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Research article

A MICROARRAY GENE ANALYSIS OF PERIPHERAL WHOLE BLOOD IN NORMAL ADULT MALE RATS AFTER LONG-TERM GH GENE THERAPY

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Abstract: The main aims of this study were to determine the effects of GH gene abuse/misuse in normal animals and to discover genes that could be used as candidate biomarkers for the detection of GH gene therapy abuse/misuse in humans. We determined the global gene expression profile of peripheral whole blood from normal adult male rats after long-term GH gene therapy using CapitalBio 27 K Rat Genome Oligo Arrays. Sixty one genes were found to be differentially expressed in GH gene-treated rats 24 weeks after receiving GH gene therapy, at a two-fold higher or lower level compared to the empty vector group ($p < 0.05$). These genes were mainly associated with angiogenesis, oncogenesis, apoptosis, immune networks, signaling pathways, general metabolism, type I diabetes mellitus, carbon fixation, cell adhesion molecules, and cytokine-cytokine receptor interaction. The results imply that exogenous GH gene expression in normal subjects is likely to induce cellular changes in the metabolism, signal pathways and immunity. A real-time qRT-PCR analysis of a selection of the genes confirmed the microarray data. Eight differently expressed genes were selected as candidate biomarkers from among these 61 genes. These 8 showed five-fold higher or lower expression levels after the GH gene transduction ($p < 0.05$). They were then validated in real-time PCR experiments using 15 single-treated blood samples and 10 control blood samples. In summary, we detected the gene expression profiles of rat peripheral

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Abbreviations used: GH – growth hormone; GHD – growth hormone deficiency; rAAV – recombinant adeno-associated virus; rAAV2/1 – recombinant adeno-associated viral vectors pseudotyped with viral capsids from serotype 1

whole blood after long-term GH gene therapy and screened eight genes as candidate biomarkers based on the microarray data. This will contribute to an increased mechanistic understanding of the effects of chronic GH gene therapy abuse/misuse in normal subjects.

Key words: Microarray, Peripheral whole blood, Growth hormone gene therapy, Biomarker genes

INTRODUCTION

Growth hormone (GH) therapy has yielded favorable results for childhood growth disorders and adult growth hormone deficiency (GHD) [1, 2]. However, the potential for the application of GH recombinant products in patients with growth hormone deficiency (GHD) is limited by the high cost, attendant risks, and inconvenience of the necessary exogenous s.c. injections. Gene therapy yields a better long-term therapeutic effect than traditional drugs and methods thanks to its alternative approach to the efficient delivery of proteins [3, 4]. However, this also offers the opportunity for the misuse/abuse of GH gene therapy by normal subjects seeking its anabolic or lipolytic effects [5, 6].

Exogenous GH in normal subjects can induce potentially uncontrollable GH hypersecretion, as seen in patients with GH-secreting pituitary adenomas [7]. Moreover, the mechanisms underlying the responses of normal animals to long-term GH gene therapy are not fully understood. The potential toxicity of the gene vector cannot be ignored. In addition, the product of GH gene therapy is the same as the endogenously produced protein, and therefore cannot be detected by a routine urine or blood test. The detection of the associated viral particles might be effective, but would involve muscle biopsies, which are unsuitable for normal individuals [8, 9]. Thus, gene misuse/abuse is more deceptive and even more difficult to detect than conventional drug abuse [5, 6].

The changes in certain genes detected via microarray analysis might decipher the molecular mechanisms involved in physiological and pathological processes in various cells or tissues [10, 11]. Such genes could serve as surrogate markers for the induced modifications, and could obviate the need for muscle biopsies. In this article, we determined the gene expression profiles of rat peripheral whole blood after long-term GH gene therapy using a microarray to evaluate the potential effects of GH gene abuse/misuse in animal models. Eight genes were screened as candidate biomarkers based on the microarray data and validated via real-time qRT-PCR experiments.

MATERIALS AND METHODS

Construction and production of the rAAV2/1 vector containing *GH1*

GH1 is a human GH gene (GenBank accession No. NM_000515). A 677-bp segment of *GH1*, including the 651-bp cds sequence, was cloned from a polymerase chain reaction (PCR) product using the primers 5'-GCCA

GAATTCGCCACCATGGCTACAGGCTCCCGG-3' (forward primer) and 5'-CTGCGTCGACGAAGCCACAGCTGCCCTC-3' (reverse primer; the *EcoR*I and *Sal*I restriction sites are underlined) from the template of a pUC19 plasmid DNA containing the *GH1* cds sequence (Xinxiang Medical University, Henan Province, China). The *GH1* DNA fragment was digested with *Sal*I/*EcoR*I and inserted into the *Sal*I/*EcoR*I sites of the pSNAV2.0 vector (AGTC Gene Technology Co. Ltd., Beijing, China). Large-scale rAAV2/1 production and purification were done as previously described [12]. The viral genome particle titer (approximately 1.0×10^{12} v.g. ml^{-1}) was determined by a quantitative DNA dot blot method [13].

Animals and sample preparation

Forty nine Sprague Dawley adult male rats (350 ± 10 g, body weight) were obtained from the Animal Resource Center, General Hospital of PLA, Beijing, China. The rats were randomly divided into three groups. The rats in the GH gene therapy group (GH1 group; n = 23) received 2.5×10^{11} particles of rAAV2/1-CMV-GH1 delivered via an intramuscular injection into both of the hind limbs. The negative control group (NC group; n = 13) received placebo (saline) injections. The rats in the empty vector group (EV group; n = 13) were injected with a single dose of the empty rAAV2/1 vector.

Three rats from each group were selected randomly and sacrificed 6 weeks after the injections. The rest of the rats were sacrificed 24 weeks after the injections. Samples of rat peripheral whole blood were collected by cardiac puncture into EDTA vacutainer tubes (Becton Dickinson) and stored at 4ºC until processing (within 8 hours). Once the blood collection was finished, the fresh livers, hearts, kidneys, spleens and quadriceps were quickly removed, snap-frozen between blocks of dry ice and stored at -80ºC.

Detection of *GH1* mRNA expression

The total RNA was extracted from the quadriceps, livers, kidneys, spleens, and hearts using RNeasy Mini kits (QIAGEN). 5 ng of total RNA was subjected to semi-quantitative RT-PCR using the following primers for the *GH1* gene: 5'-ATCCAGGCTTTTTGACAA-3' (forward primer) and 5'-TGGAGGGTG TCGGAATAGAC-3' (reverse primer). Thermal cycling was performed as follows: 94ºC for 5 min, 35 cycles at 94ºC for 30 s, 57ºC for 25 s and 72ºC for 30 s, and a final step of 72ºC for 7 min. 6 µl of the 25-µl final PCR volume were analyzed by electrophoresis on 2.5% agarose gels. All of the values were normalized to an internal GAPDH standard.

Western blot analysis of human GH protein expression

Soluble protein was extracted from the collected quadriceps, livers, kidneys, spleens, and hearts using a protein extraction reagent (Pierce, USA), and the final protein concentrations were determined using a BCA assay (Pierce, USA) according to the manufacturer's instructions. Appropriate amounts of the protein extracts were fractionated by electrophoresis in 12% SDS-polyacrylamide gels

and transferred to nylon membranes. The nylon membranes containing the transferred proteins were pretreated with 1.0% nonfat dried milk in 50 mM Tris (pH 8.0) and then incubated overnight with a primary mouse monoclonal antibody against human growth hormone (1:1000 dilution, NeoMarkers, UK). The membranes were washed and incubated with anti-mouse secondary antibodies and the hGH immunoreactivity was visualized by exposing x-ray film to blots incubated with ECL reagent (Amersham Biosciences, USA). Gray-scale analyses were performed using Image-Pro Plus version 6.0.

Serum IGF-1 and haematology

The serum concentrations of IGF-1 (insulin-like growth factor 1) were quantified using specific enzyme-linked immunosorbent assay (ELISA) kits designed for rats (Uscnlife, China) as per the manufacturer's instructions. Each sample was assayed in duplicate.

Total and differential white blood cell (WBC) counts were performed using an ADVIA 120 (Bayer Diagnostics, Switzerland).

Microarray analysis

Whole blood RNA was isolated and purified with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and a NucleoSpin® RNA clean-up kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. The RNA concentration was evaluated via A260/A280 measurement. Only samples with an A260/A280 between 1.7 and 2.2 were considered suitable for use. The RNA quality was assessed by 1.0% agarose gel electrophoresis.

Five RNA samples from each treatment group were combined into one pool (with equal amounts of RNA from each sample). The untreated controls (samples from the NC group) were used as the baseline expression data. Fluorescence-labeled RNA fragments were produced from 1 μg of RNA by first synthesizing double-stranded cDNA, followed by *in vitro* transcription and fragmentation reactions. A hybridization cocktail, containing the fragmented cRNA, probe array controls, bovine serum albumin and herring sperm DNA, was prepared and hybridized at 42ºC for 16 h to the CapitalBio 27 K Rat Genome Oligo Array (CapitalBio, Beijing, China). The arrays were scanned with a confocal LuxScan™ scanner (CapitalBio Corporation, China) and the data from the obtained images was extracted with LuxScan 3.0 software (CapitalBio Corporation, China), employing the LOWESS normalization method. Dye swap experiments were performed to improve the accuracy of the data analysis, and the average ratio from the reciprocal replicates was used to evaluate the expression levels, which were then log transformed and calculated with the CapitalBio® Molecule Annotation System V4.0 (MAS 4.0).

Real-time quantitative RT-PCR confirmation

Fifteen candidate genes were randomly selected from the differently expressed genes that showed 2-fold higher or lower expression levels ($p < 0.05$) and

Tab. 1. The list of primer sets used for real-time PCR validation.

Gene	Accession No.	Primer sequence 5'→3'	Amplification size (bp)
Rp113	BC086577	F: GAATCGCTGTACTGTCTT G	183
		R: CTTGGGTCCACGGAGATGC	
Gpr109a	AB103062	F: GCTCCTCTACAAACACAACG	174
		R: CGAAGGCAACGGTTGATGC	
Hexim1	BC087133	F: GAAGCTGGTGAGGAAGACG	161
		R: CACTTCTCCAGCTCCAGGTAC	
Nfkbia	XM_343065	F: GCTTGGCGAAGTTCTAGGAAT	218
		R: TGCTGTGGTGCTAAGTATAC	
G0s2	H32223	F: GATGGAAAGTGTGCAGGAG	157
		R: AGCTGTGAAAGGGCTGCA	
Ddit3	BC100664	F: GAAACGGAAACAGAGTGGTCAG	255
		R: TGATGGTGCTGGGTACACT	
Ets1	L20681	F: GCTCCATTGTTTTCCCAGAG	247
		R: CAGAGAAAGCGGCATGC	
Lyar	BC079008	F: GAA GCGGCAAAAGCACTC AG	217
		R: CGCATGGTACTGAGCTATAAC	
RT1-Db1	NM_001008884	F: TGACAGTGGGAGAACGCCTT	298
		R: TCTATGCAGCAGACTGGGAGC	
Ncoa4	NM_001034008	F: GTT TCAGGACCACTCTTAG	263
		R: CACTGTACTTTTGGAGGTTC	
Mylk2	J03886	F: GGCTCCCACCTTCTGTTTGT	236
		R: CCGACGGCTTTCATACTCCT	
RT1-T24-1	NM_001008858	F: CCCAGGTTTTCACCAATCAG	107
		R: GTGGAAGCCGTCTGCTCTGT	
Plekhb1	BG666307	F: GAATGGAGCTCACAAGAGAG	185
		R: CATCGTGCCTTTATCCAGC	
Pttg1	U73030	F: GAA AAGCCAGTGAAGAGTAG	200
		R: AGTGAGATCTGGTGCTCTTCA	
Lyl1	BC081864	F: GAGAGCTGGACTTGGTTGA	167
		R: CACCTCGTTCTTGCTCAGCTT	
β-Actin		F: GTACCCAGGCATTGCTGACA	169
		R: CTCCTGCTTGCTGATCCACATC	

validated via real-time quantitative RT-PCR experiments. The primer sequences are listed in Tab. 1. The real-time quantitative RT-PCR was performed using

a LightCycler-FastStart DNA Master SYBR Green I Kit (Roche Applied Science) according to the manufacturer's instructions. The program was as follows: initiation with 10 minutes of denaturation at 95ºC, followed by 40 cycles of amplification with 15 s of denaturation at 95ºC, 5 s at 56ºC for annealing, and 15 s of extension at 72ºC. The melting curves were produced and 1.5% agarose gel electrophoresis was performed to validate the specificity of the amplified products. The comparative threshold cycle (CT) method was used to calculate the degree of amplification. β-actin was used as a reference control gene to normalize the expression value of each gene. The experiment was performed in triplicate for each gene, and the average expression value was computed for subsequent analysis. The results were compared using the $2^{-\Delta\Delta CT}$ method [14] and are expressed as $\log_2$ means ± SD.

The 8 genes that showed 5-fold higher or lower expression levels ($p < 0.05$) in the GH1 group compared with the EV group were selected as the gene panel to distinguish the GH therapy group from the control groups. We examined the relative mRNA ratios of these genes in 15 single rat whole blood samples from the GH1 group, using 5 whole blood samples from the NC and EV group rats mixed as controls via real-time qRT-PCR. The primer sequences are listed in Tab. 2.

Tab. 2. The primer sets for the candidate biomarkers used for real-time PCR validation.

Gene	Accession No.	Primer sequence 5'→3'	Amplification size (bp)
Pla2g2a	NM_031598	F:GGAAAGGAAGCCGCACTCAGTTAT	139
		R: CACATCCACGTTTCTCCAGACGTT	
Gpr109a	AB103062	F: GCTCCTCTACAAACACAACG	174
		R: CGAAGGCAACGGTTGATGC	
Hexim1	BC087133	F: GAAGCTGGTGAGGAAGACG	161
		R: CACTTCTCCAGCTCCAGGTAC	
Nfkbia	XM_343065	F: GCTTGGCGAAGTTCTAGGAAT	218
		R: TGCTGTGGTGCTAAGTATAC	
Ilk	NM_133409	F: AGGTGCCCTTTGCTGACCTTTCTA	156
		R: TGTCAAACTTGGGTCGCTTTGCAG	
Birc3	NM_023987	F: AAGCTTTCCCTCAGACCCTGTGAA	168
		R: TAGAATCCTGCTTTGGCCAGCTCT	
Rap1b	NM_134346	F: CGGACAAGGCTTTGCGTTGGTTTA	174
		R: TGACCTTGTTCCTTCCCGACAACT	
Ptgs1	BC081816	F: TGTGTGTGACTTGCTGAAGGAGGA	146
		R: TGAGCTGCAGGAAATAGCCACTCA	
β-Actin		F: GTACCCAGGCATTGCTGACA	169
		R: CTCCTGCTTGCTGATCCACATC	

Statistical analyses

The data was analyzed by ANOVA with post hoc analysis using Bonferroni's method in the SPSS statistical package 13.0 (SPSS 13.0; SPSS Inc., Chicago, IL, USA), and was considered statistically significant at $p < 0.05$.

A receiver operating characteristic (ROC) curve analysis was done to determine whether the gene panel could discriminate the GH therapy group from the control groups. The area under the receiver operating characteristic curve (AUC) was calculated, using binary logistic regression analysis in SPSS 13.0. The results are reported as the SE of regression (SE), significance (Sig.), 95% confidence interval (95% CI), positive and negative predictive values (PPV/ NPV), true positive rate (TRP), and false positive rate (FRP).

RESULTS

Animal models and verification of GH1 transduction *in vivo*

A 215-bp band of the *GH1* gene was detected by semi quantitative RT-PCR in the injected quadriceps of rats after the injection of rAAV2/1-CMV-GH1. There was almost no expression of *GH1* in the liver, heart, kidneys and spleen in any group in this study prior to the injection of the viral vector or saline control. The expression of *GH1* mRNA was sustained for almost six months (Fig. 1).

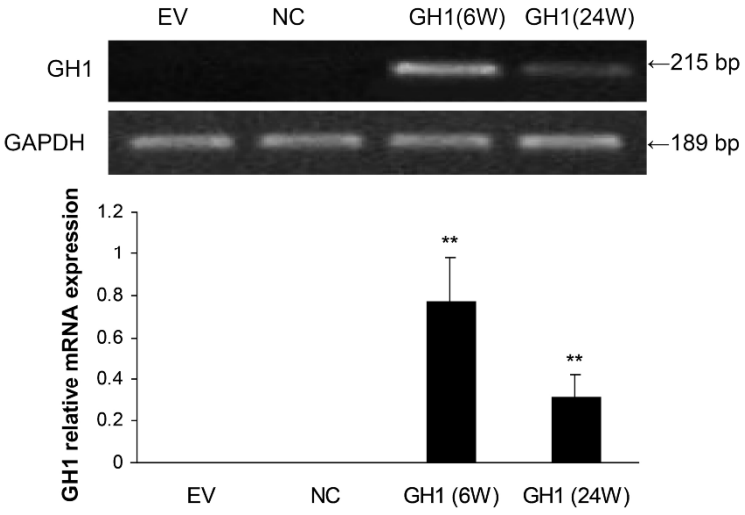

Fig. 1. The relative levels of *GH1* mRNA expression in the skeletal muscle of the hind limbs, 6 and 24 weeks after the injection of the rAAV2/1 virus containing the *GH1* expression vector. **$p < 0.01$ versus the NC group. The error bars correspond to the standard deviation (SD). NC – negative control rats; EV – group rats; GH1 (6W) – the GH1 group rats 6 weeks after the injection of rAAV2/1-CMV-GH1; GH1 (24W) – the GH1 group rats 24 weeks after the injection of rAAV2/1-CMV-GH1. The number of samples for each group is 3.

Western blot analysis demonstrated the presence of the 22-kDa secreted hGH protein in the quadriceps of the GH1 group rats, and showed the specific GH1 expression in the GH1 group rats, a trend that was confirmed by the results of the RT-PCR experiments (Fig. 2). The subsequent experiments were based on the validation of the specific *GH1* gene expression in the muscles of the animal models.

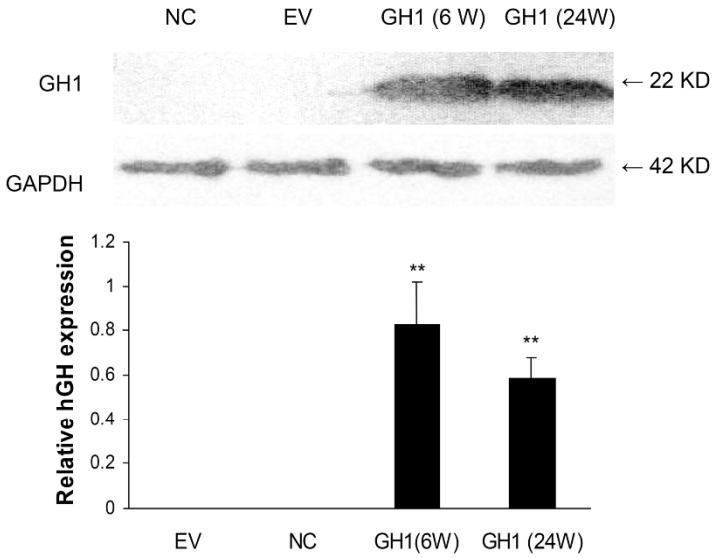

Fig. 2. A Western blot analysis of the relative hGH expression levels in the skeletal muscle of the hind limbs 6 and 24 weeks after the injection of rAAV2/1-CMV-GH1. **p < 0.01 versus the EV group. The error bars correspond to the standard deviation (SD). NC – negative control rats; EV – group rats; GH1 (6W) – GH1 group rats 6 weeks after the injection; GH1 (24W) – GH1 group rats 24 weeks after the injection of rAAV2/1-CMV-GH1. The number of samples for each group is 3.

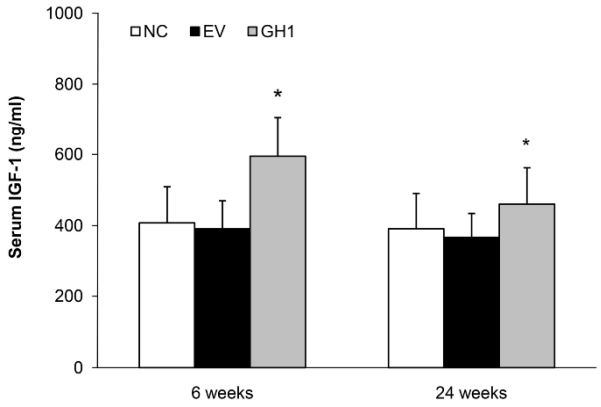

Fig. 3. The serum concentrations of IGF-1 24 weeks after the injection. *p < 0.05 versus the EV group. The error bars correspond to the standard deviation (SD).

The serum levels of IGF-I were consistently significantly higher in the GH1 group during the 24 weeks after the injection compared with the levels for the NC and EV groups ($p < 0.05$, Fig. 3). There were no notable differences in the serum levels of IGF-1 between the NC and EV groups.

Tab. 3 shows that the white blood cell (WBC), monocyte and neutrophil counts in the peripheral blood in the GH1 group increased significantly 6 weeks after the injection of *GH1*. The increase in the WBC count was mainly due to the increase in the neutrophil count.

Tab. 3. The absolute numbers of the white blood cells, lymphocytes, neutrophils and monocytes in the peripheral blood. *$p < 0.05$ versus the EV group. The number of samples for each group is 5.

Group	Mean value ± SD for the various groups			
	WBC (10^3 μl^{-1})	Lymphocyte counts (10^3 μl^{-1})	Monocyte counts (10^3 μl^{-1})	Neutrophil counts (10^3 μl^{-1})
EV	7.46 ± 1.15	5.43 ± 1.14	0.21 ± 0.05	1.26 ± 0.15
NC	7.17 ± 1.24	5.28 ± 1.21	0.19 ± 0.04	1.21 ± 0.18
GH1 (6W)	9.62 ± 1.10*	5.99 ± 1.23	0.49 ± 0.06*	2.37 ± 0.45*
GH1 (24W)	7.88 ± 1.29	5.66 ± 1.28	0.24 ± 0.06	1.58 ± 0.22

Global changes in the whole blood gene expression after injection

We detected 107 genes that were upregulated and 482 genes that were downregulated in the EV group compared with the NC group, while 168 genes were upregulated and 331 genes were downregulated in the GH1 group compared with the EV group. 8 of the differentially expressed genes that showed 2-fold higher or lower expression levels in the EV group satisfied the criterion of $p < 0.05$ compared to the NC group. Of these 8 altered genes, 6 were upregulated and 2 were downregulated. Sixty one genes showed 2-fold higher or lower expression levels in the GH1 group ($p < 0.05$) relative to the EV group. Twenty five of the 61 genes were upregulated and 35 were downregulated.

The functional classification of each altered gene was obtained by searching the Gene ontology (GO) database in the CapitalBio® Molecule Annotation System V4.0 (MAS 4.0). The distributions of the functions of the differently expressed genes in the GH1 group rats 24 weeks after the injection of rAAV2/1-CMV-GH1 compared with the EV group are shown in Fig. 4. These genes were predominantly in the molecular function, regulation of biological process, and cellular component classes. The biological interpretation of the gene clusters was completed using Ingenuity Pathways Analysis (IPA) and the KEGG databases in the MAS 4.0 software package.

As shown in Tab. 4, in the GH1 group rats 24 weeks after the injection of rAAV2/1-CMV-GH1, the genes that were upregulated or downregulated by more than 2-fold when compared with the EV group ($p < 0.05$) were associated

with the Jak-STAT signaling pathway, cardiac hypertrophy, the insulin signaling pathway, type I diabetes mellitus, the adipocytokine signaling pathway, the cell cycle, arachidonic acid metabolism, glycerophospholipid metabolism, carbon fixation, cytokine-cytokine receptor interaction, the MAPK signaling pathway, angiogenesis, oncogenesis, apoptosis, cell adhesion molecules (CAMs), and the Wnt signaling pathway. Tab. 5 defines the immune networks in the peripheral blood of the GH1 group 24 weeks after the injection of GH1. These were identified by IPA when compared to the EV group ($p < 0.05$). Tab. 6 shows the genes that were differently expressed by more than 2-fold in the EV group rats compared with the NC group rats ($p < 0.05$).

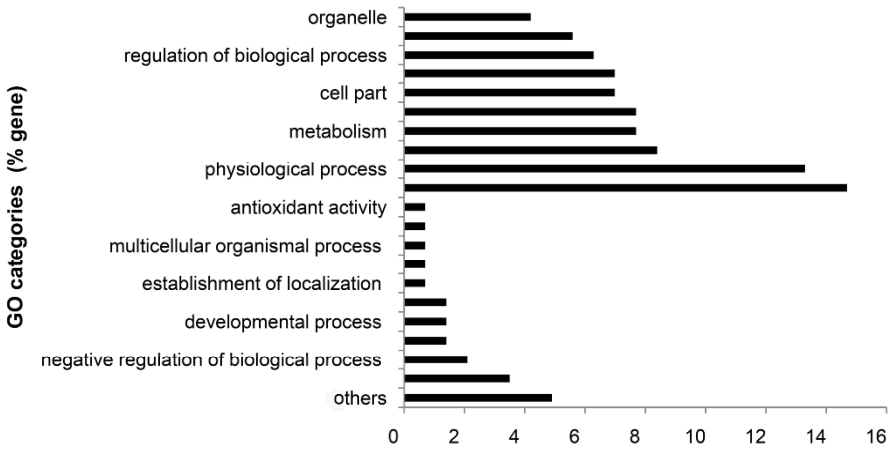

Fig. 4. The distributions of the functions and the expression levels of the genes that were differently expressed in the GH1 group rats 24 weeks after the injection of rAAV2/1-CMV-GH1. The number of samples is 5.

Tab. 4. The genes that were upregulated or downregulated by 2-fold or higher in the peripheral whole blood of the GH1 group rats 24 weeks after the injection relative to the EV group rats ($p < 0.05$).

Gene symbol	Gene function and gene description	Accession No.	Fold change
	Jak-STAT signaling pathway		
Ptpn6	Tyrosine-protein phosphatase non-receptor type 6	U77038	2.6↑
Ifngr	Interferon gamma receptor 2	XM_340967	2.1↓
Pim1	Proto-oncogene serine/threonine-protein kinase Pim-1	X63675	2.1↓
Myc	Myelocytomatosis viral oncogene homolog	NM_012603	3.2↑
Il4ra	Interleukin-4 receptor alpha chain precursor	AB015746	2.1↑
Jak2	Janus kinase 2	U13396	2.3↓
Sos2	Son of sevenless homolog 2	XM_234263	2.1↑

Gene symbol	Gene function and gene description	Accession No.	Fold change
	Insulin signaling pathway		
Gsk3b	Glycogen synthase kinase 3 beta	X73653	2.3↓
Pygl	Liver glycogen phosphorylase	BC070901	2.1↓
Prkar2b	Protein kinase, cAMP dependent regulatory, type II beta	M12492	2.3↓
Sos2	Son of sevenless homolog 2	XM_234263	2.1↑
	Type I diabetes mellitus		
RT1-Ba	RT1 class II, locus Ba	X14879	4.3↓
RT1-T24-1	RT1-149 protein	NM_001008858	3.3↓
RT1-Db1	RT1 class II histocompatibility antigen, D-1 beta chain precursor	NM_001008884	3.2↓
	Cardiac hypertrophy		
Mylk2	Myosin light chain kinase 2, skeletal/cardiac muscle	J03886	2.2↓
RT1-S3	RT1-149 protein	NM_001008826	3.3↓
Hexim1	Cardiac lineage protein 1 (Clp1)	BC087133	5.0↑
	Adipocytokine signaling pathway		
Nfkb1	Nuclear factor NF-kappa-B p105 subunit	XM_342346	4.3↓
Prkcq	PKC theta protein	XM_341553	2.1↓
Jak2	Tyrosine-protein kinase JAK2	U13396	2.8↓
Gpr109a	Nicotinic acid receptor	AB103062	17.2↑
Nfkbia	NF-kappaB inhibitor alpha	XM_343065	3.2↓
	Arachidonic acid metabolism		
Pla2g2a	Phospholipase A2	NM_031598	43.1↓
Ptgs1	Prostaglandin G/H synthase 1 precursor	BC081816	5.8↓
Alox15	Arachidonate12-lipoxygenase, leukocyte-type	NM_031010	3.1↓
	Pyruvate metabolism		
Hagh	Hydroxyacylglutathione hydrolase	BC097301	2.1↓
	Cytokine-cytokine receptor interaction		
Csf1r	Colony-stimulating factor 1 receptor	NM_001029901	2.1↓
Ccr7	Chemokine (C-C motif) receptor 7	NM_199489	2.1↓
Ltbr	Lymphotoxin B receptor	BC085880	2.4↓
Il4ra	Interleukin-4 receptor alpha chain precursor	AB015746	2.1↓
	Glycerophospholipid metabolism		
Pla2g2a	Phospholipase A2, membrane-associated precursor	NM_031598	43.1↓
Pafah1b2	Platelet-activating factor acetylhydrolase IB beta subunit	NM_022387	3.4↓
	Cytokine-cytokine receptor interaction		
Csf1r	Colony-stimulating factor 1 receptor	NM_001029901	2.1↓
Ccr7	Chemokine (C-C motif) receptor 7	NM_199489	2.1↓
Ltbr	Lymphotoxin B receptor	BC085880	2.4↓

Gene symbol	Gene function and gene description	Accession No.	Fold change
	Carbon fixation		
Aldoa	Fructose-bisphosphate aldolase A	BC064440	2.1↑
	Cell cycle		
	Angiogenesis-, oncogenesis- & apoptosis-related		
S100a9	Calgranulin B	CB728702	3.4↑
Cxcl1	Growth-regulated alpha protein precursor	NM_030845	3.3↑
Ccnl1	Cyclin-L1	BC085686	3.3↑
Nfkbia	NF-kappaB inhibitor alpha	XM_343065	3.2↓
Pcna	Proliferating cell nuclear antigen	NM_022381	3.2↑
G0s2	G0/G1 switch gene 2	H32223	3.2↑
Ddit3	DNA-damage inducible transcript 3	BC100664	3.0↑
Ets1	C-ets-1 protein	L20681	2.1↑
Rpl13	Ribosomal protein L13	BC086577	2.0↑
Ier2	Immediate early response 2	BC061717	3.0↑
Ier3	Immediate early response 3	BC099831	3.0↑
Ier5	Immediate early response 5	BE113744	3.2↑
Lyar	Cell growth-regulating nucleolar protein	BC079008	2.2↑
Il1b	Interleukin 1 beta	BC091141	4.0↑
Pcna	Proliferating cell nuclear antigen	NM_022381	3.2↑
Cdc25b	Cathepsin B precursor	D16237	3.3↑
Ywhah	14-3-3 protein eta.	BC081825	2.1↑
	Cell adhesion molecules (CAMs)		
Ilk	Integrin-linked kinase	NM_133409	5.5↓
	MAPK signaling pathway		
Cdc25b	M-phase inducer phosphatase 2	D16237	2.3↓
Map2k3	Map2k3_predicted protein	XM_239239	2.2↓
Nfkb1	Nuclear factor NF-kappa-B p105 subunit	XM_342346	4.3↑
Rap1a	Ras-related protein Rap-1A	BC083813	3.1↓
Pla2g2a	Phospholipase A2	NM_031598	43.1↓
Sos2	Son of sevenless homolog 2	XM_234263	2.1↑
Rap1b	Ras-related protein Rap-1b	NM_134346	12.2↓
	Wnt signaling pathway		
Nfkb1	Nuclear factor NF-kappa-B p105 subunit	XM_342346	2.7↑
Birc3	Inhibitor of apoptosis protein 1	NM_023987	7.8↓
Cd3g	CD3 antigen, gamma polypeptide	XM_217136	2.5↑

↑ upregulated genes, ↓ downregulated genes

Tab. 5. The GH immune networks in the peripheral blood of the GH1 group 24 weeks after the injection of GH1 relative to the EV group rats.

Gene symbol	Gene function and gene description	Accession No.	Fold change
	Antigen processing and presentation		
Hspa8	Heat shock cognate 71-kDa protein	BC061547	2.3↑
Hspa5	78-kDa glucose-regulated protein precursor	BC062017	4.0↑
RT1-T24-1	RT1-149 protein	NM_001008858	3.4↓
RT1-Db1	RT1 class II histocompatibility antigen, D-1 beta chain precursor	NM_001008884	3.2↓
RT1-S3	RT1-149 protein	NM_001008826	3.3↓
Ctsb	Cathepsin B precursor	NM_022597	3.3↓
Cd74	CD74 antigen	NM_013069	2.1↓
	T-cell receptor signaling pathway;B-cell receptor signaling pathway		
Fos	Proto-oncogene protein c-fos	NM_022197	3.5↑
Ptpn6	Tyrosine-protein phosphatase non-receptor type 6	U77038	2.6↑
Nfkbia	NF-kappaB inhibitor alpha	XM_343065	3.2↓
Nfkb1	Nuclear factor NF-kappa-B p105 subunit	XM_342346	4.3↓
Cd81	CD 81 antigen	BC060583	2.2↓
Ctla4	Cytotoxic T-lymphocyte-associated protein 4	U37121	2.4↑

↑ upregulated genes, ↓ downregulated genes

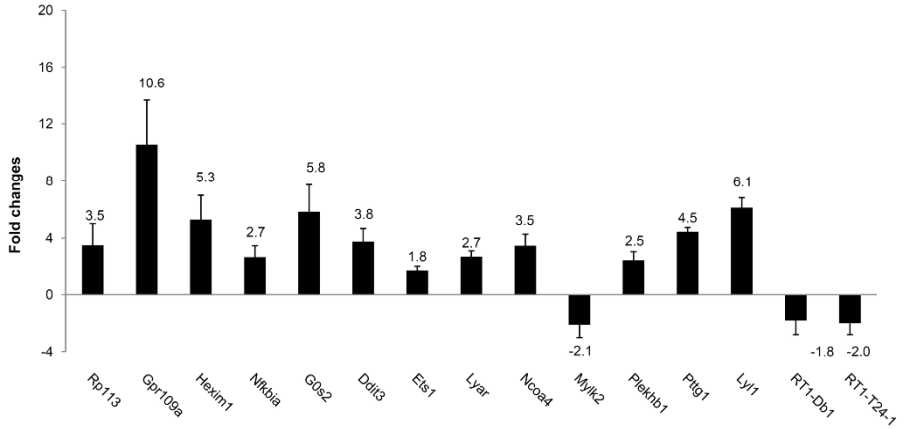

Fig. 5. Real-time RT-PCR experiments confirmed the microarray data and showed the differentially expressed genes including Rpl13, Gpr109a, Hexim1, Nfkbia, G0s2, Ddit3, Ets1, RT1-T24-1, RT1-Db1, Mylk2 and Lyar in the GH1 group; and Ncoa4, Plekhb1, Pttg1 and Lyl1 in the EV group 24 weeks after the injection. The fold change is displayed relative to normal adult male rat blood samples. The error bars correspond to the standard deviation (SD). The number of sample for each group is 5.

Real-time quantitative RT-PCR was used to verify the expression levels of 15 differently expressed genes selected on the basis of their functional properties and the fold change in their expression levels (at least 2-fold higher or 2-fold lower) among the three different groups at a significance level of $p < 0.05$ (Fig. 5). Although there were small differences in the fold-change values between the microarray and the real-time quantitative RT-PCR, the real-time quantitative RT-PCR results agreed with the microarray data.

Tab. 6. Genes that were upregulated or downregulated by 2-fold or higher in the peripheral whole blood of the EV group rats at 24 weeks relative to the NC control rats ($p < 0.05$).

Gene symbol	Gene function and gene description	Accession No.	Fold change
	Angiogenesis-, oncogenesis- & apoptosis-related		
Lyl1	Lymphoblastic leukemia-derived sequence 1	BC081864	2.7↑
Pttg1	Securin (pituitary tumor-transforming protein 1).	U73030	2.6↑
Plekhb1	Pleckstrin homology domain-containing family B member 1	BG666307	2.3↑
Rps29	40S ribosomal protein S29	BF557769	2.2↑
Pcna	Proliferating cell nuclear antigen	NM_022381	2.2↑
Ncoa4	Beta-microseminoprotein precursor	NM_001034008	2.0↑
	Antigen processing and presentation		
RT1-T24-1	RT1-149 protein	NM_001008858	2.1↓
Ctss	Cathepsin S precursor	BC059142	2.1↓
RT1-Ba	RT1 class II, locus Ba	X14879	4.2↓
	Cell adhesion molecules (CAMs)		
RT1-T24-1	RT1-149 protein	NM_001008858	2.1↓
Ppp1cc	Serine/threonine protein phosphatase PP1-gamma catalytic subunit	XM_346435	2.0↓

↑ upregulated genes, ↓ downregulated genes

Potential biomarker genes for the detection of *GH1* abuse/misuse

Eight genes that showed at least 5-fold higher or lower expression levels between the GH1 group and the controls at $p < 0.05$ were selected as candidate biomarker genes for the detection of GH gene therapy abuse/misuse. We studied their expressions using real-time PCR in a total of 15 rat whole blood samples from the GH1 group, 5 from the NC group, and 5 from the EV group using β-actin mRNA as an endogenous quantity control. To correctly assess this issue, we omitted the 15 rats (5 from each group) on which we had done the original microarray analysis. The 8 genes showed considerable concordance between the array and the quantitative real-time data in each sample (Tabs 4, 5; Fig. 6). The nonparametric receiver operator characteristic (ROC) analysis was performed based on the PPV and NPV to determine whether the gene panel could discriminate the GH therapy group from the control groups. For each gene, the

area under the curve (AUC) and its associated 95% confidence interval (CI) were reported (Tab. 7). The ROC curve is shown in Fig. 7.
The AUC for the 8-gene panel was 1.00. Tab. 7 and Fig. 7 together show that the ROC analysis of the 8-gene panel could distinguish the GH1-treated rats from the control rats.

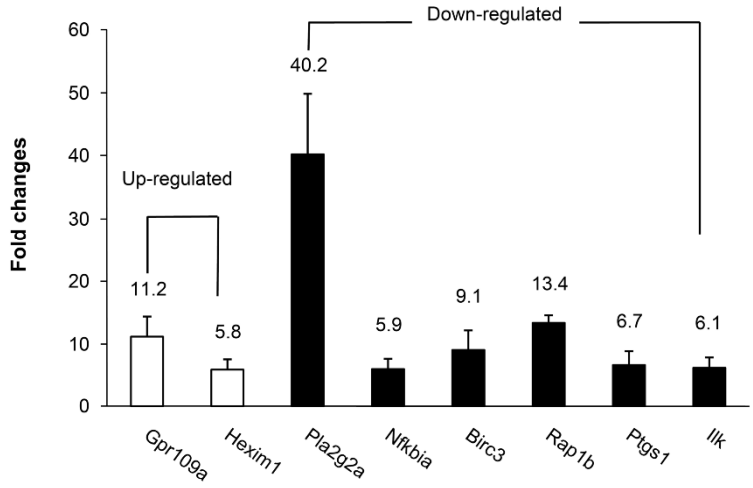

Fig. 6. The validation of candidate biomarker genes by real-time PCR. The fold change is displayed relative to normal adult male rat blood samples. The error bars correspond to the standard deviation (SD). The number of samples for each group is 5.

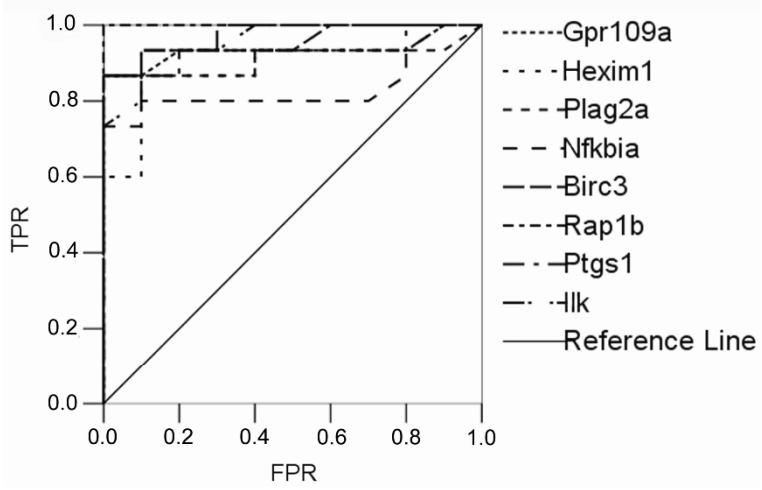

Fig. 7. The receiver operator characteristic curves (ROC) for the gene panel (8 potential biomarker genes) for GH1 abuse/misuse detection.

Tab. 7. The receiver operator characteristic (ROC) analysis predicting GH1 abuse/misuse based on the PPV and NPV.

Gene	AUC	PPV	NPV	SE	Sig.	95% CI
Gpr109a	0.93	86.7%	100.0%	0.06	0.00	(0.82, 1.04)
Hexim1	0.89	93.3%	100.0%	0.07	0.01	(0.76, 1.02)
Plag2a	0.91	86.7%	100.0%	0.09	0.01	(0.78, 1.04)
Nfkbia	0.83	80.0%	100.0%	0.04	0.06	(0.66, 1.00)
Birc3	0.96	93.3%	100.0%	0.04	0.00	(0.88, 1.04)
Rap1b	1.00	100.0%	100.0%	0.00	0.00	(1.00, 1.00)
Ptgs1	0.97	86.7%	100.0%	0.03	0.00	(0.91, 1.03)
Ilk	0.96	93.3%	100.0%	0.04	0.00	(0.89, 1.03)

DISCUSSION

Thymic function can be significantly enhanced by GH therapy to produce new T-lymphocyte cells and may therefore reverse some T-lymphocyte dysfunction. Our microarray experiments showed that Ctsb, Hspa8, RT1-Db1, RT1-S3, RT1-T24-1 Fos, Ptpn6, Nfkbia, Nfkb1, Cd81, Ctla4 and Hspa5, which are involved in the T-cell receptor signaling pathway, B-cell receptor signaling pathway, and antigen processing and presentation, were expressed differentially, at a significance level of $p < 0.05$. Furthermore, GH gene therapy was found to significantly increase the absolute number of WBCs and neutrophils. The above results indicated that the changes in the immune networks regulated by GH could improve bodily immune function, which raises the possibility that rhGH may form part of an immune-based therapeutic programme tailored to the treatment of HIV/AIDS, cystic fibrosis (CF), inflammatory bowel disease (IBD), juvenile rheumatoid arthritis (JRA) and chronic renal insufficiency (CRI).

However, the altered immunity suggested that there is a potential risk of the development of anti-GH antibodies that could blunt the effect of a high level of exogenous GH in animals. Meanwhile, RT1-T24-1, Ctss and RT1-Ba were found to be downregulated more than 2-fold in the EV group, which might reflect changes in the cellular immune responses arising from the viral vector itself.

GH acts through its receptor on the cell surface, which is a cytokine class I receptor with multiple tyrosines on the intracellular domain. Binding the hormone to the receptor induces receptor tyrosine phosphorylation with intracellular signaling through a number of pathways, such as the signal transducer and activator of transcription 5 (STAT5), mitogen-activated protein kinase (MAPK), phosphoinositide-3 kinase (PI3K), and Janus kinase 2 (JAK2), which yield differential gene expression and changes in the physiological response [15, 16]. Here, we detected the changes in the blood gene expression between *GH1*-treated rats and control rats using the CapitalBio 27 K Rat Genome Oligo Array (CapitalBio, Beijing, China), which comprises 26,962 70-mer oligo probes from the Operon Rat Genome Version 3.0.5 library, representing

27,044 gene transcripts corresponding to over 22,012 rat genes. The results suggested that the differentially expressed genes were involved in pathobiologically and therapeutically relevant processes such as angiogenesis, oncogenesis, apoptosis, cardiac hypertrophy, hematopoietic cell lineage, type I diabetes mellitus, carbon fixation, the cell cycle, cytokine-cytokine receptor interaction, focal adhesion, glycerophospholipid metabolism, and signaling pathways. The results demonstrated that exogenous GH gene expression in normal subjects is likely to induce cellular changes in the metabolism, signalling pathways and immunity. Of the 8 candidate biomarker genes, Pla2g2a, Gpr109a and Rap1b showed over 10-fold higher or lower gene expression levels in the GH1 group rats than in the NC controls.

Pla2g2a, which encodes the Pla2 group II A secreted phospholipase A2, showed an over 40-fold lower expression in whole blood after the injection of rAAV2/1-CMV-GH1 than in the NC controls ($p < 0.05$). Pla2g2a plays diverse roles in human diseases, including colon cancer, coronary artery disease and inflammation [17-21]. The mRNA expression of Gpr109a showed an over 10-fold increase in rat whole blood samples after the injection of rAAV2/1-CMV-GH1 ($p < 0.05$). It is a G-protein-coupled receptor for nicotinic acid, and is most noted for its anti-lipolytic effects in adipocytes [22, 23]. Rap1b, an abundant small GTPase in the platelets, showed an over 10-fold lower expression. The results of recent studies suggest that Rap1b is involved in a common critical step required for platelet activation [24]. The above results demonstrated that although there was no certain evidence of tumorigenesis in our research, the changes in the gene expressions in whole blood induced by an elevated GH concentration could increase the risks of colon cancer and other diseases.

In this study, we were limited by our small sample sizes. Furthermore, the molecular mechanisms involved in GH gene therapy abuse/misuse are complex. Gene expression may be confounded by the duration and dosage of the rAAV2/1-CMV-GH1 treatment. In addition, using whole blood RNA could not rule out the heterogeneity of the cell population or the potential diversity of cell-specific responses, though we could not detect any common abnormality in the CBC and leukocyte subpopulations of the studied cases.

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