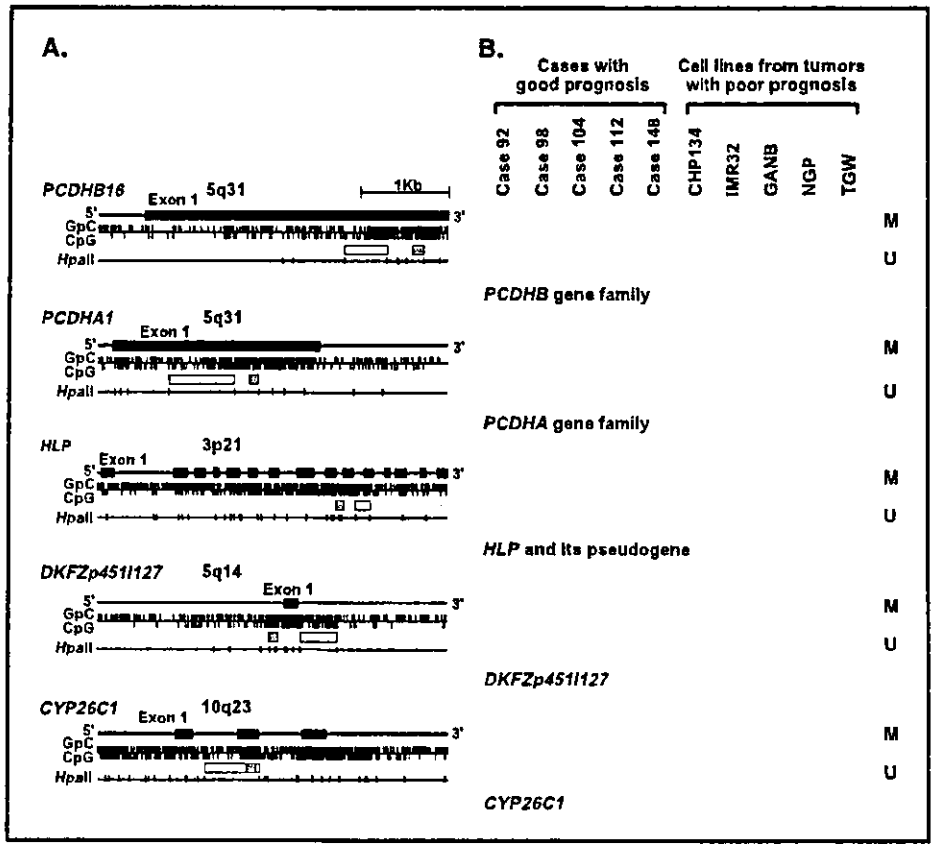

Figure 1. Five CGIs isolated by MS-RDA and their methylation statuses in the samples used for MS-RDA. *A*, genomic structures of the five CGIs. GpC, CpG, and *Hpa*II recognition sites (5′-CCGG-3′) are shown by ticks. *Closed boxes*, exons; *open boxes*, clones isolated by MS-RDA; *shaded boxes*, regions analyzed by MSP. *B*, methylation statuses analyzed by MSP. *M*, MSP using primers specific to methylated DNA; *U*, MSP using primers specific to unmethylated DNA. All the five CGIs were found to be differentially methylated between the two groups used for MS-RDA.

was also done. For each series of MS-RDA, 96 clones were analyzed for redundancy, and nonredundant clones were sequenced. Their genomic origins were examined using BLASTN software http://www.ncbi.nlm.nih.gov/BLAST/).

Sodium Bisulfite Modification and Methylation-Specific PCR. One microgram of DNA underwent sodtlbium bisulfite modification (15), and was suspended in 20 μL of TE buffer. For methylation-specific PCR (MSP), 1 μL of the solution was used for PCR with primers specific to methylated or unmethylated sequences. Using DNA from normal human bronchial epithelial and DNA methylated with *Sss*I methylase, annealing temperatures specific for methylated and unmethylated primers were determined. Quantitative MSP was done separately for methylated DNA molecules and for unmethylated DNA molecules. Standard DNA was prepared by cloning PCR products amplified by methylated and unmethylated primers into a vector, respectively. The numbers of methylated and unmethylated molecules in a test sample were determined by comparing their amplification with those of standard samples containing 10 to 10^6 molecules. The "methylation index" was calculated as the fraction of methylated molecules in the total DNA molecules (no. methylated molecules + no. unmethylated molecules). Each sample was analyzed twice, blind to clinical information, and high reproducibility was confirmed (correlation coefficient = 0.98).

The *protocadherin* β (*PCDHB*) family consists of 16 genes with single exons and three pseudogenes on 5q31, and their CGIs are located in the gene bodies. MSP primers were designed to recognize 17 of the 19 members (all except for the *PCDHB1* gene and the *PCDHB19* pseudogene). The *protocadherin* α (*PCDHA*) family consists of 15 genes and one pseudogene having unique first exons and shared exons 2 to 4 on 5q31, and their CGIs are located in exon 1. MSP primers were designed to recognize 13 of the 16 members (all except for the *PCDHAC1* and *PCDHAC2* genes and the *PCDHA14* pseudogene). The *hepatocyte growth factor-like protein* (*HLP/MSP/MST1*) gene is highly homologous to *macrophage stimulating, pseudogene 9* (*MSTP9*), and MSP primers were designed to recognize both of these. For *DKFZp451I127*, *FLJ37440*, *Zinc finger protein 297* (*ZNF297*), and *Cytochrome p450 CYP26C1* (*CYP26C1*), MSP primers were designed to recognize each of them specifically. The primers and PCR conditions are shown in Supplementary Table 1.

Semiquantitative and Quantitative Reverse Transcription-PCR. cDNA was synthesized from 3 μg of total RNA treated with DNase using a Superscript II kit (Invitrogen Co., Carlsbad, CA). For semiquantitative reverse transcription-PCR (*PCDHB1-PCDHB15*), multiple cycles of PCR were tested for each gene, and numbers giving a wide dynamic range were determined. The primers and PCR conditions are shown in Supplementary Table 2. For quantitative reverse transcription-PCR (*PCDHB16*), the number of cDNA molecules was determined by quantitative PCR, as in quantitative MSP, and the copy number was normalized to that of *GAPDH*.

Chromatin Immunoprecipitation Assay. From 1×10^6 cells, DNA/histone complexes were immunoprecipitated, and DNA was eluted in 30 μL of TE after reversing cross-linking. Copy numbers of DNA molecules of the *PCDHB16* exon, *RASSF1A* promoter, and *GAPDH* promoter in 1 L of the eluate were determined by quantitative PCR (primer sequences in Supplementary Table 3), and normalized to the copy numbers in the input. Anti-acetyl-histone H3 antibody (AcH3) and anti-dimethylated-histone H3 (lysine 9; MetH3K9) were purchased from Cell Signalling (Beverly, MA).

Statistical Analysis. Associations between methylation levels among CGI groups were examined using the Pearson correlation coefficient and Fisher's exact test. Survival time was measured from the date of initial diagnosis to the date of death or last contact. Kaplan-Meier analysis and log-rank tests were done to compare survival between the groups defined by methylation levels. Hazard ratio (HR) between groups and dose-response relationships between methylation levels and survival were estimated by the Cox proportional hazard model. Kaplan-Meier curves were drawn with the help of Aabel software (Gigawiz. Ltd. Co., Tulsa, OK) and other analyses were conducted using SAS version 8.2 (SAS Institute, Inc., Cary, NC).

Results

Genome-Scanning for Differentially Methylated CpG Islands. MS-RDA was done using five primary neuroblastomas with a good prognosis and five neuroblastoma cell lines established from cases with a poor prognosis. Seven DNA fragments, derived from CGIs of *PCDHB16*, *PCDHA1*, *HLP*, *DKFZp451I127*, *FLJ37440*, *ZNF297*, and *CYP26C1*, were isolated as methylated in the latter samples. No DNA fragments were isolated as methylated in the former samples. Methylation statuses of (i) 17 CGIs of the *PCDHB* family (detailed structure in Supplementary Fig. 1), (ii) 13 CGIs of the *PCDHA* family, (iii) *HLP* and its pseudogene, and (iv) other four unique CGIs were examined by MSP. This revealed that the *PCDHB* family (5q31), the *PCDHA* family (5q31), *HLP* (3p21) and its pseudogene (1p36), *DKFZp451I127* (5q14), and *CYP26C1* (10q23) were specifically methylated in the latter samples (Fig. 1*A* and *B*).

Close Association between Methylation and Poor Prognosis in 140 Independent Primary Samples. To analyze the significance of the differential methylation of the above five CGI (groups) in primary neuroblastomas, 140 primary samples, all different from the initial five samples, were analyzed by quantitative MSP. When distributions of methylation indices were analyzed (Fig. 2), a clear bimodal distribution was observed for (i) the CGI group in the *PCDHB* family (17 CGIs), (ii) the CGIs of *HLP* and its pseudogene, and (iii) the *CYP26C1* CGI. The results thus indicated that the cases could be classified into two groups, one with high methylation and the other with low methylation. The dose-response relationships between high *PCDHB* methylation and poor prognosis were analyzed by the Cox proportional model using the methylation index as a continuous value, and the association was confirmed with a trend $P < 0.0001$. Normal adrenal medulla had a methylation index of 4%.

According to the bimodal distribution, the effect of high methylation was assessed by dichotomous groups. For the *PCDHB* family, cutoff values of 30%, 40%, 50%, 60%, 70%, and 80% were tested, and HRs of 16.8 [95% confidence interval (95% CI), 4.0-70.9], 22.1 (95% CI, 5.3-93.4; Fig. 3), 13.1 (95% CI, 4.5-37.9), 9.1 (95% CI, 3.8-23.4), 7.0 (95% CI, 3.1-15.8), and 7.8 (95% CI, 3.4-17.6), respectively, were obtained ($P < 0.001$ for all cutoff values). This showed that cases can be classified into two groups with distinct prognoses, and we adopted a cutoff value of 40%, which gave the highest HR, for convenience in the following analysis.

The dose-response relationships were also confirmed for other four CGI (groups), *PCDHA* ($P = 0.004$), *HLP* ($P < 0.0001$), *DKFZp451I127* ($P = 0.02$), and *CYP26C1* ($P < 0.0001$). Cutoff values were similarly tested, and those for *PCDHA*, *HLP*, *DKFZp451I127*, and *CYP26C1* were set at 80%, 10%, 20%, and 70%, respectively, with HRs of 5.7 (95%CI, 1.4-24.0; $P = 0.07$), 21.7 (95% CI, 5.1-91.4; $P < 0.0001$), 3.2 (95% CI, 1.0-10.5; $P = 0.045$), and 8.7 (95% CI, 4.1-18.1; $P < 0.0001$), respectively (Fig. 3).

Existence of the CpG Island Methylator Phenotype in Neuroblastomas. Methylation of the different CGI (groups) had shown close associations with each other (Table 1). When correlation was analyzed as a continuous value, Pearson correlation coefficients between *PCDHB* and *PCDHA*, *HLP*, *DKFZp451I127* and *CYP26C1* were 0.55, 0.70, 0.26 and 0.77, respectively. This showed that multiple CGIs were simultaneously methylated in

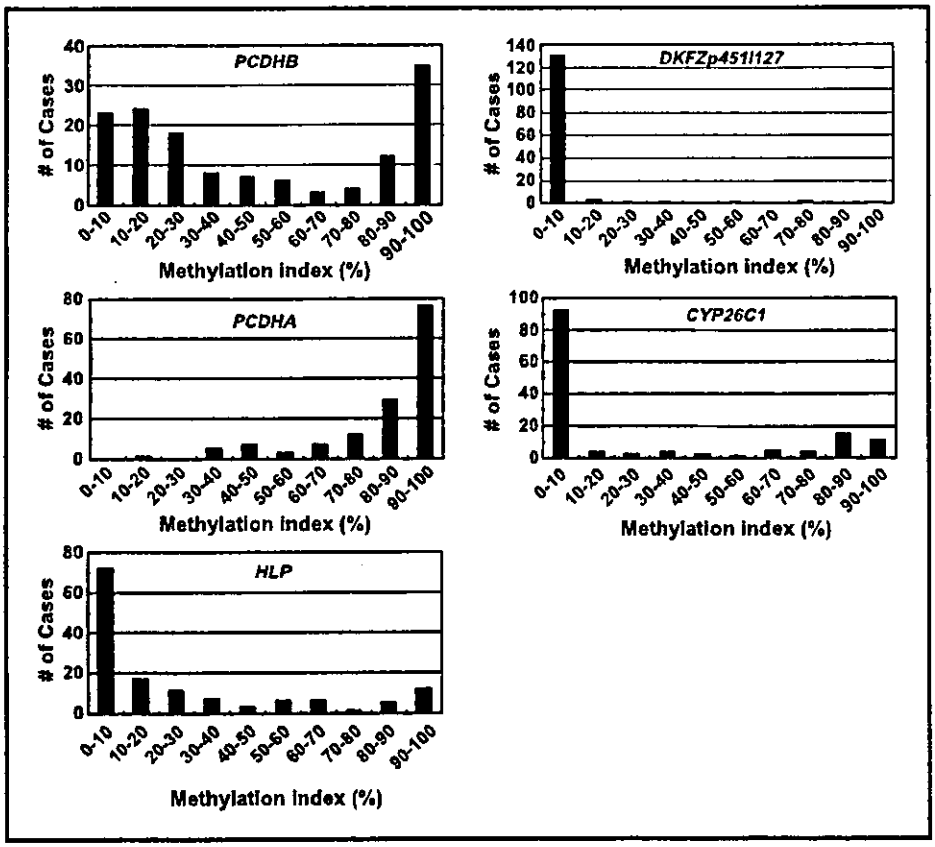

Figure 2. The distribution of methylation indices among the 140 cases analyzed: (*i*) 17 CGIs of the *PCDHB* family, (*ii*) 13 CGIs of the *PCDHA* family, (*iii*) CGIs of *HLP* and its pseudogene, (*iv*) *DKFZp451I127*, and (*v*) *CYP26C1*.

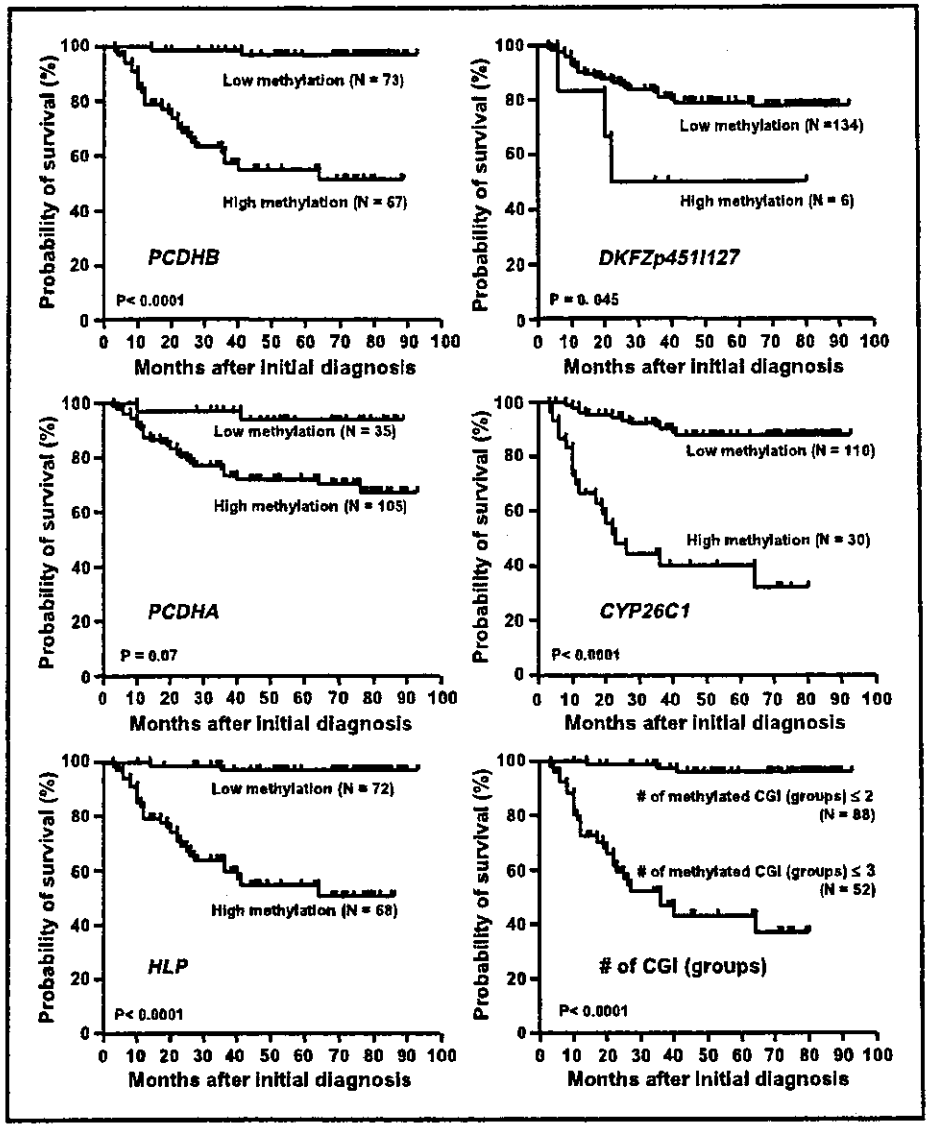

Figure 3. Predictive powers of methylation of the five CGI (groups) identified, and their multiple methylation: (*i*) 17 CGIs of the *PCDHB* family, (*ii*) 13 CGIs of the *PCDHA* family, (*iii*) CGIs of *HLP* and its pseudogene, (*iv*) *DKFZp451I127*, (*v*) *CYP26C1*, and (*vi*) methylation of three of these or more were analyzed by the Kaplan-Meier method using 140 primary samples. The *PCDHB* family, *HLP*, *DKFZp451I127*, *CYP26C1*, and methylation of multiple CGI (groups) had significant influence on survival.

neuroblastomas with a poor prognosis (Supplementary Fig. 2*A*). The simultaneous methylation of (i) 17 CGIs of the *PCDHB* family, (ii) 13 CGIs of the *PCDHA* family, (iii) CGIs of *HLP* and its pseudogene, (iv) *DKFZp451I127* CGI, and (v) *CYP26C1* CGI conformed with the concept of the CpG island methylator phenotype (CIMP; ref. 16).

Associations between CIMP and poor prognosis were examined by defining CIMP as cases with methylation of two CGI (groups) or more, those with three or more, those with four or five, and those with five. When CIMP was defined as cases with methylation of three CGI (groups) or more, the largest association with poor prognosis was observed, with a HR of 25.4 (95% CI, 7.6-84.5; Fig. 3). However, the HR (22.1) given by 17 CGIs of the *PCDHB* gene family approximated to this, and the *PCDHB* methylation level closely correlated with the number of methylated CGI (groups; Supplementary Fig. 2*B*). Therefore, for simplicity of analysis, we defined CIMP in neuroblastomas on the basis of high methylation of the *PCDHB* family, tentatively with a cutoff value of 40%.

Predictive Power of CIMP, Compared with Known Prognostic Factors. Univariate analyses showed that N-*myc* amplification, low *TrkA* expression, DNA diploidy, and an age no younger than 1 year gave HRs of 9.5 (95% CI, 4.4-20.5), 3.9 (95% CI, 1.7-9.3), 4.2 (95% CI, 1.65-10.8), and 12.3 (95% CI, 3.7-41.7). Cases were stratified by these known factors (Table 2). In those without N-*myc* amplification, CIMP also showed an influence with a HR of 12.4 (95% CI, 2.6-58.9), but almost all cases with N-*myc* amplification (37 of the 38 cases) showed CIMP. It was suggested that cases with N-*myc* amplification were contained in the cases with CIMP. CIMP was independent from *TrkA* overexpression, DNA ploidy, and age at diagnosis. Stage seemed to be a stronger prognostic factor. Notably, even when limited to cases in stages III and IV without N-*myc* amplification, which are classified into the intermediate risk group and clinically important, CIMP gave a HR of 4.8 (95% CI, 1.0-23.0; $P = 0.048$).

Multivariate analyses were finally done taking all the five known prognostic factors into account. Although CIMP gave a HR of 5.0 (95% CI, 0.47-52.7), it was not significant ($P = 0.18$), possibly due to limitation in the number of cases.

Table 1. Association between the *PCDHB* methylation and methylation of other CGIs

Variables	Methylation level of *PCDHB* family gene		*P**
	High (≥40%)	Low (<40%)	
No. cases (n = 140)	67	73	
Methylation of CGIs outside promoter regions (n = 140)			
PCDHA gene family (exon 1)†	65/67	41/73	<0.0001
HLP (exons 2-13)‡	52/67	16/73	<0.0001
CYP26C1 (exon 2)§	30/67	0/73	<0.0001
p41Arc (intron 8)	1/67	1/73	0.48
SIM2 (exon 2)	0/67	0/73	
Methylation of CGIs in promoter regions (n = 140)			
DKFZp451I127‖	6/67	0/73	0.011
RASSF1A	51/67	10/73	<0.0001
BLU	25/67	3/73	<0.0001
p16	0/67	0/73	
hMLH1	0/67	0/73	
PCDHB1	0/67	0/73	
TAF7	0/67	0/73	
p41Arc	0/67	0/73	
SIM2	0/67	0/73	

*Fisher's exact test.
†Boundaries for high methylation and low methylation of *PCDHA* gene family were set at 80% of the methylation index.
‡Boundaries for high methylation and low methylation of *HLP* were set at 10% of the methylation index.
§Boundaries for high methylation and low methylation of *CYP26C1* were set at 70% of the methylation index.
‖Boundaries for high methylation and low methylation of *DKFZp451I127* were set at 20% of the methylation index.

Effects of PCDHB Methylation on Gene Expression and Chromatin Structure. The CGIs of the *PCDHB* family were located in their gene bodies, whose methylation generally does not block gene transcription (17). The actual effects of methylation on expression were examined for 16 genes of the *PCDHB* family using 10 primary neuroblastomas with low methylation and five primary neuroblastomas with high methylation. The methylation was not associated with loss of expression (a representative result is shown in Fig. 4*A*). The effect of methylation of the *PCDHB16* CGI on the histone modification was further examined by chromatin immunoprecipitation assay. It was found that DNA methylation of the *PCDHB16* CGI did not induce histone H3 lysine 9 methylation or histone H3 deacetylation (data not shown).

Association between CIMP and Promoter Methylation. High methylation of *PCDHB* CGIs, a sensitive surrogate marker of CIMP in neuroblastomas, did not repress gene expression or induce histone modification. This indicated that CIMP is involved in the poor prognosis of neuroblastomas by causing methylation of promoter CGIs, although it is known that promoter CGIs are resistant to *de novo* methylation (18, 19).

Among the five CGI (groups) identified in this study, only that of *DKFZp451I127* was located in a promoter region. Although its methylation was infrequent, the methylation was observed only in neuroblastomas with CIMP (Table 1), and was associated with expression loss (Fig. 4*B*). To make the association clearer, methylation statuses were analyzed for eight additional CGIs in promoter regions. It was shown that methylation of promoter CGIs of *RASSF1A* (3p21) and *BLU* (3p21) was far more frequently observed in neuroblastomas with CIMP (Table 1, $P < 0.0001$). At the same time, there was a preference for CGIs affected by CIMP among CGIs in promoter regions, and also among those outside promoter regions (Table 2).

Discussion

Extensive methylation of multiple CGIs, conforming with the concept of CIMP, was here found specifically present in neuroblastomas with a poor prognosis and could be sensitively detected by focusing on the *PCDHB* family. *PCDHB* methylation did not suppress gene expression or induce histone modification. However, CIMP was associated with promoter methylation of *RASSF1A* and *BLU* genes and one of the mechanisms underlying the poor prognosis of neuroblastomas seemed to be silencing of these and possibly other tumor suppressor genes and genes important for differentiation.

CIMP was originally identified in colon cancers (16), but there has been some dispute over its presence (20). The clear correlation between CIMP and a poor prognosis found here for neuroblastomas was unequivocal and presumably reflects an intrinsic tendency for methylation of CGIs. This is because, first, neuroblastomas have a much shorter history than colon cancers, and the accumulated number of methylated CGIs in neuroblastomas is expected to parallel the speed of occurrence of

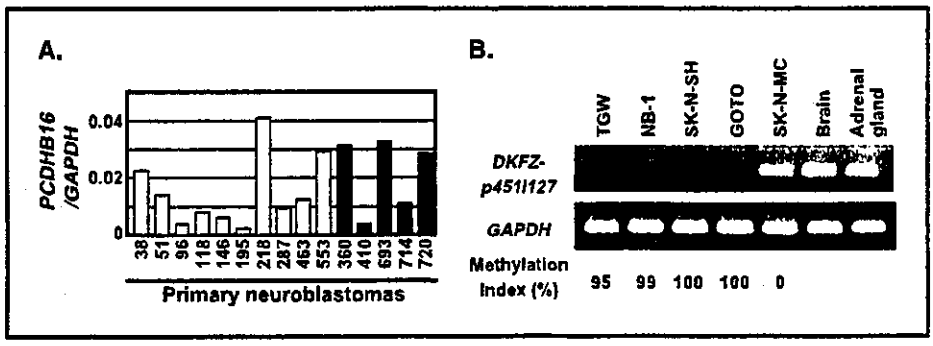

Figure 4. Effects of methylation of the *PCDHB* family and *DKFZp451I127* on gene expression. *A*, *PCDHB16* expression was analyzed by quantitative RT-PCR in 10 primary samples with low methylation (*open columns*) and five primary samples with high methylation (*closed columns*), and no difference was observed between the two groups. *B*, silencing of *DKFZp451I127* by methylation of its promoter CGI. The CGI was methylated in four cell lines, TGW, NB-1, SK-N-SH, and GOTO, whereas it was unmethylated in one cell line, SK-N-MC. *DKFZp451I127* was expressed in SK-N-MC, but not expressed at all in the four cell lines with the promoter methylation.

Table 2. HRs of death by *PCDHB* methylation status in subgroup of known prognostic factors

Stratified by		*PCDHB* methylation	No. cases	No. deaths	HR* (95% CI)	$P^{\dagger}$
Overall		High	67	1	22.1 (5.3-93.4)	< 0.0001
(n = 140)		Low	73	2	1	
N-*myc* amplification	No	High	30	8	12.4 (2.6-58.9)	0.002
(n = 140)		Low	72	2	1	
	Yes	High	37	20	NE	—
		Low	1	0		
TrkA overexpression	Yes	High	20	6	18.3 (2.2-152.6)	0.007
(n = 130)		Low	49	1	1	
	No	High	40	19	NE	
		Low	21	0		
DNA ploidy	Aneuploid	High	17	5	18.3 (2.1-156.7)	0.008
(n = 125)		Low	49	1	1	
	Diploid	High	38	17	NE	
		Low	21	0		
Clinical stages	Stages I, II,	High	8	0	NE	—
(n = 140)	and IVS	Low	52	0		
	Stages III	High	59	28	7.4 (1.8-31.3)	0.006
	and IV	Low	21	2	1	
Age at diagnosis	<1	High	11	3	NE	
(n = 140)	≥1	Low	59	0		
		High	56	25	4.5 (1.1-18.9)	0.043
		Low	14	2	1	

*HR of death for a case with high *PCDHB* methylation compared with a case with low methylation. NE shows not estimable due to no events in at least one category.

†Significance level for a high *PCDHB* methylation to low methylation using Cox proporitonal model.

methylation. Second, methylation of the *PCDHB* family did not affect gene expression, and there should have been no selection of cells with the *PCDHB* methylation, in contrast to the case of promoter methylation of tumor suppressor genes. Investigation into the mechanism of the intrinsic tendency for methylation of multiple CGIs is necessary. Furthermore, alleviation of the intrinsic tendency could block progression of neuroblastomas and have potential therapeutic value.

Among the six CGI (groups) outside promoter regions analyzed here, CIMP in neuroblastomas preferentially affected four CGI (groups); those of the *PCDHB* family, the *PCDHA* family, *HLP*, and *CYP26C1*. Unexpectedly, three CGIs that are known to be frequently methylated in human colon cancers with CIMP, MINT1, MINT2, and MINT17 (16) were not methylated in neuroblastoma cell lines (data not shown). Among the nine CGIs in promoter regions analyzed, CIMP in neuroblastomas affected only three, those of *RASSF1A*, *BLU*, and *DKFZp451I127*. The nine CGIs were selected based upon previous reports as tumor suppressor genes (*RASSF1A*, *BLU*, *p16*, and *hMLH1*; refs. 21–23), the chromosomal location flanking the *PCDHB* family (*PCDHB1* and *TAF7*), our previous report on the fidelity in inheriting methylation patterns (*p41Arc* and *SIM2*; ref. 19), and the findings here (*DKFZp451I127*). Because gene expression and possibly chromatin structures affect the frequency of *de novo* methylation (24, 25), the available data suggest that CGIs useful to sensitively detect CIMP might vary according to the tumor type.

The influence of CIMP on prognosis was here found to be comparable to that of the currently most reliable marker, N-*myc* amplification, and stronger than *TrkA* overexpression and DNA ploidy on univariate analysis. Subgroup analysis showed that the influence was independent of *TrkA* overexpression, DNA ploidy and age at diagnosis and CIMP had influence even in cases without N-*myc* amplification and in advanced stages. These points strongly indicated CIMP to be a promising new prognostic marker. However, the cutoff values adopted here are tentative, and the HRs obtained could have been overestimated. A validation study using independent samples is necessary for further evaluation. The fact that cases with CIMP contained almost all the cases with N-*myc* amplification suggested that a common molecular mechanism caused both alterations, or that CIMP may lead to N-*myc*

amplification. Whatever the case, the findings might provide clues to molecular mechanisms of neuroblastoma development.

In summary, the present study showed that CIMP is present specifically in neuroblastomas with poor prognosis and that can be sensitively detected by focusing on *PCDHB* methylation. CIMP seems to be a promising new prognostic marker, and its evaluation and investigations into the mechanisms underlying CIMP in neuroblastomas seem warranted.

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Active and passive smoking and breast cancer risk in middle-aged Japanese women

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To examine the hypothesis that tobacco smoke is associated with the risk of female breast cancer, we estimated the relative risks of active and passive smoke in middle-aged Japanese women in a population-based prospective study. The cohort consisted of residents in 4 public health center areas, aged 40 to 59 years. A self-administered questionnaire survey was conducted in 1990. This analysis included 21,805 subjects, 180 of whom had developed breast cancer by December 31, 1999. When the reference was defined as never-active smokers without passive smoking, adjusted relative risks (RRs) were 1.9 (95% confidence interval [CI] = 1.0–3.6) in current active smokers, 1.2 (95% CI = 0.4–4.0) in ex-active smokers and 1.2 (95% CI = 0.8–1.6) in never-active smokers with passive smoking. The elevated risk for ever-smokers was clearly observed in premenopausal women at baseline (RR = 3.9, 95% CI = 1.5–9.9) but not in postmenopausal women (RR = 1.1, 95% CI = 0.5–2.5). In never-active smokers, the adjusted RR for passive smoking, residential or occupational/public tobacco smoke exposure was 1.1 (95% CI = 0.8–1.6). In premenopausal women, passive smoking increased the risk (RR = 2.6; 95% CI = 1.3–5.2) but not in postmenopausal women (RR = 0.7; 95% CI = 0.4–1.0). We conclude that tobacco smoking increases the risk of female breast cancer in premenopausal women.

Key words: breast neoplasms; smoking; passive smoking; cohort study

Because most established risk factors for female breast cancer cannot be modified, the etiological role of tobacco smoking has been of interest in the public health field. As shown in a recent general comment by WHO's Executive Director, the link between smoking and breast cancer has been elusive; some studies have suggested a positive link, others found no relationship and a few have suggested that smoking has protective effects.[1] A positive association has been observed in some previous case-control studies.[2–7] In contrast, little relationship has been reported by cohort studies.[8–11] Theoretically, a cohort study provides better evidence compared to a case-control study, but the limitations, *e.g.*, reference category and misclassification of smoking habits, in recent cohort studies are still under dispute.[12–15]

Tobacco smoke is well known to contain numerous possible carcinogens.[16] Although they do not directly contact mammary cells, many studies utilizing biomarkers have demonstrated that tobacco-related carcinogens reach human breast tissue.[17–19] On the other hand, antiestrogenic effects of tobacco smoke have been suggested by many published observations.[20–23] Thus, the exposure may decrease the breast cancer risk, especially in postmenopausal women.[24,25]

The objective of our study was to examine the hypothesis that tobacco smoking is associated with the risk of female breast cancer. We estimated the risks of active and passive smoking among middle-aged Japanese women in a population-based cohort study. The influence of tobacco smoke as a breast cancer risk was elucidated by menopausal status at the baseline survey of the study.

Material and methods

Study cohort

The study cohort is part of the Japan Public Health Center (JPHC)-based prospective study on cancer and cardiovascular diseases (JPHC Study, cohort I) established on January 1, 1990. The study population was defined as Japanese residents aged 40–59 years, 27,063 men and 27,435 women, in 14 administrative districts in 4 PHC areas across Japan.[26] After the initiation of the study, 37 women were found to be ineligible and were excluded, leaving 27,398 women eligible for the study. Study procedures were approved by the ethics committee of the National Cancer Center, Tokyo, Japan.

Baseline survey

A self-administered questionnaire was distributed mostly by hand and partly by mail to the subjects in 1990. They were asked about their personal and familial medical histories, smoking habit, alcohol consumption, dietary habits and other lifestyle factors. A total of 22,482 women responded to the survey (82.1% response rate). Although the date of questionnaire completion ranged from January 1990 to May 1992, 54% responded between February 1990 and March 1990. Only 4% of questionnaires were completed after October 1990. The questions on active smoking consisted of current and former smoking status, age at initiation of smoking, average number of cigarettes smoked per day and age at cessation of smoking for former smokers. Questions on passive smoking were in 2 parts: a) "Have you lived with any regular smokers?" and age at exposure ($\leq$ 20 years old, $>$ 20 years old, both) and b) " In places outside the home, *e.g.*, at work, how often are you exposed to environmental tobacco smoke $\geq$ 1 hr/day?" (almost never, 1 to 3 days/month, 1 to 4 days/week, almost everyday).

Follow-up and identification of breast cancer

We followed the subjects from recruitment until December 31, 1999. In Japan, all death certificates are submitted to a local government office and forwarded to the PHC in the area of residence. Mortality data are then sent to the Ministry of Health, Labour and Welfare and coded for inclusion in the National Vital Statistics. The registration of deaths in Japan is required by the Family Registration Law and is theoretically complete. Therefore, all deaths of the subjects were based upon death certificates from each PHC, when they remained in the original area. Changes in residence status were identified annually through the residential registry in each area. Collection of cancer incidence data and migration data was described in a previous report.[27] Briefly, on January 1, 1990, a specific cancer registry for the JPHC Study was

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established to collect cancer incidence data on the study subjects living within the study area *via* voluntary reports from local major hospitals, on-site visits to the hospitals and records from the prefecture-wide population-based cancer registry, if available (Akita and Nagano Prefectures do not have a prefecture-wide cancer registry). Cancer incidence data were collected only for subjects who were living within the study area. Site of origin and histologic type were coded using the International Classification of Disease for Oncology, second edition (ICD-O-2). By December 31, 1999, 226 new breast cancer cases had been identified. Twelve carcinoma *in situ* were not included among these breast cancer cases. A diagnosis of breast cancer was histologically confirmed in 97% of the cases. The incidence/mortality ratio in the cancer registration was 5.4, and no cases were ascertained by death certificate alone [Death Certificate Only (DCO)]. In 1.1% of cases the subjects' death certificates were used as a supplementary information source for the registry [Death Certificate Notification (DCN)]. The estimated completeness of the registration was 91.8%, which suggested that the completeness for this cohort was reasonably high.[28,29]

Migration data were obtained from residential registries. Among non-case study subjects, 1,837 (6.7%) moved out of the study area and 34 (0.1%) were lost to follow-up within the study period.

Data analysis

From the 22,482 subjects, we excluded 612 more (including 12 breast cancer cases) with a past history of cancer in any site. Consequently, after excluding still another 53 subjects who submitted incomplete information on active or passive smoking status, a total of 21,805 subjects, 180 of whom developed breast cancer, were included in this analysis. Person-years of follow-up were counted from the date of questionnaire completion until the dates of a diagnosis of breast cancer, migration out of the study areas, death or the end of the study (December 31, 1999), whichever came first.

The relative risk (RR) and 95% confidence interval (CI) were estimated by the Cox proportional hazards model, adjusting for age and area according to the SAS PHREG procedure (SAS Institute, Inc., Cary, NC). For further adjustment, we incorporated additional possible confounders into the model; education level (≥ high school and < high school), employment status (employed and unemployed), body mass index (< 22, < 25, and ≥ 25), family history of breast cancer in mother or sisters, history of past benign breast disease, age at menarche, number of births (0, ≥ 1), menopausal status (pre and post), hormone use and alcohol consumption per week (< once/week, < 250 g/week, ≥ 250 g/week). Concerning body mass index and the number of births, influence on the estimates was similar between the categorical and continuous variables. Height, weight, fruit and vegetable intake and physical activity had little influence on the estimates and thus were omitted from the adjustment in the final analysis. Breast-feeding was not incorporated in the adjustment factors because it was not included in the questionnaire. We coded current occupations recorded in an open-end column in the questionnaire according to a major occupational category (Standard Occupational Classification for Japan, the third revision of 1997, Statistic Bureau, The Ministry of Public Management). The occupational categories consisted of professionals and technicians; managers; clerks; shop and market sales workers; service workers; security workers; agricultural, forestry and fishery workers; transport and communication workers; assemblers and manual laborers; workers unclassified and unemployed. Most agricultural, forestry and fishery workers were farmers. In the analysis concerning active smoking, passive smoking was defined as a history of exposure to residential sidestream smoke in any period or exposure to sidestream smoke (almost everyday) in any occupational and/or public setting.

After excluding from the analyses 6 cases whose pathological information was uncertain, we obtained results similar to those presented.

Results

Among the 21,805 women, the prevalence of current, ex- and never-active smokers was 5.7%, 1.7% and 92.6%, respectively. Among never-active smokers, 69% reported that they had been exposed to sidestream smoke (Table I). Table II compares known risk factors and possible confounders for breast cancer among 4 categories of smoking status. These factors included characteristics reported in the literature to be risk factors, and most of them served as adjustment factors in further statistical analyses. Table III shows RRs of incidence according to active smoking. Without taking account of passive smoking in the reference category, the adjusted RR for current active smokers was 1.7 (95% CI = 1.0–3.1). When the reference condition was defined as never-active smokers without passive smoking, a 2-fold risk was observed among current active smokers (adjusted RR = 1.9; 95% CI = 1.0–3.6). Stratified analyses by employment status showed the following adjusted RRs; 1.0 (95% CI = 0.5–2.0) for unemployed women with passive smoking, 0.8 (95% CI = 0.2–3.9) for unemployed women with active smoking, 1.2 (95% CI = 0.8–1.9) for employed women with passive smoking and 2.3 (95% CI = 1.1–4.8) for employed women with active smoking. After omitting the first 3 years after the study baseline to exclude possibly ill subjects, we observed similar results (data not shown).

In premenopausal women at baseline, ever-active smokers showed a 4-fold increased risk (adjusted RR = 3.9; 95% CI = 1.5–9.9); never-active smokers with passive smoking also exhibited a significantly increased risk (adjusted RR = 2.6; 95% CI = 1.3–5.2)compared to never-active smokers without passive smoking. Stratified analyses by employment status showed increased risk for active and passive smoking in both unemployed and employed women; adjusted RR = 4.4 (95% CI = 0.6–34.6) for unemployed women with passive smoking; 7.9 (95% CI = 0.7–90.8) for unemployed women with ever-active smoking, 2.3 (95% CI = 1.1–4.9) for employed women with passive smoking and 3.3 (95% CI = 1.2–9.4) for employed women with ever-active smoking.

In postmenopausal women at baseline, no significant increased risk was observed for ever-active smokers (adjusted RR = 1.1; 95% CI = 0.5–2.5). Stratified analyses by employment status showed the following adjusted RRs; 0.6 (95% CI = 0.3–1.3) for unemployed women with passive smoking, 0.3 (95% CI = 0.04–2.6) unemployed women with ever-active smoking, 0.7 (95% CI = 0.4–1.2) for employed women with passive smoking and 1.5 (95% CI = 0.6–3.9) for employed women with ever-active smoking. When ex-smokers were eliminated from the statistical model because of the small number of cases and person-years, the risk of smoking remained essentially unchanged (data not shown).

TABLE I – SMOKING STATUS IN FEMALE STUDY SUBJECTS; JPHC STUDY COHORT 1

Passive smoking	Active smoking		
	Never-smokers (n = 20169)	Ex-smokers (n = 374)	Current smokers (n = 1238)
Residential passive smoking (%)[1]			
Never	6175 (31.0)	79 (21.4)	234 (19.1)
Ever			
Before age 20	2231 (11.2)	54 (14.6)	225 (18.4)
After age 20	6957 (35.0)	136 (36.8)	444 (36.3)
Both	4536 (22.8)	101 (27.3)	320 (26.2)
Passive smoking in occupational and/or public settings (%)[2]			
Almost never	13626 (68.0)	199 (53.6)	553 (44.8)
1–3 days/month	1534 (7.7)	29 (7.8)	76 (6.2)
1–4 days/week	1057 (5.3)	25 (6.7)	76 (6.2)
Almost everyday	3811 (19.0)	118 (31.8)	529 (42.9)

[1]Missing and unavailable answers were omitted from the calculation; 270 in never-smokers, 4 in ex-smokers, 15 in current smokers.– [2]Missing were omitted from the calculation; 141 in never-smokers, 3 in ex-smokers, 4 in current smokers.

TABLE II - DISTRIBUTION OF KNOWN RISK FACTORS AND POSSIBLE CONFOUNDERS FOR BREAST CANCER BY SMOKING STATUS: JPHC STUDY COHORT 1

	Never-smokers		Ex-smokers (n = 374)	Current smokers (n = 1238)	p for trend[1]
	Without passive smoking (n = 5660)	With passive smoking (n = 14533)			
Age (mean)	49.9	49.6	49.1	48.6	<0.0001
Occupation, farmer (%)[2]	1281 (23.4)	3,014 (21.2)	46 (12.5)	131 (10.9)	<0.0001
Occupation, unemployed (%)[2]	2850 (52.1)	6,423 (45.2)	164 (44.6)	494 (41.2)	<0.0001
Education (> high school, %)[2]	597 (10.9)	1,746 (12.4)	68 (18.7)	140 (11.8)	0.02
Height (mean)	151.1	151.8	152.3	152.2	<0.0001
Weight (mean)	54.3	54.2	55.8	54.2	0.58
Body mass index (mean)	23.7	23.5	24.1	23.3	<0.0001
Family history of breast cancer in mother or sisters (%)[2]	18 (0.3)	90 (0.6)	3 (0.8)	5 (0.4)	0.18
History of past benign breast disease (%)[2]	455 (8.0)	1,525 (10.5)	40 (10.7)	98 (7.9)	0.08
Age at menarche (mean)	14.7	14.6	14.4	14.8	0.30
Parous women (%)[2]	4,922 (93.3)	13,063 (95.2)	307 (89.5)	1,043 (90.7)	0.04
Age at first delivery among parous women (mean)	25.0	24.9	25.5	24.5	<0.0001
Number of deliveries among parous women (mean)	2.9	2.9	2.8	2.9	0.29
Menopausal status (postmenopausal, %)[2]	3,045 (55.2)	7,734 (54.2)	189 (51.9)	602 (49.4)	<0.001
Previous and/or current hormone use (%)[2]	1,114 (21.0)	2,786 (20.4)	82 (23.0)	258 (22.1)	0.58
Alcohol consumption per week (mean grams)	79.2	115.7	164.0	239.3	<0.0001

[1]p for trend was calculated by Cochran-Mantel-Haenszel test.– [2]Missing were omitted from the calculation; 619 in occupation, 743 in education, 53 in family history of breast cancer, 53 in history of past benign breast disease, 1,369 in child birth, 473 in menopausal status and 1,369 in hormone use.

TABLE III - RELATIVE RISK OF FEMALE BREAST CANCER ACCORDING TO ACTIVE SMOKING; 10-YEAR FOLLOW-UP IN JPHC STUDY COHORT 1

Exposure	Number of case	Person-years	RR[1] (95% CI)	RR[2] (95% CI)
Pre-and post-menopausal women at baseline:				
Never-smoker	162	187,063	1.0	1.0
Ex-smoker	4	3,344	1.4 (0.5 to 3.8)	1.1 (0.4 to 3.5)
Current smoker	14	10,901	1.5 (0.9 to 2.6)	1.7 (1.0 to 3.1)
Pre-and post-menopausal women at baseline:				
Never-smoker without passive smoking	40	52,884	1.0	1.0
Never-smoker with passive smoking	122	134,178	1.2 (0.8 to 1.7)	1.1 (0.8 to 1.6)
Ex-smoker	4	3,344	1.6 (0.6 to 4.5)	1.2 (0.4 to 4.0)
Current smoker	14	10,901	1.7 (0.9 to 3.1)	1.9 (1.0 to 3.6)
Premenopausal women at baseline:				
Never-smoker without passive smoking	9	22,982	1.0	1.0
Never-smoker with passive smoking	68	60,272	2.9 (1.4 to 5.8)	2.6 (1.3 to 5.2)
Current- + ex-smoker	11	6,907	4.1 (1.7 to 9.9)	3.9 (1.5 to 9.9)
Postmenopausal women at baseline:				
Never-smoker without passive smoking	31	28,583	1.0	1.0
Never-smoker with passive smoking	52	71,602	0.7 (0.4 to 1.0)	0.6 (0.4 to 1.0)
Current- + ex-smoker	7	7,056	0.9 (0.4 to 2.1)	1.1 (0.5 to 2.5)

[1]Relative risks adjusted for public health center (4 areas) and age (4 5-year age groups).– [2]Relative risks adjusted for public health center, age, employment status (employed and unemployed), education level (≥high school and <high school), body mass index (<22, 22 ≤<25 and ≥25), family history of breast cancer in mother or sisters, history of past benign breast disease, age at menarche, number of births (0 and ≥1), menopausal status (pre and post), hormone use and alcohol consumption per week (<once/week, <250 g/week and ≥250 g/week).

Table IV shows RRs of incidence according to passive smoking status. Adjusted RR for any passive smoking was 1.1 (95% CI = 0.8–1.6). In premenopausal women at baseline, those with any passive smoking revealed a significantly increased risk (adjusted RR = 2.6; 95% CI = 1.3–5.2), and exposure to sidestream smoke in occupational and/or public settings itself showed increased risk (adjusted RR = 2.3; 95% CI = 1.4–3.8). Concerning passive smoking in occupational and/or public settings in premenopausal women, a dose-dependent increase was found (adjusted RR = 1.0 for "almost none"; 0.6 [95% CI = 0.4–2.4] for "1 to 3 days/month", 2.2 [95% CI = 1.4–3.7] for "≥ 1 days/week", p for trend 0.002). Past exposure to sidestream smoke at home did not show an increased risk. Among postmenopausal women at baseline, RRs for passive smoking were 0.7 (95% CI = 0.4–1.0), and those exposed to sidestream smoke in an occupational and/or public setting showed a marginal decreased risk (adjusted RR = 0.5; 95% CI = 0.2–1.0).

Discussion

In the present population-based prospective study of middle-aged Japanese women, an increased risk for active premenopausal smoking women was observed, especially when the reference was defined as never-active smokers without exposure to sidestream smoke. A subgroup analysis revealed that only premenopausal women at the study baseline showed increased risks from passive smoking. These findings were independent of reproductive risk factors and other potential confounders. In previous case-control studies, the risk for active and passive smoking was equivalent,[3,4,6,7] which seems to be implausible. However, the estimated risk for active smoking was larger than that for passive smoking in our study.

Breast cancer risks differ based on menopausal status.[30] Thus, the risk factors and the magnitude of their risk may be different before and after menopause. The etiological roles of endogenous

TABLE IV – RELATIVE RISK OF FEMALE BREAST CANCER ACCORDING TO PASSIVE SMOKING IN FEMALE NEVER-SMOKERS; 10-YEAR FOLLOW-UP IN JPHC STUDY COHORT 1

	Passive smoking			
	Never	(A) Past residential exposure (in any period)	(B) Occupational and/or public exposure (everyday)	(A) or (B)
All never-smokers				
Number of cases	40	114	37	122
Person-years	50,662	127,309	35,258	134,299
RR[1] (95% CI)	1.00	1.1 (0.8 to 1.5)	1.3 (0.9 to 1.8)	1.2 (0.8 to 1.7)
RR[2] (95% CI)	1.00	1.0 (0.7 to 1.4)	1.3 (0.9 to 1.9)	1.1 (0.8 to 1.6)
Premenopausal women at baseline:				
No. of cases	9	61	28	68
Person-years	22,263	56,896	17,884	60,320
RR[1] (95% CI)	1.00	1.7 (1.0 to 3.0)	2.1 (1.3 to 3.4)	2.9 (1.4 to 5.8)
RR[2]	1.00	1.6	2.3	2.6
(95% CI)		(0.9 to 2.7)	(1.4 to 3.8)	(1.3 to 5.2)
Postmenopausal women at baseline:				
Number of cases	31	51	8	52
Person-years	27,345	68,364	16,625	71,674
RR[1] (95% CI)	1.00	0.7 (0.4 to 1.1)	0.5 (0.3 to 1.1)	0.6 (0.4 to 1.0)
RR[2] (95% CI)	1.00	0.7 (0.4 to 1.1)	0.4 (0.2 to 1.0)	0.7 (0.4 to 1.0)

[1]Relative risks adjusted for public health center (4 areas) and age 4 5-year age group).– [2]Relative risks adjusted for public health center, age, employment status (employed and unemployed), education level (≥high school and <high school), body mass index (<22, 22 ≤<25 and ≥25), family history of breast cancer in mother or sisters, history of past benign breast disease, age at menarche, number of births (0 and ≥4), menopausal status (pre and post), hormone use and alcohol consumption per week (<once/week, <250 g/week and ≥250 g/week).

hormones admit of no doubt, and a causal model of breast cancer suggested that hormones increased the breast cancer risk in adults by increasing cell proliferation and the number of target cells, and also heightened the risk of the retention of spontaneous somatic mutations.[31] Therefore, higher levels of estrogens in premenopausal women may act jointly with exogenous carcinogens in breast carcinogenesis. The carcinogenic effects of tobacco smoke may result from a balance between its carcinogenic and anti-estrogenic effects.[6] Therefore, premenopausal women are likely to be affected by tobacco carcinogens because their estrogen levels are higher, thereby possibly canceling out the anti-estrogenic effects of tobacco smoke.

Smoking was reported to be associated with a decrease in the incidence of endometrial neoplasia in postmenopausal women.[23] The net effect of tobacco smoke may be antiestrogenic in the endometrium. However, available evidence, excluding 1 prospective study in Japan,[32] indicates that smoking has no beneficial effects in the breast. We did not observe statistically significant beneficial effects in the present study. However, our data suggest that at least the carcinogenic effects of tobacco smoke are not present in postmenopausal women.

Active and passive smoking are influenced by socioeconomic status.[33,34] Occupation is in fact related to smoking habits especially in women; working women generally smoke more and are exposed to sidestream smoke more frequently. Indeed, smoking status differed among several occupation-related factors in this cohort. A stratified analysis by employment status revealed interesting findings. In postmenopausal women, increased risk was observed only in employed women, although the small numbers of cases in the subgroup analyses precluded firm conclusions. Their pack-years were comparable (employed 10 ± 11 and unemployed 13 ± 13). These findings suggest that there were unknown residual confounders or different smoking behavior in these 2 groups. Risks for passive smoking were not increased in either employed or unemployed postmenopausal women. However, in premenopausal women, risks for active or passive smoking were increased in both employed and unemployed women. These findings suggest that any tobacco smoke exposure elevated the risk in premenopausal women no matter what their occupation. Educational level can be a surrogate indicator of socioeconomic status and has been reported as one of the important risk factors for breast cancer. Although we incorporated employment status and educational level into our statistical models, unknown residual confounders concerning socioeconomic status might not necessarily have been excluded from our analysis.

In our study, past exposure to sidestream smoke at home showed different effects from those by the occupational/social exposure. Residential exposure was defined as "a smoker(s) who had lived with a subject", although the current occupational/social exposure was assessed semi-quantitatively by self-report. Intensity or duration of daily exposure could not be estimated for the residential exposure. Previous cohort studies in Japanese women also used the smoking status of husbands as an index of passive smoking and did not observe elevated risk.[32,35]

The limitations of previous case-control studies were that recall and selection bias would tend to produce spurious positive association.[11] On the other hand, the limitations of previous cohort studies including misclassification of exposure and reference category have also been pointed out.[12–15] However, a well-designed prospective study is known to provide persuasive evidence. Our prospective study design also has some advantages in estimating the risks of smoking. Although recall bias may exist with information concerning passive smoking in a case-control study, there was no recall bias in our study because of its prospective nature. Never-active smokers without passive smoking were assigned to the reference, allowing for more accurate classification of exposure. Nonresidential passive smoking, *i.e.*, occupational or public exposure to tobacco smoke, was taken into account in the analyses. Subgroup analyses concerning menopausal status were done because the combined analyses may dilute the risk estimation.

On the other hand, there are some admitted limitations. Because the exposure assessment was done at 1 point (at baseline), a misclassification of the exposure might have occurred, thereby diluting the effects if some smoking women had quit smoking during the follow-up period. Information on the menopausal status was obtained at baseline. Therefore, we did not examine the risks for pre- and post-menopausal cancer. The relatively small number of incidence cases precluded further subgroup analyses. Results of the subgroup analyses according to menopausal status in this report should be confirmed by continued follow-up.

Different effects of active or passive smoking regarding breast cancer risk had been shown in premenopausal and postmenopausal women.[7,36] In a recent study, the risk of breast cancer among smokers has been clearly reported to be elevated in premenopausal women.[36] Immature breast cells are suggested to have especially increased susceptibility to smoking-related carcinogens.[6] In our

study, 94% of subjects had delivered children, but the effect of smoking in strata defined by age of full-term birth could not be examined. On the other hand, in postmenopausal women, the risk of breast cancer among smokers has been reported not to be elevated.[36] These previous observations are consistent with our observations regarding both active and passive smoking. Race is also an important factor in the interpretation of our results. To our knowledge, this is the first prospective study to link active smoking to breast cancer risk in Asian women, although recent large-scale cohort studies in America did not detect any increased risk of breast cancer.[10,11] Genetic differences concerning important metabolic enzymes, for example, higher frequency of a variant allele of cytochrome P450 1A1 gene, were reported,[37] and endogenous estrogen levels and the number of estrogen receptors have been reported to differ between Japanese and Caucasians.[38,39] Thus, an association between smoking and breast cancer might appear more readily in Japanese. The incidence of breast cancer among premenopausal women (88/90,161 person-year) was almost the same as that among postmenopausal women (90/107,241 person-year), and the association observed in premenopausal women was strong. These might be why we observed an elevated risk due to tobacco smoking in the overall subjects.

In conclusion, tobacco smoking increases the risk of female breast cancer in premenopausal women. Both active and passive smoking are promising targets in the prevention of breast cancer.

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CLINICAL INVESTIGATION **Esophagus**

NONRANDOMIZED COMPARISON BETWEEN DEFINITIVE CHEMORADIOTHERAPY AND RADICAL SURGERY IN PATIENTS WITH $T_{2-3}N_{any}$ M_0 SQUAMOUS CELL CARCINOMA OF THE ESOPHAGUS

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Purpose: To compare the treatment results between radical surgery and definitive chemoradiotherapy for resectable squamous cell carcinoma of the esophagus and to identify useful clinicopathologic and biologic markers to select better treatment.

Methods and Materials: Between August 1992 and April 1999, 98 consecutive patients were selected for this study; 53 were treated with chemoradiotherapy and 45 with surgery. The patients in the chemoradiotherapy group received 5-fluorouracil combined with cisplatin plus 60 Gy of radiation, and those in the surgery group received an esophagectomy with radical node dissection. Biologic markers were investigated immunohistochemically using pretreatment biopsy specimens.

Results: The baseline clinical TNM stage was more advanced in the chemoradiotherapy group than in the surgery group. With a median follow-up period of 43 months, the 5-year survival rate was 46% in the chemoradiotherapy and 51% in the surgery group, without statistical significance ($p = 0.47$, log–rank test). Cox regression analysis for prognosis revealed that epidermal growth factor receptor positivity, high microvessel density, and cyclin D1 positivity yielded a low value for relative risk (0.66, 0.54, and 0.62, respectively), which favored chemoradiotherapy over surgery, without statistical significance.

Conclusion: This nonrandomized study showed a trend for the chemoradiotherapy in the treatment of esophageal carcinoma, but the results need to be confirmed by additional study.

Esophageal cancer, Chemoradiotherapy, Surgery, Biologic marker.

INTRODUCTION

Esophageal cancer is a relatively uncommon but virulent disease. Surgical resection has been widely accepted as the standard treatment for esophageal cancer, with techniques improving during the past decades. However, long-term survival after resection of thoracic esophageal carcinoma is generally poor, with a 20–42.4% 5-year survival rate (1–4).

Some reports on chemoradiotherapy have indicated various advantages in managing esophageal cancer (5). Oncologists advocate that a nonsurgical approach with definitive chemoradiotherapy may be the standard for this disease (6–9). We have also reported that definitive chemoradiotherapy has a curative potential for locally advanced esophageal carcinoma (10, 11). In resectable esophageal cancer with T2–3 Nany M0 (tumor invading muscularis propria [T2] or adventitia [T3], no regional lymph node metastasis [N0], or regional lymph node metastasis [N1], and no distant metastasis [M0]), several reports (6, 7, 12) have shown the curability with chemoradiotherapy for squamous cell carcinomas of the thoracic esophagus. However, it is uncertain whether definitive chemoradiotherapy achieves comparable treatment outcomes to surgery, because a randomized controlled study has not yet been undertaken. The performance of a clinical randomized trial is quite difficult because of the differing treatment characteristics.

Recently, many reports identifying prognostic factors for esophageal cancer, including some biologic markers, have been published. We have previously reported that microves-

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sel density (MVD) is a prognostic factor for advanced esophageal cancer patients treated with definitive chemoradiotherapy (13). These results showed that patients in the high MVD group survived longer than those in the low MVD group, contrary to results published previously in patients who underwent surgery. These results suggest that some biologic markers may indicate optimal treatment modalities.

The purpose of this retrospective study was to compare the treatment outcomes of patients undergoing definitive chemoradiotherapy with those of patients undergoing radical surgery in patients with T2–3 Nany M0 squamous cell carcinoma of the esophagus. Additionally, we investigated the baseline clinicopathologic factors and expression of various biologic markers in both groups in attempt to define the factors indicative of the optimal treatment of choice.

METHODS AND MATERIALS

Selection criteria and pretreatment evaluation

The source of the study was the database of patients treated in our institution. Patients who had histologically proven squamous cell carcinoma of the thoracic or abdominal esophagus and clinically diagnosed T2–3 Nany M0 disease, as defined by the criteria of the International Union Against Cancer (14), were selected for the subjects of this analysis. The selection criteria also included the following: age ≤75 years, Eastern Cooperative Oncology Group performance status of 0–2, white blood cell count ≥3000/μL, platelet count ≥100,000/μL, aspartate aminotransferase and alanine aminotransferase levels within three times of the normal upper limit, serum bilirubin level ≤2.0 mg/dL, creatinine level ≤1.5 mg/dL, creatinine clearance ≥50 mL/min, and normal electrocardiogram. Patients with serious complications, a history of ischemic heart disease, or any carcinoma at another site other than early stage were also excluded from this study.

The routine pretreatment evaluation included barium esophagography, esophagoscopy, and cervical, chest, and abdominal CT scans. Endoscopic ultrasonography (EUS), bronchoscopy, and cervical ultrasonography were optional. The following patients were excluded because they were considered to have clinical Stage T4: tumor that extended into the lumen or caused a deformity of the tracheobronchial tree or tumor that was attached to organs at a contact angle of ≥90° in the thoracic aorta, as observed on CT scan. The differential diagnosis between Stage T2 and T3 was done by EUS. If we could clearly diagnose the T stage by esophagoscopy, esophagography and CT scan, we did not perform EUS. Positive lymph nodes were defined if they were ≥1 cm in size on any of the images. These evaluations on CT scan for staging were reviewed and judged by the diagnostic radiologist (M.H.), who did not have any clinical information.

Chemoradiotherapy

The choice of treatment was decided by the patients after having been given medical information from both the medical and the surgical oncologists. Chemotherapy consisted of protracted infusion of 5-fluorouracil (5-FU) 400 mg/m^2/d on Days 1–5 and 8–12, combined with cisplatin (CDDP) 40 mg/m^2/d on Days 1 and 8, repeated twice every 5 weeks. Concurrent radiotherapy was started on Day 1 at 2 Gy/d for 5 d/wk. The total radiation dose was 60 Gy, with a 2-week break after a dose of 30 Gy. For patients who showed an objective response to treatment, additional chemotherapy consisted of protracted infusion of 5-FU 800 mg/m^2/d on Days 1–5 and CDDP 80 mg/m^2/d on Day 1. This treatment was repeated every 4 weeks. The details of the treatment schedule have been described in previous reports (10, 11, 13). The patients who achieved a complete response underwent CT scanning and esophagoscopy every 3 months during the first 1 year and every 6 months thereafter.

Surgery

Total or subtotal thoracic esophagectomy through a right or left thoracotomy with radical node dissection was performed. Lymphadenectomy included the mediastinum, abdomen, and neck (so-called three-field dissection). However, two-field node dissection and left thoracotomy was indicated for patients with a high risk of complications. Esophagectomy and lymphadenectomy were followed by esophageal reconstruction, mostly using a gastric tube. No adjuvant chemotherapy or radiotherapy was added. The patients who underwent R0 resection underwent CT scan every 6 months and every 12 months thereafter.

Evaluations for response and toxicity

We used the World Health Organization response criteria for measurable diseases with a combination of CT, esophagoscopy, and sometimes esophagography and EUS. Toxicity was evaluated using the criteria defined by the Japan Clinical Oncology Group (16). The details of these criteria have been previously described (11). Late toxicity assessment for complete response patients was performed using the Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer late radiation morbidity scoring scheme. Late toxicity was defined as toxicity that occurred >90 days after treatment initiation. Surgical resectability was evaluated according to residual tumor classification in the TNM classification, which deals with tumor status after treatment. These criteria were defined as follows: R0, no residual tumor; R1, microscopic residual tumor; and R2, macroscopic residual tumor. Mortality was defined as patient death within 30 days postoperatively.

To evaluate the posttreatment status, we compared the consumed food content and body weight changes before and after treatment. Food content was classified into four groups: solid, semisolid, liquid, and none on the basis of the patients' charts. The content after treatment was determined as the best intake at any time after treatment initiation. The

body weight changes of patients were assessed using the same methods as for food content. We evaluated these factors once a week for inpatients and every visit to the hospital for outpatients until April 4, 2001.

Immunohistochemical examination and evaluation

Of the 98 eligible patients, 3 (2 in the chemoradiotherapy and 1 in the surgery group) were excluded from immunohistochemical examination, because sufficient pretreatment biopsy material was not available for study. We carried out immunohistochemical staining for 95 biopsy samples using the avidin-biotin-peroxidase complex method. The details of the immunohistochemical procedure and evaluation criteria of p53, cyclin D1, Ki-67, epidermal growth factor receptor (EGFR), and vascular endothelial growth factor have been previously described (13). In the microvessel count using an anti-CD31 antibody, positive staining was assessed by light microscopy in areas of the tumor containing the highest numbers of capillaries and small venules with lumens. The highly vascular areas were identified by scanning the tumor sections at low power (40× and 100×). The vessel count was performed on a 200× field (20× objective and 10× ocular). The tumors were classified into two groups, high MVD and low MVD, on the basis of the number of 50 vessel counts by making the histogram of MVD results.

Statistical analysis

The survival time was calculated from the date of treatment initiation to that of death from any cause or to the last date of confirmation of survival. Survival was updated on April 4, 2001, with a minimal follow-up period of 22 months. We estimated survival curves using the Kaplan-Meier method and compared them with the log-rank test. Relative risks and their 95% confidence intervals (CIs) of chemoradiotherapy to surgery were estimated using the univariate and multivariate Cox regression model adjusting for gender, age, performance status, tumor location, tumor length, T stage, and N stage. To evaluate the influence of biologic markers on survival according to the treatment modalities (chemoradiotherapy or surgery), the relative risks and their 95% CIs were calculated using the univariate and multivariate Cox regression model adjusting for N stage, p53, Ki-67, EGFR, cyclin D1, MVD, and vascular endothelial growth factor. The relative risk of each marker with <1 means that the survival of patients treated with chemoradiotherapy was more favorable than after surgery. Statistical analyses were performed using Statistical Analysis System software (SAS Institute, Cary, NC).

RESULTS

Patient characteristics

Between August 1992 and April 1999, a total of 348 esophageal cancer patients (209 patients treated with chemoradiotherapy and 139 patients with surgery) were treated at the National Cancer Center Hospital East, Kashiwa. Of these patients, 98 were finally selected as the subjects for this study. The reasons for excluding 250 patients were as follows: 7 because of different histologic findings, 15 because of prior treatment, 9 because of treatment with planned neoadjuvant chemoradiotherapy, 1 because of treatment with adjuvant radiotherapy, 32 because of advanced cancer stage at other sites, 6 because of medical complications, 6 because of age >75 years, 3 because of a poor performance status, and 172 because of other TNM stages.

Of the 98 selected patients, 53 patients were in the chemoradiotherapy group and 45 in the surgery group. The patient characteristics of both groups are shown in Table 1. Because this study was a nonrandomized comparison, some imbalances resulted in the baseline characteristics between the two groups. The chemoradiotherapy group included more female and older patients than the surgery group (p =

Table 1. Patient characteristics

Characteristic	CHRT	Surgery	p*
Gender (*n*)			
Male	45	44	0.04
Female	8	1	
Age (y)			
Median	64	58	0.08
Range	38–75	40–75	
PS (*n*)			
0	38	28	0.44
1	15	16	
2	0	1	
Location (*n*)			
Upper	4	0	0.03
Middle	33	22	
Lower	16	23	
Length (cm)			
Median	4.5	4.0	0.20
Range	1.8–20	1.5–8.0	
Macroscopic type (*n*)			
0	0	6	0.009
1	2	6	
2	35	22	
3	15	11	
4	1	0	
Histologic type (*n*)			
W/D SCC	2	0	0.63
M/D SCC	36	32	
P/D SCC	15	13	
T stage (*n*)			
T2	10	15	0.11
T3	43	30	
N stage (*n*)			
N0	22	31	0.008
N1	31	14	
Stage (*n*)			
IIA	22	31	0.04
IIB	6	3	
III	25	11	

Abbreviations: CHRT = chemoradiotherapy; PS = performance status; SCC; squamous cell carcinoma, W/D = well differentiated; M/D = moderately differentiated; P/D = poorly differentiated.

* Fisher's exact test.

Table 2. Comparison of treatment results between chemoradiotherapy and surgery

Chemoradiotherapy	*n* (%)	Surgery	*n* (%)
Treatment completion		Lymphadenectomy	
RT dose of 60 Gy	52 (98)	Three fields	35 (78)
Additional 2 courses of CHT	30 (57)	Less than two fields	10 (22)
Response rate		Curability	
CR	37 (70)	R0	44 (98)
PR	16 (30)	R1,2	1 (2)
Toxicity (Grade 3 or 4)		Morbidity	
WBC count	27 (51)	Negative	15 (36)
Platelets	8 (15)	Positive	29 (64)
Creatinine	2 (4)		
Esophagitis	3 (6)		
Stomatitis	2 (4)		
Treatment-related death	0	Mortality	2 (4)

Abbreviations: CHT = chemotherapy; CR = complete response; PR = partial response; WBC = white blood cell.

0.04 and 0.08, respectively). Of the macroscopic type, more patients had type 0 (i.e., superficial cancer) in the surgery group ($p = 0.009$). For T stage, 10 (19%) and 43 (81%) patients had T2 and T3 disease, respectively, in the chemoradiotherapy group and 15 (33%) and 30 (67%), respectively, did so in the surgery group ($p = 0.16$). Additionally, 22 (42%) and 31 (58%) had N0 and N1 disease, respectively, in the chemoradiotherapy group and 31 (69%) and 14 (31%) in the surgery group ($p = 0.04$). For clinical stage before treatment, patients in the chemoradiotherapy group had a more advanced stage than those in the surgery group ($p = 0.008$).

Treatment results

A summary of the treatment results for the chemoradiotherapy and surgery groups is shown in Table 2. Fifty-two (98%) of the 53 patients treated with chemoradiotherapy completed at least the chemoradiotherapy segment, with a total radiation dose of 60 Gy. Seven (13%) received an additional course of chemotherapy, and 26 (49%), 2 (4%), and 2 (4%) received two, three, and four additional courses, respectively. One patient who did not complete the chemoradiotherapy segment because of severe radiation dermatitis, achieved a complete response, despite the early termination of treatment, with a radiation dose of 46 Gy. Of the 53 patients, 37 (70%) achieved a complete response. No patients received salvage surgery after definitive chemoradiotherapy.

All 45 patients in the surgery group underwent esophagectomy through right or left thoracotomy. Lymphadenectomy was carried out in 35 patients (78%) with three-field dissection; 10 patients (22%) underwent two field or less dissection. An "R0" resection was achieved in 44 patients (98%).

Toxicity and morbidity

The major hematologic toxicity of chemoradiotherapy was leukopenia and thrombocytopenia. Grade 3 or 4 leukopenia and thrombocytopenia was seen in 27 (51%) and 8 (15%) of the 53 patients, respectively. In respect to nonhematologic toxicity, esophagitis was seen in 34 patients (64%), including 3 (6%) with Grade 3 or 4. Stomatitis and diarrhea were seen in 9 (17%) and 5 (9%) patients, respectively. For late toxicity, Grade 3 or 4 pericarditis, pleural effusion, and radiation pneumonitis was seen in 4 (11%), 5 (14%) and 3 (8%) of the 37 complete response patients, respectively. No treatment-related deaths occurred in the chemoradiotherapy group.

Of the 45 patients who underwent esophagectomy, the morbidity rate was 64% (29 of 45). The major complication was anastomotic leakage in 13 patients (29%), including one followed by reoperation and another by drainage to the leakage cavity. The mortality rate of surgery was 4% (2 of 45). One patient died of acute myocardial infarction 3 days after surgery and another of severe mediastinitis because of anastomotic leakage.

Overall survival

Figure 1 shows the survival curves of both the chemoradiotherapy and the surgery groups. With a median follow-up period of survivors in both groups of 43 months, the median survival time was 33 months in the chemoradiotherapy and had not been reached in the surgery group. The 3- and 5-year survival rate was 49% and 46% in the chemoradiotherapy group and 61% and 51% in the surgery group, respectively. No statistically significant difference in overall survival was found between the two groups ($p = 0.47$, relative risk = 1.24, 95% CI 0.69–2.21). After adjusting for clinical factors, no statistically significant difference was detected between the groups ($p = 0.70$, relative risk 0.88; 95% CI 0.45–1.72).

Figure 2 shows the survival curves of the patients' subgroup according to N stage. In the patients with Stage N0, the median survival was 37 months in the chemoradiotherapy group and was not reached in the surgery group. The patients treated with surgery had a tendency to survive longer than those who underwent chemoradiotherapy, without statistical significance ($p = 0.30$). In Stage N1, the

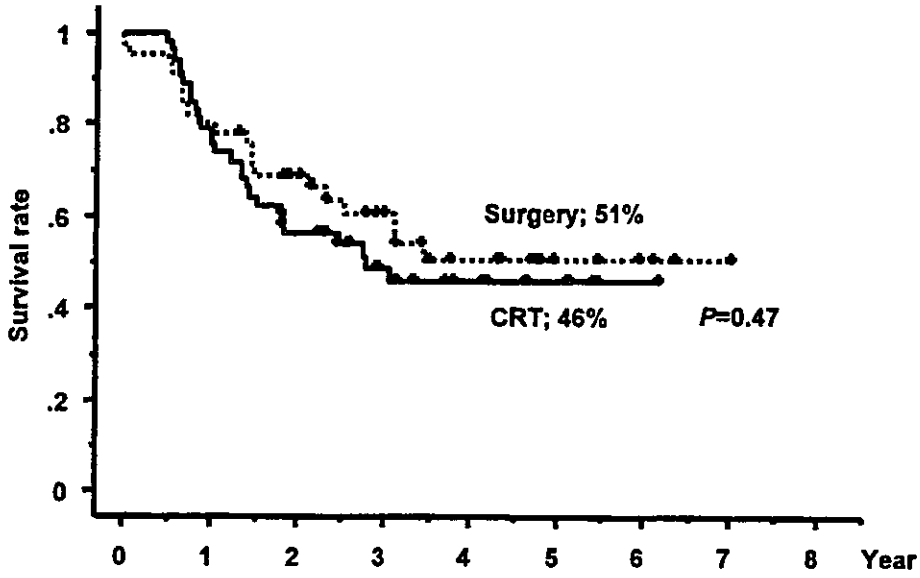

Fig. 1. Survival curves of chemoradiotherapy group (CRT, solid line) and surgery group (dotted line) with Kaplan-Meier method. Median survival was 34 months in chemoradiotherapy group and not reached in surgery group. No statistical significant difference was found in both groups in overall survival ($p = 0.47$).

median survival was 30 months in the chemoradiotherapy group and 18 months in the surgery group. In contrast to those with Stage N0, the patients treated with chemoradiotherapy had a tendency to survive longer than those in the surgery group, without significance ($p = 0.37$).

Food content and body weight after treatment

Table 3 presents the comparison of the oral intake status of the patients before and after treatment. Although the status of all 27 patients treated with chemoradiotherapy who had been able to take solid food at baseline was unchanged in food content, the status of 10 (36%) of the similar 28 patients treated with surgery had changed to lower levels after treatment ($p < 0.01$). Additionally, 23 (88%) of the 26 patients who had had impairment in taking solid content at baseline were able to take solid food after chemoradiotherapy, and only 10 (59%) of the 17 patients with a similar status in the surgery group had improvement in food content status ($p = 0.03$).

With regard to body weight changes, when comparing before and after treatment, 31 (76%) of 41 patients (3 were not assessable) in the surgery group and 16 (31%) of 51 patients in the chemoradiotherapy group had body weight loss. Table 4 shows the average body weight change ratio in both groups. The ratio of the chemoradiotherapy group was significantly greater than that in the surgery group ($p < 0.01$).

Analyses of prognostic factors, including biologic markers

Table 5 shows the univariate and multivariate Cox regression analyses on survival in each category, including N stage and biologic markers. In the univariate analysis, the relative risk of N1 was low, at 0.67, and that of N0, cyclin D1-negative, and low MVD showed high values (1.64, 3.86, and 1.82, respectively). No statistical significant differences were observed in all variables. In multivariate analysis, the factors EGFR positivity, high MVD, and cyclin D1 positivity yielded relative risks of 0.66, 0.54, and 0.62, respectively, with the 95% CI ranging from 0.30 to 1.47, 0.22 to 1.31, and 0.30 to 1.30, respectively. Because the number of subjects was small, no statistically significant differences were found despite the low relative risk. The association of other variables with survival was negligible.

DISCUSSION

In the present study, although the same selection criteria as used in other clinical trials were applied to both groups, a selection bias might still remain. In our institution, the preferred treatment was chosen by the patients after having been given information from the medical and surgical oncologists. Despite these circumstances, imbalances were present in the baseline patient characteristics between these groups: patients referred to chemoradiotherapy were older and had a more advanced stage than those opting for surgery. These imbalances might have been caused by the bias in both patients and oncologists who were likely to avoid radical surgery for patients with any risks, although this tendency seems to be usually seen in general practice. Whatever the cause, these imbalances seemed to be in favor of the surgery group rather than the chemoradiotherapy group.

In our results, no statistically significant difference was noted in overall survival between both groups, particularly in the difference in the 5-year survival rate, which was only 5%. These results suggest that definitive chemoradiotherapy

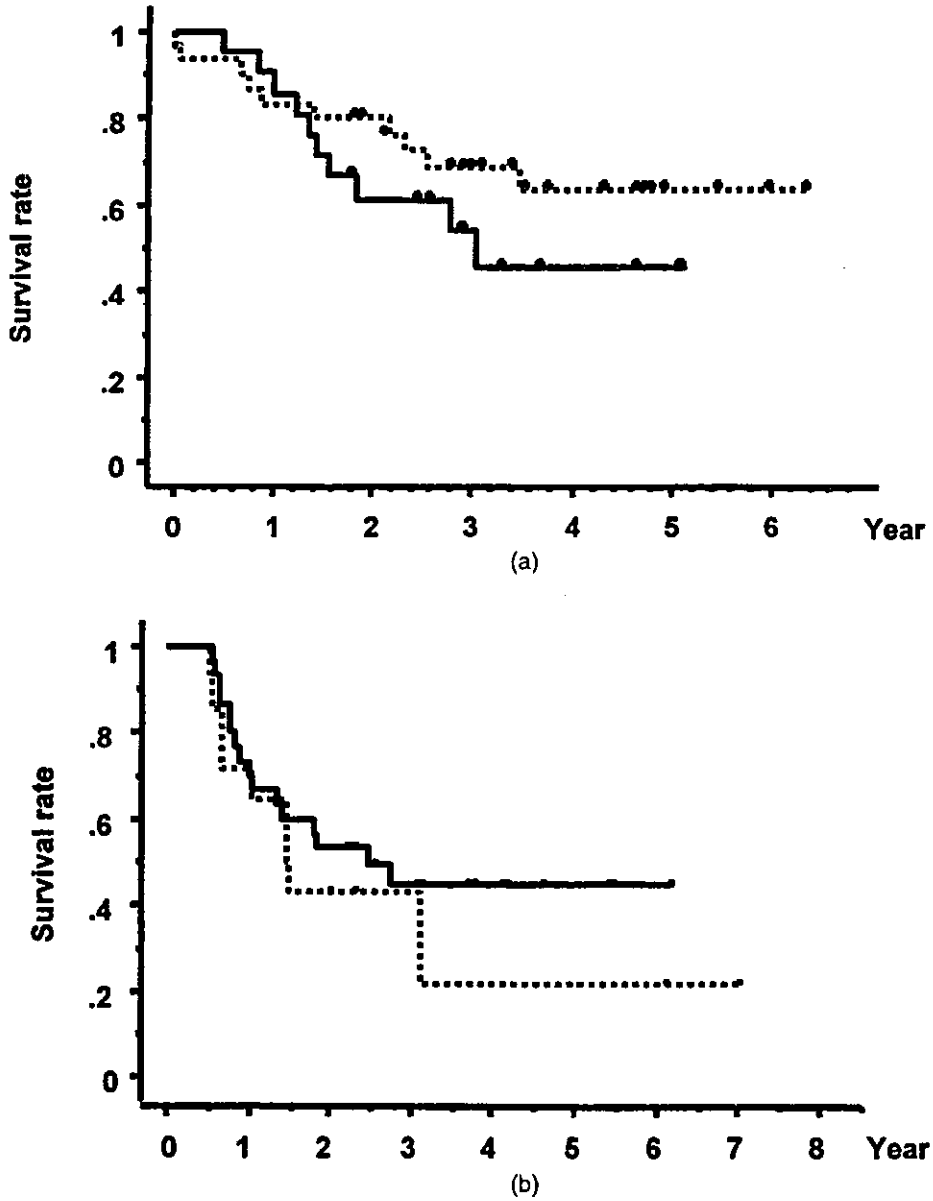

Fig. 2. (a) Survival curves of patients with N0 disease treated with chemoradiotherapy (solid line) or surgery (dotted line). Patients treated with surgery had a tendency to survive longer than those treated with chemoradiotherapy, without statistical significance ($p = 0.30$). (b) Survival curves of patients with N1 disease treated with chemoradiotherapy (solid line) or surgery (dotted line). Patients treated with chemoradiotherapy had a tendency to survive longer than those treated with surgery, without statistical significance ($p = 0.37$).

provides at least comparable survival as surgery in the treatment of squamous cell carcinoma of the esophagus. According to the National Cancer Institute "Cancer Gov.," definitive chemoradiotherapy is one of the treatment options

Table 3. Comparison of oral intake status of patients before and after treatment

Baseline status	Treatment	*n*	Improved	No change	Worse	*p*
Solid	CHRT	27	—	27 (100)	0	<0.01
	Surgery	28	—	18 (64)	10 (36)	
Semisolid, liquid, or none	CHRT	26	23 (88)	3 (12)	0	0.03
	Surgery	17	10 (59)	7 (41)	0	

Abbreviation: CHRT = chemoradiotherapy.

Table 4. Ratio of body weight change before and after treatment

Treatment	Ratio (%)	*p*
CHRT	+1.5 ± 6.6	<0.01
Surgery	−5.3 ± 6.5	

Abbreviation: CHRT = chemoradiotherapy.

for esophageal cancer. However, this policy has not yet been accepted worldwide, because no prospective randomized trials comparing the two modalities have yet been reported. Our study may present evidence to support the treatment policy purported by "Cancer Gov."

A possible criticism of this study is that the surgical outcomes were inferior to those in other institutions. However, in the treatment results of the surgery group, the 5-year survival rate (50%) was fairly comparable with that of published results. Ando *et al.* (1) have reported that the 5-year survival rate for Stage T2 and T3 patients treated with esophagectomy was 30.1% and 27.1%, respectively. Müller *et al.* (2) also reported that the mean 3-year survival rate after esophagectomy was 25% in a literature review. Compared with these reports, our surgical data seem superior to those in other institutions. One reason for this result was that fewer patients with advanced stage were included in our study; other reasons included recent advances in imaging diagnosis before treatment, surgical technique, and perioperative management.

In the chemoradiotherapy group, the survival outcomes in this study seemed to be superior to those of other reports. Cooper *et al.* (7) reported that the 5-year survival rate was 26% in the randomized definitive chemoradiotherapy group and 14% in the nonrandomized combined modality group. Araujo *et al.* (8) reported that the 5-year survival rate of concomitant radiotherapy and chemotherapy was 16% in Stage II esophageal cancer patients. Because this study was nonrandomized, there might have been selection bias, which may have led to better outcomes. The determination of the T stage in both groups was performed by the same diagnostic radiologist, and when unsure of whether it was T2–T3, we diagnosed strictly to T2–T3 by CT scan and EUS. Additionally, our treatment schedule for chemoradiotherapy was different from that of other published schedules. Chemotherapy was administered for 2 weeks with one-half dose of standard 5-FU/CDDP in the chemoradiotherapy segment to prolong the time of synergistic effect between 5-FU/CDDP and radiation. In our institution, the planning target volume for carcinoma of the upper or middle third esophagus included the primary tumor with a 3-cm craniocaudal margin, metastatic nodes with a 1- to 1.5-cm margin, supraclavicular fossa, and mediastinum. For carcinoma of the lower third esophagus, the field was extended to include the perigastric nodes but excluding the supraclavicular fossa if the cervical nodes tested negative. Such issues as selection bias, strict staging, synergistic effect, and wide radiation

Table 5. Univariate and multivariate analyses of relative risks of chemoradiotherapy against surgery on survival in 95 esophageal cancer patients

		Univariate analysis			Multivariate analysis*		
Category	*n*	RR†	95% CI	*p*	RR†	95% CI	*p*
N stage							
0	51	1.64	0.68–3.96	0.27	1.01	0.38–2.71	0.98
1	44	0.67	0.30–1.47	0.32	0.81	0.35–1.87	0.62
p53							
Negative	34	1.46	0.51–4.11	0.48	0.77	0.33–1.80	0.55
Positive	61	1.10	0.54–2.23	0.79	0.96	0.48–1.94	0.91
Ki-67							
≤64	34	1.35	0.49–3.73	0.57	0.77	0.31–1.93	0.58
>64	61	1.10	0.53–2.26	0.81	0.94	0.48–1.85	0.85
EGFR							
Negative	44	1.23	0.55–2.78	0.61	1.26	0.62–2.57	0.53
Positive	51	1.20	0.52–2.79	0.68	0.66	0.30–1.47	0.31
Cyclin D1							
Negative	31	3.86	0.86–17.4	0.08	3.25	0.68–15.5	0.14
Positive	64	0.91	0.45–1.84	0.80	0.62	0.30–1.30	0.21
MVD							
≤50	56	1.82	0.75–4.38	0.19	1.35	0.51–3.55	0.55
>50	39	0.74	0.34–1.64	0.46	0.54	0.22–1.31	0.17
VEGF							
Negative	51	1.24	0.53–2.94	0.62	1.04	0.43–2.50	0.94
Positive	44	0.97	0.43–2.19	0.94	0.77	0.33–1.79	0.54

Abbreviations: RR = relative risk; CI = confidence interval; EGFR = epidermal growth factor receptor; MVD = microvessel density; VEGF = vascular endothelial growth factor.

* Adjusted by N stage, p53, Ki-67, EGFR, cyclin D1, MVD, and VEGF.

† When relative risk >1, surgery had better survival than chemoradiotherapy.

field may therefore have led to a better outcome than those in other reports.

Many studies have reported on chemoradiotherapy followed by surgery vs. surgery alone. Recent randomized studies found conflicting results. Bosset *et al.* (17) did not find any overall survival benefit with the addition of preoperative CDDP and 37 Gy of radiation to esophagectomy in squamous cell carcinoma of the esophagus. Urba *et al.* (18) also reported no statistically significant difference in survival between preoperative chemoradiotherapy vs. surgery alone for resectable esophageal cancer. Burmerster *et al.* (19) also reported no difference in overall survival between preoperative chemoradiotherapy followed by surgery and surgery alone. Our results also showed that definitive chemoradiotherapy had comparable survival to radical surgery alone for esophageal cancer. Thus, it is uncertain whether preoperative chemoradiotherapy may provide a better survival for esophageal cancer patients than surgery or definitive chemoradiotherapy alone. Additional studies are needed to clarify these controversies in the treatment of esophageal cancer.

The site of failure in the chemoradiotherapy group was different from in the surgery group. In the chemoradiotherapy group, it was local in 18 patients, local and regional lymph nodes in 1, and distant metastasis in 4 patients. In the surgery group, it was local in 1 patient, regional lymph nodes in 4, and distant metastasis in 14. It is uncertain whether the future combination of these modalities will obtain better survival for esophageal cancer patients, because of the more frequent complications of salvage surgery after chemoradiotherapy with a radiation dose of 60 Gy and frequent distant metastasis after surgery.

We investigated the food content and body weight of the patients in terms of the patients' status both before and after treatment. In our results, the status after treatment was better for the chemoradiotherapy group than for the surgery group, with a statistically significant difference. It has been reported that 90% of patients after esophagectomy lost weight compared with their preoperative status at 3 months, and only 10% of the patients noted additional weight loss at 1 year postoperatively, with postprandial fullness, diarrhea, and dumping symptoms (20). In our study, 76% of the patients in the surgery group had body weight loss because of these reasons. However, in the chemoradiotherapy group, only 31% of the patients had body weight loss. These results support the possibility that definitive chemoradiotherapy may become one of the standard treatments for resectable esophageal cancer.

Additionally, we examined the clinical backgrounds and biologic markers using pretreatment biopsy specimens by immunohistochemical staining to identify a better treatment for each individual. According to N stage, the Stage N1 patients treated with chemoradiotherapy had a tendency to survive longer than Stage N1 patients undergoing surgery, and the reverse phenomenon was observed for Stage N0. Many reports have indicated that lymph node metastasis is an independent poor prognostic factor in patients treated with surgery including radical node dissection (1, 21, 22). In contrast, chemoradiotherapy in this study seems to have had an equal antitumor effect and survival in patients with or without lymph node metastasis. Patients with EGFR positivity, cyclin D1 positivity, and high MVD tumors showed a very low relative risk in multivariate analysis, which favored chemoradiotherapy for survival. Because of the limitations of using a small number of subjects, our data showed no statistical significance. Additional studies, including prospective ones, are needed to confirm the usefulness of these biologic markers.

CONCLUSION

This retrospective, nonrandomized study showed a trend for better outcome after chemoradiotherapy in the treatment of squamous cell carcinoma of the esophagus. Although confirmative studies in randomized trials are needed, this trial, designed as surgical vs. nonsurgical, was hard to conduct because of difficulties in acquiring informed consent. In this circumstance, stratifying the different modalities for suitable patients by using baseline clinical and biologic markers may be a useful approach.

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