Table 2. Toxicity profiles

Toxicity	JCOG grade					Grade 4 (%)
	0	1	2	3	4	
Hematological toxicity						
Leukopenia	11	13	7	4	2	5.4
Neutropenia	17	5	5	6	4	10.8
Anemia	7	6	15	9	–	–
Thrombocytopenia	32	3	1	1	0	0
Non-hematological toxicity						
Nausea/vomiting	13	14	10	0	–	–
Diarrhea	25	4	6	2	0	0
Stomatitis	30	7	0	0	0	0
Alopecia	35	2	0	–	–	–
Allergic reaction	36	1	0	0	0	0
Fever	18	10	9	0	0	0
Peripheral neuropathy	36	1	0	0	–	–
Total bilirubin	20	–	8	8	1	2.7
AST	16	16	5	0	0	0
ALT	16	16	5	0	0	0
Alkaline phosphatase	16	17	2	2	0	0
Creatinine	33	2	0	2	0	0
Hyponatremia	12	17	7	0	1	2.7
Hypokalemia	21	12	4	0	0	0

response to chemotherapy. The most frequent reason for treatment termination was disease progression (27 patients, 73%). Other reasons for treatment termination were no response after 8 weeks from initiation of treatment in two, patient refusal in two, severe toxicity in two, death in three (one due to disease progression and two treatment-related) and medical judgment by the investigators in one.

Toxicity

The toxicity observed in the study period is summarized in Table 2. The major toxicity was myelosuppression and gastrointestinal toxicity. Grades 3 and 4 neutropenia occurred in 16 and 11% of the patients, respectively. Severe thrombocytopenia was infrequent. The incidence of grade 3 diarrhea was 5%. Mild nausea and vomiting (grades 1 and 2) were frequently experienced (65%). An increase in total bilirubin of grade 4 was observed in one patient (2.7%) and was diagnosed as obstructive jaundice caused by the development of lymphadenopathy from the primary disease. An increase in total bilirubin grade 3 was observed in eight patients, three cases of which were judged to be treatment-related. An increase in serum creatinine grade 3 was observed in two patients (5.4%). One patient experienced grade 4 hyponatremia due to loss of oral intake associated with primary disease. Early death, which was defined as death within 30 days from the last administration of anti-cancer drugs, occurred in five patients. The causal relationship between the death and the study treatment was 'unlikely' in three of those five patients. However, the remaining two deaths were assessed to be treatment-related. One patient died of severe neutropenia and rapidly progressive disseminated intravascular coagulation (DIC), which was complicated with respiratory dysfunction, and the other patient died of progressive neutropenic sepsis.

Efficacy

The efficacy-related data are summarized in Table 3. Only two of 35 response-assessable patients achieved objective partial response (response rate 5.7%; 95% confidence interval: 0.7–19.2%). However, in terms of the response of ascites, three disappearances and 10 decreases of ascites were obtained (response rate 35.1%; 95% confidence interval: 20.2–52.5%). The median duration of response of ascites was 103 days with a range of 52–337 days. The median survival time of all patients was 155 days (95% confidence interval: 131–225 days) and the 1 year survival rate was 16.2% (95% confidence interval: 4.3–28.1%).

DISCUSSION

Although unresectable or metastatic gastric cancer is potentially incurable, there is significant evidence that adding systemic chemotherapy to the best supportive care could provide benefits in survival and quality of life as compared with best supportive care alone (1–3). However, it has been difficult to assess which of many available regimens is the most effective, although several regimens have been tested in randomized controlled trials. Some randomized trials failed to demonstrate the superiority of 5FU-based combination regimens as compared with 5FU-monotherapy (19–21). A recent randomized controlled trial showed that three commonly used combination regimens, 5FU/adriamycin/MTX (FAMTX), 5FU/cisplatin (FP) and etoposide/leucovorin/5FU (ELF), have only modest activity and that there were no significant differences in overall survival among these regimens (22). More recently, infusional 5FU in combination with cisplatin and epirubicin (ECF) showed significant superiority over FAMTX in terms of response rate, quality of life and survival, suggesting that the ECF could be a new standard treatment for future clinical trials (23). However, regarding the median survival time in those large-scale trials, there was little substantial difference among the various regimens. Therefore, in general, 5FU-based or cisplatin-based combinations are widely accepted as a possible standard therapy (24). In clinical practice, oncologists need to select a regimen considered to be the most appropriate for each individual patient based on the medical condition of each patient, including such factors as age, performance status, organ function and extent of disease. The cisplatin-based regimens are usually inappropriate to be used for patients having peritoneal dissemination and retention of ascites, because such patients have potential renal impairment or poor performance

Table 3. Responses to treatment (total of 355 administrations of the sequential MTX/5FU therapy)

	No. of evaluable patients	CR	PR	NC	PD	NE	Response rate (%)	95% CI (%)
Objective response	35	–	2	21	6	6	5.7	0.7–19.2
Response in ascites	37	3	10	15	6	3	35.1	20.2–52.5

CR, complete response; PR, partial response; NC, no change; PD, progressive disease; NE, not evaluable; CI, confidence interval.

status, which makes it difficult to tolerate the large volume hydration for the prevention of cisplatin-induced renal injury. Among several 5FU-based regimens, sequential MTX/5FU therapy is widely used because this regimen has definite anti-tumor activity against advanced gastric cancer with acceptable toxicity even in high-risk patients. The purpose of the present phase II study was to evaluate the efficacy and toxicity of the sequential MTX/5FU regimen in patients with unresectable gastric cancer with peritoneal dissemination accompanied by malignant ascites and to assess whether further investigation in a phase III setting is warranted.

Progression to peritoneal dissemination is very common in advanced gastric cancer and is frequently a component of the first episode of failure after surgery for primary gastric cancer (25). Therefore, intraperitoneal chemotherapy has previously been investigated for peritoneal dissemination for the purposes of palliation and the prevention of peritoneal metastasis after surgery in high-risk patients. The pharmacokinetic rationale for intraperitoneal therapy is that drug concentrations within the peritoneal cavity are several-fold to 1–2 logs higher than concentrations that can be achieved after oral or intravenous treatment (26,27). In ovarian cancer, a large randomized trial demonstrated a small but statistically and clinically significant survival advantage in patients receiving intraperitoneal therapy (28). However, generally the efficacy of intraperitoneal chemotherapy is considered to be modest because the penetration of intraperitoneally injected drug into submesothelial tissue is too limited to achieve anti-tumor activity. Moreover, intraperitoneal chemotherapy sometimes induces systemic adverse events similar to systemic chemotherapy in addition to local complications such as chemical peritonitis. No definite data are currently available to specify which treatment option, intraperitoneal or systemic chemotherapy, is more suitable for patients with peritoneal dissemination in terms of benefit regarding survival and quality of life.

When we perform systemic chemotherapy in patients who have fluid retention such as ascites or pleural effusion, we have to consider the pharmacokinetic alterations of the anti-tumor agents administered. Intravenously administered MTX penetrates the ascites or pleural effusion and the clearance rate of MTX from ascites and plasma is ~5 and ~120 ml/min, respectively (29). Therefore, the retention of body fluid prolongs the terminal plasma half-life of intravenously administered drug owing to the slow re-entry of the sequestered drug into the bloodstream. Such phenomena should be associated with both favorable anti-tumor activity against peritoneal or pleural dissemination and with the potential risk of systemic toxicity. In another phase II study of sequential MTX/5FU therapy against unresectable or metastatic gastric cancer previously conducted by the JCOG, in which the same dosage and schedule as in the present study were utilized but the patients having ascites were ineligible for entry (JCOG 9207 study), none of 56 enrolled patients experienced grade 3 or 4 neutropenia (data not shown). In the present study, grades 3 and 4 neutropenia were observed in six (16 %) and two patients (11 %), respectively. The incidence of leukopenia, anemia, increase in total bilirubin and increase in serum creatinine of grade 3 or 4 tended to be more frequent in the present study than in the JCOG 9207 study (data not shown). Therefore, the toxicity of the sequential MTX/5FU therapy might be more severe in patients with malignant ascites than in those without. Two treatment-related deaths were observed in the present study. These two patients developed progressive neutropenic sepsis, which is a major cause of death. Although these two patients had met the eligibility criteria required in the study, both patients were retrospectively shown to be at high-risk for neutropenic infection, because pretreatment serum CRP values were highly elevated in both patients and leukocytosis was also observed at the baseline in one patient. Therefore, we consider that patients with apparent inflammatory signs such as elevation of CRP or leukocytosis should be excluded from future studies to prevent neutropenic sepsis. It is known that the different methods of administration of 5FU, either as a bolus or by infusion, represent different efficacy and toxicity profiles, thus infusional 5FU has more clinical benefit in efficacy (response rate) and safety in metastatic colorectal cancer. At present, however, we do not have sufficient data to establish whether these clinical observations hold true in patients with peritoneal dissemination with malignant ascites and it seems to be important to investigate the infusional 5FU-based regimens in this clinical setting, which may contribute to reducing the toxicity.

It is difficult to evaluate the efficacy of chemotherapy against peritoneal dissemination in clinical trials as well as in clinical practice, because most disseminated tumor cells do not form a measurable mass but rather constitute a diffuse lesion. Clinicians have to assess the efficacy of treatment and disease status in each patient based on the integration of clinical information such as clinical imaging, tumor markers and clinical symptoms. In the present study, the therapeutic efficacy was assessed according to the specific criteria for the study based on the change in the volume of ascites visualized by abdominal CT scan or ultrasonography as a surrogate marker. Using these criteria, we found that the ascites disappeared or was decreased by the MTX/5FU therapy in 35% of the patients. Konishi et al.

also reported that ascites disappeared in 50% (8/16) of patients with peritoneal-disseminated gastric cancer after MTX/5FU therapy (16). These results show that sequential MTX/5FU therapy is effective in controlling malignant ascites and also suggest that this regimen is effective against peritoneal dissemination from advanced gastric cancer.

Although the present study was originally planned as a phase II study involving 50 patients, patient enrollment had been delayed and finally terminated before the projected number of patients was achieved. The delay in patient enrollment was probably caused by the eligibility criteria for this study. Although peritoneal dissemination of advanced gastric cancer is very common in clinical practice, most patients with peritoneal dissemination accompanied by malignant ascites tend to have relatively poor performance status and impaired organ function, which was considered to be a critical issue delaying patient enrollment. The JCOG monitoring committee accepted the investigators' decision that the objectives of this study, which were to calculate the response rate in ascites and to evaluate the safety of sequential MTX/5FU therapy for decision-making to pursue further investigation in a phase III study, were achieved even with the actual sample size of 37 patients and that the response rate in ascites of 35% (95% confidence interval: 20.2–52.5%) observed in this study was positive.

It is well known that peritoneal dissemination of gastric adenocarcinoma occurs more commonly as the histologically diffuse type than the intestinal type. Konishi et al. reported that sequential MTX/5FU therapy was more effective against undifferentiated gastric cancer (i.e. histologically diffuse type) than differentiated gastric cancer (i.e. histologically intestinal type), with a response rate of 32% (9 PRs/28 patients) vs 0% (0 PRs/10 patients) (16). A similar tendency was observed in the present study, namely that the response rate of ascites was higher for the histologically diffuse type than for the intestinal type (44%, 11 responders among 25 patients, versus 17%, two responders among 12 patients). The difference in the efficacy of the sequential MTX/5FU therapy depending on the histological type might be explained by the difference in the activities of two enzymes, thymidylate synthetase and thymidine kinase, in the various histological types of gastric cancer (30). However, other reports have suggested that there were no significant differences according to the histological type. (15)

In conclusion, the findings of the present study suggest that sequential MTX/5FU therapy is effective in controlling malignant ascites from gastric cancer with overall acceptable toxicity and that further investigations are warranted. However, the present study also suggests that severe toxicity may occur more frequently in patients with malignant ascites than in those without malignant ascites. Whether there is true clinical benefit in this regimen for patients with peritoneal dissemination from advanced gastric cancer should be evaluated in future randomized clinical trials. Since the peritoneal dissemination from gastric cancer is considered to be an incurable disease, the patient's survival and quality of life will be important endpoints to be assessed in the future clinical trials. Recently, various new drugs with different mechanisms of action have been developed. However, since the patients whose main diseases are peritoneal dissemination are usually excluded from the phase II trials of new drugs or new combination regimens because of the lack of measurable lesions in those patients, the available data as to the efficacy against peritoneal dissemination are very limited unless we conduct trials specifically designed for this purpose as the present study. We think it is important to assess the roles of new drugs from the viewpoint of how we can maximize the potential value of each drug or regimen in disease-specific clinical situations. In this study we have focused on peritoneal dissemination with malignant ascites from advanced gastric cancer, which is very common and a major clinical problem. At present, any 5FU-based combination chemotherapies cannot prolong overall survival compared with 5FU alone. However, the present study brought us to the hypothesis that if we choose an appropriate regimen and administer it to the appropriate patient population (for example, to choose MTX/5FU therapy for the patients with peritoneal dissemination), survival may be prolonged compared with 5FU alone. We think that MTX/5FU therapy is the most reasonable regimen to be tested as a first-line chemotherapy in patients with peritoneal dissemination from advanced gastric cancer. From this clinical standpoint, a phase III randomized controlled trial comparing sequential MTX/5FU therapy with infusional 5FU-monotherapy (800 mg/m^2 of 5FU continuous infusion over 5 days every 4 weeks) in patients with advanced gastric cancer who have peritoneal dissemination with or without ascites is currently being carried out by the Japan Clinical Oncology Group (JCOG 0106-MF study). As a final note, we suggest that in future trials we should investigate the therapeutic strategy not only with newer cytotoxic drugs including irinotecan, taxanes and oxaliplatine, but also with new molecular targeting drugs such as antibody, VEGF drugs and EGF drugs, to bring about a breakthrough in this dire clinical condition.

Acknowledgments

This work was supported by a Grant-in-Aid for Cancer Research (8S-1, 11S-3, 11S-4) and for the Second Term Comprehensive 10-year Strategy for Cancer Control (H10-Gan-027, H12-Gan-012) from the Ministry of Health, Labor and Welfare Japan.

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Jpn J Clin Oncol 2005;35(3)162–164
doi:10.1093/jjco/hyi046

Special Report

Learning from a Visit to the JNCI Editorial Office

Katsumi Hashimoto[1] and Seiichiro Yamamoto[2,3]

[1]Managing Editor of the Japanese Journal of Clinical Oncology, [2]Statistical Advisor of the Japanese Journal of Clinical Oncology and [3]Biometric Research Section, Statistics and Cancer Control Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo, Japan

Received January 25, 2005; accepted January 26, 2005

INTRODUCTION

We, the Managing Editor and Statistical Advisor of the *Japanese Journal of Clinical Oncology* (JJCO), had a chance to visit the *Journal of the National Cancer Institute* (JNCI) editorial office in Bethesda, Maryland, in the USA. As is generally known, JNCI is acclaimed as the source for the most up-to-date information in the field of cancer research. It is also the most-cited academic journal in oncology. Whilst there we took the opportunity to interview the Executive Editor, Dr Rebecca Chasan, and the Managing Editor, Mr Mark Leader (Fig. 1). They graciously spent more than 3 hours with us. As the information that they shared with us is highly valuable for both editorial staff and authors, we have summarized the major points from the interview and draw comparisons between JNCI and JJCO.

OVERVIEW

JNCI is published by Oxford University Press (OUP). It is not affiliated with the United States National Cancer Institute (NCI), although originally JNCI was founded at NCI as a government publication. OUP took over ownership in 1997 mainly to develop an online version of the journal. JNCI accepts papers that give insight into mechanisms and processes involved in cancer prevention, development, screening, and treatment.

REVIEW PROCESS

Figure 2(a) and (b) indicate the review process for initial submission of manuscripts for JNCI and JJCO. At both journals, the Editor-in-Chief rejects some manuscripts on initial submission, the remainder are forwarded to Associate Editors. The Associate Editors reject some manuscripts and send others to reviewers for peer review. Those which are peer-reviewed

Figure 1. JNCI editorial office. From left to right: K. Hashimoto (Managing Editor JJCO), R. Chasan (Executive Editor JNCI), M. Leader (Managing Editor JNCI).

can be rejected or provisionally accepted, depending on the outcome of the peer review process. A notable difference between JNCI and JJCO is the existence of Senior Editors. Senior Editors do not exist at JJCO. The JNCI senior editors, all of whom have PhD degrees, are involved in the entire review process to help the Editor-in-Chief of JNCI, but their main function is to edit manuscripts to ensure that the presentation is as clear and logical as possible. They are concerned not only with scientific issues but also with the wording and presentation of the manuscript. As the journal's target audience is relatively broad, it is not necessary that the Senior Editors be medical doctors. Most have a background in molecular biology.

ONLINE JOURNAL AND ONLINE SUBMISSION/REVIEW SYSTEM

JJCO is published in electronic form on the Internet. Full-text articles can be accessed through HighWire Press

For reprints and all correspondence: Katsumi Hashimoto, Editorial Office of the Japanese Journal of Clinical Oncology, 5-1-1 Tsukiji, Chuo-ku, Tokyo, Japan 104-0045. E-mail: kahashim@info.ncc.go.jp

(a) Review Process in JNCI

Author → EA → SE →EIC → AE → SE → EA → Reviewer → SE → SR
→ SE → AE →SE → EIC, SE → EIC → Author

EA: Editorial Assistant, SE: Senior Editor, EIC: Editor-in-Chief, AE: Associate Editor
SR: Statistical Reviewer

(b) Review Process in JJCO

Author → ME → EIC, EE → AE → Reviewer → AE → EIC, EE → Author

ME: Managing Editor, EIC: Editor-in-Chief, EE: Executive Editor, AE: Associate Editor

Figure 2. (a) Review process in JNCI. (b) Review process in JJCO.

(http://jjco.oupjournals.org/). The online version of JNCI, JNCI Cancer Spectrum (http://jncicancerspectrum.oupjournals.org/), contains not only the full content of the printed journal but also weekly updated news and useful cancer information including cancer statistics, drug information, and NCI's Physician Data Query (PDQ).

Recently, the number of medical journals that employ an online submission and peer review system has increased substantially. The online system reduces the time and correspondence expense required in the reviewing process. At JNCI an online submission and peer review system was launched in January 2004. Since the start of online submission, the number of submissions has increased by approximately 20%. The transition from conventional paper submission to online submission was very smooth, and JNCI now receives very few conventional submissions. At JJCO the online submission system started in 2003. The number of submissions has dramatically increased by 50% since the online system launched.

COMMENTS FROM THE MANAGING EDITOR OF JJCO

I have been Managing Editor of JJCO for 3 years, which is my first experience as a journal editor. I have learned how to be a journal editor through trial and error and with the help of other more experienced staff; however, I have not had many opportunities to discuss editorial processes and difficulties. An interview with the editorial staff of a first-class scientific journal was a tremendous opportunity for me to assess the editorial process of JJCO. JNCI receives a much larger number of submissions than JJCO and the review process is much more complicated. Nonetheless, the editorial review process at JNCI runs smoothly without any noticeable bottlenecks. Overall, I found, through the interview, that the approach I took in managing JJCO was appropriate. For example, as is the case with JJCO, the editorial staff of JNCI emphasize the importance of receiving comments from assigned reviewers in a timely manner.

As mentioned above, the number of manuscripts submitted to JJCO has increased strikingly since online submission started, while the number of editorial staff has remained the same. The sudden growth in submissions has led to an increase in the volume of work that the editorial office has had to handle at various stages of the review process. One of the important tasks in the review process is the selection of reviewers. At JJCO, Associate Editors generally choose two reviewers from among 160 Editorial Board Members for each manuscript. This sharp rise in submissions has imposed a heavy burden on the Associate Editors and Editorial Board Members of JJCO. Therefore, the number of reviewers needs to be increased to reduce the burden. Also, a wide variety of experts' comments are important for the fair evaluation of manuscripts. JNCI has adopted an external reviewer system for peer review, and as they have a huge database of external reviewers, they are able to obtain a broad range of opinion. At JJCO we have decided to increase greatly the number of reviewers in order to improve the reviewing process. We expect to solve the workload problem to some extent in this way; however, a number of issues still remain to be considered.

The JNCI staff introduced us to a useful organization, the Council of Science Editors (CSE) (http://www.councilscienceeditors.org/index.cfm) to help find ways to alleviate our problems and to improve our skills. Members of CSE include editors of many prestigious scientific journals such as JNCI, *Lancet*, *Nature*, *Journal of the American Medical Association*, and *Journal of Clinical Oncology*. CSE improves communication in science by educating authors and editors. Also, CSE promotes effective communication practices in publishing. On a personal level I have to engage in a special effort to improve my skills as an editor through communication and experience.

COMMENTS FROM THE STATISTICAL ADVISOR OF JJCO

Editorial work on a scientific journal needs a very broad knowledge base. During our visit to the JNCI editorial office I asked the Executive Editor about the difficulties associated with dealing with broad areas of science. She stressed the importance of the participation of multidisciplinary reviewers and added that the requirement of a very broad knowledge base, by the Editor-in-Chief, about cancer research is important. This is refelected by the fact that the Editor-in-Chief, and a JNCI, Dr Barnett S. Kramer, is also the Editor-in-Chief, and a member of several PDQ editorial boards. One reason that the

editorial office of JNCI works very effectively seems to be related to their system, which is established so as to directly implement the Editor-in-Chief's ideas.

Another point I was curious about is the role of the Senior Editor. Senior Editors play a very important role in the JNCI system. A similar function is used at *Science* and at the *New England Journal of Medicine*, though it is not very common at other journals, including JJCO. As noted above, the Senior Editor serves as a bridge between authors, reviewers, and other editors. The Senior Editor's role is not only administrative, but also scientific.

I'd like to introduce my experience with the Senior Editor when our paper was published in the JNCI (J Natl Cancer Inst 2003;95:906–13). For example, my original manuscript included the following statement:

> Among the contents of soybeans, isoflavones, a group of phytoestrogens has been hypothesized to have a protective effect against the development of breast cancer.

The Senior Editor changed this as follows:

> Soybeans contain isoflavones, a group of phytoestrogens, that have been hypothesized to have reduce the risk of breast cancer.

The reason for the wording change was explained as the journal avoids use of the phrase "protective effect" unless the results are derived from a randomized, controlled trial.

In another example the original sentence said:

> Other possible mechanisms enabling soybean isoflavones to be anticarcinogenic are inhibition of protein tyrosine kinases and other enzyme activities, stimulations of sex hormone binding globulin production, antioxidant effects, and inhibition of angiogenesis.

The change by the Senior Editor included some questions to authors as follows:

> Other possible anticarcinogenic mechanisms associated with soy or isoflavones include inhibiting protein tyrosine kinases and other enzyme activities that **[Au: do what? interfere with cell growth and survival?]**, stimulating sex hormone-binding globulin production that **[Au: does what?]**, antioxidant effects protecting DNA from damage **[Au: correct?]**, and inhibiting angiogenesis.

Figure 3. JNCI editorial office. From left to right: S. Yamamoto (Statistical Advisor JJCO) and J. Watson (Senior Editor JNCI).

As shown in these examples, the role of the Senior Editor is to make sure that the manuscript is readable by the general audience. Her suggestions were generally very reasonable and improved the quality of my manuscript. Of course, some points she made were not correct in a strict scientific sense, but we were able to communicate with each other via e-mail and reached agreement without a significant time lag, which was surprising since we have a 13-hour time difference. I was very glad to meet the Senior Editor, Dr Joanna Watson (Fig. 3), who dealt with my manuscript, when I visited the JNCI editorial office.

The quality of JNCI is kept high by this efficient system and by the continuous effort of editorial staff. The visit was very fruitful in that we got to personally experience the system employed on this quality journal. It also confirmed to us that the system used on JJCO is appropriate. It is the intention of the editorial staff of JJCO to provide a high level of service to both readers and authors through the continued publication of a quality journal involving collaborative efforts with readers, authors, and the editorial staff of other journals.

Original article

Identification of risk factors for the development of complications following extended and superextended lymphadenectomies for gastric cancer

Y. Kodera[1], M. Sasako[2], S. Yamamoto[3], T. Sano[2], A. Nashimoto[4] and A. Kurita[5] on behalf of the Gastric Cancer Surgery Study Group of Japan Clinical Oncology Group

[1]Department of Gastroenterological Surgery, Aichi Cancer Centre, Nagoya, [2]Gastric Surgery Division, National Cancer Centre Hospital and [3]Cancer Information and Epidemiology Division, National Cancer Centre Research Institute, Tokyo, [4]Department of Surgery, Niigata Cancer Centre Hospital, Niigata, and [5]National Shikoku Cancer Centre, Matsuyama, Japan

Correspondence to: Dr Y. Kodera, Department of Surgery II, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 464-8550, Japan (e-mail: ykodera@med.nagoya-u.ac.jp)

Background: Extended lymphadenectomy for gastric carcinoma has been associated with high mortality and morbidity rates in several multicentre randomized trials.

Methods: Using data from 523 patients registered for a prospective randomized trial comparing extended (D2) and superextended (D3) lymphadenectomies, risk factors for overall complications and major surgical complications (anastomotic leakage, intra-abdominal abscess and pancreatic fistula) were identified by multivariate logistic regression analysis.

Results: Mortality and morbidity rates were 0·8 per cent (four of 523) and 24·5 per cent (128 of 523) respectively. Pancreatectomy (relative risk 5·62 (95 per cent confidence interval (c.i.) 1·94 to 16·27)) and prolonged operating time (relative risk 2·65 (95 per cent confidence interval 1·34 to 5·23)) were the most important risk factors for overall complications. A body mass index of 25 kg/m^2 or above, pancreatectomy and age greater than 65 years were significant predictors of major surgical complications.

Conclusion: Pancreatectomy should be reserved for patients with stage T4 disease. Age and obesity should be considered when planning surgery.

Paper accepted 24 January 2005

Published online in Wiley InterScience (www.bjs.co.uk). **DOI:** 10.1002/bjs.4979

Introduction

Despite a declining incidence in Western Europe[1] and the USA[2], gastric carcinoma remains the second commonest cause of cancer death worldwide, with over 600 000 deaths per year[3]. Given the poor outcome of irresectable disease treated by other therapeutic modalities in phase II and III trials[4,5], the curative treatment of gastric carcinoma remains primarily surgical. Although the presence of distant metastases usually precludes curative surgery, this does not necessarily apply to disease in the regional lymph nodes, which can be dissected *en bloc* with the primary lesion[6,7]. This type of resection may allow cure, provided that metastases are within the margins of dissection. Removal of a wider range of lymph nodes by extended lymph node dissection might increase the chance of cure, but is inappropriate if the cancer has spread systemically.

In Japan, gastrectomy plus extended systematic lymphadenectomy (D2 resection) has long been the standard treatment, even for superficial cancers[8]. Success with D2 resection has led to the evolution of a superextended lymphadenectomy (D3 resection) and several feasibility studies evaluating dissection of para-aortic lymph nodes have been performed[9–12]. A randomized trial (Japan Clinical Oncology Group (JCOG) 9501) was launched in 1995, primarily to explore the potential survival benefit of D3 over D2 dissection[13]. This trial has provided the opportunity to evaluate prospectively collected data on gastric cancer surgery in Japan. The present study represents a detailed analysis of risk factors for overall and surgical complications following D2 and D3 resections.

Patients and methods

Between June 1995 and April 2001, 523 patients registered in the JCOG 9501 study were allocated randomly to either D2 (263 patients) or D3 (D2 plus para-aortic lymph node dissection; 260 patients) resection. Eligibility criteria and the method of randomization have already been reported in detail[13]. In brief, patients aged less than 75 years of age with histologically proven and resectable primary gastric carcinoma with an estimated depth of SS (penetrating the muscle layer), SE (penetrating the serosa) or SI (invasion to an adjacent organ) were recruited after giving informed consent. Patients found positive for free cancer cells by cytological examination of peritoneal washes and those with Borrmann type 4 tumours (linitis plastica type) were excluded. Twelve institutions participated in the trial initially and 12 other institutions were added to increase patient recruitment.

After laparotomy, cytological examination of peritoneal washes was performed, followed by gross examination of the abdominal cavity and the primary lesion. Only patients who were negative for free cancer cells in the abdominal cavity and without evidence of gross para-aortic lymph node spread, peritoneal carcinomatosis or other distant metastasis were eligible to participate. The patients were allocated randomly to either D2 or D3 resection by the minimization method of balancing the groups according to T stage (T2 *versus* T3/T4), gross appearance (Borrmann types 1 and 2 *versus* Borrmann types 3 and 5) and institution. The surgeons were notified immediately of the allocation results and completed the operation accordingly.

Patients underwent appropriate gastrectomy with systematic lymphadenectomy as allocated. Perigastric lymph nodes (nodal stations 1, 3, 4, 5 and 6 according to the Japanese Classification of Gastric Cancer[14]) and nodes at the base of the left gastric artery (7), along the common hepatic artery (8) and at the base of the splenic artery (11) were resected routinely. Lymph nodes along the hepatoduodenal ligament and behind the pancreatic head (12 and 13) were resected when the primary lesion was located in the lower third of the stomach. Lymph nodes along the left side of the cardia (2), within the splenogastric ligament (4sa) and at the splenic hilum (10) were resected with the spleen when total or proximal gastrectomy was performed. Concurrent resection of the pancreatic tail was not routine during either D2 or D3 resection and was reserved for patients with direct invasion to the pancreas. In patients randomized to superextended lymphadenectomy, para-aortic lymph nodes from the level of the coeliac trunk down to the root of the inferior mesenteric artery (16a2 and 16b1) were dissected. The mode of reconstruction following resection was not specified.

All information on complications was extracted from the case-report forms for the trial. Anastomotic leakage, intra-abdominal abscess and pancreatic fistula were considered to be major surgical complications. Anastomotic leakage was defined as dehiscence confirmed by radiographic examination using contrast medium. Pancreatic fistula was diagnosed if there was prolonged purulent discharge containing pancreatic juice from the drainage tube.

Factors that might affect the risk of overall and major surgical complications were evaluated by univariate analysis using cross-tabulations. Variables analysed included extent of lymphadenectomy, splenectomy, pancreatectomy, type of gastrectomy, pathological (p) T category (pT2 and pT3 *versus* pT4), sex, age, body mass index (BMI), operating time, amount of blood loss and need for autologous blood transfusion. Operating time and blood loss were divided into tertiles for analysis. Two factors associated with surgical experience were also evaluated: institutions that enrolled over 20 patients *versus* those with fewer patients and first and second halves of the trial (1995–1998 *versus* 1999–2001). The χ^2 test was used to assess differences in proportions. The independent contribution of various factors was assessed by multivariate logistic regression analysis, with mutual adjustment of potential risk factors for complications. All factors analysed in the univariate analysis were included as variables in the multivariate analysis. Two-sided P values are presented. Statistical analysis was performed using SAS® version 8.12 (SAS Institute, Tokyo, Japan).

Results

Total gastrectomy was performed in 199 (38·0 per cent) of 523 patients and proximal gastrectomy in four;

Table 1 Complications

Severe abdominal complications	
Pancreatic fistula	30
Abdominal abscess	29
Anastomotic leakage	11
Other complications	
Pneumonia	16
Anastomotic stenosis	14
Bowel obstruction/ileus	16
Lymphorrhoea	10
Thoracic effusion requiring thoracic drainage	7
Severe feeding problem requiring prolonged hyperalimentation	6
Wound abscess	5
Postoperative bleeding	3
Severe diarrhoea	3
Urinary tract infection	3
Catheter-induced sepsis	3
Pulmonary embolism	2
Cardiac failure	1
Cholecystitis requiring percutaneous drainage	1

the remaining patients underwent distal gastrectomy. Splenectomy was performed in 191 patients (36·5 per cent) and distal pancreatectomy in 22 (4·2 per cent). There was no significant difference in the type of gastrectomy and incidence of combined resection between the two groups. Details of patient demographics and tumour stages have been reported previously[13].

There were four hospital deaths (0·8 per cent), two in each group. Two patients suffered from rapid disease progression and died 3 and 5 months after surgery without being discharged from hospital. One patient died from pneumonia at 46 days and another died from massive bleeding from the gastroduodenal artery 24 days after operation. Complications were identified in 128 patients (24·5 per cent) and major surgical complications in 49 patients (9·4 per cent) (*Table 1*).

The results of univariate analyses of risk factors for overall postoperative complications are summarized in *Table 2*. Only pancreatic resection ($P = 0·001$) and

Table 2 Univariate and multivariate analysis of risk factors for overall complications

			Univariate analysis		Multivariate analysis	
	n	No. with complications	Relative risk	*P*	Relative risk	*P*
Extent of lymphadenectomy						
D2	263	55	1		1	
D3	260	73	1·48 (0·99, 2·21)	0·057	0·93 (0·58, 1·51)	0·776
Splenectomy						
No	332	64	1		1	
Yes	191	64	2·11 (1·41, 3·17)	< 0·001	2·05 (0·52, 8·01)	0·304
Pancreatectomy						
No	501	115	1		1	
Yes	22	13	4·85 (2·02, 11·63)	< 0·001	5·62 (1·94, 16·27)	0·001
Extent of gastrectomy						
Distal	320	62	1		1	
Total or proximal	203	66	2·01 (1·34, 3·00)	< 0·001	0·84 (0·22, 3·27)	0·804
Invasion to adjacent organs						
T2, T3	501	123	1		1	
T4	22	5	0·90 (0·33, 2·50)	0·846	0·37 (0·11, 1·24)	0·107
Sex						
M	358	94	1		1	
F	165	34	0·73 (0·47, 1·14)	0·163	0·73 (0·45, 1·19)	0·207
Age (years)						
< 56	160	33	1		1	
56–65	207	48	1·16 (0·70, 1·92)	0·557	1·26 (0·73, 2·17)	0·403
> 65	156	47	1·66 (0·99, 2·77)	0·053	1·63 (0·92, 2·89)	0·092
Body mass index						
< 25	446	101	1		1	
≥ 25	77	27	1·85 (1·10, 3·10)	0·019	1·75 (0·99, 3·08)	0·054
Operating time (min)						
< 240	167	23	1		1	
240–297	179	43	1·98 (1·13, 3·46)	0·016	1·77 (0·96, 3·25)	0·068
> 297	177	62	3·38 (1·97, 5·78)	< 0·001	2·65 (1·34, 5·23)	0·005
Blood loss (ml)						
< 395	174	27	1		1	
395–710	174	42	1·73 (1·01, 2·97)	0·045	1·05 (0·58, 1·90)	0·886
> 710	175	59	2·77 (1·65, 4·64)	< 0·001	1·11 (0·58, 2·12)	0·754
Blood transfusion						
Yes	408	87	1		1	
No	115	41	2·04 (1·31, 3·20)	0·002	1·53 (0·92, 2·56)	0·102
Case volume*						
< 20	147	41	1		1	
≥ 20	376	87	0·78 (0·51, 1·20)	0·256	0·83 (0·51, 1·34)	0·437
Period						
1995–1998	295	75	1		1	
1999–2001	228	53	0·9 (0·59, 1·33)	0·566	0·87 (0·56, 1·35)	0·539

Values in parentheses are 95 per cent confidence intervals. *No. of patients registered.

Table 3 Univariate and multivariate analysis of risk factors for major surgical complications

			Univariate analysis		Multivariate analysis	
	n	No. with major complications	Relative risk	*P*	Relative risk	*P*
Extent of lymphadenectomy						
D2	263	23	1		1	
D3	260	26	1·16 (0·64, 2·09)	0·623	0·67 (0·32, 1·39)	0·279
Splenectomy						
No	332	20	1		1	
Yes	191	29	2·79 (1·53, 5·09)	< 0·001	1·08 (0·15, 7·56)	0·941
Pancreatectomy						
No	501	43	1		1	
Yes	22	6	3·99 (1·49, 10·74)	0·003	6·90 (1·86, 25·58)	0·004
Extent of gastrectomy						
Distal	320	19	1		1	
Total or proximal	203	30	2·74 (1·50, 5·03)	< 0·001	2·15 (0·31, 15·20)	0·442
Invasion to adjacent organs						
T2, T3	501	47	1		1	
T4	22	2	0·97 (0·22, 4·26)	0·964	0·37 (0·067, 2·01)	0·246
Sex						
M	358	38	1		1	
F	165	11	0·60 (0·30, 1·21)	0·150	0·57 (0·25, 1·27)	0·169
Age (years)						
< 56	160	7	1		1	
56–65	207	20	2·34 (0·96, 5·67)	0·061	3·06 (1·15, 8·20)	0·026
> 65	156	22	3·59 (1·49, 8·66)	0·005	4·04 (1·48, 11·02)	0·006
Body mass index						
< 25	446	34	1		1	
≥ 25	77	15	2·93 (1·51, 5·69)	0·001	3·32 (1·54, 7·12)	0·002
Operating time (min)						
< 240	167	8	1		1	
240–297	179	14	1·69 (0·69, 4·13)	0·252	1·60 (0·60, 4·27)	0·350
> 297	177	27	3·58 (1·58, 8·12)	0·002	2·96 (1·03, 8·55)	0·045
Blood loss (ml)						
< 395	174	10	1		1	
395–710	174	11	1·11 (0·46, 2·68)	0·822	0·47 (0·17, 1·30)	0·145
> 710	175	28	3·12 (1·47, 6·65)	0·003	0·86 (0·32, 2·31)	0·767
Blood transfusion						
Yes	408	29	1		1	
No	115	20	2·75 (1·49, 5·08)	< 0·001	1·99 (0·97, 4·08)	0·061
Case volume*						
< 20	147	16	1		1	
≥ 20	376	33	0·79 (0·42, 1·48)	0·457	0·76 (0·36, 1·57)	0·454
Period						
1995–1998	295	30	1		1	
1999–2001	228	19	0·80 (0·44, 1·47)	0·475	0·83 (0·43, 1·61)	0·575

Values in parentheses are 95 per cent confidence intervals. *No. of patients registered.

prolonged operating time (patients in the upper tertile for whom the operating time was more than 297 min; $P = 0·005$) were identified as significant independent risk factors for overall complications (*Table 2*). A BMI of 25 or more was close to significance ($P = 0·054$).

The results of univariate analyses of risk factors for major surgical complications are summarized in *Table 3*. Multivariate analysis identified BMI ($P = 0·002$), pancreatic resection ($P = 0·004$), age (56–65 years, $P = 0·026$; over 65 years, $P = 0·006$) and operating time over 297 min ($P = 0·045$) as significant independent risk factors for major surgical complications (*Table 3*).

Discussion

Gastrectomy plus extended systemic lymphadenectomy (D2 resection) is the standard procedure for gastric carcinoma in Japan. This approach has resulted in superior stage-by-stage survival than that observed in most Western countries and has led to cure for a

proportion of patients with nodal disease beyond the perigastric region, although this has not been confirmed in Western randomized trials[15,16]. Although long-term follow-up revealed significantly better disease-free survival for the D2 group in the subset with node-positive cancer[17], this difference did not extend to all patients in the trial, in part owing to the unacceptably high mortality rate associated with D2 resection[8]. JCOG 9501, a Japanese multi-institutional prospective randomized trial comparing D2 with more extended resection, has superior quality control of surgical procedures and reliability of data[13] than retrospective Japanese studies and Western prospective trials.

The most significant risk factor for both surgical and overall complications in the present study was pancreatic resection, although it should be noted that this was performed in only 4·2 per cent of patients, compared with 30·3 and 15·2 per cent in the UK Medical Research Council (MRC) and Dutch trials respectively[15,16]. The rate of pancreatectomy was lower in the present series because a pancreas-preserving technique[18,19] was generally used, whereas distal pancreatectomy and splenectomy were integral parts of D2 dissection in the Dutch trial unless cancer was located in the distal stomach. The low morbidity rate in the present study may well be related to pancreas preservation[18,19]. The success of this approach has also been reported in a multicentre phase II trial of D2 dissection in Northern Italy[20].

Splenectomy, on the other hand, was not an independent determinant of risk, possibly because it was never performed with distal gastrectomy in the present series. In the Dutch randomized trial a high mortality rate after distal gastrectomy was attributed in part to necrosis of the remnant stomach as a result of splenectomy and division of the short gastric arteries[21]. The survival benefit of splenectomy performed solely to facilitate dissection of lymph nodes close to the splenic hilum has been questioned, however, and a randomized trial to explore this issue is ongoing[22].

Age was not an independent risk factor for overall complications in this study, in contrast to the Dutch trial in which age over 65 years was a significant risk factor for hospital death and overall complications[21]. This discrepancy may be attributed to the fact that only patients aged 75 years or less were eligible for inclusion in the JCOG 9501[13], whereas other trials have included older patients[15,16]. Japanese patients were, on average, 8 years younger than Dutch patients[23]; consequently the proportion of patients over 65 years of age was 29·8 per cent in the present series as opposed to 51·3 per cent in the Dutch trial[16]. This age distribution may account for the very low incidence of perioperative cardiovascular events in the present series, another factor that may have influenced the low morbidity and mortality rates.

Extended lymph node dissection may be hampered by excess bodyweight[24–26] and in the present study BMI was a significant risk factor for major surgical complications. Caucasians in general have a higher BMI than Japanese and the incidence of morbid obesity is significant among patients in the USA and Europe. Only 14·7 per cent of the present patients had a BMI of 25 kg/m^2 or greater, whereas one-third of the US population is obese (BMI over 27 kg/m^2)[27]. These data suggest that the patients' physique favours Japanese patients when major gastric cancer surgery is performed.

The extent of lymph node dissection (D2 *versus* D3), surgical volume and the period in which the operation was performed had no impact, suggesting that there were no learning curve issues. Although D2 resection has long been a standard procedure in Japan, all surgeons in the trial were experts from specialized centres who had sufficient experience with D3 resection through numerous other studies. Of the variables reflecting difficulties encountered during surgery, prolonged operating time was identified as a significant independent risk factor for both overall and major surgical complications. However, amount of blood loss and blood transfusion were significant only in univariate analysis; this may be attributable to multicolinearity, as these two factors are closely related.

Gastrectomy with extended lymphadenectomy is feasible and safe in Japan, provided that older patients with co-morbidity are excluded and pancreatectomy is reserved for lesions with direct invasion to the pancreas. Obese patients should be treated with caution, however, as they have a significant risk of developing major surgical complications. Hopefully, with careful patient selection, appropriate surgical expertise and pancreas and spleen preservation[8] where possible, equally good results, rarely achieved previously[20,28], will be realized in the West.

Acknowledgements

The authors thank Dr Yoshimura for help with the statistical analysis, Ms Hongo for data management and Ms Sugimoto for secretarial assistance. This study was supported by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare and the Second Term Comprehensive 10-year Strategy for Cancer Control by the Ministry of Health and Welfare, Japan.

Participating institutions and chief participants: National Cancer Center Hospital (M. Sasako, T. Sano), Niigata Cancer Centre Hospital (A. Nashimoto, H. Yabuzaki), National Shikoku Cancer Centre (A. Kurita, Y. Kubo), Osaka Medical Center for Cancer and Cardiovascular Diseases (M. Hiratsuka, I. Miyashiro), Osaka National Hospital (K. Kobayashi, T. Tsujinaka), National Cancer Centre Hospital East (T. Kinoshita), Tokyo Metropolitan Komagome Hospital (K. Arai, Y. Iwasaki), Aichi Cancer Centre (T. Kito, Y. Yamamura), Osaka Medical College (K. Okajima, M. Tanigawa), International Medical Centre of Japan (O. Kobori, T. Shimizu), Sakai City Hospital, Kanagawa Cancer Centre (H. Furukawa, H. Imamura), Tokyo Metropolitan Bokuto Hospital (M. Kitamura, S. Inoue), Nagaoka Chuo General Hospital (T. Yoshikawa, T. Shimizu), Niigata City General Hospital (K. Aizawa), Cancer Institute Hospital (K. Ota, S. Oyama), Kyoto Second Red Cross Hospital (H. Tokuda, S. Takahashi), Saitama Cancer Centre, Hiroshima City Hospital (Y. Tanaka, K. Uchida), Kanazawa University (K. Miwa, T. Fujimura), Gifu Municipal Hospital (H. Tanemura, H. Oshita), Kagoshima University (T. Aiko, S. Hokita), Iwate Medical University (M. Terashima, K. Saito) and Okayama University (H. Isozaki).

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CpG Island Methylator Phenotype Is a Strong Determinant of Poor Prognosis in Neuroblastomas

Masanobu Abe,[1,2] Miki Ohira,[3] Atsushi Kaneda,[1] Yukiko Yagi,[1] Seiichiro Yamamoto,[4] Yoshihiro Kitano,[5] Tsuyoshi Takato,[2] Akira Nakagawara,[3] and Toshikazu Ushijima[1]

[1]Carcinogenesis Division, National Cancer Center Research Institute; [2]Department of Oral and Maxillo Facial Surgery, University of Tokyo Graduate School of Medicine; [3]Biochemistry Division, Chiba Cancer Center Research Institute; [4]Information Division, Research Center for Cancer Prevention and Screening, National Cancer Center; and [5]Department of Pediatric Surgery, National Center for Child Health and Development, Tokyo, Japan

Abstract

Neuroblastoma, one of the most common pediatric solid tumors, is characterized by two extreme disease courses, spontaneous regression and life-threatening progression. Here, we conducted a genome-wide search for differences in DNA methylation that distinguish between neuroblastomas of the two types. Three CpG islands (CGI) and two groups of CGIs were found to be methylated specifically in neuroblastomas with a poor prognosis. By quantitative analysis of 140 independent cases, methylation of all the five CGI (groups) was shown to be closely associated with each other, conforming to the CpG island methylator phenotype (CIMP) concept. The presence of CIMP was sensitively detected by methylation of the *PCDHB* CGIs and associated with significantly poor survival (hazard ratio, 22.1; 95% confidence interval, 5.3-93.4; $P < 0.0001$). Almost all cases with N-*myc* amplification (37 of 38 cases) exhibited CIMP. Even in 102 cases without N-*myc* amplification, the presence of CIMP (30 cases) strongly predicted poor survival (hazard ratio, 12.4; 95% confidence interval, 2.6-58.9; $P = 0.002$). Methylation of *PCDHB* CGIs, located in their gene bodies, did not suppress gene expression or induce histone modifications. However, CIMP was significantly associated with methylation of promoter CGIs of the *RASSF1A* and *BLU* tumor suppressor genes. The results showed that neuroblastomas with CIMP have a poor prognosis and suggested induction of silencing of important genes as an underlying mechanism. (Cancer Res 2005; 65(3): 828-34)

Introduction

Epigenetic abnormalities, especially alterations in DNA methylation, are intimately involved in development of various human tumors (1). Aberrant methylation of promoter CpG islands (CGI) causes inactivation of tumor suppressor genes. Genomic instability is caused by genomic hypomethylation and is associated with hypermethylation (2, 3). Identification of epigenetic abnormalities in human cancers is expected to lead not only to discovery of novel disease mechanisms but also to development of new diagnostic markers. Therefore, we previously developed a genome-wide scanning method, methylation-sensitive representational difference analysis (MS-RDA), for detecting differences in DNA methylation (4, 5). This technique analyzes unmethylated, CpG-rich regions of the genome and has already identified genes silenced in human lung, stomach, breast, and pancreatic cancers (6–9).

Neuroblastoma derived from primitive cells of the sympathetic nervous system is one of the most common solid tumors in childhood, characterized by two extreme disease courses, spontaneous regression, and life-threatening progression (10, 11). The clinical outcome is associated with disease stage, age at diagnosis, histologic classification, N-*myc* amplification, DNA ploidy, and *TrkA* overexpression (10–12). These characteristics are therefore used to classify cases into low-, intermediate-, and high-risk groups. However, especially in the cases with intermediate risk, prediction of prognosis and therapeutic decision-making are still difficult, and development of new markers is an urgent priority. Moreover, the molecular bases underlying the two distinct clinical courses are still unknown, and their clarification is needed to allow development of novel therapeutics.

In the present study, considering the major involvement of epigenetic machinery in embryonic development (13, 14), we searched for differences in DNA methylation between neuroblastomas with a good prognosis and counterparts with a poor prognosis by MS-RDA.

Materials and Methods

Tissue Samples and Cell Lines. Tumor samples were obtained from 145 nonrecurrent cases between 1995 and 1999 and were used under approval of institutional review boards. The mean age at initial diagnosis was 27 months (range, 0-216 months). Their clinical stages were determined according to the International Neuroblastoma Staging System, and 40, 17, 20, 60, and 8 cases belonged to stages I, II, III, IV, and IVS, respectively. Normal adrenal medulla tissue was collected from a case undergoing nephrectomy for a renal cancer. Neuroblastoma cell lines were obtained from the American Type Culture Collection (Manassas, VA), the Japanese Collection of Research Bioresources (Tokyo, Japan), and the RIKEN Bio Resource Center (Tsukuba, Japan). GANB was established by A.N. and normal human bronchial epithelial cells were purchased from Cambrex (East Rutherford, NJ). High molecular weight DNA and total RNA were extracted as previously described (7). Total RNAs of brain and adrenal glands were purchased from Clontech (Palo Alto, CA).

MS-RDA and Database Search. MS-RDA was done as previously described (4, 5). Genomic DNA of primary neuroblastomas with a good prognosis (cases 92, 98, 104, 112, and 148) and neuroblastoma cell lines established from cases with a poor prognosis (CHP134, IMR32, GANB, NGP, and TGW) were digested with *Hpa*II, and then two pooled DNA samples were prepared. Although use of cell lines is highly recommended for MS-RDA (5), no cell lines were available for neuroblastomas with a good prognosis, and therefore we used the primary samples. To isolate CGIs that were hypermethylated in the latter, the cell line pool was used as the tester, and the primary tumor pool as the driver. MS-RDA in the opposite direction

Note: Supplementary data for this article are available at Cancer Research online (http://cancerres.aacrjournals.org/).

Requests for reprints: Toshikazu Ushijima, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Phone: 133-547-5240; Fax: 135-565-1753; E-mail: tushijim@ncc.go.jp.

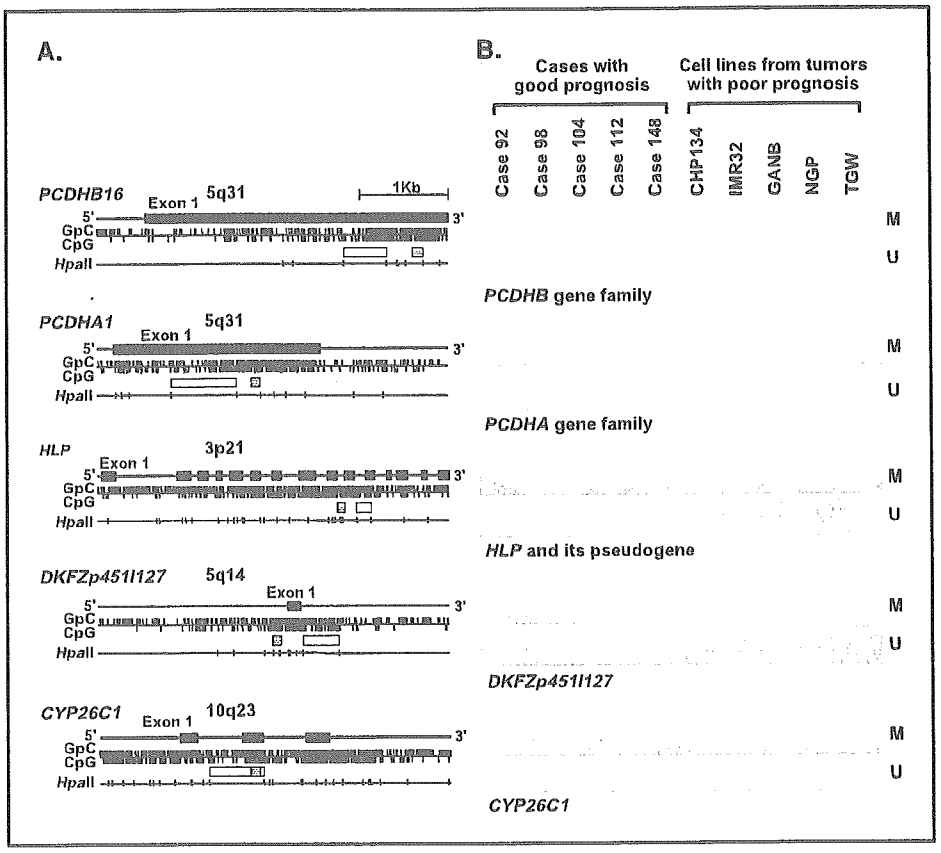

Figure 1. Five CGIs isolated by MS-RDA and their methylation statuses in the samples used for MS-RDA. *A*, genomic structures of the five CGIs. GpC, CpG, and *Hpa*II recognition sites (5′-CCGG-3′) are shown by ticks. *Closed boxes*, exons; *open boxes*, clones isolated by MS-RDA; *shaded boxes*, regions 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 *SssI* 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

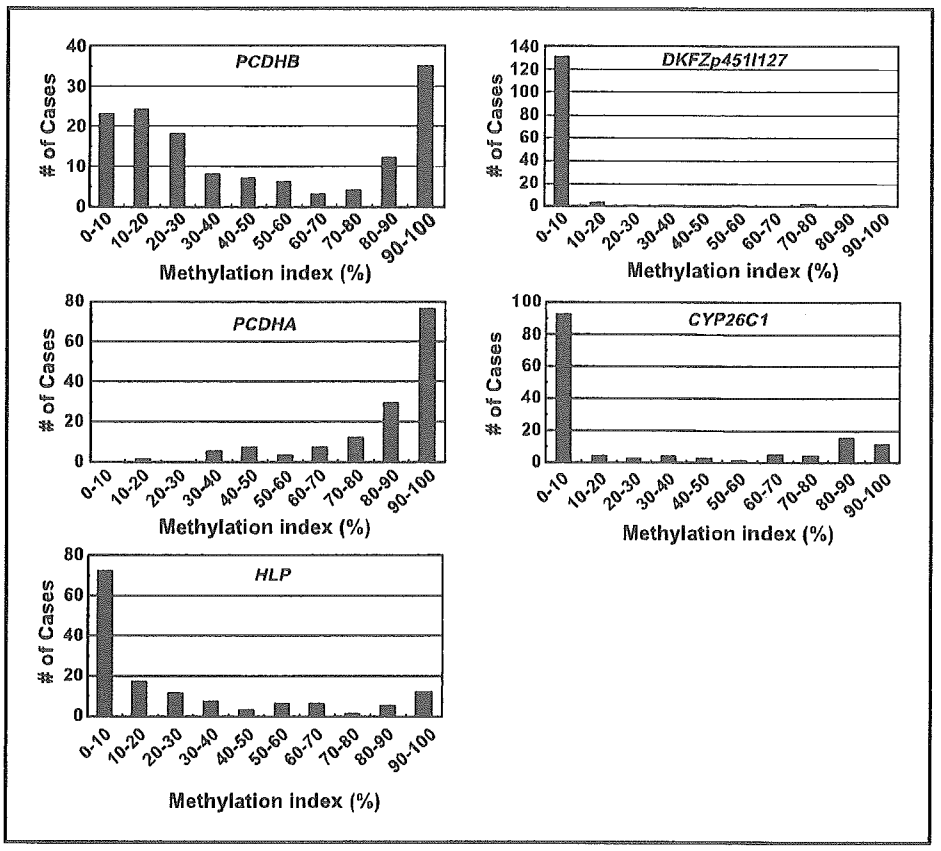

Figure 2. The distribution of methylation indices among the 140 cases analyzed: (*i*) 17 CGIs of the *PCDHB* family, (*ii*) 13 CGIs of the *PCDHA* family, (*iii*) CGIs of *HLP* and its pseudogene, (*iv*) *DKFZp451I127*, and (*v*) *CYP26C1*.

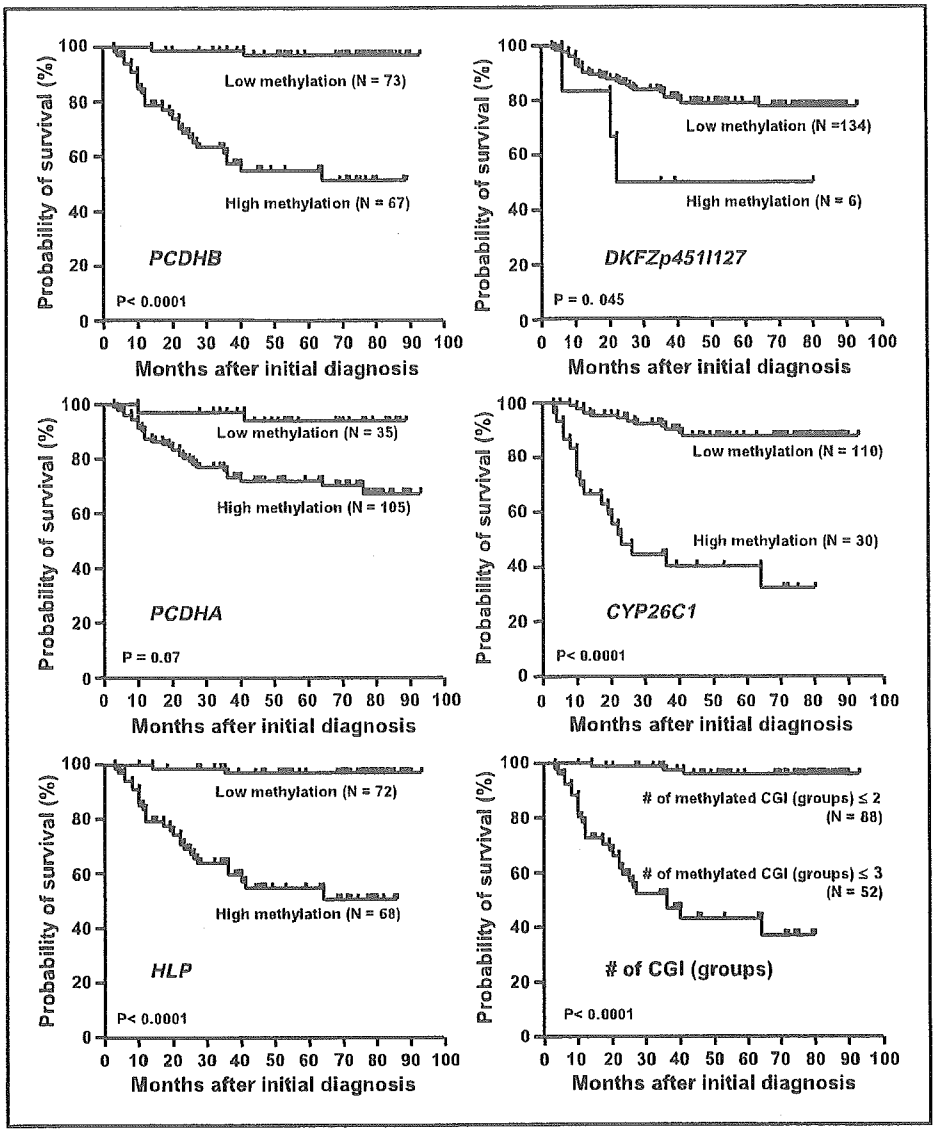

Figure 3. Predictive powers of methylation of the five CGI (groups) identified, and their multiple methylation: (*i*) 17 CGIs of the *PCDHB* family, (*ii*) 13 CGIs of the *PCDHA* family, (*iii*) CGIs of *HLP* and its pseudogene, (*iv*) *DKFZp451I127*, (*v*) *CYP26C1*, and (*vi*) methylation of three 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

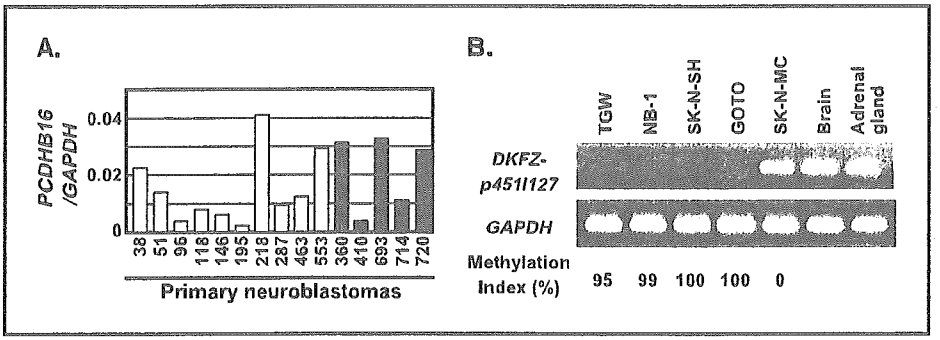

Figure 4. Effects of methylation of the *PCDHB* family and *DKFZp451I127* on gene expression. *A*, *PCDHB16* expression was analyzed by quantitative RT-PCR in 10 primary samples with low methylation (*open columns*) and five primary samples with high methylation (*closed columns*), and no difference 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*