Transcriptome reveals the overexpression of a kallikrein gene cluster (KLK1/3/7/8/12) in the Tibetans with high altitude-associated polycythemia

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Abstract. High altitude-associated polycythemia (HAPC) is a very common disease. However, it the disease is still unmanageable and the related molecular mechanisms remain largely unclear. In the present study, we aimed to explore the molecular mechanisms responsible for the development of HAPC using transcriptome analysis. Transcriptome analysis was conducted in 3 pairs of gastric mucosa tissues from patients with HAPC and healthy residents at a similar altitude. Endoscopy and histopathological analyses were used to examine the injury to gastric tissues. Molecular remodeling was performed for the interaction between different KLK members and cholesterol. HAPC was found to lead to morphological changes and pathological damage to the gastric mucosa of patients. A total of 10,304 differentially expressed genes (DEGs) were identified. Among these genes, 4,941 DEGs were upregulated, while 5,363 DEGs were downregulated in the patients with HAPC (fold change ≥2, P<0.01 and FDR <0.01). In particular, the kallikrein gene cluster (KLK1/3/7/8/12) was upregulated >17-fold. All the members had high-score binding cholesterol, particularly for the polymers of KLK7. The kallikrein gene cluster (KLK1/3/7/8/12) is on chromosome 19q13.3-13.4. The elevated levels of KLK1, KLK3, KLK7, KLK8 and KLK12 may be closely associated with the hypertension, inflammation, obesity and other gastric injuries associated with polycythemia. The interaction of KLKs and cholesterol maybe play an important role in the development of hypertension. The findings of the present study revealed that HAPC induces gastric injury by upregulating the kallikrein gene cluster (KLK1/3/7/8/12), which can bind cholesterol and result in kallikrein hypertension. These findings provide some basic information for understanding the molecular mechanisms responsible for HAPC and HAPC-related diseases.

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Introduction

Patients with high altitude-associated polycythemia (HAPC) often have excessive erythrocytosis [generally, females have hemoglobin (Hb) levels >19 g/dl and males have Hb levels >21 g/dl]. The disease affects the majority of individuals residing at a high altitude (1). More red blood cells (RBCs) are produced to carry oxygen to the lungs (2,3). The number of RBCs reaches a high level in the majority of individuals following long-term exposure to high-altitude situations. However, the number of RBCs continues to increase and this results in serious complications.

Normally, gastric mucosal lesion (GML) is a gastrointestinal disorder which is often associated with HAPC is hard to overcome. GMLs usually lead to serious clinical complications in the gastric system and extend from the mucosa into the submucosa. There is evidence to indicate that blood flowing through capillaries (4,5), arterioles (6) and collecting venues (7) is important for maintaining the normal structure and functions of most organs. Generally, the gastric micro-circulates are bypassed by arteriovenous shunting, which results in severe injuries to the gastric mucosa (8). It can be hypothesized that HAPC-related GML is linked with gastric mucosal ischemia, which is caused by microvascular thrombosis due to polycythemia. Additionally, under normal conditions, the physiological balance is often kept between the secretion of peptic acid and the defense mechanisms of the gastroduodenal mucosa. Mucosal injuries and subsequent peptic ulcers can occur when the balance between the factors of aggression and the defense mechanisms is disrupted. The decrease in the defense system is often caused by a number of factors, such as hypoxia (9). Hypoxia is a main reason for the pathophysiological change at high-altitude situations. Hypoxia usually decreases the blood flowing to the gastric tissues, resulting in ischemia and the subsequent destruction of the mucosal linings. Rat models of hypoxia-ischemia have been widely used to explore the molecular mechanisms through which hypoxia-ischemia often causes brain or neonatal injuries (10-14). However, there are limited data available on the effects of hypoxia-ischemia on HAPC-related GML (15).

In this study, to better understand the molecular mechanisms responsible for the development of HAPC-related GML, we compared the gene expression profiles from patients with HAPC and healthy residents at high altitudes in an aim to identify more candidate molecules which are involve in the development of HAPC.

Materials and methods

Study participants. All experimental protocols were conducted based on the ethical guidelines of the Helsinki Declaration and approved by Human Ethics Committee at the People's Hospital of Tibet Autonomous Region (Lasha, China). Written informed consent was obtained from all participants. In June 2014, 3 patients living at altitudes of 3,600 to 4,800 m (Tibetan Plateau), diagnosed with HAPC were recruited as the HAPC group. At the same time, 3 healthy residents, also living at altitudes of 3,600 to 4,800 m (Tibetan Plateau) and receiving gastrointestinal endoscopy for the re-examination of gastric submucosal injuries at the same time, were enrolled as the control group. All participants were life-long residents at Lhasa or Nagqu, and/or Rigaze of Tibet. Each patient with HAPC was matched to a healthy resident in regards to factors, such as gender, birthplace, age, lifestyle, diet, body mass index (BMI), altitude and occupation. All subjects were native male Tibetans and residents and were 40 to 45 years of age.

Prior to endoscopic examinations, peripheral venous blood was obtained using vacuum tubes. The oxygen saturation of arterial blood was measured using pulse oximetry. the inclusion criteria were as follows: i) HAPC was diagnosed as defined at the 2004 Qinghai International High Altitude Medicine Conference, namely concentrations of Hb >21 g/dl for males and >19 g/dl for females (1); ii) the clinical complications included the metabolic disturbance-related headaches (16), cachexia, polycythemia and hypercalcemia (17), digestive disorders (18) and difficulty sleeping (19). Finally, 30 HPAC patients and 30 matched healthy subjects (with a similar lifestyle, living altitude, age and gender distribution as the HPAC patients) received endoscopy detection.

The exclusion criteria were as follows: i) chronic pulmonary disorders, such as emphysema (20), bronchitis (21), alveolar fibrosis and lung cancer (which may be caused by smoking) (22); ii) chronic respiratory polycythemia; iii) severe other disorders, such as heart, brain, liver and kidney disease; iv) alcohol and drug abuse, mental injury or disease, which may make it difficult to perform gastroscopy; v) pregnant or lactating women; vi) an obstructed gastrointestinal tract; and vii) medical history, such as recent gastrointestinal bleeding. Chronic gastritis was diagnosed based on the Chinese Consensus on Chronic Gastritis, which was established in Shanghai in 2006.

Endoscopic detection. A rigorous endoscopic surveillance was conducted in all participants, as previously described (23). Ten milliliters viscous lidocaine hydrochloride mucilage (Jiangsu Jichuan Pharmaceutical Co., Ltd., Jiangsu, China) was orally administered to all subjects. A gastroscope (Olympus GIF-260; Olympus, Tokyo, Japan) was used to observe the gastric tissues. All subjects were examined by the same endoscope and one endoscopist. The light source strength and type of endoscopic lamp used was also same in the study. The color change of the gastrointestinal mucosa (the esophagus, cardia, gastric fundus, gastric antrum, duodenal bulb and the descending portion) was also overserved in all persons.

Bile influx measurement. One-channel MI catheters with unique sensor arrays were used to traverse the working channels of the upper endoscopes. Bile influx was measured in all subjects. Motility index was tested at the sites of 2, 5 and 10 cm above the squamocolumnar junction. The motility index values were compared at different levels along the esophageal axis between the patients with HAPC and the healthy subjects.

Histological analysis. The mucosa was collected at the Endoscopy Surgery Department, the People's Hospital of the Tibet Autonomous Region (Tibet, China). We analyzed 12 gastric antrum biopsies, including 6 biopsies from patients with HAPC and another 6 from the control subjects. The samples were frozen in liquid nitrogen as soon as the surgical excision was completed, and they were stored at -80˚C. Prior to histological analyses, the gastric mucosa tissues were treated formalin and embedded in paraffin. The treated tissues were cut into 5-μm-thick sections, and stained with hematoxylin and eosin (Sigma, St. Louis, MO, USA). The results were analyzed by two experts in a double-blinded manner. The mucosa was considered injured if one or more of the following were observed: non-continuous surface, expanded gland, hemorrhage, or cells with destructed morphology (24).

RNA extraction. Gastric samples from the 3 patients with HAPC and 3 control subjects were collected. All samples were treated with 3 ml TRIzol (Invitrogen, Carlsbad, CA, USA), and homogenized with a homogenizer. Following extraction with chloroform, RNA was precipitated with isopropanol. The resultant pellets were re-suspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Following DNase digestion, the quantification and purity RNA was measured at 260/280 nm using an Agilent-Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA integrity and genome DNA contamination was identified using agarose gel electrophoresis.

Microarray data analysis. Single-color gene expression profiles were created to compare data between the patients with HAPC patients and the healthy controls. These profiles was constructed using 4x44 K oligonucleotide microarrays (Agilent Technologies). The human genome microarray has 41,000 genes and transcripts. The representative sequences can be found at public databases. The RNA samples were amplified and then labeled using a labeling kit (Agilent Technologies) and hybridized with a human genome microarray in the chambers of Agilent's SureHyb. Following hybridization and washing, the slides were scanned using a DNA microarray scanner (G2505B; Agilent Technologies) and analyzed using Feature Extraction Analytics software (version 9.5.3; Agilent Technologies). All the procedures were performed at KangChen Bio-Tech (Shanghai, China). The Agilent GeneSpring GX software (version 7.3; Agilent Technologies) was used to analyze the signal intensities, which represent the gene levels. For data analysis, fold changes were used to explore the differentially expressed genes (DEGs) at the 2-fold change cut-off. Genes were regarded as upregulated genes with changes in expression of ≥2-fold compared to the controls. By contrast, genes were regarded as downregulated

Table I. Primers and product sizes of genes that were examined by RT-qPCR.

Gene	Length	Forward primer	Reverse primer
KLK1	140 bp	GCTCTGTACCATTTCAGCAC	GCTGTGTTTTCGTCGTCAAA
KLK3	160 bp	ATCCTGTCTCGGATTGTGGG	AGATCACGCTTTTGTTCCTG
KLK7	150 bp	GCAGGAGAAGAAGCCCAGGG	GTGGGCGGCAGTGAGCACCC
KLK8	140 bp	GCCTGGGCAGGACACTCCAG	CAGTTGCCACCTACAAGGAC
KLK12	151 bp	GAGGGCACCAGCCTGCGCTG	AGCCGCTGTGCCGGATCTGC

genes with changes in expression of <2-fold. Changes in expersion of 0.50-1.99-fold were not regarded as significant.

Gene Ontology (GO) analysis. The GO database was referred to in order to analyze the DEGs involving biological functions and signaling pathways. The GO project has a controlled vocabulary to show gene functions in any organism (http://www.geneontology.org). The ontology has three different domains: biological processes, cellular components and molecular functions. Fisher's exact test was used to determine whether there were overlaps between the DEGs list and the GO annotation. A value of P<0.05 indicate that there were statistically significant differences in GO enrichment terms in the DEGs.

Reverse transcription-quantitative PCR (RT-qPCR). The microarray results were repeatedly confirmed by RT-qPCR for top DEGs, including KLK1, KLK3, KLK7, KLK8, KLK12 and the actin gene was used as a loading control. In order to compensate the shortage of the sample size of transcriptome analysis, total RNA was isolated from gastric mucosal samples from another 20 patients with HAPC patients and another 20 healthy subjects living at the same altitude. RNA (5 μg) was reverse-transcribed using a reverse transcriptase reaction kit (Takara, Dalian, China). Using the primers shown in Table I, PCR was conducted in triplicate using SYBR-Green PCR Master Mix and the 7500 Fast real-time PCR detection system (ABI Biosystems, Foster City, CA, USA) with the amplified conditions: 95˚C for 10 min, 45 cycles of 95˚C for 10 sec, 60˚C for 34 sec and 60˚C for 60 sec. The relative expression values were calculated using the $2^{-\Delta\Delta T}$ method.

DEGs on chromosome locations. Chromosomes may be related to HAPC-induced GML (25,26), which can be reflected by DEGs on different chromosomes. Thus, in this study, all the DEGs were marked on 24 chromosomes to visualize their distributions on all chromosomes.

Remodeling the interaction between KLKs and cholesterol. Cholosterol has been widely reported to be associated with hypertension (27,28), while kallikrein hypertension is also well known (29,30). Thus, in this study, we explored the interaction between KLKs and cholesterol using the software PyMOL v1.8 (https://www.pymol.org) and AutoDock 4.2.6 (http://autodock.scripps.edu).

Statistical analysis. Data are presented as the means ± SD. Hierarchical cluster analysis was performed between HPAC and healthy groups using transcriptional data and SPSS software. The Chi-square and Student's t-test were used to calculate the statistical significance of paired data where appropriate. A value of P<0.05 was considered to indicate a statistically significant difference.

Table II. Baseline characters between HPAC patients and healthy subjects.

	HPAC patients (n=30 cases)	Healthy controls (n=30 cases)	P-values
Male, n (%)	13 (43.3)	13 (43.3)	1
Age, years	42.5±2.5	42.5±2.5	1
Smokers, n (%)	15 (50.0)	15 (50.0)	1
Drinkers, n (%)	12 (40.0)	12 (40.0)	1
Spouse, n (%)	21 (70.0)	21 (70.0)	1
Living altitude (m)	4,200±600	4,200±600	1
Bile reflux, n (%)	26 (86.7)	5 (16.7)	0.00
Score of gastric mucosal damage	3.04±0.31	0.26±0.05	0.00
Hyperemia and bleeding, n (%)	54 (90.0)	5 (8.3)	0.00

In HAPC and healthy groups, all patients received endoscopy detection, 3 cases were used for transcriptome analysis and 20 cases were used for RT-qPCR analysis, respectively. None were examined by both transcriptome analysis and RT-qPCR analysis. Hyperemia and bleeding was calculated as 2 cases in each subject. The Chi-square statistic and Student's t-test were performed to determine differences between 2 groups. A value of P<0.05 indicates significant differences.

Results

Endoscopic findings of the upper gastrointestinal tract. The patients had a similar gender distribution, age, living altitude and lifestyle with the healthy subjects (Table II). In the HAPC group, the endoscopic findings reveaked darker colors in the upper gastrointestinal tract when compared with the controls, including the esophagus (N1, brown; P1, dark red), cardia (N2, thin brown; P2, dark red brown), gastric fundus (N3, thin brown; P3, dark red brown), gastric antrum and body (N4, brown; P4, red), the duodenal bulb (N5, brown; P5, dark brown) and descending portion (N6, brown; P6, dark brown) (Fig. 1). All the N-marked numbers indicate the gastrointestinal tract from healthy subjects, whereas all the P-marked numbers indicate the gastrointestinal tract from patients with HAPC. Furthermore, in the HAPC group, the mucosa was thin

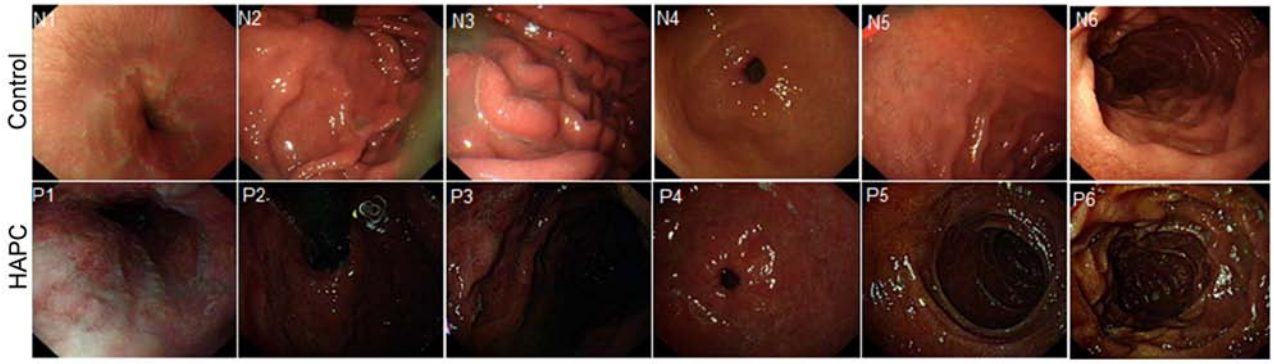

Figure 1. Endoscopic findings of the upper gastrointestinal tract. The upper gastrointestinal tract includes the esophagus (N1/P1), cardia (N2/P2), gastric fundus (N3/P3), gastric antrum and body (N4/P4), the duodenal bulb (N5/P5) and descending portion (N6/P6). 'N' indicates normal healthy subjects. 'P' indicates patients with polycythemia.

and red, with a fine meshwork of vessels (Fig. 1, P1 and P4). Furthermore, congestion was detected in areas where veins were slightly wider. Additionally, due to the significantly red appearance of the esophageal mucosa, there were no evident boundaries that were distinguished between the esophageal mucosa and gastric mucosa. The color was orange below the line. Diffuse hyperemia, edema and changes of congestion, were observed in HAPC group, whereas no such symptoms were observed in the control group. In addition, the duodenal bulb was slightly enlarged with marked hyperemia and swelling in the HAPC group when compared with the control group (Fig. 1).

Abnormal contraction and relaxation only occurred in approximately 12.5% of the subjects, and most pyloric antra contracted and relaxed normally in the control group (P<0.01). Additionally, in the HAPC group, bile was observed in both the fundus and body of the stomach, as well as in the pyloric antrum and varying degrees of bile reflux were detected in approximately 86.7% of the patients. By contrast, in the control group, gastric bile was observed in only 16.7% of the subjects (P<0.001) (Table II).

Histopathological changes. As shown in Fig. 2A, histopathological analysis revealed that HAPC induced severe congestion, edema and multiple hemorrhagic erosions in the gastric antrum mucosa from patients with HAPC. By contrast, no significant damage was observed in the gastric mucosa from the control group subjects. On the other hand, the mean score of gastric mucosal damage was 3.04±0.31 in the patients with HAPC, which was higher than the score of 0.26±0.05 in the control subjects (P<0.01) (Table II).

Under a microscope, a variety of changes in vessels within the gastric mucosa was observed in the HAPC group, such as dilation and distortion accompanied by hyperemia and bleeding; these changes were greater than those of the control group, with statistically significant differences (90.0 vs. 8.3%, P<0.001) (Table II). Under high magnification, the number of vessels/cm^2 was significantly higher in the HAPC group than in the control group (24.68±4.38 vs. 11.79±2.43, P<0.05) (Fig. 2C). Similarly, the average vessel diameter was significantly higher in the HAPC group than in the control group (39.2±11.5 vs. 15.9±4.5, P<0.05) (Fig. 2D). Furthermore, the RBC counts were also higher in the HAPC group than in the control group (160.91±62.53 vs. 30.33±15.98, P<0.05) (Fig. 2E).

Hierarchical cluster analysis of the DEGs in the gastric mucosa of patients with HAPC. We analyzed the expression spectrum of the DEGs in the gastric mucosa of patients with HAPC and the control group subjects. Transcriptome analysis revealed mRNA subtype-specific and clinical subtype-specific patterns of DEGs. DEGs with >2-fold changes in expression at the transcriptional level were considerably more diverse in the HAPC group than in the control group. However, the number of overall upregulated DEGs was higher in the control group than in the HAPC group.

Hierarchical cluster analysis revealed 4,341 upregulated DEGs and 5,363 downregulated DEGs in the gastric mucosa of patients with HAPC. In particular, the kallikrein gene cluster (KLK1/3/7/8/12) was upregulated >17-fold in the patietns with HAPC compared to the controls (Fig. 3).

Results of RT-qPCR. Transcriptome analysis revealed the changes in all KLK members and 10 members were DEGs (Fig. 3A). Among these members, KLK1/3/7/8/12 were significantly upregulated, KLK11 and KLK15 were downregulated, and KLK4/5/10 were slightly upregulated. No significant differences were observed in the other members (KLK2/6/9/14) as shown by RT-qPCR analysis of these genes in 3 pairs of tissues from patients wtih HAPC-induced GML; KLK1 and KLK3 were upregulated >30-fold, while KLK5 and KLK11 were downregulated 3-fold in the tissues from patients with HAPC-induced GML compared with the healthy tissues. To confirm the microarray results, RT-qPCR was performed to measure 5 top upregulated genes from 20 HAPC patients and 20 healthy subjects, including the 3 pairs of patients and healthy subjects analyzed by transcriptome analysis. The results revealed that the KLK1, KLK3, KLK7, KLK8 and KLK12 expression patterns (Fig. 3B) were similar to those observed in the microarray experiments (Fig. 3A).

Biological function analysis of DEG. The identified differential genes were annotated in the GO format for biological function classification and in the pathway analysis format for the elucidation of whole chains of events in the gastric mucosa tissues from patients with HAPC compared with the controls. In the GO analysis, the upregulated DEGs were involved in responses to tissue injuries, inducing smooth muscles, increasing vascular permeability and complex formation, and others. The downregulated DEGs were involved in intrinsic GTPase activity,

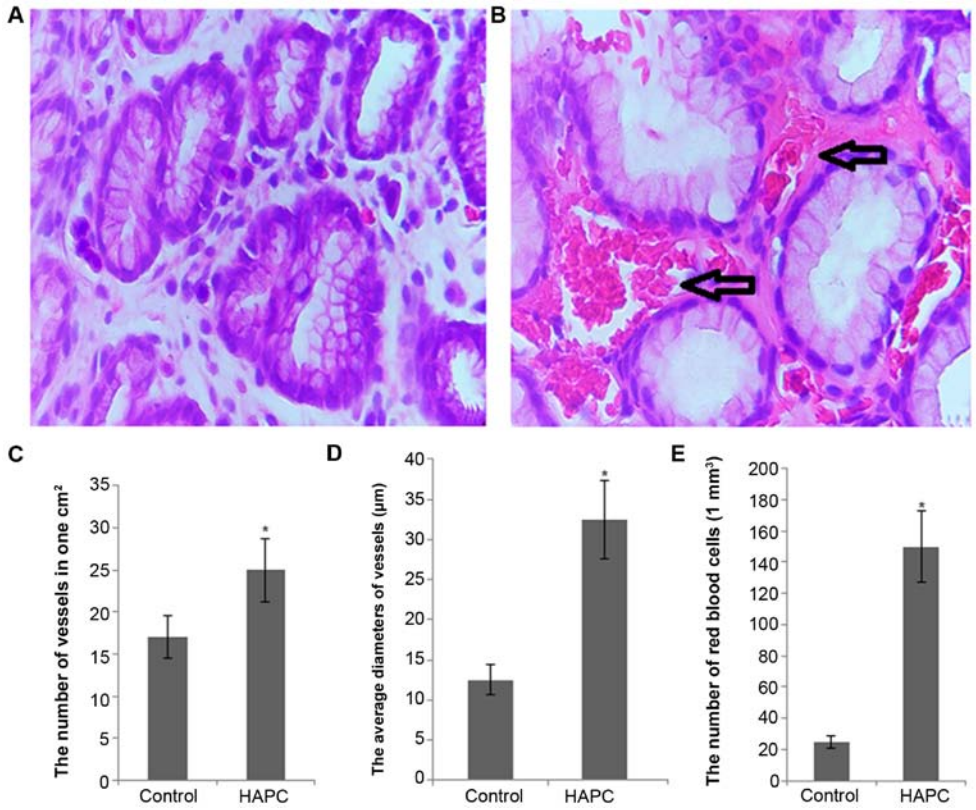

Figure 2. Histopathological examination of gastric mucosa tissue sections. (A) Results of hematoxylin and eosin staining showing the appearance of the gastric mucosa in the antrum region of the control group (x400 magnification). (B) Results of hematoxylin and eosin staining showing the appearance of the gastric mucosa in the antrum region of patients with high altitude-associated polycythemia (HAPC) (x400 magnification). (C) Number of vessels in 1 cm^2. (D) Average diameters of vessels (μm). (E) The number of red blood cells (1 mm^3). The arrows indicate a high concentration of red blood cells. All data are presented as average values ± SD and n=3/group. *P<0.05 vs. controls.

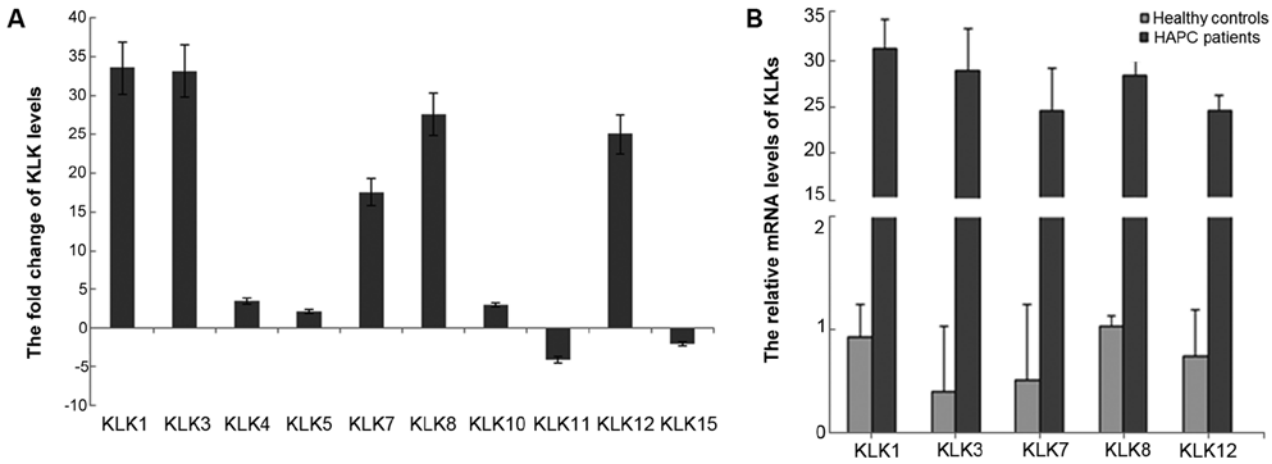

Figure 3. Changes in the mRNA levels of KLK genes between patients with high altitude-associated polycythemia (HAPC) and healthy subjects. (A) Microarray analysis of the fold changes in the levels of KLK genes in HAPC patients when compared with healthy subjects (n=3 patients with HAPC and n=3 healthy controls). The data are presented as the mean values in each group. (B) RT-qPCR analysis of the top 5 upregulated KLK genes. The results represent the quantification of the mRNA levels relative to β-actin. Normalized expression values were obtained by RT-qPCR (n=20 patients with HAPC and n=20 healthy controls). The data are presented as the mean values ± SD.

cellular processes, recruiting PH domain proteins, cell growth and proliferation, as well as others (data not shown).

The map of DEGs on 24 chromosomes. All DEGs were marked on 24 chromosomes (Fig. 4). KLK1, KLK3, KLK7, KLK8 and KLK12 were all located on chromosome 19q13.3-13.4 and were highly upregulated in the HAPC group compared with the controls.

Interaction between KLK members and cholesterol. AutoDock analysis revealed that 5 members (KLK1/2/7/8/12) had high-score binding ability with cholesterol (Table III).

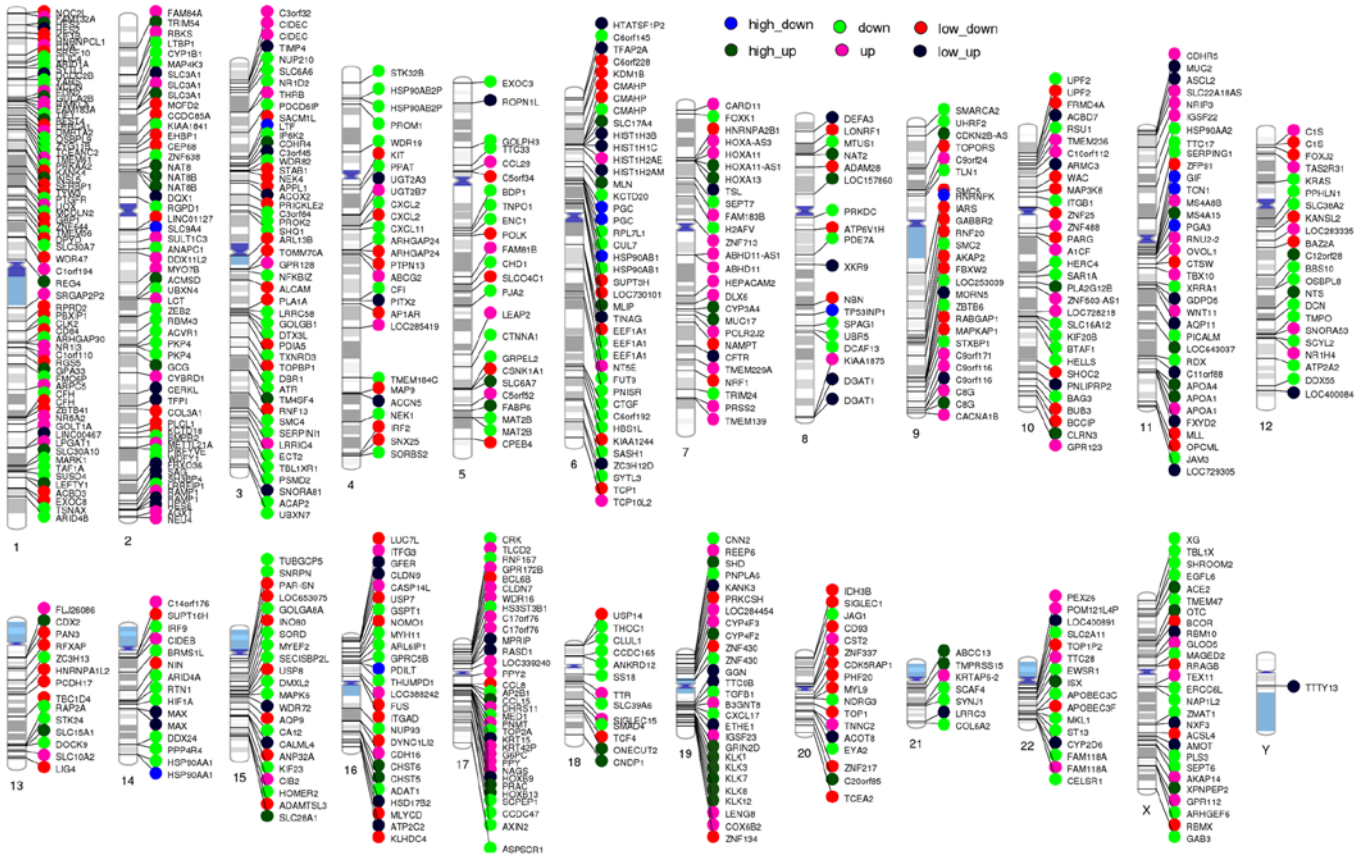

Figure 4. Whole chromosome view of the expression levels of the 400 top differentially expressed genes (DEGs) mapped to 24 chromosomes. Each circle represents one DEG. Expression levels are normalized to six grades (low_up/4-6-fold, up/7-10-fold, high_up/>10-fold; low_down/4-6-fold, down/7-10-fold, high_down/>10-fold).

All the members had different binding sites with cholesterol. Cholesterol tended to bind the sites near the C-terminal KLK1and KLK2 sequences, the N-terminal KLK7 and KLK12 sequences and the middle KLK8 sequence (Fig. 5). Using the polymer of KLK7 as the model, the results revealed that the trimers tended to have high score for binding cholesterol (Fig. 5 and Table III).

Discussion

The Qinghai-Tibetan Plateau is the largest and highest plateau, which contains the largest high-altitude population worldwide. Due to the increase in the concentrations of RBC, there is a significant increase in blood viscosity and both microcirculation disturbances and systemic disorders can occur. However, no effective prevention and control measures have yet been utilized. Generally, the incidence of HAPC increases with the elevation of altitude. However, due the varieties of living environments such as different altitudes, and diverse ethnic groups, the incidence of HAPC is differs around the world (31).

The pathogenesis of HAPC is complex and it is difficult to overcome. It has been widely reported that the increased synthesis and release of erythropoietin, induced by long-term exposure to high-altitude hypoxic conditions, is a key factor in the development of HAPC (32). In this study, Hb concentrations, RBC counts, the number of microvessels and the diameter of microvessels in the HAPC group were significantly higher than those in control group. This was also associated with the notion that the initiating factor of HAPC was the high-altitude hypoxia-induced enhancement of bone marrow erythropoiesis, which induced RBC hyperplasia and related clinical manifestations. In addition, the oxygen saturation of arterial blood in the HAPC group was significantly lower than that in control group, indicating that this may be associated with excessive RBC hyperplasia, as well as increased blood viscosity and flow resistance in patients with HAPC.

Studies have shown that in the presence of high altitude-induced hypoxia, the dynamic equilibrium between *in vivo* nitric oxide and endothelin is disrupted, causing enhanced vasoconstriction and increased systemic peripheral resistance. This leads to the dilation of mucosal vessels. Moreover, HAPC caused by high altitude-induced hypoxia leads to increased blood viscosity, decreased blood flow and severe local mucosal congestion, resulting in severe vascular hyperemia and even rupture. As a consequence, microcirculation disturbances occur in the gastric mucosa (15). Considering the environment contributing to long-term high altitude-induced hypoxia, the increase in the numbers of RBC can cause damage to the gastrointestinal mucosa and compromise normal physiological functions, such as digestion and absorption.

The Whole Human Genome Oligo Microarray is a broad view that represented all known genes and transcripts in the human genome. Sequences were compiled from a broad source survey, and then verified and optimized by alignment to the

Table III. Interaction between the KLK1/2/7/8/12 gene cluster members and cholesterol.

KLK members	Score	Area	Atomic contact energy	Transformation
KLK1	4284	487.40	-339.50	2.36, -0.29, -2.37, 121.70, 37.95, -11.19
KLK2	4944	553.90	-204.99	2.48, 0.98, 0.14, -13.00, 16.86, 13.76
KLK7	4386	481.70	-205.67	-3.04, -0.67, 2.14, -0.62, 10.67, 10.93
KLK8	4342	456.00	-305.37	0.29, -0.67, -0.59, -11.48, 21.89, 4.73
KLK12	4734	564.50	-252.11	-1.68, -0.14, -0.24, -24.08, -16.63, -7.34
KLK7 complex	5534	613.60	-62.14	2.90, -0.66, 2.05, -27.73, -5.61, 25.19

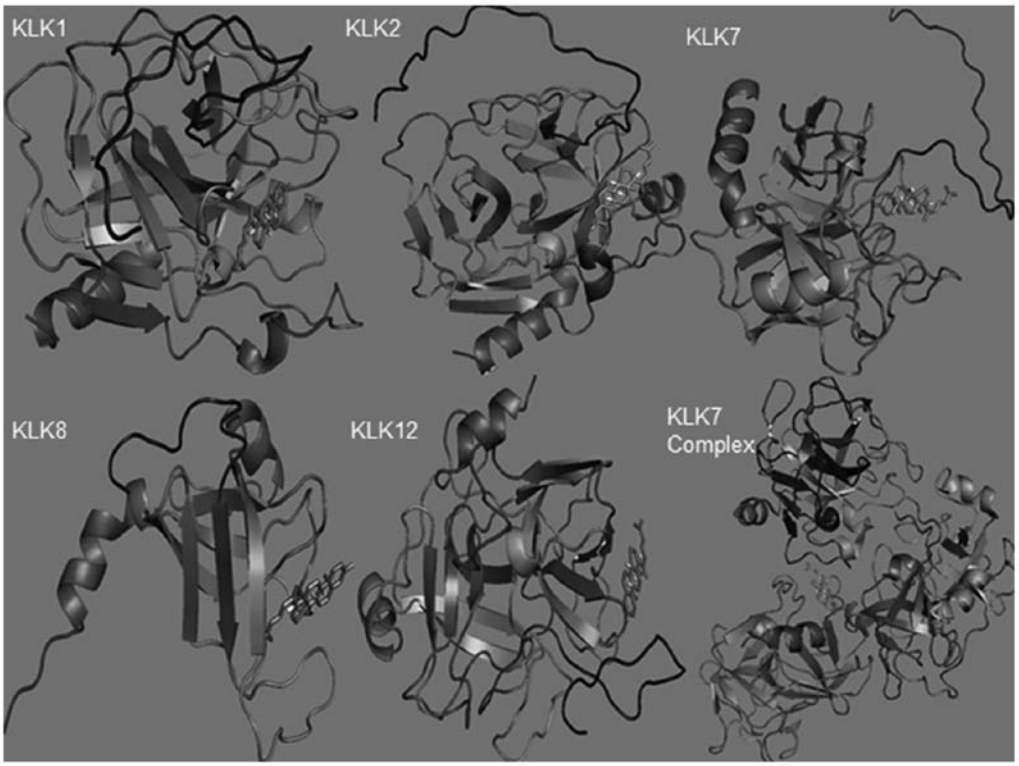

Figure 5. AutoDock remodeling of the interaction between KLK1/2/7/8/12 gene cluster members and cholesterol. Cholesterol tended to bind the sites near the C-terminal of KLK1 and KLK2 sequences, the N-terminal of KLK7 and KLK12 sequences and the middle of the KLK8 sequence. The trimers of KLK7 were also formed to bind cholesterol.

assembled human genome. In this study, gastric mucosa tissues samples from patients with HAPC and healthy controls were analyzed using genome microarrays, in which 4,941 genes (fold change ≥2) were upregulated and 5,363 genes (fold change ≤0.5) were downregulated in the patients with GML. Using GO and pathway analyses, we then examined the function of DEGs. Our results indicated that HAPC-induced GML was a process involving multiple genes and pathways.

Certainly, there are some limitations to the present study. Firstly, the use of only 6 subjects seems to be a small population with which to explore the molecular mechanisms of GML. Furthermore, the more detailed molecular mechanisms remain to be explored. The pathogenesis of HAPC-induced GML was not explored in this study this was one of initial aims. The disease can be elucidated *in vitro* using gastric cells from GML-related induced pluripotent stem (iPS) cells (33).

Kallikreins are a group of 15 serine proteases, which are encoded by the largest gene cluster of proteases. KLK loci have been described in the human (34), chimpanzee (35), mouse (36) and rat (37) genomes (Fig. 6). Generally, KLK loci consist of a single copy of KLK4-KLK15 genes with different numbers of classical KLK genes and pseudogenes, which are caused by gene duplication. In humans, the KLK loci span approximately 300 kb on chromosome 19 in the cytogenic region 13.3-13.4. The KLK genes are clustered together and are not intervened by other genes. The three KLK genes (KLK1, KLK2 and KLK3) are clustered within KLK15, while KLK4-KLK14 and the ΨKLK1 pseudogene are located telomeric to KLK2. The transcriptional direction of KLK genes is from telomere to centromere exception of KLK2 and KLK3.

Over the past decades, a number of studies have demonstrated important pathophysiological roles for these kallikreins in various tissues (38). Therefore, kallikreins are considered as attractive targets for the development of novel therapies for airway disorders (39), cardiovascular disease (40), brain injury (41), skin inflammation and allergy (42) and neoplastic

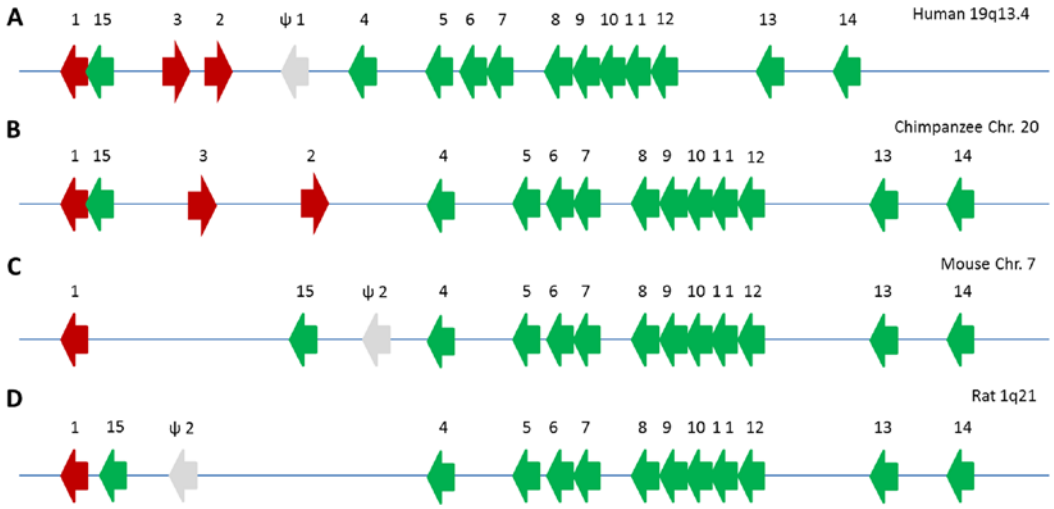

Figure 6 Distribution of kallikrein gene loci. (A) Distribution of kallikrein gene loci in humans. (B) Distribution of kallikrein gene loci in the chimpanzee. (C) Distribution of kallikrein gene loci in the mouse. (D) Distribution of kallikrein gene loci in the rat genome. Arrowheads indicate the transcriptional direction of KLK genes. Red arrowheads indicate classical kallikrein genes (KLK1/KLK2/KLK3). Green arrowheads indicate non-classical kallikrein genes from KLK4 to KLK15. Grey arrowheads indicate kallikrein pseudogenes.

disorders (43). Increasing evidence indicates that many kallikreins are implicated in carcinogenesis (44) and are utilized as potential biomarkers for head and neck squamous cell carcinoma (45) and colorectal adenocarcinoma (46). There are 15 kallikrein members located on chromosome 19. These proteins have conserved functions in their capacities to release the vasoactive peptide (47) and Lys-bradykinin from low-molecular-weight kininogen (48).

KLK1 belongs to a member of the peptidase S1 family and is a serine protease, which involves kinin production for the normal functions of cardiac and arterial tissues. A previous study demonstrated that KLK1 is involved in the normal development of uterine endometrial tissues (49). Bradykinin released from the endothelium plays a critical role in the regulation of the human cardiovascular system. Endothelial cells significantly express *de novo* kallikrein, and it plays an important role in the generation of vasoactive kinins (50). Kallikrein-kinin system has been shown to be involved in some functions of kidneys, such as salt homeostasis. Therefore, reduced levels of KLK1 contribute to salt-sensitive hypertension in Dahl salt-sensitive animal models (51). In this study, we found increased levels of KLK1 in patients with HPAC, which may play a protective role in preventing the development of hypertension. Polycythemia innovles an increase in the amount of RBC which circulate in the blood stream. Thus, these changes increase the viscosity of the blood and result in high blood pressure.

KLK3 is also known as prostate-specific antigen or γ-seminoprotein, which is a glycoprotein enzyme encoded by the KLK3 gene. PSA is secreted by the epithelial cells from the prostate gland and is a widely used biomarker for prostate cancer (52). Elevated levels of serum PSA concentrations are often found in patients with prostate cancer compared with healthy individuals. According to associations found in healthy adults between the seminal plasma and serum concentrations of PSA, the mutants of KLK2 and KLK3 can be benefical to adjust the cut-off value in the PSA model for prostate cancer (53). Obese individuals have been observed to have low levels of serum PSA. Delayed early detection in obese men may result in the risk of prostate cancer (54). Of note, patients with polycythemia tend to have a higher risk for cancer (55). Moreover, the association between polycythemia and obesity has been widely reported (56).

KLK7 is a serine protease encoded by the KLK7 gene located on chromosome 19q13. KLK7 is initially purified from the epidermis and is a stratum corneum chymotryptic enzyme (57). KLK7 has been shown to be aberrantly expressed in colon cancer and to be involved in cell proliferation *in vitro* and *in vivo*. Thus, KLK7 is a potential therapeutic target for human colon cancer (58). KLK7 specifically participates in pathophysiological events associated with skin disorder, gastrointestinal tract injury and central nervous system diseases (59). In this study, KLK7 was found to be highly expressed in gastric tissue and it was found that i may cause gastric injury, as indicated by gastrointestinal endoscopy.

KLK8 is a tryptic serine protease with a few types of substrates. KLK is expressed in many developing organs, while its expression is restricted to limited regions such as the hippocampus. In the hippocampus, KLK8 is involved in activity-dependent synaptic changes, including long-term potentiation, which can be suppressed in KLK8-knockout mice. KLK8 is expressed in oligodendrocytes following injury to the central nervous system. KLK8 is also highly expressed in the epidermis of the skin and is involves in desquamation by the degradation of adhesive molecules which connect layers of the epidermis. Therefore, KLK8 may play a role in tissue development and rearrangement (60). On the other hand, KLK8 has been shown to be associated with the progression of inflammation (61). In our study, we demonstrated that high levels of KLK8 may contribute to the development of gastric inflammation.

The kallikrein family consists of 15 genes, most of which have been found to be differentially expressed in various types of cancer and may be used as biomarkers for cancer prognosis. The levels of KLK5, KLK6, KLK12 and KLK13 are consistently related the risk of prostate cancer and tumor aggressiveness (62). Furthermore, KLK12 plays a critical role in angiogenesis by modulating the bioavailability of proangiogenic factor and activating the kinin-receptor-B2 signaling pathway. However,

the proangiogenic activity of KLK12 is different from KLK1 and is not related to kinin release in lung endothelial cells (63). Although little is known about angiogenesis and polycythemia, our results suggest that elevated levels of KLK12 cause angiogenesis which may be associated with polycythemia.

Most importantly, KLKs are associated with hypertension (29,30), while cholesterol is also associated with hypertension (27,28) which is an important clinical symptoms of HAPC. Thus, in this study, we explored the interaction between KLKs and cholesterol using AutoDock remodeling. The results suggested that all five members (KLK1/2/7/8/12) had high-score binding ability with cholesterol by binding it from different sites. Moreover, the trimer of KLK7 was used to find that polymers could bind cholesterol well. All the findings implied that KLKs are important proteins for binding cholesterol through different mechanisms, causing signs of KLK hypertension, which may be closely associated with HPAC-induced GML.

As already stated above, there are some limitations to the present study. Firstly, only three subjects were selected in each group for transcriptome analysis and some bias may exist. To avoid this bias, RT-qPCR was performed using tissues from 20 healthy subjects and 20 patients with HAPC residing at the same high altitudes, and the results revealed the same trend: KLK1, KLK3, KLK7, KLK8 and KLK12 were all upregulated in patients with HAPC compared with the controls. Thus, the trend was not caused by group bias. Secondly, the exact functions of KLK1, KLK3, KLK7, KLK8 and KLK12 were not determined in the present study. Animal models should be used to confirm our findings. Thirdly, the interaction of these molecules was not identified. Finally, only a few crystal structures of KLKs are available, thus the interaction of polymers of KLK1/2/8/12 and cholesterol cannot be explored by molecular remodeling. Thus, further research is required in order to fully understand the molecular mechanisms responsible for the development of HAPC and for HAPC-related diseases.

In conclusion, as demonstrated by the above-mentioned data, the elevated levels of KLK1, KLK3, KLK7, KLK8 and KLK12 may be closely associated with hypertension, inflammation, obesity and other gastric injuries associated with polycythemia. From the interacting partners of KLK members (Table III), the five members, KLK1, KLK3, KLK7, KLK8 and KLK12, may have multiple functions. The interaction of KLKs and cholesterol may play an important role in hypertension in patients with HAPC, which results in gastric injuries in these patients. The results of this study originated from the whole genome microarray data comparing patients with HAPC with healthy controls, providing evidence of the molecular mechanisms responsible for HAPC-induced GML. Therefore, the present findings indicated that HAPC-induced GML leads to the activation of protective responses through the upregulation of the levels of the KLK1/3/7/8/12 gene cluster. These results may also have implications in the treatment of gastric lesions.

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