厚生労働省科学研究補助金研究報告書

難治性疾患克服事業

遺伝性出血性末梢血管拡張症(オスラー病)に関する遺伝疫学的検討と診療ガイドラインの作成

平成２１年度　総括・分担研究報告書

研究代表者　塩　谷　隆　信

平成 22(2010)年５月

目　　次

［Ⅰ］総括・分担研究報告

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研究代表者　塩谷隆信　秋田大学大学院医学系研究科保健学専攻理学療法学講座　教授

研究要旨
A. 研究目的
遺伝性出血性末梢血管拡張症（Hereditary hemorrhagic telangiectasia: HHT, オスラー病）は，多臓器疾患であるために臨床症状が極めて多岐にわたり，患者は内科のみならず，外科，耳鼻咽喉科，皮膚科，歯科など極めて多くの科を初診する．さらに，合併する脳動静脈奇形あるいは，肺動静脈奇形の破裂により時に致死的となることも稀ではない．本研究は，日本におけるオスラー病の発生頻度や罹病率について遺伝疫学的に検討を行ない，本疾患による致死的合併症の予防，治療のための診療ガイドライン作成，さらに将来的には遺伝子治療の足がかりを探ろうとするものである．

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B. 研究方法
昭和54年ー平成21年までの30年間に秋田大学医学部第二内科（現呼吸器内科）でHHTと診断した7家系137症例（男性67例，女性70例）を対象として，後方視的に臨床像の解析を行った．HHT家系調査から有病率を疫学的に検討し，さらにHHTの責任遺伝子の解析を行なった．
（倫理面への配慮）
ヒト遺伝子解析研究については，科学技術会議生命倫理委員会「ヒトゲノム研究に関する基本原則」を十分に認識し，平成16年文部科学省・厚生労働省・経済産業省共同告示による「ヒトゲノム・遺伝子解析研究に関する倫理指針」および平成13年文部科学省研究振興局長通知（12文科振第266号）を遵守して実施した．さらに，本疫学研究に際しては，平成19年文部科学省・厚生労働省による疫学研究に関する倫理指針および文部科学省研究振興局長通知（14文科振第123号）を遵守して行った．本研究は，臨床研究に関する倫理指針（平成20年厚生労働省告示第1号）を遵守して行った．
本課題については，秋田大学および京都大学

の倫理審査委員会に申請して承認を得た．秋田大学および京都大学の倫理委員会は3省合同指針に示された委員会の満たすべき要件を備えている．情報の漏洩に備えStand Alone で使用するコンピューターを用いアクセスはパスワードで厳重に管理した．

ヒト遺伝子解析研究については，科学技術会議生命倫理委員会「ヒトゲノム研究に関する基本原則」を十分に認識し，平成16年文部科学省・厚生労働省・経済産業省共同告示による「ヒトゲノム・遺伝子解析研究に関する倫理指針」および平成13年文部科学省研究振興局長通知（12文科振第266号）を遵守して実施した．さらに本疫学研究に際しては，平成19年文部科学省・厚生労働省による疫学研究に関する倫理指針および文部科学省研究振興局長通知（14文科振第123号）を遵守して行った．本研究は，臨床研究に関する倫理指針（平成20年厚生労働省告示第1号）を遵守して行った．

C. 研究結果

7家系137例中43例(31%)がオスラー病と診断された．2家系で遺伝子連鎖解析が施行され，HHT1(encoding endoglin; ENG) との連鎖が示唆された．オスラー病の5家系でENGの4つの変異(G→C transversion, base pair insersion(2 type), base pair deletion)が確認された（表1）．

7家系の遺伝疫学的検討から，本邦の有病率は1:8,000〜5,000 と推定された（図1）．4家系において家族性PAVMがみられ，HHT43例中17例(40%)においては，肺動脈静脈奇形(Pulmonary arteriovenous malformation; PAVM)が合併した．PAVM合併HHTの1例で脳動静脈奇形の破裂，2例で脳膿瘍の致死的合併症が併発した．多発性PAVM 6例に経カテーテル肺動脈塞栓術が施行された．

表1　　HHTの責任遺伝子

Pedigree	Site	Description	Nonemenclature
SB1	Intron3	A splice donor site mutation	Inv3+1G>C
SB2 &SB3	Exon 7	Insertion of A	c.828-829 insA
SB4	Exon 8	A 4-bp deletion	c.1120-1123 del AAAG
SB7	Exon 11	Insertion A	c.1470-1471 ins A

SB5, SB6においてはENG, ALK1ともに該当なし　　Nomenclature by Antonarakis, et al (1998)

Sequence; Exon 8 AAAG deletion

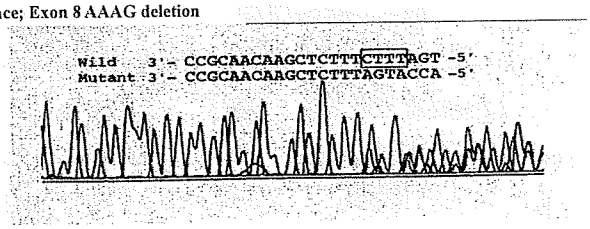
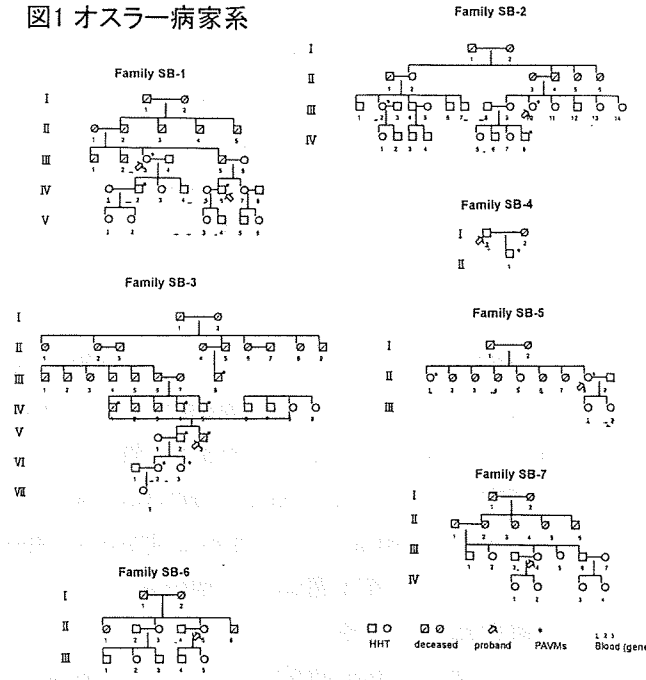


図1 オスラー病家系



D. 考察

遺伝性出血性末梢血管拡張症（オスラー病）は，従来，欧米に多い疾患であり，本邦における疫学的な頻度は低いと報告されてきたが，その理由のひとつとして，本症の従来の診断規準の精度の低さが考えられる．

HHT の新しい診断規準として以下を提案したい．

1．繰り返す鼻出血
2．皮膚粘膜の末梢血管拡張
3．肺，脳，肝臓，脊髄，消化管の動静脈奇形
4．一等親以内の同一患者の存在

以上のうち，3 つ以上の存在で確診，2 つで疑診，1 つ以下は可能性が低い．

E. 結論

遺伝性出血性末梢血管拡張症（オスラー病）は本邦において稀ならず存在し，その頻度は 1:8,000ー5,000 と推定された．さらに，責任遺伝子のひとつは，第 9 染色体 9q(ENG)の変異に連鎖する．オスラー病の約 30%に PAVM を合併する．オスラー病では致死的合併症予防のため PAVM 治療が不可欠であり，治療法の第一選択は経カテーテル肺動脈塞栓術である．

F. 健康危険情報

なし

G. 研究発表

1. 論文発表

投稿準備中

2. 学会発表

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今後は遺伝性出血性末梢血管拡張症（オスラー病）の有病率，発生率に関して，日本全国における疫学調査を行なう必要性がある．さらに，本症の責任遺伝子に関して解析を行なう必要がある．次に，上述の診断規準を用いた遺伝性出血性末梢血管拡張症（オスラー病）の診療ガイドラインの作成と普及が必要である．診療ガイドラインの作成にあたっては，全国の血液内科，呼吸器内科，消化器内科，放射線科，脳外科，耳鼻咽喉科など，本症の診断と治療にかかわる診療科にアンケート調査を行い，現在までの診断と治療に関するデータを集積する必要がある．本データと現在まで報告されている文献を収集解析した資料と対比して，本症の治療方針に関するガイドラインを作成し普及する必要がある．

Respirology 14(Suppl 3); A177, 2009.

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H. 知的財産の出願・登録状況
1. 特許取得（申請中を含む）
なし

2. 実用新案登録
なし
3. その他
なし

［Ⅱ］参考資料

Respirology (1998) **4**, 277–280

CASE REPORT

Transcatheter embolization of pulmonary arteriovenous malformations in Rendu–Osler–Weber disease

TAKANOBU SHIOYA[1], MASA-AKI SANO[2], MANABU KAGAYA[2], NOBUAKI ITO[2], AKIKO WATANABE[2], KAZUHIRO SATO[2], TAKEHUMI ITO[2], MASAHIRO SASAKI[2], MANABU HASHIMOTO[3] AND MAMORU MIURA[2]

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Transcatheter embolization of pulmonary arteriovenous malformations in Rendu–Osler–Weber disease
SHIOYA T, KAGAYA M, SANO M, ITO N, WATANABE A, SATO K, ITO T, SASAKI M, HASHIMOTO M, MIURA M. *Respirology* 1998 **3**: 277–280
Abstract Interest in the treatment of the pulmonary arteriovenous malformations (PAVMs) that occur in approximately one-third of patients with Rendu–Osler–Weber (ROW) disease (hereditary haemorrhagic teleangiectasia) has recently been renewed. PAVMs can now be occluded safely by the transvenous placement of detachable balloons or metal coils, thus avoiding the many potential complications of thoracotomy. This study analyses the treatment of eight PAVMs in four ROW patients by transcatheter embolization using detachable balloons or metal coils. After embolization, the mean right-to-left shunt fraction significantly decreased from $39.1 \pm 5.1\%$ to $11.9 \pm 1.1\%$ ($P < 0.05$) and PaO_2 significantly increased from 53.3 ± 7.8 torr to 76.2 ± 8.4 torr ($P < 0.05$). No serious complications occurred. One detachable balloon was deflated, but no recanalization occurred. We conclude that transcatheter embolization is a safe and efficacious treatment for PAVMs associated with ROW disease. Long-term studies are now needed to determine the risk of recanalization in this treatment.

Key words: hereditary haemorrhagic telangiectasia, pulmonary arteriovenous malformations, Rendu–Osler–Weber disease, transcatheter embolization.

INTRODUCTION

Rendu–Osler–Weber (ROW) disease (hereditary haemorrhagic telangiectasia) is an autosomal dominant disease that is characterized by systemic capillary dilation and bleeding diathesis.[1,2] Pulmonary arteriovenous malformations (PAVMs) are rare, mostly congenital, abnormalities of the pulmonary circulation, often associated with ROW disease. They involve direct communications between pulmonary arteries and veins via enlarged, tortuous vascular spaces.[3,4] The right-to-left shunt causes hypoxaemia which may give rise to decreased exercise capacity, dyspnoea and cyanosis. PAVMs can cause two serious complications: haemoptysis or haemothorax from the abnormal vessels, and thrombo-emboli that might result in fatal systemic emboli.[5] Thus, nearly half of all ROW patients with PAVMs report a history of stroke or transient ischaemic attack.[6,7] Brain abscess occurs in 5–14% of ROW patients with PAVM as a result of septic emboli.[8,9]

It is generally accepted that these risks justify the treatment of even asymptomatic PAVMs provided the diameter of the feeding vessels is more than 3 mm.[10] Disadvantages of surgical treatment are loss of the normal lung tissue surrounding the PAVMs and morbidity associated with thoracotomy. Currently, one accepted mode of treatment is the transcatheter embolization of feeding vessels using detachable balloons or metal coils.[11,12] Although some reports concerning embolization of PAVMs have been published, the data on long-term results are limited.[5,13]

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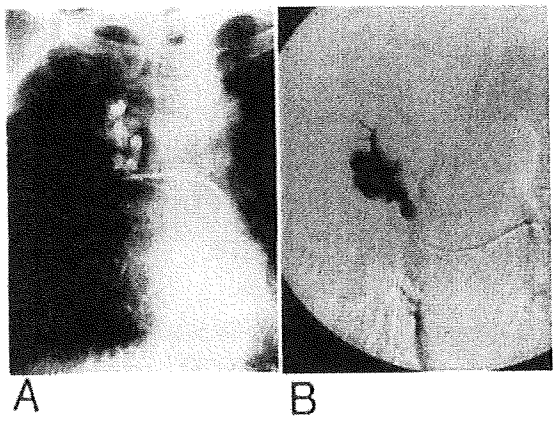

Figure 1 Case 1. (a) Pulmonary angiogram showing large PAVM in the right upper lung. (b) After embolization with two detachable balloons, the pulmonary angiogram shows occlusion of the PAVM and filling of several branches to the normal lung which were not apparent on the original angiogram due to the steel effect of the PAVM.

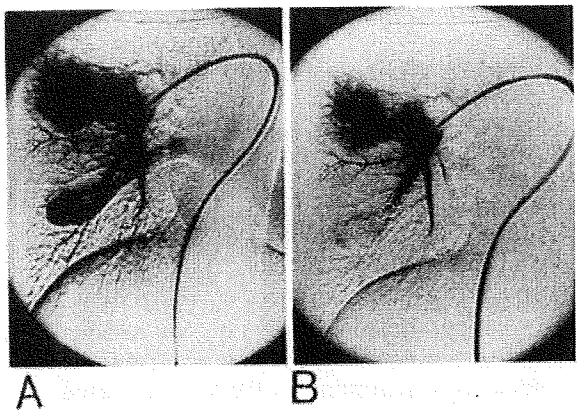

Figure 2 Case 2. (a) Pulmonary angiogram showing PAVM in the right basal lung. (b) Pulmonary angiogram after embolization with a steel coil showing occlusion of PAVM in the right basal lung.

In Japan, only 144 ROW patients and 126 ROW families have been reported to date, with one-third of the ROW patients being complicated by PAVMs.[14, 15] The first choice of treatment is still considered to be surgical resection. To help clarify the treatment of such cases, we therefore present our results of transcatheter embolization of PAVMs associated with ROW disease.

METHODS AND SUBJECTS

Over the last four years, in the Second Department of Internal Medicine at Akita Uiversity School of Medicine, transcatheter embolizations were used to treat four ROW patients with PAVMs. The embolizations were all performed by the same radiologist (M.H.). Following introduction via the femoral vein, pulmonary angiography was performed using a digital subtraction technique. Feeding vessels with a diameter of more than 5 mm were selectively cannulated and embolized with metal coils of

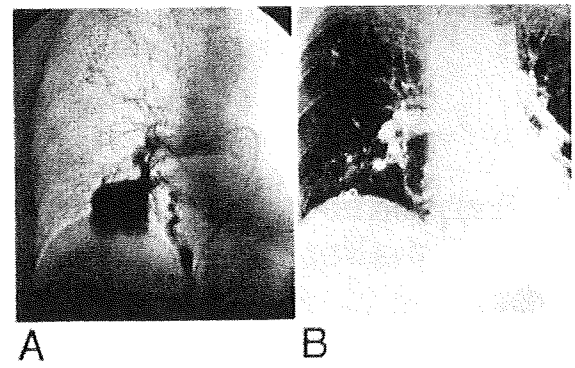

Figure 3 Case 3. (a) Pulmonary angiogram showing two PAVMs in the right basal lung. (b) Pulmonary angiogram after embolization with steel coils showing occlusion of the PAVMs in the right basal lung.

appropriate size (Target Therapeutics, Fremont, California). Additional coils were placed until there was no further flow from the pulmonary circulation. Latex detachable balloons (NYCOMED, Paris, France) with non-polymerizing liquids (radio-opaque agents) were placed using coaxial catheters in one patient (case 1). All PAVMs with accessible feeding vessels larger than 5 mm were embolized. The outcomes were evaluated between the 7th and 14th day after embolization.

The right-to-left shunt fraction was calculated using inhalation of 100% and the following formula:

$$\frac{QS}{QT} = \frac{0.003(P_AO_2 - PaO_2}{0.003(P_AO_2 - PaO_2) + (CaO_2 - CvO_2)}$$

in which P_AO_2 is the ideal alveolar oxygen tension, PaO_2 is the arterial oxygen tension, CaO_2 is the arterial oxygen content, and CvO_2 is the mixed venous oxygen content.

All data are expressed as means ± the standard errors of the means (SEM), and were analysed using Student's *t* test (two-tailed) for paired samples. *P* values of 0.05 or less were considered significant.

RESULTS

Individual results of transcatheter embolization treatment are given in Table 1.* Patients 1 and 2 had a single PAVM and patients 3 and 4 had multiple PAVMs. After embolization, the mean PaO_2 significantly increased from 53.3 ± 7.8 torr to 76.2 ± 8.4 torr ($P < 0.05$), and the mean right–left shunt significantly decreased from 39.1 ± 5.1% to 11.9 ± 1.1% ($P < 0.05$). In patient 1, one detachable balloon deflated on the 7th operative day; however, no recanalization occurred.

* Pulmonary angiograms before and after embolization are shown in Figure 1 through Figure 4.

Table 1 Results of embolization in individual patients

Patient age (years) and sex	Number of PAVMs treated	PaO_2 before treatment (torr)	PaO_2 after treatment (torr)	Shunt before treatment (%)	Shunt after treatment (%)
1 43, M	1 (balloon)	64.4	83.6	35.0	13.2
2 19, F	1 (coil)	65.7	86.5	33.1	12.7
3 58, F	2 (coil)	32.1	51.2	NT	NT
4 30, M	4 (coil)	51.1	83.5	49.1	9.8
Mean ± SEM		53.3 ± 7.8	76.2 ± 8.4	39.1 ± 5.1	11.9 ± 1.1

NT, not tested.

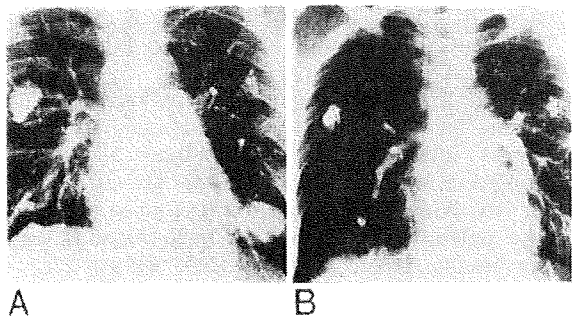

Figure 4 Case 4. (a) Pulmonary angioram showing four PAVMs in both lungs. (b) Pulmonary angiogram after embolization with steel coils showing occlusion of PAVMs in both lungs.

DISCUSSION

The first acount of PAVMs, attributed to Churton,[16] is a pathological report. The name of ROW disease derives from three subsequent authors.[2,17–19] Rendu[18] described familial epistaxis in patients with angiomas of the skin and mucous membranes. Osler[2] presented three cases of lesions with epistaxis and clearly distinguished capillary pathology from other causes of epistaxis that could be attributed to clotting disorders. Finally, Weber[19] described the skin and nasal manifestations of ROW disease. Pulmonary complications were not recognized clinically until Wilkens[20] gave a description of a cyanotic 16-year-old girl who was found to have multiple PAVMs at autopsy. Dines *et al.*[6] reported on 63 cases of PAVMs; 36% with ROW disease. Deaths in the medically treated group were primarily from cerebrovascular accidents. Dines *et al.* later reported on a further 38 cases including 47% with ROW disease. These pooled data indicate a 40% incidence of ROW disease in cases of PAVMs.[3] All reviews have emphasized an autosomal dominant pattern of genetic transmission. However, in at least one case of possible homozygosity, the affected stillborn offspring exhibited extensive angiomatous malformations of the internal organs that were more severe than the clinical findings in the heterozygous parents.[1,17]

Race has been reported as a factor in the prevalence of ROW disease, which is rare in black people and Arabs. In Japan, 144 ROW patients and 126 ROW families have been described and approximately one-third of the ROW patients have been associated with PAVMs.[15] Thus, ROW disease is less rare than previously believed in Japan.

Surgical excision of part or all of the lung was the usual treatment for PAVMs from 1939 until recently.[11,12] Silicone and latex balloons were first introduced for neurosurgical procedures in Europe. Servinenko temporarily occluded 304 different cerebral vessels using this method,[21] although none of his patients were reported to have ROW disease. His work inspired White *et al.* to initiate research with detachable silicone balloons in swine. Since then, White *et al.*[4] have reported a series of 17 patients with 91 PAVMs. Balloon embolization is thus an new important approach to the treatment of PAVMs, and its implications for the reduction of morbidity and mortality in ROW disease are considerable.[22]

Coil embolization has also been applied for PAVMs by Taylor *et al.*[11] More recently, Dutton *et al.*[23] have reported the results of treatment with coil embolization in 53 PAVM patients, concluding that the technique is effective, well tolerated, and associated with few complications. The correct choice of coil size is critical: too small a coil may pass through the venous portion of the malformation into the left atrium and thence into the systemic circulation, potentially with disastrous consequences, while too large a coil may displace the catheter tip from the feeding vessels and risk occlusion of the more proximal normal pulmonary arterial branches. A variety of methods, including the use of calibrated catheters, have been used to measure the feeding vessels supplying the malformation and thus determine the correct coil size.[5,23]

Detachable balloons are preferred by some researchers for the embolization of PAVMs, since these devices, unlike conventional steel coils, can be retrieved if they are too small for the vessel being occluded, and fewer normal vessels are occluded by the balloon technique than the coil technique.[5,22] Balloon embolization does, however, require multiple catheter exchanges for embolization of more than one vessel. Also, when non-polymerizing liquids are used for the balloon inflation, as in our case 1, there is a risk that the balloon will deflate prematurely and migrate into the systemic circulation.[5,19] Finally, when the feeding vessel to a PAVM is particulary large, the length of balloon required to achieve occlusion may compromise the more proximal vessels to the normal lung,[5] whereas the use of steel coils usually preserves

these vessels.[5,21] Thus, we consider metal coil therapy to be an easier and safer technique than detachable balloon therapy for PAVMs associated with ROW disease. However, further prospective clinical studies will be needed to determine the relative merit of the two techniques.

CONCLUSION

PAVMs associated with ROW disease are multiple in occurrence and may appear in succession. Treatment of PAVMs is crucial in ROW disease because the rupture of the PAVM or systemic emboli via PAVMs may be lethal. Percutaneous transcatheter embolization is the procedure of choice for the treatment of PAVMs associated with ROW disease. This technique is safe and well tolerated and is associated with excellent symptomatic and objective improvement. The advantages and disadvantages of embolization by detachable balloons and metal coils have been discussed above. Long-term studies will be needed to determine the risk of recanalization in these treatments.

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Hereditary Hemorrhagic Telangiectasia (HHT) in Akita Prefecture, Japan

We read with interest the article by Hisamatsu et al (1) indicating the case of Osler-Rendu-Weber disease (hereditary hemorrhagic telangiectasia, HHT) associated with hepatic arteriovenous malformation. HHT was first described in 1864 by Sutton and later recognized and reported by Rendu, Osler, and Weber, and is thus known as Rendu-Osler-Weber disease or Osler-Rendu-Weber disease (2, 3). This is an autosomal dominant disorder characterized by multiple telangiectatic lesions involving the skin and mucous membranes associated with epistaxis and other bleeding complications. HHT has been reported to occur across a wide geographic distribution throughout Europe and North America (2, 3). However, few cases have been reported in Asian countries. Also, there have been no epidemiological studies about the incidence or the prevalence of HHT in Japan.

We have treated 7 families and 10 patients who had pulmonary arteriovenous malformations (PAVMs) since 1978. Ten patients (4 males, 6 females, aged 56±12 years) with HHT were admitted for evaluation and treatment at the Second Department of Internal Medicine at Akita University School of Medicine during from 1978 to 1999. We interviewed their families to obtain precise information. The clinical criterion for the diagnosis of HHT was the presence of any two of the following: recurrent epistaxis, telangiectases elsewhere than in the nasal mucosa, evidence of autosomal dominant inheritance, and visceral involvement (4). Pedigrees of 7 families and family members are shown in the Fig. 1. Fifty-one persons out of 208 family members (24.8%) were diagnosed to be HHT, and 17 HHT patients (8.2%) had PAVMs. The percentage of HHT patients associated with PAVMs was 33.3%, which is close to the percentages (20–30%) that were reported in Europe and North America (2).

HHT has an estimated prevalence of 1 in 2,351 members of the population in the eastern France area of Ain, in 3,500 on the Danish island of Funen, in 5,155 in the Leeward Islands, 1 in 16,500 in Vermont, and 1 in 39,216 in northern England (2, 3). The population of Akita prefecture in 1998 was reported to be 1,221,720. Therefore, if 51 HHT patients at our university are hypothesized to be the total number, the prevalence of HHT in Akita Prefecture is roughly estimated at 1:24,000, a figure close to those reported in Vermont and northern England.

Recently, the condition has been shown to be a family of disorders caused by mutations in various genes, and the genes responsible for two forms have been identified (5, 6). Genetic linkages to HHT have been established to chromosome 9q33-q34 in some families and to chromosome 12q in others (5). This discovery of genetic heterogeneity should bring a re-evaluation of the natural history of these disorders, because the incidence of the many clinical manifestations may vary widely among the various forms. A current multicenter effort is analyzing the correlations between genotype and phenotype (6). Multicenter cooperation may also lead to randomized prospective trials to determine the efficacy of various therapies. The development of a functional assay to provide presymptomatic diagnosis appears possible. The finding that a protein binding transforming growth factor β (TGF-β) has a key role in the disease should help elucidate the pathophysiologic features. Therapeutic advances, including gene replacement, may now be a realistic possibility given the ease of access through the bloodstream to endothelial cells, the target tissue (5, 6). Although the understanding of HHT is expanding rapidly as stated above, there have been no such studies reported so far among Japanese HHT patients. Thus, the national epidemiological survey for HHT and genetic analysis of this disease in Japan is very urgent.

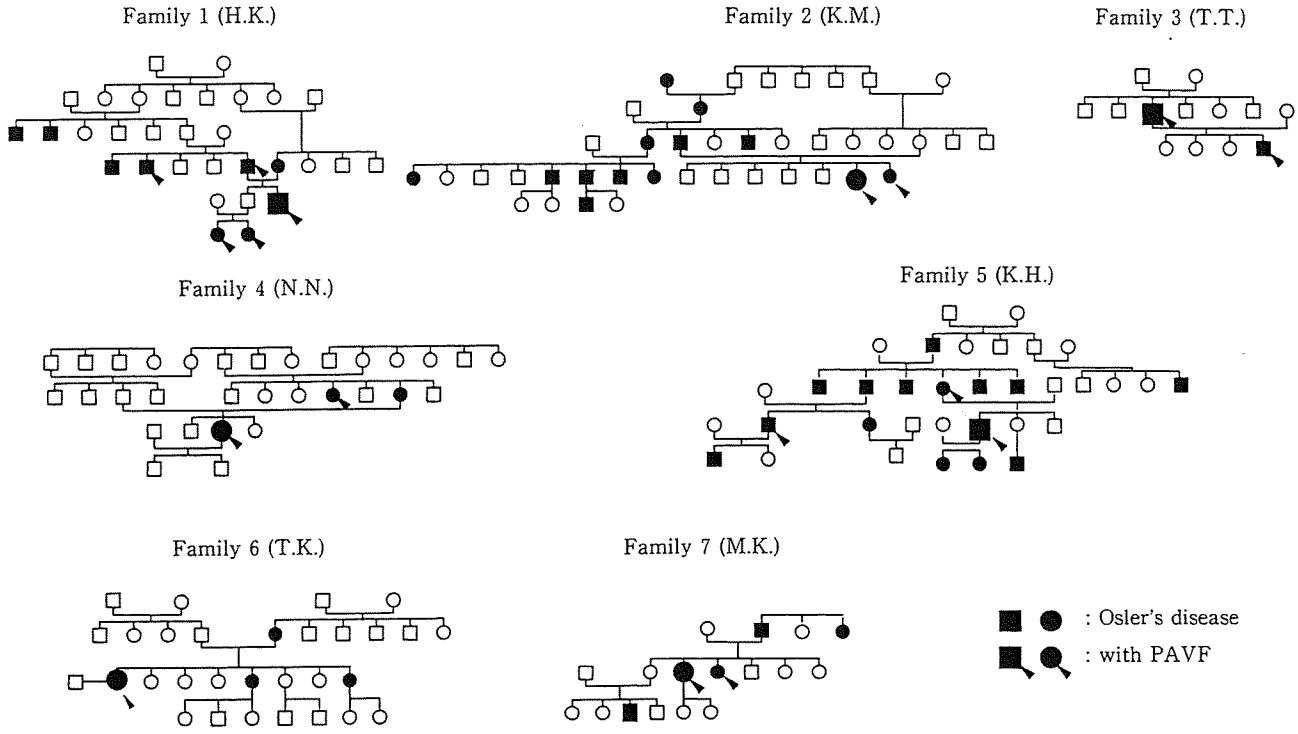

Figure 1. Pedigree of HHT patients in Akita Prefecture. Large symbols indicate the proband of the family. Closed symbols indicate HHT patients, and arrows indicate the patients associated with PAVMs.

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HUMAN MUTATION 19:140–148 (2002)

RESEARCH ARTICLE

Genetic Epidemiology of Hereditary Hemorrhagic Telangiectasia in a Local Community in the Northern Part of Japan

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Communicated by David Yandell

Hereditary hemorrhagic telangiectasia (HHT or Rendu-Osler-Weber syndrome) is an autosomal dominant disorder characterized by aberrant vascular development. We report here a genetic epidemiologic study in a county, A, in the Akita prefecture (population 1.2 million) located in northern Japan. Nine HHT patients who had been referred to tertiary-care hospitals were located in and near the study county. A total of 137 pedigree members were traced of which 81 were alive and 32 were affected by HHT. Complications associated with cerebral or pulmonary arteriovenous malformations were proven in six out of seven families. Linkage analysis in two large families revealed a weak yet suggestive linkage to the *HHT1* locus (encoding *endoglin*; ENG). Three novel mutations were found in four families, all of which led to a frameshift: a G to C transversion at the splicing donor site of intron 3 (Inv3+1 G>C) in one family, one base pair insertion (A) at nucleotide 828 (exon 7) of the *endoglin* cDNA in two large families (c.828–829 ins A), and a four base pair deletion (AAAG) beginning with nucleotide 1120 (exon 8) of the *endoglin* cDNA (c.1120–1123 delAAAG) in one family. The insertion of A in exon 11 (c.1470–1471 insA) mutation found in one family has also been reported in a European family. No *endoglin* gene mutations were found in two families. The population prevalence of HHT in the county was estimated to be 1:8,000~1:5,000, roughly comparable with those reported in European and U.S. populations, which is contradictory to the traditional view that HHT is rare among Asians. We recommend that families with HHT be screened for gene mutations in order that high-risk individuals receive early diagnosis and treatment initiation that will substantially alter their clinical course and prognosis. Hum Mutat 19:140–148, 2002.

KEY WORDS: ACVRL1; ALK1; hereditary hemorrhagic telangectasia; HHT; endoglin; ENG; genetic epidemiology; vascular complications; Japanese

DATABASES:
ENG – OMIM: 131195, 187300 (HHT); GDB: 137193; GenBank: AH006911
ACVRL1 – OMIM: 601284, 600376 (ORW2); GDB:230240; GenBank: AH005451

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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

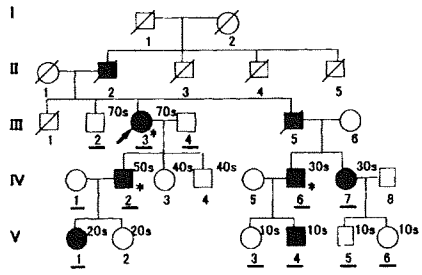
We examined nine patients with HHT who had been referred to tertiary-care hospitals. Pedigree structures for these patients were constructed as shown in Figure 1. A total of 137 pedigree members were traced of which 81 were alive and 32 affected by HHT. Of the seven pedigrees, four families (SB-1, SB-2, 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.

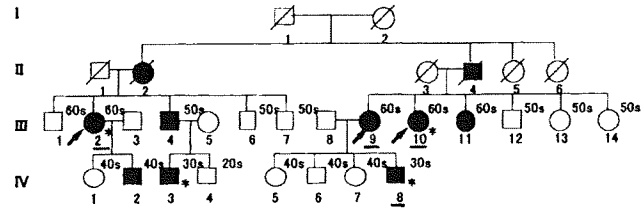
Mutation search. Due to the weak yet suggestive linkage with *HHT1* locus we searched for mutations of *ENG* in the patients. In the SB-1 pedigree we found a novel mutation, a G to C transversion at the splice donor site of intron 3 (Inv3+1 G>C) (Table 2). cDNA analysis of this mutation indicated mRNA 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



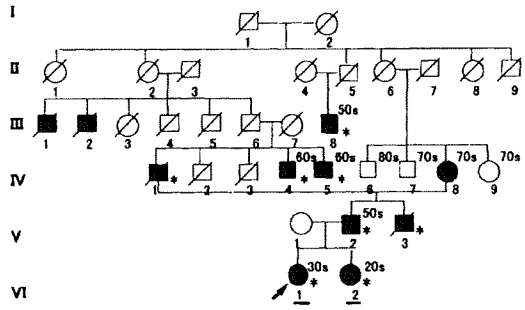
I-1: Died by unknown cause at unknown age
I-2: Died by unknown cause at unknown age
II-1: Died by unknown cause at unknown age
II-2: Died by unknown cause at unknown age
II-3: Died by unknown cause at unknown age
II-4: Died by unknown cause at unknown age
II-5: Died by unknown cause at unknown age
III-1:Died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

Family SB-2



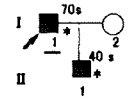
I-1 : Died by unknown cause at unknown age
I-2 : Died by unknown cause at unknown age
II-1: Died by unknown cause at unknown age
II-2: Died by unknown cause at unknown age
II-3: Died by unknown cause at age 73
II-4: Died by cerebral stroke at age 60
II-5: Died by unknown cause at unknown age
II-6: Died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

Family SB-3

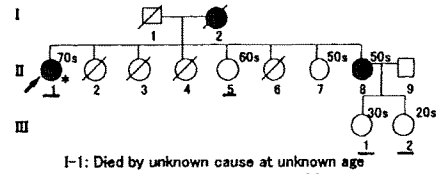


I-1 : Died by unknown cause at unknown age
I-2 : Died by unknown cause at unknown age
II-1 : Died by unknown cause at age 80
II-2 : Died by unknown cause at age 82
II-3 : Died by unknown cause at unknown age
II-4 : Died by unknown cause at unknown age
II-5 : Died by cerebral stroke at age 70
II-6 : Died 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

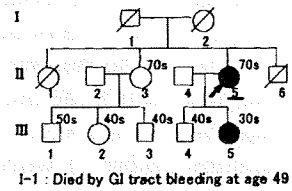


Family SB-5



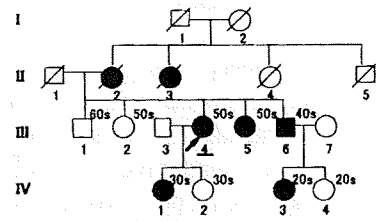
I-1: Died by unknown cause at unknown age
I-2: Died by cerebral stroke at age 65
II-2: Died by unknown cause at age 40
II-3: Died by unknown cause in infancy
II-4: Died by cancer at age 42
II-6: Died by unknown cause at age 32

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