              |
|-------------------------------------------------------------------|--------------|
| No. of males (%)                                                  | 24 (52.2)    |
| Age (y)                                                           |              |
| Mean (SD)                                                         | 49.8 ± 16.4  |
| Race [n (%)]                                                      |              |
| Chinese                                                           | 34 (73.9)    |
| Malay                                                             | 6 (13.0)     |
| Indian                                                            | 3 (6.5)      |
| Others                                                            | 3 (6.5)      |
| No. intubated (%)                                                 | 39 (84.8)    |
| APACHE II                                                         |              |
| Mean ± SD                                                         | 19.2 ± 9.6   |
| Length of ventilation (days)                                      |              |
| Median (IQR)                                                      | 12 (9-22)    |
| PaO <sub>2</sub> /FiO <sub>2</sub> ratio on ICU admission (mm Hg) |              |
| Median (IQR)                                                      | 88 (60-128)  |
| Lowest PaO <sub>2</sub> /FiO <sub>2</sub> ratio (mm Hg)           |              |
| Median (IQR)                                                      | 59.5 (44-95) |
| Days from symptom onset to hospitalisation                        |              |
| Median (IQR)                                                      | 5 (2-6)      |
| Days from symptom onset to ICU admission                          |              |
| Median (IQR)                                                      | 8 (6-10)     |
| Length of stay in hospital                                        |              |
| Median (IQR)                                                      | 23.5 (15-36) |
| Length of stay in ICU                                             |              |
| Median (IQR)                                                      | 14.5 (7-22)  |

TABLE II: SYMPTOMS EXPERIENCED BY ICU COHORT

| Symptom             | n  | (%)     |
|---------------------|----|---------|
| Fever               | 46 | (100.0) |
| Shortness of breath | 41 | (89.1)  |
| Cough               | 37 | (80.4)  |
| Myalgia             | 19 | (41.3)  |
| Diarrhoea           | 16 | (34.8)  |
| Malaise             | 15 | (32.6)  |
| Nausea/vomiting     | 14 | (30.4)  |
| Headache            | 12 | (26.1)  |
| Sore throat         | 9  | (19.6)  |
| Anorexia            | 6  | (13.0)  |

TABLE III: LIST OF CO-MORBIDITIES

| Co-morbidity               | n  | (%)    |
|----------------------------|----|--------|
| Asthma                     | 2  | (4.4)  |
| Diabetes mellitus          | 7  | (15.2) |
| Ischaemic heart disease    | 6  | (13.0) |
| Hypertension               | 14 | (30.4) |
| Congestive cardiac failure | 3  | (6.5)  |
| Chronic renal failure      | 3  | (6.5)  |
| Cerebrovascular accident   | 1  | (2.2)  |
| Peptic ulcer               | 4  | (8.7)  |
| Others                     | 22 | (47.8) |

ratios were 5.6 (95% CI: 1.7 to 18.1) and 9.5 (95% CI: 2.0 to 46.4) among those with APACHE II scores 15 to 30 and >30, as compared to those with a score of <15, after adjusting for sex and presence of CCF. The hazard ratio among patients with CCF was 21.7 (95% CI: 1.7-274.7) as compared to those without CCF, after adjusting for sex and APACHE II score. The Schoenfeld's test indicated that the assumption of proportional hazards was not violated for the overall model ( $p = 0.627$ ). The assumption was also not violated for each of the covariate in the model.

## Discussion

This coronavirus has no gender or racial predilection. Critically ill patients tend to be in their late middle age and required ICU admission in the second week of illness. All the patients required ICU admission because of respiratory failure, with median (inter-quartile range, [IQR]) PaO<sub>2</sub>/FiO<sub>2</sub> of 88 (60 to 128) mm Hg. Eighty five percent of our ICU patients required intubation for mechanical

TABLE IV: LABORATORY VALUES WITHIN FIRST 5 DAYS OF ICU ADMISSION

| Laboratory values                  | Mean ± SD      |
|------------------------------------|----------------|
| Highest serum LDH (U/L)            | 1429.4 ± 515.9 |
| Lowest serum sodium (mmol/L)       | 131.20 ± 4.07  |
| Lowest serum potassium (mmol/L)    | 3.4 ± 0.5      |
| Lowest ALC (uL)                    | 445.9 ± 186.0  |
| Highest TWC (x 10 <sup>9</sup> /L) | 12.1 ± 3.5     |
| Highest PTT (s)                    | 50.9 ± 18.4    |
| Highest PT (s)                     | 14.9 ± 2.2     |

TABLE V: LIST OF COMPLICATIONS

| Complications            | n (%)     | 95% CI      |
|--------------------------|-----------|-------------|
| Septicaemia              | 12 (26.1) | (14.3-41.1) |
| Secondary pneumonia      | 24 (52.2) | (36.9-67.1) |
| Pneumothorax             | 8 (17.4)  | (7.8-31.4)  |
| Pulmonary embolism       | 7 (15.2)  | (6.3-28.9)  |
| Deep vein thrombosis     | 11 (23.9) | (12.6-38.8) |
| Cerebrovascular accident | 4 (8.7)   | (2.4-20.8)  |
| Acute renal failure      | 9 (19.6)  | (9.4-33.9)  |
| Pneumomediastinum        | 1 (2.2)   | (0.06-11.5) |

95% exact confidence interval from binomial distribution

TABLE VI: UNIVARIATE PREDICTORS OF MORTALITY

| Covariates                                                   | Hazard ratio | 95% CI         | p value |
|--------------------------------------------------------------|--------------|----------------|---------|
| Sex (Male)                                                   | 2.63         | (1.12, 6.18)   | 0.027   |
| Age (y)                                                      | 1.02         | (0.99, 1.05)   | 0.117   |
| Race                                                         |              |                |         |
| Chinese                                                      | 1            |                |         |
| Malay                                                        | 0.83         | (0.24, 2.82)   | 0.762   |
| Indian                                                       | 1.02         | (0.24, 4.39)   | 0.981   |
| Others                                                       | 0.47         | (0.06, 3.52)   | 0.462   |
| PaO <sub>2</sub> /FiO <sub>2</sub> ratio on admission to ICU | 0.98         | (0.97, 0.99)   | 0.012   |
| Lowest PaO <sub>2</sub> /FiO <sub>2</sub> ratio              | 0.99         | (0.97, 0.99)   | 0.040   |
| APACHE II score                                              |              |                |         |
| <15                                                          | 1            |                |         |
| 15-30                                                        | 3.83         | (1.25, 11.72)  | 0.019   |
| >30                                                          | 18.18        | (4.66, 71.00)  | <0.001  |
| Highest serum LDH                                            |              |                |         |
| Up to 1000 (U/L)                                             | 1            |                |         |
| >1000 (U/L)                                                  | 3.33         | (0.98, 11.29)  | 0.053   |
| Lowest serum sodium                                          |              |                |         |
| >136 (mmol/L)                                                | 1            |                |         |
| Up to 136 (mmol/L)                                           | 0.80         | (0.19, 3.41)   | 0.764   |
| Lowest serum potassium                                       |              |                |         |
| >3.5 (mmol/L)                                                | 1            |                |         |
| Up to 3.5 (mmol/L)                                           | 0.73         | (0.33, 1.63)   | 0.444   |
| Lowest ALC (ul)                                              | 1.00         | (0.99, 1.00)   | 0.625   |
| Highest TWC (x10 <sup>9</sup> /L)                            |              |                |         |
| Up to 10                                                     | 1            |                |         |
| >10                                                          | 2.25         | (0.93, 5.45)   | 0.072   |
| Highest PTT (s)                                              |              |                |         |
| Up to 43                                                     | 1            |                |         |
| >43                                                          | 1.57         | (0.62, 3.96)   | 0.339   |
| Highest PT (s)                                               |              |                |         |
| Up to 14.5                                                   | 1            |                |         |
| >14.5                                                        | 1.83         | (0.82, 4.09)   | 0.14    |
| Ischaemic heart disease                                      | 2.40         | (0.89, 6.46)   | 0.083   |
| Hypertension                                                 | 1.44         | (0.63, 3.30)   | 0.385   |
| Congestive cardiac failure                                   | 79.75        | (7.81, 813.9)  | <0.001  |
| Diabetes mellitus                                            | 1.61         | (0.60, 4.32)   | 0.346   |
| Cerebrovascular accident                                     | 14.57        | (1.52, 140.13) | 0.020   |
| Peptic ulcer                                                 | 5.45         | (1.82, 16.26)  | 0.002   |

TABLE VII: MULTIVARIATE PREDICTORS FOR MORTALITY

| Covariates                 | Hazard ratio | 95% CI         | p value |
|----------------------------|--------------|----------------|---------|
| Sex (Male)                 | 3.18         | (1.20, 8.40)   | 0.019   |
| APACHE II score            |              |                |         |
| <15                        | 1            |                |         |
| 15-30                      | 5.60         | (1.73, 18.07)  | 0.004   |
| >30                        | 9.52         | (1.95, 46.44)  | 0.005   |
| Congestive cardiac failure | 21.70        | (1.71, 274.74) | 0.017   |

ventilation.

Reports from Hong Kong,<sup>11-13</sup> Canada<sup>6,9</sup> and Singapore<sup>14</sup> showed that 19% to 32% of SARS patients required ICU admission. About 13.8% to 25.3% of patients were mechanically ventilated.<sup>6,9,11,13,15</sup> Reported mortality ranges from 3.6% (at day 21 of outbreak)<sup>12</sup> to 10.1% (at day 28 after symptom onset).<sup>14</sup>

In-ICU mortality ranges from 37% (at day 28 after symptom onset)<sup>14</sup> to 34.2% (at day 28 after ICU admission).<sup>9</sup> In the ICUs in Toronto, mortality at 8 weeks was 52% for those requiring mechanical ventilation,<sup>9</sup> which is consistent with the mortality rate of ARDS patients requiring mechanical ventilation.<sup>16</sup> In our study, 61.5% (24/39) of ventilated patients died.

The overall mortality for SARS patients was 13.2% for those below 60 years and 43.3% for those aged 60 or older.<sup>11</sup> Data from Hong Kong indicated that advanced age, peak LDH and an initial elevated white cell count may carry a poor prognosis.<sup>12</sup> Unpublished data from our local experience estimated that mortality for patients below 60 years was 8.4%, and for those aged 60 or older was 50%. Age and serum LDH values on admission were predictors for ICU admission for our local SARS patients.

A paper from Toronto showed that diabetes and other co-morbid conditions were independently associated with poor outcome, but age of 60 years older was not.<sup>6</sup> Our study indicated that risk factors for ICU mortality were male gender, APACHE II score  $\geq 15$  and history of congestive cardiac failure.

Although mortality is higher in older patients particularly those with pre-existing co-morbid illness, previously young healthy persons have also succumbed to the disease. Some authors postulated this to higher viral loads or their host hyperimmune response.<sup>17</sup> To control the severe inflammatory damage, corticosteroids alone<sup>18,19</sup> or in combination with immunoglobulin have been attempted, including our centre. Such therapy, if proven to be effective, may be targeted at patients with poor prognostic risk factors.

Comparison of mortality among different cohorts is difficult because of different completeness of follow-up and case definition of SARS.<sup>6</sup> Only 6 (3%) out of 199 in our TTSH cohort remained hospitalised at the end of the study period, compared to 45% and 23% in Hong Kong<sup>12</sup> and Canada<sup>6</sup> respectively. The case fatality rate is approximately 3% to 12%, depending on whether the denominator includes both suspect and probable cases (3%) or probable cases alone (12%).<sup>7,8,12,17</sup>

The WHO case definition for probable SARS was revised on 1 May 2003 to include polymerase chain reaction to detect the virus and/or serology to detect the antibodies as criteria for defining a probable SARS case.<sup>20</sup> Singapore was declared SARS-free by WHO on 31 May 2003. The last SARS patient was discharged from hospital on 13 July 2003. The final overall case fatality in Singapore is 13.7% (33/238), following reclassification of 32 out of 600 suspect cases as probable on the basis of diagnostic laboratory tests.<sup>21,22</sup> The most recent WHO epidemiological record dated 27 June 2003 reported an overall case fatality of 15%.<sup>23</sup>

### Conclusion

Critically ill SARS patients had significant morbidity and mortality. They also consumed much ICU resources. Such information are useful for the clinicians and health administrators in planning for future outbreaks of SARS. Further studies are needed to elucidate the poor prognostic factors so that novel therapy can be targeted in such high-risk patients, to improve the outcomes and reduce the burden of the disease on the health care service.

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### REFERENCES

1. Ksiazek TG, Erdman D, Goldsmith CS, et al. A novel coronavirus associated with severe acute respiratory syndrome. *N Engl J Med* 2003; 348:1953-66.
2. Drosten C, Gunther S, Preiser W, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* 2003; 348:1967-76.
3. Chee YC. Severe acute respiratory syndrome (SARS) – 150 days on. *Ann Acad Med Singapore* 2003; 32:278-80.
4. Hsu LY, Lee CC, Green JA, et al. Severe acute respiratory syndrome (SARS) in Singapore: clinical features of index patient and initial contacts. *Emerg Infect Dis* 2003; 9:713-7.
5. Leo YS, Chen M, Heng BH, et al. Centers for Disease Control and Prevention. Severe acute respiratory syndrome. *MMWR Morb Mort Wkly Rep* 2003; 52:405-11.
6. Booth CM, Matukas LM, Tomlinson GA, et al. Clinical features and short-term outcomes of 144 patients with SARS in the Greater Toronto Area. *JAMA* 2003; 289:2801-9.
7. Poutanen SM, Low DE, Henry B, et al. Identification of severe acute respiratory syndrome in Canada. *N Engl J Med* 2003; 348:1995-2005.
8. Tsang KW, Ho PL, Ooi GC, et al. A cluster of cases of severe acute respiratory syndrome in Hong Kong. *N Engl J Med* 2003; 348:1977-85.
9. Fowler RA, Lapinsky SE, Hallett D, et al. Critically ill patients with severe acute respiratory syndrome. *JAMA* 2003; 290:367-73.
10. World Health Organization. Case definitions for surveillance of severe acute respiratory syndrome (SARS). (Assessed 20 April 2003, at <http://www.who.int/csr/sars/casedefinition/en/>)
11. Donnelly CA, Ghani AC, Leung GM, et al. Epidemiological determinants of spread of causal agent of severe acute respiratory syndrome in Hong Kong. *Lancet* 2003; 361:1761-6.
12. Lee N, Hui D, Wu A, et al. A major outbreak of severe acute respiratory syndrome in Hong Kong. *N Engl J Med* 2003; 348:1986-94.
13. Peiris JSM, Chu CM, Cheng VCC, et al. Clinical progression and viral load in a community outbreak of coronavirus-associated SARS pneumonia: a prospective study. *Lancet* 2003; 361:1767-72.
14. Lew TWK, Kwek TK, Tai DYH, et al. Acute respiratory distress syndrome in critically ill SARS. *JAMA* 2003; 290:374-80.
15. Vu TH, Cabau JF, Nguyen NT, et al. SARS in Northern Vietnam. *N Engl J Med* 2003; 348:2035.
16. Esteban A, Anzueto A, Frutos F, et al. Characteristics and outcomes in adult patients receiving mechanical ventilation: a 28-day international study. *JAMA* 2002; 287:345-55.
17. Lapinsky SE, Hawryluck L. ICU management of severe acute respiratory syndrome. *Intensive Care Med* 2003; 29:870-5.
18. So LKY, Lau ACW, Yam LYC, et al. Development of a standard treatment protocol for severe acute respiratory syndrome. *Lancet* 2003; 361:1615-7.
19. Lee N, Sung J. The use of corticosteroids in SARS. *N Engl J Med* 2003; 348:2034-5.
20. World Health Organization. Case definitions for surveillance of severe acute respiratory syndrome (SARS). (Revised 1 May 2003.) (Accessed 16 Jul 2003, at: <http://www.who.int/csr/sars/casedefinition/en/>)
21. Ministry of Health, Singapore. Singapore SARS-free for 66 days and clear of any smoldering cases. Press release 16 Jul 2003.
22. Khalik S. Hospitals to lift restrictions; last patient out of SARS ward. *The Straits Times* 17 Jul 2003; pg 1 (col 1-3).
23. World Health Organization. Severe acute respiratory syndrome (SARS): over 100 days into the outbreak. *Wkly Epidemic Rec* 2003; 78:2170-228.