Gene expression profiling of high altitude polycythemia in Han Chinese migrating to the Qinghai-Tibetan plateau

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Abstract. Chronic mountain sickness (CMS) is a condition in which the hematocrit is increased above the normal level in residents at high altitude. High altitude polycythemia (HAPC) is the most characteristic sign of CMS. However, the pathogenesis of HAPC is poorly understood. The present study aimed to investigate the gene expression profile of HAPC in Han Chinese migrating to the Qinghai-Tibetan Plateau and to identify the pathogenetic mechanisms. A total of 9 differentially expressed genes were identified in HAPC patients using microarrays: 5 were up-regulated and 4 were down-regulated. Functional analysis of the array data revealed that cell division cycle 42 (CDC42) and the human immune response may be key features underlying the mechanism and development of HAPC.

Introduction

Immigration from the plain to the plateau results in a compensatory increase in the number of red blood cells (RBC), which facilitates acclimatization to hypobaric hypoxia (1-4). Increasing the number of RBCs enables the lungs to obtain more atmospheric oxygen (3). The increased number of RBCs stabilizes at a certain level during long term-exposure to high altitudes in the majority of individuals; however, in others the number of RBCs continues to increase, causing serious clinical symptoms and signs, known as high altitude polycythemia (HAPC).

International diagnostic guidelines for HAPC were adopted by the 6th International Conference on High Altitude Medicine in 2004 (5). HAPC is characterized by excessive erythrocytosis (females, Hb ≥19 g/dl; males, Hb ≥21 g/dl) and occurs in natives or long-term residents above 2,500 m (5). Excessive erythrocytosis can increase blood viscosity, reduce blood flow velocity and aggravate hypoxemia (4). On the plateau (3,000-4,700 m), the incidence of HAPC is 2.43-37.5% and increases with elevation (6). Furthermore, the incidence of HAPC is significantly higher in migrants to a plateau than in the native population. The incidence of HAPC in males is also markedly higher than in females and is associated with work intensity (6-10).

High altitude hypobaric hypoxia is the primary cause of HAPC, but the pathogenesis is unknown. Upon exposure to high altitude, hypoxia inducible factor 1 (HIF-1) increases erythropoietin (EPO) mRNA levels by binding the EPO promoter, resulting in increased EPO secretion (11). Erythroid progenitor cells are the main targets of EPO and are very sensitive to this molecule (12). An *in vitro* study showed that erythroid progenitor cells from HAPC patients were more sensitive to EPO than those from healthy migrants (13). However, our previous investigation showed that the increase in RBCs did not always correlate with serum EPO levels (14). Under conditions of hypobaric hypoxia, the number of RBCs can increase at the same time as serum EPO levels are decreasing (14). Therefore, we speculated that the EPO-EPOR system may be associated with erythroid progenitor cell proliferation and differentiation soon after exposure to a plateau. Subsequently, other factors may also be involved in the regulation of erythropoiesis in the bone marrow, and multiple factors working together contribute to excessive RBC proliferation during long term-exposure to high altitudes. Therefore, we

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Abbreviations: HAPC, high altitude polycythemia; CMS, chronic mountain sickness; HIF-1, hypoxia inducible factor 1; EPO, erythropoietin; HSC, hematopoietic stem cell; BFU-E, erythroid blast-forming unit; CFU-E, erythroid colony-forming unit; CDC42, cell division cycle 42; RBC, red blood cell; CYP1B1, cytochrome P450, family 1, subfamily B, polypeptide 1; ERAP2, endoplasmic reticulum aminopeptidase 2; CLC, Charcot-Leyden crystal protein; JNK, c-Jun-NH2-terminal kinases; ROS, reactive oxygen species; ECM, extracellular matrix

Key words: altitude, hypoxia, polycythemia, chronic mountain sickness, gene expression profiling, microarray

Table I. Living habits and clinical data for subjects whose RNA was used for microarray analysis.

Cases	Age (years)	Birthplace (province)	Occupation	Time on plateau (months)	Qinghai CMS scores	Oxygen saturation (%)	Hemoglobin (g/dl)
HAPC1	22	Hubei	Cook	24	6	92	22.5
Control1	29	Hubei	Cook	24	4	92	18.8
HAPC2	25	Sichuan	Operator	30	8	86	21.7
Control2	31	Sichuan	Operator	30	5	92	18.9
HAPC3	24	Jilin	Operator	12	9	90	22.0
Control3	20	Jilin	Operator	12	5	89	18.6
HAPC4	23	Gansu	Maintenance worker	8	11	86	23.9
Control4	19	Gansu	Maintenance worker	8	0	90	18.1
HAPC5	27	Shanxi	Cook	12	6	84	21.8
Control5	32	Shanxi	Cook	12	5	92	18.7

compared the global gene expression profiles of leukocytes from HAPC patients with those from matched controls to identify disease-specific molecular signatures and candidate molecules involved in the pathophysiology of HAPC.

Materials and methods

Ethical approval. The investigation followed the principles outlined in the Declaration of Helsinki for the use of human blood and was approved by the Ethics Committee of the Third Military Medical University. Informed consent was obtained from all subjects. Each subject was assigned a unique patient number, which was used thereafter for the protection of privacy.

Subjects. Subjects were divided into two groups: 5 male Han Chinese patients with HAPC (diagnosed according to the International Consensus Statement on HAPC) and 5 matched controls. The subjects were migrants to the TuoTuo River area (Qinghai-Tibetan Plateau, 4,550 m) (5). Living habits and clinical data were collected and deposited into a database (Table I). Each of the 5 HAPC patients was matched to a control according to gender, nationality, birthplace, length of time on the plateau, height of location and work intensity. Anti-coagulated peripheral blood samples were obtained from subjects at 4,550 m. The hemoglobin concentration was measured three times using the HiCN method and a spectrophotometer (Ehaihuicheng Inc., Shanghai, China). After centrifuging at 1,000 rpm for 10 minutes, the leukocytes were collected and mixed with three volumes of RNA-preservation fluid (Bioteke Inc, Beijing, China) and stored in liquid nitrogen ready for RNA extraction. The gene expression profiles of the RNAs were then analyzed using a Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA). Peripheral blood and plasma samples were obtained from a second batch of subjects (13 HAPC patients and 13 matched controls) from the NaQu area (Qinghai-Tibetan Plateau, 4,610 m). Peripheral blood samples were treated as described above and the gene expression data were confirmed using qRT-PCR.

RNA extraction. RNA extraction and microarray analysis for the first-batch subjects were performed as previously described (15). Total RNA was extracted using the Trizol reagent (Invitrogen, Shanghai, China) and purified using the RNeasy MinElute Cleanup Kit (Qiagen, Beijing, China) according to the manufacturer's instructions. Total RNA from the second batch of subjects was extracted using the TRIpure LS Reagent (BioTeke, Beijing, China) for qRT-PCR. Only RNA with a 28S/18S ribosomal RNA ratio between 1.0 and 1.5 was used in the microarray assay and qRT-PCR experiments.

Microarray analysis. As previously described (15), an aliquot (2 μg) of total leukocyte RNA was used to synthesize double-stranded cDNA, which was subsequently transcribed into biotin-tagged cRNA using the MessageAmp aRNA Amplification Kit (Ambion, TX, USA). The cRNA was then fragmented to produce strands of 35-200 bases in length according to the Affymetrix protocol. The fragmented cRNA was then hybridized to the Human Genome U133 Plus 2.0 Array. Microarray hybridization was performed at 45˚C for 16 hours in an Affymetrix GeneChip Hybridization Oven. The arrays were then washed and stained with streptavidin-phycoerythrin in an Affymetrix Fluidics Station 450 and scanned with an Affymetrix GeneChip Scanner 3000 to analyze the hybridization data. The scanned images were assessed by visual inspection and then analyzed with Affymetrix GeneChip Operating Software (version 1.4). dChip software was used in a global scaling procedure to normalize the different arrays. A two-class unpaired method from the Significance Analysis of Microarrays software (SAM version 3.02, Stanford University) was used to compare differentially expressed genes in the HAPC and control groups.

qRT-PCR. qRT-PCR was performed on three genes differentially expressed in the microarray and two c-Jun-NH2-terminal kinases (JNK). The latter (not included in the microarray) may be involved in apoptosis of hemopoietic stem cells (HSCs). Total RNA extracted from the second batch of peripheral blood samples was used to confirm the microarray data by qRT-PCR. After priming with random primers at 65˚C

Table II. Primer sequences and annealing temperatures used for qRT-PCR.

Gene		Sequence (5'-3')	Annealing temperature (˚C)	Amplicon size (bp)
Major histocompatibility complex, class II, DQ β 1(HLA-DQB1)	F	GCCCTCAACCACCACAACCT	64	173
	R	GGAGTCATTTCCAGCATCACCAG		
Farnesyltransferase, CAAX box, β (FNTB)	F	AAGAGGCCTTCGACAACTGA	60	117
	R	ACTATCTGGGGGATGGGTTC		
Cell division cycle 42 (CDC42)	F	ACGACCGCTGAGTTATCCAC	60	142
	R	CCCAACAAGCAAGAAAGGAG		
JNK1	F	TAGGCTCAGGAGCTCAAGGA	60	91
	R	TGAAATGGTCGGCTTAGCTT		
JNK2	F	AGGCGAGGGATCTGAAACTT	60	150
	R	AATTGGTTTCAGCTGCTGGT		
β-actin	F	GACTTAGTTGCGTTACACCCTTTCT	62	159
	R	GCTGTCACCTTCACCGTTCC		

for 5 min followed by a 1-min incubation on ice, cDNA was synthesized from 500 ng total RNA using M-MLV reverse transcriptase (ReverTra Ace, Toyobo, Japan), RNase inhibitors and a cocktail of dNTPs in a 20 μl reaction volume at 30˚C for 10 min, followed by incubation at 42˚C for 20 min and 99˚C for 5 min. The resulting cDNA was amplified by qRT-PCR using the Opticon Monitor 3 system according to the manufacturer's instructions (MJ Research, USA). Reactions were performed in a 25-μl volume with 1 μl of primers and 12.5 μl hot start reaction mix SYBR Green (Takara, Dalin, China). The reactions were repeated three times. The PCR conditions were: 2 min at 95˚C followed by 40 cycles of 15 sec at 95˚C, 15 sec at the annealing temperature and 15 sec at 72˚C. The PCR primer sequences and the annealing temperatures are listed in Table II. Amplification specificity was checked using melting curve analysis. Gene expression was normalized against that of β-actin.

Detection of Hb, ROS, CLC and EPO. The hemoglobin concentration in the blood from the second-batch subjects was assessed as described above. The levels of plasma Charcot-Leyden crystal protein (CLC), reactive oxygen species (ROS) and EPO in the blood of the second batch subjects were measured twice using an ELISA (Uscn Life Science Inc., Wuhan, China), fluorescence labeling (GenMed Scientifics Inc., Shanghai, China) and a radioimmunoassay kit (PuerWeiye Inc., Beijing, China), respectively, according to the manufacturer's instructions.

Statistical analysis. Data were expressed as the mean ± SD. Student's t-test was used to calculate the statistical significance of unpaired data where appropriate. Statistical significance was defined as $p<0.05$.

Results

Microarray data. After SAM analysis of the microarray data obtained from the 5 HAPC patients and 5 matched controls, 9 differentially expressed genes were identified with a threshold-fold change >2.0. These genes were used for unsupervised hierarchical clustering and the results were analyzed and visualized using the TreeView program (Fig. 1). The functions of the differentially expressed genes were analyzed using a Web-based program, Molecular Annotation System 3.0 (www.capitalbio.com) and three separate open source pathway resources: KEGG, BioCarta and GenMAPP. Table III lists the significant pathways associated with hematopoiesis.

Verification of differential expression by qRT-PCR. The mRNA expression levels of 3/9 differentially expressed genes were assayed. The expression levels of the genes (as detected by real-time reverse transcriptase PCR) were consistent with the results obtained from the microarrays, although there were minor discrepancies (Table IV). Moreover, the expression level of JNK1 and JNK2 mRNA in HAPC patients was significantly lower than that in the controls.

Hb, ROS, CLC and EPO concentrations. As shown in Fig. 2, the concentration of hemoglobin in HAPC patients was significantly higher than that in the matched controls; however, the concentration of CLC was markedly lower than that in the controls. There was no significant difference in plasma ROS and EPO levels between the two groups (Fig. 2).

Discussion

The Affymetrix Human Genome U133 Plus 2.0 microarray contains 47,000 transcripts and covers the majority of known human genes. In this study, we used whole genome microarray analysis to gain a comprehensive understanding of the pathogenesis of HAPC. This is the first report analyzing global gene expression in HAPC patients; 9 differentially expressed genes were identified.

CDC42, a Rho GTPase, is associated with HSC morphology, adhesion, migration, mobilization and erythropoiesis in the bone marrow (16-20). Yang *et al* observed that

Table III. List of differentially expressed genes in HAPC patients compared with matched controls.

Probe set ID	Gene symbol	Gene name	GO ontology			Fold change
			Molecular function	Biological process	Cellular component	
209480_at 212999_x_at	HLA-DQB1	Major histocompatibility complex, class II, DQ β 1	MHC class receptor activity	Antigen processing and presentation of peptide or polysaccharide antigen via MHC class; immune response	Membrane; integral to member; MHC class protein complex	5.29699 3.78169
213831_at 236203_at	HLA-DQA1	Major histocompatibility complex, class II, DQ α 1	MHC class receptor activity	Antigen processing and presentation of peptide or polysaccharide antigen via MHC class; immune response	Membrane; integral to member; MHC class protein complex	3.26864 2.69427
227462_at	ERAP2	Endoplasmic reticulum aminopeptidase 2	Aminopeptidase activity; peptidase activity; metallopeptidase activity; zinc ion binding; metal ion binding	Proteolysis; immune response; blood pressure regulation; antigen processing and presentation of endogenous peptide antigen via MHC class I	Endoplasmic reticulum; endoplasmic reticulum lumen; endoplasmic reticulum membrane; integral to membrane	2.50872
202435_s_at 202436_s_at 202437_s_at	CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	Monooxygenase activity; protein binding; electron carrier activity; oxygen binding; heme binding; metal ion binding	Aromatic compound metabolism; visual perception; estrogen metabolism; toxin metabolism; response to organic substance; oxidation reduction	Endoplasmic reticulum; endoplasmic reticulum membrane; microsome; membrane	2.29293 2.67767 2.09686
204619_s_at 221731_x_at	VCAN	Versican	Binding; calcium ion binding sugar binding; hyaluronic; acid binding	Cell adhesion; development; cell recognition	Extracellular region; extracellular matrix (sensu Metazoa)	2.22859 2.0023
208727_s_at	CDC42	Cell division cycle 42 (GTP binding protein,	Nucleotide binding; GTPase activity; protein binding; GTP binding 25 kDa)	Establishment and/or maintenance of cell polarity; small GTPase-mediated signal transduction; actin cytoskeleton organization and biogenesis; macrophage differentiation; positive regulation of pseudopodium formation; negative regulation of protein complex assembly	Intracellular; cytoplasm; filopodium; cytosol; plasma membrane	0.4959
225851_at	FNTB	Farnesyltransferase, CAAX box, β	Farnesyltranstransferase activity; protein farnesyltransferase activity; protein binding; zinc ion binding; transferase activity; metal ion binding	Negative regulation of cell proliferation; protein amino acid farnesylation; wound healing; positive regulation of fibroblast proliferation	-	0.48858
206207_at	CLC	Charcot-Leyden crystal protein	Lysophospholipase activity; sugar binding; hydrolase activity	Development; lipid catabolism	-	0.23757
209728_at	HLA-DRB4	Major histocompatibility complex, class II, DR β 4	Receptor activity	Antigen processing and presentation of peptide or polysaccharide antigen via MHC class; immune response	Membrane; integral to complex member; MHC class protein	0.19131

Table IV. mRNA expression in HAPC patients compared with that in controls as assessed by microarray analysis and qRT-PCR.

Gene	Probe set ID	Fold change	
		Microarray	qRT-PCR
HLA-DQB1	209480_at	5.30	4.50
FNTB	225851_at	0.49	0.56
CDC42	208727_s_at	0.50	0.70
JNK1	-	-	0.58
JNK2	-	-	0.67

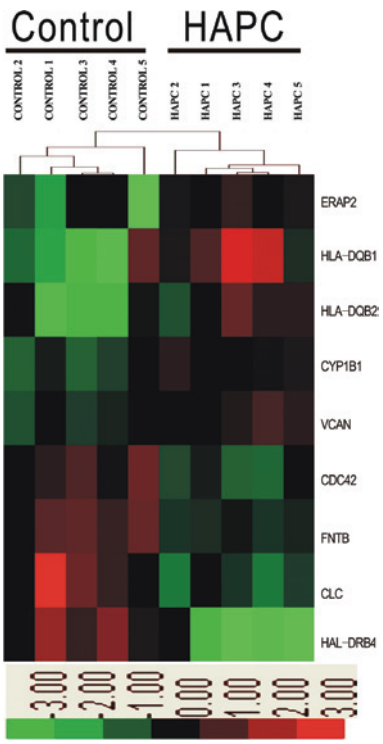

Figure. 1. Clustering display of differentially expressed genes in HAPC patients. Comparison of the nine differentially expressed genes was performed using SAM software and visualized with TreeView following hierarchical clustering. Gene symbols are shown on the right. Expression levels are represented by a color tag, with red representing the highest levels of expression and green the lowest.

CDC42$^{-/-}$ HSCs enter the active cell cycle, resulting in significantly increased numbers of stem/progenitor cells in the bone marrow (16). They also found that increased CDC42 activity caused a significant reduction in the number of HSCs and reduced erythroid blast-forming unit (BFU-E) and erythroid colony-forming unit (CFU-E) activity, which are mediated by the JNK pathway and induce apoptosis of HSCs (16). They therefore hypothesized that CDC42 activity is crucial for erythropoiesis and numerous HSC functions. In the present study, CDC42, JNK1 and JNK2 mRNA expression was significantly lower in HAPC patients than in controls. It is therefore possible that a reduction in CDC42 activity in HAPC patients may result in an increase in the number of BFU-E/CFU-E and reduced apoptosis of HSCs. The microarray and qRT-PCR data revealed that the expression levels of FNTB mRNA in HAPC patients were significantly lower than those in controls. CDC42 undergoes post-translational modification by FNTB to yield a carboxyl-terminal CAAX (where C represents cysteine, A is an aliphatic amino acid and X is any amino acid) signaling motif, which promotes proper localization to the plasma membrane and is required for biological activity (21). Failure of the CAAX modification renders CDC42 inactive due to mislocalization (22). Reduced FNTB expression in HAPC patients may therefore inactivate CDC42, leading to increased erythropoiesis. This indicates that decreased expression of CDC42 and defects in carboxyl-terminal CAAX modifications (mediated by down-regulation of FNTB) reduce CDC42 activity, which in turn promotes excess erythropoiesis in the bone marrow by affecting HSC adhesion, migration, mobilization and (possibly) apoptosis mediated by JNK1 and JNK2.

Of the 9 differentially expressed genes identified on the microarray, ERAP2, CLC, HLA-DQB1, HLA-DQA1 and HLA-DRB4 are involved in immune responses. ERAP2 is involved in the formation of HLA class I binding peptides (23-26). Suppression of ERAP2 by siRNA reduces the surface expression of HLA class I molecules and affects T cell presentation of antigenic epitopes (23). Increased expression of ERAP2 mRNA in HAPC patients may promote the expression of HLA class I molecules, which stimulate CD8$^+$ T cells to evoke an immune response. Ackerman *et al* revealed that CLC protein interacts with eosinophil lysophospholipase and inhibits its lipolytic activity (27). Furthermore, CLC is essential for the functional of CD25$^+$ Treg cells (28). Lower levels of CLC mRNA and protein in HAPC patients indicate an altered immune response. Changes in HLA class II molecule (HLA-DQB1, HLA-DQA1 and HLA-DRB4) mRNA expression in HAPC patients indicate that CD4$^+$ T cells are also involved in altered immune responses. However, it is unclear whether these altered immune responses are the cause or the result of HAPC.

Hypoxia is an environmental stressor that affects the autonomic nervous system and endocrine function (29). Several studies have shown that the plasma IL-1, IL-6 and IL-8 levels are elevated under conditions of hypoxia or exposure to high altitudes (30-32), indicating changes in immune function. However, Monterio *et al* showed that intravenous injection of CD4$^+$ T cells increased the number of activated CD4$^+$ T cells and their secreted hematopoietic regulatory factors in the bone marrow of athymic nude mice, improving their anemia (33). Alexander *et al* also showed that T cell-derived cytokines have marked effects on hematopoiesis (34).

CYP1B1, a member of the cytochrome P450 superfamily, shares activity with monooxygenase, which metabolizes various polycyclic aromatic hydrocarbons, aryl amines and retinoic acid and steroid hormones (35-37). A study of the association between CYP1B1 and peripheral blood lipid levels showed that high density lipoprotein (HDL) levels were significantly lower in patients expressing high levels of CYP1B1 than in those expressing low or medium levels (38). Similarly, high CYP1B1 expression in HAPC patients correlates with low HDL levels. HDL cholesterol levels are a significant factor for determining the lifespan of erythro-

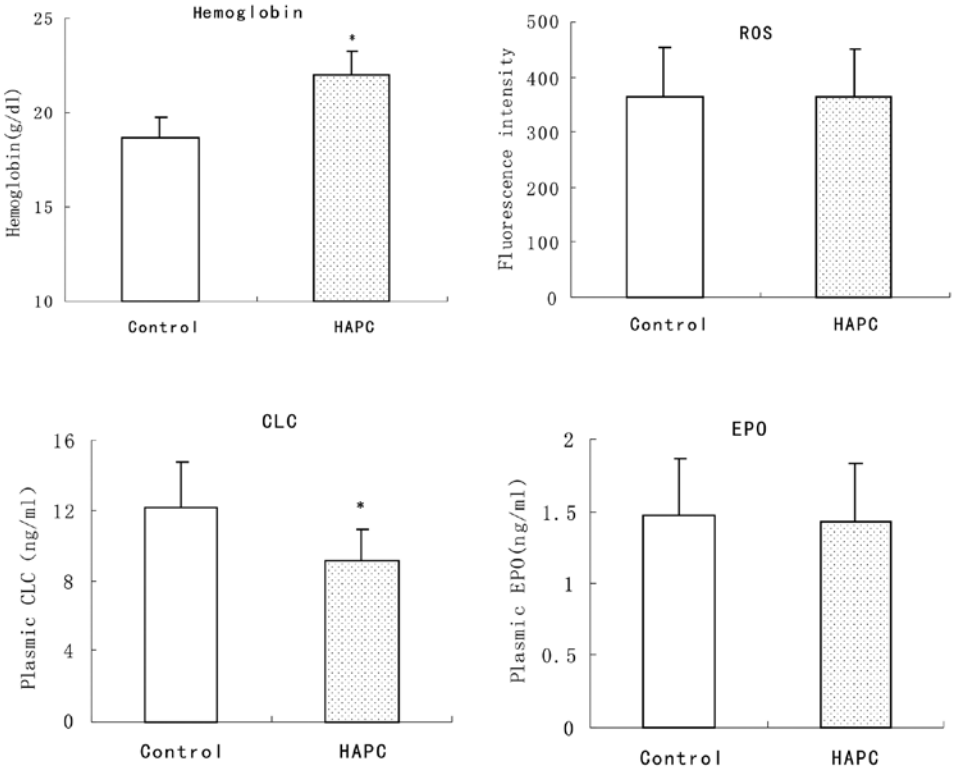

Figure. 2. Concentration of hemoglobin, ROS, CLC and EPO in HAPC patients and controls (n=13). (A) The hemoglobin concentration in the HAPC group was higher than that in the controls. (B) There was no difference in the level of plasma ROS between HAPC patients and controls. (C) Plasma CLC level was markedly lower in HAPC patients than in controls. (D) There was no difference in plasma EPO levels between HAPC patients and the controls. *p<0.05 compared with the controls.

cytes. High HDL levels cause anemia due to the decreased deformability, increased osmotic fragility and reduced lifespan of erythrocytes (39). Therefore, we propose that decreased CYP1B1 expression in HAPC patients may reduce plasma HDL levels which, in turn, results in increased erythrocyte longevity.

Versican is expressed throughout the body and endows the extracellular matrix (ECM) with hygroscopic properties, creating the loose hydrated matrix necessary to support key events in development and disease (40). Numerous cellular processes including adhesion, proliferation, apoptosis and migration are regulated by versican (41-43). Several functional studies have shown that versican increases cell motility and proliferation (44-46). Up-regulation of versican expression in HAPC patients may increase the pericellular matrix around the cells and expansion of the ECM in the bone marrow, creating a highly malleable extracellular environment that supports HSC proliferation and migration.

The present study found no difference in the plasma levels of ROS and EPO between HAPC patients and controls. This may be due to the small sample size. A larger epidemiologic survey (246 HAPC patients and 716 controls) conducted by our research team showed that plasma ROS and EPO levels were significantly higher in HAPC patients than in controls (data not shown); therefore, we propose that EPO and ROS are involved in the pathogenesis of HAPC.

In summary, nine genes were identified as differentially expressed in HAPC patients, providing clues to the molecular pathogenesis of this condition. Analysis of our array data revealed that CDC42 may play a fundamental role in erythropoiesis in the bone marrow of HAPC patients. However, the exact role of CDC42 in HAPC remains unknown. These data also reveal a complex relationship between HAPC and the immune response, which requires further study.

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