An “up, no change, or down” system: time-dependent expression of mRNAs

in contused skeletal muscle of rats used for wound age estimation

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Abstract

The combined use of multiple markers is considered a promising strategy in estimating the age of wounds. We sought to develop an “up, no change, or down” system and to explore how to combine and use various parameters. In total, 78 Sprague Dawley rats were divided randomly into a control group and contusion groups of 4-, 8-, 12-, 16-, 20-, 24-, 28-, 32-, 36-, 40-, 44-, and 48-h post-injury ($n = 6$ per group). A contusion was produced in the right limb of the rats under diethyl ether anesthesia by a drop-ball technique; the animals were sacrificed at certain time points thereafter, using a lethal dose of pentobarbital. Levels of PUM2, TAB2, GJC1, and CHRNA1 mRNAs were detected in contused muscle using real-time PCR. An up, no

change, or down system was developed with the relative quantities of the four mRNAs recorded as black, dark gray, or light gray boxes, representing up-, no change, or down-regulation of the gene of interest during wound repair. The four transcripts were combined and used as a marker cluster for color model analysis of each contusion group. Levels of PUM2, TAB2, and GJC1 mRNAs decreased, whereas that of CHRNA1 increased in wound repair ($P < 0.05$). The up, no change, or down system was adequate to distinguish most time groups with the color model. Thus, the proposed up, no change, or down system provide the means to determine the minimal periods of early wounds.

Key words: Contused skeletal muscle, forensic pathology, marker, real-time PCR, wound age estimation

Introduction

The determination of wound age is important in medicolegal traumatology, but remains a challenging application in forensic medicine. In forensic pathology, it is often important to estimate wound age as accurately as possible, especially when there are several injuries that were caused by multiple abusers or vehicles within narrow intervals, in view of the value of wound age in the reconstruction of crime scenes and the determination of the cause of death [1]. However, as yet, no parameter has been shown to be sufficiently accurate to distinguish the minimal interval of an early wound in court.

Many studies have been conducted to assess wound vitality and estimate wound

ages over several decades, and multiple parameters have been tested with respect to time- dependent appearance in wound healing [2]. Unfortunately, no reproducibility data was reported in most studies [3]. An increasing number of forensic pathologists are conscious that no single parameter alone will provide forensic safety; a combination of multiple data points has recently been considered as a promising way to narrow the margin of error when determining the age and vitality of a wound [4, 5]. However, neither a system nor a strategy has yet been proposed as to how to combine and use such markers. In previous study, the appropriate references[6] or target genes[7, 8] have been determined to estimate the wound age.

In this study, transcripts of Pumilio 2 (PUM2), transforming growth factor- β activated kinase 1 binding protein 2 (TAB2), gap junction protein gamma 1 (GJC1 or connexin 45), and the nicotinic cholinergic receptor alpha 1 (CHRNA1) were detected in contused skeletal muscle of rats using real-time fluorescence quantitative PCR (real-time PCR), the above four genes had been identified as the different expressed genes in normal and contused skeletal muscle of rats using the digital gene expression in our previously study. In order to develop a theoretical model to distinguish post-injury time periods by combination of several mRNAs, a system was developed that simplifies the relative quantity of mRNAs as up-, no change, or down-regulated before combining the four mRNAs as a marker cluster.

Materials and Methods

Animal model and tissue preparation

All procedures were performed according to the “Guiding Principles in the Use and Care of Animals” (NIH Publication No 85-23, Revised 1996) and were approved by the Institutional Animal Care and Use Committee of Shanxi Medical University of China [Batch number of rats: SCXK (Jin) 2009-0001)]. Animals received humane care in accordance with the principles of the Guide for the Care and Use of Laboratory Animals protocol, published by the Ministry of the People’s Republic of China (issued on June 4, 2004). The laboratory personnel attended and completed training on how to use experimental animals ethically and were allowed to conduct this study by the Committee above.

In total, 78 Sprague Dawley male rats, 10-12 weeks old, weighing 200–250 g, were kept pairwise in stainless steel wire cages at 22–24°C at a relatively humidity of 40–60%, with free access to food and water. The rats were divided randomly into a control group ($n = 6$) and the 4-, 8-, 12-, 16-, 20-, 24-, 28-, 32-, 36-, 40-, 44-, and 48-h contusion groups ($n = 6$ each).

After a 12/12-h light/dark cycle, the rats were anesthetized with diethyl ether. Their right posterior limbs were then shaved; residual hair was removed using a depilatory agent (Nair; Carter-Wallace, New York, NY, USA) [9]. Subsequently, the rats were placed on a foam bed. A 100-g weight fell freely onto the right posterior limb of the rats from a height of 200 cm across a clear Lucite guide tube [7]. The size of impact interface of the counterpoise was about 4.9 cm^2 . The energy transmitted to the right posterior limb was calculated to be 1.96 J as calculated by the equation: $E = mgh$. After injury, the rats were allowed to recover from the anesthetic in clean cages

with free access to food and water. The rats of control group were not contused but shaved.

The rats were sacrificed at 4-, 8-, 12-, 16-, 20-, 24-, 28-, 32-, 36-, 40-, 44-, and 48-h post-contusion under anesthesia with a lethal dose of pentobarbital (350 mg/kg body weight, intraperitoneal injection). A muscle sample (~100 mg) was dissected from the right posterior limb musculi quadriceps femoris and cut into two part. All of the contused muscles were immediately snap-frozen in liquid nitrogen for real-time PCR. Muscle specimens were also dissected from the control group.

Total RNA preparation

Total RNA was extracted from the snap-frozen muscle specimens (~50 mg) using TRIzol reagent (CoWin Biotech, Beijing, China), following the manufacturer's protocol. Subsequently, the concentration (ng/μL) and purity of the total RNA were evaluated using ultraviolet-visible (UV-Vis) spectrophotometry (Ultrospec 4300 pro: Biochrom, Cambridge, UK). Only RNAs with OD₂₆₀/OD₂₈₀ ratios of 1.8–2.0 were used in subsequent experiments. The integrity of the RNA was also estimated by 1.5% formaldehyde denaturing agarose gel electrophoresis.

Real-time PCR

The first-strand cDNA was synthesized using the Prime Script RT-PCR Kit (Takara Biotech, Dalian, China) according to the standard protocol. For the reverse transcription, 0.4 μg of total RNA was used in a final reaction volume of 10 μL.

Primers and TaqMan fluorescent probes were designed using the AlleleID 6 software (Premier Biosoft International, Palo Alto, CA, USA), checked using BLAST,

and synthesized by Invitrogen Trading Co. (Shanghai, China; Table 1). Ribosomal protein L13 (RPL13) and ribosomal protein L32 (RPL32) were taken as reference genes, which were probed with fluorescent FAM and Cy5, respectively, because both of the RPL13 and RPL32 mRNAs have been confirmed to be the most stably expressed genes in contused skeletal muscle [6]. The gene of interests of PUM2 and CHRNA1 mRNAs were probed fluorescently with HEX, and the TAB2 and GJC1 mRNAs were probed with ROX. To avoid the amplification of genomic DNA, all sequences between the sense primers and antisense primers contained genomic DNA introns.

A 25- μ L reaction mixture was prepared for real-time PCR amplification using the Premix Ex Taq Kit (Takara Biotechnology Co. Ltd., Dalian, China), containing 12.5 μ L Premix Ex Taq, 0.5 μ L RNase-free H₂O, 0.5 μ L (10 μ M) each of the TaqMan fluorescent probes and forward and reverse primers of a gene of interest (GOI) and the two reference genes, 2.5 μ L 10% DMSO, and 2.0 μ L cDNA. Amplification was performed with a Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA), with one round of pre-denaturation at 95°C for 10 s, and 40 rounds of denaturation at 95°C for 5 s followed by annealing and extension at 60°C for 40 s. Triple fluorescent signals (FAM, Cy5, and HEX or ROX) were measured synchronously at the end of each cycle. Fluorescence curves of the PCR products were evaluated using MxPro QPCR software (Stratagene).

For amplification efficiency, the synthesized cDNAs were diluted serially five-fold (1, 1:5¹, 1:5², 1:5³, and 1:5⁴) or two-fold (1, 1:2¹, 1:2², 1:2³, and 1:2⁴) in

EASY Dilution solution (Takara Bio Inc.). Sterile purified water was used as the negative control. All PCRs were repeated twice.

Histological Analysis

Another 39 rats (13 groups, $n=3$) were contused for hematoxylin and eosin (H&E) staining. The animals were sacrificed at each time point consistent with previously study, and were perfused intracardially with heparinized saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The samples were collected, processed in an automated tissue processor, and embedded in paraffin. Four-micron-thick coronal sections were cut on a microtome and stained with H&E. The sections were observed using optical microscopy (BX61, Olympus, Tokyo, Japan).

Statistical analyses

The expression levels of GOIs were quantified using the statistical model of $(1 + \text{Eff.})^{-\Delta\Delta C_t}$, based on the normalized factor of the geometric mean of the level of reference genes (RPL13 and RPL32 mRNAs) [10]. A one-way analysis of variance (ANOVA) and the Student–Newman–Keuls (SNK) test were used. Data are presented as the mean $\pm$ SD. P -values < 0.05 were considered to indicate statistical significance.

Results

Evaluation of histological changes in wound repair

The control groups showed normal histological in the quadriceps femoris of right posterior limb (Fig.1a). Tissue from animals receiving trauma showed pathological

alterations early after injury. Briefly, the skeletal muscle cell edemas were detected at first 4 hours after injury (Fig. 1b). With the continuous extension of wound, there are a large number of necrotic skeletal muscles fib, neutrophile granulocyte and mononuclear cells infiltrated in the injured tissue (Fig. 1c~i). At 36- h after injury, a small amount of fibroblasts were observed, and which were increased gradually in the injured area (Fig. 1j~m).

Amplification efficiency of the target and reference genes

The purity of the total RNA was checked by seeing that the OD₂₆₀/OD₂₈₀ ratio was within 1.8–2.0 in all samples. The signals of the 28s rRNA and 18s rRNA were also checked; the brightness of the 28s rRNA was about double that of the 18s rRNA.

Standard curves of the target and reference genes were plotted based on the amplification curves when taking the serially diluted cDNA as templates. The amplification efficiencies (Eff.) of the PUM2, TAB2, GJC1, and CHRNA1 were 94.8%, 98.3%, 100.0%, and 100.5%, respectively, all within the 80–120% range, as shown in Fig. 2; all R² (Rs_q) values were close to 1.00.

Expression levels of the PUM2, TAB2, GJC1, and CHRNA1 mRNAs in wound repair

PUM2, TAB2, GJC1, and CHRNA1 mRNAs showed changes in expression during wound repair (Fig. 3, Table 2). Compared with the control group, the levels of PUM2 mRNA were lower at 8, 20, 24, 36, and 40 h ($P < 0.05$). The levels of TAB2 mRNA were lower at 12, 20, 36, 40, and 44 h ($P < 0.05$). GJC1 mRNA showed lower levels at 4, 8, 12, 16, 20, 24, 28, 36, 40, and 44 h ($P < 0.05$). However, the levels of

CHRNA1 mRNA were higher at 16, 24, 36, 40, 44, and 48 h ($P < 0.05$).

A system of “up, no change, or down” for PUM2, TAB2, GJC1, and CHRNA1 mRNAs

The relative quantities in Table 2 were simplified to up-, no change, or downregulated for each GOI and recorded as black, dark gray, or light gray, respectively (Table 3).

The elements of black, dark gray or light gray were then combined and listed one by one. For each contusion group, there was a certain color model; for example, the color-model was dark-dark-light-dark gray at 4 h. The color model varied among the contusion groups. Except for the dark-dark-light-dark model at 4 and 28 h and the light-light-light-black model at 36 and 40 h, other color models differed from one post-injury interval to another.

Discussion

Multiple approaches and techniques have been used to determine wound vitality and time periods. In addition to proteins [11], transcripts have also been used as markers to analyze the earlier stages of vitality in recent decades [12-14]. It would seem to be more appropriate to assess mRNA, by RT-PCR, in early wound age estimations, because mRNA appears earlier than the protein it encodes [15]. However, multiple appropriate reference genes are needed to quantify targets accurately when using real-time PCR [16, 17]. Thus, we explored reference genes in a previous study; RPL13 and RPL32 were found to be the most stably expressed genes in contused skeletal muscle [6].

Thus, the geometric means of the reference genes of RPL13 and RPL32 was used as a normalization factor to quantify the GOIs in this study. Normalized to these reference genes, all of the GOIs changed in the contused muscles of rats as time elapsed. The levels of PUM2, TAB2, and GJC1 mRNAs decreased, whereas the CHRNA1 mRNA level increased during wound repair within 48-h post-injury (Fig. 3), indicating their potential for use in wound age estimation.

In this study, the expression level of PUM2 mRNA was decreased, which was likely to related to the regulation of wound healing, as mammalian Pumilio proteins could play an important role in translational regulation of embryogenesis and cell development and differentiation[18]. TAB2 is involved in stress response, inflammation, and apoptosis to protect tissues from stressors [19]. The decreased TAB2 mRNA probably reduced the anti-inflammation and promoted the degradation of contused muscles. Cx45 is involved in the process of proliferation, differentiation, and merging in muscles [20]. In this study, Cx45 mRNA was down-regulated, which was beneficial to decrease the communication of cells and limit the lesion. CHRNA1 is a kind of cholinergic receptor (AChR). Combined and activated by ACh, the CHRNA1 could evoke action potentials and excite muscle cells [21]. The increased CHRNA1 mRNA was likely to prepare for the muscle repair.

In forensic pathology, several strategies have been developed to distinguish vital from post-mortem lesions and to determine the minimal interval of early wounds. Forensic pathologists have been searching for parameters that are more objective, sensitive, specific, accurate, and stable. Multiple markers and candidates have been

detected at both the protein [22] and mRNA levels [23, 24]. Among them, cytokines and growth factors were considered especially promising [25], such as CD15, TNF α , IL-6, IL-1 β , TGF- α , and TGF- β 1 [3]. Ratios of mRNAs and positive cells have also been used as observation indexes. For example, it has been reported that a MMP-9/MMP-2 ratio of >0.84 indicates a skin wound-healing age of 5 days or less [26] and an oxygen-regulated protein 150 (ORP150) positive ratio of >50% indicates a wound age of 7–14 days [27].

Among these strategies, combinations of values have been considered promising in recent years. An increasing number of studies has proposed that combinations of markers and the use of several methods could narrow error margins and minimize ranges of wound age estimations [28]. For example, Takamiya detected 27 cytokines simultaneously with a multiplexed bead-based immunoassay to estimate the timing of cerebral wounds [29]. Kameyama assessed numerous markers involved in angiogenesis and lymphangiogenesis by real-time RT-PCR, *in situ* hybridization, and immunohistochemistry in the early stages of rat skin incision wounds for forensic wound evaluations [30]. Nevertheless, how to combine and use various markers remains unknown to date.

In this study, we developed a system that took the PUM2, TAB2, GJC1, and CHRNA1 mRNAs as a cluster of markers, and recorded their relative quantity as black, dark, or light gray boxes, representing the up-, no change, or downregulation, respectively, of a gene of interest. The system could distinguish most periods of the early wounds, because the color models differed among time groups (Table 3).

Two strategies were used in the up-, no change, or downregulation system: one was simplification of the relative quantities of mRNAs of GOIs to qualitative data and the other was that the combination of the four mRNAs were combined and used as a marker cluster, making a promising model to narrow the age of early wounds in forensic traumatology. The combination of several markers made it possible to multiply the volume of data. It should have been 81 ($3^4 = 81$) color models with a combination of four mRNAs in an up, no change or down system, whereas there were only 10 in this study (Table 3).

In fact, the up, no change, or down system was inadequate at 36 h from 40 h, because the groups shared the same color model of light-light-light-black (Table 3). Nevertheless, the wound periods of 36 h and 40 h could be distinguished when considering the relative levels of CHRNA1 mRNA, because the level of CHRNA1 mRNA was 1.99-fold at 36 h, significantly lower than 3.74-fold at 40 h, versus the control group ($P < 0.05$). Unfortunately, the 4-h and 28-h groups could not be discriminated based on the color model or relative quantities of the GOIs. More markers and/or technologies are required in this case.

Thus, the markers should have different expression levels when used in an up, no change, or down system. In the present study, the PUM2, TAB2, GJC1, and CHRNA1 mRNAs did show differing time-dependent expression patterns (Fig. 3), likely related to their differing structures and functions. PUM2 acts as a translational inhibitor in several organisms [31], TAB2 plays a role in several cell types including endothelial and immune cells [32], GJC1 is involved in the structure of gap junctions [33], while

CHRNA1 participates in neurotransmitter-gated ion channels [21]. There were 10 color-models among the 12 contusion groups by combining the four markers, which was adequate to distinguish most contusion groups (Table 3).

This study has several limitations. The expression level of the GOIs in post-mortem muscle is currently unknown. Additionally, the animal model used may not be fully reflective of the human situation. These issues should be explored in future work. Despite these drawbacks, the proposed up-, no change, or down-regulation system shows great potential in applications requiring estimation of early-wound time periods.

Conclusions

To explore how to combine and use various markers for wound age estimation, we took PUM2, TAB2, GJC1, and CHRNA1 mRNAs as a marker cluster. On the basis that the four transcripts showed differing time-dependent expression patterns in contused muscle healing, we developed an up-, no change, or down-regulation system by simplifying and combining the relative quantities of four mRNAs. The system was adequate to distinguish most contusion time periods in this study. Thus, the up-, no change, or down-regulation system developed has potential for determining the minimal periods of early wounds.

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Figure legends

Figure 1. Histological analysis of contused muscle during wound repair.

Light micrographs of sections of quadriceps femoris muscle. (a) The morphology of normal skeletal muscles as a control. (b, c) The skeletal muscle cell edemas, necrosis, and erythrocyte exudation were presented. (d, e) A lot of inflammatory cells (predominantly polymorphonuclear leukocyte) were detected in the injury area. (f, g, h, i) A large number of monocytes and neutrophils exudate, but erythrocyte exudation reduced. (j, k) Mononuclear cells gradually increased and a small amount of fibroblasts appeared. (l, m) At 48 hours after injury, fibroblasts increased further (mainly in the interstitial space), and the mount of the neutrophil decreased.

Figure 2. Standard curves of the target and reference genes

Standard curves were plotted based on the amplification of serially diluted template cDNA. The reference genes of RPL13 and RPL32 mRNAs were probed with fluorescent FAM and Cy5, respectively. PUM2 mRNA was probed fluorescently with HEX (a), TAB2 with ROX (b), GJC1 with ROX (a), and CHRNA1 with HEX (d).

The amplification efficiency of each gene of interest was similar to that of the reference genes.

Figure 3. Real-time PCR analyses of PUM2, TAB2, GJC1, and CHRNA1 mRNA expression in contused muscle

In total, 78 Sprague Dawley male rats, 10-12 weeks old, weighing 200–250 g, were divided into the control group ($n = 6$) and 4-, 8-, 12-, 16-, 20-, 24-, 28-, 32-, 36-, 40-, 44-, and 48-h contusion groups ($n = 6$ each). A contusion was produced in the right

limb of the rats before they were sacrificed at certain time points. Levels of PUM2, TAB2, GJC1, and CHRNA1 mRNAs were detected in contused muscle using real-time PCR. Compared with the control group, the levels of PUM2 mRNA were lower at 8, 20, 24, 36, and 40 h (a). The levels of TAB2 mRNA were lower at 12, 20, 36, 40, and 44 h (b). GJC1 mRNA showed lower levels at 4, 8, 12, 16, 20, 24, 28, 36, 40, and 44 h (c). However, the levels of CHRNA1 mRNA were higher at 16, 24, 36, 40, 44, and 48 h (d).

Data are presented as means $\pm$ SD; * $P < 0.05$.

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a **b** **c** **d**

Tables

Table 1. Primers and probes used for real-time PCR

Gene symbol	GenBank accession no.	Strand	Nucleotide sequence	Location	Reporter	Quencher	Size of PCR products (bp)
PUM2	NM_01106715.1	Sense	AGCTGCATTTGTGCCAAATCC	982-1002	HEX	BQH	121
		Probe	AGTGCTGCTCCTCCAGGGACCGA	1013-1035			
		Antisense	CTGAGGTGGAACCACTGCTG	1102-1083			
TAB2	NM_01012062.1	Sense	ATGACCTGCGACAAAAGTTCC	211-231	ROX	BQH	127
		Probe	AACAGCACAGCAGGCATCCAGGTT	305-282			
		Antisense	CCGTAAAGATACCTTGTACTCTCC	337-314			
GJC1	NM_001085381.1	Sense	TAACAGGGCAAACCAATTCCAC	383-404	ROX	BQH	111
		Probe	TTGGAGCTTCCTGACTCGCCTGCT	414-437			
		Antisense	TCAGCACAGTGAGCCAGATC	493-474			
CHRNA1	NM_024485.1	Sense	TCCTTGTTGATGTAGCTCAATGAG	1270-1291	HEX	BQH	163
		Probe	AACACAGCCAGCGTCCCGATGAGA	1398-1375			
		Antisense	AGAGACCATGAAGTCAGACCAG	1432-1409			
RPL32	NM_013226.2	Sense	ATCTGGCCCTTGAATCTTCTCC	99-121	Cy5	BQH	115
		Probe	TGTCGATGCCTCTGGGTTTCCGCC	182-159			
		Antisense	AGAGGACCAAGAAGTTCATCAGG	213-192			
RPL13	NM_031101.1	Sense	TCGTGAGGTGCCCTACAGTTAG	209-230	FAM	BQH	107
		Probe	CACACCAAGGTCCGGGCTGGCAG	235-257			
		Antisense	GGTGCGTGCCATTTTCTTGTG	315-295			

Table 2. Levels of PUM2, TAB2, GJC1, and CHRNA1 mRNAs in contused**skeletal muscle**

Group	PUM2	TAB2	GJC1	CHRNA1
Con	1.00±0.58	1.00±0.50	1.00±0.15	1.00±0.44
4 h	0.67±0.35	0.74±0.18	0.57±0.19*	1.20±0.56
8 h	0.15±0.10*	0.68±0.30	0.29±0.17*	0.47±0.21
12 h	0.62±0.48	0.51±0.16*	0.24±0.10*	1.45±0.66
16 h	0.65±0.25	0.68±0.32	0.54±0.17*	2.18±1.07*
20 h	0.15±0.10*	0.45±0.11*	0.32±0.16*	1.35±0.59
24 h	0.43±0.32*	0.72±0.28	0.42±0.19*	2.08±0.61*
28 h	0.80±0.45	0.66±0.26	0.61±0.19*	0.74±0.36
32 h	0.70±0.29	0.85±0.43	0.80±0.29	0.96±0.36
36 h	0.28±0.18*	0.36±0.18*	0.14±0.08*	1.99±0.64*
40 h	0.51±0.30*	0.21±0.06*	0.75±0.23*	3.74±0.92*
44 h	0.62±0.16	0.19±0.04*	0.50±0.24*	4.38±1.96*
48 h	0.99±0.54	0.70±0.46	0.78±0.30	3.19±1.43*

Note: Data are presented as means ± SD. *n* = 6 per group. **P* < 0.05.

Table 3. Up-, down- or no change system for PUM2, TAB2, GJC1, and

CHRNA1 mRNAs

Groups	Con	4 h	8 h	12 h	16 h	20 h	24 h	28 h	32 h	36 h	40 h	44 h	48 h
PUM2													
TAB2													
GJC1													
CHRNA1													

Note: The light gray box represents down-regulation, the dark gray box represents no change, and the black box represents up-regulation of the target gene compared with the control group. Data are means $\pm$ SD. * $P < 0.05$.