MUTATION IN BRIEF

DHPLC-Based Mutation Analysis of ENG and ALK-1 Genes in HHT Italian Population

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Hereditary haemorrhagic telangiectasia (HHT or Rendu-Osler-Weber syndrome) is an autosomal dominant disorder characterized by localized angiodysplasia due to mutations in endoglin, ALK-1 gene, and a still unidentified locus. The lack of highly recurrent mutations, locus heterogeneity, and the presence of mutations in almost all coding exons of the two genes makes the screening for mutations time-consuming and costly. In the present study, we developed a DHPLC-based protocol for mutation detection in ALK1 and ENG genes through retrospective analysis of known sequence variants, 20 causative mutations and 11 polymorphisms, and a prospective analysis on 47 probands with unknown mutation. Overall DHPLC analysis identified the causative mutation in 61 out 66 DNA samples (92.4%). We found 31 different mutations in the ALK1 gene, of which 15 are novel, and 20, of which 12 are novel, in the ENG gene, thus providing for the first time the mutational spectrum in a cohort of Italian HHT patients. In addition, we characterized the splicing pattern of ALK1 gene in lymphoblastoid cells, both in normal controls and in two individuals carrying a mutation in the non-invariant –3 position of the acceptor splice site upstream exon 6 (c.626-3C>G). Functional essay demonstrated the existence, also in normal individuals, of a small proportion of ALK1 alternative splicing, due to exon 5 skipping, and the presence of further aberrant splicing isoforms in the individuals carrying the c.626-3C>G mutation.

KEY WORDS: ALK1; ACVRL1; ENG; HHT; hereditary haemorrhagic telangiectasia; Rendu-Osler-Weber Syndrome; DHPLC

INTRODUCTION

Hereditary haemorrhagic telangiectasia (HHT or Rendu-Osler-Weber syndrome; MIM# 187300) is an autosomal dominant disorder characterized by localized angiodysplasia. The current prevalence is 1:8,000 (Begbie et al., 2003). Phenotypic penetrance is age-dependent and nearly complete by age 40. The expression of HHT is extremely variable with a great disparity of clinical manifestations between affected individuals, even among members of the same family. HHT is characterized by mucocutaneous telangiectases, frequent epistaxis, and gastrointestinal hemorrhages. Arteriovenous malformations affect the lung, brain, liver, and more rarely, the spinal

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cord.

Mutations in either of two identified genes (McAllister et al., 1994; Johnson et al., 1996) *endoglin* (*ENG*; MIM# 131195; XM_055188) and *ALK-1* (*ACVRL1*; MIM# 601284; Z22533) are responsible for the disease. The existence of a third locus has been reported (Cole et al, 2005). Thus far, 163 mutations of the *ENG* gene and 131 mutations of the *ALK1* gene, mainly detected with direct sequencing, have been described (Bayrak-Toydemir et al., 2004; Brusgaard et al., 2004; Letteboer et al., 2005; Abdalla et al., 2005; Kuehl et al., 2005; Schulte et al., 2005) and, with few exceptions, the mutations tend to be family-specific.

The key to the appropriate management of patients with HHT is to establish an early diagnosis; in addition, the identification of the mutation can contribute to the elucidation of the role of the critical regions of these two genes involved in different functional aspects.

Many methods for screening genetic alterations have been developed, including single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), constant denaturant gel electrophoresis (CDGE) and direct sequencing which accelerate mutational analysis at the DNA level. However, each of these methods has limitations: either a low sensitivity and reproducibility or high costs and difficulty to perform.

Denaturing high-performance liquid chromatography (DHPLC) for detection of DNA heteroduplexes was developed as a method for identifying DNA variations. The overall cost of this technique were initially considered slightly higher than that for conventional methods but due to its high sensitivity of 95-100% (Gross et al., 1999; Erlandson et al., 2000), DHPLC is now used to detect sequence variation for numerous genes. However, because of its low cost and simplicity, SSCP is still being used to detect genetic mutations. The aims of our study were to develop a DHPLC-based protocol for mutation detection in *ALK1* and *ENG* genes and to determine the mutational spectrum in a cohort of Italian HHT patients.

MATERIAL AND METHODS

Patients

A total of 66 unrelated patients (59 familial and seven sporadic cases) referred to our Interdepartmental HHT Centre at University of Bari, received a definite clinical diagnosis of HHT according to Curacao criteria (Shovlin et al., 2000). Peripheral blood was obtained with informed consent.

Mutation analysis

Genomic DNA was isolated from peripheral blood according to standard procedures. PCR was performed using the primer pairs and conditions listed in Table 1. SSCP was carried out using a GeneGel excel (Pharmacia, Gaithersburg, MD) run at 12°C on a GenePhor system (Pharmacia, Gaithersburg, MD) according to the manufacturer's instruction.

DHPLC was performed on a WAVE 1100 DNA fragment analysis system (Transgenomic, Crewe, UK). To enhance heteroduplex formation, untreated PCR products were denatured at 95°C for 5 minutes, followed by gradual reannealing to 25°C over 50 minutes. Products were automatically loaded on a DNA sep column and eluted according to manufacturer's instructions at a flow rate of 0.9 ml/min by mixing buffer A (0.1 mM TEAA) and buffer B (0.1 mM TEAA and 25% acetonitrile) with a Buffer B increase of 2% per minute for 4.5 minutes. Samples were detected by a UV-C system. The oven temperature(s) for optimal heteroduplex separation under partial DNA denaturation were determined for each amplified fragment using the WAVE Maker software (version 1.6.0) and adjusted according to experience. The criteria for optimal heteroduplex separation and assigning the presence of a sequence alteration are explained in detail in the first part of the results.

Direct sequencing was performed using BigDye chemistry (Applied Biosystems, Foster City, CA) on the ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Whenever a missense variant was found, to exclude the possibility that we were dealing with a polymorphism, 100 healthy controls were analysed, the co-segregation with the disease in other members of the family and the amino acid conservation among homologous genes were verified. All mutations were named according to international recommendations (den Dunnen and Paalmas, 2003; www.hgvs.org/mutnomen/). Nucleotide numbers derived from the GenBank reference sequences were as follows: *ENG* genomic sequence: 3513290 (AH006911.1), *ALK1* genomic sequence: 2228561 (AH005451.1), *ENG* cDNA: 33871100 (BC014271.2), *ALK1* cDNA: 4557242 (NM_000020.1).

Table 1. PCR conditions and DHPLC parameters used to analyze each *ENG* and *ALK1* coding amplicon

Amplicon Name	Size (bp)	PCR condition	Primer sequences	Reference	Elution T (°C)	Stop % Buffer B
ENG EXON 1	180	58°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: actggacacaggataaggcccag R: aatacttggggcctggtccgtg	Gallione et al., 1998	66-67	58,6
ENG EXON 2	341	58°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: cctcataaggtggctgtgatgatg R: catctgccttggagcttcctct	McAllister et al., 1994	60-62-63	64,8
ENG EXON 3	261	50°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: aacctatacaaatctgact R: acagagatggacagtagg	This work	63-64	62,4
ENG EXON 4	331	50°C, 2,5mM $MgCl_2$, 2U Taq Pol	F: ttcctgacctcctacatgg R: ctcttggtgcccaagttt	McAllister et al., 1994	62-63	64,6
ENG EXON 5	325	55°C, 1,5mM $MgCl_2$, 1U Taq pol	F: gggctctgttaggtgcag R: gggtggggctttataaggga	McAllister et al., 1994	65-66-67	64,4
ENG EXON 6	234	55°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: ctgtccgcttcagtgttccatc R: ggaaacttccctgatccagaggtt	McAllister et al., 1994	65-66	61.4
ENG EXON 7	315	58°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: gaggcctggcataaccct R: gtggccactgatccaagg	McAllister et al., 1994	62-64	64.1
ENG EXON 8	201	58°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: gccgcctggcctgcctctgcta R: tgagccagaggggcaggagtt	Shovlin et al., 1997	62-63-64	59.8
ENG EXON 9A	291	55°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: aatggctgtgacttgggaccctg R: accaaccaggctggtcctgatac	This work	63-64	63.5
ENG EXON 9B	213	58°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: ctgcaggggctcagaacaca R: ggccaggtgggttaagcacg	Gallione et al., 1998	62	60.4
ENG EXON 10	268	58°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: agattgaccaagtctccctccc R: aggctgtctccctcctgactct	McAllister et al., 1994	63-64-65	62.7
ENG EXON 11	436	55°C, 1,5mM $MgCl_2$, 2U Taq Pol	F: actcaggggtgggaactctt R: ccttccatgcaaaccacag	McAllister et al., 1994	64-65	66.5
ENG EXON 12	154	55°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: gagtaaacctggaagccgc R: gccactagaacaaacccgag	McAllister et al., 1994	58	56.7
ENG EXON 13	255	58°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: ccagcacaacagggtaggggat R: ctcagaggcttcactgggctcc	McAllister et al., 1994	63	62.2
ENG EXON 14	198	60°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: aggaccctgacctccgcc R: ctctcctgctgggcgagc	Gallione et al., 1998	62	59.6

Amplicon Name	Size (bp)	PCR condition	Primer sequences	Reference	Elution T (°C)	Stop % Buffer B
ALK1 EXON 2	266	60°C, 2,5mM $MgCl_2$, 1U Taq Pol	F: ctctgtgatttcctctgggca R: tacattctccccagcttctcaa	Berg et al., 1997	62-63-64	62.6
ALK1 EXON 3	345	62°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: agcctgggaccacagtggctga R: ggaggcaggggccaagaagat	Berg et al., 1997	66	64.9
ALK1 EXON 4	318	58°C, 2,5mM $MgCl_2$, 1U Taq Pol	F: agctgacctagtggaagctga R: ctgattctgcagttcctatctg	Berg et al., 1997	64-65-66	64.2
ALK1 EXON 5	242	66°C, 2,5mM $MgCl_2$, 1U Taq Pol	F: aggagcttgcagtgacccagca R: atgagagcccttggtcctcatcca	Berg et al., 1997	64-65	61.7
ALK1 EXON 6	294	58°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: aggcagcgcagcatcaagat R: aaacttgagccctgagtgcag	Berg et al., 1997	64	63.6
ALK1 EXON 7	388	63°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: tgacgactccagcctcccttag R: caagctccgcccacctgtgaa	Berg et al., 1997	65-66	65.8
ALK1 EXON 8	293	63°C, 2,5mM $MgCl_2$, 1U Taq Pol	F: aggtttgggagaggggcaggagt R: ggctccacaggctgattcccctt	Berg et al., 1997	65	63.5
ALK1 EXON 9	256	66°C, 1,5mM $MgCl_2$, 1U Taq Pol	F: tcctctgggtggtattgggcctc R: cagaaatcccagccgtgagccac	Berg et al., 1997	64-65	62.3
ALK1 EXON 10	197	62°C, 2,5mM $MgCl_2$, 1U Taq Pol	F: tctcctctgcacctctctcccaa R: ctgcaggcagaaaggaatcaggtgtc	Berg et al., 1997	60-64	62.7

Functional study

Total RNA was extracted from 10^6 lymphoblastoid cells from patients 503/02, 710/02 and normal controls in two separate experiments using a High Pure RNA isolation kit (Roche Diagnostic GmbH, Mannheim, Germany). A total of 11 µl of RNA from each sample (4 µg) were retro-transcribed using 200 U of MMLV-RT (Promega, Madison WI, USA), 1 µl of Random primers (Promega, Madison WI, USA) and 20 U of RNAsin RNAse Inhibitors (Promega, Madison WI, USA). The cDNA was amplified using 3.5 U of Taq Expand Long Template PCR System (Roche Diagnostic, Germany), Taq Expand Long Template Buffer 2 (Roche Diagnostic GmbH, Mannheim, Germany), and exonic primers ALK1cDNA4F and ALK1cDNA8R (Johnson et al., 1996). Bands were visualized on both polyacrylamide and agarose gel, eluted using QIAquick Gel extraction kit (QIAGEN) and cloned in a TOPOPCR2.1 TA-cloning system (Invitrogen Inc, USA). For each sample, 40 clones were sequenced as described above.

RESULTS

In the first phase of our study, the mutation screening of both *ENG* and *ALK1* genes was performed with SSCP analysis on genomic DNA from a cohort of non-related HHT patients. The disease-causing mutation was detected in 20/26 (76.9%) probands (Lastella et al., 2003 and unpublished results). As the ability to detect gene mutation has placed DHPLC technology at the forefront of genetic analysis for a wide variety of diseases, in order to set up a DHPLC-based protocol and to standardize the conditions for the *ENG* and *ALK1* gene mutation screening, first a retrospective analysis was performed and DNA samples were selected for this purpose from the 20 patients in whom the mutation had been identified. The *ALK1*-mutated-subgroup was composed of 13 DNA samples from affected individuals, each one heterozygous for one out 13 different mutations distributed along six out nine exons of the *ALK1* gene, including exon-intron junctions (Table 2A; columns a and b); the *ENG*-mutated-subgroup was composed of seven DNA samples from affected individuals, each one carrier of one out 7 different mutations scattered on seven out 15 *ENG* exons (Table 2B; columns a and b). Furthermore, 11 well-known polymorphic variants were analysed, four in the *ALK1* and seven in the *ENG* gene, respectively (Table 3). DNA samples from three normal subjects were used as controls. Initially, for each PCR product, the melting behaviour of the wild-type sequence was visualized using the WAVE^{TM} Maker algorithm. The elution for each PCR product was performed at a temperature corresponding to 80-90% α-helical fraction for each melting domain and, when necessary, the temperatures were manually adjusted with 1-2°C gradual increases (Xiao and Oefner, 2001) and then tested. The optimal quantity of the loaded PCR product corresponded to a peak of A_{260} ~4-12 mV.

With the conditions listed in Table 1, 31/31 (100%) sequence variants were detected by means of one or more additional peaks with respect to the corresponding wild-type control.

Most amplicons were composed of different melting domains, thus more than one elution temperature was needed to detect the variants. For instance, the two point mutations in *ALK1* exon 10, as well as one disease-causing and two polymorphic variants in *ENG* exon 8 fell within distinct melting domains and were detected at different temperatures. Conversely, all four mutations distributed throughout the *ALK1* exon 8 were clearly detected at 65°C.

With regard to the mutations falling in exon 3 of the *ALK1* gene, the elution pattern at 65°C of the amplicon of a DNA sample bearing only the heterozygous intron 3 polymorphism (c.313+11C>T) was virtually identical to that of amplicons also carrying pathological changes. Considering that the afore-mentioned widespread polymorphism is highly recurrent (Abdalla et al., 2005; Olivieri et al., 2002; Lesca et al., 2004), it was crucial to obtain specific reproducible chromatograms for the disease-causing alleles harboured in *ALK1* exon 3. Therefore, seven DNA samples, called the "*ALK*1-exon 3-subgroup"(see Table 4) which exhibited different genotypes including those homozygous or compound heterozygous for the SNP but bearing a disease-causing mutation at the same time, were selected to identify specific elution patterns. In the absence of any disease-causing mutation, the elution pattern of the heterozygous genotype (C/T) showed two clear additional peaks for all temperatures tested (65°C, 66°C) when compared to the single peak of both homozygous (C/C;T/T) genotypes. However, only at a temperature of 66°C did the two disease-causing mutations falling in exon 3 yield elution patterns which clearly differed from those corresponding to all the three wild-type SNP-related genotypes, irrespective of their SNP-related genotypes and whether they were in cis with either the C- or T-allele of the SNP. As shown in Figure 1, a DNA fragment carrying a mutation yielded an additional peak when homozygous for the polymorphism, and three additional peaks when compound heterozygous for the polymorphism. All patterns were absolutely reproducible.

Once the conditions to ascertain the mutation detection were optimized, DNA samples from 41 HHT individuals with unknown mutations together with six cases which remained unresolved using SSCP analysis were screened prospectively by DHPLC. The causative mutation was found in 42 out 47 patients (89.4%). By means of both retrospective and prospective DHPLC mutational screening, we were able to detect 31 different mutations in the *ALK1* gene, 15 of which are novel, and 20 in the *ENG* gene, 12 of which are novel (HHT Mutation Database, http://137.195.14.43/cgi-bin/WebObjects/hht.woa). In addition, two unpublished *ALK1* nucleotide substitutions which are predicted to be silent sequence variants, and two *ENG* amino acidic changes, one of which unreported, were found. Complete DNA sequencing for the 5 probands in whom no disease-causing mutation was detected failed to reveal any nucleotide variant.

All 42 mutations identified by prospective DHPLC analysis were tested in turn by the retrospective SSCP technique (Table 2A and 2B, columns c and d), with 10 resulting undetectable (Table 2A and 2B, columns a-d). All these ten mutations consist in a 1-bp substitution.

***ALK1* mutations**

In the present work we present 15 novel and 16 previously published mutations (Table 2A) in the *ALK1* gene. Nine novel mutations are represented by single base-pair substitutions, giving rise to missense mutations. One missense mutation in exon 3, c.121T>C (p.C41R), affects a cysteine residue which is conserved in the extracellular, ligand-binding, domain of all members of TGFβ/BMP-type I-receptor group (Klaus et al., 1998). The c.283T>C (p.C95R) disrupts the so-called "cysteine-box" region which is a peculiar feature of both type I- and type II-serine/threonine receptors. The third missense mutation in exon 3 (c.197A>C) causes a previously unreported substitution of a non-conserved histidine with a proline residue. The introduction of a proline might induce a distortion in the three-dimensional structure of the extracellular domain of the mutant protein. Even though the familial history was positive for the disease, none of the other affected members was alive. The substitution was absent in the 58-year-old healthy proband's sister. Since this change was not found in the 100 healthy controls, and no additional variant was identified in the patient, it was considered the causative mutation. It is noteworthy to note that no polymorphic variant consisting in amino acidic substitutions have ever been reported in the *ALK1* gene (Lesca et al., 2004). The remaining six missense mutations affect the intracellular Ser/Thr kinase domain. Two missense mutations were detected in exon 6. The c.656G>A transition (p.G219H) and the c.686A>T transversion (p.K229M) involve highly conserved amino acids in all *ALK1* orthologs and fall in the kinase subdomains I and II, respectively. In addition, the p.K229M mutation directly affects the ATP-binding site. Two missense mutations fall in exon 8; c.1115C>T (p.T372I) lies in a sequence of eight amino acids within subdomain VIII which forms part of a consensus specific to Ser/Thr kinases. In two apparently unrelated families (HHT12 and HHT20), the mutation c.1208T>C (p.L403P) affects a highly conserved residue, resulting in a predicted α-helix disruption (Abdalla et al., 2003). A missense mutation in exon 9, c.1280A>T (p.D427V), changes a highly conserved and positive-charged aspartate into a hydrophobic valine within subdomain X. All such mutant proteins are most likely misfolded and targeted to intracellular degradation or, alternatively,mconstitute non-functional polypeptides (Abdalla et al., 2003). One nonsense mutation, c.448C>T (p.Q150X), was identified in exon 4; the corresponding mRNA is probably unstable and subjected to nonsense-mediated-decay (NMD, Berg et al., 1997). One 2-bp insertion (c.218_219insAA) and one 4-bp duplication (c.115_118dupCCAC) were detected in exon 3. They give rise to frameshifts and premature stop codons (PTC), thus presumably representing null alleles degraded by NMD. Three mutations were identified in intronic splice sites. A complex mutation involving an adenine deletion in –4 position and an adenine-to-cytosine substitution in the invariant –2 position of the splice acceptor site, affected intron 7. A point mutation was found in the invariant +2 position of intron 8 (c.1376+2T>G). A C>G transversion of intron 5 acceptor site in the non-invariant –3 position (c.626-3C>G), was detected in a very large family (HHT5), and in one sporadic case (HHT72). Despite the close geographical location, they do not share the same haplotype, therefore, they represent distinct mutational events.

To assess whether the c.626-3C>G was a real disease-causing mutation, we investigated its consequence at the transcriptional level. RT-PCR performed on RNA from lymphoblastoid cell lines derived from controls and carriers of the wild-type *ALK1* sequence gave rise to a major band corresponding to the expected 728-bp-long splicing product (Fig. 2) and, unexpectedly, as no alternative splicing events had ever been reported regarding the *ALK1* gene, also to one additional band of significantly lower intensity. Screening and sequencing of plasmid clones containing different RT-PCR-products confirmed that the major band had the expected 728-bp-long sequence corresponding to the cDNA sequence reported in literature (Berg et al., 1997). The sequencing of the

faster band showed an exon-5-skipping event leading to a 628-bp-long isoform. The same experiment was performed on the RNA from two members of family HHT5 (patients 0503/02 and 0710/02). As shown in Figure 2, the 728-bp-long and the 628-bp-long bands were present in both mutated individuals, but two other bands also appeared, obviously as a consequence of the splice site mutation. The sequencing of plasmid clones containing RT-PCR products revealed a 581-bp-long product due to exon 6 skipping, and a 481-bp-long product caused by the simultaneous skipping of both exon 5 and exon 6. Computational analysis using the web-based software NNSPLICE0.9 (http://www.fruitfly.org/seq_tools/splice.html) indicated that both acceptor and donor splice sites of exon 5 did not show a high score (0.53 and 0.67 respectively), accounting for the small proportion of exon 5 skipping also observed in normal individuals. As far as exon 6 is concerned, the c.626-3C>G mutation abolishes the acceptor splice site score, thus predicting the observed exon 6 skipping in the mutated individuals.

***ENG* mutations**

A total of 12 novel and 8 known mutations were identified in the *ENG* gene (Table 2B). The novel mutations were four nonsense substitutions, one small frameshift insertion, one small duplication, one frameshift and one in-frame deletion, one missense and three splice site mutations. All mutations resided in the endoglin extracellular region. The four nonsense substitutions, c.97C>T (p.Q33X) in exon 2, c.889C>T (p.Q297X) in exon 7, c.1306C>T (p.Q436X) in exon 9B, and c.1513G>T (p.E505X) in exon 11, are likely to give rise to nonsense-mediated-decay (NMD) (Shovlin et al., 1997). A 1-bp insertion (c.1098_1099insT) and a 23-bp deletion (c.1097_1119del) were located in exon 8, whereas a 2-bp duplication (c.1190_1191dupAG) fell within exon 9A; they cause frameshift and premature stop codons, therefore they probably determine unstable transcripts (Gallione et al., 1998). A 3-bp deletion was identified within exon 3 in the HHT32 family (c.309_311delCAG). This mutation predicts the loss of serine 104 in the mutant polypeptide. Similar mutations have been reported to behave as null alleles in that they give rise to misfolded precursors prevented from reaching cell surface through intracellular degradation (Paquet et al., 2001). The missense mutation c.287T>C transition detected in HHT38 family changes leucine 96 to a proline. This alteration, which was not present in a panel of 100 healthy controls in the Italian population, co-segregated with the disease. Since this leucine-to-proline substitution is predicted to destroy a α-helical structure by the web-based *nnpredict* (http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html) software, the mutant protein is likely to behave as an unstable, misfolded intracellular precursor. Lastly, three mutations were identified which affected the invariant nucleotides of splice canonical sites: c.816+2T>A, c.817-2A>T, c.1683+2T>C. All these substitutions are predicted to disrupt the proper splicing process in the *ENG* gene. In addition, we identified two single-nucleotide variants which caused amino acidic substitutions (Table 3). A novel sequence variant, c.388C>T (p.P130S), was identified in the HHT55 family; it is in trans with the disease-causing mutation, c.816+2T>A, as demonstrated by segregation analysis. The already reported c.572G>A transition (p.G191D) was found in a patient of HHT65 family, who was also a carrier of the c.97C>T (p.Q33X) disease-causing mutation.

Table 2A. Mutations found in *ALK1* gene

Family No.	Intron/ Exon	DNA change	Protein change	a	b	c	d	e	f	Reference
HHT2	**Ex 3**	**c.121T>C**	**p.C41R**	**N**		**D**	**N**	**+**	**+**	**This paper**
HHT79°	**Ex 3**	**c.115-118dupCCAC**	**p.H40PfsX130**			**D**	**D**			**This paper**
HHT13	Ex 3	c.152G>A	p.C51Y	D	D			+	+	Klaus et al., 1999
HHT67	**Ex 3**	**c.197A>C**	**p.H66P**			**D**	**D**			**This paper**
HHT25	Ex 3	c.200G>A	p.R67Q	D	D			+	+	Berg et al., 1997
HHT44	Ex 3	c.200G>A	p.R67Q			D	D	+	+	Berg et al., 1997
HHT39	Ex 3	c.200G>A	p.R67Q			D	D		+	Berg et al., 1997
HHT50	**Ex 3**	**c.218-219insAA**	**p.H73QfsX50**			**D**	**D**			**This paper**
HHT62	**Ex 3**	**c.283T>C**	**p.C95R**			**D**	**D**		+	**This paper**
HHT40	Ex 4	c.430C>T	p.R144X	D	D					Abdalla et al., 2003
HHT15	**Ex 4**	**c.448C>T**	**p.Q150X**			**D**	**D**			**This paper**
HHT5	**Int 5**	**c.626-3C>G**	*****	**D**	**D**			+		**This paper**
HHT72°	**Int 5**	**c.626-3C>G**	*****			**D**	**D**			**This paper**
HHT51	**Ex 6**	**c.656G>A**	**p.G219H**			**D**	**D**	**+**	**+**	**This paper**
HHT34	**Ex 6**	**c.686A>T**	**p.K229M**	**D**	**D**			**+**	**+**	**This paper**
HHT27	Ex 7	c.858C>A	p.Y286X	D	D					Olivieri et al., 2002
HHT14	Ex 7	c.858C>A	p.Y286X			D	D			Olivieri et al., 2002
HHT53	Ex 7	c.924C>A	p.C308X			D	D			Berg et al., 1997
HHT54	Ex 7	c.961C>T	p.Q321X			D	D			Letteboer et al., 2005
HHT1	Ex 7	c.988G>T	p.D330Y	D	D			+	+	Olivieri et al., 2002
HHT35	**Int 7**	**c.1048-4_1048-2 delACAinsCC**	**r.spl?**			**D**	**D**			**This paper**
HHT8	**Ex 8**	**c.1115C>T**	**p.T372I**	**D**	**D**			**+**	**+**	**This paper**
HHT11	Ex 8	c.1120C>T	p.C374W	N		D	N	+		Berg et al., 1997
HHT52	Ex 8	c.1120C>T	p.C374W			D	N			Berg et al., 1997
HHT43	Ex 8	c.1135G>A	p.E379K	D	D				+	Lesca et al., 2004
HHT24	Ex 8	c.1135G>A	p.E379K			D	D	+	+	Lesca et al., 2004
HHT22	Ex 8	c.1135G>A	p.E379K			D	D	+	+	Lesca et al., 2004
HHT20	**Ex 8**	**c.1208T>C**	**p.L403P**	**D**	**D**			**+**	**+**	**This paper**
HHT12	**Ex 8**	**c.1208T>C**	**p.L403P**			**D**	**D**		**+**	**This paper**
HHT36	Ex 8	c.1218G>T	p.W406C			D	D	+	+	Letteboer et al., 2005
HHT4	Ex 8	c.1231C>T	p.R411W	D	D			+		Trembath et al., 2001
HHT9	Ex 8	c.1231C>T	p.R411W			D	D			Trembath et al., 2001
HHT76°	Ex 8	c.1232G>A	p.R411Q			D	D			Johmson et al., 1996
HHT63	**Int 8**	**c.1376+2T>G**	**r.spl?**			**D**	**N**			**This paper**
HHT73°	Ex 9	c.1275C>G	p.F425L	N		D	N		+	Lesca et al., 2004
HHT18	**Ex 9**	**c.1280A>T**	**D427V**			**D**	**D**		**+**	**This paper**
HHT17	Ex 9	c.1321G>A	p.V441M			D	D	+	+	Abdalla et al., 2005
HHT29	Ex 10	c.1435C>T	p.R479X	D	D					Lesca et al., 2004
HHT10	Ex 10	c.1450C>T	p.R484W	D	D			+	+	Trembath et al., 2001
HHT42	Ex 10	c.1450C>T	p.R484W			D	D	+	+	Trembath et al., 2001
HHT33	Ex 10	c.1468C>T	p.Q490X	N		D	N			Trembath et al., 2001

a: SSCP prospective analysis; **b:** DHPLC retrospective analysis; **c:** DHPLC prospective analysis; **d:** SSCP retrospective analysis; **e:** familial co-segregation; **f:** amino acid conservation among homologues. Novel mutations in bold.
°: sporadic case; **D:** detected; **N:** not detected; ***: r.=, r.626_772del, r.526_772del**
Nucleotide numbers derive from GenBank reference sequence: 4557242 (NM_000020.1).

Table 2B. Mutations found in *ENG* gene

Family No.	Intron/ Exon	DNA change	Protein change	a	b	c	d	e	f	Reference
HHT47	Ex 1	c.1A>G	p.M1?			D	D	+		Letteboer et al., 2005
HHT26	Ex 1	c.63delC	p.T22QfsX21	D	D					Lastella et al., 2003
HHT65	**Ex 2**	**c.97C>T**	**p.Q33X**			**D**	**D**			**This paper**
HHT45°	Ex 3	c.229C>T	p.Q77X	D	D					Lastella et al., 2003
HHT38	**Ex 3**	**c.287T>C**	**p.L96P**			**D**	**D**	**+**		**This paper**
HHT32	**Ex 3**	**c.309_311delCAG**	**p.S104del**			**D**	**D**	**+**		**This paper**
HHT19	Ex 4	c.511C>T	p.R171X	D	D					Shovlin et al., 1997
HHT21	Ex 5	c.591_619del	p.R199LfsX125	D	D					Lastella et al., 2003
HHT54	Ex 6	c.771dupC	p.Y258LfsX76			D	D			Lesca et al., 2004
HHT71°	Ex 6	c.790G>A	p.D264N			D	N		+	Letteboer et al., 2005
HHT55	**Int6**	**c.816+2T>A**	**r.spl?**	**N**		**D**	**N**			**This paper**
HHT59	**Int 6**	**c.817-2A>T**	**r.spl?**			**D**	**N**			**This paper**
HHT49	**Ex 7**	**c.889C>T**	**p.Q297X**			**D**	**D**			**This paper**
HHT23	Ex 7	c.909_929del	p.R304_I310del	D	D					Lastella et al., 2003
HHT31	**Ex 8**	**c.1097_1119del**	**p.D366EfsX22**			**D**	**D**			**This paper**
HHT28	**Ex 8**	**c.1098_1099insT**	**p.A367CfsX29**	**D**	**D**					**This paper**
HHT46	**Ex 9A**	**c.1190_1191dupAG**	**p.D398RfsX24**	**D**	**D**					**This paper**
HHT3	**Ex 9B**	**c.1306C>T**	**p.Q436X**			**D**	**D**			**This paper**
HHT57	**Ex 11**	**c.1513G>T**	**p.E505X**	**N**		**D**	**N**			**This paper**
HHT70	**Int 11**	**c.1683+2T>C**	**r.spl?**			**D**	**N**			**This paper**

a: SSCP prospective analysis; **b:** DHPLC retrospective analysis; **c:** DHPLC prospective analysis; **d:** SSCP retrospective analysis; **e:** familial co-segregation; **f:** amino acid conservation among homologues. Novel mutations in bold.
°: sporadic case; **D:** detected; **N:** not detected.
Nucleotide numbers derive from GenBank reference sequence: 33871100 (BC014271.2).

Table 3. Polymorphic changes found in *ALK1* and *ENG* genes; novel variants in bold

Intron/Exon	Polymorphic change	Ref. when first reported
ALK1		
Intron 1	c.1-38C>T	Abdalla et al., 2005
Intron 3	c.313+11C>T	Olivieri et al., 2002
Intron 5	c.626-59_626-60delGGinsT	Lesca et al., 2004
Exon 6	c.747G>A, p.V249V	Abdalla et al., 2005
Exon 7	**c.933G>A, p.A311A**	**This work**
Exon 8	**c.1092C>T, p.I364I**	**This work**
ENG		
Exon 1	c.14C>T, p.T5M	Shovlin et al., 1997
Exon 2	c.207G>A, p.L69L	Shovlin et al., 1997
Intron 2	c.67+25G>T	Lastella et al., 2003
Exon 4	**c.388C>A, p.P130S**	**This work**
Exon 5	c.572G>A, p.G191D	Abdalla et al., 2005
Intron 7	c.991+25_991+26insCCTCC	Lesca et al., 2004
Exon 8	c.1029C>T, p.T343T	Shovlin et al., 1997
Exon 8	c.1096G>C, p.D366H	Lesca et al., 2004
Exon 12	c.1724T>C, p.I575T	Lastella et al., 2003

Table 4. The "*ALK1*-exon 3 subgroup" genotypes

Sequence in exon 3	Genotype at c.313+11 locus
Wild-type	CC
Wild-type	*CT*
Wild-type	*TT*
c.200G>A, **p.R67Q**	*CC*
c.200G>A, **p.R67Q**	*CT*
c.152G>A, **p.C51Y**	*TT*
c.152G>A, **p.C51Y**	*CT*

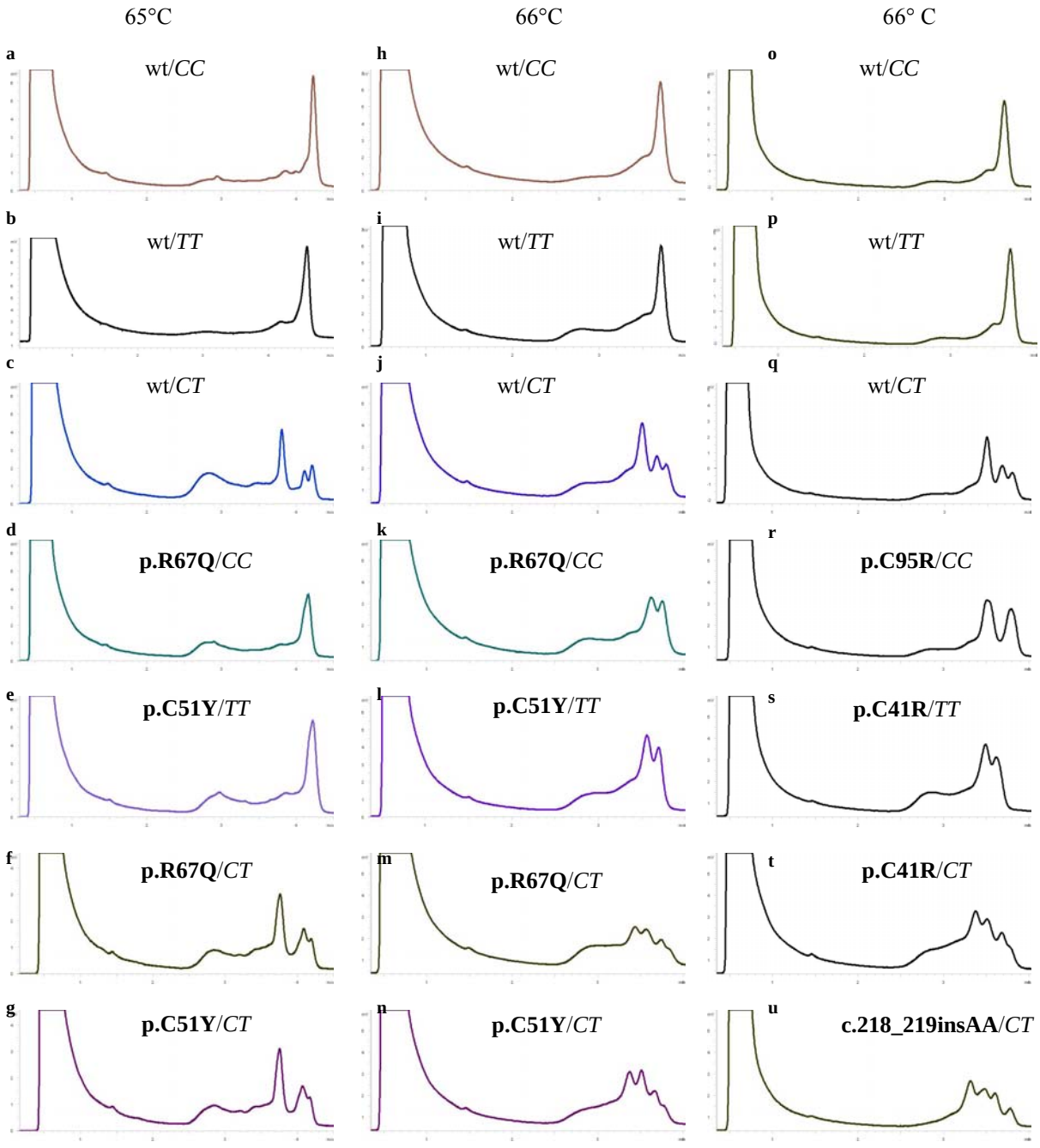

Figure 1. DHPLC elution profiles of the *ALK1* exon 3 samples from wild type individuals, carriers of different genotypes (italics) for the most common polymorphism (c.313+1C>T) and patients, carriers of different genotypes for the polymorphism and mutation (in bold) at same time.
1a-g: elution profile at 65°C of the "*ALK1*-exon 3-subgroup" samples reported in Table 4 (retrospective analysis).
1h-n: elution profile at 66°C of the same "*ALK1*-exon 3-subgroup" samples (retrospective analysis).
1o-u: elution profile at 66°C of PCR products from DNA samples of some patients reported in Table 2A (prospective analysis).

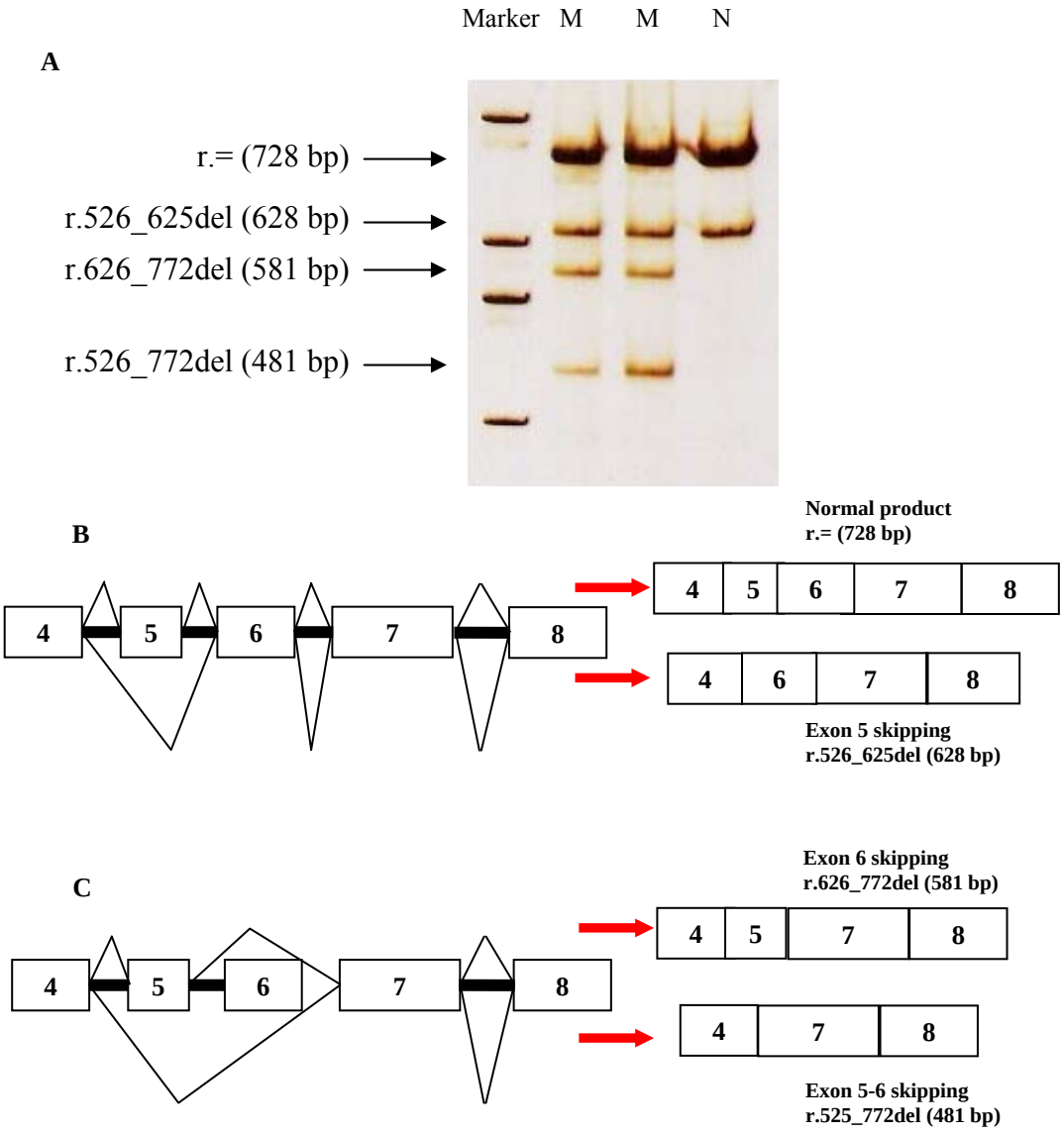

Figure 2. A: *ALK1* cDNA analysis in a normal control (N) and in two carriers of the c.626-3C>G mutation (M). Polyacrylamide gel of the RT-PCR products obtained from lymphoblastoid cells showing two bands in a normal control (N) and two additional bands due to aberrant splicing in mutated individuals (M).

B: Diagrammatic representation of the most abundant normal splicing isoform and exon 5 skipping observed in both mutated and healthy individuals.

C: Diagrammatic representation of exon 6 skipping and exon 5-exon 6 skipping observed only in mutated individuals.

DISCUSSION

In the present study, the DHPLC for mutational screening of the two known HHT-causing genes, *ENG* and *ALK1* was standardized for the first time. A mutation detection rate of 100% was obtained in a retrospective screening performed on known sequence variants and a 89.3% rate in a prospective screening performed on 47 probands with a definite diagnosis, with a cumulative mutation detection rate of 92.4% (61/66). Since no further nucleotide variations were detected with direct sequencing in the five DHPLC-negative probands, DHPLC revealed a100% sensitivity compared to that for direct sequencing, which is considered the gold standard in mutational analysis (Xiao and Oefner, 2001); however, DHPLC is much less laborious, time-consuming and expensive. Our results agree with data reported by other groups on DHPLC analysis performed on many other genes, such as *BRCA* (Gross et al., 1999), *NF1* (De Luca et al., 2004), *MLH1* and *MSH2* (Holinski-Feder et al., 2001). Furthermore, our prospective DHPLC mutation detection rate (89.3%) is very similar to that obtained by direct sequencing on *ENG* and *ALK1* by other groups (Letteboer et al., 2005; Schulte et al., 2005). Ten different mutations, all consisting in 1-bp substitutions, were undetectable by SSCP analysis. This suggests that DHPLC is particularly suitable for detecting *ALK1* mutations, since 1-bp substitutions account for 89 of the 131 *ALK1* mutations reported thus far (Bayrak-Toydemir et al., 2004; Brusgaard et al., 2004;Letteboer et al., 2005; Abdalla et al., 2005; Kuehl et al., 2005; Schulte et al., 2005). The detection rate for transitions and transversions were similar with the two methods.

The lack of highly recurrent mutations, the locus heterogeneity, the presence of mutations in almost all coding exons of the two genes make the screening for mutations time-consuming and costly. DHPLC proved to be highly sensitive, rapid, specific and reproducible; therefore, we feel that it may become the elective technique for routine molecular diagnosis in HHT.

Our study provides the first description of the mutational spectrum of the two known HHT-causing genes in the Italian population. Among the Italian patients, we found a high prevalence of *ALK1*-mutation-carrier probands (41) compared to that for the *ENG*-mutation-carrier probands (20). Such results, in agreement with the French (Lesca et al., 2004) and Spanish data (Fernandez-Lopez et al., unpublished data reported in the 6th HHT Scientific Conference, Lyon, 21-23 April 2005), are in contrast with studies on other populations (Letteboer et al., 2005; Abdalla et al., 2005; Kuehl et al., 2005; Schulte et al., 2005**).** In the French population described by Lesca et al. (2004), the higher prevalence of ALK1 mutations was due to both the founder effect and presence of mutational hot-spots which occurred in ALK1 but not in ENG since the authors observed 36 and 34 different mutations in ALK1 and ENG, respectively. In our study, the difference in prevalence cannot completely be explained by the existence of recurrent mutations in *ALK1,* as we observed 31 different mutations in *ALK1* and 20 different mutations in *ENG*. Therefore, the prevalence of *ALK1* mutations seems to be a feature of some Mediterranean populations, such as Italy and Spain, as it has already emerged in preliminary results from another Italian group (Olivieri et al., 2002 and unpublished data reported at the 6th HHT Scientific Conference, Lyon, 21-23 April 2005).

In the current study, no disease causing mutations were detected in one sporadic and four familial cases. Some of the unidentified mutations might consist in whole-exon deletions or duplications which require different screening techniques, such as Southern blotting, RT-PCR, QMPCR (Cymerman et al., 2003) or MLPA (Letteboer et al., 2005). Mutations in intronic or regulatory sequences may account for other unidentified mutations, although no such mutations have ever been reported thus far. Finally, involvement of the still unidentified HHT3 locus (Cole et al., 2005) cannot be ruled out as none of the four mutation-negative families was large enough to allow linkage analysis. Based on the absence of polyps in the gastrointestinal tract of our patients, the *MADH4* gene (Gallione et al., 2004) mutations can be excluded.

RT-PCR-based experiments showed that a small proportion of the *ALK1* gene transcript undergoes alternative splicing consequent to exon 5 skipping, also in the lymphoblastoid cell lines of normal individuals. Alternative splicing has never been described in the *ALK*1 coding region while several alternative splicing isoforms of the *ALK4* gene (an *ALK1* paralogous) were reported in human pituitary adenoma tumoral tissue (Alexander al., 1996), but their functional meaning is not understood. The r.526_625del isoform seems to escape NMD, in spite of a PTC subsequent to frameshift, and is predicted to encode a truncated 223-aa protein. The function of the exon-5-skipped isoform is not obvious and requires further experiments as it was detected in very low amounts in lymphoblastoid cell lines, which do not represent a physiological cellular environment.

It is noteworthy that no alternative isoforms involving exon 6 were detected in normal individuals, even though it has a non-canonical GC-donor site (Berg et al., 1997) and may be expected to behave as a "weak" exon (Clark and Thanaraj, 2002). Conversely, the presence of the c.626C>G mutation affecting the acceptor site immediately upstream does determine both exon 6 skipping (r.626_772del) and exon 5-exon 6 skipping (r.526_772del). The r.626_772del isoform does not disrupt the original reading frame and is predicted to encode a polypeptide lacking 49 amino acid residues (p.K210_L258del), which includes the entire ATP-binding site. Nonetheless, its expression in a more physiological context, such as endothelial cells, at both transcriptional and translational level needs to be confirmed.

In conclusion, we have developed a rapid and reliable method for mutation screening in HHT patients. Compared to other more conventional strategies, the DHPLC-based protocol is faster, less costly and would thus facilitate the genetic counselling for affected families.

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