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Brief Communication

# Gene response of mouse skin to pressure injury in the neck region

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**Abstract**

We analyzed the gene expression pattern in mouse skin following compression of the neck by fluorescent mRNA differential display (FDD-PCR). RNA was isolated from the skin tissue immediately or 30 min after ligation at the neck for 25 min resulting in death (Group A-0, Group A-30). Control mice underwent no compression of the neck and were killed by decapitation (Group C-0, Group C-30). FDD-PCR and sequence analysis revealed that the faciogenital dysplasia gene (Rho member families) and secreted frizzled related protein 1 (modulator of Wnt networks) were enhanced only in the Group A-30. In addition, common salivary protein 1 and mouse 0 day neonate skin cDNA clone z4631433E12 from the RIKEN full-length enriched library were also induced in Groups A-0 and A-30. These findings were consistent with the results of statistical analysis by ANOVA following quantitative real-time PCR. No differences in band pattern were observed between Group C-0 and Group C-30. Therefore, our findings suggested that the altered expression of genes was associated with signal transduction. The results may contribute to clarifying the pathophysiology of compression of the skin and may be useful in the diagnosis of suffocation.


*Keywords:* Suffocation; Pressure injury; Differential display-PCR; Gene expression; RT-PCR; Mouse skin

## 1. Introduction

In forensic practice, the diagnosis of compression on the neck is very complex and controversial, as clear and unambiguous findings at autopsy are frequently absent. In such cases, it is easy to diagnose compression of the neck using 'the compression marks reaction'. In 1955, Gotoda [1] first reported that compressed skin lesions showed metachromasia together with irregular patterns of elastic fibres, which could be applied to the practice of forensic medicine, especially in the diagnosis of strangulation cases. And he termed this phenomenon 'the compression marks reaction'. Furthermore, several forensic medicine textbooks have noted that histological examination using Azan or Weigert's stains is useful for the detection of pressure on the neck when a compressive neck injury is suspected [2]. However, it is sometimes difficult to evaluate the findings obtained from these staining methods, since pseudo-positive reactions may be found on uncompressed skin lesions. In addition, as Gtoda described [1], this method has fatal fault that positive-reactions by this method are observed even in strangulated skin after death.

Although there are no reports that mechanical compression induce gene expression, we expected that a mechanical compression might induce some transcriptional responses in diverse cells of the skin tissues. Differential display (DD), which was first developed by Liang et al. [3], detects mRNA variations among many different kind of tissues and cells at the same time, compared with the subtraction method or micro array analysis. Furthermore, as DD uses polymerase chain reaction, the technique is more highly sensitive than the other methods and we speculated that we could detect some slightly alterations of gene expression in compressed skin tissue using this method. In this study, we investigated gene expression after pressure on the neck using highly efficient experimental procedures involving fluorescence differential display PCR (FDD-PCR) and quantitative real-time PCR.

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## 2. Materials and methods

### 2.1. Animal treatment and RNA isolation from mouse skin

Seven-week old male BALB/c mice were obtained from SLC, Inc (Sizuoka, Japna). These mice were anaesthetized by an intra-peritoneal injection of pentobarbital (50 mg/kg), and, thereafter, compression was applied to the neck by ligation with a string (tuzurihimo, Yamautiseihimo), for 25 min resulting death. The neck skin was dissected either immediately after death (Group A-0) or 30 min later (Group A-30). Control mice were killed by decapitation without undergoing neck compression and the neck skin was then dissected as described (Group C-0 and Group C-30). The Animal Care Committee of Nagasaki University approved this research protocol. Total RNA was extracted from the skin using ISOGENE (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Possible DNA contamination was removed by treatment with RNase-free DNase I (Invitrogen Corp., Carlsbad, CA, USA).

### 2.2. Histological examination

The skin was also taken from the compressed neck for the purposes of comparative histochemical examination. The tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and 8 mm-thick sections were prepared. Azan stain was employed to evaluate compression.

### 2.3. Fluorescence differential display PCR

Fluorescence differential display PCR (FDD-PCR) was performed by the method of Yoshikawa et al. [4] with a slight modification using Enzyme Set-FDD (TAKARABIO INC., Otsu, Japan). Three mice were used in each group. The total RNA (400 ng) was reverse transcribed using two ROX-labelled 3′-anchored oligo-dT primers (T13-15GA and T13-15GG). The 48 different sets of primers—the combination of 24 arbitrary primers (TAKARABIO INC., Otsu, Japan; No 1, GATCATAGCC; No 2, CTGCTTGATG; No 3, GATCCAGTAC; No 4, GATCGCATTG; No 5, CTTGATTGCC; No 6, AGGTGACCGT; No 7, GATCATGGTC; No 8, TTTTGhGCTCC; No 9, GTTTTCGCAG; No 10, GTTGCGATCC; No 11, GATCTGACAC; No 12, CTGATCCATG; No 13, TGGATTGGTC; No 14, GGAACCAATC; No 15, GATCAATCGC; No 16, TCGGTCATAG; No 17, GATCTGACTG; No 18, TCGATACAGG; No 19, TACAACGAGG; No 20, GATCAAGTCC; No 21, GATCTCAGAC; No 22, AGCCAGCGAA; No 23, CAAACGTCGG and No 24, CTTTCTACCC) and the 3′-anchored oligo-dT primers (see above)—were used for PCR amplification of cDNA. PCR amplification was carried out in a total volume of 20 μl, according to the manufacturer's instructions, using a DNA thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, CA, USA). The PCR cycling conditions were 94 °C for 2 min, 40 °C for 5 min and 72 °C for 5 min, then 34 cycles of 95 °C for 30 s, 40 °C for 2 min and 72 °C for 1 min, followed by 72 °C for 5 min. The amplified cDNAs were separated on 4% polyacrylamide gel containing 7 M urea using a 200×475 mm glass plate. After electrophoresis, fluoroimages were scanned with an FMBIO II Multi-View fluoroimage analyzer (TAKARABIO INC., Otsu, Japan). The bands showing differential expression were cut out.

### 2.4. Re-amplification and rapid selection of the desirable cDNA fragments

The excised bands of interest were eluted in 50 μl of TE buffer at 100 °C for 10 min. The solutions (2 μl each) were re-amplified in the same reaction mixture as in the first PCR with 10 cycles and 10 μl of each product were loaded on a 3% agarose gel containing H.A.-yellow (TAKARABIO INC., Otsu, Japan). The cDNA fragment was recovered from the band on the gel and re-amplified again and a third round of PCR was performed with 10 μl of each product and then separated on a 3% agarose gel containing H.A.-Red (TAKARABIO INC., Otsu, Japan). The main product was recovered from the gel and the recovered fragment was purified, using QIAEX II (QIAGEN, GmbH, Germany).

### 2.5. Sequencing of differentially displayed products

The direct sequence primed with an arbitary primer was performed using BigDye® Terminator Cycle Sequencing Ready Reaction Kit V3.0 (Applied Biosystems, CA, USA). The nucleotide sequences were tested for homology, to known sequences in the DNA database of the DDBJ by BLAST search and unknown sequences were electrically cloned by the method of the NCBI by UniGene.

### 2.6. Quantitative real-time PCR

Quantitative detection of GAPDH and four differentially expressed genes (S1–S4) was performed with ABI PRISM 7900HT (Applied Biosystems, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) according to the manufacturer's instructions. Five mice were used in each group. DNA-free total RNA (3.5 mg) were reverse transcribed using Superscript II and oligo (dT) 12–18. The primer sequences and conditions for each gene are summarized in Table 1. Before quantitative real-time PCR, agarose gel electrophoresis and the direct sequence following PCR were carried out to confirm the specificity of each primer set. All reverse transcription and quantitative real-time PCR assays were performed in duplicate for each mouse. The intra-assay variability was $<7\%$.