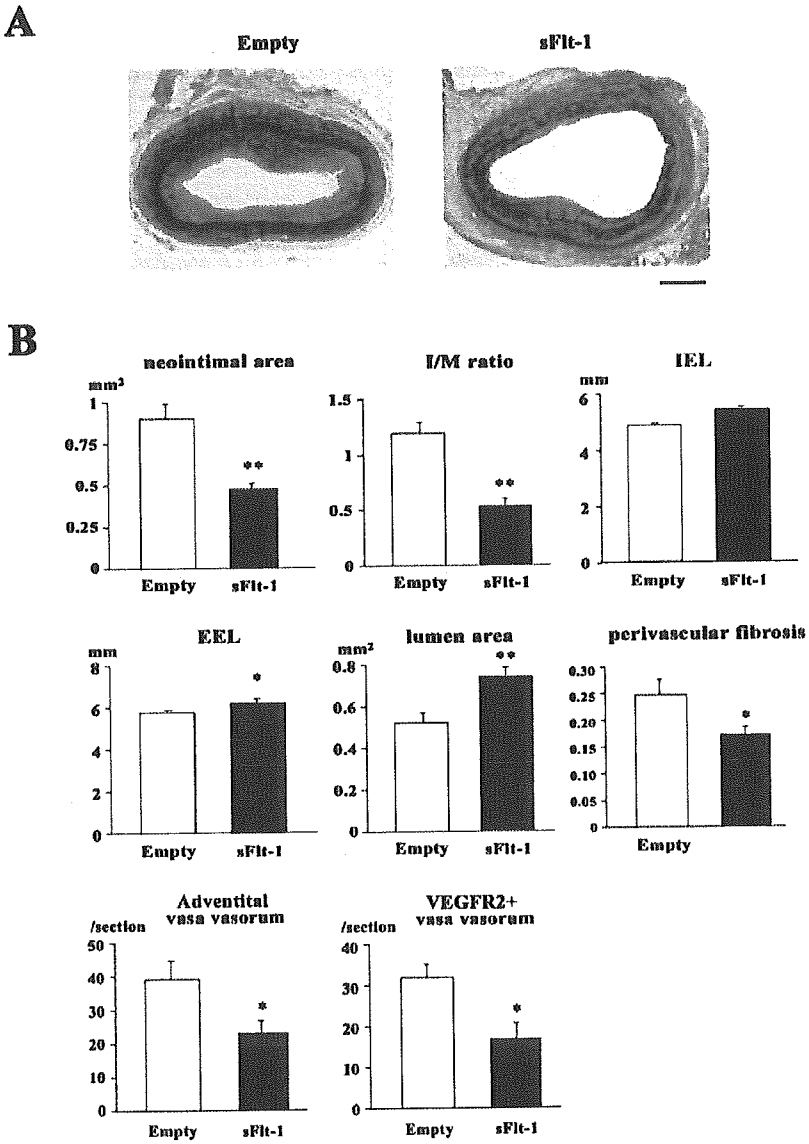

Figure 3. Inhibitory effect of *sFlt-1* gene transfer on restenotic changes (neointimal formation and negative remodeling) in rabbits. A, Carotid artery sections from empty-plasmid and *sFlt-1* groups 28 days after balloon injury stained with elastica van Gieson's stain. Bar=200 μm. B, Neointimal formation (neointimal area and intima-media ratio), negative remodeling (length of internal elastic lamina, length of external elastic lamina, and lumen area), perivascular fibrosis, adventitial vasa vasorum (number of CD31-positive vessels in adventitia), and VEGFR-2–positive vasa vasorum (number of VEGFR-2–positive vessels in adventitia) on day 28 after balloon injury are shown. *$P<0.05$, **$P<0.01$ vs empty-plasmid group, n=8 or 9.

transfected mice (Figure 6A and 6B). Wire injury also increased recruitment of bone marrow–derived monocytes (CD31-positive and c-Kit–positive) and circulating monocytes (Mac-1–positive) into peripheral blood (Figure 6C). *sFlt-1* gene transfer attenuated such changes in cell distribution, suggesting that wire injury increased such cell lineages in peripheral blood through VEGF. Plasma and femoral arterial concentrations of VEGF increased after wire injury, which was partly attenuated by *sFlt-1* transfection (online Tables III and IV).

Effects of $VEGF_{165}$ Gene Transfer on Neointimal Formation and Adventitial Angiogenesis in Rabbits

A recombinant adenoviral vector containing human $VEGF_{165}$ or the *LacZ* gene was produced. Gene transfer was performed by administering adenovirus solution (1 mL, 2×10^9 plaque-forming units) by a channel balloon catheter (Remedy, Boston Scientific Inc) immediately after balloon injury of rabbit carotid arteries (online Data Supplement and Figure). There were no significant differences between the empty-plasmid and *VEGF*-transfected groups in terms of neointimal formation, perivascular fibrosis, and negative remodeling (smaller lumen size, internal elastic lamina, and external elastic lamina) on day 28. In contrast, the number of adventitial vasa vasorum (the degrees of adventitial angiogenesis) was markedly increased in the *VEGF*-transfected group.

Discussion

VEGF has conventionally been thought to be an endothelial cell–specific growth factor and that it inhibits vascular pathological processes by accelerating endothelial proliferation and regeneration through endothelial VEGFR-2. If so, blockade of VEGF would suppress endothelial regeneration

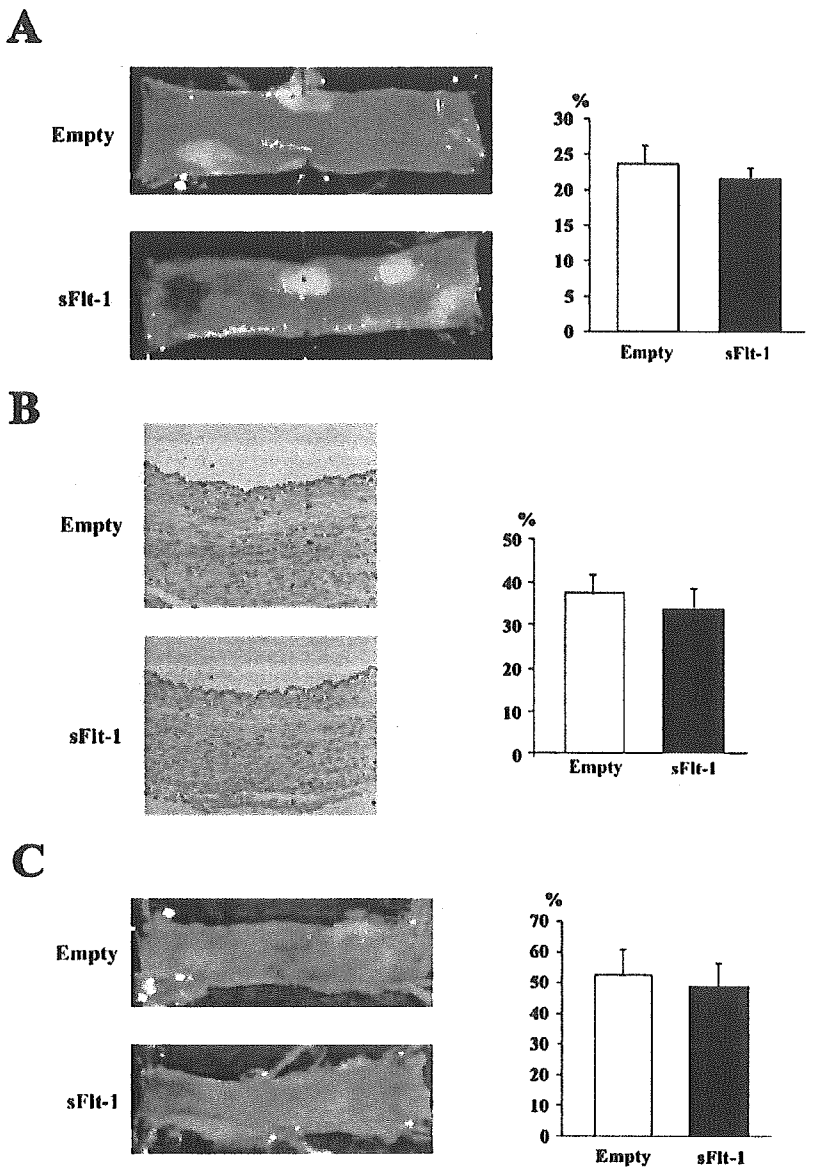

Figure 4. No significant effect of *sFlt-1* gene transfer on endothelial regeneration. A, Reendothelialization of rabbit carotid artery determined by Evans blue staining (deendothelialized areas are stained with blue) 7 days after balloon injury in rabbits. Endothelial regeneration of injured arteries was identified by intravenous injection of Evans blue dye 30 minutes before harvesting of vessels from rabbits and mice. Ratios of surface area covered by endothelium to total area in empty-plasmid and *sFlt-1* groups (n=7 each) are shown. B, Cross sections of rabbit femoral arteries stained with CD31 antibody 7 days after injury. Degrees of endothelial recovery (length of CD31-positive layer, length of internal elastic lamina in cross sections) in both groups are also shown (n=7 each). C, Reendothelialization of mouse femoral arteries 14 days after injury as determined by Evans blue staining (n=9 each).

and enhance neointimal formation after injury. In contrast to the conventional assumption, we here demonstrated that blockade of VEGF by *sFlt-1* gene transfer attenuated neointimal formation in rabbits, rats, and mice, indicating the essential role of VEGF in experimental restenosis.

As previously reported by others,[5–7,15] we demonstrated persistent increases in VEGF in arterial wall cells after injury. Emerging evidence suggests expression of functional VEGFR-1 and VEGFR-2 in cell types other than endothelial cells. We showed herein an increased expression of VEGFR-1 in lesional monocytes and medial smooth muscle cells during the early stage and in smooth muscle cells in the neointima and media during later stages. Increased VEGFR-2 expression was noted only at later stages. *sFlt-1* gene transfer attenuated the increased expression of inflammatory and growth factors such as VEGF, MCP-1, IL-1β, and so forth at early stages. Expression of VEGFR-1 in monocytes mediates chemotaxis,[9] and VEGFR-1 in smooth muscle cells mediates migration.[27] VEGFR-1 has been shown to act as an important mediator of VEGF-induced inflammation.[9,10,13] More recently, Yamada et al[28] showed that VEGF-mediated angiogenesis and inflammation are mediated by MCP-1. We also demonstrated the central role of MCP-1 in the mechanism of neointimal formation after vascular injury.[23–25,29] It is likely, therefore, that VEGF might cause vascular inflammation and migration of vascular smooth muscle cells and thus, cause neointimal formation after injury. Further studies are needed to elucidate the relative roles of VEGFR-1 and VEGFR-2 in the mechanisms of neointimal formation.

This study also demonstrated in rabbits the role of VEGF in the development of negative remodeling, another major cause of human restenosis after balloon angioplasty.[30] Fibro-

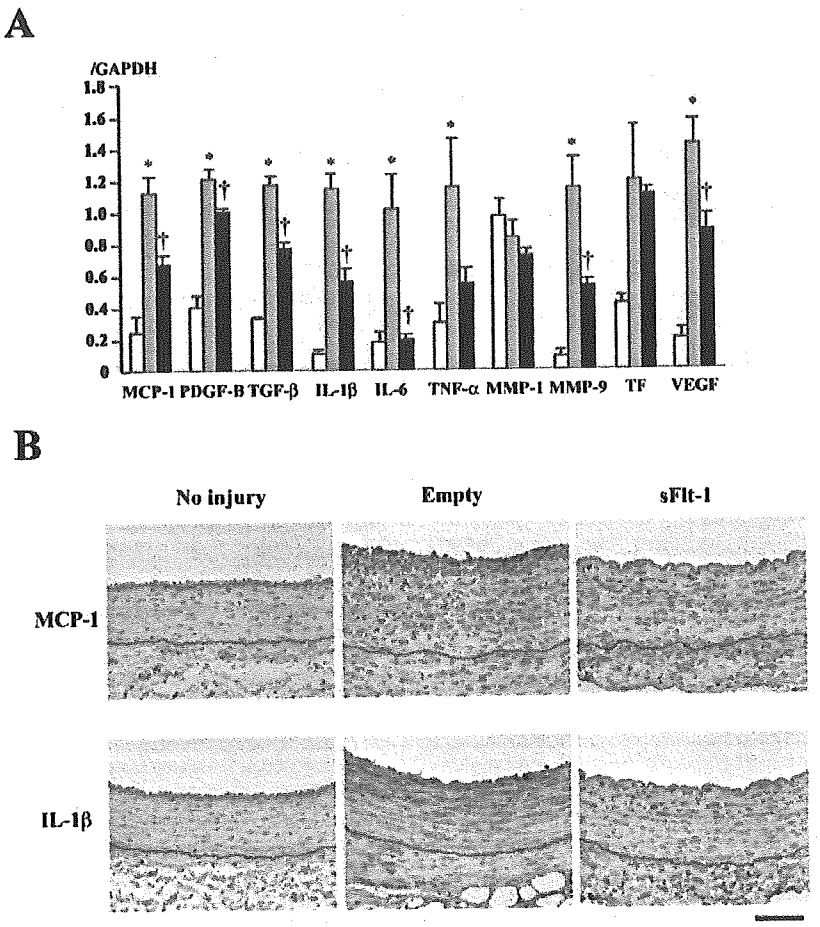

Figure 5. Inhibitory effect of *sFlt-1* gene transfer on expression of various inflammatory factors in rabbits. A, Effect of *sFlt-1* gene transfer on mRNA levels of various proinflammatory factors 1 day after injury. Quantitative real-time PCR was performed. *$P<0.01$ vs uninjured control (uninjured) artery; †$P<0.05$, ††$P<0.01$ vs empty-plasmid group. B, Carotid artery sections from control uninjured animals and those from empty-plasmid and *sFlt-1* groups 7 days after balloon injury stained immunohistochemically with MCP-1 and IL-1β. Internal and external elastic layers are highlighted with blue and black lines, respectively. Bar=100 μm. Immunohistochemical experiments were repeated 5 times, all with representative results.

sis and vasa vasorum in the adventitia have been implicated to be the central pathological processes leading to constrictive negative remodeling after balloon injury. Therefore, our present data suggest that *sFlt-1* gene transfer inhibited the development of negative constrictive remodeling by limiting adventitial fibrosis and angiogenesis.

VEGFR-1 has been shown to be an important mediator of stem cell recruitment and differentiation.[13] Sata and colleagues[26] have shown that a considerable proportion of neointimal and medial cells were bone marrow–derived progenitor cells. However, the role of VEGF in the recruitment and differentiation of progenitor cells into neointimal cells after vascular injury has not been addressed. We showed here that *sFlt-1* gene transfer inhibited recruitment of bone marrow–lineage cells into the peripheral circulation and injured arterial wall and thus, reduced neointimal formation after injury. These data suggest that VEGF might contribute to neointimal formation by recruiting bone marrow–derived and circulating monocytes.

Surprisingly, *sFlt-1* gene transfer did not affect endothelial regeneration after endothelial injury, suggesting a minor role of endogenous VEGF in endothelial regeneration after endothelial injury. It remains to be determined whether inhibition of VEGFR-2–mediated activity of endothelial regeneration by *sFlt-1* gene transfer might have been overshadowed by other stimuli (eg, basic fibroblast growth factor, angiopoietins, etc). On the contrary, adenovirus-mediated gene transfer of *VEGF* enhanced adventitial angiogenesis but did not reduce neointimal formation after balloon injury in rabbits. The latter observation is consistent with previous reports demonstrating that exogenous VEGF does not reduce neointimal formation in animals and humans.[15–17] Taken together, the role or mechanisms of action of VEGF may differ between exogenous and endogenous VEGF and between angiogenesis and neointimal formation.

This study may have significant clinical implications. First, *sFlt-1* gene transfer might be an attractive anti-VEGF therapy for inflammatory vascular disease and other inflammatory disorders. However, local delivery of *sFlt-1* must be preferable for clinical use to avoid potential side effects by systemic delivery. Second, our finding indicates that more research is required, especially on the safety of VEGF, before translational clinical research proceeds. Deleterious effects associated with overexpression of VEGF have recently been reported: (1) injection of VEGF-expressing skeletal muscle myoblasts into the murine heart caused the formation of hemangiomas and lethagenic heart failure[31] and (2) *VEGF* gene transfer into rabbit carotid arteries induced neointimal

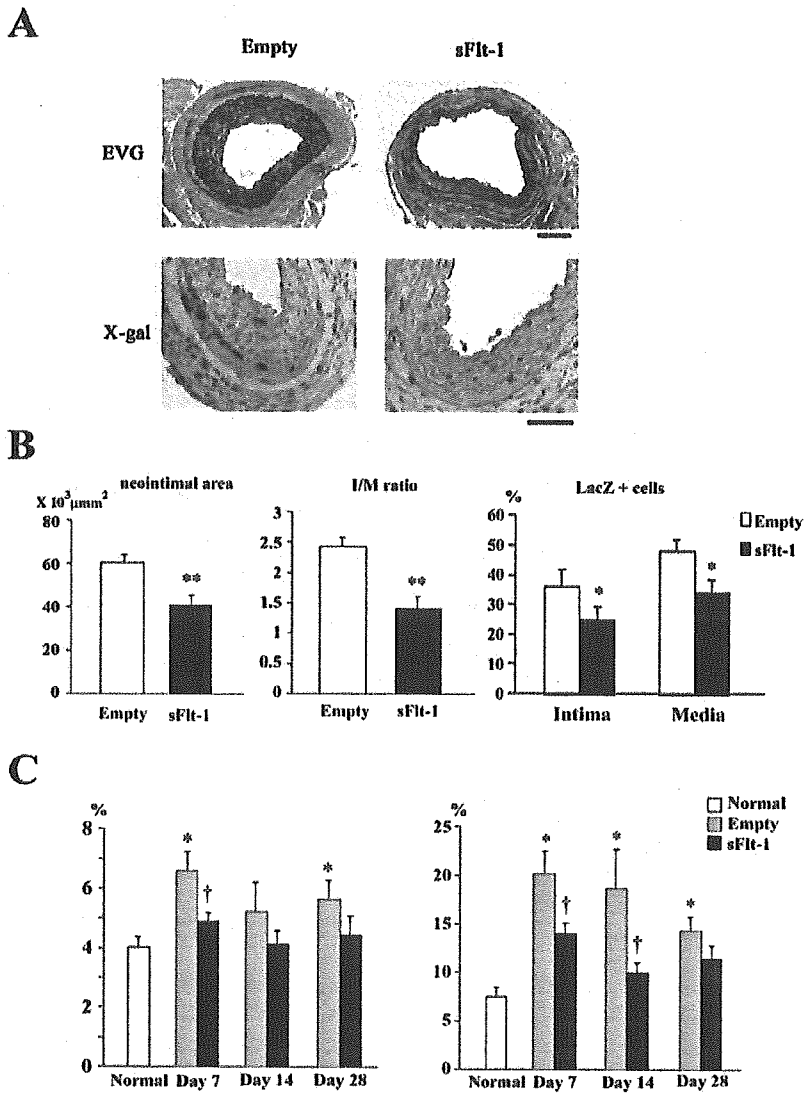

Figure 6. Contribution of bone marrow-derived cells to effect of *sFlt-1* gene transfer in mice. A, Arterial sections from empty-plasmid and *sFlt-1* groups 28 days after wire injury stained with X-gal or elastica van Gieson's (EVG) stains. Bar=100 μm. B, Inhibitory effects of *sFlt-1* gene transfer on neointimal formation (neointimal area and intima-media ratio) and percentage of LacZ-positive cells (100×LacZ-positive nuclei/total nuclei) in neointima and media. *$P<0.05$, **$P<0.01$ vs phosphate-buffered saline, n=8 each. C, Summary of fluorescence-activated cell sorting analysis of recruitment of bone marrow-derived monocytes (left) and circulating monocytes (right) into peripheral circulation in normal mice and mice transfected with empty plasmid or *sFlt-1* after wire injury, n=7 or 8. *$P<0.05$ vs normal mice (no injury); †$P<0.05$ vs empty-plasmid group.

formation with angiomatoid proliferation of endothelial cells.[12] These studies highlight the potential side effects or toxicity that would against the clinical use of VEGF for therapeutic angiogenesis and restenosis.

In conclusion, this study has provided direct in vivo evidence suggesting that increased expression and activity of VEGF are essential for the development of experimental restenosis after intraluminal injury by recruiting monocyte-lineage cells. Although there is no clinical evidence suggesting enhancement of atherosclerosis or neointimal formation by VEGF therapy,[16,17] our present data raise the question of whether VEGF therapy is useful without serious risks in patients with advanced atherosclerosis.

Acknowledgments

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c-kit and PDGFRA Mutations in Extragastrointestinal Stromal Tumor (Gastrointestinal Stromal Tumor of the Soft Tissue)

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Abstract: Extragastrointestinal stromal tumor (EGIST) is a unique tumor that occurs outside the gastrointestinal tract. EGIST shows a c-kit expression and histologic appearance similar to those of gastrointestinal stromal tumor (GIST). Most GISTs have gain-of-functional mutation of the c-kit gene, and some have mutation of the platelet-derived growth factor receptor-alpha (PDGFRA) gene. However, the frequency of mutation of those genes in EGISTs remains unclear. We examined the clinicopathologic features, prognostic factors, and c-kit and PDGFRA mutation in 39 cases of EGIST. Tumors with high mitotic counts (≥5/50 high power fields) or a high Ki-67 labeling index (≥10%) were significantly correlated with worse prognoses. The c-kit mutation was found in the juxtamembrane domain (exon 11) and the extracellular domain (exon 9) in 12 of 29 cases (41.4%) and 2 of 29 cases (6.9%), respectively. The PDGFRA gene mutation was found at the juxtamembrane domain (exon 12) and the tyrosine kinase domain (exon 18) in one case each. The pattern of kit and PDGFRA mutation in EGIST was essentially similar to that in GIST. Our results suggest that the c-kit and PDGFRA mutations play an important role in the tumorigenesis of EGIST. High mitotic counts and a high Ki-67 labeling index may be useful for predicting the aggressive biologic behavior in EGIST. Furthermore, STI-571, targeting c-kit and PDGFR tyrosine kinase, seems to be a possible therapeutic strategy for EGISTs, especially advanced cases.

Key Words: extragastrointestinal stromal tumor, soft tissue, gastrointestinal stromal tumor, c-kit, PDGFRA, STI-571

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Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract.[8,21] GIST is typically characterized by immunohistochemical expression of c-kit. The interstitial cell of Cajal also expresses c-kit and CD34.[11,28] Therefore, GIST is considered to show differentiation along the lines of interstitial cell of Cajal. Since Hirota et al first described the c-kit gene mutation in GIST,[11] several studies have revealed various types of c-kit oncogene mutations, including exon 11 encoding the juxtamembrane domain, exon 9 encoding the extracellular domain, and exons 13 and 17 encoding the tyrosine kinase domain.[3,7,12,16–18,23,26,27,30,31,34] Moreover, recent studies have described the mutations of PDGFRA at the juxtamembrane domain (exon 12) and tyrosine kinase domain (exon 18) in some populations of GIST.[9,10] In the earlier literature, GIST was classified as a smooth muscle tumor variously termed leiomyoma, epithelioid leiomyoma, leiomyoblastoma, leiomyosarcoma, epithelioid leiomyosarcoma, or malignant leiomyoblastoma. GIST of the digestive tract is now considered to be the distinctive entity, distinguished from leiomyoma, leiomyosarcoma, schwannoma, and other mesenchymal tumors.[20] Recently, Miettinen et al have reported c-kit positive tumors primarily in the omentum and mesentery.[22] Reith et al also have reported this kind of tumor, named extragastrointestinal stromal tumor (EGIST).[25] Furthermore, Sakurai et al have described a c-kit gene mutation at exon 11 in 5 cases of GISTs primarily in the omentum.[29] However, the frequency of c-kit and PDGFRA mutations, or the clinicopathologic importance of such mutations in a large series of EGISTs, is still poorly understood.

STI-571 (imatinib mesylate, [Gleevec]; Novartis Pharmaceuticals Corp, East Hanover, NL) is an anti-tyrosine kinase drug that was originally developed for bcr-abl in chronic myeloid leukemia.[6] Recent studies have revealed the anti-tumor effect of STI-571 in GIST.[4,14,32,33] In this study, we aimed to characterize the clinicopathologic features in a large series of EGISTs, and then elucidated the frequency of kit and PDGFRA abnormalities and their clinicopathologic importance for the targeted therapeutic strategy by STI-571.

MATERIALS AND METHODS

Case Materials

We reviewed leiomyomas, leiomyoblastomas, leiomyosarcomas, and other spindle cell tumors of the abdominal cavity, retroperitoneum, and pelvic cavity that were stored in the files of our department. Leiomyosarcomas were diagnosed as tumors showing histologically recognizable smooth muscle differentiation as shown by interlacing fascicles of spindle cells with eosinophilic cytoplasm and blunt-ended nuclei. All leiomyosarcomas were positive for smooth muscle actin, sometimes for desmin, and entirely negative for c-kit. We defined EGISTs as tumors fulfilling the following characteristics: 1) tumors that originate from the soft tissues of the abdominal cavity, retroperitoneum, and pelvic cavity, but display no connection to the wall of the gastrointestinal tract and the pelvic organs; 2) tumors showing histologic resemblance to homologous GIST; and 3) tumors with c-kit immunopositivity. In this study, the majority of EGISTs were diffusely and strongly positive for c-kit. Six cases with weak c-kit positivity were included in this study because they showed weak but diffuse immunoreactivity for c-kit, diffuse expression of CD34, and similar histologic features to those of other EIGSTs with strong immunoreactivity for c-kit.

Finally, 39 cases of EGIST were obtained. Each EGIST was evaluated for clinicopathologic and histologic features, including cell type (epithelioid or spindle), cellularity (low, moderate, or high), mitoses, and the presence of necrosis and hemorrhage. Mitoses were counted and summed from 50 high power fields (HPF).

Immunohistochemistry

Formalin-fixed paraffin-embedded samples were used for the immunohistochemical examination. Immunohistochemical staining was performed using the following primary antibodies: c-kit (polyclonal c-19, dilution; 1/200, Santa Cruz Biochemistry, CA.), CD34 (QB-end-10, dilution; 1/50, Novocastra, Newcastle, UK), alpha-smooth muscle actin (1A4, dilution; 1/5000, Sigma BioSciences, St. Louis, MO), muscle-specific actin (HHF35, dilution; 1/200, Biomedia, Foster City, CA), desmin (D33, dilution 1/100, DAKO, Carpinteria, CA), S-100 (polyclonal, dilution; 1/400, DAKO), and Ki-67 (MIB-1, dilution 1/100, DAKO). The subsequent development of antibody-bridge labeling was performed by the streptavidin-biotin-peroxidase method (Histofine SAB-PO Kit, Nichirei, Tokyo, Japan) with hematoxylin counterstaining.

Polymerase Chain Reaction Single-Strand Conformation Polymorphism (PCR-SSCP) for c-kit and PDGFRA

Mutations of exons 9, 11, 13, and 17 of the c-kit gene and those of exons 12 and 18 of the PDGFRA gene were examined in 29 cases of EGIST, according to the previously described PCR-SSCP methods.[35] Ten randomly selected cases of leiomyosarcomas were examined together. Genomic DNA was extracted from paraffin-embedded tissue by using standard proteinase K digestion and phenol/chloroform extraction. The DNA sequences for each exon were amplified for the first PCR with each primer for 40 cycles (94°C for 1 minute, 52–56°C for 1 minute, and 72°C for 1 minute) by using a thermal cycler (T Gradient, Biometra, Goettingen, Germany.). The annealing temperature and sequences of each primer are summarized in Table 1. The PCR products were electrophoresed through 2.0% agarose gel with ethidium bromide to confirm the correct amplification. The reamplified products were diluted 1:1 in loading buffer (94% formamide, 10 mg bromphenol blue, 0.05% xylene cyanol), denatured by heating at 98°C for 3 minutes, and snap frozen on ice, then loaded onto 12.5% polyacrylamide gel. Electrophoresis was carried out for 90 minutes at a constant power of 600 V using an electrophoretic apparatus (GenePhor System, Amersham Pharmacia Biotech, Tokyo, Japan) under the three different temperature conditions. After electrophoresis, the gels were stained using a DNA silver staining kit (Hoefer Automated Gel Stainer, Amersham Pharmacia Biotech). The samples showing abnormal migration bands by PCR-SSCP were reamplified for 25 cycles under the same conditions. The amplified products were then purified by centrifugal filter devices of Microcon (Millipore, Bedford, MA). After the purification, a direct sequencing was carried out by the dideoxy chain termination method using a Perkin Elmer ABI Prism 310 sequence analyzer (Applied Biosystems, Foster City, CA). If the sample did not show the abnormal migration bands in SSCP, direct sequencing was carried out.

Statistical Analysis

The correlation between the presence of mutation and the clinicopathologic parameters was analyzed by Fisher exact test and Student's *t* test. We analyzed disease-specific survival and disease-free survival. The end points included any relapse (local recurrence and/or distant metastasis) of EGIST for the analysis of disease-free survival and death from EGIST for the analysis of tumor-specific survival. Univariate analyses of both survivals were performed by the Kaplan-Meier method with a log-rank test.

RESULTS

Clinical Findings

The clinicopathologic findings of EGIST are summarized in Table 2. The 39 patients comprised 15 men and 24 women, ranging in age from 30 to 88 years (mean, 59 years). The tumors were located in the abdominal cavity (11 cases), omentum (3 cases), mesentery (3 cases), retroperitoneum (17 cases), and pelvic cavity (5 cases). Of 11 abdominal cases, 2 cases (case nos. 21 and 38) showed omental tumors with peri-

TABLE 1. Primer Sequences and Annealing Temperature for PCR

Exon	Primer Sequence	Annealing (°C)
c-kit		
9F	5′-TTT GGA AAG CTA GTG GTT CA-3′	52
9R	5′-ATG GTA GAC AGA GCC TAA AC-3′	
11F	5′-CTA TTT TTC CCT TTC TCC CC-3′	54
11R	5′-TAC CCA AAA AGG TGA CAT GG-3′	
13F	5′-GCT TGA CAT CAG TTT GCC AG-3′	56
13R	5′-AAA GGC AGC TTG GAC ACG GCT TTA-3′	
17F	5′-TTT CTC CTC CAA CCT AAT AG-3′	56
17R	5′-CCT TTG CAG GAC TGT CAA GC-3′	
PDGFRA		
12-1F	5′-CCA GTT ACC TGT CCT GGT CAT-3′	53
12-1R	5′-TGG AAA CTC CCA TCT TGA GTC-3′	
12-2F	5′-AAA TTC GCT GGA GGG TCA TT-3′	53
12-2R	5′-GGA GGT TAC CCC ATG GAA CT-3′	
18F	5′-AGT GTG TCC ACC GTG ATC TG-3′	53
18R	5′-GTG TGG GAA GTG TGG ACG TA-3′	

toneal dissemination, 1 case (case no. 4) showed a predominantly omental tumor with invasion to the mesocolon, 1 case (case no. 11) showed an omental tumor with invasion to the abdominal wall, 2 cases (case nos. 31 and 35) showed tumors located between the mesocolon and retroperitoneum, and 1 tumor (case no. 39) was located between the transverse colon and duodenum. As for the remaining abdominal tumors (case nos. 10, 18, and 29), further details of their origin were not available, but they did not display any definite connection to the gastrointestinal wall proper. Of the 5 pelvic tumors, 2 cases (case nos. 9 and 24) were located between the rectum and urinary bladder, 2 cases (case nos. 1 and 30) between the rectum and sacrum, and the other (case no. 36) at the right side of the uterus. Surgical excision was the primary treatment of all of the cases.

Follow-up information was available for 33 patients, and the follow-up period ranged from 5 to 192 months (median, 43 months). Fourteen patients (42.4%) were alive without any evidence of disease after the initial operation. Three patients (9.1%) were alive with a recurrent tumor. Distant metastasis was seen in 8 patients (24.2%), including liver metastasis (3 cases), lung metastasis (2 cases), both liver and lung metastasis (2 cases), and bone metastasis (1 case). Sixteen patients (48.5%) had died of the tumor. The cause of death was related to EGIST in all patients who died. The estimated 5-year tumor-specific and recurrence-free survival rate was 52.5% and 42.9%, respectively.

Pathologic Findings

The tumors ranged from 3 to 35 cm in size (mean, 13.5 cm). Grossly, most tumors presented as circumscribed or lobulated firm masses. Cystic change was recognized in several cases; in particular, 2 cases were present as a huge cystic mass. Histologically, 21 cases (54%) were predominantly of the spindle cell type and 18 (46%) were of the epithelioid type. The EGISTs of the spindle cell type consisted of short or ill-developed fascicles of spindled cells (Fig. 1A). These cells usually had short plump nuclei; these features are distinct from those of conventional leiomyosarcoma characterized by well-developed fascicles of spindle cells with blunt-ended nuclei and eosinophilic fibrillary cytoplasms. The EGISTs of the epithelioid cell type consisted of a sheet of rounded to polygonal cells, showing the features of benign or malignant leiomyoblastoma (Fig. 1B). Mild nuclear pleomorphism was observed in 13 cases (Fig. 1C, D). Some cases showed cytoplasmic vacuolization, resulting in signet-ring cell formation. Skenoid fibers were not observed in any cases. Mitotic counts varied from 0 to 100 per 50 HPF. Immunohistochemically, 33 tumors were strongly and diffusely positive for c-kit (Fig. 1E). Six cases showed weak but diffuse staining for c-kit. CD34 was positive in 24 (62%) cases. Alpha-smooth muscle actin, desmin, and S-100 protein were positive in 11 (31%), 2 (5%), and 2 cases (5%), respectively.

c-kit Mutation

Of 29 cases of EGIST available for molecular study, 12 (41.4%) showed the c-kit mutations at exon 11. Deletions ranging from 6 to 60 bp in exon 11 were found in 7 cases, and point mutations were found in 5 cases (Fig. 2). Two cases (6.9%) showed a mutation at exon 9, which was a 6-bp (GCCTAT) insertion at codon 504, resulting in duplication of Ala and Tyr (Fig. 3). None of the EGISTs had a mutation in

TABLE 2. Clinicopathologic Findings in 39 Cases of EGIST

Case No.	Age (yr)	Gender	Size (cm)	Site	Cell Type	Cellularity	Nuclear Pleomorphism
1	64	M	11	Pelvic cavity	Epithelioid	Moderate	+
2	80	F	15	Retroperitoneum	Spindle	Moderate	−
3	84	F	25	Mesentery	Epithelioid	Moderate	−
4	52	M	30	Abdominal cavity	Epithelioid	Moderate	+
5	46	F	7	Retroperitoneum	Epithelioid	High	−
6	69	M	18	Retroperitoneum	Spindle	High	+
7	64	F	4	Mesentery	Epithelioid	Moderate	−
8	62	F	11	Omentum	Epithelioid	Moderate	−
9	58	M	22	Pelvic cavity	Spindle	Moderae	−
10	54	M	15	Abdominal cavity	Epithelioid	Moderate	−
11	38	M	6	Abdominal cavity	Epithelioid	High	−
12	68	F	15	Retroperitoneum	Spindle	High	+
13	45	F	10	Retroperitoneum	Spindle	High	−
14	63	M	6	Retroperitoneum	Spindle	Moderate	−
15	54	M	15	Omentum	Epithelioid	Moderate	+
16	45	M	3	Retroperitoneum	Epithelioid	Moderate	−
17	50	F	14	Retroperitoneum	Spindle	High	−
18	45	F	25	Abdominal cavity	Epithelioid	High	−
19	49	F	17	Omentum	Epithelioid	Moderate	−
20	30	F	14	Retroperitoneum	Epithelioid	Low	−
21	80	M	10	Abdominal cavity	Epithelioid	Moderate	+
22	67	F	10	Retroperitoneum	Spindle	Moderate	+
23	56	F	12	Retroperitoneum	Spindle	High	+
24	88	M	15	Pelvic cavity	Epithelioid	Moderate	−
25	48	F	11	Retroperitoneum	Spindle	High	−
26	52	F	10	Retroperitoneum	Spindle	High	+
27	54	M	35	Retroperitoneum	Spindle	High	−
28	57	F	9	Retroperitoneum	Spindle	Moderate	−
29	62	F	5	Abdominal cavity	Spindle	High	−
30	65	F	8	Pelvic cavity	Spindle	High	−
31	55	M	15	Abdominal cavity	Spindle	High	−
32	54	M	15	Abdominal cavity	Spindle	High	+
33	74	F	14	Retroperitoneum	Spindle	Moderate	+
34	75	F	8	Retroperitoneum	Spindle	Moderate	+
35	79	F	10	Abdominal cavity	Spindle	Moderate	−
36	56	F	15	Pelvic cavity	Spindle	Moderate	−
37	54	F	16	Mesentery	Epithelioid	High	−
38	56	M	10	Abdominal cavity	Epithelioid	Moderate	+
39	63	F	15	Abdominal cavity	Epithelioid	Moderate	−

exons 13 or 17. In total, 14 of 29 (48.2%) cases of EGISTs had c-kit gene mutations. None of the 10 leiomyosarcomas had the kit mutation.

PDGFRA Mutation

The mutation of PDGFRA exon 12 was found in 1 case (case no. 3), showing GTC to GAC transition at codon 561 (V561D) (Fig. 4). One case (case no. 19) had a mutation at exon 18 with a 12-bp deletion of codon 842 to 845 (Del DIMH) (Fig. 4). In total, 2 of 29 (6.9%) EGISTs, or 2 of 15 (13.3%) kit-wild EGISTs, had PDGFRA mutations. Two cases with PDGFRA mutation had epithelioid morphology, a large tumor size (25 cm or 17 cm), low mitotic activity, and a low Ki-67 labeling index. One case with the exon 12 mutation (case no. 3)

TABLE 2. (Continued)

Case No.	Mitosis (/50 HPF)	Ki-67 LI	c-kit Mutation	PDGFRA Mutation	Local Recurrence (mo)	Metastasis (mo)	Final (mo)	Follow-up
1	4	10.8	–	–	+, 9	Lung, 7	AWD	22
2	9	3.9	–	–	–	–	NED	9
3	4	3.3	–	Exon 12	+, 5	–	DOD	5
4	4	2.3	–	–	–	–	NED	60
5	1	2.3	Exon 11	–	–	–	NED	86
6	12	17.2	–	–	+, 10	–	DOD	37
7	4	1	Exon 11	–	–	–	NED	192
8	3	1.2	–	–	–	–	NED	6
9	4	0.5	–	–	–	–	NED	126
10	5	0.5	–	–	–	–	NED	161
11	2	2.3	ND	ND	+, 5	–	DOD	5
12	5	3.2	ND	ND	+, 101	–	DOD	136
13	19	10	–	–	–	Lung, 24	DOD	50
14	4	4.1	Exon 11	–	+, 79	–	DOD	96
15	3	1	ND	ND	–	–	NED	62
16	1	2	Exon 11	–	–	–	NED	86
17	10	1	–	–	+, 52	Liver, 44	DOD	68
18	100	12.7	–	–	–	Spine, at present	DOD	13
19	1	2	–	Exon 18	–	–	NED	48
20	9	1.8	Exon 11	–	+, 10	–	DOD	21
21	17	6.6	Exon 9	–	+, 36	Liver, 55	AWD	55
22	20	33.9	Exon 11	–	–	Liver, 6	AWD	6
23	20	12.1	ND	ND	–	Liver, lung, 46	DOD	48
24	5	7	Exon 11	–	–	–	NED	13
25	50	1	Exon 11	–	+, 10	–	DOD	28
26	25	11.5	–	–	+, 54	Liver, lung, 54	DOD	60
27	0	2	–	–	–	–	NED	94
28	5	4.2	ND	ND	+, 9	–	DOD	9
29	25	7.5	–	–	–	–	NED	6
30	1	1	Exon 11	–	–	–	NED	175
31	15	8	Exon 11	–	+, 10	–	DOD	22
32	28	11.7	Exon 11	–	+, 10	–	DOD	21
33	4	1.9	ND	ND	+, 9	–	DOD	24
34	10	2.7	ND	ND	NA	NA	NA	
35	4	4	Exon 9	–	NA	NA	NA	
36	3	NA	ND	ND	NA	NA	NA	
37	24	8.1	ND	ND	NA	NA	NA	
38	8	NA	Exon 11	–	NA	NA	NA	
39	100	12.3	ND	ND	NA	NA	NA	

ND, not done; NA, not available; NED, no evidence of disease; AND, alive with disease; DOD, die of disease; LI, labeling index.

was malignant with short survival, whereas the other case with the exon 18 mutation (case no. 19) was free of recurrence for 4 years. The PDGFRA mutation was not found either in kit-mutant EGISTs or in leiomyosarcomas.

Statistical Analysis for Prognosis

Follow-up information was available for 33 patients, of which 27 were available for analysis of the c-kit gene and PDGFRA gene mutation. The tumors with the c-kit mutation

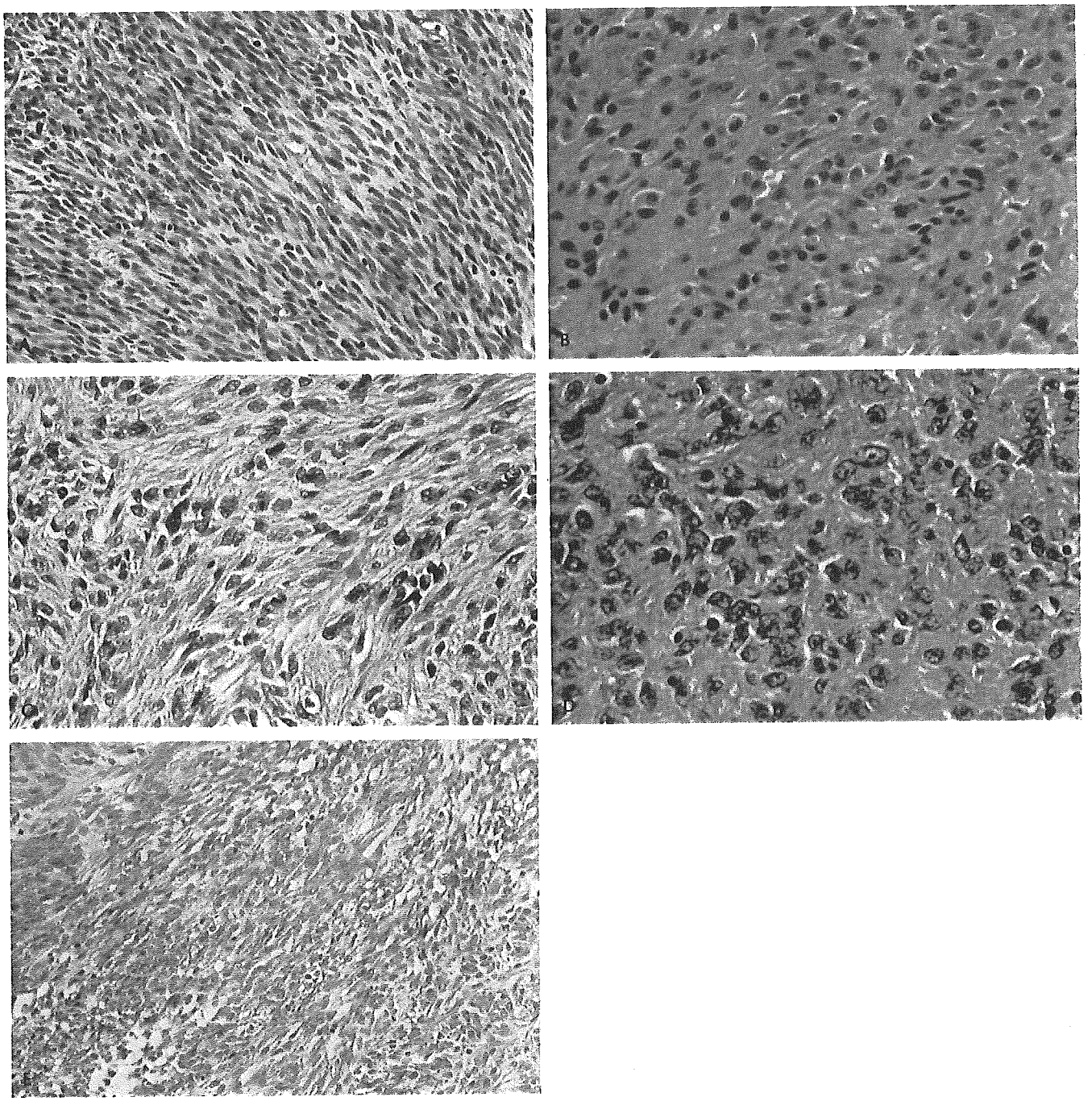

FIGURE 1. Histologic appearance of EGIST. **A:** Spindle cell type. Spindle tumor cells are arranged in fascicles with palisading pattern. **B:** Epithelioid cell type. Tumor cells with rounded nuclei are arranged in sheets. **C:** Spindle cell type with cellular atypia. Nuclear enlargement and irregularity are noted. **D:** Epithelioid cell type with cellular atypia. Nuclear enlargement and irregularity are noted. **E:** c-kit expression in EGIST.

were smaller in size than those without the mutation (P = 0.008). No other clinicopathologic factors correlated with the c-kit mutation in the 27 cases (Table 3). Even when moderate and high cellularity were combined, the cellularity (low vs. moderate, high) was not correlated with the presence of the c-kit mutation. The presence of the c-kit mutation was not correlated with either disease-specific survival or disease-free survival (P = 0.86 and P = 0.90, respectively). There was no significant correlation between the presence of gene mutation in either c-kit or PDGFRA and any clinicopathologic factors,

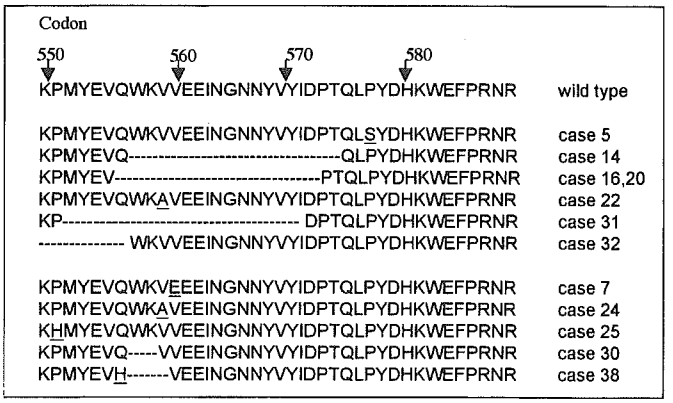

FIGURE 2. c-kit mutation at exon 11 and predicted amino acid sequence. -----, deletion. Point mutation is underlined.

including the prognosis. In 33 cases, a high mitotic rate (≥5/50 HPF) was significantly associated with both shorter disease-specific survival ($P = 0.015$) and shorter disease-free survival ($P = 0.014$) (Fig. 5). A high Ki-67 labeling index (≥10% HPF) also had significantly shorter disease-specific survival and shorter disease-free survival than those with a low index (<10%) ($P = 0.025$ and $P = 0.024$, respectively) (Fig. 5). We defined three categories on the basis of a combination of the mitotic rate and MIB-1 labeling index: the high-risk group (≥5/50 HPF with ≥10% Ki-67), the intermediate-risk group (≥5/50 HPF with <10% Ki-67, or, <5/50 HPF with ≥10% Ki-67), and the low-risk group (<5/50 HPF with <10% Ki-67).

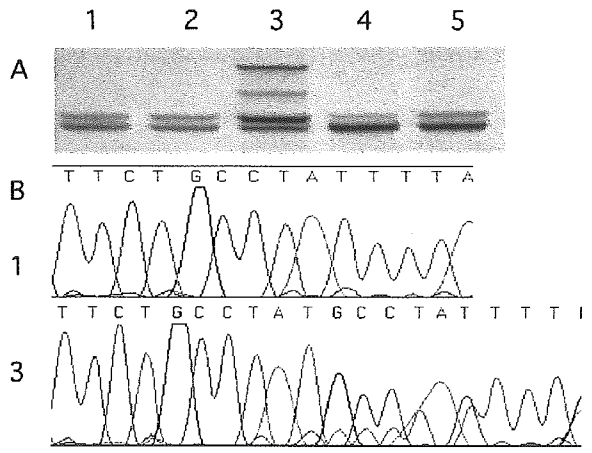

FIGURE 3. c-kit mutation at exon 9. **A:** The result of SSCP. Lane 3 (case no. 21) shows abnormally migrated bands corresponding to a 6-bp insertion. Lane 1, normal tissue (wild type); lane 2, EGIST, case no. 20 (exon 11 mutation-positive); lane 3, EGIST, case no. 21 (exon 9 mutation-positive); lane 4, case no. 1 (kit mutation-negative); lane 5, leiomyosarcoma. **B:** The results of DNA sequencing. The case in the lane 3 has a 6-bp (GCCTAT) insertion at codon 504. The sequence of lane 1 represents wild type of exon 9.

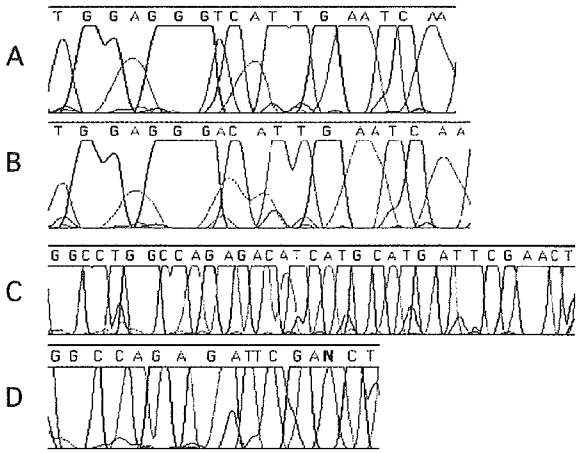

FIGURE 4. PDGFRA mutation. **A:** Wild type of exon 12. **B:** GTC to GAC transition at codon 561 (V561D) in exon 12 (case no. 3). **C:** Wild type of exon 18. **D:** A 12-bp deletion of codon 842 to 845 (Del DIMH) in exon 18 (case no. 19).

Based on the above grading system, 7 cases were classified as high risk, 12 as intermediate risk, and 14 as low risk. The risk-grade was significantly associated with disease-specific survival and disease-free survival ($P = 0.018$ and $P = 0.006$, respectively). None of the other factors, including tumor size, site, cellularity, nuclear pleomorphism, and cell type, correlated with both types of survival.

DISCUSSION

In the current study, we found the frequent c-kit expression and gene mutations in EGIST. Moreover, to the best of our knowledge, this is the first report of PDGFRA mutation in EGIST. Histologically, EGISTs had an identical appearance to GISTs that were located in the gastrointestinal tract proper. In addition, conventional leiomyosarcomas lacked c-kit expression and c-kit gene mutation in our previous and current studies, which are in accordance with the results reported by other laboratories.[13,15,17] Those data suggest that EGIST is the distinctive entity, distinguished from leiomyosarcoma. In the current series of EGISTs, the c-kit mutations were found to have the point mutation and deletion at exon 11 (juxtamembrane domain) in 12 of 29 cases (41.4%), and the identical tandem duplication of Ala and Tyr at codon 504 of exon 9 (extracellular domain) in 2 of 29 cases (6.9%), but not in the kinase domain (exons 13 and 17). The pattern of c-kit mutation found in our EGISTs was similar to that found in GISTs. These mutations have been demonstrated to result in ligand-independent phosphorylation and activation of the c-kit tyrosine kinase in GISTs.[11,12] Therefore, it is suggested that c-kit mutations play an important role in the tumorigenesis of EGISTs. Several large studies in which more than three regions of the c-kit gene were investigated revealed the mutations in approximately 40% to 90% of GISTs, including the mutations in exon 11

TABLE 3. Clinicopathologic Findings of 27 Cases of EGISTs With and Without c-kit Mutation

Variable	c-kit Mutation Positive (n = 12)	c-kit Mutation Negative (n = 15)	*P*
Age (yr)	58.8 ± 16	58.7 ± 12	NS
Gender			
M	6	6	NS
F	6	9	
Site			
Abdomen	4	7	NS
Retro and pelvic	8	8	
Size (cm)	9.8 ± 4.3	17.5 ± 8.3	0.008
Cell type			
Epithelioid	6	7	NS
Spindle	6	8	
Cellularity			
Low, moderate	7	8	NS
High	5	7	
Nuclear pleomorphism			
None	9	11	NS
Present	3	4	
Mitoses			
<5/50 HPF	5	7	NS
≥5/50 HPF	7	8	
MIB-1 labeling index			
<10%	10	10	NS
≥10%	2	5	
Risk grade			
Low	5	6	NS
Intermediate	5	5	
High	2	4	

NS, not significant.

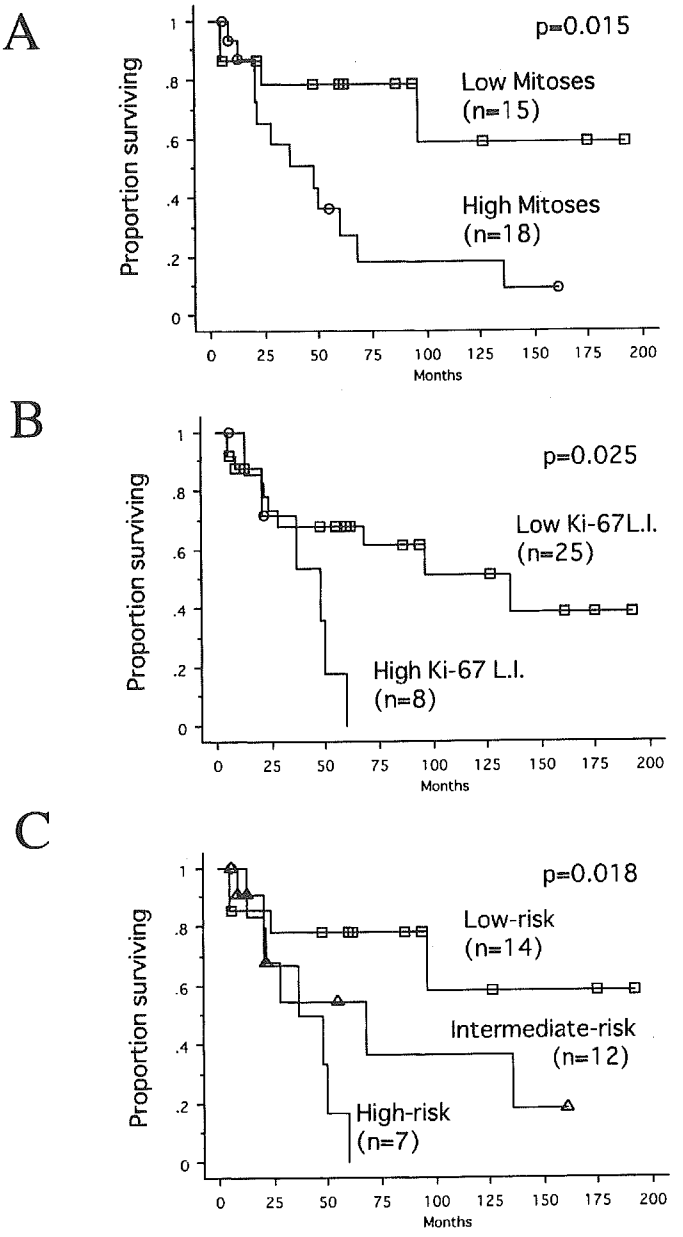

FIGURE 5. Kaplan-Meier analysis for survival. **A:** Tumors with high mitotic counts (≥5/50 HPF) have significantly shorter disease-specific survival than those with low counts (<5/50 HPF) ($P = 0.015$). **B:** Tumors with a high Ki-67 labeling index (≥10% HPF) also have a significantly shorter disease-specific survival than those with a low index (<10%) ($P = 0.025$). **C:** The high-risk group has a significantly worse prognosis ($P = 0.018$).

(31%–71%), exon 9 (3%–13%), exon 13 (0%–4%), and exon 17 (0%–4%).[1,16,26,30,34] The percentage of c-kit mutations, in particular exon 11, varies among the groups, although it is clear that the exon 11 mutation is the most frequent event in the kit gene alternations in GIST. In addition, the frequency (48%) of c-kit mutation in our EGISTs seems to be lower than that in GISTs, particularly compared with those in recent reports.[1,26] One possible explanation is the difference in the type of tissue used for DNA extraction. Two large studies using frozen specimens have shown exon 11 mutation in about 70% of GISTs[1,26]; in contrast, several studies using formalin-fixed paraffin-embedded tissue have reported it in 50% to 60%.[16,34] Another potential factor is the methodologic differences among the studies. Rubin et al[26] examined the whole sequence of c-kit cDNA by RT-PCR and the direct sequencing method, showing kit mutation in 92% of GISTs, which is the highest percentage among the large studies. Corless et al suggested the advantage of a denaturing high-pressure liquid chromatography (D-HPLC) to detect the kit mutation.[3] To prevent to miss the mutation, we performed the electrophoresis for the SSCP analysis under three different temperature conditions (data not shown). However, the anatomic site of tumors might influence

the rate of kit mutation. A majority of gastric epithelioid GISTs have been reported to be the wild type for kit.[1,34] Likewise, EGIST might be a subtype with less frequent kit mutation, although the cell type (epithelioid vs. spindle) was not correlated with the frequency of kit mutation in EGIST.

PDGFRA is similar in structure to other receptor tyrosine kinase, such as c-kit and FLT3. Recent studies have described a gain-of-functional mutation of PDGFRA at the juxtamembrane domain (exon 12) and tyrosine kinase II domain (exon 18).[9,10] Heinrich et al have reported the PDGFRA mutation in 14 of 40 (35%) kit-wild GISTs.[9] Of the 14 cases, 10 had exon 18 mutations, including D842V (n = 8), Del DIMH 842-845 (n = 1), and Del HDSN 845-848P (n = 1), and 4 had exon 12 mutations, including V561D (n = 1) and the other types (n = 3). Hirota et al also reported the PDGFRA mutation in 5 of 8 KIT-wild GISTs, including 3 cases with D842V and 2 cases with V561D.[10] According to their data, D842V at exon 18 seems to be the most frequent mutation in the PDGFRA mutations of GIST. In the current study, two EGISTs had the PDGFRA mutation; one had V561D, a second hot spot in GIST, and the other had Del DIMH842-845, a minor type of mutation in GIST. This result suggests that the PDGFRA mutation may play an important role in the tumorigenesis of the small population of EGISTs.

However, there is still a question about the tumorigenetic mechanism of EGISTs lacking detectable c-kit and PDGFRA gene mutations. Rubin et al reported high-level c-kit phosphorylation and activation in GISTs with or without mutations.[26] In addition, Hirota et al showed that a small population of GISTs lacked both the kit and PDGFRA mutation.[10] It is likely that alternative oncogenic mechanisms other than c-kit and PDGFRA may be present in some populations of EGISTs. However, further details remain to be clarified.

In this study, 6 cases showed weak positivity for c-kit with consistent histologic and clinicopathologic characteristics of EGIST. Immunoreactivity for CD34 was strong in those cases, suggesting that weak immunoreactivity for c-kit is not due to concerns about the formalin fixation. As mentioned above, this subset might have molecular abnormalities other than kit activation. Of the 6 cases, 1 had the PDGFRA exon 12 mutation, which is compatible with the result of the recent immunoblot study showing that PDGFRA-mutant GIST tended to express a lower level of c-kit protein than kit-mutant GIST.[9]

In the current study, the c-kit gene mutations were not correlated with the prognosis of the patients with EGISTs. As for GISTs, several authors have discussed the importance of the c-kit gene mutation in predicting the prognosis. In the earlier literature, some groups suggested that the c-kit gene mutation might be an important prognostic factor.[7,23,31] However, most of the studies published from 1998 to 1999 have investigated only exon 11. However, more recent studies investigating the extracellular and kinase domains, as well as the juxtamembrane domain, reported no significant association between the presence of c-kit mutation and biologic behavior.[3,30] The clinicopathologic importance of the PDGFRA mutation is still unclear. In the current study, 2 cases of EGIST with the PDGFRA mutation had epithelioid morphology, a large tumor size (25 cm and 17 cm), low mitotic activity, and a low Ki-67 labeling index. One case with the exon 12 mutation (case no. 3) was malignant with short survival, whereas the other case with the exon 18 mutation (case no. 19) was free of recurrence for 4 years. Further study with a larger number of GISTs and EGISTs is expected to determine the correlation between the PDGFRA mutation and clinicopathologic factors or biologic behaviors.

In this study, a high mitotic rate (>5/50 HPF) and a high Ki-67 labeling index (>10%) were each significantly associated with an adverse outcome. However, Reith et al have suggested that more than 2 mitoses per 50 HPF may be useful in predicting the biologic behavior in EGISTs.[25] However, the shortness of the follow-up period in the Reith et al[25] series (median, 24 months) may lead a bias of their data. Although GISTs >5 cm in maximum diameter tend to be more aggressive,[5,19,24] this was not the case with EGISTs in our series. Furthermore, most of EGISTs were >5 cm in size. This may be explained by the anatomic site of EGISTs; alternatively, EGISTs appear to have enough space to grow and may only present clinical symptoms over a long term. Therefore, a grading system defined by a combination of mitotic rate and tumor size, which is commonly used in GISTs of the gastrointestinal tract, may not be applicable to EGISTs. Our results suggest that the tumor grade, defined by a combination of the mitotic rate (>5 HPF) and the MIB-1 labeling index (>10%), may be useful in predicting the aggressive biologic behavior in EGIST. Moreover, this grading system might be helpful in predicting the biologic behavior of GISTs of unknown origin (the digestive tract proper vs. soft tissue). However, we still cannot clarify the finding that EGISTs with a kit mutation were smaller in size than those without a mutation.

It is clinically important that EGIST have c-kit expression and c-kit gene mutations because c-kit is an important molecule, not only for the pathologic diagnosis but also as a therapeutic target. STI-571 (Gleevec) is a small molecule that selectively inhibits the tyrosine kinase activity of the abl (bcr-abl), platelet-derived growth factor receptor (PDGFR), and c-kit.[2] Although originally designed to target the bcr-abl of chronic myeloid leukemia, STI-571 can regulate the cell growth and lead to apoptosis of the GIST cell line.[32] Furthermore, a remarkable therapeutic effect on a patient with a metastatic GIST has been reported.[14] Recent phase I and II clinical trials of STI-571 in GISTs have demonstrated relatively good efficiency so far.[4,33] Our results suggest that the application of STI-571 could be a therapeutic strategy for EGISTs because EGISTs have kit abnormality at the molecular level. The effect of STI-571 for PDGFRA-mutant GISTs has not yet been established. However, PDGFRA may become a molecular thera-

peutic target of STI-571 in at least some populations of EGISTs and GISTs. We expect further clinical investigation.

In summary, we have demonstrated that EGISTs had c-kit mutations at the juxtamembrane domain and extracellular domain, as well as the PDGFRA mutation. Furthermore, STI-571 could be an effective therapy for EGISTs, as well as for GISTs, based on the similarity of their clinicopathologic and molecular characteristics.

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PAPER

Ten year recurrence after first ever stroke in a Japanese community: the Hisayama study

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Background: Very few population based cohort studies have focused on the long term recurrence of stroke.
Objective: To examine 10 year cumulative recurrence rates for stroke in a Japanese cohort according to pathological type and clinical subtype of brain infarction.
Methods: During a 32 year follow up of 1621 subjects ≥40 years of age, 410 developed first ever stroke. These were followed up prospectively for 10 years after stroke onset.
Results: During follow up, 108 (26%) experienced recurrent stroke. The cumulative recurrence rates were 35.3% at five years and 51.3% at 10 years. The 10 year recurrence rates of subarachnoid haemorrhage (SAH), brain haemorrhage, and brain infarction were 70.0%, 55.6%, and 49.7%, respectively; the difference between SAH and brain infarction was significant (p=0.004). Most recurrent episodes after SAH or brain haemorrhage happened within a year after the index stroke, whereas recurrence of brain infarction increased consistently throughout the observation period. Cardioembolic stroke had a higher recurrence rate (75.2%) than lacunar infarction (46.8%) (p=0.049). The 10 year risk of stroke recurrence increased with age after lacunar or atherothrombotic brain infarction, but not after the other types or subtypes. After atherothrombotic brain infarction, cardioembolic stroke, or SAH, the type and subtype of most recurrent strokes were the same as for the index stroke, but recurrence after lacunar infarction or brain haemorrhage showed divergent patterns.
Conclusions: Japanese people have higher recurrence rates of stroke than other populations. Recurrence rate after a first brain infarct increases consistently through the next 10 years.

Japanese people have high rates of morbidity and mortality from stroke.[1] Among stroke survivors, recurrence is common, resulting in cumulative disability and cognitive dysfunction.[2] Consequently, precise information is needed on the long term rates and determinants of recurrence after first stroke, so that clinical trials can be designed and health care policies for primary and secondary stroke prevention can be established. Most studies on stroke recurrence, reported mainly from Western countries, have been based on stroke registries[3–11] or on series of patients referred to hospitals.[12 13] A truly representative assessment of stroke recurrence in a community would require a prospective cohort of a defined population and an exhaustive follow up system. The Framingham study is the only cohort based examination of both initial and recurrent stroke, but it refers to the recurrence of thrombotic brain infarction only.[14] Stroke is divided into several pathological types. Among them, brain infarction is further classified into several clinical subtypes.[15–17] Very few studies, however, have accurately defined types and subtypes while also evaluating the long term risk of stroke recurrence.[3]

Since 1961, we have been carrying out a prospective cohort study of cardiovascular disease in the town of Hisayama, Japan.[18 19] The most outstanding features of this study are that the causes of death were verified by necropsy and that most of the stroke patients were examined morphologically at necropsy or, before death, by brain imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI). Our aim in this study was to estimate 10 year cumulative recurrence rates after first ever stroke in the community of Hisayama, using data stratified by sex, age, stroke type, and, in cases of brain infarction, the clinical subtype.

METHODS

Subjects and follow up surveys

In 1961, we carried out a screening examination among Hisayama residents and established a cohort consisting of 1621 stroke-free subjects aged ≥40 years (88.1% of the total population in this age range). These subjects were then followed up for 32 years, from 1 November 1961 to 31 October 1993. A detailed description of the study methods has been published previously.[18 19] In brief, we collected information about new cardiovascular events through a daily monitoring system established by the study team, local practitioners, and the town government. When we suspected a patient was having a new neurological symptom or a new deterioration of an already existing symptom, one of the physicians participating in the study would carefully evaluate the subject and try to obtain information by further diagnostic examinations, including lumbar puncture, cerebral angiography, or recent brain CT or MRI. During the 32 year period, all but two subjects were followed up and 1063 subjects died. Of those who died, 861 (81.0%) underwent necropsy.

The study was conducted with the approval of the human ethics review committee of Kyushu University Graduate School of Medical Sciences.

First ever stroke

Stroke, defined as the sudden onset of a non-convulsive and focal neurological deficit persisting for over 24 hours, was classified into four pathological types: brain infarction, brain haemorrhage, subarachnoid haemorrhage, and undetermined. Brain infarction was further divided into four clinical subtypes: lacunar infarction, atherothrombotic brain

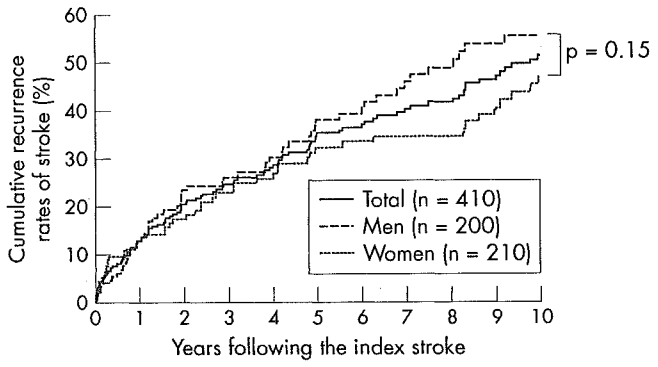

Figure 1 Kaplan–Meier estimates of cumulative recurrence rates of stroke for all subjects and for all subjects divided by sex. Deaths without stroke recurrence were censored.

infarction, cardioembolic stroke, and undetermined. These types and subtypes were defined on the basis of the *Classification of Cerebrovascular Disease III* proposed by the National Institute of Neurological Disorders and Stroke (USA).[15] The subtypes of ischaemic stroke were classified by TOAST (trial of Org 10172 in acute stroke treatment)[16] and by the Cerebral Embolism Task Force.[17] A detailed method of classifying stroke has been published previously.[19] The diagnosis and classification of stroke in our study were based on clinical history, neurological examination, all available clinical information (including brain CT or MRI), and necropsy findings.

During the 32 year follow up, we identified 410 first ever stroke events (200 men and 210 women, mean (SD) age, 73.9 (10.1) years), and divided them into 298 cases of brain infarction, 73 of brain haemorrhage, 35 of subarachnoid haemorrhage, and four undetermined. The cases of brain infarction by subtype consisted of 167 lacunar infarcts, 62 atherothrombotic brain infarcts, 56 cardioembolic strokes, and 13 undetermined.

Recurrent stroke

The definition of recurrent stroke was the same as that of index stroke, but with an additional criterion: there had to be either a new focal neurological deficit or a new deterioration of a previous deficit that was not attributed to brain oedema, haemorrhagic transformation after ischaemia, intercurrent illness, or iatrogenesis. This definition included recurrence in the early stage after the preceding stroke or recurrence in the same vascular territory as the preceding stroke.

We followed up the 410 patients with index stroke from the time of stroke onset until death or 31 August 2003. Under those conditions, all patients completed the follow up period. In the 10 years after the index stroke, 108 patients developed recurrent stroke. Of these, 88 had one recurrent stroke, 13 had two, six had three, and one had four. However, the end point of this study for each subject was the first recurrence.

Morphological evaluation

Brain imaging, including CT or MRI, was carried out in 153 (37%) of the 410 subjects with index stroke and in 43 (40%) of the 108 subjects with recurrent stroke. Necropsy findings were available in 332 (84%) of the 394 deceased stroke patients. As a result, morphological evaluation, including brain imaging or necropsy, was undertaken in 376 (92%) of the index stroke patients and 102 (94%) of the recurrent stroke patients until 31 August 2003.

Because we began collecting data on stroke subjects in 1961, imaging examinations of the brain and heart were non-existent in the early study period. However, we compensated

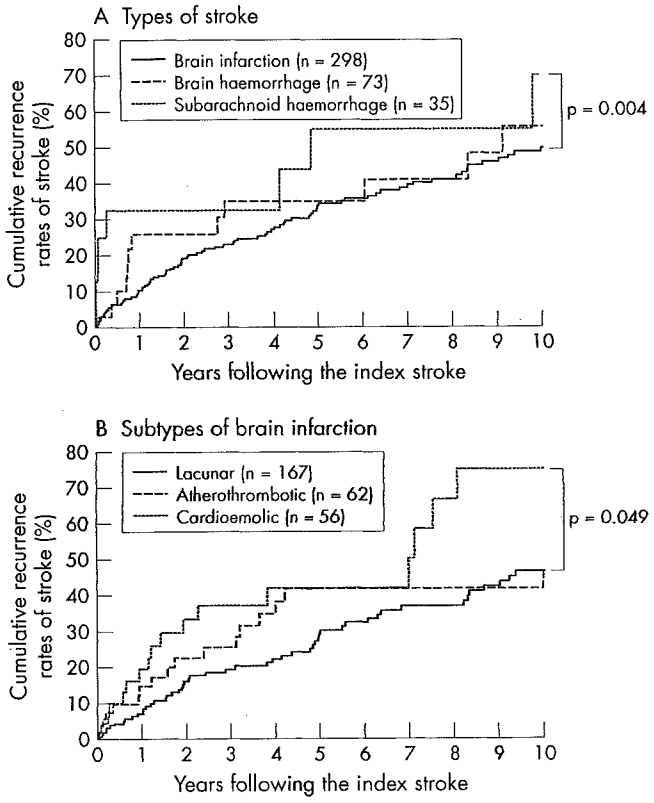

Figure 2 Kaplan–Meier estimates of cumulative recurrence rates of stroke according to stroke type (A) and, in cases of brain infarction, the subtype (B). Deaths without stroke recurrence were censored.

for this disadvantage by carrying out necropsy examinations on the vast majority of deceased patients. We reviewed the brains to evaluate the site, size, and pathological features of the stroke. We also investigated the heart and major vessels in detail—including the aorta, carotids, vertebrobasilar arteries, and the circle of Willis—in order to identify atherothrombotic stenotic lesions and embolic sources. In cases where the necropsy was carried out a long time after stroke onset, it was important to distinguish brain haemorrhage from brain infarction with haemorrhagic transformation. The latter was usually the result of a cardioembolic mechanism. When an infarcted area was surrounded by deposition of haemosiderin—with either no or mild atherosclerosis of the responsible artery, and given the presence of the embolic source—we considered the stroke lesion to be a brain infarct with haemorrhagic transformation. An old lesion that looked like a slit was considered to indicate a brain haemorrhage, especially if found in the basal ganglia or thalamus.

To classify the subtypes of brain infarction, we considered important the size and location of the infarcted area, the presence of stenosis or occlusion of a responsible cerebral artery, and the embolic source, in addition to clinical information including the disease course. Where multiple asymptomatic infarctions were present, we considered an infarct to be the lesion responsible for the stroke when it was most closely in accord with the neurological findings and disease course in the acute period of the stroke. The criteria for diagnosing brain infarction subtypes were given in full detail in our previous report.[19] When sufficient clinical and morphological information was obtained, a diagnosis of subtype was defined as "definite"; on the other hand, when either type of information was insufficient, the diagnostic level was defined as "probable." Among 298 cases of brain infarction, 272 were definite and 26 probable. In this study,

we present the data on the definite and probable cases together, as these combined data were almost identical to the data for definite cases only.

Statistical analysis

SAS software (version 6.12) was used for statistical analysis. The cumulative recurrence rates of stroke and the 95% confidence intervals (CI) were estimated by the Kaplan–Meier product limit method. The Cox proportional hazards model was used to test differences in recurrence rates as well as to estimate relative risks (RR) and 95% CIs of stroke recurrence.

RESULTS

Recurrence rates of stroke

Figure 1 shows the Kaplan–Meier estimates of cumulative recurrence rates of stroke for all subjects and for all subjects divided by sex. The recurrence rates (95% CI) at 1, 5, and 10 years were 12.8% (8.9% to 16.6%), 35.3% (29.0% to 41.5%), and 51.3% (43.8% to 58.9%), respectively, for all subjects. For men, these rates were 12.9% (7.3% to 18.5%), 38.1% (28.9% to 47.2%), and 55.6% (44.9% to 66.4%); for women the rates were 12.5% (7.3% to 17.6%), 32.3% (23.8% to 40.9%), and 47.1% (36.5% to 57.6%). The recurrence rates were slightly higher for men than for women, but the overall difference was not statistically significant ($p = 0.15$).

Figure 2, panel A, shows cumulative recurrence rates of stroke by type of index stroke. The recurrence rates at 1, 5, and 10 years were 10.0% (6.3% to 13.8%), 34.1% (27.3% to 40.9%), and 49.7% (41.4% to 57.9%) after brain infarction; 25.6% (9.0% to 42.2%), 34.9% (16.0% to 53.8%), and 55.6% (32.2% to 79.1%) after brain haemorrhage; and 32.5% (10.3% to 54.6%), 55.0% (25.6% to 84.4%), and 70.0% (39.0% to 100%) after subarachnoid haemorrhage, respectively. The 10 year recurrence rate of subarachnoid haemorrhage was significantly higher than that of brain infarction (RR = 2.89 (95% CI, 1.40 to 5.97); $p = 0.004$). Also, brain haemorrhage recurred at a slightly higher rate than brain infarction, but the difference was not statistically significant ($p = 0.52$). Annual recurrence rates after brain infarction were about 10% per year in the first two years and consistently about 4% per year afterward. On the other hand, 58.3% of recurrent episodes took place within a year after brain haemorrhage, and 66.7% within three months after subarachnoid haemorrhage.

Figure 2, panel B, shows the cumulative recurrence rates of stroke by clinical subtype of brain infarction. The recurrence rates at 1, 5, and 10 years were 7.2% (3.1% to 11.2%), 30.4% (22.1% to 38.7%), and 46.8% (36.6% to 56.9%) after lacunar infarction; 14.8% (4.5% to 25.0%), 42.0% (25.5% to 58.5%), and 46.9% (29.2% to 64.5%) after atherothrombotic brain infarction; and 19.6% (6.3% to 32.8%), 42.2% (23.8% to 60.6%), and 75.2% (52.6% to 97.8%) after cardioembolic stroke, respectively. Cardioembolic stroke had a significantly higher risk of 10 year recurrence than lacunar infarction (RR = 1.76 (95% CI, 1.00 to 3.11); $p = 0.049$). The recurrence rate of atherothrombotic brain infarction was slightly higher than that of lacunar infarction, but the difference was not statistically significant ($p = 0.59$).

Figure 3 Kaplan–Meier estimates of cumulative recurrence rates of stroke for all subjects divided by age. Deaths without stroke recurrence were censored.

Figure 3 shows the cumulative recurrence rates of stroke by age. The 10 year risk of stroke recurrence was lowest in the youngest age group (40 to 59 years) and increased with age. Table 1 shows the relative risks of stroke recurrence among age groups during 10 years for each type and subtype of index stroke. The 10 year risk of stroke recurrence after brain infarction was lowest in the youngest age group and increased with age. For brain haemorrhage or subarachnoid haemorrhage, on the other hand, there was no significant relation between age and recurrence rates. Among the subtypes of brain infarction, the 10 year risk of recurrence after lacunar and atherothrombotic brain infarction was lowest in the youngest age group and increased with age, whereas for cardioembolic stroke there was no significant relation between age and recurrence rates.

Patterns of stroke recurrence

To evaluate patterns of stroke recurrence, table 2 shows the numbers and frequencies of first recurrent stroke by pathological types and clinical subtypes according to the type of index stroke. Most recurrent strokes after atherothrombotic brain infarction, cardioembolic stroke, or subarachnoid haemorrhage were the same type or subtype as the index stroke. On the other hand, recurrence after lacunar infarction or brain haemorrhage showed divergent patterns. The 51 patients who had recurrent stroke after lacunar infarction were divided as follows: 18 cases (35%) had a second lacunar infarction, 16 (31%) had atherothrombotic brain infarction, nine (18%) had brain haemorrhage, and six (12%) had cardioembolic stroke. Among the 12 recurrent cases of brain haemorrhage, seven (58%) had a second brain haemorrhage, three (25%) had lacunar infarction, and two (17%) had atherothrombotic or cardioembolic infarction.

DISCUSSION

One of the strengths of our study is that we investigated almost all stroke events occurring in a community based prospective cohort. Our study design eliminated the selection bias encountered in stroke registries or in series of hospital inpatients. Another strength is that recurrence rates were estimated up to 10 years after a subject's first ever stroke.

Recurrence rates of stroke

Three previous reports from stroke registries in Australia[3] and Britain[4 5] have reported five year cumulative stroke recurrence rates of 16.6% to 29.5%. In comparison, our study's five year cumulative stroke recurrence rate was 35.3%. There might be several reasons for this difference. First, there was a difference in methodology. The studies of the other three stroke registries all used a single set of criteria, which excluded vascular events occurring in the first 21 days after the index stroke unless such an event was clearly in a different vascular territory.[3–5] On the other hand, our study excluded neither early recurrence (10 cases within 21 days) nor recurrence in the same vascular territory. Second, race might greatly influence stroke recurrence. In our study,

Table 1 Relative risks and 95% confidence intervals of stroke recurrence during 10 years by age in each type or subtype of index stroke

Index stroke	Age group (years) 40 to 59 RR	60 to 69 RR (95% CI)	70 to 79 RR (95% CI)	80 and over RR (95% CI)	p Value for trend
All types of stroke	1.0	1.3 (0.5 to 3.0)	1.6 (0.7 to 3.8)	2.2 (0.9 to 5.4)	0.016
Brain infarction	1.0	2.0 (0.6 to 6.5)	2.5 (0.7 to 8.1)	3.9 (1.1 to 13.1)	0.002
Lacunar infarction	1.0	2.2 (0.5 to 9.4)	2.6 (0.6 to 11.1)	4.8 (1.0 to 22.2)	0.022
Atherothrombotic brain infarction	1.0 *		1.8 (0.4 to 7.5)	4.7 (1.2 to 18.6)	0.001
Cardioembolic stroke	1.0	0.8 (0.1 to 7.3)	1.4 (0.2 to 12.3)	0.4 (0.0 to 4.1)	0.51
Brain haemorrhage	1.0	0.6 (0.0 to 6.3)	1.2 (0.2 to 10.3)	2.1 (0.2 to 24.3)	0.71
Subarachnoid haemorrhage	1.0	1.0 (0.2 to 6.0)	0.7 (0.1 to 4.4)	0.0	0.60

*Two age groups (40 to 59 and 60 to 69) were combined, as there were no recurrences after atherothrombotic brain infarction in the 40 to 59 age group.
CI, confidence interval; RR, relative risk.

haemorrhagic stroke—including brain haemorrhage and subarachnoid haemorrhage—recurred at higher rates than brain infarction, and the proportion of haemorrhagic stroke (26%) among all types was higher than those found in the three registries in Western countries (14% to 19%).[3–5] In addition, as Asians, including Japanese, have a higher stroke incidence than Europids,[1] they might also have higher rates of stroke recurrence.

In our study, most recurrent episodes occurred within a year after the index haemorrhagic stroke. This may indicate the importance of controlling risk factors and of treating the patient to prevent recurrence without delay in the first days and months after the onset of haemorrhagic stroke. On the other hand, cumulative recurrence rates after brain infarction, especially lacunar infarction, increased steadily during our 10 year study period. The Oxfordshire Community Stroke Project[6] also showed that the recurrence rate after lacunar infarction was low and almost constant throughout the follow up period. Arteriosclerosis, which is thought to progress consistently for a long period, may be related to recurrent thrombotic infarction. Thus careful observation and adequate treatment to prevent recurrence are needed for a long time after brain infarction.

Several studies have focused on the relations between brain infarction subtypes and the risks of recurrent stroke,[3 7–10 12] but their findings are equivocal. Some of those studies have claimed that the subtype of brain infarction is not a predictor of long term recurrence,[3 7 8] while others showed that the highest risk of recurrence is with atherothrombotic brain infarction.[9 10 12] In our study, cardioembolic stroke had the highest risk of recurrence among the three major subtypes of brain infarction. This is probably attributable to our inclusion of early recurrent episodes, which were often observed after cardioembolic stroke.[20 21]

In some studies,[3 11] aging was found to be a predictor of stroke recurrence. In the present study, the risk of recurrence after first ever lacunar or atherothrombotic brain infarction was lowest in the youngest age group and then increased with age. Aging would accelerate atherosclerotic changes in major cerebral arteries and arteriolosclerotic changes in penetrating arteries, thus increasing the risk of recurrent stroke.

Patterns of stroke recurrence

In the present study, the types or subtypes of most recurrent strokes after atherothrombotic brain infarction, cardioembolic stroke, or subarachnoid haemorrhage were the same as those of the index stroke. On the other hand, recurrence after lacunar infarction or brain haemorrhage showed divergent patterns. This finding was also emphasised in some previous reports.[4 13]

Several aetiological mechanisms for lacunar infarction have been proposed[22–24]: lipohyalinosis or microatheroma in a penetrating artery; branch-atheromatous disease, which is located in basilar or middle cerebral arteries and occludes the origins of one or more penetrating arteries; and microembolism from carotid or cardiac disease. These multifactorial aetiologies would support divergence in the type and subtype of recurrent stroke after lacunar infarction. Our findings denote the importance of evaluation to detect any large vessel disease or embolic source, even in patients with lacunar infarction.

Table 2 The numbers and frequencies of first recurrent stroke by pathological types and clinical subtypes according to type of index stroke

Type or subtype of index stroke	Type or subtype of recurrent stroke: All BI	Subtype of BI: LA	AT	CE	UND-BI	BH	SAH	UND	Total
Brain infarction	74 (85%)					10 (11%)	–	3 (3%)	87 (100%)
Lacunar infarction		18 (35%)	16 (31%)	6 (12%)	–	9 (18%)	–	2 (4%)	51 (100%)
Atherothrombotic brain infarction		1 (6%)	14 (82%)	–	1 (6%)	1 (6%)	–	–	17 (100%)
Cardioembolic stroke		–	–	16 (94%)	1 (6%)	–	–	–	17 (100%)
Undetermined subtype of BI (UND-BI)		–	–	–	1 (50%)	–	–	1 (50%)	2 (100%)
Brain haemorrhage	5	3 (25%)	1 (8%)	1 (8%)	–	7 (58%)	–	–	12 (100%)
Subarachnoid haemorrhage	2	1 (11%)	1 (11%)	–	–	1 (11%)	6 (67%)	–	9 (100%)
Undetermined type of stroke	–	–	–	–	–	–	–	–	0 (0%)

Percentages are the proportions of types or subtypes of recurrent stroke calculated using the numbers of total recurrent stroke as the denominators.
AT, atherothrombotic brain infarction; BH, brain haemorrhage; BI, brain infarction; CE, cardioembolic stroke; LA, lacunar infarction; SAH, subarachnoid haemorrhage; UND, undetermined.

Hypertension is a major risk factor for both lacunar infarction and brain haemorrhage, and lesions of all lacunar infarcts and most brain haemorrhages in our patients were located in brain areas that have the common feature of penetrating arteries, such as the basal ganglia, thalamus, and pons. These similarities would support the overlap between lacunar infarction and brain haemorrhage in recurrent stroke types.

Study limitations

There are several potential limitations to the findings in our study. First, we enrolled stroke cases that developed among an inception cohort during 32 years of follow up. The prevalence of cardiovascular risk factors and the risk of stroke recurrence may have changed widely during this long term observation period.[25] Secular trends in stroke recurrence should be examined, and we will do so in another study. Second, the study did not consider the effects of cardiovascular risk factors or those of medical or surgical treatment. Thus our estimates for the risk of stroke recurrence are probably quite conservative. Third, brain imaging was available in only 37% of the index stroke cases. However, we collected available clinical information on both index and recurrent strokes in minute detail and carried out necropsies on 84% of deceased stroke patients. We believe that our exhaustive and careful evaluation of the clinical information, as well as the high rate of necropsy, improved the quality and validity of the diagnosis as well as the stroke classification in our study.

Conclusions

Our findings show higher recurrence rates of stroke in a Japanese community than in Western populations. The divergent patterns of stroke recurrence after index lacunar infarction or brain haemorrhage are of interest and importance for the prevention of recurrent stroke, because the Japanese are characterised by high morbidity of lacunar infarction and brain haemorrhage. The consistent increase in cumulative recurrence rates during the long observation period and the higher recurrence rates after index brain infarction among older patients are both important for medical care. We believe that these findings will contribute to a better understanding of stroke recurrence in the Japanese, who are considered to be at greater risk of stroke than other populations.

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Competing interests: none declared

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