Table 2. Clinico-Pathological and Epidemiological Characteristics of Patients by *BRAF*^{V600E} Mutation Status

	All patients†			Exposed patients		
BRAF^{V600E} mutation status	Present ($n=38$)	Absent ($n=26$)	*P*-value	Present ($n=26$)	Absent ($n=21$)	*P*-value
Median radiation dose (mGy, range)	18.5 (0–2,758)	156.9 (0–2,304)	0.022*	104.9 (0.4–2,758)	333.4 (0.7–2,304)	0.025*
Median age at the time of atomic-bombing (yr, range)	24 (1–52)	22.5 (3–49)	0.8*	29.5 (1–47)	24 (3–49)	0.9*
Median age at diagnosis (yr, range)	54 (20–89)	51 (29–70)	0.2*	54 (20–89)	51 (29–70)	0.2*
Latency period (yr, range)	—	—		29 (15–46)	21 (11–36)	0.014*
Histology						
Conventional PTC (n)	38	23	0.062‡	26	18	0.082‡
Follicular variant (n)	0	3		0	3	
Gender						
Male (n)	3	3	0.7‡	2	3	0.6‡
Female (n)	35	23		24	18	

†Patients consist of 17 nonexposed and 47 exposed patients.
*Wilcoxon rank-sum test.
‡Fisher's exact test.

Table 3. Logistic Regression Analysis of *BRAF*^{V600E} Mutation Status*

	β[a]	*P*-value
Radiation dose ($\log_{10}$ transformed)	−0.979	0.039
Latency period	0.124	0.010
Age at the time of atomic-bombing	0.031	0.4
Gender	−0.685	0.7
Histology	−23.948	1.0

*Analysis was performed only for the 47 exposed patients.
[a]Regression coefficients in the logistic regression model.

post-Chenobyl children and from patients with a history of radiation therapy [31–33]. In addition, *AKAP9-BRAF* rearrangement was recently found in PTCs in post-Chernobyl children [22]. Moreover, in vitro and in vivo experiments have revealed that external X-ray irradiation can induce rearrangement of *RET/PTC1* and *RET/PTC3* in tumor cell lines and human normal thyroid tissue transplanted in scid mice [37–39]. These findings suggest that chromosomal rearrangements may be important in the development of radiation-associated papillary thyroid cancer. Thus, we hypothesize that radiation exposure may influence the selection of an early genetic event; a genetic event other than *BRAF*^{V600E} mutation in the MAP kinase signaling pathway including chromosomal rearrangements may be involved in the development of PTCs among A-bomb survivors, specifically those exposed to high radiation dose.

We found a significant association of *BRAF*^{V600E} mutation with latency period (years from A-bombing to diagnosis) among exposed patients (Table 2). Notably, latency period was positively associated with *BRAF*^{V600E} mutation in logistic regression analysis including age at the time of A-bombing or age at diagnosis as a covariable (Table 3). The low frequency of *BRAF*^{V600E} mutation in PTCs with short latency period also suggests that a molecular event other than *BRAF*^{V600E}mutation in the MAP kinase signaling pathway may play a major role in the development of PTC among A-bomb survivors.

This study has several limitations: PTC specimens resected before 1956 are unavailable, rendering it impossible to assess PTC developed within 10 yr after the A-bombing. Other molecular events, specifically *RET/PTC* rearrangement and *RAS* mutations, need to be analyzed with an increased number of study subjects. Our findings in this study therefore argue for the need to do further studies to clarify the mechanisms of radiation-associated PTC.

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ORIGINAL PAPER

Mutation profile of *EGFR* gene detected by denaturing high-performance liquid chromatography in Japanese lung cancer patients

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Abstract
Purpose *EGFR* mutations in lung cancer increase sensitivity to an EGFR tyrosine kinase inhibitor, gefitinib. Mutation analysis of *EGFR* is essential for prediction of gefitinib response and avoidance of the coincidental severe side effects for the unresponsive population. The purpose of the present study is to apply DHPLC as a screening system of detection of *EGFR* mutations for large scaled population.
Methods *EGFR* mutations were detected by both DHPLC procedure and direct sequencing using lung cancer tissue samples obtained from 97 patients (81 surgical specimens and 16 pleural effusions of non-resectable lung cancer patients).
Results DHPLC analysis detected *EGFR* mutations in 5 h as opposed to 18 h by direct sequencing for ten samples, and it costs eightfold more expensive by direct sequencing than DHPLC. In addition, DHPLC analysis was sixfold more sensitive than sequencing analysis for detection of the point mutation of exon 21, L858R. Using this system, *EGFR* mutations in exons 18, 19 and 21 were found in 34 of 97 patients (36%). Thirteen of the 15 patients with exon 21 mutations (87%) were female non-smokers, who were diagnosed with adenocarcinomas with the feature of BAC. Eight of the 18 patients with exon 19 mutations (44%) were 7 male and 1 female current or former smokers, and BAC feature was observed in 61% (8/18).
Conclusion DHPLC analysis for screening followed by sequencing analysis appears to be more sensitive and accurate, as well as easier and faster. In addition, these results suggest different mutagenesis and carcinogenesis pathways for mutations.

Keywords Lung cancer · EGFR mutation · DHPLC · BAC

Abbreviations

AAH	Atypical adenomatous hyperplasia
BAC	Bronchioloalveolar carcinoma
CT	Computed tomography
DHPLC	Denaturing high-performance liquid chromatography
EGFR	Epidermal growth factor receptor
TEAA	Triethylammonium acetate

Introduction

Lung cancer is the most common cause of cancer death among males in Japan, accounting for 23.1% of all male cancer deaths, and it ranks third among females (12.6%) ("Cancer Statistics in Japan" Editorial Board 2005). Despite significant progress in the treatment of lung

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cancer, its overall prognosis is poor: a 5-year survival rate of 14%, and especially poor prognosis for non-resectable advanced cases (Mountain and Dresler 1997). Among new therapeutic strategies, a quinazoline-derived EGFR tyrosine kinase inhibitor, gefitinib, showed profound response in a subgroup of non-small cell lung cancer, most of whom were female non-smokers with adenocarcinomas (Lynch et al. 2004; Kim and Choy 2004).

EGFR, a member of the ErbB family for receptor tyrosine kinases, is a 170 kDa transmembrane glycoprotein which contains an extracellular ligand-binding region, a hydrophobic transmembrane domain, a tyrosine kinase domain and a carboxyl autophosphorylation region. Normally, ligand binding to EGFR causes receptor dimerization and tyrosine kinase activation, resulting in regulation of cell proliferation though the RAS/MAPK pathway, and cell survival and transformation through the PI3K/AKT pathway (Jorissen et al. 2003). Recently, somatic mutations of *EGFR* in lung cancer tissues were observed: The mutations enhanced its tyrosine kinase activity and increase the sensitivity of the tumor to gefitinib, an EGFR-tyrosine kinase inhibitor (Lynch et al. 2004; Paez et al. 2004). These mutant EGFRs selectively activated the AKT and STAT signaling pathways, which contribute to cell survival (Sordella et al. 2004). Various mutations in exons 18 and 21 have subsequently been reported, and these mutations are clustered near the ATP cleft of the tyrosine kinase domain (Pao et al. 2004; Kosaka et al. 2004; Tokumo et al. 2005; Yang et al. 2005). Functional analysis of the mutations showed stabilization in their interaction with both ATP and gefitinib based on evidence of increased receptor activation after ligand binding and enhanced inhibition induced by gefitinib (Lynch et al. 2004). In fact, the response rates of gefitinib among patients with *EGFR* mutations were high: 65% (11/17) in Korea and 89% (8/9) in Japan (Tokumo et al. 2005; Han et al. 2005). The frequency of *EGFR* mutations among gefitinib or erlotinib responsive patients was 71% (12/17) in USA, and 78% (7/9) in Taiwan (Pao et al. 2004; Huang et al. 2004). Detailed DNA sequence analysis has recently revealed a second point mutation, T790M, using a tumor biopsy specimen obtained at the time of relapse (Kobayashi et al. 2005). In contrast to the response of gefitinib, severe adverse effects, such as acute lung injury, have also been reported (AuAstrazeneca 2004, in-house data). A multicenter prospective review in Japan showed that gefitinib-induced interstitial lung disease occurred in 215 of the 3,222 cases (5.8%), of with 83 (2.6%) died. Therefore, it is clear that the DNA sequence analysis of *EGFR* in lung cancer is essential for prediction of gefitinib response.

Direct sequencing approach for detection of *EGFR* mutations, which is a standard method, is not easy and is time-consuming. To seek easier and more sensitive approach, we applied Denaturing high-performance liquid chromatography (DHPLC) and compared the sensitivity, cost-effectiveness and analysis time with direct sequencing method. In this paper, we show that analysis time by DHPLC was one-forth of that by direct sequencing, and running cost was low. In addition, DHPLC analysis was sixfold more sensitive than sequencing analysis for detection of the point mutation of exon 21, L858R. Thus, DHPLC followed by direct sequencing for detection of mutation type is more accurate and cost-effective. Using this approach, we investigated the clinicopathological relationship of *EGFR* mutations and showed the different characteristics between exons 19 and 21 mutations among Japanese lung cancer patients. Considering these evidences, dependable method for detection of *EGFR* mutations is urgently needed for not only prediction of response to gefitinib, but also detailed analysis of lung carcinogenesis associated with *EGFR* mutations. In this paper, we show the clinical application of DHPLC as a highly sensitive and useful screening system for detection of *EGFR* mutations in lung cancer tissues.

Materials and methods

Tissue samples, pleural effusion

Tissue samples were obtained from 81 surgical specimens of lung cancer patients, and pleural effusions were obtained from 16 non-resectable lung cancer patients. Study patients had been admitted to Saga Medical School Hospital between 2000 and 2005, and had not received any anticancer chemotherapy or thoracic irradiation. Clinical stage was determined by the criteria of the International Union Against Cancer, and informed consent was obtained from all subjects. Histological subtype and tumor content were confirmed by HE staining using adjacent tumor samples. Pleural effusion used for analysis was assessed as class V by pathologist. A total of 97 study patients were investigated on EGFR mutation status by both DNA direct sequencing and DHPLC.

DNA extraction and sequencing analysis

DNA was isolated from fresh frozen tissues from tumors using QIAamp® DNA mini kit (QIAGEN, Hilden, Germany) as manufacturer's instructions. The mutations of exons 18, 19 and 21 were determined by

PCR-based direct sequencing. The primers for EGFR previously described were used. PCR amplification was performed in 20-μl volume using Discoverase™ DHPLC DNA polymerase (Invitrogen Inc., CA) at 95°C for 10 min, then 40 cycles at 95°C for 30 s, 58°C for 30 s, 72°C for 1 min and a final extension at 72°C for 10 min. The amplified product was isolated using Microcon YM-50 (Millipore Inc. MA), and sequenced directly using Applied Biosystems PRISM dye terminator cycle sequencing method with ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Denaturing high-performance liquid chromatography

PCR amplification was performed as described above. The primer sets used for DHPLC were as follows: sense: 5′-CAACCAAGCTCTCTTGAGGATC-3′ and antisense: 5′-CCCAGCCCAGAGGCCTGT-3′ (amplicon size, 115 bp) for exon 18; sense: 5′-GCAGCATGT GGCACCATCTC-3′ and antisense: 5′-AGAGCCAT GGACCCCCACAC-3′ (amplicon size, 197 bp) for exon 19; sense: 5′-TCTGTCCCTCACAGCAGGGTCT-3′ and antisense: 5′-GCTGGCTGACCTAAAGCCAC C-3′ (amplicon size, 218 bp) for exon 21. Amplification was confirmed by agarose gel electrophoresis of PCR products followed by staining with ethidiumbromide. The PCR products were denatured at 94°C for 4 min and gradually cooled to 25°C for a heteroduplex formation. Unpurified PCR samples were separated on a heated C18 reverse phase column using 0.1 M TEAA in water and 0.1 M TEAA in 25% acetonitrile at a flow rate of 0.9 ml/min. Sample analysis temperatures were predicted using the WAVEMaker™ software (Transgenomic Inc., NE). Predicted melting temperatures for exons 18 and 21 were 61.4 and 61.2°C for heteroduplex separation, respectively. The analysis for exon 19 was undergone by size-dependent separation at 50°C.

Statistical analysis

The association between *EGFR* mutations and clinicopathological parameters were examined by chi-square test for contingency tables.

Results

One patient with exon 21 mutation detected only by DHPLC, but not by direct sequencing

Although the detection of *EGFR* mutations is pivotal for prediction of gefitinib response as well as investigation of the mechanisms of lung carcinogenesis, the sequencing analysis is not easy and time-consuming. To find an easier and faster method for detection of *EGFR* mutations, we performed DHPLC along with sequencing analysis for exons 18, 19 and 21 using 97 lung cancer patients. A total of 97 lung cancer patients, 14 squamous cell carcinomas, 76 adenocarcinomas and 7 others (6 large cell carcinomas and 1 carcinosarcoma), were studied. Representative results of direct sequencing and DHPLC with *EGFR* mutations are shown in Fig. 1 and 2. *EGFR* mutations in exons 18, 19 and 21 were detected in 34 of 97 lung cancer patients (36%) (Table 1). A mutation in exon 18 was observed in a patient, and mutations in exon 19 were observed in 18 patients (15 surgical specimens and 3 pleural effusions) by DHPLC. These results were consistent with those by sequencing analysis (Table 2). Mutations in exon 21 were found in 15 patients (14 surgical specimens and 1 pleural effusion): 14 patients showed the mutations by both sequencing and DHPLC analyses, whereas one patient showed a mutation only by DHPLC. To confirm this mutation, we conducted sequencing analysis using fractionated samples obtained from a retention time of 5–6 min by DHPLC (Fig. 3). A mutation of exon 21, L858R, was apparently observed by sequencing analysis using fractionated samples, although the mutation was not detected by sequencing analysis using non-fractionated samples. A chest computed tomography (CT) scan and pathological finding of the patient are shown in Fig. 4. CT scan of the chest showed 2 cm lung mass in diameter, and pathological type was adenocarcinoma with the feature of BAC. The tumor specimen obtained for *EGFR* mutation analysis contained papillary adenocarcinoma with central scar formation, and the number of lung cancer cells was less than 20% (Fig. 4b).

Clinicopathological comparison of EGFR mutations between exons 19 and 21

The clinicopathological characteristics of lung cancer patients with *EGFR* mutation are shown in Table 1. Clear associations of *EGFR* mutations with gender (female), smoking status (non-smoker) and pathological typing (adenocacinoma) were observed. Looking at the pathological type of lung cancer, the frequency of *EGFR* mutations was 43% (33/76) for adenocarcinoma, whereas no *EGFR* mutations were observed in squamous cell carcinoma. A mutation of exon 19 was detected in one carcinosarcoma. The frequency of *EGFR* mutations in female adenocarcinoma patients was 66% (25/38), whereas that in male adenocarcinoma patients was 24% (9/38), suggesting that both

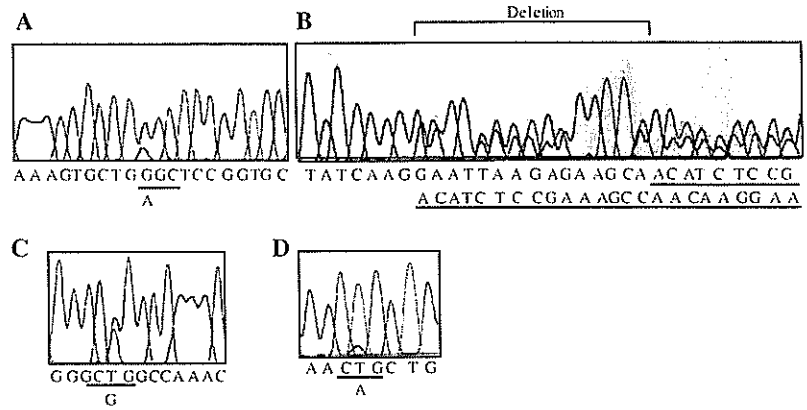

Fig. 1 *EGFR* mutations of exons 18, 19 and 21 in lung cancer tissue. **a** Heterozygous missense mutation (*underline*) of exon 18 (G719S). **b** Heterozygous in-frame deletion in exon 19 involving 15 bp from codons 746–750. Heterozygous missense mutation (*underline*) of exon 21 [L858R (**c**) and L861Q (**d**)]

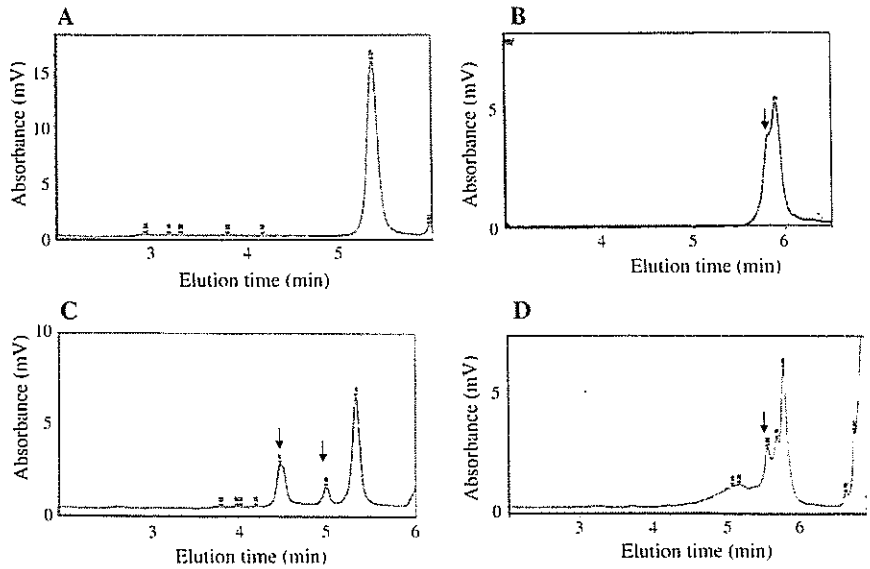

Fig. 2 Detection of *EGFR* mutations using DHPLC. The mixture of PCR amplicons of wild type and mutant are denatured and re-annealed. Heteroduplexes and homoduplexes are isolated by different retention times in reverse-phase chromatography. Heteroduplexes are eluted earlier than homoduplexes, resulting in detection of the mutations. Wild type of EGFR exon19 is shown in **a**. Mutations of exon 18, G719S (**b**), and 21, L858R (**d**), shows double-peak pattern (*arrows*). Mutation of exon 19, del E746-A750, was detected by size-dependent separation at 50°C, and short sized DNA fragments are eluted earlier (*arrows*) as shown in **c**

gender (female) and pathological type (adenocarcinoma) impacted *EGFR* mutations. One mutation in exon 18 and 15 mutations in exon 21 were point mutations (1 G719S, 14 L858R and 1 L861Q) (Table 4, Fig. 1), while 18 mutations in exon 19 were in-frame deletions of 9–24 bp around codons 746–759 (Table 3, Fig. 1). Thirteen of the 15 patients (87%) with exon 21 mutations were female non-smokers; the pathological types were all adenocarcinomas with features of BAC except for 2 patients (Table 4). A patient with exon 18 mutation was a female non-smoker with BAC. On the other hand, 8 of the 18 patients with exon 19 mutations were current or former smokers; 8 patients were men and lung cancer tissues of 7 did not reveal the feature of BAC (Table 3). The differences of pathological type, including BAC or not, gender and smoking status between exons 21 and 19 were statistically significant (Table 5). These results suggest that different mechanisms of mutagenesis and carcinogenesis may exist between exons 19 and 21 mutations. Among these patients, seven patients, one with exon 21 mutation and six with exon 19 mutations, were treated with 250 mg gefitinib per day, and all patients responded, and gefitinib-induced lung injury was not observed.

Proposed screening system of EGFR mutations

To examine the sensitivity of DHPLC compared with sequencing analysis, we used H1975 cells with a point mutation of L858R. The point mutation was detected

Table 1 Characteristics of patients with *EGFR* mutations

Age±SD (yrs)			
	With mutations	67.4±11.1	
	Without mutations	67.5±8.5	
Sex			
	Male	10/59 (17%)	P<0.01
	Female	24/38 (63%)	
Smoking			
	Smoker	10/57 (18%)	P<0.01
	Non-smoker	24/40 (60%)	
Histology			
	Squamous cell carcinoma	0/14 (0%)	P<0.01
	Adenocarcinoma	33/76 (43%)	
	Others	1/7 (14%)	
Stage*			
	I	24/58 (41%)	
	II	1/12 (8%)	
	III	6/14 (43%)	
	IV	3/13 (23%)	
	Total	34/97 (36%)	

*Postoperative pathological stage (clinical stage in 16 patients)

Table 2 Comparison between DHPLC and sequence analysis on *EGFR* mutations

Exon 18			Exon 19			Exon 21		
Patient no.	DHPLC	Seq	Patient no.	DHPLC	Seq	Patient no.	DHPLC	Seq
1	M	M	1	M	M	1	M	M
			2	M	M	2	M	M
			3	M	M	3	M	
			4	M	M	4	M	M
			5	M	M	5	M	M
			6	M	M	6	M	M
			7	M	M	7	M	M
			8	M	M	8	M	M
			9	M	M	9	M	M
			10	M	M	10	M	M
			11	M	M	11	M	M
			12	M	M	12	M	M
			13	M	M	13	M	M
			14	M	M	14	M	M
			15	M	M	15	M	M
			16	M	M			
			17	M	M			
			18	M	M			

Seq direct sequencing, *M* mutation

by direct-sequencing method when it contained more than 30% of H1975 cells, whereas the mutation was detected in the presence of, as little as, 5% of H1975 cells by DHPLC (Fig. 5). Comparison between DHPLC and direct sequencing in terms of analysis time for ten samples, *EGFR* mutations are detected in 5 h by DHPLC as opposed to 18 h by direct sequencing. In addition, it costs eightfold more expensive by direct sequencing than DHPLC. Thus, DHPLC is more sensitive, cost-effective and easier than direct sequencing. Based on these evidences, we proposed the screening system shown in Fig. 6. *EGFR* mutations are screened by DHPLC, and subsequently direct sequencing is performed for confirmation of the mutations and analyses of the mutation types using only samples in which *EGFR* mutations are detected by DHPLC. This system enables us to provide quick results and DHPLC analysis using fractionated samples reveals more obvious results without subcloning technique.

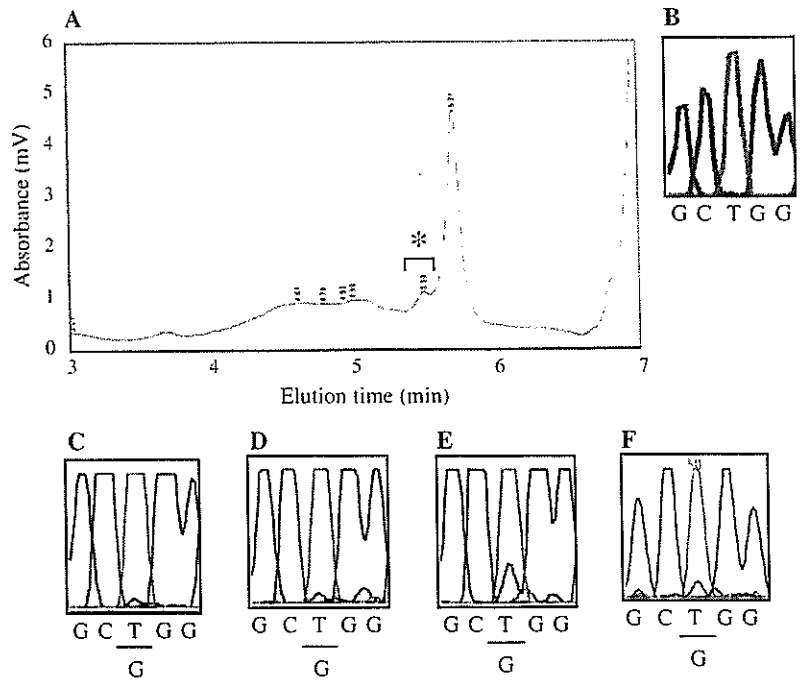

Fig. 3 Detection of *EGFR* mutation, L858R, using sequencing analysis of fractionated samples obtained by DHPLC system. Genomic DNA isolated from lung cancer tissue of patient no.3 with exon 21 mutation was conducted by DHPLC (**a**), followed by sequencing analysis using fractionated samples with retention time of 5–6 min. The results of sequencing analysis in the indicated period (*asterisks*) are shown in **c–f**, and a result of that using non-fractionated sample is shown in **b**

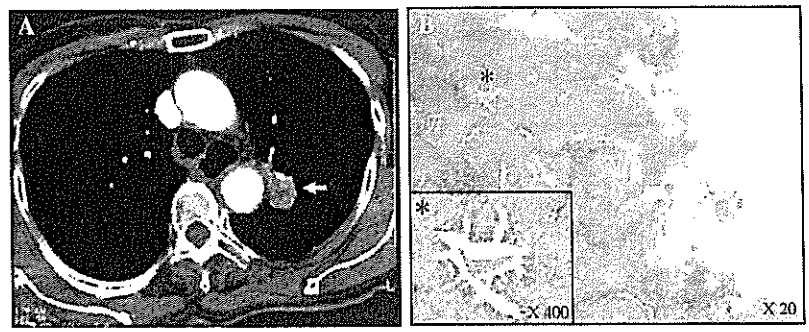

Fig. 4 A case of lung cancer in which *EGFR* mutation of exon 21, L858R, was detected by only DHPLC and not direct sequencing. **a** CT scan of the chest showed 2 cm lung mass in left lung (*white arrow*). **b** The tumor specimen obtained for mutation analysis, which contained papillary adenocarcinoma shown by 400-fold magnification (*asterisks*), with central scar formation

Discussion

In this paper, we proposed new screening system for detection of *EGFR* mutations by DHPLC analysis. DHPLC analysis can detect mutations within 2–3 min by comparing two chromosomes as a mixture of denatured and reannealed PCR amplicons revealing discrete retention of homo- and heteroduplex DNA (Xiao and Oefner 2001). Since hot spots for mutations are present in the *EGFR* gene, which are associated with gefitinib response, DHPLC is a useful method for detection. In our study, results using DHPLC analysis were consistent with those of direct sequencing. In addition, DHPLC analysis was sixfold more sensitive than sequencing analysis for detection of the point mutation of exon 21, L858R. It is difficult to detect the mutation by direct sequencing analysis using whole tissue samples when the number of lung cancer cells is low. Indeed, patient no. 3 with exon 21 mutation had a pure BAC pattern, and the number of lung cancer cells was low in tumor samples. The sequencing analysis did not detect a point mutation of exon 21, but DHPLC did. In addition to these benefits, DHPLC is cost-effective, which costs eightfold less expensive than direct sequencing. We therefore propose DHPLC for screening followed by sequencing as a more sensitive and accurate, as well as easier and faster, method.

Using this system, we also demonstrated differences in clinicopathological characteristics between the *EGFR* mutations of exons 19 and 21. Eighty seven per-

Table 3 *EGFR* mutations of exon 19 in lung cancer tissues

No.	Sex	Mutation	Pathological type	BAC	Stage[a]	Smoking[b]	Gefitinib treatment
1	F	del L747-S752	ad	+	IA	0	Yes
2	M	del E746-A750	ad	–	IIIB	1,000	No
3	M	del L747-S752	ad	+	IIIB	50	No
4	F	del T751-E758	ad	+	IA	0	No
5	M	del E746-A750	ad	ND[c]	IIIB	15	Yes
6	M	del E746-A750	ad	–	IA	200	No
7	M	del E746-A750	Carcinosarcoma	–	IB	400	No
8	M	del L747-E749	ad	+	IA	200	No
9	F	del E746-A750	ad	+	IB	0	No
10	F	del L747-K754	ad	–	IB	0	No
11	F	del K745-E746	ad	ND	IV	300	Yes
12	M	del L747-T751	ad	ND	IV	760	Yes
13	F	del E746-A750	ad	+	IIIB	0	Yes
14	F	del E746-A750	ad	+	IA	0	No
15	F	del E746-A750	ad	+	IB	0	No
16	M	del E746-A750	ad	–	IA	0	No
17	F	del E746-A750	ad	–	IIB	0	Yes
18	F	del E746-A750	ad	–	IIIB	0	No

M male, *F* female, *ad* adenocarinoma, *BAC* bronchioloalveolar carcinoma, *ND* not determined

[a] Postoperative pathological stage (clinical stage in patient no. 5, 11, 12)

[b] Smoking index: (number of cigarettes smoking/days) × years

[c] Diagnosis was made only from cytology of pleural effusion and biopsy specimen was not obtained

Table 4 *EGFR* mutations of exon 21 in lung cancer tissues

No.	Sex	Mutation	Pathological type	BAC	Stage[a]	Smoking[b]	Treatment of gefitinib
1	F	L858R	ad	+	IA	0	No
2	F	L858R	ad	+	IA	0	No
3	F	L858R	ad	+	IA	0	No
4	F	L858R	ad	+	IA	0	No
5	F	L858R	ad	+	IA	0	No
6	F	L858R	ad	+	IA	0	Yes
7	F	L858R	ad	+	IA	0	No
8	F	L858R	ad	+	IA	0	No
9	F	L858R	ad	ND[c]	IV	0	No
10	F	L861Q	ad	+	IA	0	No
11	M	L858R	ad	–	IB	1,000	No
12	F	L858R	ad	+	IA	0	No
13	F	L858R	ad	+	IIIB	0	No
14	M	L858R	ad	+	IA	200	No
15	F	L858R	ad	+	IA	0	No

M male, *F* female, *ad* adenocarinoma, *BAC* bronchioloalveolar carcinoma, *ND* not determined

[a] Postoperative pathological stage (clinical stage in patient no. 9)

[b] Smoking index: (number of cigarettes smoking/days) × years

[c] Diagnosis was made only from cytology of pleural effusion and biopsy specimen was not obtained

cent of the patients with exon 21 mutations were non-smokers and/or women, and their pathological types were adenocarcinoma with the feature of BAC. On the other hand, 44% of those with exon 19 mutations had a smoking history and included eight male patients, with BAC feature not observed in 39% of these patients. Another group also reported that gender difference was observed for mutational location dominance of exon 19 for males and exon 21 for females among Japanese lung cancer patients (Tokumo et al. 2005). These results suggest discrete mechanisms of mutagenesis in *EGFR* gene and carcinogenesis pathway between exons 19 and 21.

EGFR mutations, including both exons 19 and 21, were recently reported to be observed even in histologically normal bronchial and bronchiolar epithelium of

Table 5 Comparison between exons 19 and 21 mutations in lung cancer samples

	Female		Non-smoker		BAC*	
Exon 21	13/15 (87%)	p=0.05	13/15 (87%)	p=0.05	13/14‡ (93%)	p=0.02
Exon 19	10/18 (56%)		10/18 (56%)		8/15‡ (53%)	

BAC bronchioloalveolar carcinoma

*Adenocarcinoma with BAC

‡The cases whose diagnosis was made only from cytology of pleural effusion were excluded

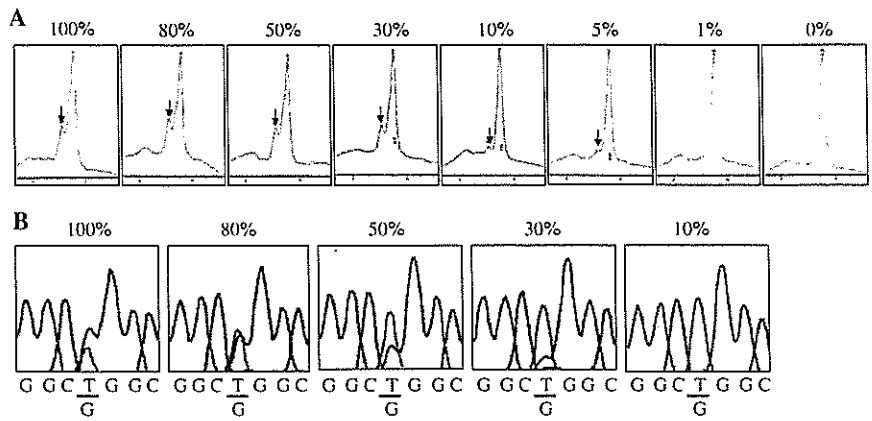

Fig. 5 Comparison of the sensitivity to *EGFR* mutation in exon 21 between DHPLC (**a**) and sequencing analysis (**b**). H1975 cells, which have a point mutation of L858R, were used for the comparison. The indicated ratio of mixture with A549 cells, which do not have the mutation, was conducted with DHPLC or sequencing analysis

lung cancer patients with *EGFR* mutations in tumor cells (Tang et al. 2005), suggesting that both *EGFR* mutations are early events of lung carcinogenesis. However, it is possible that the factors inducing *EGFR* mutations are different, depending on the mutation types. It has also been reported that the patterns of *TP53* mutations depend on smoking status: G:C-to-T:A transversions and A:T-to-G:C transitions were consistently associated with smoking, whereas G:C-to-A:T transitions were associated with never-smokers, who had been highly exposed to occupational and environmental lung mutagenesis (Le Calvez et al. 2005). *KRAS* mutations are also frequently observed in adenocarcinoma, even in atypical adenomatous hyperplasia (AAH), which is the premalignant lesion of adenocarinoma (Le Calvez et al. 2005; Westra et al. 1996). However, these mutations were associated with former smoker (Le Calvez et al. 2005), and the populations with *KRAS* mutations and *EGFR* mutations were discrete (Kosaka et al. 2004). Considering these evidences, we assume that types of *EGFR* mutations could depend on the etiology; mutations in exon 19 are partly related with smoking and those in exon 21 are related with the other factors. In addition, we found different pathological types carrying *EGFR* mutations: exon 21 mutations had adenocarcinoma with the feature of BAC, and exon 19 mutations had adenocarcinoma without BAC. Noguchi et al. (1995) subdivided small, early adenocarinomas into two groups: replacing growth type and non-replacing type. Their concept was that peripheral adenocarinoma of the lung undergoes sequential progression from AAH through localized BAC to advanced localized BAC with fibroblastic proliferation. The accumulation of multiple allelic losses was observed during the sequential progression of replacing growth type (Aoyagi et al. 2001), whereas the non-replacing type was thought to be de novo adenocarcinomas without stepwise progression (Noguchi et al. 1995). Recently, Yatabe et al. (2005) reported that *EGFR* mutations in exon 21 were observed in two cases of AAH. These results suggest that the process of lung carcinogenesis may be different between two groups of *EGFR* mutations, exons 19 and 21.

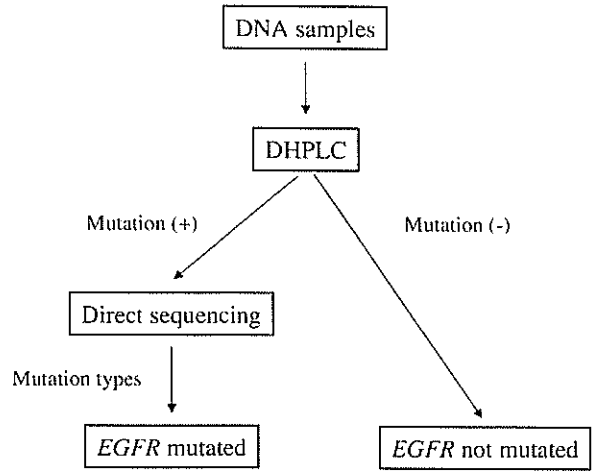

Fig. 6 Proposed screening system of *EGFR* mutations. *EGFR* mutations are screened by DHPLC. When *EGFR* mutations are detected by DHPLC, direct sequencing is subsequently performed for confirmation of the mutations and analyses of the mutation types

We show in this paper that the response to gefitinib was observed in all patients with *EGFR* mutations, and the response rates of gefitinib among patients with *EGFR* mutations were from 80 to 90% in other reports (Tokumo et al. 2005; Han et al. 2005; Huang et al. 2004), suggesting the usefulness of detection of *EGFR* mutations for the prediction of gefitinib response. Analysis of *EGFR* mutations is necessary when the patients have some risk factors of acute lung injury by gefitinib treatment (Seto and Yamamoto 2004). Considering high response rate of gefitinib in contrast with high frequency of gefitinib-induced severe lung injury in Japan (AuAstrazeneca 2004, in-house data; Fukuoka et al. 2003), the precise, sensitive methods for detection of *EGFR* mutations are urgently needed especially for Japanese population. Although *EGFR* mutations observed in lung cancer tissues were located between exons 18 and 21, all types do not have equal effects in response to gefitinib (Shigematsu and Gazdar 2006). G719A and A859T were detected in lung cancer patients who experienced progressive diseases after treatment of gefitinib (Han et al. 2005), and T790M was observed at the time of relapse (Kobayashi et al. 2005; Pao et al. 2005). These results indicate that identification of mutation typing is indispensable. A screening system by DHPLC and subsequent direct sequencing for detection of the mutation typing, a quick, sensitive and cost effective method, is useful for not only prediction of gefitinib but also analysis of gefitinib resistance.

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Development of a Novel Method to Detect *Helicobacter pylori cagA* Genotype from Paraffin-Embedded Materials: Comparison between Patients with Duodenal Ulcer and Gastric Cancer in Young Japanese

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Key Words
Helicobacter pylori · *cagA* gene · Gastric cancer · Duodenal ulcer · Paraffin-embedded section

Abstract
Background/Aim: *cagA* gene polymorphism of *Helicobacter pylori* contributes to clinical outcome of patients. We investigated the implication of the *cagA* polymorphism in young Japanese patients using paraffin-embedded sections. ***Methods:*** We studied 71 young patients with gastric cancer or with duodenal ulcer. *H. pylori* infection was confirmed by sections with Giemsa staining and immunohistochemical staining and the degree of gastritis was evaluated. DNA was extracted from paraffin-embedded sections of 20 patients both from the gastric corpus and the antrum. A portion of *cagA* gene was amplified with polymerase chain reaction, followed by direct sequencing of the fragment. ***Results:*** We established a novel method to determine the *cagA* subtype using paraffin-embedded sections. We found that all the samples possessed East-Asian type *cagA* both in the corpus and the antrum, not only in patients with gastric cancer but also with duodenal ulcer. Although the *cagA* gene sequence was completely identical between the gastric corpus and the antrum in all patients, the corpus gastritis was more prominent in patients with gastric cancer than those with duodenal ulcer. ***Conclusions:*** *cagA* polymorphism can be evaluated with the use of paraffin-embedded sections. The degree of corpus gastritis may not be regulated by *cagA* diversity only.

Introduction

Helicobacter pylori infection is a critical factor for promoting atrophic gastritis [1]. Long-term infection of *H. pylori* results in glandular atrophy and intestinal metaplasia. Since corpus atrophic gastritis is a fundamental and essential status for human gastric carcinogenesis, *H. pylori* is regarded as an important carcinogen in the devel-

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opment of human gastric cancer [2]. Indeed, it has been accepted that there is a strong association between *H. pylori*-associated gastritis and gastric cancer [3–6]. A clinical study by Uemura et al. [8] confirmed a strong association between the occurrence of gastric cancer and *H. pylori* infection followed by corpus gastritis. Atrophic gastritis and gastric cancer are very common diseases in Japan, since the high prevalence of *H. pylori* infection has especially been confirmed in elderly people [9]. However, not all patients with *H. pylori* infection have gastric cancer, so it is clinically important to select the population with a high risk for gastric cancer development [10].

H. pylori cagA protein is an important virulent factor for gastric mucosa injury. Huang et al. [11] demonstrated the strong association between anti-cagA seropositivity and development of gastric cancer, suggesting an importance of cagA for gastric carcinogenesis. Recent studies have clarified the molecular mechanism of gastric mucosal injury induced by cagA protein. cagA protein produced in the bacterial cell is translocated into the host cell by the type IV secretory system, followed by tyrosine phosphorylation by src-family kinases and activation of SHP-2 phosphatase [12, 13]. Recent studies revealed that cagA protein showed diversity and was subclassified into two types, namely Western type and East-Asian type. The latter type was reported to have a high affinity to SHP-2 and was thus regarded as a more harmful form than the Western type [14]. In fact, diversity of cagA protein was assessed and showed a tight relationship between its diversity and the clinical outcome [15].

However, until now, most studies have been performed with the use of isolated colonies, resulting in the uncertainty of whether it really reflects the original character of *H. pylori* in the stomach. In addition, it is clinically important to investigate the topography of gastric inflammation, which may be influenced by the heterogeneous distribution of *H. pylori*. Then, in the present study, we investigated the cagA subtype of *H. pylori* with the use of DNA samples extracted from paraffin-embedded sections that reflect the original character of the bacteria. Moreover, we studied the diversity in the samples from the gastric corpus and the antrum separately to discuss the heterogeneity of the cagA subtype in the stomach.

Methods

Patients

We studied 32 patients with gastric cancer (20 men, mean age 26.6 years; 12 women, mean age 25.6 years) and 39 patients with duodenal ulcer (36 men, mean age 25.1 years; 3 women, mean age 25.6 years). All patients were <30 years and underwent gastrectomy. The resected stomach was fixed in buffered formalin and embedded in a paraffin block. Except for the pathological lesions, non-neoplastic gastric mucosae in the lesser curvature were cut and embedded in paraffin sections in the same manner. These non-neoplastic mucosas were used for the present examinations. All patients had histological gastritis either in the gastric corpus and antrum, and were confirmed as being *H. pylori*-positive by Giemsa staining. Patients who received eradication therapy were not included in this study. We obtained informed consent from all patients and the Ethical Committee of Hiroshima University approved our protocol.

Evaluation of Histology of Gastritis

In each patient, histological gastritis of the lesser curvature of the corpus and antrum were evaluated with the use of the sections stained with hematoxylin and eosin (HE). We scored the degree of gastritis (mononuclear infiltration and activity; from 0 to 3) with the criteria of the updated Sydney System [16]. Two specialists (M.I. and K.H.) who independently scored the degree of gastritis were blind to the clinical information.

DNA Extraction from Paraffin-Embedded Sections

We randomly selected 20 patients (10 with gastric cancer and 10 with duodenal ulcer) and conducted DNA extraction from the tissue sections of the patients. Tissue sections of 4 μm in thickness were placed on glass slides and stained with HE. The tissue sections were then dehydrated in graded ethanol solutions and dried without a cover glass. Tissues were scraped from the slides with sterile needles. DNA was extracted from the tissues by incubation in 20 μl of extraction buffer (100 m*M* Tris-HCl; 2 m*M* EDTA, pH 8.0; 400 μg/μl proteinase K) at 55°C overnight. The tubes were boiled for 7 min to inactivate the proteinase K, and then 2 μl of the extracts was used for each polymerase chain reaction (PCR) amplification.

PCR Reaction

To determine the cagA subtype, two pairs of primers (forward: cagA01 and reverse: cagA02 or cagA05, respectively) were used for PCR amplification, yielding 117- and 92-basepair (bp) products, respectively (table 1). Each 20 μl of reaction mixture contained 0.2 μl of Pyrobest DNA polymerase (5 units/μl; Takara, Shiga, Japan), 2 μl of 10× Pyrobest buffer II, 2 μl of dNTP mixture (2.5 m*M* each), and 1 μl each of forward and reverse primers. The reaction mixtures were heated to 95°C for 5 min, followed by 50 cycles of denaturation at 95°C for 30 s, annealing at 63°C for 30 s, and elongation at 72°C for 30 s. After PCR, the products were electrophoresed on 8% polyacrylamide gels containing 1× TBE buffer (50 m*M* Trizma base, 67 m*M* borate, 1 m*M* EDTA).

Sequencing

DNA bands were excised from the gels and the DNAs were eluted and purified using QIAquick Gel Extraction kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Then, purified DNA fragments were subjected to sequence reaction using BigDye Terminators Version 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif., USA) and ABI Prism 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instruction.

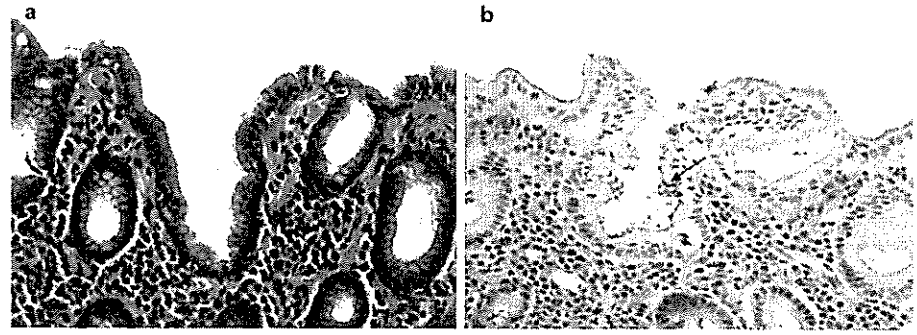

Fig. 1. Detection of *H. pylori* in the gastric mucosa from the corpus by HE section (**a**) and by immunohistochemical staining (**b**) in a 26-year-old male patient. Arrow indicates the bacterium just on the epithelial cells.

Table 1. Histology in the corpus and antrum of the patient groups

	Corpus			Antrum		
	GC (n = 30)	DU (n = 36)	p	GC (n = 32)	DU (n = 39)	p
Infiltration						
Grade 0 or 1, n (%)	10 (33.3)	32 (88.9)		8 (25.0)	6 (15.4)	
Grade 2 or 3, n (%)	20 (66.7)	4 (11.1)	<0.001	24 (75.0)	33 (84.6)	>0.3
Activity						
Grade 0 or 1, n (%)	20 (66.7)	35 (97.2)		19 (59.4)	20 (51.3)	
Grade 2 or 3, n (%)	10 (33.3)	1 (2.8)	0.002	13 (40.6)	19 (48.7)	>0.4

Immunohistochemistry

Four-micrometer sections of formalin-fixed paraffin-embedded tissues were used for immunohistochemical staining. After deparaffinization and hydration, internal peroxidase was blocked by incubation with 0.3% H_2O_2 in methanol for 15 min. After incubation with 5% skim milk/PBS for 20 min, the sections were reacted with the primary antibody (diluted with PBS) for 2 h at room temperature. The primary antibody used was anti-*H. pylori* polyclonal antibody (dilution of 1:50; Dako, Kyoto, Japan). Antigen retrieval was carried out with microwave treatment before reacting with primary antibody.

Statistics

Statistical analysis was performed by χ^2 test and Fisher's exact test with SPSS Version 11.5J software (SPSS Inc., Chicago, Ill., USA). A p value of <0.05 was considered statistically significant.

Results

Immunohistochemical Detection of H. pylori

We examined the *H. pylori* status by immunohistochemical staining. As shown in figure 1, we could hardly detect the bacteria in HE sections. In most sections the mucous layer over the mucosal epithelium had been washed out and could not be found in the sections. In immunohistochemical analysis, several bacteria could be detected just on the epithelium. The image is not different between sections from the two groups (gastric cancer and duodenal ulcer).

Comparison of Histology in Gastritis between Gastric Cancer and Duodenal Ulcer Patients

First, we compared the grades of gastritis between sections from young patients with gastric cancer and with duodenal ulcer. As shown in table 1, the degree of antral gastritis was not statistically different in neutrophil activity and chronic inflammation between these two groups. On the other hand, in the gastric corpus the degree of gastritis was statistically more prominent (activity, $p = 0.002$, and chronic inflammation, $p < 0.001$) in sections with gastric cancer than in those with duodenal ulcer.

Establishment of Amplification Method of cagA *Gene Using Paraffin-Embedded Sections*

Since DNA samples were degraded in various degrees and the amount of *H. pylori* DNA relative to the human DNA was small, many experimental improvements

cagA Analysis from Paraffin-Embedded Section

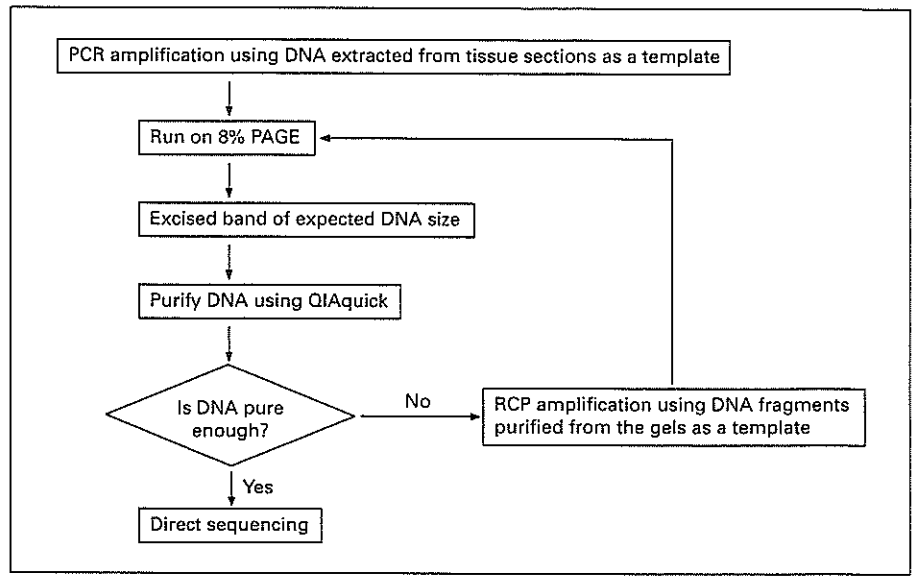

Fig. 2. The method from PCR amplification to direct sequence.

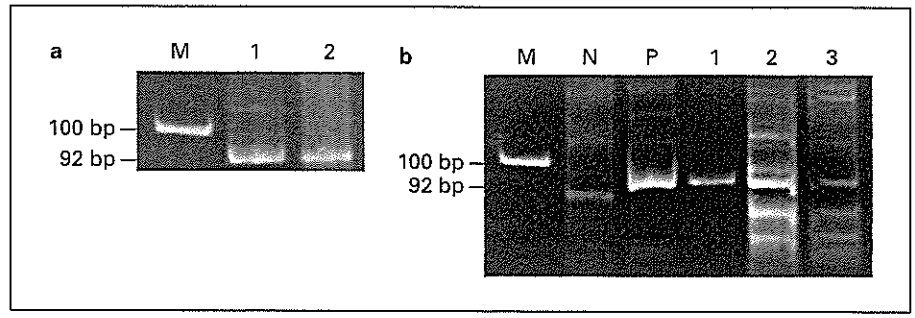

Fig. 3. Detection of PCR products. **a** Products with single amplification (lane 1) and those by repeated PCR three times (lane 2). **b** Final products of amplified *H. pylori* DNA from 3 patients (lanes 1–3). M = Marker; N = negative control; P = positive control.

Table 2. Oligonucleotides used to detect the CagA

Gene	Primer	Primer sequence	Size, bp
cagA	cagA01	5′-TAGCCCTGAACCCATTTACG-3′	(01-02) 117
	cagA02	5′-TGTTCCCTTGAAAGCCCTAC-3′	
	cagA05	5′-TGAGATCACTAACTGCAGCAC-3′	(01-05) 92

were needed to obtain PCR products of good quality capable for conducting direct sequence reaction (fig. 2). First, we found that PCR products should not exceed 100 bp in size to obtain reproducible amplification. Primers that were used in this study are shown in table 2. For the PCR amplification, Pyrobest DNA polymerase possessing proofreading activity was suitable for our experiments. Since the first PCR products contained additional DNA fragments (fig. 3a, lane 1), the amplified fragments were separated and purified from the 8% PAGE and the purified DNA fragments were used as a template for second or third PCR to obtain a single band of *cagA* fragment (fig. 3a, lane 2). In order to confirm that obtained PCR fragments were specific for *cagA*, we always included negative controls in each reaction (fig. 3b). As for the reproducibility of this method, we confirmed that we could obtain completely identical results by repeating the examination with the same section (data not shown).

Comparison of cagA Subtype between Gastric Cancer and Duodenal Ulcer Patients

Using direct sequencing, we were able to obtain the *cagA* DNA sequences from all the tested samples. The

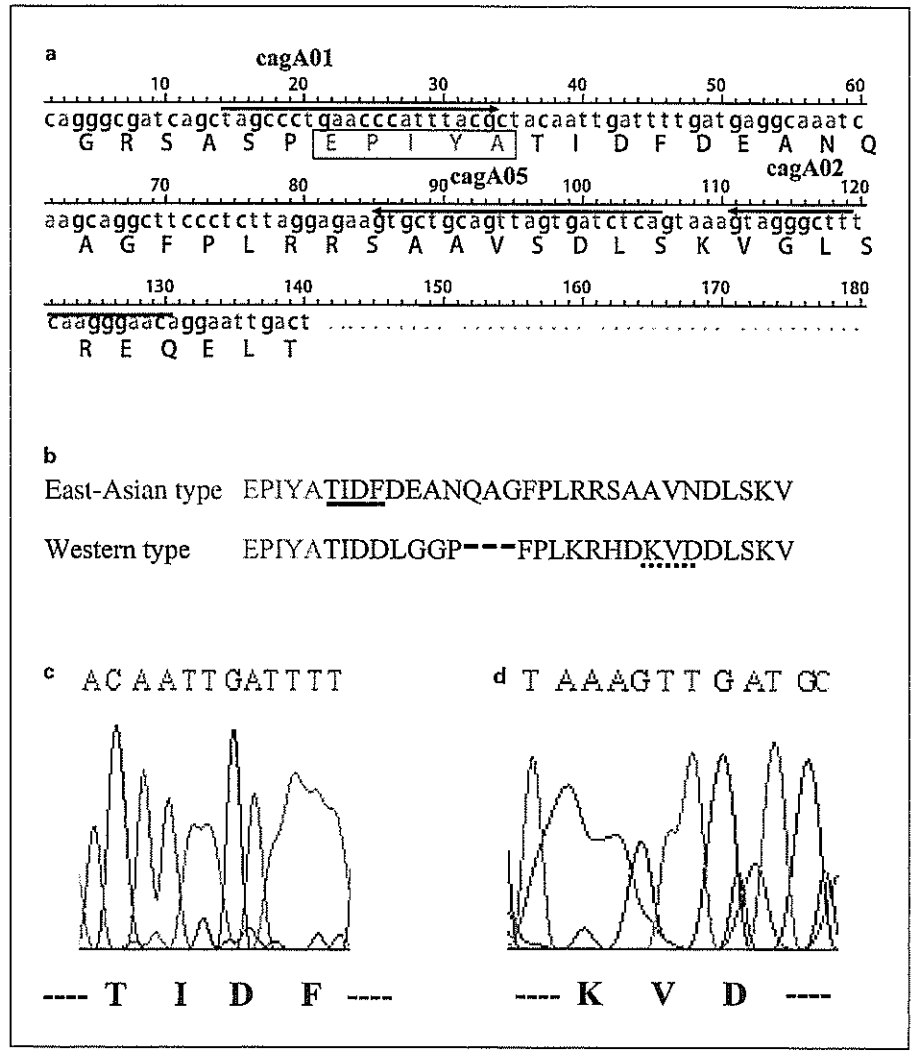

Fig. 4. **a** The information about the place of primers (cagA 01, 02 and 05). **b** Representative amino acid sequence of East-Asian and Western type cagA protein [from 14]. Results of direct sequence of DNA extracted from paraffin-embedded section with the use of primers cagA01-02 (**c**, **d**). The results of direct sequence of *cag*A DNA (upper) and encoding amino acids cagA protein (lower) were demonstrated. **c** The sequence of East-Asian cagA (#974–977, +2 to +5 from Y; black line in **b**). **d** The sequence of Western cagA (+17 to +19 from Y; dotted line in **b**). The patient used in figure 4d was not included in our study.

information about the place of primers used in our study is demonstrated in figure 4a, and typical amino acid sequences of two cagA subtypes are also demonstrated in figure 4b. The representative results are shown in figure 4c. In gastric cancer patients, we detected only East-Asian type cagA both in the gastric corpus and antrum. In duodenal ulcer patients, the same results were obtained not only in the gastric corpus but also in the antrum. Although we detected only East-Asian type cagA in the present study, we confirmed that the Western type cagA could be detected in another examination and denied the possibility that our system could only be applicable for detecting East-Asian type cagA (fig. 4d).

cagA Subtype Heterogeneity between the Gastric Antrum and the Corpus

Then, we compared all the DNA sequences encoding amino acids of cagA protein between those from the gastric corpus and from the antrum. The DNA sequence was completely identical between the DNAs extracted from the different sites.

Discussion

East-Asian cagA is supposed to be more virulent to epithelial injury and is regarded as being more carcinogenic for gastric mucosa than the Western type. In fact, the international distribution of the gastric cancer incidence could be explained by the diversity of this harmful

type of cagA [17]. However, in Japanese patients, almost all bacteria had cagA protein and have revealed that its subtype was the East-Asian type [18, 19]. Therefore, it is difficult to explain the difference in clinical outcome induced by *H. pylori* infection within Japanese patients only by the diversity of cagA status.

Focus must be placed on the methodology of these studies. In most studies, samples were extracted from isolated colonies of *H. pylori*. In the status, it is controversial whether the biological characters of isolated colonies reflect the original character of the bacteria in vivo, since the presence of metastability of the *H. pylori* could not be completely denied [20]. Another important problem is the topography of gastric inflammation. In gastric carcinogenesis, corpus-predominant gastritis showed a tighter relationship with gastric cancer, whereas antral-predominant gastritis is a negative factor for gastric carcinogenesis [8]. It is difficult to explain the heterogeneous distribution of gastritis inflammation only from the difference of solitary bacteria.

In the present study, we examined and compared the difference in the status of gastritis between two groups: the first included gastric cancer patients <30 years whose *H. pylori* was expected to be virulent in gastric carcinogenesis and the other included duodenal ulcer patients who were considered to have *H. pylori* that is less potential in gastric carcinogenesis. We have previously reported the tight association between *H. pylori* infection and the occurrence of gastric cancer in young patients [21, 22]. We then compared the status of *H. pylori* cagA status in these two groups, and the improved points in this study were (1) to use the paraffin-embedded section to avoid the metastability of the bacteria, and (2) to examine the status of gastric corpus and antrum separately in each patient.

In the present study, we could confirm that corpus gastritis was more prominent in gastric cancer patients than in duodenal ulcer patients, whereas antral gastritis was not different between these two groups. These findings are completely compatible with the report by Uemura et al. [8]. However, unexpectedly, the cagA status was not different between these two groups not only in the gastric antrum but also in the gastric corpus. This suggests that the status of gastritis, especially in the gastric corpus, is not affected by the status of the cagA protein in Japanese subjects. The main cause is still unclear but this might be regulated in the step of bacterial adhesion or of intracellular signaling after SHP-2 activation. A previous report has demonstrated that intragastric status including acid secretion may be a key factor for the mechanism of corpus inflammation induced by the cagA infusion system [23], but some controversial results have also been published. It is still controversial whether *H. pylori* in the stomach is monotonous or heterogeneous. We previously studied the bacterial resistance for antibiotics and demonstrated that the heterogeneity of *H. pylori* could be found in approximately 30% of the patients [24]. In the present study, the sequence of the cagA hot spot is completely identical between gastric corpus and the antrum in all cases examined. It is unlikely that the heterogeneous distribution as for cagA subtype could be detected in Japanese patients. Differing from the drug resistance gene, *cagA* gene may be highly conserved and not be under the status of easy metastability.

The main advantage in this study is the establishment of a methodology to examine the cagA status using paraffin-embedded sections. Fortunately, by using the paraffin-embedded surgical sections, we succeeded in reducing the influence of *H. pylori* floating in the gastric mucous. Previously, only cagA status (positive or negative) was determined by using paraffin-embedded sections, and no report could be found as for the direct sequencing of *cagA* gene [25, 26]. Our method seems to be time- and money-consuming comparing the analysis with the use of fresh biopsy specimens. However, in old and rare cases, such as gastric carcinoma in young patients, often we only have to use the paraffin-embedded sections as a starting material. This is the main purpose of our study and we believe our method may contribute to the further extension in this field including retrospective studies using old samples.

However, our protocol may be laborious since it requires the repeated PCR reactions to obtain a high-quality result. For example, as in our experience, the size of the PCR product was limited to 100 bp in length and three times repeated PCR was needed for most cases. In addition, our experience showed that the results were affected by the conditions of sample preparation including the fixation procedure. An immediate fixation after resection may be important for the maintenance of the good quality of *H. pylori* DNA, and the use of buffered formalin seemed to be essential. Long-term washing of fixed samples may result in extreme reduction of bacterial number and should be avoided. Further technical improvement of our method may be required for application to the clinical examination in practice. In the next step, it may be possible to distinguish two types of *cagA* gene without sequencing. Actually, the size of the PCR product in each subtype was different (117 vs. 108 bp) when we used cagA 01-02 primers. As demonstrated in figure 3, we found

some additional bands and these made it difficult to distinguish the cagA pattern only by evaluating the pattern after electrophoresis. However, it is important to try to make better primers for this purpose.

Taken together, this is the first report demonstrating the cagA status of gastric cancer and duodenal ulcer in young patients using paraffin-embedded sections. Our results will provide the next strategy to clarify the difference in corpus gastritis between these patients. The clarification of bacterial adhesion including the intragastric condition may be a next step in this matter.

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