INTRODUCTION

Hereditary hemorrhagic telangiectasia (HHT, or Rendu-Osler-Weber syndrome; MIM# 187300) is an autosomal dominant disorder characterized by aberrant vascular development. The clinical manifestations of HHT include epistaxis, mucocutaneous and gastrointestinal telangiectases, and large arteriovenous malformations of the lung, brain, and liver [Guttmacher et al., 1995; Shovlin and Letarte, 1999; Matsubara et al., 2000; Garcia-Tsao et al., 2000].

Mutations in at least two genes have been shown to be associated with HHT: *endoglin* (ENG; MIM# 131195) on chromosome 9 [McAllister et al., 1994] and *ALK-1* (approved symbol, ACVRL1; MIM# 601284) on chromosome 12 [Johnson et al., 1996]. Molecular–genetic analyses of HHT have identified disease loci on chromosomes 9 (HHT1) and 12 (HHT2 or ORW2; MIM# 600376) and at least one other *HHT* locus has been predicted [Piantanida et al., 1996; Wallace and Shovlin, 2000]. The disease gene on chromosome 9q encodes Endoglin (ENG) [Shovlin and Letarte, 1999] which is expressed predominantly in endothelial cells and associates with TGF-β signaling receptors [Yamashita et al., 1994; Zhang et al., 1996; Lastres et al., 1996]. The HHT disease gene on chromosome 12 encodes an activin receptor-like kinase (ALK-1) which also encodes a receptor of the TGF-β receptor family [Johnson et al., 1996].

A significant proportion of HHT patients (~30%) with *ENG* mutations have pulmonary and cerebral vascular involvement [Guttmacher et al., 1995; Berg et al., 1996; Moussouttas et al., 2000; Matsubara et al., 2000]. These manifestations are often silent and present as pulmonary arteriovenous malformation (PAVMs) and cerebral arteriovenous malformation which often cause considerable morbidity and mortality if left untreated.

The population prevalence of HHT has been suggested to be 1 in 50,000 to 100,000 [Tuente, 1964]. Porteus et al. [1992] investigated the prevalence of HHT in the northern region of England and reported a minimum prevalence of 1 in 40,000. Guttmacher et al. [1994] suggested a much higher incidence of 1 in 16,500 based on a genetic epidemiological study in Vermont. Clustering of HHT has also been reported in various areas with strikingly high prevalence: 1 in 2,300 in Jura Valley in France [Bideau et al., 1992] and comparable prevalences in other ethnic groups [Vase et al., 1985; Jesserun et al., 1993]. These results suggest a higher prevalence of HHT than originally thought and a higher heterogeneity in prevalence among areas.

HHT displays age-related penetrance with manifestations developing throughout life and varying between affected individuals even within the same pedigree. The key to appropriate management of patients with HHT is to establish an early diagnosis. Therefore, more sensitive screening methods should be established for early diagnosis to reduce the number of cases overlooked.

We performed a population genetic epidemiological study of HHT in a local community (county A) in the northern part of Japan where clustering of HHT is suspected [Shioya et al., 2000]. We first searched for a putative founder mutation of the *ENG* gene in this community which would provide a molecular tool for early detection of affected members in high-risk families. Since the prevalence of HHT is more common in Caucasians than in Asians or other ethnic groups [Haitjema et al., 1996], we evaluated the population prevalence in this community to test whether prevalence of HHT is as small as traditionally reported.

MATERIALS AND METHODS

Patients

All studies were performed with the approval of the Ethical Committee of Akita University for Research on Human Subjects. The study was conducted in county A of Akita prefecture (population 1.2 million) which is located in the northern part of Japan. Nine patients of northern-Akita ancestry in county A were referred to tertiary-care hospitals (Akita University Hospital, Nakadouri General Hospital, Senboku Kumiai General Hospital) in the local community for therapy. These three tertiary-care hospitals, located in Akita city or Ohmagari city, cover patients from counties bordering on the city of Akita. The potential HHT cluster was located in county A (population 170,000), an area bordering the south of the city of Akita. Diagnosis criteria included the presence of three of four key features: spontaneous and recurrent epistaxis, teleangiectasia, visceral manifestations, and affected first degree relatives [Shovlin and Letarte, 1999]. HHT status for decreased an-

cestors was judged by the presence of recurrent epistaxis alone when no other information was available. The pedigree structures were constructed based on interviews. Clinical evaluations, including history, physical examination, and portable oximetry to screen for PAVMs [Shovlin and Letarte, 1999], were performed on all available family members of each proband. Additional medical history was obtained from patient records.

Genetic Analysis

Isolation of DNA and cDNA synthesis from mRNA. Genomic DNA was isolated from peripheral blood using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). mRNA was isolated, using the PolyATtract System 1000 (Promega, Madison, WI), from monocytes prepared from fresh peripheral blood with Ficoll Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). cDNA was transcribed by cDNA Synthesis Kit (TaKaRa, Kyoto, Japan) with an oligo(dT)$_{18}$ primer.

ENG primer sequences. *ENG* cDNA primers were designed from *ENG* cDNA sequences (Gene Bank accession number NM000118) except primers ENGRT-F and ENGRT-R which were derived from the work of Pace et al. [1997]. *ENG* genomic primers were derived from the genomic sequence of *ENG* (GenBank accession number AF035753, AF036969-71, U37439, U37441-2, and U37445-7) except primers for exons 5 and 7. Primers used to amplify exon 5 and exon 7 were designed from the sequence data reported by McAllister et al. [1994]. All primers used in this study are shown in Table 1.

ENG mutation screens. For amplification of genomic DNA, PCR was carried out in a 15 μl volume containing 60 ng genomic DNA, 0.6 μM forward and reverse primers, 1.7 mM MgCl2, 0.2 mM each dNTP, 1 unit AmpliTaq Gold (Perkin Elmer, Foster City, CA) and 1.5 μl of 10× GeneAmp Gold Buffer (Perkin Elmer). Amplification was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Watertown, MA) using a program consisting of an initial denaturation step of 95°C for 9 min followed by 40 cycles of denaturation at 94°C for 45 sec, annealing at 53°C for 45 sec, and extension at 74°C for 1 min and a final extension step at 72°C for 7 min. cDNA reverse transcribed from mRNA was amplified by PCR in a 30 μl volume containing 5 μl of cDNA, 0.6 μM of forward and reverse primers, 1.5 mM of MgCl2, 0.2 mM of each dNTP, 1.5 U of AmpliTaq Gold, 3.0 μl of 10× GeneAmp Gold Buffer. Amplification was performed according to the same PCR program used for genomic DNA with the exception of the cycle extension time, which was for 2 min. The quality of PCR-amplified products was assessed by agarose-gel electrophoresis. The bands were excised and purified using a Prep-A Gene DNA Purification Kit (Bio-Rad, Hercules, CA). An ABI PRISM dRhodamine Terminator

TABLE 1. Primers Used in This Study

Exon	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Product size
Primers for PCR amplifying genomic *ENG* DNA			
1	ENGex1F : CCACTGGACACAGGATAAG	ENGex1R : GGCTTTCTTTCAACACTGA	311bp
2	ENGex2F : ACGTTTGGAAAGTAGGAGTC	ENGex2R : AAATGCCACCTCTTATGG	393bp
3	ENGex3F : AGGGTGGCACAACCTAT	ENGex3R : CAGAGATGGACAGTAGGGA	270bp
4	ENGex4F : CAAATTACTTCCTGACCTCC	ENGex4R : CAGAACCTGGCATATTCC	456bp
5	pENGex5F: GGGCTCTGTTAGGTGCAG	pENGex5R: GGGTGGGGCTTTATAAGGGA	294bp
6	pENGex6F: CTGTCCGCTTCAGTGTTCCATC	pENGex6R: GGAAACTTCCCTGATCCAGAGGTT	230bp
7–8	ENGex7F : CTGTGGCACAGACTGTGT	ENGex8R : CTAGGACCCCAAGAGTCTT	810bp
9a–9b	ENGex9F2: CAGTGCCTCCTGATGGT	ENGex9R : GGCCAGGTGGGTTAAGC	487bp
10–11	ENGex10F: ATGATGCCTGTTCCTCC	ENGex11R: GTCCCTTCCATGCAAAC	864bp
12	ENGex12F: GATCTTCCAGGACTCACC	ENGex12R: CACCTTGCCATGTGCTA	317bp
13–14	ENGex13F: ACAACAGGGTAGGGGAT	ENGex14R: ATTCTGGGTCGAGTGGA	538bp
Primers for PCR amplifying cDNA			
1–7	ENGC1F : GCCACTGGACACAGGATAAGG	ENGC1R : GAGCTTGAAGCCACGAATGTT	926bp
4–11	ENGC3F : ACCACAGAGCTGCCATCCTT	ENGC5R : TCTGACCTGCACAAAGCTCTG	1032bp
6–13	ENGC2F : ACCACAACATGCAGATCTGGA	ENGC2R : TGTACCAGAGTGCAGCAGTGA	1041bp
10–14	ENGC6F : ATGGACAGCCTCTCTTTCCAG	ENG3U2R : ATTGGTGGTGAATACACAGGG	1427bp
2–4	ENGRT-F : GAGAGGGGCGAGGTGACATAT	ENGRT-R : CTCTTGGAAGGTGACCAGGC	276
Primers for sequencing[a]			
11	ENGex11F: ATTTGAAGGCAGCAGGT		

[a]For sequencing exons except exon 11, forward or/and reverse primers for PCR amplifications were used.

Cycle Sequencing Ready Reaction Kit (Perkin Elmer) was used to sequence the purified PCR products. Sequence analysis was performed with a 310 Genetic Analyzer (Perkin Elmer).

Enzyme digestion. The PCR products of exon 3 were digested with BsaAI (New England BioLabs, Beverly, MA). Reaction mixtures containing 5 μl of PCR product, 5 units of enzyme, and 2.2 μl of 10× NE Buffer in a total volume of 22 μl were incubated at 37°C for 5 hr. Fragments were analyzed by agarose gel electorophoresis.

Linkage analyses. Linkage analysis was performed to confirm that the prevalent type of HHT was HHT1 using eight microsatellite markers, ordered D9S1690, D9S1677, D9S1776, D9S1682, (*ENG*), D9S290, D9S164, D9S1826, and D9S158, spanning 50 cM on chromosome 9q. To exclude linkage with *HHT2* locus we used eight polymorphic markers, ordered D12S310, D12S1617, D12S345, D12S85, *ALK1*, D12S368, D12S83, D12S326, and D12S351, spanning 60 cM on chromosome 12q. Linkage studies were performed as using GENEHUNTER [Kruglyak et al., 1996].

RESULTS

Patients

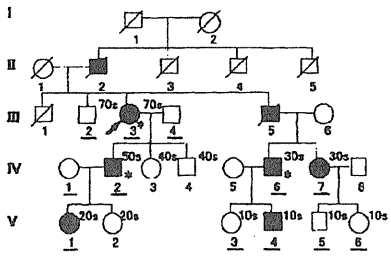
We examined nine patients with HHT who had been referred to tertiary-care hospitals. Pedigree structures for these patients were constructed as shown in Figure 1. A total of 137 pedigree members were traced of which 81 were alive and 32 affected by HHT. Of the seven pedigrees, four families (SB-1, SB-2, SB-3, and SB-6) had lived in county A for more than five generations. The other families had lived in other counties which all bordered county A. Three patients (III-2, III-9, and III-10 in SB-2) were found to originate from the same family while the other patients were from unrelated families (III-3 in SB-1, VI-1 in SB-3, I-1 in SB-4, II-1 in SB-5, II-5 in SB-6, and III-4 in SB-7) (Fig. 1). The total number of HHT patients currently alive and confirmed as being affected in county A was 23, giving an approximate population incidence of 1:8,000 in this county. Interviews revealed affected members and the presence of PAVMs in each pedigree which were confirmed by the medical records in hospitals. About 50% (16/32) of the individuals interviewed in all families had PAVMs except families SB-6 and SB-7. Although family SB-7 did not have PAVMs as determined from family history, cerebral arteriovenous malformation was confirmed by examination of medical records in IV-1 in SB-7. Other complications associated with HHT were incidence of strokes at a young age; age 44 in SB-1 III-5 and age 40 in SB-3 V-3. Although several other symptoms, gastrointestinal bleeding, liver cirrhosis, and sudden death, were reported in the interviews to have occurred in families, these could not be confirmed by medical records or could not be judged whether they were associated with HHT.

Genetic Analysis

Linkage analysis. Linkage analysis was conducted in two pedigrees (SB-1 and SB-2) from which a sufficient number of subjects participated in this study: III-2, III-3, III-4, IV-1, IV-2, IV-6, IV-7, V-1, V-3, V-4, V-5, and V6 in the SB-1 pedigree and III-2, III-9, III-10, and IV-8 in the SB-2 pedigree. Linkage with *HHT1* locus was tested using eight microsatellite markers (D9S1690- D9S1677-D9S1776- D9S1682- *Edoglin*-D9S290-D9S164-D9S1826-D9S158, in order). Linkage analyses showed suggestive linkage with *HHT1* locus on chromosome 9. The maximum two-point LOD score was obtained at D9S1682 ($\theta=0$): LOD Score 2.4 for pedigree SB-1 and LOD score 1.1 for pedigree SB-2, and a maximum multipoint LOD score was obtained at 38 cM: LOD Score 2.4 for pedigree SB-1 and LOD score 1.1 for pedigree SB-2 (data not shown). Next, the linkage with *HHT2* locus was tested using eight markers (D12S310-D12S1617-D12S345-D12S85-*ALK1*-D12S368-D12S83-D12S326-D12S351, in order) which resulted in linkage with *HHT2* being excluded: LOD scores obtained by multipoint linkage analyses were less than –2.4 for the SB-1 pedigree and –2.2 for the SB-2 pedigree and the two-point LOD scores < -2 for each family ($\theta=0$) at D12S85 and D12S368 in the two pedigrees.

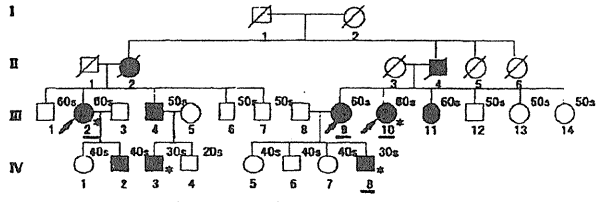
Mutation search. Due to the weak yet suggestive linkage with *HHT1* locus we searched for mutations of *ENG* in the patients. In the SB-1 pedigree we found a novel mutation, a G to C transversion at the splice donor site of intron 3 (Inv3+1 G>C) (Table 2). cDNA analysis of this mutation indicated mRNA lacking exon 3 (Data not shown). We tested the concordance between phenotype and the presence of mutation by restriction enzyme polymophism using BsaAI in SB-1 family. The results showed a complete concordance between HHT phenotype and the presence of mutation (Data not shown). Another

Family SB-1



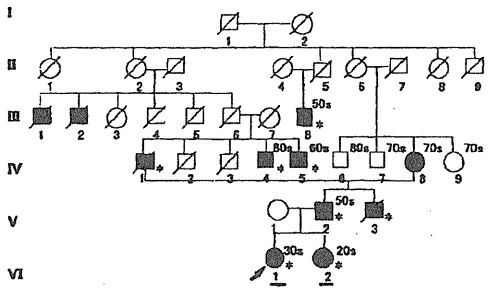
I-1: Died by unknown cause at unknown age
I-2: Died by unknown cause at unknown age
II-1: Died by unknown cause at unknown age
II-2: Died by unknown cause at unknown age
II-3: Died by unknown cause at unknown age
II-4: Died by unknown cause at unknown age
II-5: Died by unknown cause at unknown age
III-1:Died by unknown cause at age 60
III-5:Died by cerebral stroke at age 44

Family SB-2



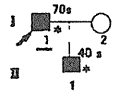
I-1 : Died by unknown cause at unknown age
I-2 : Died by unknown cause at unknown age
II-1: Died by unknown cause at unknown age
II-2: Died by unknown cause at unknown age
II-3: Died by unknown cause at age 73
II-4: Died by cerebral stroke at age 60
II-5: Died by unknown cause at unknown age
II-6: Died by unknown cause at unknown age

Family SB-3

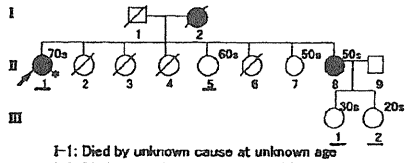


I-1 : Died by unknown cause at unknown age
I-2 : Died by unknown cause at unknown age
II-1 : Died by unknown cause at age 80
II-2 : Died by unknown cause at age 82
II-3 : Died by unknown cause at unknown age
II-4 : Died by unknown cause at unknown age
II-5 : Died by cerebral stroke at age 70
II-6 : Died by unknown cause at age 60
II-7 : Died by unknown cause at age 62
II-8 : Died by cancer at age 61
II-9 : Died by unknown cause at age 80
III-1: Died by unknown cause at age 66
III-2: Died by cerebral stroke at age 42
III-3: Died by unknown cause at age 70
III-4: Died by cancer at age 37
III-5: Died by unknown cause at age 50
III-6: Died in World war II at age 40
III-7: Died by cerebral stroke at age 37
IV-1: Died by unknown cause at age 62
IV-2: Died by unknown cause at age 60
IV-3: Died by cerebral stroke at age 50
V-3 : Died by cerebral stroke at age 40

Family SB-4

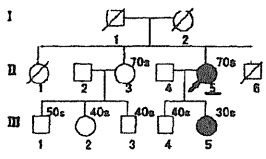


Family SB-5



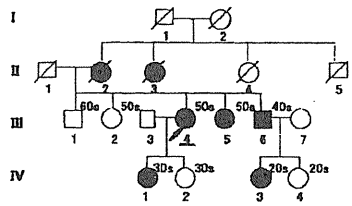
I-1: Died by unknown cause at unknown age
I-2: Died by cerebral stroke at age 65
II-2: Died by unknown cause at age 40
II-3: Died by unknown cause in infancy
II-4: Died by cancer at age 42
II-6: Died by unknown cause at age 32

Family SB-6



I-1 : Died by GI tract bleeding at age 49
I-2 : Died by renal disease at age 47
II-1: Died by unknwon cause in infancy
II-6: Died by unknown cause in infancy

Family SB-7



I-1 : Died by unknown cause at unknown age
I-2 : Died by unknown cause at unknown age
II-1 : Died by unknown cause at unknown age
II-2 : Died by unknown cause at age 78
II-3 : Died by unknown cause at unknown age
II-4 : Died by unknown cause at unknown age
II-5 : Died by unknown cause at unknown age
IV-1: Cerebral arteriovenous malformation

FIGURE 1. Patients and pedigrees. Solid symbols indicate affected members. Arrows indicate patients who regularly visit tertiary hospitals. Asterisks indicate patients with PAVMs. Those people who participated and donated their blood are underlined.

TABLE 2. Mutations in *Endoglin* Found in This Study

Pedigree	Site	Description	Nomenclature[a]
SB-1	Intron 3	A splice donor site mutation leading skipping exon 3	Inv3 + 1 G>C
SB-2 and SB-3	Exon 7	Insertion of A	c.828–829 ins A
SB-4	Exon 8	A 4-bp deletion	c.1120–1123 del AAAG
SB-7	Exon 11	Insertion A	c.1470–1471 ins A

[a]Nomenclature is based on a rule proposed by Antonarakis et al. [1998].

novel mutation was a one base pair insertion (A) at nucleotide 828 (exon 7) of the endoglin cDNA (c.828-829 ins A) which was found in all affected participants from SB-2 and SB-3 families by direct sequencing (Data not shown). This mutation causes a frameshift that results in a premature stop codon. Still another novel mutation was a 4 bp deletion (AAAG) beginning with nucleotide 1120 (exon 8) of the endoglin cDNA (c.1120–1123 del AAAG) found in SB-4 (Data not shown). A frameshift mutation, insertion of A (c.1470–1471 ins A), was found in the SB-7 pedigree. However, we found no mutations in the *ENG* gene of the SB-5 pedigree or the proband of SB-6.

Since both the SB-2 and SB-3 pedigrees shared the same mutation we investigated whether this mutation was derived from a common ancestor. The haplotype analysis revealed a common haplotype around the *Endoglin* gene spanning 24.3 cM from D9S1690 to D9S290 suggesting an ancestral origin of the mutation derived from a common founder in the two pedigrees (Data not shown).

DISCUSSION

Gastrointestinal bleeding, pulmonary arteriovenous malformations, and cerebral involvement (arteriovenous malformation and cerebral abscess) are the major morbidities and mortalities of HHT associated with the *ENG* mutation [Shovlin and Letarte, 1999]. These complications, however, can be prevented by appropriate follow-up of cases with family histories of HHT and who have symptoms suggestive of HHT1. High-risk patients are, however, often overlooked due to three major factors. First, the expression of the disease is seen with a wide disparity of clinical features even among members of the same family. Second, phenotypic penetrance is age-dependent, although nearly complete by age 40. Third, there are large variations in HHT phenotypes: in some individuals clinical symptoms are mildly expressed and thus, may easily be overlooked [Porteus et al., 1992; Plauchu et al., 1989]. An alternative method for appropriate long-term care, therefore, is to establish a molecular diagnosis of HHT for high-risk subjects.

Endoglin is a homodimeric integral membrane protein [Gougos and Letarte, 1988, 1990] and is expressed primarily in the vascular endothelial cells [Cheifets et al., 1992; Gougos and Letarte, 1990]. To date, more than 30 different mutations have been reported in *ENG* [Shovlin and Letarte, 1999]. Here, we report three novel mutations in HHT patients: one mutation led to exon skipping (SB-1), and the other two mutations were insertion of A in exon 7 (SB-2 and SB-3) and a 4 bp deletion in exon 8. The remaining mutation was insertion of A in exon 11, which has also been reported in European kindred [Cymerman et al., 2000]. All of these mutations led to frameshifts. Since all families with proven mutations exhibited vascular complications, mutation-specific phenotypes seemed not to occur for *ENG*. The homogeneity of clinical profiles of the families in the present study with different mutations was consistent with the haploinsuficiency model [Shovlin et al., 1997; Lux et al., 2000].

Genetic epidemiological data revealed several founder mutations of various genes in the current study population around county A—suggesting that this population is genetically isolated [Hirano et al., 1996]. In the families from county A and surrounding counties the mutations were found in a single family and were not common to multiple families (Table 2) with the exception of the mutation in exon 7 (c.828-829 ins A). Since four mutations were found in a single geographically defined HHT population (from a total Akita city catchment area of less than 30 km) the lack of common ancestral changes indicated that most mutations had arisen in recent generations. This significant level of new mutations should be balanced with reduction in fitness unless the prevalence of *ENG* mutations

steadily increases. One possibility by which HHT mutations may result in reduction of fitness, especially in women, would be lower reproductive fitness. Interestingly, vascular complications were shown to be exacerbated during pregnancy [Néau et al., 1988; Gammon et al., 1990; Revelaqua et al., 1992]. Another implication of the significant level of mutations is that *ENG* may have a relatively high mutation rate. At present, however, we do not have definitive evidence regarding the mutation rate of *ENG* in this population.

The variability of mutations indicates that we should screen for family-specific mutations in places of community-specific mutations for accurate diagnosis. Mutational searches to detect family-specific mutations failed in two families. For one of two families a family history of vascular complications was not proven and thus, HHT of this kindred may not be affected by a *ENG* mutation but rather by an *ALK1* mutation as reported previously [Johnson et al., 1996] or another type of HHT [Piantanida et al., 1996; Wallace and Shovlin, 2000]. Alternatively, the absence of mutations may suggest technological or other unknown difficulties, also as reported previously [Shovlin et al., 1997]. Therefore, as suggested previously [Cymerman et al., 2000], additional effort to detect *ENG* expression levels may be necessary. Although it was demonstrated that two major founder mutations can explain the high prevalence of HTT in Netherlands Antilles [Gallione et al., 2000], we believe that approaches using known mutations with the assumption of a founder mutation cannot be applied for screening purposes even in isolated populations due to large false negative rates. Our current conclusion is in accord with an observation on *ALK-1* mutations, which reported more than two mutations in a local cluster of HTT in the county of Fyn, Denmark [Kjeldsen et al., 2001].

We found 23 affected cases in a cluster in county A that included, however, only populations traceable by family interview and did not include exact numbers of offspring in each pedigree. Therefore, the number of patients was likely to be underestimated. A conservative estimate of the total population affected by HHT in this county could be obtained by assuming that an affected adult (age 30 years and above) has two children, one of whom would be affected by HHT. The number of children is based on the typical birth rate in Japan in the 1990s. Based on this assumption, five cases from SB-2, seven cases from SB-3, and one case from SB-6 may be added, leading to an estimated number of 36. Thus, from these cases we postulated that the population prevalence of HHT ranges from 23 (1:8,000) to 36 (1:5,000) of 170,000 people in county A. This estimated prevalence is roughly comparable to those reported in European and U.S. populations [Porteus et al., 1992; Guttmacher et al., 1994; Kjeldsen et al., 1999]. The present results contradict the traditional view that HHT is rare among Asians [Haitjema et al., 1996] and suggest that this view may be associated with poor recognition of HHT by physicians. In support of the concept that HHT is as common in Japan as in Europeans is the early work of Miyoshi et al. [1976] from the Southern prefecture of Tokushima. These authors conducted clinical genetic studies from five families with HHT and estimated a prevalence rate of 2–9 affected individuals per 100,000 population in Tokushima. We believe that approaches based on clinical epidemiology and genetics are critical to trace high-risk subjects in families with HHT. Such systematic follow-up will substantially improve the clinical course and prognosis by preventing unnecessary morbidity and mortality of affected persons.

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［Ⅲ］研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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塩谷隆信，佐竹將宏，守田亮，三浦肇，小高英達，佐藤一洋，佐野正明，橋本　学，伊藤宏	家族性肺動静脈奇形を合併した遺伝性出血性末梢血管拡張症(オスラー病)	日本内科学会雑誌	99(10)	141-144	2010

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