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Protective effect of ischaemic preconditioning in total knee arthroplasty

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Abstract. – OBJECTIVE: To investigate the genomic response induced by ischaemic preconditioning (IPC) in muscle biopsies taken from the operative leg of total knee arthroplasty patients.

MATERIALS AND METHODS: The gene expression profile GSE21164 was extracted from Gene Expression Omnibus (GEO) database. Patients undergoing primary knee arthroplasty were randomized to control and treatment (IPC) groups. Muscle biopsies were taken from the quadriceps muscle of the operative knee at the immediate onset of surgery (T0) and at 1 hour into surgery (T1). Limma package of R language was used to identify the differentially expressed genes (DEGs) between control and treatment group. To find out specific genes, DEGs at T0 were compared with DEGs at T1. Scansite was used to find out the binding domain for specific DEGs. Functional enrichment analysis was done by DAVID.

RESULTS: Of the genes queried on the Affymetrix Human Genome U133 Plus 2.0 microarray, we identified 263 (T0) and 266 (T1) DEGs compared to the control group. Down-regulation of DEGs related with regulation neuron apoptosis was observed at T1. The most significant function of DEGs at T0 was related with neurological system process. The most specific DEG was FAM125B at T0 and T1 time points. Its common binding domain was SH3.

CONCLUSIONS: The protective effect of IPC was associated with altered expression of genes involved in neurological system process and regulation of neuron apoptosis. The dynamic expression of FAM125B can be a supervised marker during the surgery. IPC may be of potential benefit in this and other musculoskeletal conditions.

Key Words:
Ischaemic preconditioning, Total knee arthroplasty, Differentially expressed gene, Function enrichment analysis, Motif analysis.

Introduction

Total knee replacement (TKR) is a reliable treatment for end-stage arthritis of the knee, resulting in pain relief and return of function. By the year 2030 it has been predicted that 3.48 million primary TKRs will be performed annually in the United States[1].

During TKR, events such as tourniquet inflation and deflation, cementing, and preparation of the bones cause circulatory, respiratory, neurological, and hematological changes. Surgeons use tourniquets during knee procedures to create a relatively bloodless surgical field. However, using tourniquets can result in not only systemic hemodynamic changes but also local side effects such as neurovascular injury. The severity of these effects depends on cuff pressure and the duration of inflation.

Exsanguination of both lower limbs with tourniquets causes translocation of around 800 mL of blood volume to the central circulation[2], causing major hemodynamic consequences. Deflation of the tourniquet causes blood to shift back into the lower limbs along with release of metabolites back into the systemic circulation, resulting in peripheral vasodilatation. Most embolic events during TKR occur when the tourniquet is deflated, as a result of absorption of bone marrow, fat, and surgical debris into medullary vasculature[3]. Cerebral emboli may occur either via opening of recruitable pulmonary vasculature or patent foramen ovale (PFO)[3]. Kato et al[4] observed that embolization occurred during femoral reaming in 27% of patients with tourniquet use and 4% of patients without tourniquet. They also found that embolic events occur even with an inflated tourniquet.

Free radicals are released during joint replacement, predominantly during reperfusion following release of the tourniquet. Excess production of the free radicals is associated with cardiopulmonary complications, acute respiratory distress syndrome, fat embolism syndrome, and postoperative mental changes[5]. Studies have attempted to lower the inflammatory response with low-dose steroids preoperatively and postoperatively[5] or ischemic limb preconditioning. The beneficial effect of ischemic limb preconditioning was noted by Cheng et al[6].

Ischaemic preconditioning (IPC) has emerged as an extremely powerful method of protecting

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tissue against ischaemia-reperfusion (IR) injury[7]. It is an innate protective mechanism that increases a tissue's tolerance to prolonged ischaemia when it is first subjected to short bursts of ischaemia and reperfusion. It is thought to provide this protection by increasing the tissue's tolerance to ischaemia, thereby, reducing oxidative stress, inflammation and apoptosis in the preconditioned tissue. The protective effects of IPC have been demonstrated in animal models[8-11] and are now being investigated in human trials[12-14].

The complex mechanism through which IPC provides protection has only been partially elucidated. Understanding how gene expression is regulated by IPC will allow the development of translational therapeutics to combat IR injury in a variety of clinical settings. cDNA microarray technology has made it possible to characterize gene expression *in vivo*. Accordingly, we sought to investigate the effect of IPC in patients undergoing total knee arthroplasty (TKA). The primary objective of this study was to investigate the genomic response induced by IPC in muscle biopsies taken from the operative leg of TKA patients using microarray analysis.

Materials and Methods

Source of Data

We extracted the microarray expression profile from the study of Murphy et al[15], which was deposited in GEO (Gene Expression Omnibus) database: GSE21164. This study was carried out to investigate the genomic response induced by IPC in muscle biopsies taken from the operative leg of TKA patients in order to gain insight into the IPC mechanism.

Patients undergoing primary knee arthroplasty were randomized to control and treatment groups. Patients in the treatment group received a (IPC) immediately prior to surgery. Muscle biopsies were taken from the quadriceps muscle of the operative knee at the immediate onset of surgery (T0) and at 1 hour into surgery (T1). Total RNA was extracted from biopsies of four control and four treatment patients and hybridised to the Affymetrix Human U133 2.0 chip.

Data Preprocessing and Differentially Expressed Genes (DEGs) Analysis

The probe-level data in CEL files were converted into expression measures and the missing values were completed[16]. All the data were normalized before statistical analysis[17].

Limma package[18] of R language was used to identify the DEGs between control and treatment group. The genes with a |logFC (fold change)| > 1 and a p-value < 0.01 were considered differentially expressed.

Comparing the Difference of DEGs Expression at Different Time Points

According to the principle that the expression of the same gene under different conditions is different, we compared the DEGs at the onset of surgery (T0) and at 1 hour into surgery (T1) to choose the most specific DEGs at the 2 time points.

Gene Set Enrichment Analysis for DEGs

Individual gene analysis (IGA) evaluates the significance of individual genes between two groups of samples compared. The main problems of IGA originate from the use of the cutoff threshold value. First, the final result of IGA is significantly affected by the selected threshold, which is normally chosen arbitrarily. Second, many genes with moderate but meaningful expression changes are discarded by the strict cutoff value, which leads to a reduction in statistical power. Gene set analysis (GSA) methods, free from the problems of the "cutoff-based" methods. GSA directly scores pre-defined gene sets for differential expression and especially aims to identify gene sets with 'subtle but coordinated' expression changes that cannot be detected by IGA methods. The key principle is that even weak expression changes in individual genes gathered to a large gene set can show a significant pattern.

To assess the function of the interesting gene sets, we conducted functional enrichment tests using the online tool DAVID (Database for Annotation, Visualization, and Integrated Discovery) for modules. DAVID bioinformatics resources consist of an integrated biological knowledgebase and analytic tools aimed at systematically extracting biological meaning from large gene or protein lists.

We mapped the DEGs at T0, DEGs at T1 and the common DEGs in T0 and T1 into DAVID respectively for function analysis. False discovery rate (FDR) < 0.05 was set as threshold.

Search for Motif and Phosphorylation Sites for FAM125B

Post-translational modifications (PTMs) occur on almost all proteins analyzed to date. The function of a modified protein is often strongly affected by these modifications and therefore increased

knowledge about the potential PTMs of a target protein may increase our understanding of the molecular processes in which it takes part. As a pervasive regulatory mechanism, reversible protein phosphorylation plays an important role in signaling networks[19]. Annotation of phosphorylation and other modification sites in proteomes is a critical first step toward decoding protein function and downstream regulatory networks

Phosphorylation is the most important post-translational modification of proteins, orchestrates most of biological processes and regulates cellular dynamics and plasticity. Usually, a member of protein kinase (PK) superfamily only modifies limited substrates by mainly recognizing special sequence/structural profiles around modified residues (S/T or Y), to ensure the signaling fidelity. In this regard, identification of phosphorylation sites, especially kinase-specific phosphorylation sites, is fundamental for understanding the molecular mechanisms of phosphorylation and elucidating dynamic interactions between PKs and their substrates.

To predict non-specific or kinase-specific phosphorylation sites, a widely adopted hypothesis is that a PK could recognize distinct sequence patterns/motifs of substrates by its kinase domain for modification[20].

Obenauer et al[21] as regards as scansite identifies short protein sequence motifs that are recognized by modular signaling domains, phosphorylated by protein Ser/Thr- or Tyr-kinases or mediate specific interactions with protein or phospholipid ligands. Each sequence motif is represented as a position-specific scoring matrix (PSSM) based on results from oriented peptide library and phage display experiments. Threshold values need to be assigned when scanning query proteins with the Motif Scan programs to decide which scores are likely to suggest real interactions. There are two parameters: scores and Scoring percentiles[21].

In the present study, we used Scansite for finding out the binding domain on the sequence of FAM125B.

Results

DEGs Analysis

We obtained publicly available microarray dataset GSE21164 from GEO database. Patients undergoing primary knee arthroplasty without IPC were set as control group. After preprocessing and normalization, at the threshold of $p < 0.01$ and $|logFC| > 1$, we got 263 significant DEGs at T0 time point as compared to the control group. A total of 266 genes were differentially expressed at 1 hour into surgery compared to the control group.

Comparing the DEGs Between T0 and T1

In order to find the difference of DEGs expression between T0 and T1, we performed intersec-

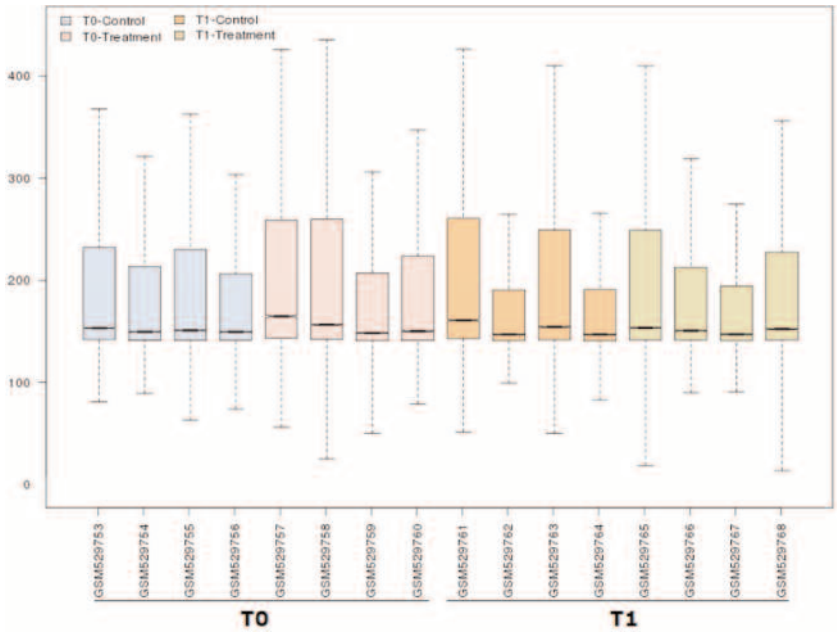

Figure 1. The box plots were generated using the normalized data of muscle biopsies taken from the operative knee at the immediate onset of surgery (T0) and at 1 hour into surgery (T1). T0 is the left column and T1 is the right column. Black line in each box represents the median of each sample. All the black lines are almost in the same position indicating minimum variability in these datasets.

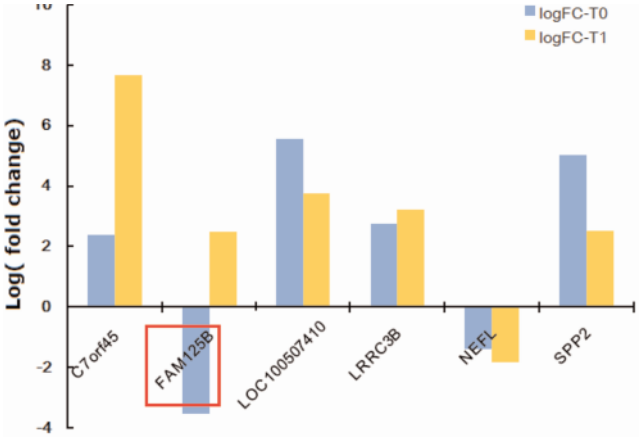

Figure 2. The fold changes of the common DEGs at T0 and T1 time points. C7or145, LOC100507410, LRRC3B and SSP2 were up-regulated at the 2 time points. NEFL was down-regulated at the 2 time points. FAM125B was down-regulated at T0 whereas it was up-regulated at T1.

tion analysis for the 2 sets of DEGs. The intersection analysis produced 2 types of results: a subset of each original set (T0, T1), a matrix representing the specific, unique groups of loci which intersected across all data sets (T0 and T1). There were 6 common DEGs between T0 and T1 (Figure 2). Among the common 6 DEGs, C7orf45, LOC100507410, LRRC3B and SPP2 were up-regulated at the two time points. NEFL was down-regulated at the two time points. The expression of FAM125B at T0 was down-regulated which was opposite to T1.

Functional Enrichment Analysis for DEGs Cluster

In the up step, we got 3 clusters of DEGs: clusters of each original set (T0, T1), cluster of common DEGs between T0 and T1. We mapped the 3 clusters of DEGs into DAVID respectively for functional enrichment analysis. The results are shown in Table I. As for the DEGs at T0, the most significant function was neurological system process. As for the DEGs at T1, the most significant function was regulation of neuron apoptosis. No significant function was enriched for the common DEGs.

The Phosphorylation Sites for FAM125B

Because the expression of FAM125B at T0 is different to T1, we use Scansite for finding out its phosphorylation sites. Our results showed that there were 5 motifs which could bind with FAM125B protein domains (Figure 3). They were src Homology 3 (SH3) group, basophilic serine/threonine kinase group, DNA damage kinase group, acidophilic serine/threonine kinase group and kinase binding site group. The domain binding with SH3 was the most universal in FAM125B protein. There were 7 genes which could bind with SH3 domain of FAM125B. They were ABL1, ITK, NCK1, GRB2, PLCG1, AMPH and SORBS1 (Figure 4). The SH3 (Src Homology 3) domain is a representative of the rapidly growing family of modules that recognize proline-rich ligands. The SH3 domains regulate protein localization, enzymatic activity and often participate in the assembly of multicomponent signaling complexes[22].

Discussion

IPC has been shown to protect against ischaemia-reperfusion injury in both animal mod-

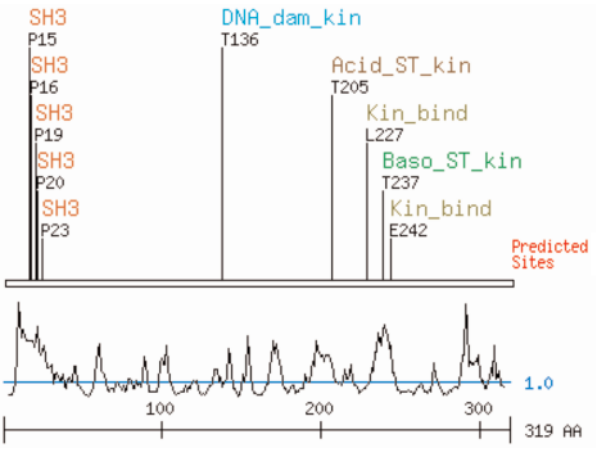

Figure 3. Description of elements in Motif Scan graphical output. The protein query (FAM125B) is represented schematically as a line. Labels with different colors above the protein indicate where motifs were found and identify the motif family. The ruler at the bottom marks numbered intervals along the protein sequence.

Src homology 3 group (SH3)

Abl SH3			Gene Card ABL1	
Site	Score	Percentile	Sequence	SA
P20	0.2664	0.006 %	PPPPQPPPPPPQRGT	3.132

Itk SH3			Gene Card ITK	
Site	Score	Percentile	Sequence	SA
P20	0.3971	0.003 %	PPPPQPPPPPPQRGT	3.132

Nck 2nd SH3			Gene Card NCK1	
Site	Score	Percentile	Sequence	SA
P16	0.4306	0.140 %	RSRDPPPPQPPPPPP	3.508

Grb2 SH3			Gene Card GRB2	
Site	Score	Percentile	Sequence	SA
P15	0.4360	0.101 %	RRSRDPPPPQPPPPP	3.508

PLCg SH3			Gene Card PLCG1	
Site	Score	Percentile	Sequence	SA
P19	0.4758	0.061 %	DPPPPQPPPPPPQRG	3.508

Amphiphysin SH3			Gene Card AMPH	
Site	Score	Percentile	Sequence	SA
P19	0.4988	0.180 %	DPPPPQPPPPPPQRG	3.508

Cbl-Associated protein C-SH3			Gene Card SORBS1	
Site	Score	Percentile	Sequence	SA
P23	0.5661	0.085 %	PQPPPPPPQRGTDQS	2.844

Basophilic serine/threonine kinase group (Baso_ST_kin)

Akt Kinase			Gene Card AKT1	
Site	Score	Percentile	Sequence	SA
T237	0.4680	0.144 %	TFRGRNSTRTDYEYQ	3.494

DNA damage kinase group (DNA_dam_kin)

DNA PK			Gene Card PRKDC	
Site	Score	Percentile	Sequence	SA
T136	0.4133	0.117 %	PIQETVDTQEVAFRK	0.913

Acidophilic serine/threonine kinase group (Acid_ST_kin)

GSK3 Kinase			Gene Card GSK3A	
Site	Score	Percentile	Sequence	SA
T205	0.4759	0.184 %	HDSSQPTTPSQSSAA	2.649

Kinase binding site group (Kin_bind)

PDK1 Binding			Gene Card PDPK1	
Site	Score	Percentile	Sequence	SA
E242	0.5411	0.170 %	NSTRTDYEYQHSNLY	3.835

Erk D-domain			Gene Card MAPK1	
Site	Score	Percentile	Sequence	SA
L227	0.5485	0.110 %	PRHISLTLPATFRGR	0.507

Figure 4. Motif Scan's output table. For each motif family with a site on the graphical output (Figure 3), details about the best matching domain motifs and the position of the site in the query are shown. The score, percentile and sequence of the site are indicated, as is the calculated surface accessibility for that site (labeled SA).

Table I. Lists of the functional enrichment analysis for DEGs at T0 and T1.

Term	Count	FDR
a).T0		
GO:0007268–synaptic transmission	10	0.0063
GO:0019226–transmission of nerve impulse	11	0.0059
GO:0019953–sexual reproduction	11	0.0326
GO:0032504–multicellular organism reproduction	11	0.0463
GO:0045893–positive regulation of transcription, DNA-dependent	11	0.0412
GO:0045944–positive regulation of transcription from RNA polymerase II promoter	11	0.0087
GO:0048609–reproductive process in a multicellular organism	11	0.0463
GO:0051254–positive regulation of RNA metabolic process	11	0.0432
GO:0007610–behavior	13	0.0063
GO:0010628–positive regulation of gene expression	13	0.0294
GO:0045935–positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	13	0.0467
GO:0007267–cell-cell signaling	14	0.0168
GO:0006357–regulation of transcription from RNA polymerase II promoter	15	0.0327
GO:0050890–cognition	18	0.025
GO:0050877–neurological system process	26	0.0019
b).T1		
GO:0043523–regulation of neuron apoptosis	5	0.0163
GO:0051960–regulation of nervous system development	7	0.0179
GO:0051129–negative regulation of cellular component organization	6	0.0189
GO:0051385–response to mineralocorticoid stimulus	3	0.0194
GO:0007017–microtubule-based process	8	0.0199
GO:0050767–regulation of neurogenesis	6	0.034
GO:0006575–cellular amino acid derivative metabolic process	6	0.034
GO:0001952–regulation of cell-matrix adhesion	3	0.034
GO:0035270–endocrine system development	4	0.0385

els[8-11] and human studies[12-14]. To date, relatively little data describing the genomic response to IPC in humans has been reported. Therefore, to identify the genomic response induced by IPC, we analyzed gene expression patterns in a cohort of TKA patients from the study of Murphy et al[15]. Of these genes queried on the Affymetrix HG-U133A Plus 2.0 microarray, we identified 263 DEGs at T0 and 266 DEGs at T1 respectively compared to their control groups.

Functional enrichment analysis showed that the most significant function of those DEGs at T0 was related with neurological system process. The most significant function of DEGs at T1 was related with regulation of neuron apoptosis and all the DEGs are down-regulated. Our results indicate that nervous system may contribute to the protective signaling provided by IPC. The release of neuropeptides during IPC suggests that the activation of sensory and autonomic nerves may contribute to the protective signaling provided by IPC[23,24]. Calcitonin gene-related peptide (CGRP) is one of the most powerful endogenous vasodilator peptides yet described[25], and the release of CGRP from capsaicin-sensitive primary sensory neurones could be a direct consequence of hypoxia in hypoperfusion states[26]. Specifically, the chemosensitive C-fibre afferents expressing the capsaicin (transient receptor potential vanilloid type-1 receptor, TRPV1) receptors are activated and/or sensitized by many chemical stimuli released during IR, such as lactate, protons and inflammatory mediators[27]. Moreover, some of the remote protective effects of IPC might also be attributed to afferent C-fibre-derived mediator release due to an enhanced NO synthesis[28] and the inhibition of tumor necrosis factor-alpha production[24].

The periosteum is densely innervated by nociceptive C-fibre afferent nerves[29]. Hartmann et al[30] have revealed that activation of the chemo- (capsaicin-) sensitive afferent nerves is involved in the mechanisms of microcirculatory anti-inflammatory protection provided by limb IPC. Controlled activation of chemosensitive C-fibres or the CGRP receptors by the induction of IPC or other means may furnish therapeutic benefit by ameliorating the periosteal microcirculatory consequences of tourniquet ischaemia. Ogawa et al[31] also clearly showed that IPC pre-treatment protects kidneys against ischaemia-reperfusion injury, and the effects are, at least in part, mediated by sympathetic nerves, as the protective effects were abolished by denervation.

The most interesting result in the current study is that the expression of FAM125B at T0 is different from T1. The protein MVB12 encoded by FAM125B is a component of the ESCRT-I complex, a heterotetramer, which mediates the sorting of ubiquitinated cargo protein from the plasma membrane to the endosomal vesicle. ESCRT complexes are responsible for the endosomal sorting of ubiquitinated membrane proteins into multivesicular bodies (MVBs) en route to their lysosomal degradation[32]. Mvb12 is a novel member of ESCRT-I involved in cargo selection by the multivesicular body pathway[33]. Sorting of endocytosed cell surface proteins through the MVB pathway plays an essential role in maintaining proper cell surface protein composition. In addition, this pathway acts to quickly and dramatically change the protein composition of the cell surface during processes such as differentiation and adaptation. For example, growth factor signaling is regulated in part by the controlled endocytosis and degradation of growth factor receptors, a process that is disrupted in pathological states of uncontrolled cellular proliferation in certain types of cancer. In addition to protein degradation, the MVB pathway also functions in the targeting and transport of lysosomal resident proteins[34]. Furthermore, MVBs have been shown to play an essential role in the immune response of mammals where they function in antigen presentation by dendritic cells and in the formation of exosomes[35]. In the present study, the up-regulation of FAM125B at T1 may be a protective mechanism which can degrade some harmful proteins produced during IPC through binging with other proteins. Our motif scan results showed that SH3 domain of FAM125B can bind with ABL1, ITK, NCK1, GRB2, PLCG1, AMPH, and SORBS1.

Conclusions

The findings of this study show that IPC induces a protective genomic response in TKA patients. The protective effect of IPC was associated with altered expression of genes involved in neurological system process and regulation of neuron apoptosis. The dynamic expression of FAM125B can be a supervised marker during the surgery. Results of this study indicate that IPC may be of potential benefit in this and other musculoskeletal conditions.

Acknowledgments

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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