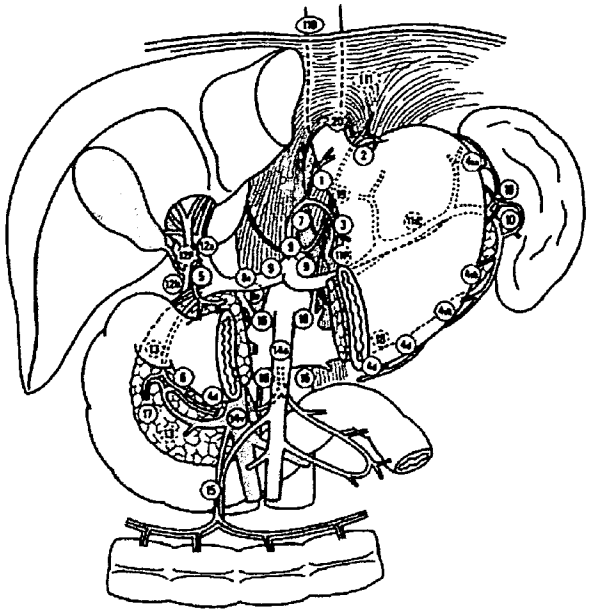

Figure 1. Schema of the location of the gastric regional lymph node stations (by Japanese Classification (5)) (please note that a colour version of this figure is available as supplementary data at http://www.jjco.oxfordjournals.org).

standard surgical procedure for gastric cancer (8,9), and more radical surgery with extended para-aortic lymph node dissection (PAND) has been practiced to improve the survival for advanced gastric cancer in some specialized centers (10–13). Because PAND was controversial, a randomized controlled trial, the Japan Clinical Oncology Group Study 9501, was launched in 1995 to explore the potential survival benefit of D2 plus PAND over D2 dissection.

In the present study, we focused on 260 gastric cancer patients in the experimental treatment arm of JCOG9501 who underwent curative gastrectomy with D2 plus PAND, to identify the risk factors for PAN metastasis and the most likely route of metastasis to PAN.

PATIENTS AND METHODS

We used data obtained from the JCOG9501 study. The details of this phase III trial have been described elsewhere (14). Briefly, the eligibility criteria were histologically proven adenocarcinoma of the stomach, T2(subserosa)-T4, M0, no macroscopic metastasis to the PAN, negative lavage cytology, adequate organ function, and age $\leq$75 years. Linitis plastica ('Bormann type 4') was excluded. All of the patients gave written informed consent to the study. Randomization and data handling were performed by the JCOG Data Center, a government-sponsored organization to perform multicenter clinical trials. Approval of the institutional review board was obtained at all participating institutions. The 24 institutions belonging to the Gastric Cancer Surgical Study Group of the JCOG participated in the trial.

From June 1995 to April 2001, 523 gastric cancer patients were randomized, and 260 patients were assigned to an experimental treatment arm and underwent D2 plus PAND surgery. In this group, PAN were dissected from the level of the celiac trunk down to the root of the inferior mesenteric artery (stations No. 16a2 and No. 16b1).

All the data were recorded according to the 12th Edition of the GRGCS (6) which was available at the start of the study. Although the 13th Edition (1998) with new nodal classification (N1–N3) is currently available (5), we used the original data description in the present study.

The clinicopathological parameters that could be identified pre- or intra-operatively to decide the indication for PAND were compared between patients with and without PAN metastasis. The Fisher's exact test or χ^2 test were used to assess the differences in proportion. To assess the association of various factors with PAN metastasis, multivariate logistic regression analysis was used with backward elimination procedure for variable selection with $\alpha = 0.20$. Next, the association between the histological status of 17 regional lymph node stations and the proportion of PAN metastatis were evaluated with odds ratio. In addition, to assess the relative strength of the association between lymph nodes and the PAN metastasis, all the 17 nodal stations were included in the multivariate logistic regression with backward elimination procedure for variable selection with $\alpha = 0.20$.

Table 1. Categories of the gastric regional lymph nodes divided by the location

Category*	Tumor location			
	Lower third	Middle third	Upper third	Whole stomach
N1	3, 4sa, 4sb, 4d, 5, 6	1, 3, 4sa, 4sb, 4d, 5, 6	1, 2, 3, 4sa, 4sb	1, 2, 3, 4sa, 4sb, 4d, 5, 6
N2	1, 7, 8a, 9	2, 7, 8a, 9, 10, 11	4d, 5, 6, 7, 8a, 9, 10, 11, 20	7, 8a, 9, 10, 11
N3	2, 8p, 10, 11, 12, 13, 14v, 17, 18	8p, 12, 13, 14v, 17, 18	8p, 12, 13, 14v, 17, 18, 19, 110, 111	8p, 12, 13, 14v, 17, 18, 20, 110, 111
N4	14a, 15, 16, 19, 20	14a, 15, 16, 19, 20	14a, 15, 16	14a, 15, 16, 19

*Categories of the regional lymph nodes were classified according to the 12th Edition of the Japanese General Rules for Gastric Cancer Study (6).

Table 2. Association between clinicopathological factors and histological metastasis of para-aortic lymph nodes (PAN)

Factors	Category	Proportion of PAN metastasis (%)	Univariate		Multivariate	
			Odds ratio (95% CI)	*P* value	Odds ratio (95% CI)	*P* value
Sex	Male	9.9% (18/182)	2.0 (0.7–6.2)	0.24	2.4 (0.7–7.7)	0.16
	Female	5.1% (4/78)				
Body mass index	<25	9.0% (20/221)	1.8 (0.4–8.2)	0.55	–	–
	25	5.1% (2/39)				
Macroscopic type	3, 5	9.9% (15/151)	1.6 (0.6–4.1)	0.37	–	–
	0, 1, 2	6.4% (7/109)				
Tumor location	Lower	10.9% (12/110)	5.6 (0.7–45.5)	0.19	–	–
	Middle	8.7% (9/103)	1.3 (0.5–3.2)		–	
	Upper	2.1% (1/47)				
Tumor size	5 cm	12.7% (21/165)	13.7 (1.8–103.6)	<0.001	8.2 (1.1–64.5)	0.045
	<5 cm	1.1% (1/95)				
Histological type	Undifferentiated	11.7% (18/154)	3.4 (1.1–10.3)	0.025	2.7 (0.8–8.8)	0.093
	Differentiated	3.8% (4/106)				
T stage	T3, T4	10.8% (18/167)	2.7 (0.9–8.2)	0.10	–	–
	T2(SS)	4.3% (4/93)				
N stage*	N2, N3, N4	20.5% (17/83)	8.9 (3.1–25.0)	<0.001	6.9 (2.4–20.0)	<0.001
	N0, N1	2.8% (5/177)				

*Macroscopic N stage was classified according to the 12th Edition of the Japanese General Rules for Gastric Cancer Study (6).

Two-sided *P* values were calculated and are presented. Statistical analysis was performed using SAS version 8.12 software (SA5 Institute, Tokyo, Japan).

In order to validate reproducibility of the predictive factors detected in this prospective study, we analyzed a retrospectively collected data set consisting of 158 patients who had undergone gastrectomy with PAND at Osaka Medical College between 1978 and 1999.

RESULTS

The patients ranged in age from 27 to 75 years (mean age, 61.0 years) and included 182 men and 78 women. In 47 of the 260 patients, the tumor was located in the upper third of the stomach, while it was in the middle third in 103 and the lower third in 110. Total gastrectomy was performed in 97 patients, distal gastrectomy in 160, and proximal gastrectomy in three.

PAN metastasis was histologically found in 22 (8.3%) of 260 patients. The association between the possible risk factors and PAN metastasis is shown in Table 2. Tumor size $\geq$ 5 cm, undifferentiated type of histology, and macroscopic N2–4 stage at surgery showed significant association in univariate analysis. After adjustment of other variables, macroscopic N stage and tumor size showed statistically significant association. The proportion of PAN metastasis stratified with macroscopic N stage is shown in Table 3. There were no significant associations in sex, body mass index, macroscopic tumor type, tumor location or macroscopic T stage.

In the independent data set from Osaka Medical College, the above results were reproduced; in macroscopically N0/N1 cases, PAN metastasis was found in 1.6% (1/64), while in macroscopically N2 or N3/4, PAN metastasis was found in 9.8% (6/61) and 42.4% (14/33), respectively (Table 3).

We next examined the associations between the histological status of 17 regional lymph node stations and PAN metastasis (Table 4). Most nodal stations except for those along the greater curvature of the stomach (No. 2, 4sa, 4sb, 10) and No. 13, had significant association with PAN

Table 3. Proportion of histological metastasis of para-aortic lymph nodes (PAN) stratified with macroscopic N stages

N stage*	Proportion of PAN metastasis (%)	
	JCOG9501	Osaka Medical College
N0	1/42 (2.4)	0/13 (0)
N1	4/135 (3.0)	1/51 (2.0)
N2	12/72 (16.7)	6/61 (9.8)
N3–4	5/11 (45.5)	14/33 (42.4)

*Macroscopic N stage was classified according to the 12th Edition of the Japanese General Rules for Gastric Cancer Study (6).

Table 4. Association between histological metastasis of 17 regional lymph node stations and that of para-aortic lymph nodes (PAN)

Lymph node station	Histological metastasis	Proportion of PAN metastasis (%)	Odds ratio (95% CI)	*P* value
1	+	27.5% (11/40)	7.2 (2.9–18.1)	<0.001
	−	5.0% (11/220)		
2	+	20.0% (2/10)	1.9 (0.4–10.0)	0.61
	−	11.7% (12/103)		
3	+	18.0% (21/117)	31.1 (4.1–234.8)	<0.001
	−	0.7% (1/143)		
4sa	+	20.0% (2/10)	2.2 (0.4–11.5)	0.31
	−	10.4% (11/106)		
4sb	+	13.3% (2/15)	1.7 (0.4–8.2)	0.37
	−	8.2% (20/245)		
4d	+	16.0% (12/75)	3.3 (1.4–8.0)	0.012
	−	5.4% (10/184)		
5	+	24.2% (8/33)	4.8 (1.8–12.6)	0.003
	−	6.2% (14/225)		
6	+	21.8% (17/78)	9.8 (3.5–27.6)	<0.001
	−	2.8% (5/180)		
7	+	45.5% (15/33)	26.2 (9.5–72.5)	<0.001
	−	3.1% (7/227)		
8a	+	28.6% (12/42)	8.3 (3.3–20.9)	<0.001
	−	4.6% (10/218)		
8p	+	40.0% (4/10)	8.5 (2.2–32.9)	0.006
	−	7.3% (17/233)		
9	+	35.3% (6/17)	7.7 (2.5–23.6)	0.001
	−	6.6% (16/243)		
10	+	25.0% (2/8)	3.3 (0.6–18.6)	0.20
	−	9.3% (9/97)		
11	+	33.3% (8/24)	7.9 (2.9–21.7)	<0.001
	−	5.9% (14/236)		
12	+	50.0% (3/6)	11.9 (2.2–63.5)	0.010
	−	7.7% (18/233)		
13	+	40.0% (2/5)	6.4 (1.0–40.7)	0.084
	−	9.5% (17/179)		
14v	+	37.5% (3/8)	6.6 (1.4–29.9)	0.030
	−	8.4% (17/203)		

metastasis ($P < 0.05$). Among those 12 stations, No. 3 and No. 7 showed much higher odds ratios than others. When we entered the histological status of all N1 or N2 stations to the multivariate logistic regression model, any stations except No. 7 were removed owing to the variable selection with $\alpha = 0.20$. Station No. 7 was shown to be statistically significant ($P = 0.002$) with the odds ratio of 41.0 (95% confidence interval (CI), 4.0–425.3). When we used the histological status of station No. 7 as the diagnostic factor of PAN metastasis, the sensitivity and specificity were calculated at 68.2 and 92.4%, respectively.

DISCUSSION

In the present study, the incidence of PAN metastasis was significantly higher in patients with undifferentiated tumor, large tumor and tumor with macroscopic N2–4. Similar results have been reported in retrospective studies by other researchers (15,16). Among these factors, macroscopic N stage ($P < 0.001$) and tumor size ≥ 5 cm ($P < 0.045$) were significant risk factors for PAN metastasis after adjusting for other variables. Only one tumor smaller than 5 cm had PAN metastasis, while 12.5% of larger tumors had metastasis.

The incidence of PAN metastasis was clearly different between the N0–1 group and the N2–4 group (2.8% versus 20.5%), and its odds ratio was 8.6 (95% CI, 3.1–24.2). The results were reproduced in an independent validation dataset.

As for the regional lymph node status, most of them were associated with PAN metastasis but station No. 7 was the only significant indicator to PAN metastasis after adjusting for other variables. The diagnostic sensitivity and specificity of station No. 7 for PAN metastasis were as high as clinically useful and this may be a convenient diagnostic indicator for PAN metastatis. Although station No. 9 around the celiac artery is located between station No. 7 and PAN, the histological status of station No. 9 did not show statistical significance in this multivariate analysis. It might be due to the high correlation between No. 7 and No. 9 status. Actually, all six cases with metastases in both station No. 9 and PAN were also positive in No. 7 station. This result indicated that the pathological status of No. 7 was considered to be the confounding factor between No. 9 and PAN status. Another explanation is that metastatic cancer cells that left No. 7 nodes enters PAN through the celiac route but sometimes without being trapped by No. 9 nodes. Or else, while the No. 7 lymph nodes along the left gastric artery can easily be identified during surgery, metastatic nodes at No. 9 station may be missed or misclassified at post-operative nodal retrieval.

This finding also helps us to study the pattern of lymphatic flow to the nodes surrounding the abdominal aorta. Lymphatic flow is thought to reach the para-aortic nodes via the following possible routes: (i) directly from the left para-cardial lymph nodes, (ii) from the lymph nodes along the splenic artery, (iii) from the lymph nodes around the celiac artery, (iv) from the lymph nodes along the superior mesenteric artery, and (v) from the lymph nodes on the posterior surface of the pancreatic head and the nodes along the posterior common hepatic artery (3,4). In this study, 15 of the 22 patients with PAN metastasis had involvement of lymph node No. 7, which is located by the celiac trunk. This suggests that the most likely route for PAN metastasis is from the left gastric artery nodes passing by the celiac artery.

JCOG9501 had superior quality control of surgical procedures and should provide more reliable data than previous retrospective studies. This also provides us with reliable information about metastasis to PAN, although the number of patients with PAN metastasis was not large ($n = 22$). The possible survival impact of PAND should be clarified in further analyses.

In conclusion, this study indicated that macrosocpic N staging and tumor size ≥ 5 cm were important and independent risk factors for PAN metastasis, and that the lymphatics accompanying the celiac artery seem to be the most frequent route for metastasis to PAN. Station No. 7 was the most diagnostic lymph node for indicating the status of PAN.

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Conflict of interest statement

None declared.

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Clonal and Parallel Evolution of Primary Lung Cancers and Their Metastases Revealed by Molecular Dissection of Cancer Cells

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Abstract **Purpose:** Several models of cancer progression, including clonal evolution, parallel evolution, and same-gene models, have been proposed to date. The purpose of this study is to investigate the authenticity of these models by comparison of accumulated genetic alterations between primary and corresponding metastatic lung cancers.

Experimental Design: A whole-genome allelic imbalance scanning using a high-resolution single nucleotide polymorphism array and mutational analysis of the *p53*, *EGFR*, and *KRAS* genes were done on eight sets of primary and metastatic lung cancers. Based on the genotype data, the natural history of each case was deduced, and candidate metastasis suppressor loci were determined.

Results: Five to 20 chromosomal regions showed allelic imbalance in each tumor. Accumulated genetic alterations were similar between primary and corresponding metastatic tumors, and the majority(>67%) of genetic alterations detected in metastatic tumors was also detected in the corresponding primary tumors. On the other hand, in seven of the eight cases, there were genetic alterations accumulated only in metastatic tumors. Among these alterations, allelic imbalances at chromosome 11p15 and 11p11-p13 regions were the most frequent ones (4 of 8, 50%). Likewise, four cases showed genetic alterations detected only in primary tumors.

Conclusions: The natural history of each case indicated that the process of metastasis varies among cases, and that all three models are applicable to lung cancer progression. According to the clonal and parallel evolution models, it is possible that a metastasis suppressor gene(s) for lung cancer is present on chromosome 11p.

Metastasis is a principal event leading to death in individuals with cancer. However, the molecular basis of metastasis is still unclear. A generally accepted model for tumor progression is the "clonal evolution" model. This model is well illustrated in colorectal carcinogenesis (1) and holds that more malignant cells with additional genetic alterations predominate in a tumor cell population. In this model, metastasis represents the end stage of evolution, and the presence of genetic alterations responsible for the metastatic ability of tumor cells is predicted. The finding that primary tumor cell populations are comprised of cells with different metastatic abilities supports this model (2). If tumor cells with such genetic alterations consist of a small subpopulation among the primary tumor cells, these alterations can be detected only in metastatic tumors but not (or hardly) detected in the corresponding primary tumor (3–5). In fact, we and others identified several genetic alterations that were detected only in metastatic tumors but not in the corresponding primary tumors (6–12), supporting the authenticity of this model.

Recently, other models for tumor progression and metastasis were also proposed. One is "the parallel evolution model," which proposes early occurrence of metastasis and parallel evolution of primary and metastatic tumors (13). This was based on the report of Schmidt-Kittler et al. that genetic alterations in breast cancer cells that disseminated into the bone marrow of patients generally do not resemble those in the corresponding primary tumors (14). The parallel evolution model has been very applicable to the metastatic pattern of several solid epithelial tumors (15–17). In addition, information provided by global gene expression profiling of primary tumor cells led to the proposal of another model, "the same-gene model" by Bernards and Weinberg (18). This model holds that genetic alterations acquired early in carcinogenesis confer not only a selected replicative advantage but also a proclivity to metastasize on cancer cells. This was based on a report of van't

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Table 1. Characterization of eight cases of primary and metastatic lung cancers

Case	Age*	Gender	Smoking	Histology (differentiation, stage†)	Sample (interval time‡)	Genotype call (%)
1	48	F	–	ADC (M/D, IIIA)	N	96.4
					P	89.2
					B-M (26)	91.9
2	43	F	–	ADC (M/D, IIA)	N	89.0
					P	87.9
					B-M1 (18)	84.5
					B-M2 (33)	89.1
3	59	M	+	ADC (W/D, IIB)	N	88.0
					P	86.5
					B-M1 (40)	92.7
					B-M2 (64)	93.1
4	51	M	+	SCC (M/D, IIIA)	N	81.9
					P	90.5
					B-M (35)	89.6
5	40	M	+	LCC (IIB)	N	95.0
					P	92.3
					B-M (3)	92.2
6	67	M	+	SCLC (IIIA)	N	84.1
					P	90.7
					MLN-M (0)	89.6
7	55	F	–	SCLC	N	88.6
					P	92.2
					Pu-M	91.2
					Li-M	90.9
8	79	M	+	SCLC	N	87.8
					PI	88.0
					HLN-M	88.9
					PI-M	88.9
					Li-M	88.8
					PaLN-M	90.8

Abbreviations: ADC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large cell carcinoma; W/D, well differentiated; M/D, moderately differentiated; P/D, poorly differentiated; N, adjacent normal lung tissue; P, primary lung tumor; M, metastasis; B, brain; HLN, hilar lymph node; PI, pleural; Li, liver; PaLN, paraaortic lymph node; Pu, pulmonary; MLN, mediastinal lymph node.

*Age at diagnosis.

†Pathologic stage at surgery of primary lung tumor.

‡Months from the surgery of primary lung tumor.

§Number and % of common allelic imbalance = number and percentage of regions with allelic imbalance common to primary and metastatic tumors including partial overlap.

‖Number of accumulated allelic imbalance = number of regions with allelic imbalance specific to primary or metastatic tumor.

¶$P < 0.05$ for difference against fraction of error call.

Veer et al. that prognosis of patients with breast cancer can be predicted by gene expression profiles of primary tumors (19). Ramaswamy et al. then showed that a subset of primary tumors resembled metastatic tumors with respect to gene expression signature (20). In this model, it was hypothesized that there are no genes and genetic changes specifically and exclusively involved in orchestrating the process of metastasis.

In the present study, we investigated the authenticity of the tumor progression models above by comparison of primary and corresponding metastatic tumors obtained from eight patients with lung cancer. We focused on genetic alterations rather than expression profiles because genetic alterations are stable and irreversible and, thus, can be used as a "molecular footprint" of cancer progression (4). There have been several studies using this strategy (6, 7, 9–12), including the one by Schmidt-Kittler et al. as described above (14). However, in most of these studies, only a limited number of genetic loci and/or genes were examined. Recent progress in array technology has enabled us to accomplish not only high-resolution analysis of expression status but also that of genetic status. Thus, in this study, we used a high-resolution single nucleotide polymorphism (SNP) array, mapping 10k, which covers 11,560 loci throughout the whole human genome in 210-kb mean intervals, for the allelic imbalance scanning to obtain comprehensively information on allelic status. Furthermore, to obtain more precise data, the laser capture microdissection method was used to enrich cancer cell components in five non-small cell cancer (NSCLC) cases because various fractions of noncancerous cells are often contaminated in macrodissected NSCLC samples. In addition to allelic imbalance scanning, mutation analysis of the *p53*, *EGFR*, and *KRAS* genes was done because these genes are frequently mutated in lung cancer.

Materials and Methods

Patients and tissues. Seven primary lung tumors, their 12 corresponding metastases (seven brain, one liver, one pleural, and three lymph node), and seven corresponding normal lung tissues were

Table 1. Characterization of eight cases of primary and metastatic lung cancers (Cont'd)

Fraction of allelic imbalance (%)	Fraction of error calls (%)	Fraction of different calls (%)	No. of allelic imbalance regions (no. and % of common allelic imbalance[§])	No. of accumulated allelic imbalance[‖] (no. of extended allelic imbalance)
63.0	2.0		13	0
70.5	0.7	9.2[¶]	14 (13, 93%)	1 (0)
58.2	4.5		18	1 (1)
69.4	5.4	25.2[¶]	19 (17, 89%)	5 (3)
70.4	3.9	18.1[¶]	20 (18, 90 %)	4 (2)
70.9	7.2		16	0
73.3	8.4	5.3	16 (16, 100%)	0
72.4	9.0	5.0	16 (16, 100%)	0
31.8	25.8		14	0
37.2	26.3	4.0	14 (14, 100%)	3 (3)
51.2	0.3		15	3 (1)
47.8	0.3	13.6[¶]	16 (13, 81%)	4 (1)
33.8	8.1		10	2 (0)
45.0	6.8	9.7[¶]	12 (8, 67%)	6 (2)
55.2	2.7		13	0
57.7	2.8	6.3[¶]	14 (13, 93%)	1 (0)
59.0	3.0	2.3	16 (13, 81%)	3 (0)
35.0	4.9		10	1 (0)
32.3	5.1	2.1	9 (9, 100%)	0
28.4	4.7	2.1	8 (8, 100%)	0
19.8	4.9	8.3[¶]	8 (8, 100%)	2 (1)
25.5	4.9	8.6[¶]	7 (6, 86%)	2 (1)

obtained at surgery or autopsy from seven patients who were treated at the National Cancer Center Hospital, Tokyo, Japan. One primary lung tumor, two corresponding metastases (one pulmonary and one liver), and a corresponding normal lung tissue were also obtained by autopsy at Tokushima University Hospital, Tokushima, Japan (case 7 in Table 1). In total, 30 DNA samples from the eight cases were subjected to DNA array analysis (Table 1). These cases were histologically classified as three adenocarcinomas, one squamous cell carcinoma, one large cell carcinoma, and three small cell carcinomas (SCLC) according to the WHO criteria (21). In five NSCLC cases (cases 1-5), normal lung tissues were obtained at surgery from regions >5 cm distance from tumors and showing macroscopically normal morphology in the resected lobes of the lung. Tumors and normal lung tissues were then separately fixed with methanol and embedded in paraffin. Cancerous and noncancerous cells of these five cases were obtained by the laser capture microdissection method using the Pixcell Laser Capture Microdissection system (Arcturus Engineering, Mountain View, CA). Noncancerous cells were isolated from a normal lung tissue section but not from a region surrounding the tumor of a tumor tissue section under microscopic observation. Genomic DNAs were extracted as described previously (22). In an SCLC case (case 6), normal lung tissue was also obtained at surgery from a region >5 cm distance from the primary tumor and showing macroscopically normal morphology in the resected lobe. In two other SCLC cases (cases 7 and 8), normal lung tissues were obtained at autopsy from lobes without tumors macroscopically. In these three SCLC cases, tumors and normal lung tissues were stored at −80°C without fixation until DNA extraction. Cancerous and noncancerous cells of the three SCLC cases (cases 6-8) were macrodissected, and genomic DNAs were prepared as described previously (23, 24). This study was undertaken according to the guidelines for medical research in Japan. All the samples were analyzed after declaring anonymity to keep the privacy of individuals.

SNP array analysis. High-resolution SNP array, mapping array 10k (Affymetrix, Santa Clara, CA), was used according to the manufacturer's protocol with some modifications as described below. In the original protocol, PCR of 35 cycles is undertaken against 250 ng DNA to obtain 20 μg of whole-genome amplicon. However, due to the small number of cells obtained by laser capture microdissection, 10 to 50 ng DNAs were subjected to PCR of 35 to 45 cycles to obtain 20 μg of the amplicon. Twenty micrograms of the amplicon were purified, labeled, and hybridized to the array, and genotype calls were obtained as described previously (25).

The accuracy of the genotype calls obtained by the modified protocol was estimated as follows. A noncancerous lung tissue of another large cell carcinoma patient was subjected to macrodissection and laser capture microdissection, and genomic DNAs were extracted from both materials. Two hundred fifty nanograms of DNA from the macrodissected material were subjected to DNA array analysis according to the standard protocol (i.e., 35 cycles of PCR). Five or 50 ng of DNA from the laser capture microdissection material were subjected to DNA array analysis according to the modified protocol (i.e., 35, 40, and 45 cycles of PCR). The concordance of genotype calls among these preparations was >99%.

Statistical analyses for allelic imbalance in primary and metastatic tumors. The fraction of allelic imbalance for each tumor sample was calculated as the fraction of SNP probes for which noncancerous cell DNA was called as heterozygous and cancer cell DNA was called as homozygous. The fraction of error calls for each tumor sample was calculated as the fraction of SNP probes for which noncancerous cell DNA was called as homozygous and for which cancer cell DNA was called as heterozygous. The fraction of different calls between primary and metastatic tumors was calculated as the fraction of SNP probes for which primary tumor cell DNA was called as homozygous and heterozygous and for which the metastatic tumor cell DNA was called

Table 2. Allelic imbalance and mutations in eight cases of primary and metastatic lung cancers

Chromosome and gene	Case 1		Case 2			Case 3			Case 4		Case 5		Case 6	
	P	B-M	P	B-M1	B-M2	P	B-M1	B-M2	P	B-M	P	B-M	P	MLN-M
1									2					
2														
3									2	2				
4														
5														
6														
7														
8														
9														
10														
11														
12														
13														
14														
15														
16														
17														
18														
19														
20														
21														
22														
p53														
EGFR									ND	ND	ND	ND	ND	ND
KRAS									ND	ND	ND	ND	ND	ND

NOTE: ▒, presence of allelic imbalance or mutation. ■, presence of additional alteration in metastatic tumor(s). ■, presence of additional alteration in primary tumor. □, absence of allelic imbalance or mutation. 2, allelic imbalance in two independent regions.
Abbreviations: ND, not determined; P, primary lung tumor; M, metastasis; B, brain; HLN, hilar lymph node; Pl, pleural; Li, liver; PaLN, paraaortic lymph node; Pu, pulmonary; MLN, mediastinal lymph node.
*Number of cases with common allelic imbalance/mutation between primary tumor and at least one metastatic tumor(s).
†Number of cases with ■.
‡Number of cases with ■.

as heterozygous and homozygous, respectively. The statistical significance for the excess of fraction of allelic imbalance in primary and metastatic tumors and of different calls between primary and metastatic tumors over the fraction of error calls was calculated by the χ^2 test. A level of $P < 0.05$ was considered statistically significant.

Definition of regions of allelic imbalance. When a locus was called "homozygous" in tumor DNA and "heterozygous" in the corresponding normal tissue DNA, such a locus was judged as being an "allelic imbalance" in the tumor. On the other hand, when a locus was called "heterozygous" both in the tumor and corresponding normal tissue DNA, such a locus was judged as being "not allelic imbalance" in the tumor. By taking the call error in the SNP array analysis into account, regions containing at least six consecutive "allelic imbalance" (or "not allelic imbalance") loci were defined as the ones of allelic imbalance (or not allelic imbalance). If the region of allelic imbalance in a primary tumor overlapped that in the corresponding metastatic tumor(s) and the overlapping region contained more than six consecutive allelic imbalance loci both in the primary and metastatic tumors, such a region was judged as a common region of allelic imbalance. In contrast, if a region judged as allelic imbalance in a primary tumor (or metastatic tumors) contained more than six consecutive "not allelic imbalance" loci in metastatic tumors (or primary tumor), such a region was judged as a unique region of allelic imbalance in either primary or metastatic tumor. If allelic imbalance at the same region was due to a gain or loss of different alleles between primary and metastatic tumors, such a region was also judged as being a unique region of allelic imbalance in respective primary and metastatic tumors. If there was a common region between primary and metastatic tumors, but the region in the metastatic tumor was wider (or narrower) than that in the corresponding primary tumor, allelic imbalance in the metastatic tumor was judged to have occurred independently of allelic imbalance in the primary tumor.

Microsatellite analysis. Microsatellite markers were chosen based on the chromosomal locations mapped in the human genome-wide screening set version 9 (Research Genetics, Inc., Huntsville, AL) or the Japan Biological Information Research Center genome database (http://www.jbirc.aist.go.jp/gdbs/). Two hundred picograms to 1 ng DNA was

Table 2. Allelic imbalance and mutations in eight cases of primary and metastatic lung cancers (Cont'd)

Case 7			Case 8					Common*	M specific†	P specific‡
P	Pu-M	Li-M	P	HLN-M	PI-M	Li-M	PaLN-M			
								1	2	0
								6	0	1
								7	1	1
								6	2	0
								5	2	0
								4	1	0
								5	0	2
								6	1	0
								7	0	0
								7	1	0
								3	4	1
								4	2	0
								7	0	0
								3	1	0
								3	1	1
								3	1	0
								8	1	0
								5	2	0
								5	0	0
								3	1	0
								1	0	0
								3	1	1
								7	0	0
ND	ND	ND	ND	ND	ND	ND	ND	3	0	0
ND	ND	ND	ND	ND	ND	ND	ND	0	0	0

used for PCR of 40 cycles with a set of primers labeled with FAM or TET. PCR products were run through an ABI Prism 310 DNA Sequencer (Applied Biosystems, Foster City, CA) and analyzed by the ABI PRISM GeneScan and Genotyper software. A reduction >75% of an allele in tumor was determined as allelic imbalance.

Mutation analysis of the p53, EGFR, *and* KRAS *genes.* All eight cases subjected to the SNP array analysis were examined for mutations in exons 4 to 8 of the *p53* gene. Three adenocarcinoma cases were previously examined for mutations in exons 1 to 2 of the *KRAS* gene and exons 18 to 21 of the *EGFR* gene (26). Two hundred picograms to 1 ng of DNA was subjected to PCR amplification followed by sequencing as described previously (27).

Results

Detection of allelic imbalance in primary and metastatic tumors. Eight primary lung tumors, eight corresponding normal lung tissues, and 14 metastases were subjected to the SNP array analysis (Table 1). Genotype calls were obtained in 81.9% to 96.4% (average = 89.7%) of the 11,560 SNP sites on the array; 1,439 to 3,001 loci were informative (i.e., heterozygous in noncancerous tissues) for detection of allelic imbalance in the tumors, and fractions of allelic imbalance ranged from 19.8% to 73.3% (Table 1). Fractions of error calls for the tumors were estimated as being 0.3% to 26.3% (see Materials and Methods; Table 1) and were significantly lower than the fractions of allelic imbalance ($P < 0.05$). Therefore, it was indicated that all tumors analyzed had allelic imbalances.

Fractions of different calls between primary and metastatic tumors ranged from 2.1% to 25.2% (Table 1) and were much smaller than fractions of allelic imbalance in primary tumors in all cases. Therefore, it was indicated that the majority of genetic alterations are common between primary and metastatic tumors. However, in 8 of the 14 metastatic tumors, fractions of different calls against primary tumors were significantly higher than fractions of error calls ($P < 0.05$), indicating the presence of allelic imbalances that differentially occurred between primary and metastatic tumors. In the remaining six metastatic tumors, fractions of different calls were not significantly higher but lower than fractions of error calls. Thus, the allelic status of these metastatic tumors might not have been different from that of respective primary tumors. Alternatively, differences in the allelic status between them might have been masked by the error calls.

Common genetic alterations among eight cases. Regions of allelic imbalance were next searched for along the genomes of the 8 primary and 14 metastatic tumors. Fractions of error calls in the present SNP array analysis were estimated as being up to

26.3%. Therefore, by taking the call error into account, regions containing at least six consecutive allelic imbalance loci were defined as allelic imbalance regions because the appearance of such loci by the call error was <1 even for case 4 for which the highest probability of call error (26.3%) was inferred at a SNP locus [i.e., 1,849 informative loci × $(0.263)^6$ = 0.61]. The number of allelic imbalance regions defined by this criterion ranged from 5 to 20 among 22 tumors analyzed. This was not the same between primary and metastatic tumors in six of the eight cases (cases 1, 2, and 5-8) and was the same in the remaining two cases (cases 3 and 4). The number of allelic imbalance regions in metastases was higher than that in primary tumors in five of the six cases (cases 1, 2, 5, 6, and 7), whereas it was lower in the remaining one case (case 8). These results indicate the presence of allelic imbalance only in either metastases or primary tumors. Chromosomes showing allelic imbalance and *p53/EGFR* mutations in each tumor are indicated in gray in Table 2. There were various regions showing allelic imbalance commonly both in primary and metastatic tumors in all eight cases, and such regions were distributed among all chromosomes. *p53* mutations were detected in seven of the eight cases, whereas *EGFR* mutations were detected in all three adenocarcinoma cases (cases 1-3). No *KRAS* mutations were detected in them. All the *p53* and *EGFR* mutations were detected in primary tumors, and the same types of mutations were detected in their corresponding metastatic tumors. The chromosome 17p11-p13 region was the most common region of allelic imbalance detected both in primary and metastatic tumors (8 of 8, 100%). Allelic imbalances of chromosome regions 3p11-p26, 10q23-q26, and 13q12-q31 were the next common ones (7 of 8, 88%). All allelic imbalances defined as common were gains or losses of the same allele between primary and metastatic tumors. The allelic status of several loci that showed allelic imbalance in tumors by SNP array analysis was confirmed by microsatellite analysis. All the loci examined showed allelic imbalance as indicated by the SNP array analysis (data not shown).

Table 3. Chromosomal regions of additional allelic imbalances in primary or metastatic tumors

Case	Sample	Region of additional allelic imbalance
1	P	None
	B-M	1p31-p35
2	P	7p14-p22
	B-M1	5p15, 11p15, 11p13-q13, 12q11-q22, 16q11-q24
	B-M2	5p15, 11p15, 11p13-q14, 16q11-q24
3	P	None
	B-M1	None
	B-M2	None
4	P	None
	B-M	1q11-q25, 6q11-q14, 10q22-q23
5	P	2q11-q37, 3q24-q29, 7p22-q21, 7q22-q36
	B-M	8q11-q21, 11p11-p15, 12q11-q24, 17q11-q21
6	P	15q11-q26, 22q12-q13
	MLN-M	4p16-q12, 5p15, 11p15-q25, 15q11-q26, 18p11, 22q11-q13
7	P	None
	Pu-M	11p15-q25
	Li-M	4q11-q21, 4q31-q35, 20p11-p12
8	P	11q11-q25
	HLN-M	None
	Pl-M	None
	Li-M	3q26-q29, 18p11-q23
	PaLN-M	14q11-q13, 18p11-q23

Abbreviations: P, Primary tumor; M, metastasis; B, brain; HLN, hilar lymph node; Pl, pleural; Li, liver; PaLN, paraaortic lymph node; Pu, pulmonary; MLN, mediastinal lymph node.

In these eight cases, there were also various chromosomal regions showing allelic imbalance only in metastases but not in primary tumors (indicated in red in Table 2) or those only in primary tumors but not in metastases (indicated in blue in Table 2). Seven of the eight cases, except case 3, had chromosomal regions with allelic imbalance only in metastases, and four of the eight cases (cases 2, 5, 6, and 8) had regions with allelic imbalance only in primary tumors. Such regions were widely distributed among diverse chromosomes among the cases, but several regions were common in multiple cases. Chromosomal regions showing allelic imbalance only in primary tumors or in metastases are listed in Table 3. Allelic imbalance of chromosomal regions 11p15 and 11p11-p13 was detected only in metastases in four of the eight cases (cases 2, 5, 6, and 7). These regions were the most common ones showing allelic imbalance only in metastases in this study. 11q11-q13 was the next common region (three cases), and 4q11-q12, 5p15, 12q11-q22, and 18p11 showed allelic imbalance only in metastases in two cases, respectively. On the other hand, allelic imbalance of the 7p14-p22 region was observed only in primary tumors but not in metastases in two cases (cases 2 and 5). Allelic imbalance of other chromosomal regions detected only in primary tumors or metastases was observed in a single case.

Similarity of genetic alterations between primary and metastatic tumors. The numbers of regions with allelic imbalance detected in each tumor are summarized in Tables 1 and 2. In case 1, 13 regions were defined as allelic imbalance in the primary tumor, and 14 regions were defined as allelic imbalance in the corresponding metastatic tumor. In this case, 13 regions were the same between primary and metastatic tumors (13 of 14, 93%), and one region on chromosome 1 (1p31-p35) showed allelic imbalance only in the metastatic tumor (1 of 14, 7%). The metastatic tumor of case 4 displayed 14 regions with allelic imbalance, and all of these regions were common between primary and metastatic tumors (14 of 14, 100%). However, among them, three regions were largely extended in the metastatic tumor (number of extended region is indicated in parenthesis in Table 1). Fractions of common allelic imbalance regions (number of common allelic imbalance regions / number of allelic imbalance regions) in metastatic tumors were 13 of 16 (81%) in case 5 and 8 of 12 (67%) in case 6. Likewise, fractions of common allelic imbalance regions in multiple metastatic tumors were 17 of 19 (89%) and 18 of 20 (90%) in case 2, 13 of 14 (93%) and 13 of 16 (81%) in case 7, and 9 of 9 (100%), 8 of 8 (100%), 4 of 5 (80%), and 6 of 7 (86%) in case 8. Interestingly, regions of allelic imbalance in case 3 were completely the same among the primary tumor and two metastatic tumors. These results further indicated that the majority of genetic alterations are common between primary and metastatic tumors in lung cancer. Furthermore, a considerable fraction of allelic imbalance (15 of 38, 40%) detected only in primary tumor or metastasis was caused by extension of

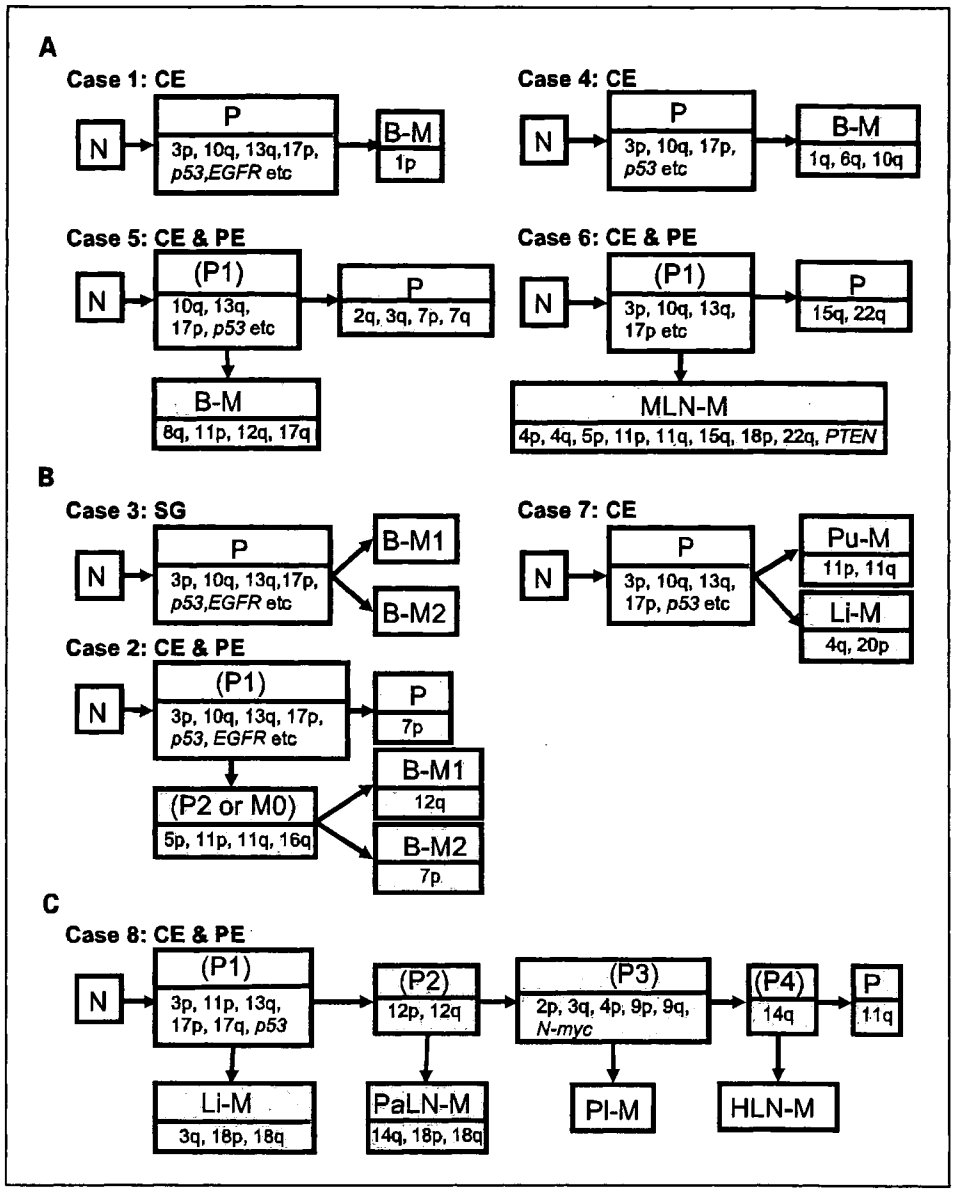

Fig. 1. Natural history of eight lung cancer cases. *A*, single metastases were available for analysis in cases 1, 4, 5, and 6. *B*, two metastases in cases 2, 3, and 7. *C*, four metastases in case 8. N, normal lung tissue; P, primary lung tumor; M, metastasis; B, brain; MLN, mediastinal lymph node; Pu, pulmonary; Li, liver; PaLN, paraaortic lymph node; PI, pleural; HLN, hilar lymph node. Putative tumors that served as precursors for the next evolution are indicated in parenthesis (*P1-P4* and *M0*). Allelic imbalance of a whole or partial chromosome arm is indicated by a chromosome arm, whereas *p53*, *EGFR*, and *PTEN* mutations and *N-myc* amplification are indicated by gene names. Alterations detected in all tumors (*white boxes*); alterations specifically observed in primary or metastatic tumors (*gray boxes*). Alteration of the *PTEN* and *N-myc* genes were detected in previous studies (20, 25, 26). Models for cancer progression supported by relationships of primary and metastatic tumors are indicated. CE, clonal evolution model; PE, parallel evolution model; SG, same-gene model.

allelic imbalance regions in paired tumors (indicated as extended allelic imbalance in Table 1).

Natural history of cancer progression deduced from the genotype differences between primary and metastatic tumors. Single metastatic tumors were available in four cases (cases 1, 4, 5, and 6; Fig. 1A). Our previous study showed a *PTEN* mutation only in the metastatic tumor but not in the primary tumor of case 6 (28). In cases 1 and 4, all genetic alterations detected in primary tumors were also detected in the respective metastatic tumors, and the metastatic tumors had additional allelic imbalances that were not observed in the primary tumors. On the other hand, in cases 5 and 6, there were regions with allelic imbalance that were detected only in primary tumors, and there were also regions with allelic imbalance that were observed only in metastatic tumors. Allelic imbalance of chromosomes 15 and 22 detected in case 6 were a loss or gain of different alleles between primary and metastatic tumors.

Two metastases were available in three cases (cases 2, 3, and 7; Fig. 1B). In case 3, no differences in the status of allelic imbalance were detected among the primary tumor and two metastases. On the other hand, two metastases in case 7 carried all allelic imbalances detected in the primary tumor and had additional allelic imbalances that were not detected in the primary tumor. Interestingly, allelic imbalances detected only in two metastases in case 7 did not overlap each other. In case 2, there were regions with allelic imbalances that were detected only in the primary tumor, in addition to regions with allelic imbalances that were observed only in metastatic tumors. Furthermore, two brain metastases had common regions of allelic imbalance in addition to unique regions of allelic imbalance in one of two metastases.

Four metastases were available in case 8 (Fig. 1C). The primary tumor of this case was previously shown to be composed of two different areas, and the *N-myc* gene was heterogeneously amplified in these areas (29). Genetic alterations

detected in all tumors were a *p53* mutation and allelic imbalances of chromosomes 3p, 11p, 13q, 17p, and 17q. Primary tumor, liver metastasis, and paraaortic lymph node metastasis had unique genetic alterations, whereas all genetic alterations detected in pleural metastasis and hilar lymph node metastasis were also detected in the primary tumor. Because common genetic alterations in the primary tumor were fewer in liver and paraaortic lymph node metastases than in pleural and hilar lymph node metastases, it is likely that metastases to the liver and paraaortic lymph node occurred earlier than those to pleural and hilar lymph nodes.

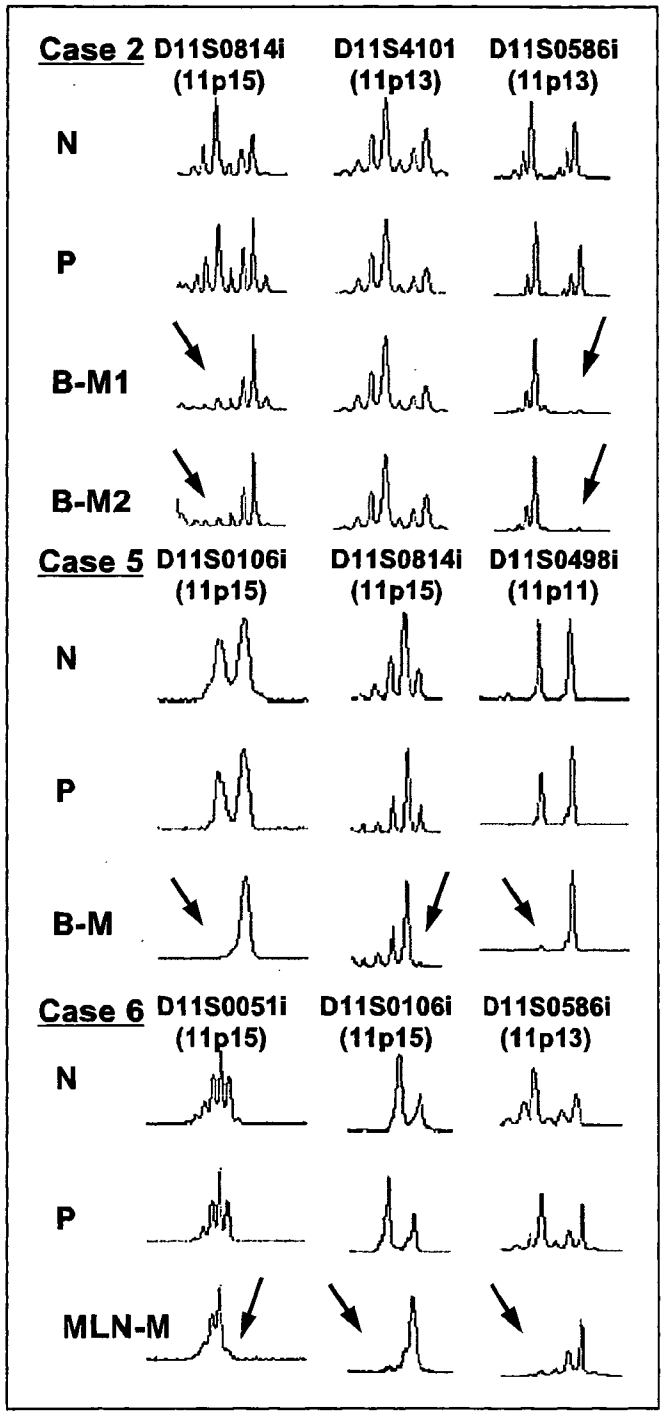

Fig. 2. Microsatellite analysis of chromosome 11p. Representative results of cases 2, 5, and 6. Names of microsatellite markers and their locations (*top*). Alleles (*two peaks*) and losses of one allele (*arrows*). N, normal lung tissue; P, primary lung tumor; B-M, brain metastasis; MLN-M, mediastinal lymph node metastasis.

Common regions of allelic imbalance on 11p in lung cancer. Frequent occurrence of allelic imbalance on chromosome 11p in metastatic tumors prompted us to examine the allelic status of this chromosome arm in primary and metastatic tumors by microsatellite analysis (Fig. 2). Two metastatic brain tumors of case 2 showed allelic imbalance at the *D11S0814i* (11p15) and *D11S0586i* (11p13) loci, whereas the corresponding primary tumors did not show allelic imbalance at these loci. Because the *D11S0149i*, *D11S0368i*, and *D11S4101* loci between the *D11S0814i* and *D11S0586i* loci did not show allelic imbalance in these metastases, the regions of allelic imbalance accumulated only in metastases of case 2 were determined as two different ones on chromosome 11p (Figs. 2 and 3). Cases 5, 6, and 7 also showed allelic imbalance of chromosome 11p only in metastases, and allelic imbalance was observed at all loci examined in these metastases, suggesting the occurrence of whole chromosome arm deletions only in metastases. Three other cases showed allelic imbalance at 11p15 both in primary and metastatic tumors (cases 1, 3, and 8). In two of them (cases 1 and 3), allelic imbalance was extended to the 11p11-p13 region both in primary and metastatic tumors. Thus, the 11p15 and 11p11-p13 regions were confirmed as being common for allelic imbalance in metastases. As indicated in Figs. 2 and 3, the result of the SNP array analysis was concordant with the result of microsatellite analysis.

Discussion

The present study indicates a high similarity of genetic alterations between primary and metastatic tumors. That is, metastatic tumors carried the majority of the genetic alterations present in the corresponding primary tumors. All the *p53* and *EGFR* mutations were detected in primary tumors and were retained in their corresponding metastatic tumors. This indicates that metastases have occurred at a late stage of lung cancer progression. The fact that genetic alterations in primary and metastatic tumors resembled each other went along well with previous results that expression profiles of metastatic tumors were similar to those of the corresponding primary tumors (19, 20). However, in seven of the eight cases, there were allelic imbalances detected only in metastases but not in primary tumors. To explain this result, we should consider two possibilities. The first possibility is that there were few cells carrying these additional genetic alterations in primary tumors; thus, we could not detect these alterations in the analysis of primary tumors (3–5). However, those few cells with these alterations selectively metastasized to distant organs or lymph nodes; thus, we were able to detect them in metastases. If this assumption is correct, the results imply that cancer cells in primary tumors are heterogeneous for accumulated genetic alterations. Because the analysis in this study looked at primary tumors in aggregate, we could not detect the alterations present in a few cells. Thus, taking the heterogeneity of cancer cells in primary tumors into consideration, the results of this study are consistent with those of a previous study (2) and match the clonal evolution model (1). From the viewpoint of this model, genetic alterations detected only in metastases might be

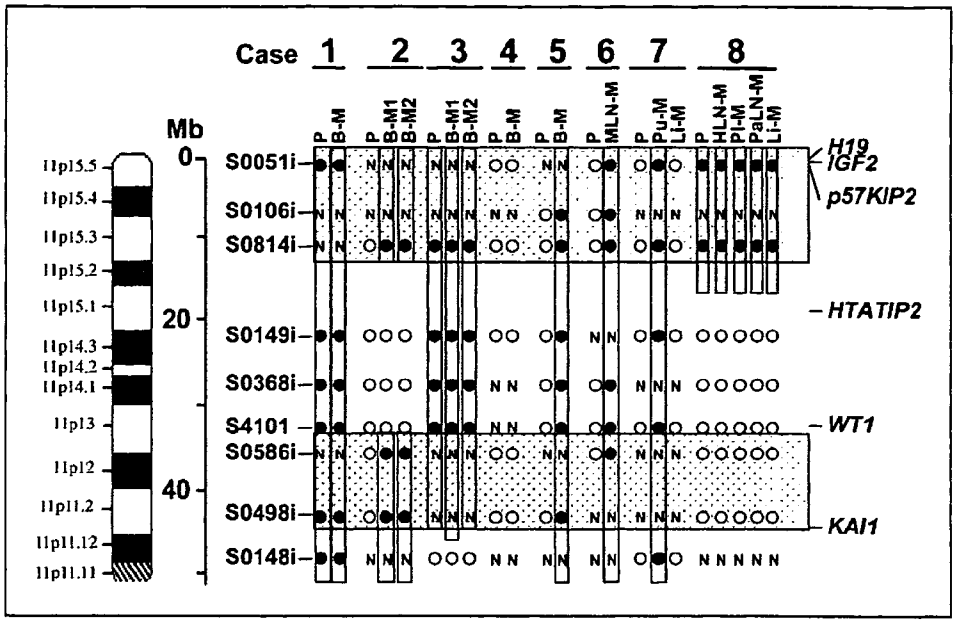

Fig. 3. Allelic imbalance on chromosome 11p. Physical position of chromosome 11p and locations of microsatellite markers (*left*). Cases and tumors (*top*). ▣, common region of allelic imbalance; □, region of allelic imbalance defined by DNA-array analysis; O, locus without allelic imbalance defined by microsatellite analysis; ●, locus of allelic imbalance defined by microsatellite analysis; N, not informative. Locations of cancer-related genes (*right*). P, primary lung tumor; M, metastasis; B, brain; MLN, mediastinal lymph node; Pu, pulmonary; Li, liver; PaLN, paraaortic lymph node; Pl, pleural; HLN, hilar lymph node.

responsible for controlling metastatic ability of cancer cells, such as detachment, invasion, survival in circulation, attachment extravasation, proliferation, induction of neovasculature, and evasion of host defenses (5, 30). The other possibility is that these additional alterations have occurred after metastasis, and these alterations conferred the growth advantage on the cells in metastatic sites. This is in agreement with the same-gene model in which genetic alterations specifically involved in metastasis do not exist (18). The results of case 3, in which all tumors showed completely the same genetic alterations, also match this model. The results can also explain the result of previous reports that analysis of primary lung and breast tumors can predict metastasis (19, 20). From the viewpoint of this model, additional alterations might confer some growth advantage on the cells in a metastatic site but not confer metastatic ability on the cells in the primary site.

In the present study, four of the eight cases showed the genetic alterations detected only in primary tumors but not in corresponding metastases. This is consistent with the concept of parallel evolution because cancer cells in primary tumors in the four cases, as well as breast cancer cases reported by Schmidt-Kittler et al., have acquired additional genetic alterations after the occurrence of metastasis (13, 14). Genetic alterations specific in primary tumors were detected by the analysis in primary tumors in aggregate; thus, cells with these alterations might comprise the majority of cells in primary sites, suggesting that these alterations conferred the cells in primary sites some growth advantage.

In a case of SCLC (case 8), we were able to analyze both lymph node metastases and distant metastases. The result indicates that liver metastasis occurred before lymph node metastasis at an early stage in SCLC progression. Thus, as indicated in breast cancer progression, it is possible that SCLC cells bypass the lymph nodes and disseminate directly through the blood to distant organs. Accordingly, the cascade model with hematogenous dissemination can be applicable to the process of SCLC progression (15). Because SCLC is the most aggressive type of lung cancer with early and wide dissemination, such aggressiveness would be well explained by this model.

As described above, the process of metastasis in each lung cancer is diverse among cases. All three progression models were applicable in lung tumor progression. Genetic alterations detected specific to primary tumors or metastases might have some biological significance, such as growth advantage and/or metastatic ability. Thus, allelic imbalances at chromosome 11p15 and 11p11-p13 regions, the most frequent alterations detected only in metastases, should be further analyzed in association with the biological behavior of lung cancer cells. According to the clonal and parallel evolution models, a metastasis suppressor gene(s) was predicted to be present in these chromosomal regions. It was previously reported that complementation with chromosome 11 induced growth inhibition in lung cancer cells (31, 32). The possible involvement of allelic imbalance on 11p in the progression of lung cancer was also indicated by the loss of heterozygosity analysis (33, 34). The 11p15 region contains a candidate tumor suppressor gene $p57^{KIP2}$, and the 11p11-p13 region contains a candidate metastasis suppressor gene *KAI1*. $p57^{KIP2}$ is a member of the cyclin-dependent kinase inhibitor family, and overexpression of $p57^{KIP2}$ causes a cell cycle arrest of SAOS-2 and mink lung epithelial cells in G_1 phase (35, 36). $p57^{KIP2}$ is known to be imprinted, and the occurrence of selective loss of the expressed allele for the $p57^{KIP2}$ gene in lung cancer cells has been reported (37). *KAI1* encodes a protein with an ability to suppress metastasis of several types of cancer cells (38, 39). In fact, *KAI1* protein expression was reported as being preferentially decreased in metastatic lung tumors rather than primary tumors (40). Because RNA and protein of the metastatic tumors analyzed in this study were not available, we could not examine the expression of these genes. Biological analyses of these genes in relation to metastatic ability and/or growth advantage of lung cancer cells as well as genetic analyses of 11p alterations in additional sets of primary and metastatic lung tumors will be necessary to elucidate how genes on 11p are involved in lung cancer progression and metastasis.

Based on the genotype data of primary and metastatic lung tumors obtained from eight patients, the natural history of each

case was deduced and candidate metastasis suppressor loci were determined. However, in this study, only a small number of paired samples were analyzed; tumors of different histologic types with and without chemotherapy were compared together, and lymph node metastases and distant metastases were compared together. Therefore, further analyses with larger sets of paired primary and metastatic tumors will give us more comprehensive information on genetic alterations involved in lung cancer progression and metastasis.

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新薬展望 2007

第Ⅰ部 治験を取り巻く環境変化

医師主導型治験の今後のあり方

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平成15年7月，いわゆる改正薬事法の施行により，医師主導型治験の実施が可能となった。医師による厚生労働大臣への治験計画届の提出が許可されたことにより，これまで承認後の市場の小ささにより医薬品・医療機器メーカーが開発に着手しなかったものの医学的には必要性の高い治療法の導入を，医師が主導して行うことが可能となった。一方，治験計画届を提出する医師には薬事法上の責務も加わり，医療機関の負担も大きくなった。

本稿では，医師主導治験で新たに生じる責務と業務について概説し，医療機関における今後の課題について紹介する。

■キーワード：医師主導治験，GCP，薬事法，CRC

1 はじめに

従来の薬事制度においては，医薬品・医療機器メーカーが医療機関に依頼して治験（薬事法第2条に定義される用語）を行う場合のみ，「医薬品の臨床試験の実施の基準に関する省令（GCP：good clinical practice）」（平成9年厚生省令第28号）の遵守のもとで，未承認の医薬品や医療機器の医療機関への提供が認められていた。一方，旧厚生省は平成11年に「適応外使用に係る医療用医薬品の取扱いについて」（研第4号医薬審第104号：いわゆる2課長通知）の中で，「公的な研究事業の委託研究等により実施されるなどその実施に係る倫理性，科学性及び信頼性が確認し得る臨床試験の試験成績がある場合」は，その結果を承認申請の際に提出する資料とできるという姿勢を示しながらも，医師が臨床試験の計画の時点から未承認の医薬品や医療機器の承認申請を目指し，厚生労働大臣への治験計画届等を提出して自ら治験を実施することは認められなかった。

しかし，平成15年7月30日に「薬事法及び採血及び供血あっせん業取締法の一部を改正する法律（平成14年法律第96号：いわゆる「改正薬事法」）」が施行となり，「医師主導型治験」の実施が可能となった。つまり，医師自ら（以下，自ら治験を実施する者）による厚生労働大臣への治験計画届等の提出が認められ，「医薬品の臨床試験の実施の基準に関する省令」の一部を改正する省令（平成15年6月12日　厚生労働省令第106号）（以下，**改正GCP**）を遵守すれば，未承認の医薬品または医療機器の提供を受けて（あるいは購入して），国内未承認薬もしくは新たな効能・効果の追加を目的とした臨床試験が可能となったのである。

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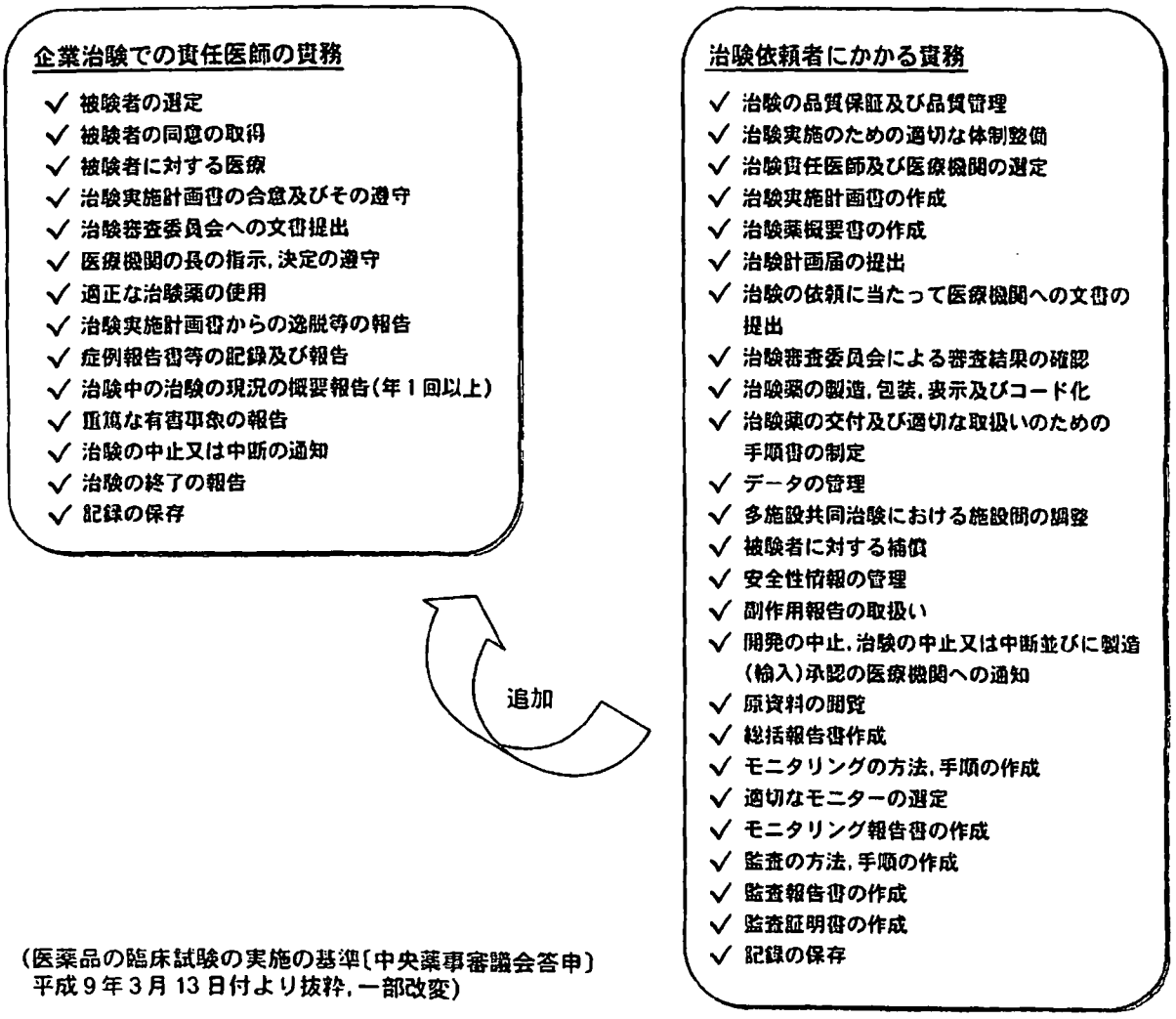

（医薬品の臨床試験の実施の基準〔中央薬事審議会答申〕平成9年3月13日付より抜粋，一部改変）

図1 自ら治験を実施する者の責務

自ら治験を実施する者の責務には，企業治験における責任医師の責務に加えて，治験依頼者の責務も加わる．

医師主導型治験が制度化され，それまで，承認後の市場の小ささなどにより医薬品・医療機器メーカーが開発に興味を示さなかった，がん領域などの罹患数の少ない領域にも，医学的には必要性の高い治療の導入を医師自らが推進していくことが可能となった。しかし，一方では，自ら治験を実施する者は薬事法上の責務を負うことになった。（図1）。

国立がんセンター中央病院の医師が自ら治験を実施する者として実施している医師主導型治験は，平成16年11月2日に治験計画届を提出した「再発あるいは治療抵抗性のc-kitあるいはPDGFR[注]陽性肉腫に対するイマチニブの第Ⅱ相試験」（以下，医師主導型治験イマチニブ）を始めとして，現在実施中の治験2本，準備段階の治験4本である。筆者らは，これら6本の医師主導型治験に，治験調整医師として，また治験調整事務局や自ら治験を実施する者をサポートする臨床研究コーディネーター（CRC）として携わっている。そこで，本稿では，筆者らが行っている医師主導型治験に関連する業務を紹介するとともに，医薬品あるいは医療機器メーカーから依頼を受けて実施する治験（以下，企業治験）と比べて新たに生じる医師の責務，それに伴う業務のいくつかについ

PDGFR：platelet-derived growth factor receptor；血小板由来増殖因子受容体

■表1　医師主導型治験に関連する法令・通知（抜粋）　（平成18年12月末現在）

1）薬事法
（昭和35年法律第145号）
2）薬事法施行規則
（昭和35年厚生省令第1号）
治験の届出，副作用報告等に関して薬事法の詳細を定めたもの
薬事法施行規則の一部を改正する省令
（平成17年12月28日付　厚生労働省令第178号）
医師主導治験の副作用等の報告に係る改正（薬事法施行規則第273条）
3）医薬品の臨床試験の実施の基準に関する省令
（平成9年厚生省令第28号）
いわゆる「GCP省令」
医薬品の臨床試験の実施の基準に関する省令の一部を改正する省令
（平成18年4月1日薬食発第0401001号）
いわゆる「改正GCP」と呼ばれているもの　「医師主導型治験」に関するGCPの規定，治験審査委員会の質及び機能の向上が記述されている。
4）医薬品の臨床試験の実施の基準の運用について
（平成18年9月21日付　薬食審査発第0921001号　厚生労働省医薬食品局審査管理課長通知）
改正GCPの運用に関する詳細な規定。
5）医薬品の臨床試験の実施の基準の運用における必須文書の構成について
（平成16年10月18日付厚生労働省医薬食品局審査管理課事務連絡）
GCPでの必須文書に関する説明と合理化の例を示したもの
6）薬事法及び採血及び供血あっせん業取締法の一部を改正する法律の一部の施行について
（平成15年5月15日付医薬発第0515017号厚生労働省医薬局長通知）
治験計画届書などの記載要領について説明
7）「薬物に係る治験の計画の届出等に関する取扱いについて」の一部改正について
（平成15年6月12日付医薬審発第0612004号厚生労働省医薬局審査管理課長通知）
上記6）の医薬発第0515017号の解説
8）自ら実施する薬物に係る治験の計画の届出等に関する取扱いについて
（平成15年6月12日付医薬審発第0612001号厚生労働省医薬局審査管理課長通知）
自ら治験を実施しようとする者の治験の計画の届出に関する規定
「自ら実施する薬物に係る治験の計画の届出等に関する取扱いについて」の一部改正について
（平成17年10月25日薬食審査発第1025001号　厚生労働省医薬食品局審査管理課長通知）
9）治験薬の製造管理及び品質管理基準及び治験薬の製造施設の構造設備基準（治験薬GMP）について
（平成9年3月31日付薬発第480号厚生省薬務局長通知）
いわゆる「治験薬GMP」
自ら治験を実施する者は，使用する治験薬が「治験薬GMP」に準拠したものであることを証明する必要がある。
10）独立行政法人医薬品医療機器総合機構に対する治験副作用等報告について
（平成16年3月30日付薬食発第0330001号厚生労働省医薬食品局長通知）
自ら治験を実施した者による治験副作用等報告の取扱いについて
（平成17年10月25日付薬食審査発第1025017号厚生労働省医薬食品局審査管理課長通知）
「独立行政法人医薬品医療機器総合機構設立後の自ら治験を実施した者による治験副作用等報告について」の改正について
（平成17年10月25日付薬食審査発第1025005号厚生労働省医薬食品局審査管理課長通知）
「独立行政法人医薬品医療機器総合機構に対する治験副作用等報告に関する報告上の留意点等について」の改正について
（平成17年10月25日付薬食審査発第1025013号厚生労働省医薬食品局審査管理課長通知）

図表1 医師主導型治験に関連する法令・通知（抜粋）（つづき）

治験副作用等報告に関する報告上の留意点等について
（平成18年4月26日付 薬食審査発第0426001号 厚生労働省医薬食品局審査管理課長通知）

11) 薬物に係る治験に関する副作用等の報告に係る薬事法施行規則の一部を改正する省令の施行について
（平成17年12月28日付 薬食発第1228001号厚生労働省医薬食品局長通知）

12)「療担規則及び薬担規則並びに療担基準に基づき厚生労働大臣が定める掲示事項等」及び「選定療養及び特定療養費用に係る厚生労働大臣が定める医薬品等」の制定に伴う実施上の留意事項について」の一部改正について
（平成17年3月31日付保医発第0331011号厚生労働省保険局医療課長通知）

13) 自ら治験を実施する者による医薬品の臨床試験の実施の基準に関するQ&Aについて
（平成17年10月25日 付事務連絡）

14) 医薬品GCP実地調査の実施要領について
（平成18年1月31日付 薬食審査発第0131006号 厚生労働省医薬食品局審査管理課長通知）

15) 新医薬品の承認申請資料に係るGCP実地調査の実施手続きについて
（平成18年2月15日付 独立行政法人医薬品医療機器総合機構 信頼性保証部長 事務連絡）

16) 治験の総括報告書の構成と内容に関するガイドラインについて
（平成8年5月1日付薬審第335号厚生省薬務局審査課長通知）

て紹介してみたい。

2 医師主導治験における自ら治験を実施する者の責務

医師主導型治験では改正GCPの中で，自ら治験を実施する者に，企業治験における治験依頼者（医薬品・医療機器メーカー）と全く同じ事務手続きと品質保証（監査）・品質管理（モニタリング）を要求されており，実施に際しては表1に一部抜粋したような，医師主導型治験に関係する法律（薬事法），政令（薬事法施行令），省令（薬事法施行規則，改正GCP），通知（局長通知，課長通知），事務連絡に十分に目を通して，その内容を理解しておく必要がある。以下に医師主導型治験において新たに加わる責務の概略を紹介する。

1．治験薬の確保（改正GCP第26条の2）

医師主導型治験の法制上の整備はされたが，実際にひとつの治験を実施するためには当然のことながら対象となる治験薬（または医療機器）の確保が必須である。しかし，製造工場を持たない医師には製造管理及び品質管理規則（GMP）を遵守した治験薬の製造は不可能である。実際には，医薬品メーカーから提供（もしくは購入）をうけることが必要となる。医師主導型治験イマチニブでは，まず，プロトコールコンセプトの策定とイマチニブを製造・販売している日本法人との接触を開始し，海外本社のProtocol Review Committeeによるプロトコールコンセプトの審査をパスし，ようやく治験薬の提供を受けることができた。また，他の治験では，すでに当該医薬品の国内承認から長い年月が経過しており当該治験薬を保有する医薬品メーカー内でも企業治験は実施していないため，治験薬製造ラインが確保できず，改正GCP第26条の2の要求を満たす治験薬の提供（いわゆる白箱提供）を受けることができなかった。対応策として，市販薬の容器および被包を変更をして提供を受ける方法を検討中である。

2．治験の準備段階（改正GCP第2章第2節）

1）標準業務手順書（SOP）作成

準備段階で，最も大変な作業は，改正GCP第15条の2に規定される各種のSOPの作成である。医師主導型治験イマチニブ開始前に作成したSOP一覧を表2に示す。このSOPの作成は病院の体制に関するSOPから当該治験特有のSOPまで多岐にわたる。筆者の着任前であるが，当院においても医師主導型治験イマチニブ開始前に，当時の病院長である野村和弘院長の指示により「医師主導型治験に対する国立がんセンターの対応に関する検討会」が設置され（事務局長は西條長宏薬物療法部長〔当時〕が担当），運営部，看護部，

表2　医師主導治験イマチニブの研究申込前に作成した業務手順書一覧

医療機関における業務手順書（1 ～ 6）は，当院最初の医師主導治験実施前に作成し，その後の医師主導治験では共通の手順書として使用している。個々の治験のために作成する業務手順書は，治験実施計画書に合わせて作成する（7 ～ 22）。

研究申込前に作成した業務手順書一覧

【医療機関における業務手順書等】

①国立がんセンター医師主導治験取扱規程
②国立がんセンター医師主導治験標準業務手順書
③医師主導治験における監査の受入に関する標準業務手順書
④医師主導治験におけるモニタリングの受入に関する標準業務手順書
⑤医師主導治験における国立がんセンター治験審査委員会標準業務手順書
⑥国立がんセンター医師主導治験審査予備調査会規定

【医師主導治験イマチニブのために作成した業務手順書】

⑦治験実施計画書および症例報告書の作成に関する標準業務手順書
⑧治験薬概要書の作成に関する標準業務手順書
⑨安全性情報に関する標準業務手順書
⑩被験者の補償に関する標準業務手順書
⑪治験薬の取り扱い標準業務手順書
⑫モニタリングに係る標準業務手順書
⑬監査に関わる標準業務手順書
⑭自ら治験を実施する者に係る標準業務手順書
⑮治験調整医師に係る標準業務手順書
⑯効果安全性評価委員会に係る標準業務手順書
⑰効果判定委員会に係る標準業務手順書
⑱病理中央診断実施手順書
⑲登録業務に関する標準業務手順書
⑳データ取扱いに関する標準業務手順書
㉑記録の保管に関する標準業務手順書
㉒総括報告書の作成に関する標準業務手順書

薬剤部も含めたメンバーにより医師主導型治験実施をめぐる実務的な問題点の洗い出しと，それらへの対応策の検討を行ったと聞いている。次に続く医師主導型治験からは，これらのSOPを雛型とするそれぞれの治験特有の手順書作成に作業は絞られ負担は軽減した。とはいえ，自ら治験を実施しようとする者は，医師主導型治験の実施は初めての場合が多く，これらのSOPを臨床現場で働く医師自らが作成することは大変な作業である。

2）医療機関の長への研究申込

医師主導型治験の特徴のひとつに，薬事法第80条の2の2に規定されている厚生労働大臣への治験計画の届出に先だって，自ら治験を実施する者が，あらかじめ改正GCP第15条の7に規定された文書を実施医療機関の長に提出し，治験の実施の承認を得なければならない点がある。当院で，医療機関の長への研究申込の際に提出した資料の一覧を表3に示す。企業治験では，治験審査委員会とのやり取りにおいて，審議資料の作成から申請までを企業の臨床開発担当者に大きく依存してしまうのが常であるが，医師主導型治験では，医師やCRC，治験事務局等の医療機関で医師主導型治験実施に携わる者たち自身がすべてその業務を担うことになる。文書の作成が最も大変な作業であることは当然であるが，表3で示した文書を体裁を整えて一塊の資料を作成するにも，相当な作業が発生する。実際，当院では，研究申込時には，数名のCRCと事務担当者が1日がかり

図表3 研究申込時提出資料

研究申込時提出資料
（1）研究委託申込書（様式3）……1部
（2）治験審査用資料（ファイリングされたもの）……10部（予備調査用）
16部（審査委員会用）

【ファイル内容】
① 治験実施計画書
② 症例報告書の見本
③ 治験薬概要書
④ 被験者への支払い（支払いがある場合）に関する資料
⑤ 被験者の健康被害に対する補償に関する資料
⑥ 医師主導治験経費見積書
⑦ 治験責任医師及び治験協力者リスト
⑧ 治験責任医師及び治験分担医師の履歴書
⑨ 同意文書及びその他の説明文書の案（補償の概要・手順）
⑩ 被験者の募集手順（広告等）に関する資料（ある場合）
⑪ モニタリングに関する手順書
⑫ 監査に関する計画書及び業務に関する手順書
⑬ 治験薬の管理に関する事項を記載した文書
⑭ 治験実施に関する業務手順書
（安全性情報に関する標準業務手順書
自ら治験を実施する者に係る標準業務手順書
治験調整医師に係る標準業務手順書 等）
⑮ 被験者の安全に係る報告（必要時）
⑯ 治験の現況の概要に関する資料（必要時継続時等）
⑰ 治験審査委員会が必要と認める資料（必要時）

で，資料のコピー，インデックスの作成，ファイリング等の作業を行い，厚さ5cm強の申請資料26冊を作成した。治験責任医師（自ら治験を実施する者）のみならず，治験事務局等の担当者やCRCらの協力と，皆が自ら治験を実施するという意識を持っていないと，これらの業務を完遂することは不可能である。

3）治験薬概要書の作成

前述した治験薬の確保とも関連するが，治験薬概要書の作成も大きな課題である。平成15年6月12日 医薬発第0612001号（表1）Ⅱ．2．（4）においては，「治験薬提供者は必要に応じ，必要な資料又は情報を提供すること。」と記述されているため，既存の医薬品を用いる治験の場合には，製薬企業からの治験薬概要書の提供をお願いするのが得策であろう。ただし，前述したとおり当該治験薬に対して治験薬提供者内で企業治験を実施していない場合，治験薬概要書の提供を得られないこともある。その場合，被験薬の物理的，化学的及び製剤学的性質，製剤組成，薬理，毒性，薬物動態，薬物代謝に関連する非臨床試験の成績を，それぞれ，様々なソースから情報として提供を受け，自らそれらをまとめて治験薬概要書を作成することも必要となる可能性がある。

3．治験の計画の届出（薬事法第80条の2第2項，薬事法施行規則第269条）

各医療機関の長から治験の実施の承認が得られたら，当該治験に参加する自ら治験を実施する者それぞれから厚生労働大臣宛に治験の計画の届出（治験計画届書の提出）を行う。さらに治験計画変更届書，治験中止届書，治験終了届書，開発中止届書の提出も医師主導型治験においては自ら治験を実施する者が担当する。このような業務を各医療機関内で誰がどう担当していくのか，事前に入