ACTA ABP BIOCHIMICA POLONICA

Vol. 69, No 4/2022
781–785
https://doi.org/10.18388/abp.2020_6066

Regular paper

Up-regulation of HSPA1A and HSPA1B in the blood of tophi patients and its clinical significance

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Objective: Poorly treated gout can cause tophi, which can lead to serious and potentially fatal complications. This study aimed to find the potential diagnostic value of blood levels of HSPA1A and HSPA1B for tophi patients. Methods: 58 tophi patients and 61 healthy controls were enrolled in this study, and the whole venous blood samples of all subjects were collected for microarray analysis to identify differentially expressed genes associated with tophi. Meanwhile, KEGG and GO analysis were used to filtrate the enriched different expression genes. The mRNA expression levels of HSPA1A, as well as HSPA1B, were measured by the RT-qPCR method, the correlation between which and the severity of the disease were analyzed. Finally, the receiver operating characteristics curve (ROC) analysis has been performed to the diagnostic value of HSPA1A as well as HSPA1B. Results: Bioinformatic analysis results suggested that both HSPA1A and HSPA1B are abnormally expressed in tophi. Then, it was observed that HSPA1A and HSPA1B were dramatically increased in the blood samples of tophi patients compared with healthy controls and were further linked with the severity of tophi. Moreover, the area under the curve (AUC) of HSPA1A for the diagnosis of ACI was 0.8999 (95% confidence interval (CI), 0.8338 to 0.9661) while of HSPA1B was 0.9093 (95% confidence interval (CI), 0.8550 to 0.9635), suggesting that blood level of HSPA1A, as well as HSPA1B, are sensitive markers to distinguish tophi patients from the healthy people. Conclusion: HSPA1A and HSPA1B were over-expressed in the blood of tophi patients and may be potential diagnostic markers for tophi.

Keywords: tophi, HSPA1A, HSPA1B, diagnosis

Received: 03 December, 2021; revised: 06 April, 2022; accepted: 10 October, 2022; available on-line: 14 December, 2022

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Acknowledgements of Financial Support: This research was suppoeted by Natural Science Foundation of Jilin(No. 20200201382JC).
Abbreviations: AUC, area under the curve; CI, confidence interval; HSPs, heat shock protein; ROC, receiver operating characteristics curve; VAS, Visual Analogue Scale

INTRODUCTION

Gout is a metabolic rheumatic disease directly related to hyperuricemia, which occurs in young men and postmenopausal women (Dalbeth *et al.*, 2021). Tophi is one of the advanced clinical stages of gout and is a crystal-related arthropathy caused by the deposition of monosodium urate in the joints or surrounding tissues, and the erosion and destruction of cartilage, synovium, tendons, ligaments and other tissues (Han *et al.*, 2017; Jud *et al.*, 2018). Current effective treatment for tophi is limited to surgical removal of large tophi to ameliorate some of the dysfunctions (Vargas-Santos & Neogi, 2017; Schlesinger & Lipsky, 2020). The synovial membrane and joints damaged by tophi cannot be repaired. Furthermore, the disabling effect of tophi on patients is irreversible (Carcione *et al.*, 2020). Therefore, it is very necessary to restrain the occurrence and development of gout and alleviate the formation of tophi.

Gout is a polygenic genetic disease affected by various related genes and the environment (Richette & Bardin, 2010). For instance, the abnormal expression of lncRNA AJ227913 in gout may be involved in the inflammatory process of gout and the metabolic balance of uric acid (Zhong *et al.*, 2019). The miR-146a alleviated inflammation in patients with acute gout as a brake therapeutic target (Dalbeth *et al.*, 2015). However, the domestic research on gout gene detection is limited to the influence of a single locus on the disease, and no summary or overall analysis of the differential expression of gout mRNAs has been found.

In this study, we aimed to research differential expression of mRNAs in tophi and found the uric acid metabolism and related control tophus gene expression of small molecule compounds to develop drugs to treat gout stone related.

MATERIALS AND METHODS

Patients

This study was conducted following the "Declaration of Helsinki" and has been approved by the Ethics Committee of Jilin Province People's Hospital. A total number of 58 patients diagnosed as tophi in the department of Hand and Foot Microsurgery, Jilin Province People's Hospital from March 2019 to March 2021 have been included in this study, as well as 61 healthy volunteers as controls. Written informed consent was obtained from all patients. Inclusion criteria were as follows: (1) Blood uric acid levels exceeded 7 mg/ dL in males and 6 mg/ dL in females; (2) X-ray tests of hands and feet showed a mass in soft tissue or bone with local osteogenesis.

Evaluation of the severity of tophi

We used the Visual Analogue Scale (VAS) to examine the clinical condition of patients. Patients were assessed through VAS, using a 10 cm-long straight line, with labels at each end to fasten the scale. Patients were requested to indicate the extent of their perception of pain on the scale, with 0 cm denoting no pain and 10 cm denoting unbearable pain. The patients were asked to at-

tend pain tests on the day of venous blood collection (Klarenbeek *et al.*, 2011).

Microarray analysis of clinical samples

The whole venous blood samples of all subjects were collected and were entrusted to CapitalBio Technology (Beijing, China) for transcriptome chip detection according to Affymetrix protocols (Thermo Fisher, California, USA). All RNA was extracted by RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), and qualified RNA was used for reverse transcription synthesis of DNA. Biotin binding cRNAs were purified by magnetic beads to remove impurities, and the cRNAs were quantified. After the cRNA fragments were reduced to a suitable size for hybridization, they were hybridized with the microarray in a 45°C furnace for cyclic hybridization. After 16 h of hybridization, the confocal scanner was used to scan the brightness of the probe and read out the initial data. After that, the relative gene expression data were obtained by the gene chip processing software.

Meta-analysis of microarray data

Graphic files obtained following chip hybridization were analyzed using the R package oneChannelGUI. Raw signals were normalized utilizing the GCRMA algorithm. Meanwhile, KEGG and GO analysis were used to filtrate the enriched different expression genes related to molecular functions as well as pathways linked to tophi.

Detection of gene expression

Gene expressions were determined by RT-qPCR. Total RNA has been isolated from blood samples using TRIzol Reagent from Life Technologies (Maryland, USA). Qualified RNA (1 μg) has been reverse-transcribed into cDNA using the ReverTra Ace qPCR RT Kit (Toyobo life science, Osaka, Japan) according to the manufacturer's guidelines. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed on a StepOnePlus™ Real-Time PCR System (Thermo Fisher, California, USA) under the guidance of the manufacturer's instructions. The human HSPA1A primer sequence was designed by Mingzu Guo et al. (Guo *et al.*, 2021) while the human HSPA1B primer sequence was designed by Huikang Tao et al. (Tao *et al.*, 2021). Each sample was tested in duplicate. The RT-qPCR conditions consisted of an initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 30 s. The conditions of the melting curve analysis were one cycle of denaturation at 95°C for 10 s, followed by an increase in temperature from 65 to 95°C at a rate of 0.5°C/s. The relative mRNA expression levels were calculated according to the following equation: abundance=$2^{-(\text{threshold cycle})}$. The results of each sample were normalized to GAPDH expression. The primer sequences used are as follows: HSPA1A forward, 5'-CCTTCACCGATACCGAGCG-3' and reverse, 5'-AAGGCCACTCCTTCATGTCC-3'; HSPA1B forward, 5'-GGGAGGACTTCGACAACAGG-3' and reverse, 5'-GACAAGGTTCTCTTGGCCCG-3'; GAPDH forward, 5'-CGGAGTCAACGGATTTGGTCGTAT-3' and reverse, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'.

Statistical analysis

GraphPad Prism version 7.00 (GraphPad, California, United States) has been used for statistical analysis. Two-tailed Students' *t*-test was applied for analyzing the difference between the two groups, meanwhile, all data were presented as mean ± standard deviation (S.D.). The ROC curve was used to evaluate the diagnostic value of HSPA1A and HSPA1B in different groups. The correlation analysis consistent with the normal distribution using Pearson analysis, otherwise the Spearman test was used. $p<0.05$ was considered statistically significant.

RESULTS

Identification of differential expression of genes in blood samples between patients with topics and healthy controls

As indicated in Fig. 1A, according to the microarray analysis, a total number of 1,239 genes resulted in being significantly modulated in tophi compared to negative controls, including 688 up-regulated genes and 551 down-regulated genes (Fig. 1A). The GO analysis revealed that over 700 genes may lead to a regulatory function on the intracellular part (Fig. 1B). What is more, MAPK signaling pathway, Herpes simplex infection, as well as cell cycle, were associated with genes enriched in the blood of tophi according to KEGG analysis (Fig. 1C). Finally, 87 genes were related to various transcription factors, among which, 34 genes were enriched in the ZF-C2H2 transcription factor (Fig. 1D). HSPA1A and HSPA1B were found to be aberrantly expressed in tophi.

Over-expression of HSPA1A and HSPA1B in blood samples of tophi patients

Next, the mRNA expression of HSPA1A and HSPA1B in tophi patients' blood samples were evaluated, and the clinical characteristics of the patients and controls were shown in Table 1. The results indicated in Fig. 2 showed that both HSPA1A and HSPA1B were significantly up-regulated compared with healthy controls ($p<0.01$).

Correlation between the HSPA1A and HSPA1B and disease severity in tophi patients

The association between the expression of HSPA1A and HSPA1B and VAS score was evaluated, and our data suggested that the concentration of either HSPA1A or HSPA1B was proportional to the VAS score of the patients (Fig. 3).

Table 1. Patient characteristics between gout and health controls.

Variables	Healthy control (n=58)	Gout (n=61)	*p*
Age (years)	41.19±5.11	41.03±6.78	0.8851
Sex (male/female)	38/20	42/19	0.8453
BMI (kg/m2)	25.92±3.61	26.18±3.39	0.6861
Obesity (yes/no)	19/39	18/43	0.8432
Smoking history (yes/no)	30/28	34/27	0.7149
History of alcohol intake (yes/no)	31/27	35/26	0.7143

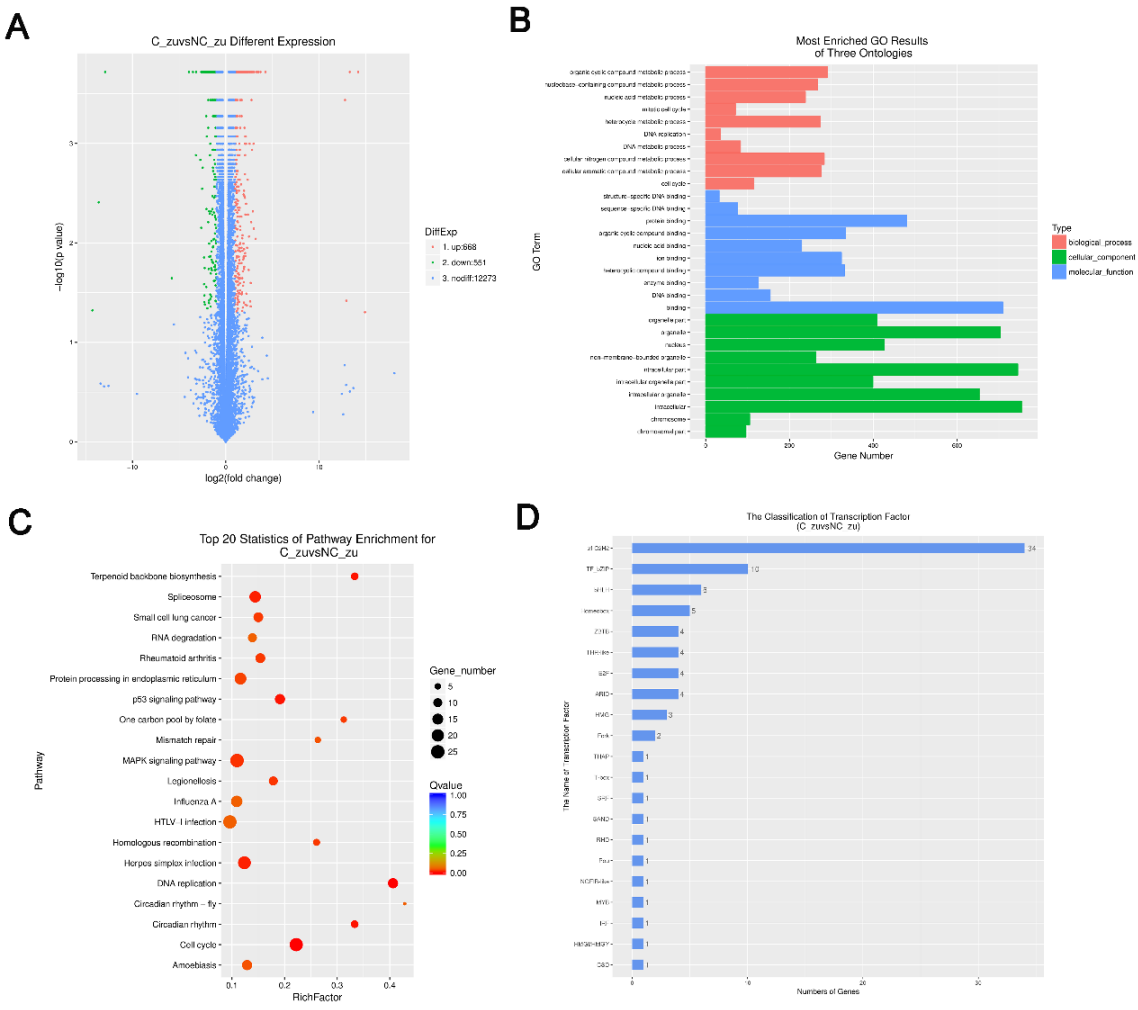

Figure 1. Differently expressed genes in tophi.
(**A**) Volcano map of differentially expressed genes in tophi. (**B**) Go analysis of enriched different expressed genes. (**C**) KEGG analysis of enriched different expressed genes. (**D**) The classification of a transcription factor in tophi.

HSPA1A along with HSPA1B may serve as early diagnostic markers for tophi

Next, the receiver-operating characteristic curve (ROC) has been drawn to evaluate the sensitivity and specificity of HSPA1A and HSPA1B in blood for the diagnosis of tophi. As shown in Fig. 4, the area under the curve (AUC) of HSPA1A was 0.8999 (95% confidence interval (CI), 0.8338 to 0.9661) and HSPA1B was 0.9093 (95% confidence interval (CI), 0.8550 to 0.9635), suggesting that both HSPA1A and HSPA1B in the blood are sensitive biomarkers for the diagnosis of tophi.

DISCUSSION

In this study, HSPA1A and HSPA1B were found to be over-expressed in bioinformatic analysis and blood samples of tophi patients compared with healthy people. Our finding suggested that HSPA1A along with HSP-

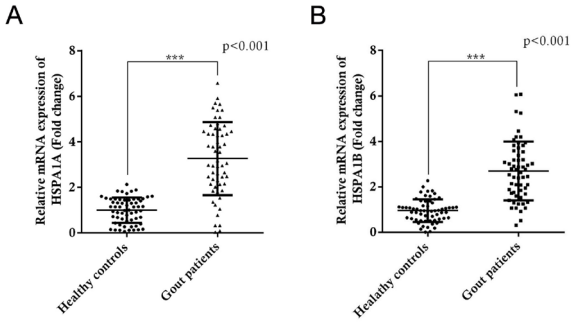

Figure 2. mRNA expression of HSPA1A and HSPA1B in blood samples of tophi patients.
***$p<0.001$.

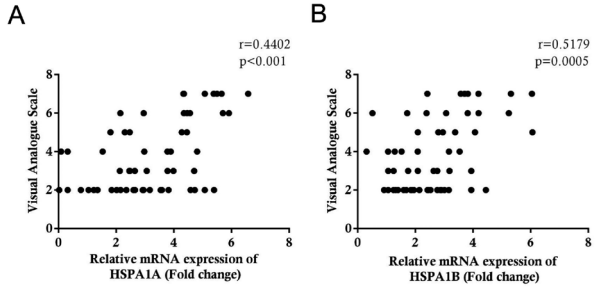

Figure 3. Correlation between mRNA expression of HSPA1A and HSPA1B and VAS of the patients.

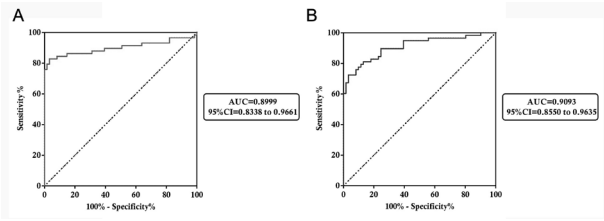

Figure 4. Results of ROC analysis to evaluate the sensitivity and specificity of HSPA1A (A) and HSPA1B (B) for the diagnosis of tophi.

A1B are potent to be biomarkers for the diagnosis of patients with tophi.

With the development of molecular biology, it has been found that in addition to environmental factors, genetic factors also play an important role in gout (Dalbeth *et al.*, 2016). Studies have found that the risk rates of relatives within three generations of gout are significantly higher (Dalbeth *et al.*, 2016; Kuo *et al.*, 2015). Furthermore, surveys in New Zealand have shown that the prevalence of gout among Maori and Pacific people is higher and significantly higher than that among local Europeans, indicating that the occurrence of gout may be related to genetics (Winnard *et al.*, 2012). In recent years, with the development of Genome-Wide Association Studies (GWAS), we have had the opportunity to re-understand the disease process of gout from the perspective of multi-genes (Major *et al.*, 2012). Kottgen and others (Kottgen *et al.*, 2013) found 28 loci with mutations related to gout and hyperuricemia through a large GWAS study in European ancestry. Our research indicated that among the differentially expressed genes between tophi and normal samples, HSPA1A, as well as HSPA1B, were dramatically over-expressed in genes associated with tophi.

Heat shock protein (HSPs) is produced by the body to protect cell function and ensure the survival of cells when the body suffers from a variety of physiological and environmental damage (Wu *et al.*, 2017). Some HSPs are expressed inductively, that is, most are expressed under conditions of impaired stress, such as oxidative stress, heat shock, heavy metal tissue hypoxia, physical damage, and ischemia (Zininga *et al.*, 2018). HSP110 has been investigated to be associated with colorectal cancer patients with unfavorable outcomes, and knockdown of HSP110 may help to provide treatment for colorectal cancer (Gozzi *et al.*, 2020). HSPA1A and HSPA1B are two genes of the HSP70 family and are found in nearly all cell types to act in a large variety of cellular protein folding and remodeling processes (Shevtsov *et al.*, 2018; Rosenzweig *et al.*, 2019). Inducible HSP70 was dramatically increased in gout synovial fluid while the expression of which in blood samples of gout remains unclear (Martin *et al.*, 2003). Our data demonstrated that HSPA1A as well as HSPA1B were dramatically over-expressed in blood samples of tophi patients and were increased with the disease severity. Moreover, the results of ROC analysis suggested that HSPA1A and HSPA1B may function as potential diagnostic biomarkers for the early diagnosis of tophi with high specificity and sensitivity.

There are a few limitations to our study. The roles of HSPA1A and HSPA1B in tophi should be confirmed by collecting more samples and be verified in samples of other ethnic groups except for the Han.

CONCLUSION

HSPA1A and HSPA1B are up-regulated in patients with this and may function as potential biomarkers for the early diagnosis of the disease. Our research suggested the potential clinical significance of HSPA1A along with HSPA1B in blood for the diagnosis and treatment of topics.

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