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A Novel Mutation in ALK-1 Causes Hereditary Hemorrhagic Telangiectasia Type 2

ABSTRACT

Hereditary hemorrhagic telangiectasia (HHT) is an autosomal-dominant bleeding disorder and has two variants, HHT1 and HHT2, associated with mutations in the *ENG* and *ALK-1* genes, respectively. We identified one Chinese HHT2 family to investigate the pathogenic gene and its possible mechanism of action by mutation screening and functional study. One substitution mutation (1717C>T) in exon 10 of the *ALK-1* was found by sequencing of all exons of *ENG* and *ALK-1* and caused a R479X mutation in the *ALK-1* protein. *ALK-1* mRNA and plasma thrombomodulin were measured by real-time quantitative PCR and ELISA, respectively. There was no significant difference in the expression levels of *ALK-1* mRNA between patients and healthy individuals. A significantly higher level of thrombomodulin was found in HHT patients. These findings indicate that the mutation causes truncation of the ALK-1 protein at the post-transcriptional level; the plasma thrombomodulin may provide an easy diagnostic indicator in HHT patients.

KEY WORDS: hereditary hemorrhagic telangiectasia (HHT), thrombomodulin, mutation, ALK-1.

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INTRODUCTION

Hereditary hemorrhagic telangiectasia (HHT, OMIM #187300) (Osler, 1901) is an autosomal-dominant vascular dysplasia characterized by telangiectases and arteriovenous malformations of skin, mucosa, and viscera, with direct arteriovenous connections lacking capillary beds (Braverman *et al.*, 1990). HHT patients often visit dental professionals for their oral-facial bleeding, at sites such as the tongue and gingival. HHT occurs over a wide ethnic and geographic distribution. Its prevalence is high, from 1:2351 to 1:39,216 in Western countries (Plauchu and Bideau, 1984; Porteous *et al.*, 1992; Jessurun *et al.*, 1993), but the prevalence of HHT in the Chinese population has not been investigated until now.

The disease has two known variants, HHT1 and HHT2 (Johnson *et al.*, 1996; Kjeldsen *et al.*, 2001). A higher frequency of pulmonary arteriovenous malformations (PAVMs) has been reported for HHT1, while HHT2 is thought to be associated with a lower penetrance and milder disease manifestations (Shovlin *et al.*, 2000). Studies have confirmed that the pathogenic genes are the endoglin gene (ENG) and the activin receptor-like kinase-1 gene (*ALK-1*) for HHT1 and HHT2, respectively. Several mutations of the two genes have been identified in HHT patients in many countries (Johnson *et al.*, 1996; Shovlin *et al.*, 1997; Kjeldsen *et al.*, 2001); however, there is little reported for the Chinese population (Zhang *et al.*, 2004). ENG and ALK-1 are type III and type I transforming growth factor beta (TGF-β) family receptors, respectively (Lux *et al.*, 1999). Animal models have shown that these receptors are important not only for maintaining vascular integrity, but also for angiogenesis during embryonic development and tumor growth (van den Driesche *et al.*, 2003). The precise mechanisms of vascular abnormalities elicited by these mutations observed in HHT patients are still unclear, although analysis of the mechanism of intracellular signal transduction has been performed, and a change in targeted gene expression was found with the use of mutant recombinant endoglin or ALK-1 proteins (Azuma, 2000).

Thrombomodulin (TM) is a glycoprotein, present on intact endothelial cell surfaces, that plays a major role in activation of the protein C anticoagulant system (Esmon, 1995). *ALK-1* is a type I TGF-β receptor and is exclusively expressed on vascular endothelial cells (Lux *et al.*, 1999). TGF-β1 has been shown to down-regulate thrombomodulin mRNA expression in cultured human endothelial cells (Ohji *et al.*, 1995). Although many studies have suggested that the TGF-β signaling family may play a key role in HHT (Bourdeau *et al.*, 1999), the precise mechanism giving rise to the vascular abnormalities is unclear. There have been few reports on the transcriptional changes and thrombomodulin levels in HHT. In the present study, we performed mutation screening on the *ENG* and *ALK-1* genes in one Chinese family with HHT2, who visited our clinic with recurrent tongue bleeding. We measured ALK-1 mRNA and thrombomodulin to investigate the possible mechanisms of formation of the vascular abnormalities in this HHT family.

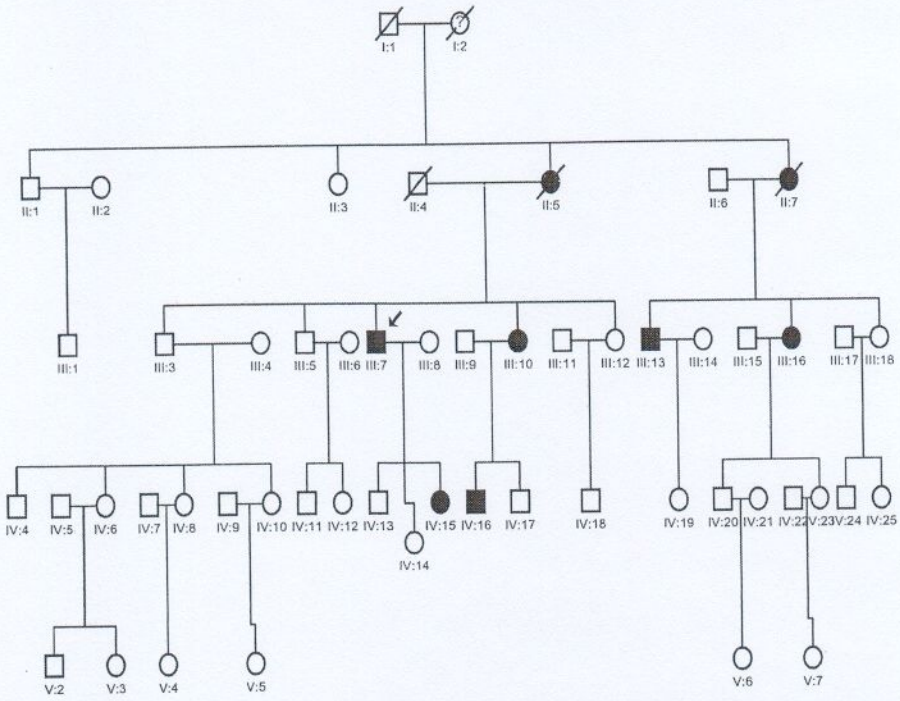

Figure 1. The pedigree diagram of this Chinese Han family with hereditary hemorrhagic telangiectasia Type 2 (HHT2). The arrowhead indicates the proband of this family, ■ indicates HHT2 patients, and ❑ indicates the normal family numbers. There are eight patients, including the proband, and 46 normal individuals in this family. It is an autosomal-dominant hereditary disease, according to genetic analysis.

MATERIALS & METHODS

Pedigree Data

The proband was a 49-year-old man who had severe bleeding on the ventral surface of the tongue. Through subsequent interviews, we extended the pedigree to three generations, including 13 individuals, six suffering from similar symptoms and seven unaffected (Fig. 1). The diagnosis of HHT2 was based on family history and the presence of cutaneous or mucocutaneous arteriovenous malformations (Shovlin *et al.*, 2000). The study was conducted with the consent of all family members and was approved by the Ethics Committee of the Peking University Health Science Center. Physical examinations were performed for all 13 family members.

Genotyping and Linkage Analysis

We collected peripheral blood samples and mixed them with sodium citrate as anti-coagulant. Genomic DNA was extracted from peripheral blood as described by Miller *et al.* (1988). Microsatellite marker information was obtained from the Genome Database (http://www.gdb.org/). PCR amplifications were carried out according to the manufacturer's instructions for the Goldentaq polymerase kit (ABI Inc., Foster City, CA, USA). PCR products were run on a commercial POP6 gel (ABI Inc.) in an ABI3700 automatic sequencing analyzer. The results were collected and analyzed by GeneMapper 3.1 software (ABI Inc.). Two-point linkage analysis was carried out with the MLINK program from the LINKAGE version 5.1 software package (Lathrop and Lalouel, 1984).

Sequence Analysis

We performed mutation screening on the ENG and ALK-1 genes on all 13 family members, with 110 normal, unrelated individuals (220 chromosomes) as controls. Intronic primers flanking the exons were used for amplifying and sequencing PCR products. Primers were designed using Primer 3 software (ABI, Inc.). PCR amplification was performed with 15 μL of a PCR reaction mix containing 50 ng of genomic DNA, 10 mM Tris (pH 8.4), 50 mM KCl, 3.0 mM $MgCl_2$, 200 μM of each dNTP, 0.6 units HotStataq™ DNA Polymerase (Qiagen, Valencia, CA, USA), and 0.3 μM of each primer. PCR thermocycles were conducted on a Perkin-Elmer GeneAmp PCR System 9700 (Perkin-Elmer, Wellesley, MA, USA). An initial denaturation was performed for 15 min at 95°C, followed by denaturation for 30 sec at 94°C, annealing for 1 min at 63°C (with the annealing temperature being decreased by 0.5°C for each cycle during the initial 15 cycles), and extension for 1 min 30 sec at 72°C. The annealing temperature then remained at 58°C for 40 sec for the subsequent 25 cycles, and the extension was reduced to 60 sec at 72°. A final extension was performed at 72°C for 10 min. The amplification products were purified with the use of a MultiScreen-PCR plate (Millipore, Billerica, MA, USA). Cycle sequencing was conducted with the use of the Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer) according to the manufacturer's instructions. All sequencing was carried out on an ABI Prism 3700 DNA Analyzer. The results were analyzed with BioEdit software and compared with sequences in the NCBI databases.

Real-time Quantitative PCR

The B-lymphocytes of peripheral venous blood from all 13 family members and 50 unrelated normal controls were infected by Epstein-Barr virus, after which immortalized cell lines were established. Total RNA was extracted and reverse-transcribed into cDNA. The ALK-1 mRNA expression level, which was quantified by the cycle threshold method, was measured with the use of a QuantiTectTM SYBR® Green PCR Kit (Qiagen), according to the manufacturer's instructions, in an ABI PRISM 7000 sequence detection system. Values were then normalized to the relative amounts of the control gene albumin (Alb, NM_000477). Primers were designed with the use of Primer 3 software, as follows: Forward and reverse primer were 5′-TCTCAGGCCTAGCTCAGATGAT-3′ and 5′-TAGGCTTCTCTGGACTGTTGCT-3′, and the sequences were 117 bp. Alb cDNA was amplified with primers: Forward primers were 5′-AATGCCCTGTGCAGAAGACT-3′, and reverse primers were 5′-CTGTGCAGCATTTGGTGACT-3′, and the sequences were 101 bp. Real-time PCR was performed by three-step cycling

under the following conditions: initial denaturation at 95°C for 15 min; and denaturation, annealing, and extension at 94°C for 30 sec, 59°C for 30 sec, and 72°C for 1 min (40 cycles). Fluorescence was measured at 59°C at the end of every cycle.

Cell Fractionation and Western Blot Analysis

The cell fractionations were performed as described previously from immortalized cell lines (Shimizu *et al.*, 1997). The proteins were separated by 10% SDS-PAGE and transferred to PVDF membrane with a semi-dry transfer apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were probed with monoclonal antibodies against ALK-1 (1:1000, R&D Systems, Minneapolis, MN, USA). For internal controls, the blots were stripped and re-probed with monoclonal antibodies against α-tubulin (1: 20,000, Sigma-Aldrich, St. Louis, MO, USA). Both Western blots were probed with mouse HRP-conjugated secondary antisera (Amersham, Buckinghamshire, UK), at a dilution of 1:3000, with proteins detected with the ECL kit (Amersham).

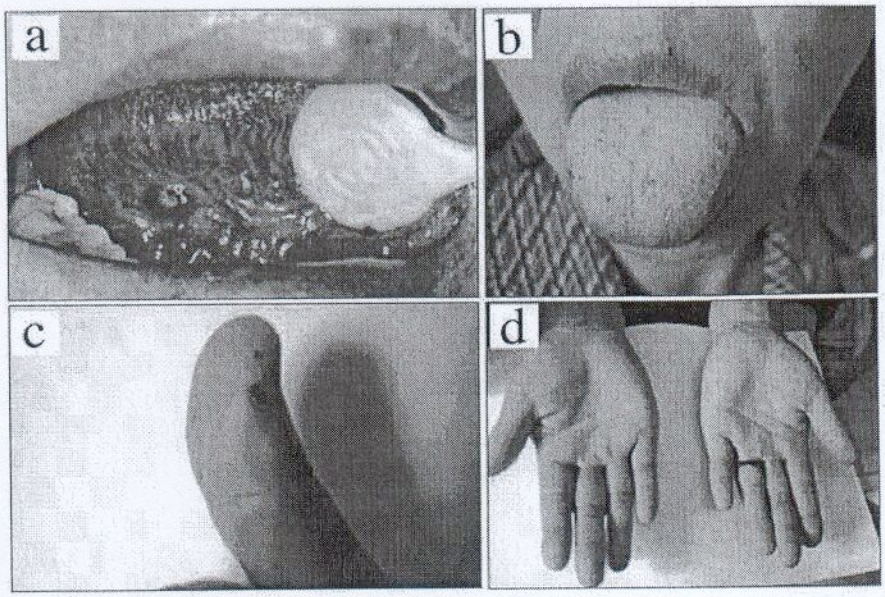

Figure 2. The lesions in patients with hereditary hemorrhagic telangiectasia type 2 in this Chinese Han family. All photographs were taken with the patients' consent. **(a)** Lesions on the ventral surface of the tongue from patient No. III:7, who had severe bleeding on the tongue; **(b,c,d)** lesions on the dorsum of the tongue, on the thumb, and on the palms of patient No. III:10, who had bleeding of the tongue and fingers.

Thrombomodulin ELISA

Peripheral venous blood samples from all 13 family members and 50 unrelated normal controls were obtained, treated with EDTA as anti-coagulant, and centrifuged at 4000 *g* for 20 min. The plasma was separated and stored at -70°C. Plasma was 50x diluted. Soluble plasma thrombomodulin was measured by a Thrombomodulin ELISA kit (Market Inc.).**(AQ)** Each sample was assayed 3 times, according to the manufacturer's instructions, using a spectrophotometer (Bio-Rad Model 550 Microplate Reader) at 450-nm wavelengths, and concentration was determined from a standard curve.

Statistics

All statistical calculations were performed with the use of GraphPad Prism 4 Demo statistical software; for real-time quantitative PCR, a χ^2 test was used, and for ELISA, one-way ANOVA was used to test for significance. $P < 0.05$ was considered statistically significant.

RESULTS

The family had six HHT patients with hemorrhagic symptoms and seven healthy individuals. The symptoms of HHT ranged from mild cutaneous and mucocutaneous telangiectasis to severe bleeding (Fig. 2), but without pulmonary arteriovenous malformation, so all patients were diagnosed as HHT2 on the basis of clinical symptoms. We examined all the exons of ALK-1 gene and found a C-to-T substitution mutation in exon 10 of *ALK-1* (1717C>T) (GI: 4557242, NM_000020.1) (Fig. 3a). This caused the base sequence to change from codon CGA to TGA (R479X) and resulted in a substantial shortening of the *ALK-1* protein in its predicted intracellular domain. Western blotting showed a smaller band for ALK-1 protein from one patient's sample and indicated the truncated ALK-1 protein. There were no significant differences in the protein expression level of ALK-1 (α-tubulin as the internal control) (Fig. 3b). Notably, all six symptomatic individuals had the same mutation. The *ALK-1* mutation was not found in any of the seven healthy relatives or the 110 normal, unrelated controls (220 chromosomes). No mutation in the endoglin gene was found in any of the 13 family members or controls. The linkage analysis results revealed that the microsatellite markers near *ALK-1*, including D12s1586 LOD_{ZMAX}, were 1.82 ($\theta = 0.0$), D12s1677 LOD_{ZMAX} was 1.74 ($\theta = 0.0$), D12s1635 LOD_{ZMAX} was 1.65 ($\theta = 0.0$), D12s368 LOD_{ZMAX} was 1.87 ($\theta = 0.0$), also supporting these results.

There were no significant differences in *ALK-1* mRNA expression levels among the patients (0.979 ± 0.011, $n = 6$), the healthy individuals of the family (0.991 ± 0.019, $n = 7$), and the normal controls (0.985 ± 0.028, $n = 50$) (Fig. 4a). Plasma thrombomodulin of the patients (1194.81 ± 71.50 ng/mL, $n = 6$) was significantly higher ($p < 0.001$) than that of healthy individuals (465.60 ± 79.91 ng/mL, $n = 7$) in this family and in normal controls (594.16 ± 224.22 ng/mL, $n = 50$). No significant difference was found between healthy individuals in this family and normal controls (Fig. 4b).

DISCUSSION

Two genes, *ENG* and *ALK-1*, are associated with hereditary hemorrhagic telangiectasia type 1 and hereditary hemorrhagic telangiectasia type 2, respectively. However, at least two kindreds appeared to have mutations in a third, as yet unknown, gene (Piantanida *et al.*, 1996; Wallace and Shovlin, 2000). To date, at least 29 and 17 different kinds of mutations in *ENG* and

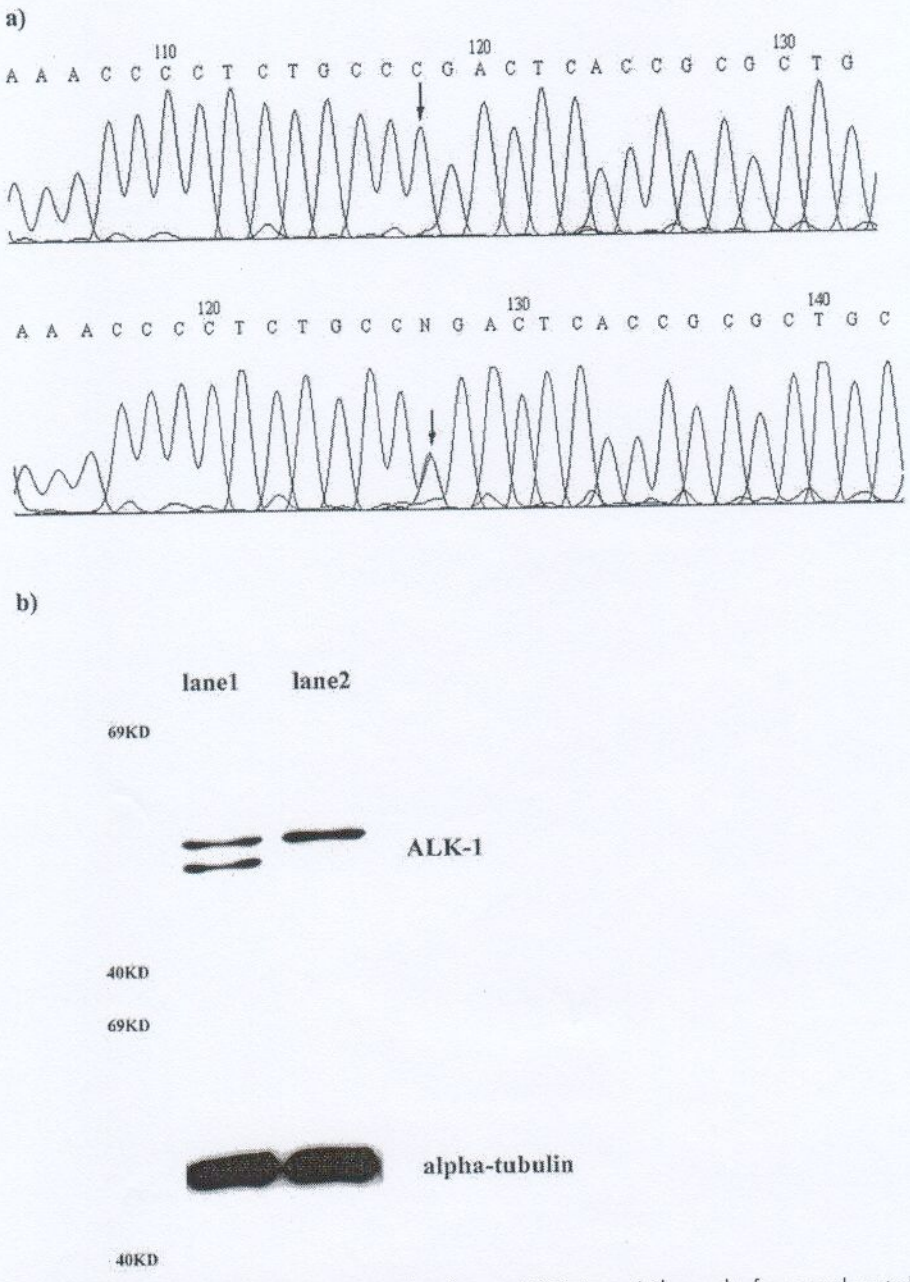

Figure 3.(AQ) (a) Exon 10 sequencing results of gene *ALK-1*. (upper) The result of a normal control; (lower) the result of the patient in this family. The arrowhead indicates the mutation base. This is a C-to-T mutation in base 1717 of *ALK-1* gene (1717C>T). This mutation resulted in an amino acid change, from Arg to stop codon (R479X) in the *ALK-1* protein. (b) Western blotting verified that the *ALK-1* protein was truncated. The protein was isolated from immortalized cells from both patients and normal family members. Lane 1 indicates the patient's sample, and lane 2 is the sample from a normal individual. A smaller band in the *ALK-1* protein from the patient's sample indicates the truncated *ALK-1* protein. The small band was not found in the sample from the normal individual. There were no significant differences in the protein expression levels of *ALK-1* (α-tubulin, the internal control).

ALK-1, respectively, have been found, including missense, nonsense, frameshift, and deletion mutations (Azuma, 2000). The most common mutation of the *ALK-1* is located on exons 8 and 3. Analysis of current data suggests that most disease-causing mutations from both *ENG* and *ALK-1* result in truncated or non-expressed proteins.

In this study, we identified a novel mutation in exon 10 of the *ALK-1* gene in one Chinese family. The substitution mutation of a C to a T at the base position 1717 leads to CGA (Arginine R) changing into TGA (termination codon, R479X). It is a novel mutation of HHT, not previously reported. The identification of *ALK-1* mutations in patients confirmed the clinical diagnosis of HHT type 2. Our results suggest that an early diagnosis of HHT by a molecular method may be useful for the early control of associated visceral involvement.

The disease manifestations are variable in severity and include epistaxis, gastrointestinal bleeding, pulmonary arteriovenous malformations, and both cerebral and liver arteriovenous malformations (Shovlin *et al.*, 1997). The nasal mucosa is the most common site for telangiectasia (95%), followed by the tongue (60%), palate, lips, buccal area, gum, pharynx, and conjunctivae (Gorlin *et al.*, 1990; Pau *et al.*, 2000). There were 27 clinical reports of HHT from 1973 to 2003 in China, wherein 232 patients were described, with 82% having a family history and 20% having pulmonary arteriovenous malformations (Su, 1999). In our cases, some of the patients exhibited epistaxis, and the index case exhibited recurrent tongue bleeding. Not only are dental clinicians likely to be the first clinicians to identify a patient with HHT, but also routine dental treatment of unidentified HHT patients, without prophylactic treatment, could be fatal.

In the present study, we identified a novel missense mutation in exon 10 of the ALK-1 gene in one Chinese family, and this mutation caused a truncated ALK-1 protein; however, there were no significant differences in *ALK-1* mRNA expression between six patients and the seven healthy members of the family, which indicated that the missense mutation occurred in genomic DNA, but did not affect ALK-1 mRNA transcription.

Thrombomodulin is a glycoprotein, present on intact endothelial cell surfaces, that plays a major role in activation of the protein C anticoagulant system. Thrombomodulin is a transmembrane receptor for thrombin (Esmon, 1995) and is

down-regulated by inflammatory cytokines. Down-regulation can be prevented by retanoic acid and cyclic AMP (Dittman, 1991). At present, thrombomodulin is increasingly used as a marker of endothelial damage in a large number of diseases, such as ulcerative colitis, sepsis, pre-eclampsia, diabetes mellitus-associated micro- or macroangiopathy (Magriples *et al.*, 1997), thrombotic-thrombocytopenic purpura, adult respiratory distress syndrome, and coronary and other atherosclerotic lesions (Boffa and Karmochkine, 1998). Increased thrombomodulin levels were also associated with disease activity in vasculitis, such as Takayasu's arteritis, Behçet disease, giant cell arthritis, polyarteritis nodosa, and Wegener's granulomatosis (Boehme *et al.*, 1997). In the present study, a significant increase in plasma thrombomodulin in patients suggested that there was damage to vascular endothelial cells in these Chinese patients with HHT2.

The level of thrombomodulin in endothelial cells has been shown to be modulated by cytokines, including transforming growth factor-β (TGF-β). Both TGF-β1 and TGF-β2 have been shown to down-regulate thrombomodulin mRNA expression in cultured human endothelial cells (Ohji *et al.*, 1995; Sandusky *et al.*, 2002), and increased TGF-β correlates with decreased thrombomodulin-containing vessels in sustained local endothelial dysfunction (Richter *et al.*, 1997). Since ALK-1 is a type I TGF-β receptor, and is exclusively expressed in vascular endothelial cells, it is suggested that the mutation found in this family produced a truncated ALK-1 protein, interrupting the receptor interaction with TGF-β1 and causing down-regulated function of TGF-β1, resulting in up-regulation of thrombomodulin, or truncated ALK-1 protein counteracted TGF-β1 directly, destoying the vascular endothelial cells and causing more soluble thrombomodulin in the blood.(AQ) The relationship between ALK-1 and thrombomodulin requires further study, but plasma thrombomodulin may provide an easy molecular marker in HHT patients.

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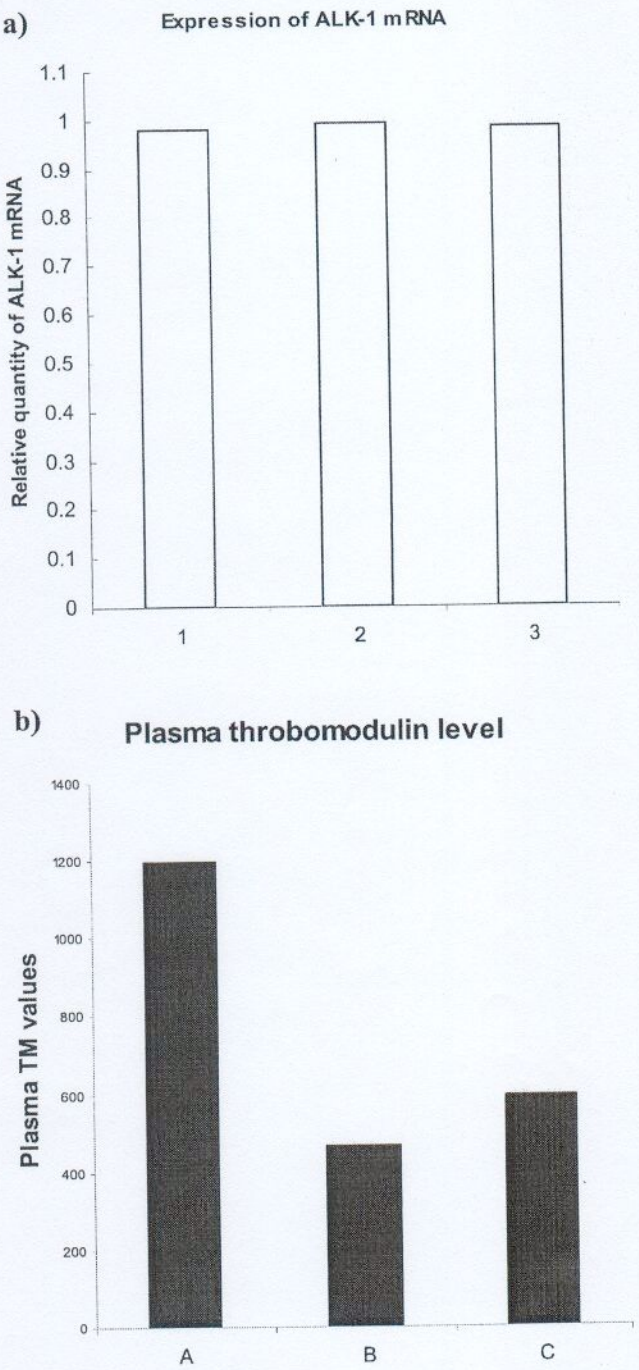

Figure 4.(AQ) (a) The expression of *ALK-1* mRNA. From the results of the real-time quantitive PCR, the relative quantity is the ratio of *ALK-1* mRNA/Alb (internal control). There were no significant differences of *ALK-1* mRNA expression level among the patients (column 1, n = 6), the healthy individuals of the family (column 2, n = 7), and normal controls (column 3, n = 50). Results are expressed as means of 3 experiments in triplicate ± SD. (b) Plasma thrombomodulin of the patients (column A, n = 6) and healthy individuals (column B, n = 7) in this family, and of normal controls (column C, n = 50). There was a significantly higher thrombomodulin level in patients ($p < 0.001$) than in healthy individuals in this family and normal controls. No significant difference(AQ) was found between healthy individuals in this family and normal controls. Results are expressed as means of 3 experiments ± SD.

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