Young Investigator's Award Finalists

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Celecoxib Enhances Brain Tumour Cell Radiosensitivity Leading to Massive Tumour Necrosis

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Proteomics of Breast Cancer: Enhanced Expression of CK19 in HER-2/*neu*-positive Tumours

LK Tai,[1]*BSc (Hons)*, DH Zhang,[2]*PhD, MSc, MBA*, LL Wong,[3]*BSc*, ESC Koay,[1,2]*PhD (USA), FRCPath (UK), FAACB (Aust)*

Introduction

Breast cancer development is a complex process which involves complicated and interlinked molecular events caused by the altered expression of both oncogenic and tumour suppressor genes and the related signal pathways. HER-2/*neu* is one of the important oncogenes in breast cancer encoding a transmembrane tyrosine kinase receptor responsible for signalling cascade activation. Its overexpression indicates an adverse prognosis effect with a more aggressive phenotype and shortened overall survival rates.

Overexpression of HER-2/*neu* is also consistently associated with high tumour grade, DNA aneuploidy, high cell proliferation rate, p53 mutation, topoisomerase IIα amplification and alterations of other molecular biomarkers of breast cancer invasiveness and metastasis.[1] However, the biological basis by which HER-2/*neu* overexpression confers the characteristically more aggressive breast tumour proliferation, invasion and metastasis associated with such tumours remains to be elucidated.

In this study, we aim to identify differentially expressed proteins which are potentially involved in the more aggressive phenotype of the subset of breast cancer associated with the HER-2/*neu* abnormality.

Materials and Methods

The breast cancer tissues which had been previously categorised as either HER-2/*neu* positive or HER-2/*neu* negative by both FISH and IHC[2] were obtained from the tumour bank of the National University Hospital of Singapore. Laser capture microdissection (LCM) was used to procure unique cell populations and 2-dimensional gel electrophoresis (2-DE), to separate the proteins between the 2 subtypes of tumours.

The differentially expressed proteins were identified using NCBinr protein database, and Mascot software based on the peptide mass fingerprinting data obtained from MALDI-ToF MS analysis.

Western blot, reverse-phase protein array and immunohistochemistry on tissue microarray sections were performed to further confirm the differential expression levels of the identified proteins in the two tumour subtypes. Semi-quantitative RT-RCR was carried out to validate the differential mRNA transcription.

Results

Seven differentially expressed proteins were unambiguously identified. Five proteins (tropomyosin 3, cytokeratin 19 [CK19], cathepsin D, aldolase A and glyoxalase I) were found to be overexpressed, whereas 2 proteins (MnSOD and serum albumin) were down-regulated in the HER-2/*neu*-positive breast tumours (Fig. 1). CK19 is a structural component of the epithelial cytoskeleton that plays an important role in cell migration and invasion. Its overexpression in the cohort of HER-2/*neu*-positive breast tumours was further analysed by reverse phase protein array and RT-PCR. As shown in Figure 2, the CK19 mRNA level in HER-2/*neu*-positive tumour cells was significantly higher compared to that in HER-2/*neu*- negative cells ($P = 0.036$). Immunohistochemical analysis on
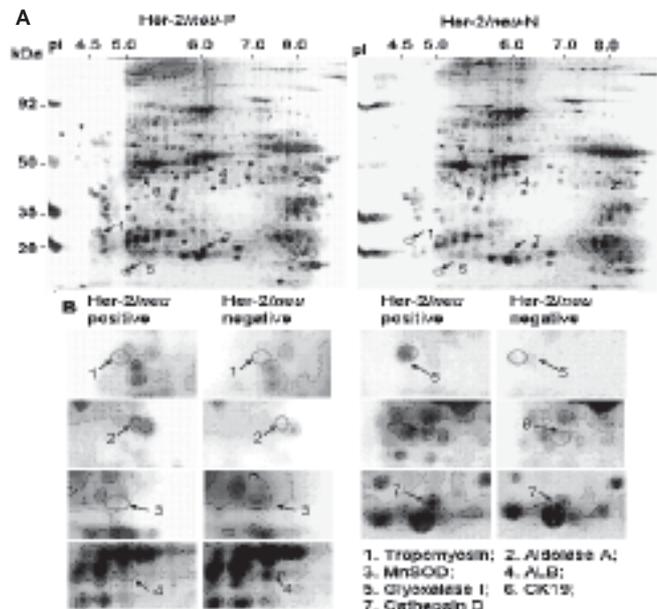

Fig. 1a. 2-D gels derived from laser-microdissected breast tumours. The pooled HER-2/neu-positive and pooled HER-2/neu-negative laser-microdissected cells were lysed and run on IPG DryStrip (pH3-10 NL). Proteins were further separated by SDS-PAGE (8.5%) gels and silver-stained. Seven differentially expressed proteins were identified by MALDI-TOF MS analysis.

Fig. 1b. Enlarged view of the 7 spots differentially expressed between HER-2/neu-positive and -negative breast tumour cells

tissue microarray sections comprising 97 breast tumours revealed that CK-19 was strongly stained in 81.5% of HER-2/*neu*-positive tumours and in 48% of HER-2/*neu*-negative tumours ($P = 0.0043$).

Discussion

The importance of proteomics techniques for clinical and diagnostic cancer research has been repeatedly addressed in the development of potential biomarkers and the documentation of the marked changes in the protein profiles during the various stages of cancer growth, invasion and metastasis. HER-2/*neu* overexpression is strongly associated with a poor prognosis and an increased likelihood of metastasis, and thus identification of the differentially expressed proteins in HER-2/*neu*-dependent breast cancer is crucial to elucidate the mechanisms of tumourigenesis. Using proteomics, we identified 2 structural proteins (tropomyosin 3, CK 19), and 3 cellular metabolic proteins (aldolase A, glyoxalase I, cathepsin D) which were overexpressed in HER-2/*neu*-positive tumours, whereas MnSOD and serum albumin were down-regulated.

Cytokeratins are intermediate filaments whose expression are often altered in epithelial cancer and are involved in the structural organisation of the cells. Differential expression of CK7, CK8, CK17, CK18 and CK19 have been reported in breast cancer and

[1] *Department of Pathology, National University Hospital*

[2] *Department of Pathology, National University of Singapore*

[3] *Oncology Research Institute, National University of Singapore*

Address of Correspondence: Dr Evelyn Koay, Department of Pathology, National University Hospital, 5 Lower Kent Ridge Road, Singapore 119074.

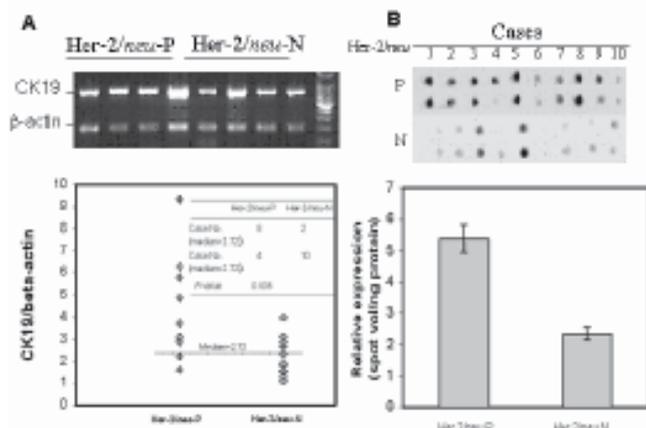*Email: patkoaye@nus.edu.sg*

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Fig. 2a. RT-PCR analysis of CK 19 expression. A representative RT-PCR from 4 HER-2/neu-positive and 4 HER-2/neu-negative tumour cells was shown (top). Median of CK19 expression in 24 different LCM cells was calculated using SPSS 12.0 software and the P-value was analysed by Fisher's exact test (bottom).

Fig. 2b. Reverse-phase protein arrays from 10 HER-2/neu-positive and 10 HER-2/neu-negative laser-microdissected tumour cells. Duplicated samples were manually spotted onto PVDF membrane (top) and arrays were probed with CK19 Ab-1 and signals were detected as described in B. Relative expression of CK19 in each sample was normalised to the signal intensity of β-actin.

other cancers. CK19 expression was found to increase proportionally with the metastatic potency of hepatocellular carcinoma (HCC) cell lines and in relation to poor differentiation.[3] A significantly higher incidence of early tumour recurrence and/or extrahepatic disease was found in patients with CK19-positive HCC than in those with CK19-negative HCC, and was attributed to the increased invasiveness of the former.[4] Serum levels of CYFRA 21-1, a CK 19 fragment, have been used as a tumour marker in a variety of cancers, such as non-small-cell lung cancer,[5] to detect occurrence of metastasis and tumour cell dissemination. In this study, we found that CK19 expression is unambiguously related to the HER-2/*neu* status in breast cancer. Overexpression of CK19 in most HER-2/*neu*-positive breast cancer is likely to increase the probability of metastasis and confer unfavourable prognosis, as found in (HCC) cell lines.[3]

The other proteins we identified, namely, tropomyosin 3, aldolases A, glyoxalase I ,and cathepsin D, are also highly likely candidates implicated in the heightened aggressiveness of HER-2/*neu*-positive breast tumours. Tropomyosin 3 transfection is reported to induce lamellipodial formation, increase cellular migration and reduce stress fibres.[6] Aldolase A is a key enzyme in glucose metabolism where it promotes cell growth when overexpressed. Glyoxalase I

catalyses the detoxification of alpha-oxoaldehydes to corresponding aldonic acids, thus protecting cells from toxic damage and inducing a pro-survival response to chemotherapy. Cathepsin D is a protease involved in protein metabolism and tissue remodelling which might support the cell proliferation and stimulate invasion. The high expression of these metabolic enzymes may explain, at least in part, the more progressive phenotype and adverse clinical outcome in patients with HER-2/*neu*-positive tumours.

In contrast, we found that serum albumin and MnSOD, were up-regulated in HER-2/*neu*-negative breast tumours. Overexpression of albumin was also reported in HER-2/*neu*-negative infiltrating ductal carcinoma, with unknown mechanism. MnSOD protects cells from oxidative stress by catalysing the dismutation of superoxide (O_2^{-}) radicals into hydrogen peroxide (H_2O_2). Our data may suggest that other protective molecules against oxidative damage, instead of other than MnSOD, may against oxidative damage be overexpressed in HER-2/*neu*-positive tumour cells.

Conclusion

Collectively, our results demonstrated that HER-2/*neu*-mediated rapid tumour growth and metastasis is a complicated process resulting from, or leading to, the imbalance of cell structural organisation, overall metabolism and resistance to stress. Arguably, proteome analysis will provide the best approach to achieve a fundamental understanding of the HER-2/*neu*-related network of metabolic and structural molecules involved in the formation and progression of breast cancer, and to move closer to the target of discovering protein biomarkers for cancer diagnosis, prognosis and therapy.

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Celecoxib Enhances Brain Tumour Cell Radiosensitivity Leading to Massive Tumour Necrosis

KB Kang,[1]*BSc (Hons), PhD*, TT Wang,[1]*MBBS, MSc*, CT Woon,[1]*BSc*, ST Cheah,[2]*FRCPath, FRCPA, FAMS*, YK Lim,[1]*BSc*, XL Moore,[1]*MBBS, PhD*, MC Wong,[1,3]*DipABPN, FRCP, FAMS*

Introduction

Radiotherapy remains integral for the treatment of glioblastoma patients who typically survive less than 18 months despite standard therapy of surgery, radiotherapy and chemotherapy. Cyclooxygenase-2 (COX-2), a rate-limiting enzyme in conversion of arachidonic acid into prostaglandins, is overexpressed in irradiated tumour cells, which is associated with radioresistance.[1] With the increasing evidence of COX-2 contributing to tumour growth and converse tumour regression following treatment with COX-2 inhibitors,[2,3] there is potential therapeutic benefit to enhance tumour radioresponse with COX-2 inhibitors. Indeed, several reports have shown enhanced tumour radiosensitivity with various COX-2 inhibitors in vitro and in vivo.[4,5] Nevertheless, in brain tumours, only 1 study showed enhanced tumour radioresponse to SC-236, a selective COX-2 inhibitor, in U251 human glioblastoma cells.[3] To date, the underlying mechanisms responsible for enhanced tumour radiosensitivity by COX-2 inhibitors remain unclear, although several mechanisms have been proposed (e.g., induction of cell apoptosis, cell cycle arrest, sublethal radiation damage and anti-angiogenesis). In this study, we determined whether the selective COX-2 inhibitor celecoxib, could enhance brain tumour radiosensitivity in vitro and in vivo, by using U87 human glioblastoma cells grown as monolayer and transplanted intracranially into nude mice. To elucidate whether this effect is due to damaged microvessels and anti-angiogenesis mechanisms, we determined the combined effect of celecoxib and irradiation by measuring tumour necrosis and microvascular density of mice brain tumours in vivo, as well as the expression of angiogenic factors vascular endothelial growth factor (VEGF), angiopoietin-1 and angiopoietin-2 proteins of U87 cells in vitro.

Materials and Methods

In vitro, U87 human glioblastoma cells were treated with celecoxib (30 mmol/L), irradiation (9 Gy), combined celecoxib and irradiation, or vehicle dimethyl sulfoxide (DMSO) for 24, 48 and 72 hours, and cell viability was assessed by 3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. After 72 hours of treatment, COX-2, VEGF, angiopoietin-1 and angiopoietin-2 proteins were quantitatively analysed by Western blot, whereas prostaglandin E_2 in the culture medium was measured by enzyme immunoassay.

In vivo, 36 nude mice were intracranially transplanted with U87 cells (5000 cells in 5 μL medium). After 7 days, the mice were treated with celecoxib (100 mg/kg for 2 weeks), irradiation (8 Gy for 3 days), combined therapy or vehicle DMSO, and the survival time recorded. Upon death, brain tumours were isolated, fixed, paraffin-embedded, coronally sectioned (4 μm) and stained with haematoxylin and eosin. The largest tumour region was measured to calculate brain tumour volume, by 0.5 x width^2 x length formula. For necrosis analysis, the largest tumour necrotic region was measured, and presented in percentage of necrosis over largest tumour area. For microvascular density, adjacent tissue of the largest tumour section was immunohistochemically stained with von Williebrand factor endothelial marker. Any positive-stained endothelial cell, or endothelial-cell cluster that was separated from tumour cells and other connective tissue elements was considered as a single, countable microvessel. All measurements were aided by Zeiss KS400 software, and performed by 2 independent observers in a blinded manner. The intra- and inter-variability of these observers were validated by simple regression analysis.

GBSTAT School Pak statistical package was used in all statistical analysis. The mice survival curves were analysed by Logrank survival test. Treatment effects on cell viability, proteins, prostaglandin E_2, necrosis and microvascular density were analysed by 1-way analysis of variance (ANOVA)-Dunnett's test, and differences between 2 treatment groups analysed by Student's t-test. Probabilities less than 0.05 ($P <0.05$) were considered to be statistically significant.

Results

Irradiation and celecoxib independently caused significant reduction (10% to 20%, and 36% to 40% respectively) in U87 cell viability at 24, 48 and 72 hours. When combined with irradiation, celecoxib significantly reduced cell viability by 54% to 63%, compared to controls (Fig. 1A). At 72 hours, COX-2 protein was increased by celecoxib and/or irradiation (Table 1A). The COX-2 metabolite, prostaglandin E_2 was increased by irradiation alone at 72 hours. However, this increase was completely blocked with the presence of

Table 1. The Effect of Celecoxib and/or Irradiation on Protein Expression (COX-2, VEGF, angiopoietin-1, angiopoietin-2) and Prostaglandin E_2 in vitro, as well as Tumour Necrosis and Microvascular Density in vivo

	Control	Irradiation	Celecoxib	Combination
A. In vitro COX-2	0.8 ± 0.2	1.6 ± 1.3**	1.4 ± 0.3*	1.7 ± 0.3*
Prostaglandin E_2 (ng/mg total protein)	208.0 ± 51.1	684.7 ± 79.4**	3.7 ± 0.5**	5.4 ± 0.5**
VEGF	0.9 ± 0.0	0.9 ± 0.0	0.8 ± 0.1**	0.8 ± 0.1*
Angiopoietin-1	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1*	0.4 ± 0.1*
Angiopoietin-2	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1*	0.8 ± 0.1**
B. In vivo				
Necrosis/ tumour area (%)	2.2 ± 1.4	1.4 ± 1.1	8.1 ± 3.7*	23.3 ± 8.5** #
Microvessels/mm²	91.2 ± 7.2	65.4 ± 4.0*	66.4 ± 4.8*	52.5 ± 2.9** #

COX-2: cyclooxygenase-2; VEGF: vascular endothelial growth factor

Table 1. Protein levels (analysed by Western blot) were presented in arbitrary units normalised against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein. Prostaglandin E_2 was measured by enzyme immunoassay. The largest tumour necrotic region was presented in percentage over largest tumour area. Microvascular density was analysed by immunohistochemical staining of von Williebrand Factor on non-necrotic tumour region. All data are expressed in mean ± standard error of mean, representative of 4 to 8 independent experiments in vitro or 8 to 10 mice in vivo. *$P <0.05$, **$P <0.01$ significantly different from control groups (1-way ANOVA, Dunnett's test). #$P <0.05$ significantly different between combination and celecoxib-treated groups (Student's t-test).

[1] *Division of Medical Science, National Cancer Centre, Singapore*

[2] *Department of Pathology, Singapore General Hospital, Singapore*

[3] *National Neuroscience Institute (Singapore General Hospital Campus), Singapore*

Address of Correspondence: Dr KB Kang, Division of Medical Science, National Cancer Centre, 11 Hospital Drive, Singapore 169610.
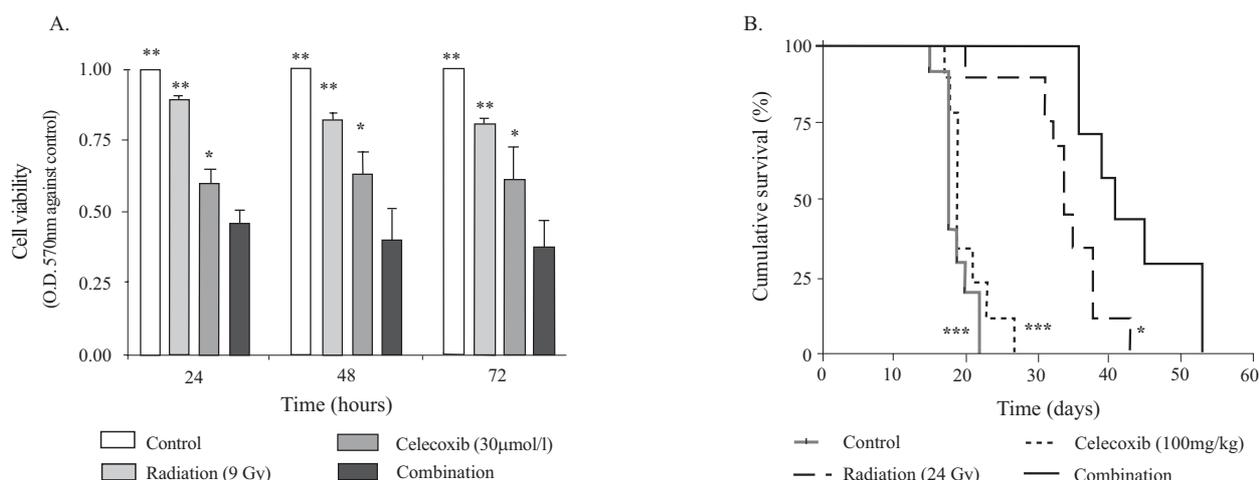
A.

B.

Fig.1. Celecoxib enhances brain tumour radiosensitivity in vitro and in vivo.
The effect of celecoxib, irradiation, combined celecoxib and irradiation, or DMSO (as control) on U87 cell viability (Fig. 1A) and the median survival of mice intracranially transplanted with U87 cells (Fig. 1B), are shown. Each group represents 7 to 8 independent experiments (Fig. 1A) or 8-10 mice (Fig. 1B). *P <0.05, **P <0.01, ***P <0.0001 significantly different from combined therapy group (Fig. 1A: 1-way ANOVA, Dunnet's test; Fig. 1B: Logrank survival test).

celecoxib (Table 1A). When combined with irradiation for 72 hours, celecoxib reduced VEGF and angiopoietin-1, but increased angiopoietin-2 proteins (Table 1A).

Irradiation extended the median survival of brain tumour mice from 18 (controls) to 33 days. Combined celecoxib and irradiation further extended the median survival to 41 days (Fig. 1B). Upon death, the average brain tumour volume of all mice was 45.2 ± 3.9 mm^3. Celecoxib-treated tumours contained significantly greater proportion of necrosis compared to irradiated and control tumours. Irradiation further increased necrosis of celecoxib-treated tumours by approximately 190% (Table 1B), validated by 2 independent observers with intra- and inter-variability correlation coefficient values of 0.99. Single therapy of irradiation or celecoxib significantly reduced tumour microvascular density (compared to controls), which was further reduced by combination therapy (Table 1B). Two independent observers validated these data with intra- and inter-variability correlation coefficient values of 0.98 and 0.90, respectively.

Discussion

Celecoxib significantly increased brain tumour radiosensitivity, as demonstrated by reduced U87 cell viability and extended survival of brain tumour mice. There is 1 study, which showed enhanced brain tumour radioresponse by SC-236 in U251 human glioblastoma cells in vitro and as xenograft in mice thighs.[3] Our novel study used celecoxib (currently prescribed for pain and inflammation with established safety profile), on U87 human glioblastoma cells in vitro and intracranially transplanted into mice brain, which mimics a direct microenvironment of brain tumours in vivo. Moreover, our

study used celecoxib 100 mg/kg/day in mice, which produced peak plasma concentration of 3 to 4 mmol/L,[6] equivalent to human plasma concentration after 800 mg/day celecoxib, a Food and Drug Administration-approved dosage.

In defining the underlying mechanism, we showed massive tumour necrosis and inhibition of angiogenesis by celecoxib to enhance brain tumour radioresponse. In addition to tumour cells, combined celecoxib and irradiation targets and destroys the tumour microvasculature, thus causing massive necrosis. This may be due to celecoxib reduction of prostaglandin E$_2$, which is known to be pro-angiogenic and pro-proliferative, thus a survival factor in irradiated tumours. Celecoxib also reduced the expression of other pro-angiogenic factors VEGF and angiopoietin-1, and increased angiopoietin-2 proteins, an antagonist of angiopoietin-1. Our findings are novel and with the proven safety profile of celecoxib, these results can potentially be translated clinically to glioblastoma patients.

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In Vitro Drug Sensitivity and Expression Profiling For Disease Prognostication in Childhood Acute Lymphoblastic Leukaemia (ALL): An Exploratory Model Using Cell Lines

XG Liu,[1]*MBBS*, SP Chen,[1]*BSc MSc*, SKY Kham,[1]*BSc*, TC Quah,[4]*MBBS, MMed*, AEJ Yeoh,[1] *MBBS, MMed*

Introduction

Poor prednisolone response, defined as the peripheral blast count $\geq$1,000/μL after 7 days induction with prednisone and 1 dose of intrathecal methotrexate, defines a high-risk group of patients with poor outcome (~38% to 50% EFS compared to 80% EFS in prednisolone good responders).[1,2] Many treatment protocols, including ours and the widely adopted German Berlin-Frankfurt-Munster clinical trials, utilise prednisolone response as an important prognostic factor. This suggests that resistance to therapy is already present at initial diagnosis and this can be exploited to improve prediction of eventual outcome.

Global gene expression profiling (GEP) using the microchip array provides a snapshot of the messenger RNA transcripts of the cells and hence divulges the pathways active in the cell. Although GEP can be used to diagnose and subgroup many different types of cancer including acute lymphoblastic leukaemia,[3] it has been unable to prognosticate who is able to do well compared to those who will relapse. We postulate that the leukaemia cells at diagnosis have no need to manifest their resistance pathways as this confers no survival advantage. This may explain why GEP at diagnosis is not helpful in prognostication. However, we suspect that, paired GEP of leukaemia cells at diagnosis and after therapy with prednisolone may unmask the resistance pathways as prednisolone therapy alone is a powerful prognostic factor.

Our aim is to identify and compare the changes in GEP of leukemic cells prior to, and after, exposure to prednisolone to define a molecular signature for prognosis. We started this proof of concept using the leukaemia cell lines.

Materials and Methods

Cell Lines

Four clinically important ALL cell lines were studied: Reh/TEL-AML1 t(12; 21), 697/E2A-PBX1 t(1; 19), Sup-B15/BCR-ABL t(9; 22) and RS4; 11/MLL-AF4 t(4; 11). All cell lines were purchased from the ATCC and cultured according to the ATCC's instructions.

MTT Assay

Prednisolone 21-hemisuccinate sodium salt was serially diluted to 6 concentrations (0.08 to 250 μg/mL).[4] Cells were harvested during log phase and resuspended to 2 x 10^6 per mL. Eight μL of cell suspension was incubated with each drug concentration in triplicate wells of a 96-well flat-bottomed microtitre plate. Cells cultured in drug-free medium were served as controls for cell survival and wells with only culture medium were used to blank the spectrophotometer. The plates were incubated for 4 days at 37°C in humidified air containing 5% CO_2. After 4 days, MTT assay was performed according to the manufacturer's instructions. Absorbance was measured at the wavelength of 570 nm in a microtiter plate reader (reference wavelength: 655 nm). The leukaemic cell survival rate was calculated with the equation: (mean OD treated wells/mean OD control wells) x 100%.

Gene Expression Profiling

The cell lines were cultured with 1.0 μg/mL and 0.4 μg/mL of prednisolone. Cells were harvested after 2 days by Ficoll density gradient centrifugation. RNA was extracted using RNeasy Kit. Total mRNA of 1 μg was reverse transcribed into cDNA and then into cRNA as per manufacturer's recommendation. The Affymetrix HG-U133A chip was used to profile the leukaemia cell lines. Interassay reproducibility was determined in duplicate and was found to be excellent.

Quantitative Real-time RT-PCR

Real-time RT-PCR using the LightCycler was used to determine the level of mRNA for 5 genes that were found to be commonly upregulated among the 3 sensitive cell lines with 0.4 μg/mL of prednisolone. ABL was used as the housekeeping gene and water as a no-template control. The ratio of specific genes copy number against that of ABL was reported. These values were used to correlate with the Affymetrix data.

Results and Discussion

Prednisolone concentration of 0.4 μg/mL was found to be the optimal concentration that separates the 4 cell lines into 3 distinct groups of sensitive, intermediate and resistant. Prednisolone concentration of 1 μg/mL was able to convert the intermediate sensitive cell line to sensitive.

By comparing the changes in gene expression profiles between day 2 treated samples and day 2 untreated controls, we found that there was an exponential increase in the number of significant genes which changed from resistant to intermediate to sensitive cell lines (Table 1).

Analysis of the genes changed, and surprisingly, we found that there were no common genes in cell line Reh (resistant) compared to the genes in the sensitive cell lines. Intriguingly, there was marked overlap in the genes that were significantly changed in the intermediate and sensitive cell lines. Specifically, 5 genes (6 probe sets) were found to be consistently elevated after treatment with 0.4 μg/mL of prednisolone among the 3 sensitive cell lines. This indicates that the

Table 1. Changes in Gene Expression Profile Observed in Cell Lines Incubated with 0.4 μg/mL versus 1.0 μg/mL of Prednisolone

Cell lines		Number of gene changes			
		0.4 μg/mL	1.0 μg/mL	Common	Percentage (0.4 μg/mL)
REH (Resistant)	Increase	4	19	0	0
	Decrease	3	18	0	0
697 (Intermediate)	Increase	11	45	9	~82%
	Decrease	3	42	0	0
Sup-B15 (Intermediate)	Increase	187	535	174	~93%
	Decrease	50	505	41	~82%
RS4; 11 (Sensitive)	Increase	391	944	292	75%
	Decrease	270	709	226	84%

[1] *Department of Paediatrics, National University of Singapore, Singapore*
Address of Correspondence: Dr Allend Yeoh, Department of Paediatrics, National University of Singapore, 5 Lower Kent Ridge, Singapore 119074.
Email: paeyej@nus.edu.sg

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(A) mRNA level

(B) Expression Signal

1 Day 0 untreated control
2 Day 2 untreated control
3 Day 2 treated sample (0.4 µg/mL of prednisolone)
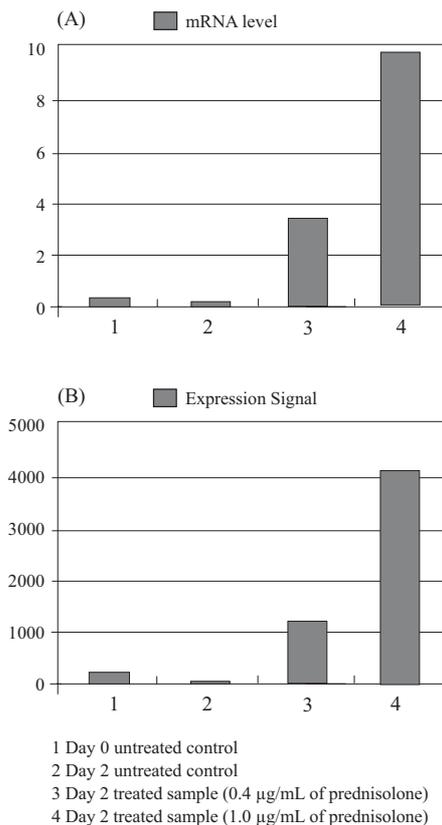4 Day 2 treated sample (1.0 µg/mL of prednisolone)

Fig. 1. Expression signal and mRNA level of gene KLF2 in cell line RS4;11 as determined by (A) Affymetrix Genechip HG-U133A and (B) Realtime Quantitative PCR respectively.

gene expressions changed in Reh cell line were random while gene expressions changed in the other 3 cell lines were due to exposure to prednisolone.

The raised genes were confirmed using real-time quantitative PCR. A typical strong correlation between GEP and real-quantitative PCR is shown in Figure 1. In addition, probe sets 204698 at and 33304 at were designed to interrogate different parts of the same gene

ISG20, and both of them turned out to be of the same prognostic value, implying that the changes in gene expressions were not random.

We hereby report that a minimum molecular signature by changes gene expression profiling that is consistently correlated to the response to prednisolone therapy in childhood ALL. This provides the basis for us to study the changes in GEP after 7 days of prednisolone compared to diagnosis in vivo in our current Singapore-Malaysia ALL trial. This will, hopefully, improve our ability to predict the outcome of ALL in children.

Conclusion

In vitro drug sensitivity and gene expression profiling are valuable for disease prognostication in childhood ALL. We hope to extend this finding to patient samples post-therapy with prednisolone in our leukaemia trial.

Acknowledgement

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Young Investigator's Award Finalist

The Effects of Exercise on the Functional Status of the Elderly Persons Living in the Long-term Care Setting of a Psychiatric Hospital

YSC Tan,[1]*SRN, BNurs, Adv Dip Gerontol Nurs*, I Alina,[1]*SRN, Adv Dip Gerontol Nurs*, SH Chua,[1]*SRN, RMN, Msc (Health Care Mgmt)*, SL Lee,[1]*SRN*, PM Kumari,[1]*SRN, RMN, Adv Dip Gerontol Nurs*, V Vasanthi,[1]*SRN, RMN, Adv Dip Gerontol Nurs*

Introduction

Exercise is an effective way of promoting wellness in people of all ages. It often slows down the process of disability in old age or reverses the decreased mobility that contributes to diseases in old age.[1] The effectiveness of exercise has not been well-evaluated in Asian countries, particularly for elder persons living in long-term psychiatric settings. This study was done to explore the effects of

exercise on the functional status of the elderly persons living in the long-term care setting of a psychiatric hospital.

Materials and Methods

The quasi-experimental design was used for this study. The subjects included in this study were inpatients of the psychiatric hospital aged 65 years and above, who are able to follow simple instructions, have been staying in the ward for more than 6 months,

[1] *Department of Nursing, Institute of Mental Health / Woodbridge Hospital*
Address of Correspondence: Ms Yvonne Deborah Tan Shu Ching, Department of Nursing, Ward 63A, Institute of Mental Health / Woodbridge Hospital, 10 Buangkok View, Singapore 539747.

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are independent in their Activities of Daily Living (ADL) and/or need moderate assistance in their ADL (semi-dependent). Subjects with physical limitations (e.g., fractures), dementia, severe communication difficulties, and who are totally dependent on nursing staff for their ADL were excluded from this study. A sample of 107 elderly who fit the criteria was taken from the psychogeriatric wards of the hospital. Those who participated in the exercise program were grouped into the experimental group, while those who did not participate in the exercise program were grouped into the control group.

Data collection was carried out using a questionnaire, where patients' demographic data, medical history, fall history, exercise involvement, Elderly Mobility Scale (EMS) and Modified Barthel Index (MBI) were recorded. All subjects recruited in the study and who had given their consent for the study were assessed using the EMS and their functional level, using MBI. This was to ascertain the patients' baseline functional and mobility status before the commencement of the study.

A simple, 20-minute exercise program, which consisted of a Range Of Motion (ROM) exercise session and walking exercise of about 60 metres, was introduced to the experimental group for 3 times a week for 3 months. No intervention was introduced to the control group. A post-test recording was taken after the exercise

Table 1. Demographic Table of the Participants (n = 107)

Characteristics	Frequency	%
Gender:		
Male	17	15.9
Female	90	84.1
Race:		
Chinese	98	91.6
Indian	3	2.8
Malay	3	2.8
Others	3	2.8
Mobility Status:		
Independent	78	72.9
Semi-dependent	29	27.1
Psychiatric problem:		
Schizophrenia	98	91.6
Depression	2	1.9
Others	7	6.5
Medical Problem:		
Yes	46	43
No	61	57

program and the results were compared with the baseline data. Data analysis was done using the Statistical Package for Social Science version 11.

Results

Of the 107 participants, 17 were males and 90 were females. The mean age of the participants was 73.9 years. All participants suffered from a mental disorder, of which schizophrenia was the commonest followed by depression. Seventy-eight of the participants were independent in their mobility status and 29 of them were semi-dependent. Fifty-two of the participants in the experimental group were more likely to be exercising before (OR = 3.4, 95% CI, 1.4-8.2).

Table 2 shows that the percentage change in MBI and EMS for those in the experimental group was higher than those in the control group. This is significant as it indicates that the simple exercise program did improve the functional status of the elderly persons. The percentage change in the MBI was also higher in those participants who were semi-dependent (11.84%).

Discussion

The study showed that there were significant changes in the MBI scores for those in the experimental group as compared to the control group ($P < 0.001$, Table 2). This is statistically and clinically significant as the Barthel Index is sensitive to change and a score of 1 or 2 may decide the level of dependence of the patient.

There was a significant change in the MBI of those who were semi-dependent as compared to those who were independent (Table 3). This could be due to participants who had a high initial score, and had improved in their scoring where it was beyond what the upper limit score could measure.[2]

Within the experimental group, there were 52 participants who had exercised before ($P = 0.004$). This group of participants was more likely to exercise because they were interested in the exercise program, enjoyed exercising or liked doing something familiar to them. The kind of exercises they did was mainly walking exercises, which was also part of the exercise program ($P = 0.030$). This could have introduced bias into the study, as participants who liked exercising would have chosen to participate in the study.

Limitations

The findings of this study cannot be generalised towards other populations as the implementation period for the exercise program

Table 2. Percentage Change in Modified Barthel Index (MBI) and Elderly Mobility Scale (EMS)

		Mean	SD	Minimum	Maximum	Median
Participated in Exercise Programme						
Yes	% change in MBI	-7.77	15.45	-104.35	.00	-2.04
No	% change in MBI	-1.09	5.74	-22.95	12.50	.00

$P < 0.001$ (MWU)

		Mean	SD	Minimum	Maximum	Median
Participated in Exercise Programme						
Yes	% change in EMS	-6.42	24.72	-180.00	17.39	00
No	% change in EMS	-.34	19.50	-80.00	61.54	.00

$P = 0.01$ (MWU)
Change in MBI = pre MBI – post MBI
Change in EMS = pre EMS – post EMS

Table 3. Percentage Change in Modified Barthel Index (MBI) in the Mobility Status Groups

		Mean	SD	Minimum	Maximum	Median
Mobility status						
Independent	% change in MBI	-2.57	7.07	-46.77	12.50	.00
Semi-independent	% change in MBI	-11.84	20.62	-104.35	9.68	-6.98

was only 3 months, as some of the patients were transferred from the psychiatric hospital to sheltered homes. Participants were also allocated according to their willingness to participate in the exercise program (experimental group) and those who did not want to participate were grouped into the control group. This could have introduced biases into the study.

Recommendation

Many studies have been conducted to explore the intensity of aerobic activity and the corresponding degree of fitness. However, the exercises were not applicable to those elderly persons who are more debilitated.[3] This study was conducted based on a simple exercise program, which is of low-to-moderate intensity. This is best for geriatric patients, where the aim of exercise is to maintain the interest of the elderly persons and avoiding injuries arising from overuse.[4]

Conclusion

The outcome of this study suggests that the effects of exercise on the functional status of the elderly persons living in the long-term care setting of the psychiatric hospital were similar to most studies that were done across countries, on subjects who are mentally capable in the community settings. The introduction of a simple exercise program will help the elderly persons in the psychiatric hospital to stay active instead of sitting around with nothing to occupy them in the dayspace of the hospital. Besides, the benefits exercise has on the individual may mean lesser burden on the caregivers, and the time and energy channelled to those who need more nursing care.

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Young Investigator's Award Finalist

The Impact of Microanastomosis of the Intramuscular Nerve Branch on the Healing of a Completely Lacerated Skeletal Muscle: A Histopathological Analysis

L Zheng,[1] *BMed,* JAC Tan,[1] *BSc,* BL Tan,[1] *BSc,* BP Pereira,[1] *MEng, BEng,* AYT Lim,[2] *FRCS, MBBS,* Lahiri A,[2] *MD,* VP Kumar,[1] *FRCS, MBBS*

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Introduction

A completely lacerated skeletal muscle that is repaired by suturing the cut muscle ends often results in an incomplete recovery. Garret et al[1] have reported histological abnormalities, characterised by muscle fibre atrophy, increased degree of fibrosis, as well as denervation of the segment distal to the laceration, which has been isolated from a nerve supply when the muscle was cut. Although various approaches have been attempted to enhance muscle regeneration and to reduce fibrosis formation, muscle function of lacerated muscles after muscle repair generally do not recover fully.[2] The surgical repair of the severed intramuscular nerve branches, concomitant to skeletal muscle lacerations, is perhaps ignored as it requires microsurgical skills and expertise.

We hypothesise that a poor neuromuscular reinnervation might, in part, account for the incomplete healing of lacerated muscle repaired by epimysial suturing alone, and that the integrity of the intramuscular nerve might play a role in the recovery process. This study aims to investigate the long-term morphologic changes in a completely lacerated muscle that is repaired with micro-anastomosis of the main intramuscular (IM) nerve, followed by epimysial suturing of the cut muscle ends.

Materials and Methods

The medial gastrocnemius (MG) in adult NZ White rabbits were used as the model in this study (n = 20). The MG of 1 limb was lacerated completely at the proximal quarter of the muscle belly, just distal to the motor point of the branch from the tibial nerve. The muscle was immediately repaired by epimysial suturing the cut muscle ends together. Two different muscle repair options were used; in 1 group only a muscle repair was done without the IM nerve repaired (non-NR group) and in the other the microanastomosis of the main IM nerve branch, before the muscle repair was done [i.e., the Nerve-Repair (NR) group]. A third group was used as a positive control, where the IM nerve was identified and left intact when the muscle was lacerated, and then the muscle belly was repaired [i.e., the Nerve-Preserved (NP) group]. The non-operated contra-lateral MG in each group serves as normal controls (NC). Removed muscles

[1] *Musculoskeletal Research Laboratories, Department of Orthopaedic Surgery, National University of Singapore, Singapore*
[2] *Department of Hand and Reconstructive Microsurgery, National University Hospital, Singapore*
Address of Correspondence: Barry P Pereira, Musculoskeletal Research Laboratories, Department of Orthopaedic Surgery, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260.
Email: dosbarry@nus.edu.sg

were snap-frozen and transversely sectioned for histochemistry and immunohistochemistry (IHC) staining. The non-NR and NR goups were analysed at the fourth and seventh month postoperatively, while the positive controls, NP group were evaluated only at 7th month after repair. Only the segment distal to the laceration site was assessed in this study.

Muscle regeneration was quantified through IHC for neonatal myosin heavy chain (MHCn), double-stained for intermediate filament, vimentin and desmin. Vimentin was used as a marker for fibrosis. This was further confirmed with Masson's-modified trichrome. Acetyl cholinesterase (AchE) staining for motor endplate was employed to evaluate the neuromuscular innervation. AchE staining was also coupled with neonatal myosin heavy chain (MHCn) immunostaining to identify the relationship between them. The cross-sectional area (CSA) of total myofibres and regenerating myofibres, area of fibrosis and proportion of muscle types, were quantified and analysed using a computerised imaging system linked to the microscopy.

The average and standard deviation of all data were compared among the different groups using one-way ANOVA for statistical analysis. Statistical significance was defined as $P < 0.05$.

Results
Muscle Regeneration (Fig.1)

After 4 months, the CSAs of the total myofibres in the NR group were noted to be significantly higher than the non-NR group. In addition, the NR group had a higher number of regenerating myofibres with larger CSAs than the non-NR group. After 7 months, the number of regenerating myofibres in the NR group decreased significantly, compared to the non-NR group. The CSAs of total myofibres and regenerating fibres in the NR group, when compared to the positive control, the NP group, at 7 months, were however smaller and fewer in number. In the NP group, the CSA of total and regenerating fibres did not statistically differ from collateral normal controls (NC).

Muscle Fibrosis (Fig. 2)

Vimentin and Masson's trichrome staining showed no difference in area of fibrosis between non-NR and NR groups at the fourth month. Fibrosis formation in both groups, however, was significantly increased, compared with contra-lateral control. In contrast, the NR group showed significantly decreased fibrosis formation in comparison with non-NR group at 7 months. There was no difference in area of fibrosis between the NR group and the NP group, and between the NP group and contra-lateral normal controls, at 7 months.

Muscle Innervation (Fig. 1)

Poorly organised, disconnected, randomly distributed AchE-positive motor end-plate spots, featured as multiple and polyneural reinnervation suggestive of nerve terminal spouting, was observed in the non-NR group, at both time-points. In contrast, the NR group demonstrated a well-organised, interconnected band of AchE-positive motor endplate spots that was similar in pattern to the positive (NP) and normal (NC) controls, but it demonstrated a less dense staining than those of the controls, at the fourth month and the seventh month. No difference was found between the NP and NC controls at 7 months.
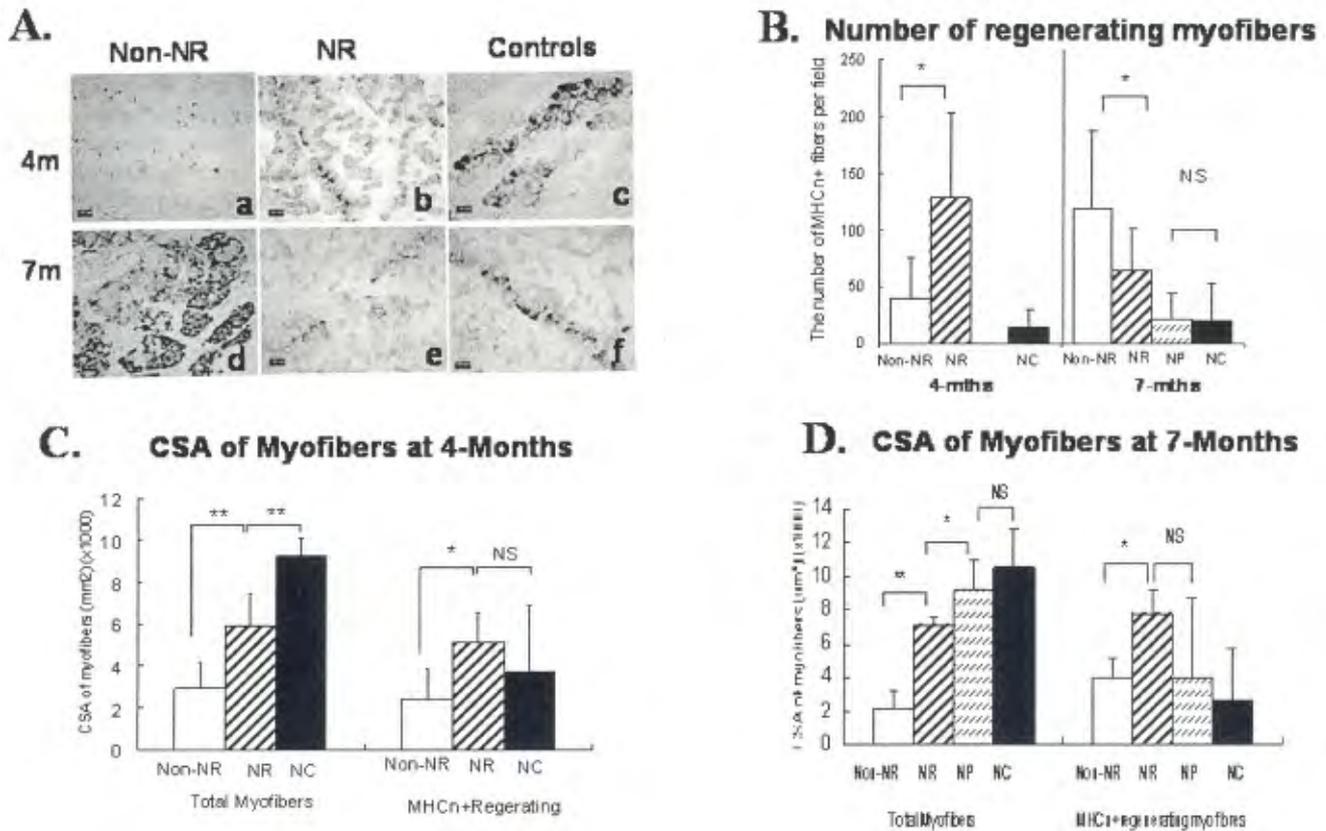

Fig. 1. Quantification of total and regenerating myofibre size and regenerating myofibre number in distal segment at 4 months (**a-b**) and 7 months after repair (**d-f**). (**A**): AchE stains (dark blue color) coupled with MHCn (brown) immunostaining. (**a,d**): Non-nerve repair (Non-NR); (**b,e**): Nerve-repair (NR); (**c**): Contra-lateral normal control (NC); (**d**): Positive control, nerve preserved (NP). *P <0.05; **P <0.01; NS: not significant. (**B**): The number of regenerating myofibres per field at 4 months and 7 months (**C**) CSA of total and regenerating myofibres at 4 months (**D**) CSA of total and regenerating myofibres at 7 months.

A.

B.
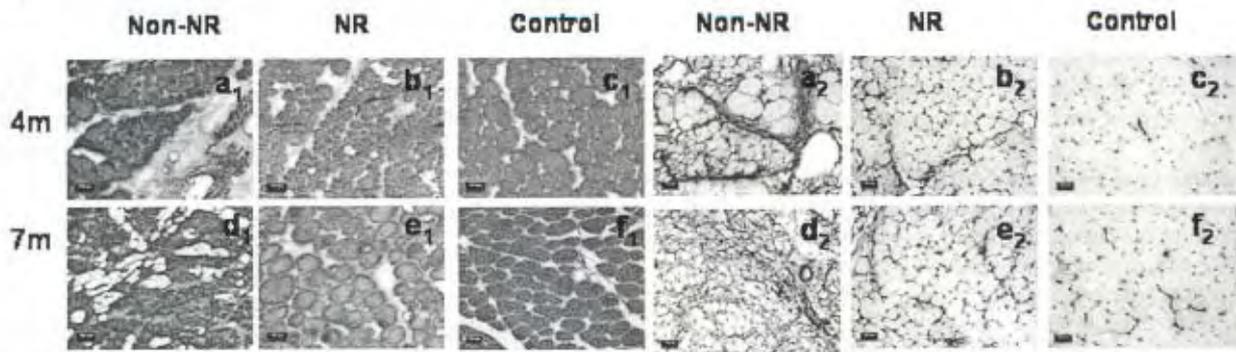
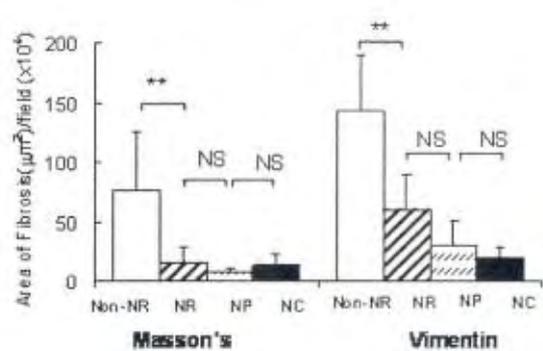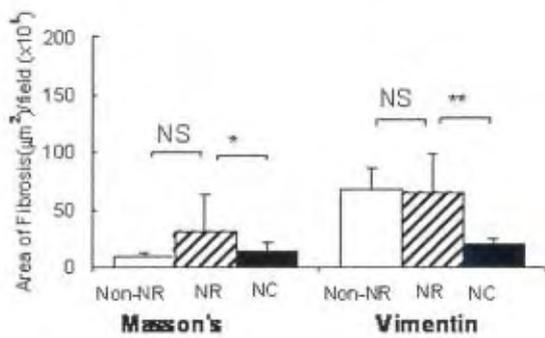*Fig. 2. Quantitative histological analysis of fibrosis formation in distal segment detected by Masson's Trichrome (a_1-f_1) and Vimentin (a_2-f_2) immunostaining, 4 months and 7 months after repair. **Top panel (A):** Masson's Trichrome (collagenous area: blue) and Vimentin immunostaining (fibrosis area: brown); **Bottom panel (B):** area of fibrosis per field. Note: Non-NR: non-nerve repair; (NR): nerve repair; NC: contra-lateral normal control (c_1,c_2) and NP: nerve preserved (f_1,f_2). *P <0.05; **P <0.01; NS: not significant*

Discussion

The NR group, where intramuscular nerve branch was repaired, demonstrated substantially improved muscle morphology with enhanced muscle regeneration and decreased fibrosis formation, especially at 7 months. Recent studies suggest that innervation plays an essential role in promoting regenerating muscle fibre maturation and blocking fibrosis formation[3,4] This was also confirmed by our findings of appropriate innervation present in both the NR and NP groups, in contrast to the poor innervation noted in the non-NR group. Clinically, the findings from this study provide a rationale for repair the intramuscular nerve branch which has further improved the recovery of lacerated skeletal muscles. Nevertheless, a slower morphologic recovery correlated to the less dense staining of motor endplates in the NR group was noted when compared to the NP group. This is more likely the result of a slower re-innervation of the muscle given the time gap difference for nerve regeneration after repair, between the 2 groups. In this context, an additional-therapy that could promise to further improve muscle recovery after a laceration would be the use of growth factors that would either promote nerve or muscle regeneration, or reduce fibrosis formation,[5] and future studies will be necessary to assess the earlier time-points so as to fully understand the mechanisms involved.

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