evaluation. Patients exhibiting high-level 24-h urinary oxalate excretion or elevated plasma oxalate concentrations (except those with secondary hyperoxaluria) receive a preliminary diagnosis of PH. Next, urinary L-glycerate and glycolate levels are measured, as GRHPR activity in blood monocytes (2). Mutation analysis follows. Lack of GRHPR activity in monocytes or liver biopsy samples, associated with elevated urinary oxalate and L-glycerate levels, allows a definitive diagnosis of PH2. Such diagnosis may be verified by genetic analysis of *GRHPR*. However, liver biopsy is of course invasive and unattractive to patients.

Currently, the National Center for Biotechnology Information for single-nucleotide polymorphism (SNP) lists at least 37 missense mutations in *GRHPR* and it has been reported that 15 mutations in *GRHPR* cause loss of enzyme expression or function (3–10). Previous studies suggested that allelic frequency varies among different PH2 populations. The most common mutation is c.103delG in exon 2, which creates a frameshift inducing premature termination at codon 451. This mutation constitutes about 40% (3, 6) of all known mutations causing PH2, and appears to have originated in a founder of Northern European or American origin (7).

In Japan, the prevalence of both primary hyperoxaluria type 1 (PH1) and PH2 is poorly known, although we reviewed literature data on 59 Japanese PH1 patients described between 1962 and 2003 (11) and reported on a single PH2 patient (9). Recently, primary hyperoxaluria type 3 (PH3; OMIM 613616) has been identified in some unclassified PH patients; this condition is characterized by mutations in the gene (*HOGA1*) encoding 4-hydroxy-2-oxoglutarate aldolase; the gene was formerly termed *DHDPSL* (12). The frequencies of mutations causing PH2 and PH3 have not been described in Asian patients. In this report, we describe the genetic features of four Japanese patients with PH2 and the ethnic distribution of *GRHPR* variants.

Materials and methods

Patient profiles are shown in Table 1. Four patients were diagnosed with PH2 using gas chromatography/mass spectrometry-based urine metabolome analysis (13). No liver biopsies were performed. Genomic DNAs from the four patients; from both parents of Patients 1, 2, 4, and 5; and from 14 patients with recurrent calcium oxalate urolithiasis but without hyperoxaluria were extracted from peripheral blood samples using QIAamp kits (Qiagen, Hamburg, Germany) according to the manufacturer's instructions. Informed consent was obtained from all patients and their parents in line with the requirements of the Institutional Review Board of Hamamatsu University School of Medicine. Polymerase chain reaction (PCR) was used to explore all splicing acceptor and donor sites using a modification (14) of a previously described method (4, 9). DNAs were sequenced on ABI Prism 377 or ABI 310 DNA platforms and the sequences were compared with the known human *GRHPR* sequence (GenBank accession no. NM_012203) using NCBI BLAST alignment.

Table 1. Clinical features of Japanese patients with primary hyperoxaluria type 2

Patient/ Gender	Age at symptom development	Symptom	Renal parenthymal calcifications	Age at diagnosis	Location of stones at diagnosis	Urinary excretion levels		Present age	Renal function	Clinical course
						Oxalate	Glycerate			
1/Male	10 months	UTI	–	7 years 2 months	Bilateral kidney	1.9, 1.8 SD	386, 473 mmol/mol Cr	7 years	WNL	SWL, 9 times
2/Male	3 years 10 months	Gross hematuria	–	4 years 8 months	Bladder	1.7, 2.2 SD	612, 587 mmol/mol Cr	25 years	WNL	Lithotripsy, twice SWL, once
3[a]/Male	7 months	Gross hematuria	–	10 months	Bilateral kidney and ureter	227±120 (mg/day/1.73 m^2)	3,032 ± 1,276 (mg/day/1.73 m^2)	20 years	WNL	SWL, 5 times
4/Male	3 years	Gross hematuria	–	21 years	Bilateral kidney	0.2 mg/mg Cr	504 mmol/mol Cr	21 years	WNL	–
5/Female	2 years	Gross hematuria	+	8 years	Bilateral kidney	1.69 mg/mg Cr	1,081 mmol/mol Cr	8 years	WNL	–

SD, standard deviation; SWL, shock wave lithotripsy; UTI, urinary tract infection; WNL, within normal limits.
[a]The details of Patient 3 have been reported in Ref. (8). Urinary excretion levels of oxalate and glycerate by Patients 1 and 2 were measured in the Division of Human Genetics, Medical Research Institute, Kanazawa Medical University, using the technique of Ref. (5). Two and three SDs of the measured glycerate levels was 7.8 and 16.6 mmol/mol Cr, respectively. Urinary excretion levels of oxalate and L-glycerate by Patients 4 and 5 (siblings) were measured in the Research Institute of Medical Mass Spectrometry, Kurume University School of Medicine, using a modification of the technique of Ref. (5). Mean ± 3SD of L-glycerate levels was 168.5 mmol/mol Cr.

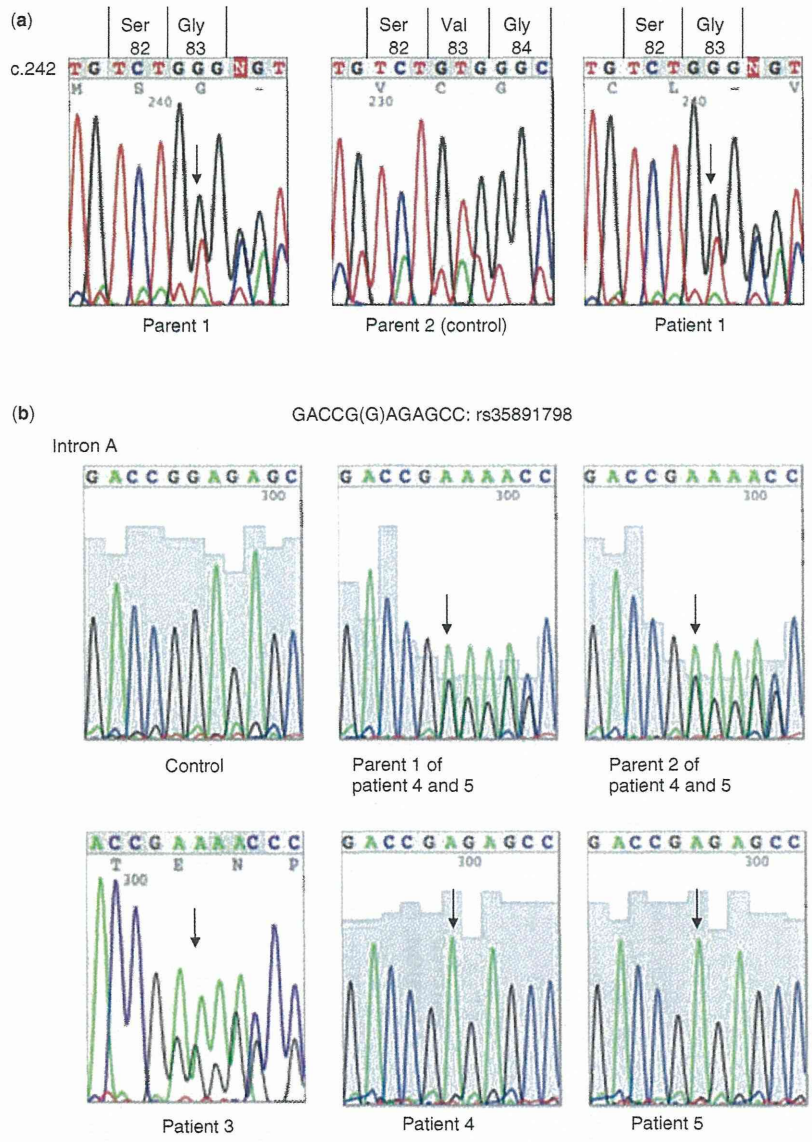

Fig. 1. Nucleotide sequences of mutated exons and introns. Isolated sections of electropherograms are shown. The deduced nucleotide sequence and *GRHPR* codons are shown above each panel. Arrows: affected nucleotide(s). All electropherograms show the forward sequence. (**a**) Electropherogram of exon 3 of Patient 1. (**b**) Electropherograms of intron A of Patients 3, 4, and 5, and the parents of Patients 4 and 5.

All published data on PH2 were reviewed. We incorporated information gained from personal communications. We also included our new *GRHPR* sequence data. The two criteria used to explore ethnic associations between *AGT* mutations and PH1 (15) were employed to explore whether particular *GRHPR* mutations might exhibit such associations. The criteria were, first, that a relevant mutation had to be largely confined to one population group or geographic area; and, second, that the mutation was associated with PH2 in at least two unrelated individuals.

To obtain the background prevalence of these genotypes in unrelated Japanese individuals, we designed Taqman^R probes to identify these genotypes in totally 237 individuals from the previous cohort in the rural city in the same prefecture (n = 125) (16) and unrelated outpatients (n = 112) without history of urolithiasis in our department.

Briefly, these DNAs were extracted from the blood samples given by the participants using a QIAamp DNA Blood Maxi kit according to the manufacturers' instructions (Qiagen). A 50 ng sample of each subjects DNA was amplified by PCR, with the primer set for using custom Taqman^R probe identifying *GRHPR* Ex8 (c.864_865del GT) and *GRHPR* intron 1 (c.83+51delG; rs35891798) by using the StepOne (Applied Biosystems, Carlsbad, CA). The Assay IDs above (Assay Names) are AH89ZKB (PH2_0001) and AHABEP4, respectively.

Table 2. The GRHPR gene mutations[a]

Exon/Intron	Nucleotide	cDNA change	Amino acid change	Allelic frequency: Cramer et al. (3) and Webster et al. (7, 8)	Cregeen et al. (4)	Lam et al. (6)	Johnson et al. (5)	Levin-laina et al. (10)	Our cases (9)	Total	%
Intron A	c.84-2A>G	Splicing error	n/a		3					3	3.3
	c.84-13_c.84-12del; c.84-8_c.84-5del	Splicing error	n/a		1					1	1.1
Exon 2	c.103delG	1 bp deletion	Asp35Thr; frameshift, aa 44X	18[b]	14		2			34	37.8
Exon 3	c.248_249delTG[c]	2 bp deletion	Val83Gly; frameshift, aa 91X						1	1	1.1
Exon 4	c.295C>T	Nonsense transition	Arg99X	4	2					6	6.7
	c.337G>A	Missense transition	Glu113Lys						1	1	1.1
	c.375delG	1 bp deletion	Leu126Cys; frameshift, aa 133X		1					1	1.1
Exon 4/Intron D	c.403_405+2 delAAGT	4 bp deletion and splicing error	n/a	2	7					9	10.0
Exon 6	c.494G>A	Missense transition	Gly165Asp	3[d]	5		6			14	15.6
	c.540delT	1 bp deletion	Leu181Cys; frameshift, aa 204X		1					1	1.1
Exon 7	c.608_609delCT	2 bp deletion	Pro204Leu; frameshift, aa 210X		3					3	3.3
Intron G	c.246-2A>G	Missense transition and splicing error	n/a	1						1	1.1
Exon 8	c.864_865delTG	2 bp deletion	Val289Asp; frameshift, aa 310X			2			7	9	10.0
Exon 9	c.904C>T	Missense transition and splicing error	Arg302Cys		1				1	2	2.2
	c.934A>G	Missense transition	Asn312Asp					2		2	2.2
	c.965T>G	Missense transversion	Met322Arg	2						2	2.2
				30	38	2	8	2	10	90	100.0

a/a, amino acid; n/a, not analyzed; X, stop codon.

[a]Sixteen mutations in all 90 alleles including two PH2 patients whom we newly performed genotyping and four those with personal communication by Dr Scott Cramer.

[b]Three homozygous patients are provided by personal communication with Dr Scott Cramer.

[c]This is a novel mutations we detected in this report.

[d]One homozygous patient is provided by personal communication with Dr Scott Cramer.

Table 3. The relationship between the mutation and ethnic origin in 45 patients with primary hyperoxaluria type 2

No. of patients	Ethnic origin	Geographic area	Allelic mutations		Authors
1	Japanese	East Asia	c.248_249delTG[a]	c.904C>T	
2	Japanese	East Asia	c.864_865delTG	c.864_865delTG	
3[b]	Japanese	East Asia	c.337G>A	c.864_865delTG	Our cases (9)
4	Japanese	East Asia	c.864_865delTG	c.864_865delTG	
5	Japanese	East Asia	c.864_865delTG	c.864_865delTG	
6	Caucasian American	America	c.103delG	c.103delG	
7	Caucasian American	America	c.103delG	c.103delG	Cramer et al. (3)
8	Caucasian American	America	c.103delG	c.103delG	
9	Caucasian American	America	c.103delG	c.103delG	
10	Caucasian Italian	Europe	c.295C>T	c.295C>T	
11	Caucasian Italian	Europe	c.295C>T	c.295C>T	
12	Caucasian Italian	Europe	c.403_404+2 delAAGT	c.403_404+2 delAAGT	
13	Indian subcontinent	Mid East	c.494G>A	c.246-2A>G	Webster et al. (7, 8)
14	Caucasian American	America	c.103delG	c.103delG	
15	African American	America	c.965T>G	c.965T>G	
16	Caucasian German	Europe	c.103delG	c.103delG	
17	Caucasian American	America	c.103delG	c.103delG	
18	Caucasian American	America	c.103delG	c.103delG	Dr Scott Cramer,
19	Caucasian American	America	c.103delG	c.103delG	Personal communication
20	Indian subcontinent (Arabic)	Mid East	c.494G>A	c.494G>A	
21	Caucasian	Europe	c.84-2A>G	c.84-2A>G	
22	Caucasian	Europe	c.103delG	c.84-2A>G	
23	Caucasian	Europe	c.103delG	c.103delG	
24	Caucasian	Europe	c.103delG	c.103delG	
25	Caucasian	Europe	c.103delG	c.103delG	
26	Caucasian	Europe	c.103delG	c.103delG	
27	Caucasian	Europe	c.103delG	c.103delG	
28	Caucasian	Europe	c.103delG	c.103delG	
29	Caucasian	Europe	c.103delG	c.904C>T	Cregeen et al. (4) and Dr
30	Caucasian	Europe	c.295C>T	c.295C>T	Gill Rumsby, Personal
31	Caucasian	Europe	c.375delG	c.608-609_delCT	communication
32	Caucasian	Europe	c.608-609_delCT	c.608-609_delCT	
33	Indian subcontinent	Mid East	c.494G>A	c.494G>A	
34	Indian subcontinent	Mid East	c.494G>A	c.494G>A	
35	Indian subcontinent	Mid East	c.403_404+2 delAAGT	c.403_404+2 delAAGT	
36	Indian subcontinent	Mid East	c.403_404+2 delAAGT	c.403_404+2 delAAGT	
37	Indian subcontinent	Mid East	c.403_404+2 delAAGT	c.403_404+2 delAAGT	
38	Indian subcontinent	Mid East	c.403_404+2 delAAGT	c.540delT	
39	Indian subcontinent	Mid East	c.494G>A	c.84-13_c.84-5delinsCTTT	
40	Chinese	East Asia	c.864_865delTG	c.864_865delTG	Lam et al. (6)
41	Indian subcontinent (Afghanistan)	Mid East	c.494G>A	c.494G>A	
42	Indian subcontinent (Afghanistan)	Mid East	c.494G>A	c.494G>A	Johnson et al. (5)
43	Indian subcontinent (Afghanistan)	Mid East	c.494G>A	c.494G>A	
44	Caucasian England	Europe	c.103delG	c.103delG	
45	Yemenite	Mid East	c.934A>G	c.934A>G	Levin-laina et al. (10)

[a]Novel mutations.
[b]This patient was reported in Ref. (8).

Written informed consents were obtained from all the participants and the study design was approved by Institutional Review Board of Hamamatsu University School of Medicine.

Results

Table 1 shows the clinical features of Japanese patients with PH2. We identified a novel *GRHPR* mutation associated with this disease. In Patient 1, a compound heterozygote for the *GRHPR* gene, we found a two-nucleotide deletion (c.248_249delTG) in exon 3 which resulted in premature termination at codon 91 (Fig. 1a). We also found a known missense transition, c.904C>T (Arg302Cys) in exon 9, which causes GRHPR activity to be only 5.6% that of the wild-type control (5). Patients 2, 4, and 5 were homozygotic for the c.864_865delTG mutation. Moreover, a SNP in intron A (rs35891798) was identified in Patient 3 (a heterozygote) (8); in the parents (heterozygotes) of Patients 2, 4 and 5; and in Patients 2, 4, and 5 (homozygotes) (Fig. 1b and Figure S1b,c, Supporting Information). The c.864_865delTG mutation was associated with the rs35891798 SNP. However, in Patient 1 and the parents of that patient (Figure S1a), and in 14 patients with recurrent urolithiasis in the absence of hyperoxaluria, c.864_865delTG was not associated with the rs35891798 SNP (data not shown).

Among the unrelated Japanese, there were no mutant allele of *GRHPR* c.864_865delTG (Figure S2a)(0/474 alleles; a DNA from one subject did not generate the clear genotype result). Nine heterozygous and one homozygous subjects were identified as a polymorphism in the intron 1, rs35891798.

Table 2 lists known *GRHPR* mutations and Table 3 shows relationship between PH2-causing mutations and patient ethnic origin; information derived from personal communications with Drs Scott Cramer and Gill Rumsby is included. The allelic frequencies of the c.103delG, c.494G>A, c.403_404+2delAAGT, and c.864_865delTG mutations were 37.8%, 15.6%, 10.0% and 10.0%, respectively. All patients with the c.103delG or c.295C>T mutations were Caucasian (European or American); all those with the c.494G>A mutation and 78% (7/9 alleles) of those with the c.403_404+2 delAAGT mutation were from the Indian subcontinent; and all patients with the c.864_865delTG mutation were Chinese or Japanese.

Sixteen *GRHPR* mutations have now been described, one of which is newly reported here. The mutations occur all along the *GRHPR* gene, with the exception of exon 1, and 44% are single-nucleotide substitutions (seven missense and one nonsense); eight small deletions have also been described.

Discussion

We explored *GRHPR* mutations in four patients diagnosed with PH2 with the aid of gas chromatography/mass spectrometry-based urine metabolome analysis (13); we did not perform liver biopsies. We found a novel mutation, c.248_249delTG, in exon 3, and also showed that the c.864_865delTG mutation in exon 8 was linked to the rs35891798 SNP of intron A. Moreover, we determined that the c.864_865delTG mutation was of Chinese or Japanese origin.

In terms of the diagnosis of primary hyperoxaluria, Rumsby et al. (17) indicated that genetic screening afforded definitive diagnosis in 34% of PH1 patients and 33% of those with PH2. In that report, however, the only candidate mutation for PH2 was c.103delG. In this study, we found a relationship between *GRHPR* mutations and ethnic distribution. The c.103delG mutation is found only in Caucasians, but c.494G>A and 78% (7/9) of c.403_404+2delAAGT mutations occur in patients of the Indian subcontinent (one such patient was an Italian Caucasian). The c.864_865delTG mutation occurs only in Chinese and Japanese. The allelic frequency of the c.864_865delTG mutation in three Japanese and one Chinese PH2 patient was 75% (9/12). Thus, our data will be of use when genetic screening for PH2 is planned, and may obviate the need for invasive biopsy.

The association between the c.864_865delTG mutation in exon 8 and the rs35891798 SNP in intron A is particularly noteworthy. As the c. 864_865delTG mutation is located toward the end of exon 8, the mutation may be associated with translational difficulty. If the observed linkage is indeed robust, diagnosis of PH2 would be facilitated. The linkage appeared to be absent in patient 1 (without the c. 864_865delTG mutation) and in 14 patients with recurrent urolithiasis but without hyperoxaluria. It is necessary to explore the frequency of the rs35891798 SNP in large number of healthy subjects with detailed information on urolithiasis and life styles including known and unknown risk factors. Our preliminary small scale survey on 230 subjects revealed 11 alleles of 460 alleles were mutants. Although detailed urological information is not available for the homozygous subject on rs35891798, the questionnaire he filled in at the interview did not include urolithial problem as the past history. The absence of c.864_865delTG mutant alleles in this unrelated Japanese, apparently not symptomatic on urolithiasis strengthens our interpretation that this functional mutation is responsible for PH2 in Japanese.

The number of PH2 patients is small compared to those with PH1. To date, only 16 mutations in *GRHPR* have been described, one of which is newly reported here. Although the *AGXT* mutations are predominantly (75%) single-nucleotide substitutions (18), a predisposition to minor deletions in *GRHPR* is evident.

Conclusions

We analyzed the *GRHPR* genes of four PH2 patients and identified a novel mutation. The c.103delG mutation (in Caucasians), the c.494G>A mutation (in those from the Indian subcontinent), and the c.864_865delTG mutation (particularly in patients of East Asian origin) should be screened when PH2 is suspected.

Supporting Information

The following Supporting information is available for this article:

Figure S1. Nucleotide sequences of intron A and exon 8. Isolated sections of electropherograms are shown. Deduced nucleotide sequences and *GRHPR* codons are shown above each panel. All electropherograms show forward sequence. (a) Control, patient 1, and parents of patient 1; (b) Patient 2 and parents of patient 2; (c) Patients 4 and 5 and their parents. Arrows, affected nucleotide(s); underlining, the first nucleotides of intron H.
Figure S2. Allele discrimination plot of c.864_865delTG (2a) and rs35891798 (2b) is shown. Blue dots indicate homozygous wild type of c.864_865delTG (2a). Green and red dots indicate heterozygous and mutant homozygous case, respectively (2b).

Additional Supporting information may be found in the online version of this article.

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FIELD OF VISION

Internal frontier: The pathophysiology of the small intestine

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Abstract

Even though the small intestine occupies a major portion of the abdominal space and is essential for life, in most pathology textbooks any chapter on small intestinal diseases, especially in human beings, is typically shorter than those for other gastrointestinal organs. Clinical and experimental investigations of the small intestine in various clinical situations, such as nutrition management, obesity interventions, and emergency care, have elucidated several important biological problems associated with the small intestine, the last frontier of gastroenterology. In this issue, a review by Professor Basson and his team at Michigan State University sheds light on the changes in the human small intestine under various conditions based on their clinical and surgical experience. With the advent of recent innovations in enteroscopy, a form of endoscopy used to examine deep within the small intestine, the issue that they highlighted, i.e., mucosal adaptation and atrophy of the human small intestine, has emerged as a major and manageable challenge for gastroenterologists in general, including the readers of the *World Journal of Gastroenterology*.

Key words: Ileum; Jejunum; Small intestine; Atrophy; Adaptation; Enteroscopy

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INVITED COMMENTARY ON HOT ARTICLES

Juan Rosai's Surgical Pathology (9th edition) contains 45 pages on the small intestine, compared with 80 pages on the large intestine[1]. The actual length of the small intestine is much longer than that of the large intestine, and the vital importance of the small intestine is well known. Why then are fewer pages devoted to diseases of the small intestine? Obviously, the access of surgical pathologists and gastroenterologists to the small intestine is limited, compared with access to the large intestine and appendix. Actually, the old version of Morson and Dawson's textbook Gastrointestinal Pathology[2] is fairer: the same number of pages is devoted to the small intestine and to the large intestine, probably because this book is a product of the century during which autopsies made major contributions to our knowledge base. This issue of the *World Journal of Gastroenterology* has a wide-ranging review article on the pathophysiology of the small intestine by Professor Basson and his colleagues[3]. These authors are surgeons, and a tremendous amount of data based on their own clinical experiences and those of others, as well as on animal experiments, are comprehensively discussed in their review. This review article addresses conditions such as starvation and parenteral nutrition in patients with severe trauma or pancreatitis and in patients with postoperative short-gut syndrome for various reasons including bariatric surgery for obesity. Although these

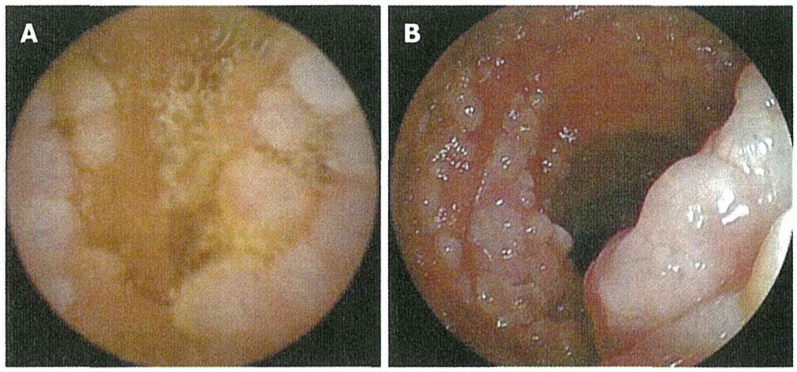

Figure 1 Endoscopy. Images obtained using capsule endoscopy (A) and double-balloon enteroscopy (B) in a 61-year-old man with follicular lymphoma who visited us because of gastrointestinal bleeding from an unknown cause and a hemoglobin concentration of 9.7 g/dL.

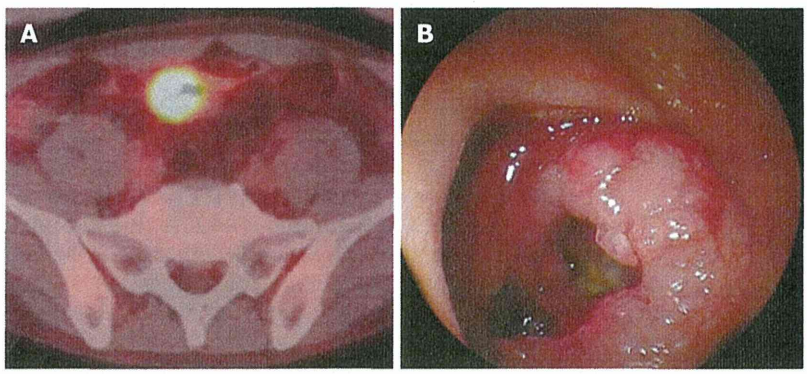

Figure 2 A mass positive by positron emission computed tomography actually was a tumor arising the mucosa of the small intestine of a 48-year-old woman who had suffered Crohn disease. A: Positron emission computed tomography/computed tomography showed an accumulation in site of wall thickening of ileum; B: Image obtained by double-balloon enteroscopy.

conditions are undoubtedly serious, recognition and explicit statements by medical professionals regarding the importance of the small intestine, and of its diseases as a "great burden of human distress", have only recently appeared in medical literature[4]. Most clinical conditions that highlight the importance of the small intestine are familiar only to experts in a particular area of medicine. These conditions are not typically a concern of non-surgical gastroenterologists, such as pathologists who are accustomed to diagnosing neoplasms of the large intestine, grading inflammatory bowel disease, and validating ischemic necrosis of the resected small intestine in cases with an acute abdomen. The small intestine is most often investigated because of its involvement in a disease originating from another organ, such as the mesothelium[5]. Changes in the mucosa of the small intestine itself are an exceptional category among the slides that pile up next to the microscope.

The situation is now changing. As avid readers of *World Journal of Gastroenterology* may have noticed[6-9], recent progress in enteroscopy has enabled areas deep within the small intestine to be reached, providing clinicians active in the wider branches of gastroenterology with the opportunity to evaluate changes in the small intestine, which have previously only been observed by surgeons. This also means that many non-surgeons must commit to the management of the small intestine, based on findings and evaluations associated with specimens of small intestine obtained using these newly developed modalities. For ordinary pathologists, for example, the concepts mainly discussed in this review, i.e., adaptation and atrophy of the small intestine, may be new and unfamiliar. These lesions were encountered only after specific "congenital or acquired disease or medical and surgical intervention" had occurred in the patients. When specimens obtained by enteroscopy become routine in the future, however, the concepts and knowledge of adaptation and atrophy of the small intestine will become an essential "must" for all practitioners including general pathologists.

One of the important conditions that the authors of this review addressed is the change in the small intestine in subjects receiving total parental nutrition. This lifesaving modality has many variations in terms of its nutritional regimen and the effects of these variations on pathophysiology, that is, on the grade of adaptation of the small intestine. These effects on the small intestinal mucosa and the consequent outcomes of individual patients have been thoroughly investigated and published in many scientific articles, but the scientific evidence in human beings remains insufficient according to the

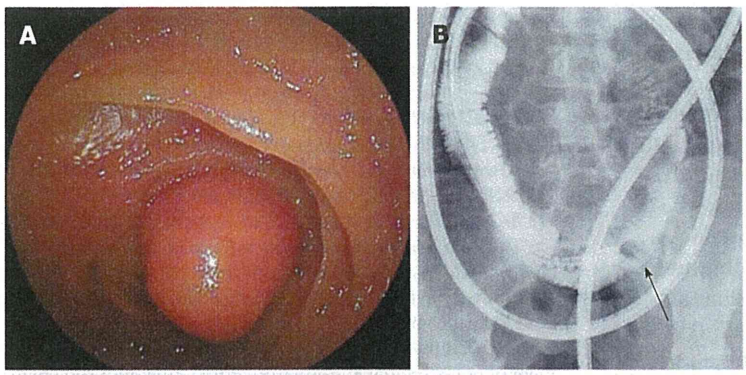

Figure 3 Identification of lipoma clarified the reason of intermittent abdominal discomfort in a 29-year-old female. Endoscopic finding (A) and selective small bowel series (B) obtained using double-balloon enteroscopy. This required surgical intervention. An arrow in the B indicates a defect by a lipoma.

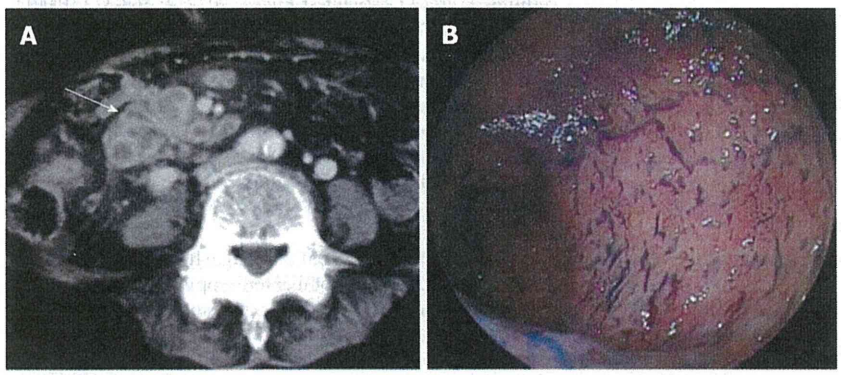

Figure 4 Intestinal tuberculosis is also revealed by double-ballon enteroscopy. A: Computed tomography showed wall thickening of the ileum with contrast enhancement (arrow); B: Double-balloon enteroscopy showed destruction of the small intestinal villi.

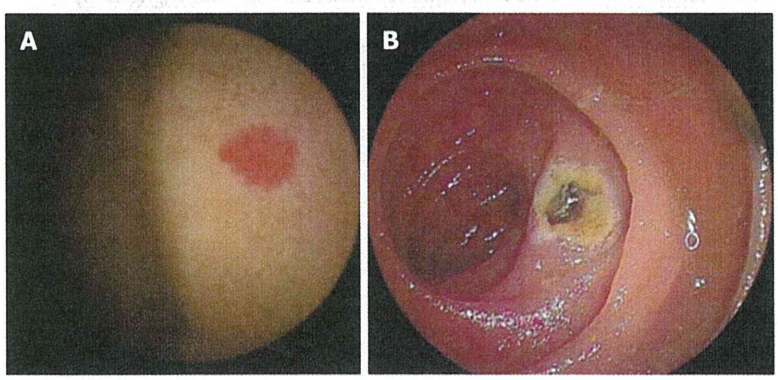

Figure 5 Angioectasia. A: Diagnosis of angioectasia was made by capsule endoscopy; B: Argon plasma coagulation successfully treated the lesion.

above-mentioned authors. The concept of mucosal adaptation[10] includes proliferation, functional augmentation, and cellular differentiation. Biochemical changes include alterations in molecules related to apoptosis, proliferation, signal transduction, and fatty acid metabolism. These meticulous frameworks in intestinal cells and tissues have so far been revealed by studies using *in vivo* and *in vitro* manipulative systems. From now on, in the era of enteroscopy, lingering questions such as "what is the reality and examples of mucosal adaptation in human clinical settings?" and "how can we validate the accumulated experimental findings in human beings and exploit them in clinical practice?" will be answered.

Small intestinal enteroscopy is now available in ordinary hospitals, thus facilitating the detection of previously unobserved pathological conditions. Capsular endoscopy, the latest wireless version of enteroscopy, has become a popular practical procedure since the publication of a seminal report on this modality a decade ago[11]. In the minds of laymen, this technique seems like a dream[12]. The use of capsular endoscopy and refined enteroscopy using a double-balloon method[13] in clinical practice have

revealed thousands of new anecdotal findings (Figure 1), and the rapid accumulation of this kind of basic knowledge of the small intestine will help to set up principles of surveillance for mucosal adaptation and atrophy of the small intestine (especially morphological changes) in various clinical conditions. For example, introduction of capsular endoscopy and a double-balloon method disclosed previously unrecognized lesions such as adenocarcinoma arising from Crohn disease in the small intestine (Figure 2)[14], submucosal lipoma (Figure 3), tuberculosis at the terminal ileum (Figure 4), and angioectasia (Figure 5). None of these lesions were accessible until the recent development of capsule endoscopy and the double balloon method. The morphology is new to pathologists and endoscopists, and these developments will critically influence the managements of the patients. The concepts that Professor Basson and colleagues have illuminated in their review will soon become an important guidepost for evaluating the histopathology of the small intestine in daily practice and for patient care by a broader range of gastroenterologists.

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EDITORIAL

Editorial: an obsession with subtyping gastric cancer

Haruhiko Sugimura

Published online: 13 March 2013

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Categorization was part of human nature even before the work of Carl von Linné, but no categorizations can be more complicated than pathological categorizations, especially those based on the microscopic morphology of cancer cells. Why do we categorize tumors? Lung cancer and gastric cancer are different, so their therapy, care, and prevention measures should also differ. Why must we differentiate between adenocarcinoma and squamous cell carcinoma among cancers arising within the same organ (such as the lung)? Again, therapy (probably), care (possibly), and prevention measures (definitely: for example, avoiding smoking to prevent squamous cell carcinoma of the lung) should be modified according to the tumor category. Then, should we also discriminate among the subtypes of adenocarcinoma of the gastrointestinal tract, such as well-differentiated versus poorly differentiated? Yes, we know of biological differences between these two subtypes, and a principle of general pathology teaches us that the latter is generally associated with a poorer prognosis. However, we are not sure whether this difference is notable when comparing subtypes at different stages (for example, early-stage poorly differentiated cancer versus advanced-stage well-differentiated cancer). Heterogeneity within a single tumor and heterogeneity among many similar-looking tumors had been known before the era of massive parallel sequencing revolutionized molecular concepts regarding human cancer.

One of my good friends, a confident pulmonary surgeon, once said to me, "No histological description is needed in a breast cancer pathology report; just 3 or 4 immunohistological scores including HER2, hormone receptors, and maybe a proliferation indicator, such as the Ki-67 labeling index." In the era of companion diagnostics, categorization based on morphology, which is often subjective, may only further complicate clinical cancer management and create an unnecessary burden on pathologists. Actually, every few years the name, subtype categories, and requirements for describing questionable attributes change for unknown reasons and without any emerging evidence but simply because they have been labeled as being a "general rule." On the other hand, as we approach the brink of personal medicine, the ultimate tumor subtyping—in which the whole genome sequencing of tumor DNA up to the single-cell level will be performed—is appearing on the horizon. The cost of such analyses is becoming less and less and is much less than that of hiring technicians who can make beautifully stained sections. In contrast to morphological classifications, the DNA sequence data can directly pinpoint target molecules that clinicians can then use as a starting point for individualized therapy. Complaints of late

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or insufficient pathology reports will disappear, along with the jobs of pathologists. How, then, should the current morphological subtyping be viewed from a molecular perspective?

The first edition of the international histological classification of tumors of the upper gastrointestinal tract, published in 1977, was led and edited by a Japanese pathologist, Dr. Kunio Oota, and pathologists in 13 countries [1]. Dr. Isamu Kino, who was one of the consulting pathologists of that issue, collected and viewed the glass slides of the gastric tumors and then discussed the diagnosis or subtyping with the other participants. The subtypes described for adenocarcinoma continue to be used today [2]: (a) papillary, (b) tubular, (c) mucinous, and (d) signet-ring cell carcinoma. However, the TNM classification prefers a simpler grading: well, moderately, and poorly differentiated [3]. The simplicity of a classification is very important for clinical practice, and the general view of tumors differs according to the stages that are typically encountered by pathologists, which in turn is influenced by the health insurance system, the surveillance system, and the proficiency of diagnostic and screening clinicians in each country. Thus, histological subtypes that can withstand the austerity of medical cost measures must have both a biological and a therapeutic relevance.

In this issue, Tomio Arai, one of the greatest pupils of the late Prof. Kino, has described biological characteristics that are closely related to the morphological subtype. Arai describes a high prevalence of microsatellite instability (MSI) in the papillary subtype of well-differentiated adenocarcinoma and in the solid subtype of poorly differentiated adenocarcinoma of the stomach [4]. Dr. Arai and his colleagues took advantage of a particular series of surgical cases at a single institute, which mainly treats the elderly, and they clearly characterized MSI-positive tumors, which tended to occur in older patients and exhibited a female preponderance and particular histological features. As previously reported, MSI in early-stage adenocarcinoma with papillary features (papillary subtype of well-differentiated adenocarcinoma) is often caused by MLH1 promoter methylation [5], and the concept of field cancerization arising from epigenetic changes has now been extended to and established for other cancers and genes [6]. Arai further showed that a solid subtype of poorly differentiated adenocarcinoma that usually appears as a component in a progressive stage also gains MSI during tumor growth. This finding in solid-subtype tumors implies that the solid subtype, which is usually assigned to a diffuse-type category, should be reassigned as an intestinal type based on the features of its molecular lineage.

Their clear demonstration and meticulous pathological analysis have brought about an end to arguments regarding MSI and histological features. Earlier reports often concluded that MSI was more prevalent in poorly differentiated, diffuse-type tumors than in intestinal-type tumors [7]. This dichotomy of gastric cancer classification, intestinal versus diffuse type, has often been used by the international community, and the morphological features of the "subtypes" of both categories are often missed. Furthermore, in advanced gastric cancer, the tissues often have a heterogeneous morphology (Prof. Kino often referred to this as a "varied structure"), and they are often MSI positive, which might reflect clonal heterogeneity because the extra bands on the gel are interpreted as evidence of CA repeat slippages [8]. The detailed description of the tissue, including an exact measurement of MSI, is often inadequate, as many laboratory investigators have experienced.

Another issue that Arai addressed is the relationship between MSI and the patient prognosis. Prejudice based on early reports of MSI-positive colon cancer might lead us to expect that MSI-positive tumors are usually associated with a biologically better prognosis [9]. The findings of a better prognosis in MSI-positive cancer cases are probably related to the fact that these MSI-positive cancer cells harbored a near-diploid pattern of DNA. Instead of chromosomal numerical abnormality (CNA), which is related to the extraordinary destruction of genetic material and is strongly correlated with an abysmal prognosis [10], authentic MSI-positive tumors often exhibit a less drastic copy number change in chromosomes [11]. The stage-adjusted analysis performed by Arai et al. did not support a better prognosis for MSI-positive cases in their study. MSI-positive, papillary (sub)type gastric cancer exhibited fewer CNA; thus, CNA information for MSI-positive tumors would be interesting.

The data Arai and his colleagues have provided here will help pathologists to understand the relevance of the subtyping of well-differentiated gastric adenocarcinoma, and this kind of basic support for attributes that many young clinical doctors wrongly believe to be scientifically sound remains scarce in the "general rules" of clinical practice, as most of the required attributes are mainly for research purposes. We should continue to make an effort to validate the significance or insignificance of these attributes so as to edit out the unnecessary ones. Even scientifically sound data, such as those reported by Arai et al., do not necessarily need to be included in conventional pathology reports. Only when these markers are recognized as determinants for the selection of therapeutic measures by several follow-up studies should such obsession with classification become a rule.

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Original Article

D2-40-positive lymphatic vessel invasion is not a poor prognostic factor in stage I lung adenocarcinoma

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The present study investigates whether lymphatic vessel invasion (LVI) detected by D2-40 staining is a prognostic factor for stage I adenocarcinoma of the lung. We retrospectively reviewed 124 patients who underwent complete resection for stage I adenocarcinoma of the lung from January 1983 to June 2003. LVI was microscopically evaluated using D2-40 immunostaining. The median follow-up was 71 months. The LVI positive rate was 37%. The 5-year cancer-specific survival rates of the D2-40 positive LVI and negative groups were 88.8% and 84.3%, respectively ($P = 0.630$). The stage I lung adenocarcinoma patients who were determined to be LVI positive based on D2-40 immunostaining did not have a significantly poorer prognosis than the LVI negative cases. Thus, lymphatic microinvasion may not be a prognostic indicator in early lung cancer, although advanced LVI does appear to correlate with survival. It is therefore unnecessary to use D2-40 immunostaining to diagnose LVI in practical settings, and Hematoxylin-Eosin and Elastica van Gieson staining should continue to be used to predict the prognosis of patients with stage I lung adenocarcinoma.

Key words: D2-40 immunostaining, Elastica van Gieson, Hematoxylin-Eosin, lung adenocarcinoma, lymphatic vessel invasion, prognostic factor

Lung cancer is the most common cancer and a major cause of cancer-related death worldwide.[1] The standard treatment of stage I non-small cell lung cancer (NSCLC) is resection. However, even with complete clinical resection, the postoperative survival rate remains unsatisfactory.[2] Various prognostic factors for lung cancer have been reported;[3] lymph node metastasis is known to be a poor prognostic factor for patients with NSCLC.[4] However, for patients with stage I disease who do not have lymph node metastasis, the prognostic factors are still unclear. Blood vessel invasion (BVI) is considered to be one of the most useful prognostic factors for advanced NSCLC.[5] However, there are only limited data regarding the relationship between lymphatic vessel invasion (LVI) and the prognosis of stage I NSCLC patients.

Recently, immunostaining with the D2-40 antibody has been reported to be more sensitive for the detection of lymphatic invasion compared with Hematoxylin-Eosin (HE) staining in several types of malignancies, such as uterine cervical, colorectal, breast, and gastric carcinomas.[6–9] However, there have been only a few reports so far demonstrating a positive correlation between the LVI detected by D2-40-positive vessels and the prognosis.[10,11] We suggest that the reason that LVI using D2-40 immunostaining has not yet been shown to be a prognostic factor in adenocarcinoma in past reports may be associated with the hypersensitivity in regard to the detection of LVI when D2-40 immunostaining was adopted, namely, D2-40 immunostaining can detect more minute microinvasion. Therefore, we assume that lymphatic microinvasion only detected by D2-40 may not be a prognostic indicator for early lung cancer, and moreover LVI, which is easily detectable by HE and Elastica van Gieson (EVG), has been shown to correlate with the patients survival.

We previously reported lymphatic vessel invasion detected by HE and EVG staining to be a significantly poor prognostic factor for stage IA lung adenocarcinoma.[12] However, the rate of LVI judging using HE and EVG in our previous reported was low, at 62%.[12] In the current study, we re-evaluated these patients with stage I adenocarcinoma of the lung by performing D2-40 immunostaining to the resected blocks in order to detect microinvasive lesions more accurately and to test the hypothesis that D2-40 positive LVI is an independent prognostic factor for stage I lung adenocarcinoma.

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PATIENTS AND METHODS

We retrospectively reviewed the records of 124 consecutive patients, admitted from January 1983 to June 2003 with stage I lung adenocarcinoma, who had undergone radical resection at the First Department of Surgery, Hamamatsu University School of Medicine. Patients who received induction chemotherapy or radiotherapy or adjuvant chemotherapy and patients with evidence of a residual tumor at the surgical margin were excluded from this study. All the resected lung cancer cases were assigned to histopathological categories according to the World Health Organization criteria.[13] For pathological staging, we used the seventh edition of the TNM classification for lung and pleural tumors.[4] Resected tumors were examined by HE and EVG stain and were sub-typed according to the 2011 International Association for the Study of Lung Cancer, the American Thoracic Society, and the European Respiratory Society (IASLC/ATS/ERS) classification system.[14]

Histological evaluation

We newly made adjacent sections from paraffin-embedded blocks, which had been stored at room temperature for more than 8 years in the Diagnostic Pathology Division in Hamamatsu University Hospital at Hamamatsu University School of Medicine. All the sections, cut 3 μm thick, were stained with D2-40, a mouse monoclonal antibody (DAKO, Carpinteria, CA, USA). Immunohistochemical studies of the sections of paraffin-embedded tissues (3 μm) were performed using a Histofine Simple Stain MAX-PO (Nichirei, Tokyo, Japan) for D2-40. Antigen retrieval was performed by heating the sections after immersion in Tris-EDTA buffer (pH 9.0) in a pressure cooker for 30 min.[15] In addition, the sections were incubated with the monoclonal antibody against D2-40 at a concentration of 1:50 (DAKO). LVI was considered to be positive when cancer calls were present, surrounded by lymphangioendothelium with D2-40 expression (Fig. 1). All histological sections were reviewed by two physicians (H.S. and K.S.) and evaluated as being either positive or negative LVI by D2-40.

Statistical analysis

The cancer-specific survival was estimated using the Kaplan–Meier method, and any differences in survival were determined by a log-rank analysis. The length of survival was defined as the interval in days between the day of pulmonary resection and date of either death or the last follow-up. An observation was censored at the last follow-up when the patients were alive or had died from a cause other than cancer. All *P*-values were two-sided, and *P*-values ≤0.05 were considered to be statistically significant. All statistical analyses were performed with the PASW statistical software package version 18 (SPSS, Inc., Chicago, IL, USA).

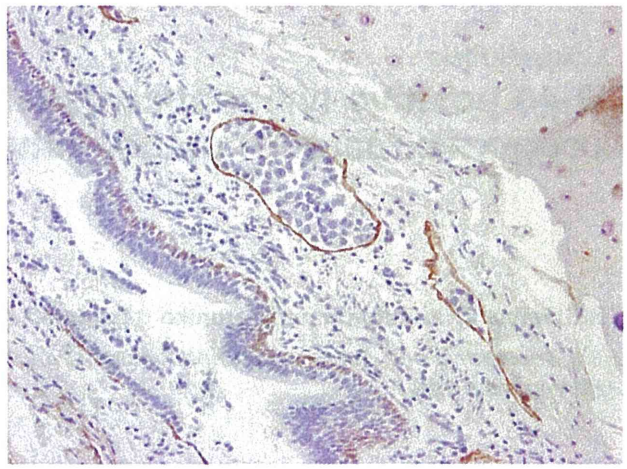

Figure 1 A representative photograph of immunohistochemical staining with D2-40 to evaluate lymphatic invasion (×20).

RESULTS

In this study, we reviewed a total of 124 patients who underwent resection of stage I adenocarcinoma of the lung. The patient characteristics are shown in Table 1. This study included 64 males and 60 females, who had a median age of 64 years (range, 38–85 years). In total 66 cases were over 65 years old, 61 cases were pathological stage T1a, 38 cases were pathological T1b, and 25 cases were pathological T2a.

The median follow-up was 71 months. A total of 46 cases (37%) were detected to have LVI with D2-40 immunostaining, and a total of 34 cases (27%) were detected to have LVI with HE and EVG. Table 2 compares the characteristics of patients with and without LVI.

The 5-year survival rate of all patients was 87.1% (Fig. 2). The 5-year survival rates of those over 65 years old and younger than 65 were 85.8% and 88.4%, respectively ($P = 0.791$). The 5-year survival rates of males and females were 80.4% and 93.7%, respectively ($P = 0.225$). The 5-year survival rates of patients with stage T1a, T1b and T2a disease were 91.7%, 81.6% and 83.7%, respectively ($P = 0.080$). The 5-year survival rates of the D2-40 positive LVI and negative LVI were 88.8% and 84.3%, respectively ($P = 0.630$) (Fig. 3). On the other hand, the 5-year survival rates of the LVI-positive group and the LVI-negative group based on HE and EVG staining was 77.5% and 90.5%, respectively ($P = 0.006$) (Fig. 4). About histological sub-typing of adenocarcinoma,

Table 1 Clinicopathological data of 124 patients with stage I lung adenocarcinoma

Characteristic	Number (n = 124)
Median age (years) (range)	64 (38–85)
<65	58
≥65	66
Gender	
Female	60
Male	64
Adenocarcinoma sub-type	
Minimally invasive adenocarcinoma	3
Lepidic	29
Acinar	9
Papillary	61
Solid predominant	22
Pathological T factor	
T1a	61
T1b	38
T2a	25
Lymphatic vessel invasion (D2-40)	
Positive	46 (37%)
Negative	78 (63%)
Lymphatic vessel invasion (HE/EVG)	
Positive	34 (27%)
Negative	90 (73%)

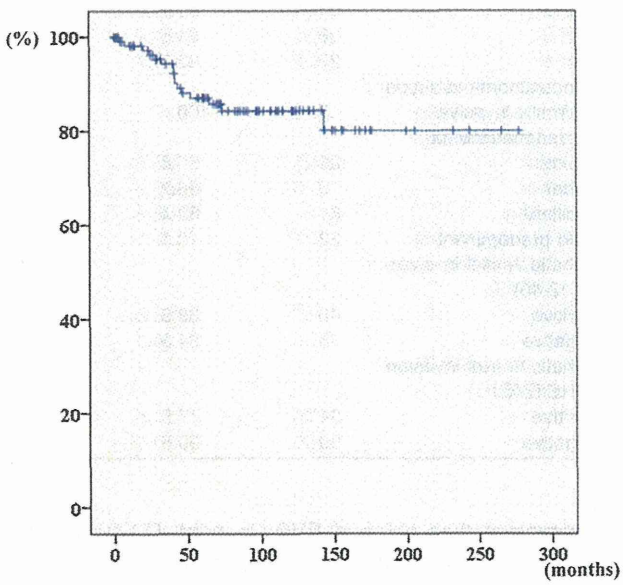

Figure 2 Overall survival rate of the 124 patients.

three (3%) were minimally invasive adenocarcinoma (MIA) and 121 (97%) were invasive adenocarcinoma, in which 29 (23%) were lepidic (LEP), 9 (7%) were acinar (ACN), 61 (49%) were papillary (PAP), 22 (18%) were solid predominant subtype (SOL). The 5-year cancer-specific survival of patients with MIA were 100%. Those of LEP, ACN, PAP and SOL were 95.8%, 66.7%, 89.4% and 78.1%, respectively (P = 0.160).

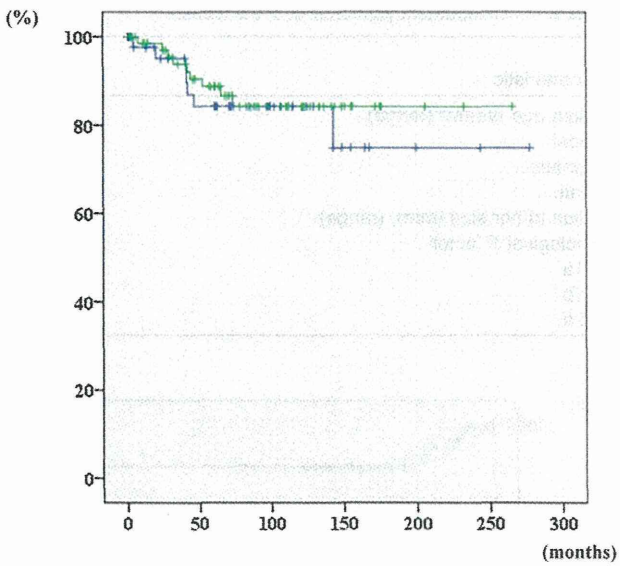

Figure 3 The5-year survival rates of the D2-40 positive LVI and negative LVI was 88.8% and 84.3%, respectively (P = 0.630). ⌐, negative; ⌐, positive.

A univariate analysis determined that LVI detected by HE and EVG (positive vs negative: 77.5% vs 90.5%; P = 0.006) was a significant prognostic factor (Table 3).

DISCUSSION

Recently, the number of patients with lung cancer has been increasing in Japan. The 5-year overall survival rates of stage IA and IB patients are 73% and 58%, respectively.[4] The treatment outcome is not satisfactory. It is necessary to identify subjects who are likely to have a poor prognosis among stage I NSCLC patients to recommend that they undergo adjuvant treatment after surgery.

For stage II through IV NSCLC patients, lymph node metastasis is one of the most important prognostic factors. Because stage I NSCLC patients do not have lymph node metastasis, it is necessary to identify other factors. One of the potential prognostic factors is LVI.[16] For detecting LVI, D2-40 immunostaining was reported to be more useful than HE staining.[17] However, there is controversy regarding the impact of D2-40 staining in NSCLC. Some have argued that D2-40-positive LVI is a poor prognostic factor in squamous cell carcinoma of the lung,[18] while others have argued the opposite.[19] In the positive study, the most common histological type was squamous cell carcinoma,[18] and there have been few reports showing a correlation between the LVI determined by D2-40 and prognosis in patients with adenocarcinoma of the lung.[20,21] We previously reported that the LVI detected by HE and EVG was a significant prognostic

Table 2 Clinicopathological data of the patients

Characteristic	Diagnosis of lymphatic vessel invasion	
	Positive (n = 46)	Negative (n = 78)
Median age (years) (range)	61 (36–83)	65 (33–82)
Gender		
Female	25	36
Male	21	42
Median tumor size (mm), (range)	23 (4–50)	23 (8–50)
Pathological T factor		
T1a	26	34
T1b	12	26
T2a	8	17

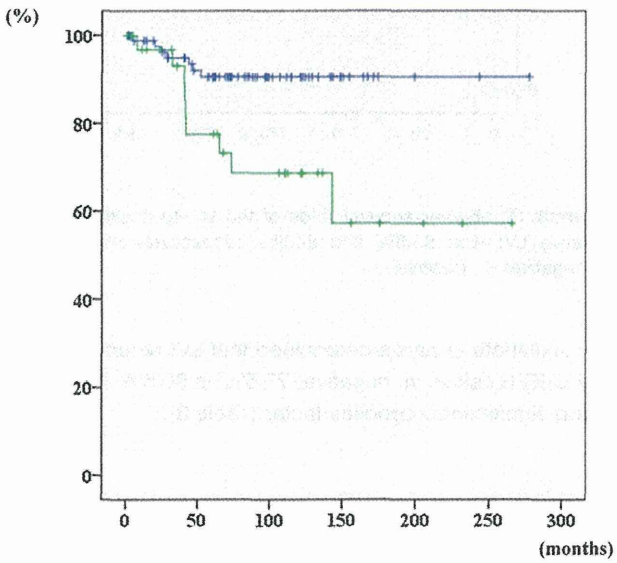

Figure 4 The5-year survival rates of LVI-positive group and the LVI-negative group based on HE and EVG staining was 77.5% and 90.5%, respectively (P = 0.006). ⌐, negative; ⌐, positive.

Table 3 The 5-year overall survival rate based on the clinical characteristics

	Number	The 5-year cancer-specific survival rate (%)	P-value
Age			0.791
<65	58	88.4	
≥65	66	85.8	
Gender			0.225
Female	60	93.7	
Male	64	80.4	
Pathological T factor			0.080
T1a	61	91.7	
T1b	38	81.6	
T2a	25	83.7	
Adenocarcinoma sub-type			0.160
Minimally invasive adenocarcinoma	3	100	
Lepidic	29	95.8	
Acinar	9	66.7	
Papillary	61	89.4	
Solid predominant	22	78.1	
Lymphatic vessel invasion (D2-40)			0.630
Positive	46	88.8	
Negative	78	84.3	
Lymphatic vessel invasion (HE/EVG)			0.006
Positive	34	77.5	
Negative	90	90.5	

factor.[12] Namely, the 5-year overall survival rate of the LVI-negative group and the LVI-positive group was 94.5% and 70.9%, respectively (P = 0.003).[12] This previous report focused on the relationship between LVI and the prognosis of stage IA adenocarcinoma based on HE and EVG staining, avoiding the use of the controversial D2-40 immunostaining. In the current study, a similar result was obtained. Namely, LVI detected by HE and EVG (positive vs negative: 77.5% vs 90.5%; P = 0.006) was a significant prognostic factor.

In the current study, we examined the correlation between D2-40 positive LVI and the prognosis of stage I lung adenocarcinoma patients who comprised patients that belonged to the same cohort as described in our previous report.[12] The LVI positive rate using D2-40 immunostaining was 37%, and the LVI positive rate using HE and EVG was 27%. Thus indicating that the LVI positive rate was higher in the D2-40 immunostaining group than the HE/EVG staining group. This shows that D2-40 immunostaining can detect more lymphatic microinvasion than HE and EVG. In brief, D2-40 immunostaining is more sensitive for detecting LVI than HE and EVG. However, lymphatic microinvasion may not be a prognostic indicator in early lung cancer, although advanced LVI does appear to correlate with survival. In fact, Hashizume *et al.*[21] reported that there was no significant difference in cancer-specific survival between patients with D2-40-positive LVI and negative groups, while there was a significant difference between patients with low grade invasion group (ly0,1) and those with high grade invasion group (ly2,3) using D2-40 immunostaining. This means that it is not important to distinguish between ly0 and ly1 when looking for a prognostic factor, that is, D2-40 immunostaining can detect more lymphatic microinvasion which may not be a prognostic indicator