inhibition of binding of cyclin D/CDK4 or cyclin D/CDK6 complexes in the Rb pathway (Serrano et al. 1995). Inactivation of p16, therefore, can result in a cellular inability to activate the Rb gene, with resultant loss of the cellular capacity to block cell cycle progression (Weinberg 1995). p16 mutations that lead to inactivation are found in many tumor types (Kamb et al. 1994; Nobori et al. 1994), but generally are lacking in colorectal cancers (Burri et al. 2001). Homozygous deletions, described in colon cancer cell lines (Guan et al. 1999; Burri et al. 2001) and hypermethylation of the promoter are alternative major mechanisms of p16 inactivation, which occurs in 40% of colon cancers while being absent in normal colonic epithelium in an earlier report (Herman et al. 1995).

The aim of the present study was to investigate aberrant p16 promoter methylation in a series of sporadic colorectal cancers and assess any association with clinicopathological features and prognostic relevance. In addition, relationships between the p16 methylation status and mRNA levels or protein expression were examined.

Materials and methods

Patients and materials

The subjects of our study were a series of 151 patients diagnosed with and undergoing surgery for colorectal carcinoma at the National Hospital Organization Sagamihara Hospital between April 1996 and March 2001. Surgically resected specimens were fixed in 10% formalin and embedded in paraffin wax, according to routine procedures, and sections were cut and stained with hematoxylin and eosin (H&E). Patients with familial polyposis coli, hereditary non-polyposis colorectal carcinoma, multiple colorectal carcinomas or inflammatory bowel disease, or those who had died within 30 days of surgery were not included in the analysis. No patient received initial chemotherapy or radiotherapy. The 151 patients comprised 95 males and 56 females, with a mean age of 65.8 years (median, 67 years; range, 39–86).

For biochemical studies of p16 transcription, fresh tumor tissues and corresponding normal mucosa distant from the tumor were obtained from 61 patients (36 males and 25 females; mean/median age, 69.8/70.5 years [range, 36–87]) between February and June 2007. Tissue blocks were prepared immediately after surgical resection, snap-frozen in liquid nitrogen, and stored at −80°C. For immunohistochemistry, another tissue block obtained simultaneously was fixed in 10% formalin and embedded in paraffin wax. Informed consent was obtained from all patients and the study was approved by our hospital's clinical research and ethics committee.

Pathological review

Slides stained with H&E were examined by an experienced gastrointestinal pathologist (HM). Proximal cancers were classified as tumors proximal to the splenic flexure and the remaining tumors were defined as distal. The depth of invasion (pT category), lymph node involvement (pN category), and pathological staging of all surgically resected tumors were assessed according to the UICC/TNM classification (International Union Against Cancer 1997). Out of 151 patients, 70 were diagnosed pathologically to have TNM stage II lesions, 57 stage III and 24 stage IV.

Adjuvant chemotherapy and follow-up

In 48 out of the 57 stage III patients, postoperative oral administration of 5-fluorouracil (5-FU) derivatives was given for at least 1 year. In 13 patients out of 24 with stage IV cases, 5-FU plus mitomycin C or leukovorin was applied by intravenous infusion biweekly after surgery. All patients were followed up regularly by physical and blood examinations with mandatory screening by colonoscopy, ultrasound, computed tomography or magnetic resonance imaging. Locoregional recurrence was defined as a tumor occurring at the anastomosis or in the locoregional lymph nodes, retroperitoneum or pelvic wall. Distant recurrences were defined as tumor manifestations outside the site of resection in the peritoneal cavity or other organs. The patients were observed for more than 5 years, and the median duration of follow-up was 79 months (range, 60–123), for the survivors were alive at the date of their last visit ($n = 87$).

DNA and RNA extraction

Genomic DNA was extracted from five 10-μm-thick formalin-fixed paraffin-embedded sections of the 151 tumors (one paraffin block representing the tumor center without normal mucosa) and 20 normal tissues (one paraffin block distant from the tumor), and from frozen sections of 61 tumors and 10 normal tissues using a DNeasy kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. In addition, DNA was extracted from a colon cancer cell line SW480 (American Type Culture Collection), in which the promoter of the p16 gene is reported to be fully methylated (Herman et al. 1995), as a positive control. DNA from peripheral blood leukocytes of a healthy volunteer was employed as a negative control for bisulfite conversion, DNA recovery, and PCR reaction.

Total RNA was extracted from frozen sections of 43 tumor tissues using a RNeasy Micro kit (Qiagen) with DNaseI (Qiagen), according to the manufacturer's protocol.

Bisulfite modification and real-time quantitative methylation-specific PCR

Bisulfite modification was conducted using an EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). Two micrograms of DNA was treated with sodium bisulfite following the manufacturer's recommendations and amplified using specifically designed primers for methylated and unmethylated p16 sequences and a *Taqman* probe. The sense and antisense primers for the methylated sequence were 5′-GTTATTAGAGGGTGGGGCGGATCG CG-3′ and 5′-CGAACCGCGACCGTAACCA-3′, respectively. These primers were used in conjunction with a *Taqman* probe [5′-(FAM)-AGTAGTATGGAGTCGGCGGCG GG-(TAMRA)-3′]. The sense and antisense primers for the unmethylated sequence were 5′-GGTTATTAGAGGGTG GGGTGGATTGTG-3′ and 5′-CCCAACCCCAAACCAC AACCATATCC-3′, respectively, used in conjunction with another *Taqman* probe [5′-(FAM)-AGGTAGTGGGTG GTGGGGAGTAGTATGGAGTTG-(TAMRA)-3′].

Real-time quantitative methylation-specific PCR (MSP) was performed with Premix Ex Taq (Takara Bio, Shiga, Japan) on a LightCycler (Roche Diagnostics, Basel, Switzerland). The PCR protocol was the same for methylated and unmethylated p16, *i.e.*, 95°C for 10 min to activate *Taq* polymerase, then 60 cycles of 95°C for 10 s and 68°C for 30 s. All PCR runs included separate reactions with templates from p16-fully methylated and the unmethylated control DNA, as well as with no template. The methylation index (MI; percentage) in a sample was calculated according to the equation: (concentration of methylated p16 sequence/concentrations of methylated plus unmethylated p16 sequence) × 100 (Kim et al. 2005).

Reverse transcription real-time quantitative PCR analysis

One microgram of total RNA was employed to synthesize cDNA using Sensiscript reverse transcriptase (Qiagen). The cDNA was then used for real-time quantitative PCR on a LightCycler. The primer pairs located in exons 1α and 2 with flanking intron 1 of the p16 gene were 5′-GAGCAG CATGGAGCCTTC-3′ and 5′-ACCGTAACTATTCGGT GCGTT-3′. The probe sequence for p16 was 5′-(FAM)-TA GAGGAGGTGCGGGCGCTGC-(TAMRA)-3′. The PCR protocol was as follows: 95°C for 10 min to activate *Taq* polymerase, then 60 cycles of 95°C for 10 s and 56°C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was used as an internal standard, its cDNA being amplified with the following primers: 5′-CAGGGA CTCCCCAGCAGT-3′ and 5′-GGCATTGCCCTCAACG ACCA-3′. These primers were used in conjunction with SYBR Green I. The PCR protocol was as follows: 95°C for 10 min to activate *Taq* polymerase, then 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 6 s. For each sample, the level of cDNA was normalized to the expression of GAPDH. SYBR Premix Ex Taq (takara Bio, Shiga, Japan) was used for the reaction.

Immunohistochemistry

We randomly selected 25 blocks from 25 tumor samples, which were prepared for immunohistochemical analysis. Immunostaining was performed with DakoCytomation Autostainer Instruments (DakoCytomation, Kyoto, Japan). Briefly, 4-μm-thick tissue sections were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. Endogeneous peroxidase activity was blocked by incubation with Peroxidase-Blocking Solution (DakoCytomation) for 5 min. Antigen retrieval consisted of autoclave treatment of sections for 30 min in Epitope Retrieval Solution. The primary antibody employed was monoclonal anti-p16 (E6H4, prediluted, DakoCytomation), with incubation for 30 min at room temperature. Using an Envision Kit, the slides were incubated with horseradish peroxidase-labeled polymer conjugated with secondary antibody for 30 min and then with Substrate-Chromogen (diaminobenzidine) Solution, followed by light counterstaining with Mayer's hematoxylin. Sections of a squamous cell carcinoma of the esophagus with known positivity for p16 were used as external positive controls. For negative controls, the primary antibodies were omitted.

Assessment of immunostaining

Image analysis using Image-Pro Plus (Version 5.1, Media Cybernetics Inc., Silver Spring, MD, USA) was performed on p16 immunostained slides; five images were captured in each case from the areas of their highest immunoreactivity at 25× magnification with a Digital Microscopy Camera (DP50-CU, Olympus Co., Tokyo, Japan) and processed with Viewfinder Lite software (Version 1.0, Pixera Corp., San Jose, CA, USA) and Adobe Photoshop software programs (Version 7.0, Adobe Photoshop Inc., San Jose, CA, USA). Distinct nuclear staining was considered to be positive, regardless of the staining intensity. When there was cytoplasmic staining, a nucleus was regarded as positive if its staining intensity equaled or exceeded that of the surrounding cytoplasm. In negative cases, sporadically decorated stromal fibroblasts served as convenient internal controls. Finally, p16-immunolabeling indices were obtained by calculating the percentage of the total area of positively stained tumor cells per whole lesion.

Statistical analysis

Categorical analysis of variables was performed using either the chi-squared test (with Yates' correction) or the

Fisher's exact test, as appropriate. Continuous data were compared with the Mann–Whitney *U*-test or Kruskal–Wallis test. Survival curves were generated by the Kaplan–Meier method. Cancer-related survival time was measured from the date of surgery to the end of follow-up or death due to colorectal cancer or other causes. Recurrence-free survival time was defined as the time from surgery to recurrent disease (alive) or death with or without recurrence. The patients who had metastases diagnosed at surgery or had incomplete resection were excluded from analyses of recurrence-free survival ($n = 127$). Associations between MI and clinicopathological features, and survival were examined using univariate and multivariate Cox's regression analyses. In the Cox's multivariate analysis, a step-wise backward variable elimination method was used with an entry limit of $P < 0.1$ and a removal limit of $P \geq 0.05$. A P value of less than 0.05 was considered statistically significant. All statistical analyses were carried out using StatView for Windows Version 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

p16 MI in normal and tumor tissue samples

The p16 MI values for normal mucosa samples ($n = 30$) ranged from 0 to 2% (mean, 0.23%; median, 0.02%), while for tumor samples ($n = 212$) they varied widely from 0 to 100% (mean, 25.7%; median, 7.1%), the difference being statistically significant ($P < 0.001$). Tumor cases were classified into three categories as follows: low (MI $\leq$ 2%), intermediate (2 < MI $\leq$ 40%), and high (MI > 40%) aberrant p16 methylation groups, accounting for 51 (34%), 54 (36%), and 46 (30%), respectively, of the 151 paraffin-embedded tumor samples.

Associations between the p16 methylation status and clinicopathological features

High aberrant p16 methylation was significantly associated with large tumor size ($P = 0.025$), but not with other clinicopathological features (Table 1).

Table 1 Associations between p16 methylation status and clinicopathological features

Variable	Aberrant p16 methylation			*P* value
	Low ($n = 51$)	Intermediate ($n = 54$)	High ($n = 46$)	
Sex				
Male	29	37	29	NS
Female	22	17	17	
Age				
<60 years	14	14	12	NS
≥60 years	37	40	34	
Size				
<5.0 cm	28	39	23	0.025*
≥5.0 cm	23	15	23	
Location				
Proximal	12	22	16	NS
Distal	39	32	30	
Differentiation				
Well	22	26	25	NS
Moderate	27	22	19	
Poor	2	6	2	
pT category				
pT3	49	52	42	NS
pT4	2	2	4	
pN category				
pN0	25	28	23	NS
pN1	22	16	16	
pN2	4	10	7	
TNM stage				
II	22	25	23	NS
III	21	20	16	
IV	8	9	7	

* Intermediate vs. high p16 methylation group

Impact on cancer-related survival

Based on univariate analysis (Table 2), patients with high p16 methylation had significantly worse ($P = 0.002$) survival than those with low or intermediate methylation (Fig. 1a). In addition, tumor differentiation ($P < 0.001$), pT category ($P = 0.003$), pN category ($P < 0.001$) and TNM stage ($P < 0.001$) had significant influence on cancer-related survival. In the Cox's regression model, high methylation group ($P < 0.001$), high pT ($P = 0.013$) and advanced TNM stage ($P < 0.001$) proved to be independent predictors of short cancer-related survival (Table 3).

Impact on recurrence-free survival

Twenty (16%) of 127 patients suffered isolated locoregional recurrence, and 20 (16%) had distant metastases. Patients with intermediate and high p16 methylation (16/127 cases, 13%) showed more frequent locoregional recurrence as compared with the low-methylation group (4/127 cases, 3%; $P = 0.009$). For distant metastases, similar differences were found in patients with metastases to the liver (intermediate and high, 9% vs. low, 2%; $P = 0.019$) and the lung (5% vs. 0%; $P = 0.029$). Other metastatic deposits were found in the peritoneum (2%), bone (1%) and brain (1%), but no significant link was apparent with aberrant methylation.

Table 2 Univariate analysis of clinicopathological features and p16 methylation status with reference to cancer-related survival

Variable	*n*	5-year survival	HR	95% CI	*P* value
Sex					
Male	95	59 (62)	1		
Female	56	34 (61)	0.894	0.526–1.521	NS
Age					
<60 years	40	21 (53)	1		
≥60 years	111	72 (65)	1.415	0.818–2.45	NS
Size					
<5.0 cm	90	56 (62)	1		
≥5.0 cm	61	37 (61)	0.979	0.580–1.651	NS
Location					
Proximal	50	34 (68)	1		
Distal	101	59 (58)	0.773	0.434–1.375	NS
Differentiation					
Well	73	48 (66)	1		
Moderate	68	43 (63)	1.108	0.636–1.929	0.7
Poor	10	2 (20)	4.121	1.848–9.189	<0.001
pT category					
pT3	143	91 (64)	1		
pT4	8	2 (25)	3.585	1.529–8.403	0.003
pN category					
pN0	76	61 (80)	1		
pN1	54	26 (48)	3.366	1.797–6.307	<0.001
pN2	21	6 (29)	4.948	2.411–10.15	<0.001
TNM stage					
II	70	61 (87)	1		
III	57	32 (56)	4.133	1.928–8.858	<0.001
IV	24	0 (0)	26.221	11.74–58.57	<0.001
Aberrant p16 methylation					
Low	51	39 (76)	1		
Intermediate	54	34 (63)	1.819	0.889–3.724	0.102
High	46	20 (62)	2.953	1.487–5.863	0.002

Values in parenthesis are percentages

HR hazard ratio, *CI* confidence interval

NS not significant

On univariate analysis of risk factors for recurrence-free survival (Table 4), patients with high methylation of p16 had a significantly worse prognosis ($P = 0.001$) than those with low or intermediate methylation (Fig. 1b). Furthermore, recurrence-free survival was also strongly related to poor tumor differentiation ($P = 0.01$) and high pN (pN1, $P = 0.002$; pN2, $P < 0.001$). Cox's regression multivariate analysis showed high p16 methylation group (intermediate, $P = 0.04$; high, $P < 0.001$) and advanced TNM stage ($P < 0.001$) to predict short recurrence-free survival (Table 5).

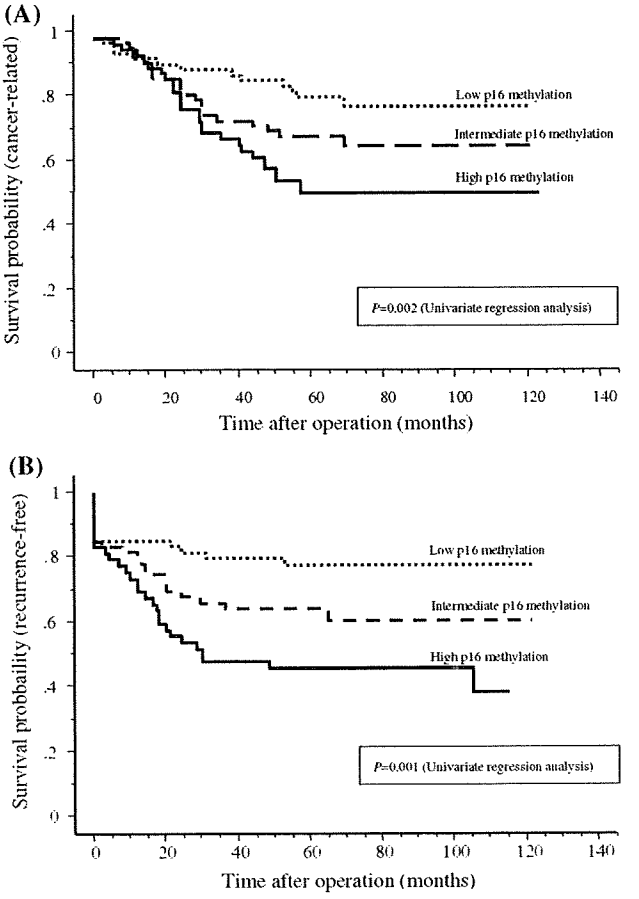


Fig. 1 Cancer-related (**a**) and recurrence-free (**b**) survival cases with reference to aberrant p16 methylation status

Table 3 Multivariate analysis of variables for cancer-related survival in the 151 patients

Variable	HR	95% CI	*P* value
Aberrant p16 methylation			
Low	1		
Intermediate	1.852	0.901–3.81	0.094
High	3.379	1.668–6.843	<0.001
pT category			
pT3	1		
pT4	3.098	1.266–7.585	0.013
TNM stage			
II	1		
III	4.955	2.292–10.71	<0.001
IV	29.33	12.89–66.71	<0.001

HR hazard ratio, *CI* confidence interval

Correlations among p16 methylation status, mRNA level and immunoreactivity in tumor samples

The tumors with high p16 methylation tended to have lower p16 mRNA levels as compared with intermediate or low

Table 4 Univariate analysis of clinicopathological features and p16 methylation status with reference to recurrence-free survival

Variable	*n*	5-year survival	HR	95% CI	*P* value
Sex					
Male	80	55 (67)	1		
Female	47	34 (72)	1.113	0.569–2.178	NS
Age					
<60 years	30	20 (67)	1		
≥60 years	97	69 (71)	1.139	0.553–2.346	NS
Size					
<5.0 cm	74	54 (72)	1		
≥5.0 cm	53	35 (66)	0.752	0.398–1.422	NS
Location					
Proximal	43	32 (74)	1		
Distal	84	57 (68)	0.805	0.399–1.624	NS
Differentiation					
Well	65	45 (69)	1		
Moderate	55	42 (76)	0.723	0.359–1.455	0.364
Poor	7	2 (28)	3.666	1.365–9.842	0.01
pT category					
pT3	122	87 (71)	1		
pT4	5	2 (40)	3.212	0.985–10.48	0.053
pN category					
pN0	70	59 (84)	1		
pN1	43	24 (56)	3.341	1.589–7.025	0.002
pN2	14	6 (43)	4.853	1.944–12.12	<0.001
Aberrant p16 methylation					
Low	51	39 (76)	1		
Intermediate	54	32 (59)	1.939	0.959–3.921	0.065
High	46	18 (39)	3.121	1.584–6.15	0.001

Values in parenthesis are percentages

HR hazard ratio, *CI* confidence interval

NS not significant

Table 5 Multivariate analysis of variables for recurrence-free survival in the 127 patients

Variable	HR	95% CI	*P* value
Aberrant p16 methylation			
Low	1		
Intermediate	2.095	1.033–4.247	0.04
High	3.962	1.987–7.903	<0.001
TNM stage			
II	1		
III	4.545	2.231–9.261	<0.001
IV	325.1	40.8–2589.8	<0.001

HR hazard ratio, *CI* confidence interval

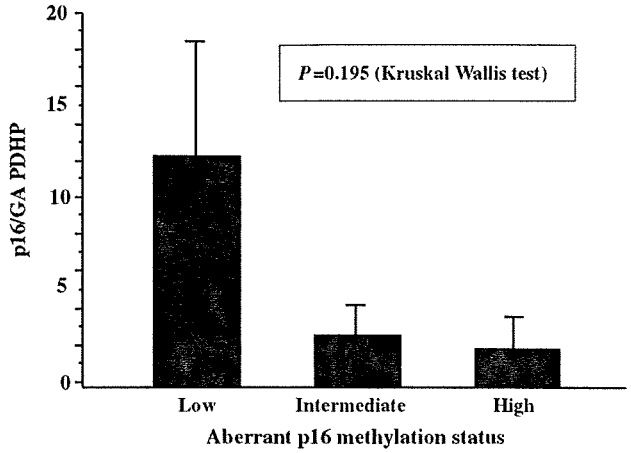


Fig. 2 Associations of p16 mRNA level and aberrant methylation status in tumor samples. The units ascribed to p16 mRNA represent the ratios of p16 to GAPDH on reverse transcription real-time quantitative PCR. Data are means ± standard errors

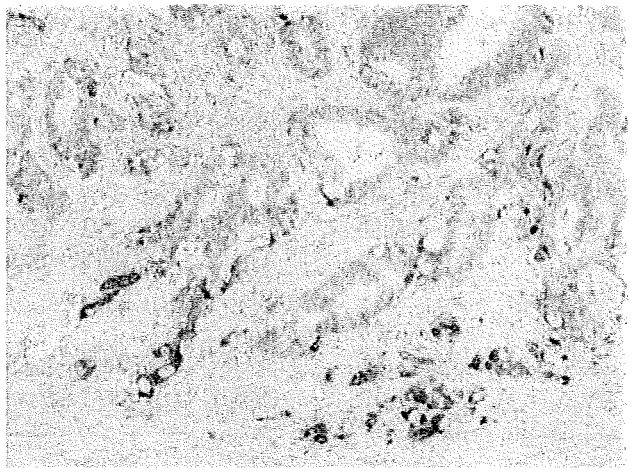


Fig. 3 Example of p16 immunostaining in a colon carcinoma. Note loss of expression in the tumor center and high intensity at the invasive front (original magnification, ×16)

methylation groups, but without any significant difference ($P = 0.195$; Fig. 2).

P16 immunostaining could be clearly discriminated and proved heterogenous, with higher expression commonly seen at invasive fronts (Fig. 3). The p16 immunolabeling indices gradually decreased from low to intermediate and high p16 methylation groups with significant intergroup variation ($P = 0.017$; Fig. 4).

Discussion

In the present study, fluorescence-based real-time quantitative MSP-detected p16 methylation was found to

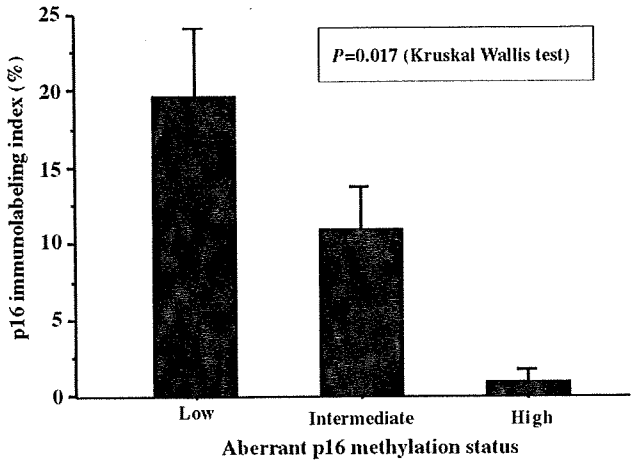


Fig. 4 Associations of p16 immunoreactivity and aberrant methylation status in tumor samples. Data are means ± standard errors

be significantly greater in tumor samples than in normal mucosa, with intermediate and high aberrant p16 methylation demonstrable in 36% and 30% of colorectal cancers, respectively. In the literature, aberrant p16 methylation was present in 18–61% of the lesions using the MSP method (Guan et al. 1999; Liang et al. 1999; Wiencke et al. 1999; Esteller et al. 2000; Burri et al. 2001; Esteller et al. 2001; Kanai et al. 2001; Yi et al. 2001; Hawkins et al. 2002; Hibi et al. 2002; Van Rijnsoever et al. 2002; Maeda et al. 2003; Norrie et al. 2003; Schneider-Stock et al. 2003; Van Rijnsoever et al. 2003; Ward et al. 2003; Kim et al. 2005; Sanz-Casla et al. 2005; Derks et al. 2006; Iacopetta et al. 2006; Ishiguro et al. 2006; Lee et al. 2006; Ogino et al. 2006; Prall et al. 2006; Goto et al. 2009). Methylation frequencies examined by other methods were rather low ranging from 17 to 40% (Herman et al. 1995; Ahuja et al. 1997; Toyota et al. 1999; Shannon and Iacopetta. 2001; Shen et al. 2007). The fluorescence-based real-time quantitative MSP used in our study does not require electrophoresis, in contrast with almost all other methods for analysis of DNA methylation. Simple MSP cannot reliably distinguish between low and high levels of methylation because it provides only a binary division (i.e., present vs. absent). In contrast, the real-time MSP method provides quantitative data, and requires only small amounts of DNA for sensitivity up to 0.1% methylated alleles in a given CpG island locus (Herman et al. 1996). Furthermore, this technique eliminates the false-positive results inherent in PCR-based approaches which rely on differential restriction enzyme cleavage to distinguish methylated from unmethylated DNA.

Our data provide evidence for wide variation in p16 MIs for tumor samples with heterogenous expression of p16 protein, in accordance with previous reports (Dai et al. 2000; Goto et al. 2009). This may be due to partial methylation, in which, as we speculate, some proportion of tumor cells contain methylated p16, whereas the remainder are unmethylated. In fact, most of p16-hypermethylated colorectal cancers presented with both methylated and unmethylated PCR products (Liang et al. 1999; Kim et al. 2005). Furthermore, Guan et al. (1999) demonstrated this to be the case with tumor samples isolated strictly by microdissection. A recent study showed some colorectal cancers to express p16 mRNA even with methylation (Kim et al. 2005). p16 is known to be heterozygously methylated in some colon cancer cell lines, p16 mRNA being detected in methylated as well as unmethylated cells (Burri et al. 2001).

Previous studies have shown that p16 methylation is a frequent phenomenon in older females (Hawkins et al. 2002; Norrie et al. 2003; Ward et al. 2003; Ishiguro et al. 2006) as well as with right-sided lesions (Hawkins et al. 2002; Van Rijnsoever et al. 2002; Norrie et al. 2003; Ward et al. 2003; Iacopetta et al. 2006), circumscribed tumor margins (Norrie et al. 2003), increased intratumoral/peritumoral lymphocytes (Hawkins et al. 2002; Norrie et al. 2003; Ward et al. 2003; Iacopetta et al. 2006), Crohn's type reactions (Norrie et al. 2003), a mucinous phenotype (Hawkins et al. 2002; Maeda et al. 2003; Norrie et al. 2003; Ward et al. 2003; Iacopetta et al. 2006), poor tumor differentiation (Shannon and Iacopetta 2001; Hawkins et al. 2002; Van Rijnsoever et al. 2002; Maeda et al. 2003; Norrie et al. 2003; Ward et al. 2003; Lee et al. 2006) and an advanced stage (Yi et al. 2001; Maeda et al. 2003; Ishiguro et al. 2006; Goto et al. 2009). This mirrors the clinicopathological features classically described with the CpG island methylator phenotype (CIMP), known to cause microsatellite instability through methylation of the hMLH1 promoter in colorectal cancers (Ahuja et al. 1997; Toyota et al. 1999; Hawkins et al. 2002; Van Rijnsoever et al. 2002, 2003; Iacopetta et al. 2006; Ogino et al. 2006). As expected, microsatellite unstable tumors frequently display methylation of p16 (Ahuja et al. 1997; Liang et al. 1999; Toyota et al. 1999; Shannon and Iacopetta. 2001; Hawkins et al. 2002; Van Rijnsoever et al. 2002, 2003; Ward et al. 2003; Iacopetta et al. 2006; Lee et al. 2006). However, high aberrant p16 methylation was here only associated with large tumor size, but not CIMP features.

In the current work, the patients with higher aberrant p16 methylation more frequently exhibited locoregional or distant metastasis and multivariate analysis indicated high p16 methylation as an independent predictor of shortened cancer-related and recurrence-free survival, in line with the results of Maeda et al. (2003). In contrast to their study providing a binary distinction (hypermethylated or not), we emphasize that three methylation levels (low, intermediate or high) have independent prognostic values. There are two other published reports of a relationship between p16 methylation and a worse prognosis on univariate analysis (Liang et al. 1999; Shen et al. 2007).

A recent study by Shen et al. (2007) confirmed that methylation of p16, $p14^{ARF}$, MINT1, or MINT31 is associated individually with shortened survival in CIMP colorectal cancers. They also mentioned that concurrent methylation of two or more genes in a CIMP-associated subset defined a group with a markedly poor prognosis. Microsatellite-unstable cancers with increasing methylation of CIMP-associated genes appeared to have better survival, whereas increasing methylation within the microsatellite-stable tumor group was associated with a worse outcome (Hawkins et al. 2002; Ward et al. 2003). These observations are somewhat paradoxical. We now need to further analyze microsatellite instability in our tumor samples.

In the present study, high p16-methylated tumor tissues displayed significantly lower p16 immunoreactivity, as compared with low- or intermediate-methylation samples. A similar tendency was observed regarding the mRNA level, in agreement with a statistically significant relationship between p16 methylation and suppression of mRNA production or loss of protein expression (Norrie et al. 2003; Schneider-Stock et al. 2003; Kim et al. 2005; Lee et al. 2006; Prall et al. 2006). Regulation at the mRNA level may be largely responsible for differences in p16 protein expression (Dai et al. 2000; Kim et al. 2005). Our data thus provide evidence that aberrant methylation may at least partly facilitate transcriptional repression of the p16 gene. We found, however, a few exceptional tumor samples that displayed relatively low p16 mRNA levels despite low MI, in accordance with the earlier observations of Kim et al. (2005). They also identified tumors with high MI expressing some mRNA. The findings imply that transcriptional regulation of p16 likely contributes not only to its own methylation but also to other processes such as *ras* pathway activation (Serrano et al. 1995).

In summary, one key finding of this study is that aberrant p16 methylation, although common in sporadic colorectal cancer, is not a reflection of the CIMP, but has potential as an independent adverse prognostic factor. The second key finding is that p16 methylation results in transcriptional silencing at the RNA level and consequent loss of protein expression.

Acknowledgments This work was supported by a Grants-in-Aid from the Japan Society for the Promotion of Science (#21590394) to H. Mitomi. We thank Y. Oya, J. Kubo and N. Anpo, Pathology Division, National Hospital Organization Sagamihara Hospital, for their expert technical assistance.

Conflict of Interest Statement None

References

Ahuja N, Mohan AL, Li Q, Stolker JM, Herman JG, Hamilton SR, Baylin SB, Issa J-PJ (1997) Association between CpG island methylation and microsatellite instability in colorectal cancer. Cancer Res 57:3370–3374

Baylin SB, Herman JG, Graff JR, Vertino PM, Issa J-P (1998) Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res 72:141–196

Burri N, Shaw P, Bouzourene H, Sordat I, Sordat B, Gillet M, Schorderet D, Bosman FT, Chaubert P (2001) Methylation silencing and mutations of the $p14^{ARF}$ and $p16^{INK4a}$ genes in colon cancer. Lab Invest 81:217–229

Dai CY, Furth EE, Mick R, Koh J, Takayama T, Niitsu Y, Enders GH (2000) $p16^{INK4a}$ expression begins early in human colon neoplasia and correlates inversely with markers of cell proliferation. Gastroenterology 119:929–942

Derks S, Postma C, Moerkerk PTM, van den Bosch SM, Carvalho B, Hermsen MAJA, Giaretti W, Herman JG, Weijenberg MP, de Bruïne AP, Meijer GA, van Engeland M (2006) Promoter methylation precedes chromosomal alterations in colorectal development. Cellular Oncol 28:247–257

Esteller M, Tortola S, Toyota M, Capella G, Peinado MA, Baylin SB, Herman JG (2000) Hypermethylation-associated inactivation of $p14^{ARF}$ is independent of $p16^{INK4a}$ methylation and *p53* mutational status. Cancer Res 60:129–133

Esteller M, González S, Risques RA, Marcuello E, Mangues R, Germà JR, Herman JG, Capellà G, Peinado MA (2001) K-*ras* and *p16* aberrations confer poor prognosis in human colorectal cancer. J Clin Oncol 19:299–304

Goto T, Mizukami H, Shirahata A, Sakata M, Saito M, Ishibashi K, Kigawa G, Nemoto H, Sanada Y, Hibi K (2009) Aberrant methylation of the *p16* gene is frequently detected in advanced colorectal cancer. Anticancer Res 29:275–278

Guan RJ, Fu Y, Holt PR, Pardee AB (1999) Association of k-*ras* with *p16* methylation in human colon cancer. Gastroenterology 116:1063–1071

Hawkins N, Norrie M, Cheong K, Mokany E, Ku S-L, Meagher A, O'Connor T, Ward R (2002) CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. Gastroenterology 122:1376–1387

Herman JG, Merlo A, Mao L, Lapidus RG, Issa J-PJ, Davidson NE, Sidransky D, Baylin SB (1995) Inactivation of the *CDK2/p16/MTS1* gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res 55:4525–4530

Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB (1996) Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93:9821–9826

Hibi K, Nakayama H, Koike M, Kasai Y, Ito K, Akiyama S, Nakao A (2002) Colorectal cancers with both *p16* and *p14* methylation show invasive characteristics. Jpn J Cancer Res 93:883–887

Iacopetta B, Grieu F, Li W, Ruszkiewicz A, Caruso M, Moore J, Watanabe G, Kawakami K (2006) APC gene methylation is inversely correlated with features of the CpG island methylator phenotype in colorectal cancer. Int J Cancer 119:2272–2278

International Union Against Cancer (UICC) (1997) TNM classification of the tumours of the colon and rectum. In: Sobin LH, Wittekind Ch (eds) TNM Classification of Malignant Tumours, 5th edn. Wiley-Liss Inc., New York, pp 59–62

Ishiguro A, Takahata T, Saito M, Yoshiya G, Tamura Y, Sasaki M, Munakata A (2006) Influence of methylated $p15^{INK4b}$ and $p16^{INK4a}$ genes on clinicopathological features in colorectal cancer. J Gastroenterol Hepatol 21:1334–1339

Kamb A, Gruis NA, Weaver-Feldhaus J, Liu Q, Harshman K, Tavtigian SV, Stockert E, Day IIIRS, Johnson BE, Skolnick MH (1994) A cell cycle regulator potentially involved in genesis of many tumor types. Science 264:436–440

Kanai Y, Ushijima S, Kondo Y, Nakanishi Y, Hirohashi S (2001) DNA methyltransferase expression and DNA methylation of CpG

islands and peri-centromeric satellite regions in human colorectal and stomach cancers. Int J Cancer 91:205–212

Kim BN, Yamamoto H, Ikeda K, Damdinsuren B, Sugita Y, Ngan CY, Fujie Y, Ogawa M, Hata T, Ikeda M, Ohue M, Sekimoto M, Monden T, Matsuura N, Monden M (2005) Methylation and expression of $p16^{INK4}$ tumor suppressor gene in primary colorectal cancer tissues. Int J Oncol 26:1217–1226

Lee M, Han WS, Kim OK, Sung SH, Cho MS, Lee SN, Koo H (2006) Prognostic value of *p16*^{INK4a} and *p14*^{ARF} gene hypermethylation in human colon cancer. Pathol Res Pract 202:415–424

Liang J-T, Chang K-J, Chen J-C, Lee C–C, Cheng Y-M, Hsu H-C, Wu M-S, Wang S-M, Lin J-T, Cheng A-L (1999) Hypermethylation of the p16 gene in sporadic T3N0M0 stage colorectal cancers: association with DNA replication error and shorter survival. Oncology 57:149–156

Maeda K, Kawakami K, Ishida Y, Ishiguro K, Omura K, Watanabe G (2003) Hypermethylation of the *CDKN2A* gene in colorectal cancer is associated with shorter survival. Oncol Rep 10:935–938

Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA (1994) Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 368:753–756

Norrie MWA, Hawkins NJ, Todd AV, Meagher AP, O'Connor TW, Ward RL (2003) Inactivation of *p16*^{INK4a} by CpG hypermethylation is not a frequent event in colorectal cancer. J Surg Oncol 84:143–150

Ogino S, Odze RD, Kawasaki T, Brahmandam M, Kirkner GJ, Laird PW, Loda M, Fuchs CS (2006) Correlation of pathologic features with CpG island methylator phenotype (CIMP) by quantitative DNA methylation analysis in colorectal carcinoma. Am J Surg Pathol 30:1175–1183

Prall F, Ostwald C, Weirich V, Nizze H (2006) $p16^{INK4a}$ promoter methylation and 9q21 allelic loss in colorectal carcinomas: relation with immunohistochemical $p16^{INK4a}$ expression and with tumor budding. Hum Pathol 37:578–585

Sanz-Casla MT, Maestro ML, Vidaurreta M, Maestro C, Arroyo M, Cerdán J (2005) p16 gene methylation in colorectal tumors: correlation with clinicopathological features and prognostic value. Dig Dis 23:151–155

Schneider-Stock R, Boltze C, Peters B, Höpfner T, Meyer F, Lippert H, Roessner A (2003) Differences in loss of $p16^{INK4}$ protein expression by promoter methylation between left- and right-sided primary colorectal carcinomas. Int J Oncol 23:1009–1013

Serrano M, Gómez-Lahoz E, DePinho RA, Beach D, Bar-Sagi D (1995) Inhibition of ras-induced proliferation and cellular transformation by $p16^{INK4}$. Science 267:249–252

Shannon BA, Iacopetta BJ (2001) Methylation of the *hMLH1*, *p16*, and *MDR1* genes in colorectal carcinoma: associations with clinicopathological features. Cancer Lett 167:91–97

Shen L, Catalano PJ, Benson AB III, O'Dwyer P, Hamilton SR, Issa J-PJ (2007) Association between DNA methylation and shortened survival in patients with advanced colorectal cancer treated with 5-fluorouracil-based chemotherapy. Clin Cancer Res 13:6093–6098

Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa J-PJ (1999) CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci USA 96:8681–8686

Van Rijnsoever M, Grieu F, Elsaleh H, Joseph D, Iacopetta B (2002) Characterisation of colorectal cancers showing hypermethylation at multiple CpG islands. Gut 51:797–802

Van Rijnsoever M, Elsaleh H, Joseph D, McCaul K, Iacopetta B (2003) CpG island methylator phenotype is an independent predictor of survival benefit from 5-fluorouracil in stage III colorectal cancer. Clin Cancer Res 9:2898–2903

Ward RL, Cheong K, Ku S-L, Meagher A, O'Connor T, Hawkins NJ (2003) Adverse prognostic effect of methylation in colorectal cancer is reversed by microsatellite instability. J Clin Oncol 21:3729–3736

Weinberg RA (1995) The retinoblastoma protein and cell cycle control. Cell 81:323–330

Wiencke JK, Zheng S, Lafuente A, Lafuente MJ, Grudzen C, Wrensch MR, Miike R, Ballesta A, Trias M (1999) Aberrant methylation of *p16*^{INK4a} in anatomic and gender-specific subtypes of sporadic colorectal cancer. Cancer Epidemiol Biomark Prev 8:501–506

Yi J, Wang Z-W, Cang H, Chen Y-Y, Zhao R, Yu B-M, Tang X-M (2001) *p16* gene methylation in colorectal cancers associated with Duke's staging. World J Gastroenterol 7:722–725

Psycho-Oncology
Psycho-Oncology (2009)
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI**: 10.1002/pon.1429

Effect of communication skills training on nurses' detection of patients' distress and related factors after cancer diagnosis: a randomized study

Sakiko Fukui*, Keiko Ogawa, Masao Ohtsuka and Naoshi Fukui
School of Nursing, Chiba University, Chiba, Japan

Correspondence to: School of Nursing, Chiba University, Inohana 1-8-1 Chuo-ku Chiba City, Chiba, Japan. E-mail: sfukui@faculty.chiba-u.jp

Received: 12 December 2007
Revised: 25 June 2008
Accepted: 5 July 2008

Abstract

***Background*: A randomized study was performed to investigate whether a communication skill (CS) training program can improve nurse's ability to detect the distress of patients who have just been informed of cancer diagnosis.**

***Methods*: Nurses were randomly assigned to the experimental or control group, and those in the former group had undergone CS training program. Nurses in both groups were then requested to support patients informed of their cancer diagnosis. Intervention consisted of one-on-one nurse interviews 3 times (on the day, 1 week, and 1 month after diagnosis). Patient's self-reported distress according to the Hospital Anxiety and Depression Scale and nurse's ratings of patient distress by Visual Analog Scale were assessed 3 times (1 week, 1 month, and 3 months after diagnosis). These two scales were compared between the nurses of the two groups to assess the impact of CS training.**

***Results*: The nurses in the experimental and control groups supported 42 and 47 patients, respectively. The analysis using mixed-effects modeling revealed no significant differences in the nurse's ability to detect patient's distress between the two groups. However, when the nurse's ratings of patient's distress and patient's self-reported distress were compared, these two scores were significantly correlated only with the nurses in the experimental group, suggesting that the nurse's ability to become aware of patient's distress had been improved in that group.**

***Conclusions*: CS training for health professionals is useful in oncology practice to improve nurse's ability to recognize the distress of patients diagnosed with cancer.**

Keywords: psychological distress; communication skills; nursing support; newly diagnosed cancer; randomized study

Introduction

Psychological distress of patients with malignancies is a normal response to cancer diagnosis, treatment, and prognosis [1–3]. Without early recognition and support for the distressed patients, there may be long-term, detrimental consequences on their quality of life [4]. Therefore, psychological distress needs to be recognized and supported appropriately by health professionals.

However, health professionals in oncology often fail to recognize patient's distress, and feel it difficult to estimate the level of such distress, because they lack knowledge about symptoms of patient's distress and often rely on superficial signs to assess it [1,5]. Patients are also sometimes reluctant to disclose their psychological concerns spontaneously [6]. It is thus important to detect distress as early as possible in the course of the disease and to refer patients for appropriate professional support. Since patient's distress is highest just after cancer diagnosis [7,8], it is particularly important for health professionals to support patients at that time.

Our previous study revealed that busy physicians at a cancer screening center have little time to support patients in their distress when they were informed of cancer diagnosis [8]. Therefore, nurses are supposed to play a key role in supporting those patients [9].

Recent studies have underscored the importance of nurse's communication skills (CS) to detect patient's distress [1,10]. Taking a brief educational course may be useful for health professionals to improve their ability to improve such distress [11–13]. However, except for one study on physicians, there are no previous studies on health professionals, which investigated the impact of such an educational course on the detection of cancer patient's distress [1].

The primary purpose of this study was to evaluate the impact of a brief educational course

on nurse's ability to detect patient's distress. In parallel, we also attempted to investigate the factors associated with a nurse's ability to detect patient's distress.

Methods

Subjects

First, we chose cancer screening institutions in east Japan, which have a follow-up system by nurses after physicians informed patients of their cancer diagnosis. At first, we approached four institutions and visited head nurses to ask them to participate. The head nurses agreed to allow us to contact nurses who worked in the follow-up system. Consent was obtained in writing from each of them.

Patients were selected from among those who had had a cancer diagnostic test and supported by nurses after a physician's consultation informing them of a cancer diagnosis. In the conventional system, nurses in charge are always present at physician consultations when patients are informed of a cancer diagnosis. The physicians then entrust nurses with the support of patients thereafter. The study protocol was reviewed and approved by the Institutional Review Board and the Ethics Committee of Tokyo Metropolitan University.

Previous studies and reviews revealed that CS training programs did not improve patient outcomes in terms of psychological distress and adjustment to cancer [14–16]. In those studies, the lack of homogeneity among the subjects could be the reason for their negative results, because homogeneity of patients is critical in studying the effects of psychological interventions [17]. Taking this into consideration, we set patient eligibility criteria for the present study as follows: (1) newly diagnosed and informed of cancer by physician's consultation after cancer diagnostic test in the study period (from January to December 2006); (2) nurse in charge present at the physician's consultation; (3) age >18 years; (4) not advanced and operable stage; (5) diagnosed with gastric, colorectal, or breast cancer, the three major sites most tested at the center, where nurses usually provide support for all these patients after cancer diagnosis; and (6) written informed consent. Patients were excluded if they had not been informed of a cancer diagnosis by physicians and had severe psychological status based on physicians' assessment.

Study design

In order to assess the efficacy of the CS training program on a nurse's ability to detect patient's distress, nurses were randomly assigned either to the experimental group or to the wait-listed control group (Figure 1). Before starting the study, nurses

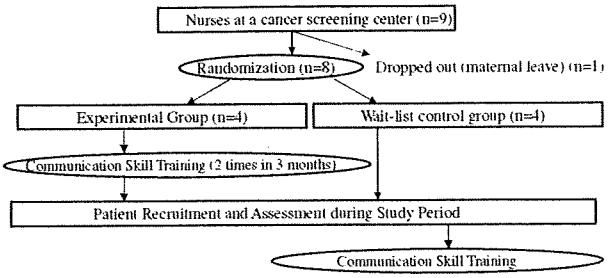


Figure 1. Nurses randomization procedure

in the experimental group attended a 1-day CS training program 2 times during a 3-month period (October and December 2005). Nurses assigned to the wait-listed control group were invited to participate in the CS training program after the study period.

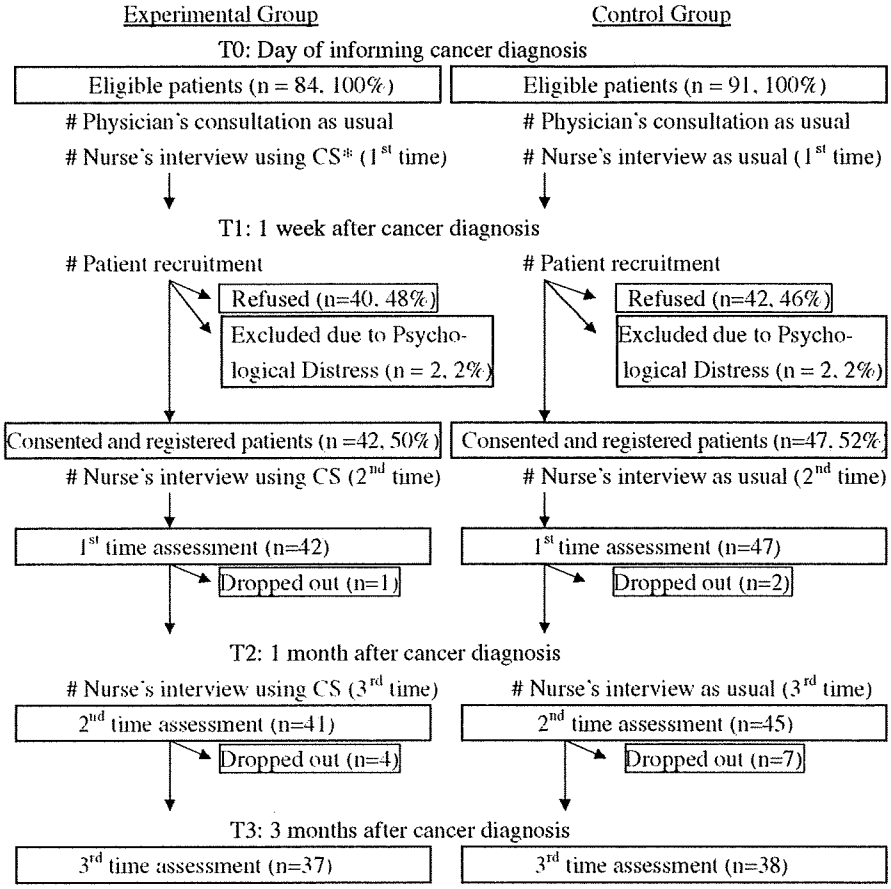
The study was explained to the patients who met the eligibility criteria, and they were consecutively asked to participate in the study by the nurses in charge. Patient recruitment and first-time assessment were started at T1 (1 week after cancer diagnosis), because the ethical manager of the institution (Tokyo Metropolitan Cancer Screening Center) did not allow us to start the patient assessment at T0 (on the day of cancer diagnosis). Each patient was interviewed 3 times by a nurse in either group, which was scheduled on the day of cancer diagnosis (T0), 1 week (T1), and 1 month after the diagnosis (T2), through one-on-one psychological and informational support (Figure 2).

CS training program for nurses

The program consisted of two workshops, one at the beginning and the other at the end of the 3-month period. The program was based on a step-by-step approach model proposed by Baile *et al.* [18] and Radziewicz *et al.* [19]. The approach consists of 6 steps, which was referred to by the acronym SPIKES: S, setting up the interview; P, assessing the patient's perception of the illness; I, obtaining an invitation by the patient to disclose information; K, giving knowledge and information to the patient; E, addressing the patient's emotions with empathic responses; and S, strategy and summary.

The program consisted of a large group meeting for 2 h and subsequent small group meetings for approximately 4 h. At the large group meeting, a brief educational course was given about the impact of communication between patients and health professionals, the principles of CS for breaking bad news, a lecture regarding psychological distress in cancer patients, and how to detect and handle it. Also, to unify the support learning effect among the nurses, original educational materials and a checklist were given to each nurse.

DOI. 10.1002/pon



CS*: Communication skills.

Figure 2. Patient recruitment and assessment procedure

At the small group meeting, nurses were divided into groups of two or three, and one or two facilitators were assigned to each group. There were three nurse facilitators in this study who had received a 2-day training on CS. In each group, one participant volunteered to play a nurse and another to play a patient in three given scenarios simulating a nurse's follow-up situation. In preparation of the scenarios, the Japanese medical system and culture were taken into account: the first is a young female diagnosed with early-stage breast cancer; the second is an adult male diagnosed with advanced-stage gastric cancer; and the third is an elderly diagnosed with severe inoperable cancer whose daughter is the main caregiver. The 'nurse' conducted a simulated interview with the 'patient' according to the SPIKES steps, while the other member of the group worked as commentators. They also discussed methods for eliciting and responding to emotions in cancer patients and how to assess and detect their distress during nurse–patient communication.

Patients' sociodemographic data

Data on patients' demographic and clinical characteristics (age, gender, employment status, marital status, family number living together, cancer site, cancer stage) were obtained by reviewing patients' records.

Patients' psychological distress

The patient's psychological distress was self-assessed at each interview using the Hospital Anxiety and Depression Scale (HADS) [20]. The HADS is a 14-item self-rating scale with each item rated on a scale of 0–3. Higher scores indicate a greater tendency to anxiety and depression. The Japanese version of HADS has been shown to have adequate validity and reliability [21].

Nurses, physicians, and interview characteristics

Data were collected on the ages of the nurses and the physicians, their genders, experience in oncology practice (years), and experience of having previous CS training. The date of notification of cancer diagnosis was recorded, and at each interview, the date and the nurse's self-reported evaluation for CS during the interview were collected.

In the experimental group, interviews were all audio-taped and transcribed. The transcripts were then assessed by two trained investigators, in order

DOI. 10.1002/pon

to determine the number of CS used within the 6-step SPIKES. The two investigators had attended the CS training together with nurse participants, and then a brief 2-day educational course on patient's distress, in which they also learned how to rate respective steps of CS during the interview. They rated each interview independently. When the rating was inconsistent between the investigators, they had a discussion to determine the final rating for the interview. In the control group, we asked the nurses about the contents of their interview with the patients.

Nurses' ratings of patient's distress

Nurses rated their patient's distress on a 100-mm-line Visual Analog Scale (VAS) immediately after the interview at each of the three assessment times (T1, T2, T3). A VAS was used because similar scales were used in previous studies to assess health professionals' ability to detect patient's distress [1,22] and because there are no suitable nurse-rated measures, to our knowledge, for the evaluation of patient's distress. Prior to the study, the nurses in both groups were also trained in the role play simulation of how to evaluate the level of distress of the patients by VAS.

Nurse's ability to detect patient's distress

In order to assess the nurse's ability to detect a patient's distress, we devised a new variable based on a previously described method [1]. For this, the patient's self-reported HADS score and nurse's VAS rating of patient's distress were first brought up to a maximal score of 100, respectively. The variable was obtained by subtracting the modified HADS score from the modified VAS rating.

Statistical analyses

Patients' demographic and clinical data and interview characteristics were tested by t-test, chi-squared test, or Mann–Whitney U-test, as appropriate, in order to assess the homogeneity of the patients involved in the study.

Patient's psychological status (patient's self-reported distress, nurse's ratings of patient's distress by VAS), time of nurse's interview, and time of physician's consultation were analyzed using repeated measures analyses of variance (MANOVAs) and chi-squared tests as appropriate. Regarding a nurse's ability to detect patient's distress, group and group-by-time changes in the new variable were also analyzed using MANOVAs. All tests were two-tailed and the alpha was set at 0.05.

Mixed-effects modeling was employed to investigate factors associated with nurse's detection of patient's distress. First, the univariate analyses were conducted to identify potential covariates using Pearson correlations and t-tests as appropriate. Factors were then entered in the multivariate model only if they satisfied P-values less than 0.10 in the univariate analyses. Group ($P = 0.54$) and time ($P = 0.25$), although they were not significant at the univariate level, were retained in the model. A linear mixed-effects model with fixed effects was used.

All data analyses were conducted using the SAS statistical software, version 9.1.

Results

Sample of institution, nurses, and physicians

A cancer institution, Tokyo Metropolitan Cancer Screening Center, consented to participate in the study. All of the nine follow-up nurses, who were always involved in the support system for patients as charge nurses, consented to participate in the study and completed the program. In this center, 18 other nurses provide technical support for potential cancer patients undergoing screening tests.

Since one nurse had a maternity leave just after attending the CS training program, eight nurses were randomly assigned to either the experimental or the control group (four nurses each) at the beginning of the study. For these nurses, the mean age $\pm$ SD was 40.8 ± 7.2, mean clinical experience (years in oncology practice) was 17.2 ± 6.8, and 7 of them were females (87.5%). None of these variables were significantly different between the experimental and control groups. Twelve physicians provided consultations for the patients who participated in the study. For them, the mean age was 42.6 ± 9.4, mean clinical experience was 16.7 ± 4.8, and 4 of them were females (33.3%). No nurses or physicians had attended a CS training workshop before this study.

Patient sample

Of the consecutive 175 eligible patients, 4 (2%) were excluded because of severe psychological distress and 82 (47%) refused to participate (57 felt the burden of participating in the study and 25 felt social barriers). Therefore, a total of 89 patients (51%) consented to participate. Of these, 45 patients were assigned to the nurses in the experimental group and 44 to those in the control groups. During the study, 11 patients (4 in experimental group and 7 in control group) dropped out after the initial assessment, and the results of 41 patients who were supported by the nurses in the experimental group and 45 patients supported by those in the control group were analyzed (Figure 2).

DOI. 10.1002/pon

Table 1. Comparison of patient variables between groups

	Experimental group $n=41$ (100%)	Control group $n=45$ (100%)	*P*-value
(a) *Patient demographic characteristics*			
Age (years)	61.4±10.8	60.9±14.3	NS
Gender			
Male	16 (39.0)	18 (40.0)	NS
Female	25 (61.0)	27 (60.0)	
Employment status			NS
Working part or full time	25 (60.9)	25 (55.6)	
Unemployed	16 (39.1)	20 (44.4)	
Marital status			NS
Married	33 (80.5)	34 (75.6)	
Single/divorced/separated/widowed	8 (19.5)	11 (24.4)	
(b) *Patient clinical characteristics*			
Cancer site			NS
Gastric	21 (51)	19 (42)	
Colorectal	10 (24)	14 (31)	
Breast	10 (24)	12 (27)	
Cancer stage			NS
I	22 (54)	24 (53)	
II	14 (34)	18 (40)	
III	5 (12)	3 (7)	
(c) *Interview characteristics*			
Time of physician's consultation of informing cancer diagnosis (min)	16.1±11.6	16.0±10.0	NS
Time of nurse's interview after physician's consultation (min)			
T0 (on the day of diagnosis)	21.9±7.6	18.3±8.0	0.04
T1 (1 week after diagnosis)	17.9±9.9	17.2±12.1	NS
T2 (1 month after diagnosis)	9.1±1.2	10.0±1.6	NS
Number of CS used during the nurse's interview (range: 0–6)[a]	4.6±1.5	—	—
Nurse's self-reported evaluation for CS during the interview (VAS, range: 0–100)	67.9±13.6	60.3±13.7	0.01
Nurse's ratings of patient's distress (VAS)			
T0 (on the day of diagnosis)	41.7±23.9	39.9±17.8	NS[b]
T1 (1 week after diagnosis)	38.4±24.0	40.0±19.9	
T2 (1 month after diagnosis)	36.7±26.5	39.8±19.0	
Patient's self-reported distress (HADS total score)			
T0 (on the day of diagnosis)	11.3±5.5	10.6±6.6	0.03[c]
T1 (1 week after diagnosis)	9.6±5.6	11.3±6.5	
T2 (1 month after diagnosis)	6.9±5.0	9.5±5.9	
Nurse's ability to detect patient's distress[d]			
T0 (on the day of diagnosis)	14.9±19.7	14.8±22.3	NS[d]
T1 (1 week after diagnosis)	15.7±20.7	13.0±20.9	
T2 (1 month after diagnosis)	19.8±21.4	17.5±20.0	

Data are shown by mean±SD or no. (%). SD, standard deviation; NS, not significant; CS, communication skills; VAS, Visual Analog Scale.
[a]Evaluation of communication skills of the nurse through the interview transcription by investigators.
[b]Repeated measures analysis of variance (MANOVA) was done.
[c]A significant change in group-by-time was shown.
[d]This value was computed as the difference between physicians' ratings of patients'distress (VAS) and patients'self-reported distress (HADS).

Between the patients who consented to participate and those who refused to participate or dropped out, statistically significant differences were not found with any of the demographic variables.

Interview characteristics

Time of nurse's interview after physician's consultation at T0 and nurse's self-reported evaluation for CS during the interview were statistically different between the experimental and control groups (Table 1). No other interview characteristics were significantly different between the groups.

In the experimental group, all four trained nurses were able to conduct the interviews along the 6-step SPIKES fairly well. The number of CS used in the interview was 4.6±1.5 (mean±SD). All 6 steps were conducted in 22 interviews out of 41 (54%), and in the remaining, those 6 steps were performed in 71–95% of the interviews, respectively (Table 2). In most interviews, the nurses successfully assessed and elicited patient's distress and responded to their emotions fairly well. Examples of nurse–patient communication are given in Table 2.

In the control group, the nurses conducted interviews mostly by reacting passively to patient's expressions. That is, the nurses mentioned the

DOI. 10.1002/pon

Table 2. Percentages and examples of each step performance in interviews within 6-step SPIKES

Step of SPIKES	Number (%)
(1) *S: Setting up the interview*	38 (93)
Ex. Before the interview, nurses carefully checked the patient status for presence of mind, possible disturbance, etc.	
(2) *P: Assessing the patient's perception of the illness*	31 (76)
Ex. Nurse: 'Did you understand what the doctor said? As your nurse, I'd be happy to explain again anything you want to confirm. Feel free to ask me anything.'	
Patient: 'I could understand my cancer diagnosis, but I am not sure what the doctor said after that. Why me with cancer? I just can't tell my wife. Now, what do I do?'	
(3) *I: Obtaining an invitation by the patient to disclose information*	29 (71)
Ex. Patient: 'I don't want to think about anything right now. I am all mixed up. (Brief silence)'	
Nurse: 'What the doctor told you is a lot to handle. Give yourself a little time. Are you OK? Let's take it one step at a time. After you have calmed down, I'd like to explain to you what the doctor said again. (Sit at the patient's side and put your hand on the patient's shoulder for a while.)'	
(4) *K: Giving knowledge and information to the patient*	39 (95)
Ex. Nurse: '(After the nurse is sure the patient has calmed down.) Let me go over again what the doctor said. The bad news is that you have breast cancer. (Carefully observe patient for a while.) The good news, however, is that we have several treatments to cure your cancer. I'm going to explain them to you while jotting them down.'	
(5) *E: Addressing the patient's emotions with empathic responses*	32 (78)
Ex. Nurse: 'Are you all right? We can introduce you to some good hospitals and doctors. And till you are hospitalized, you can call me anytime and ask me anything because I am still your nurse. Here's my name and number. I am always here for you. I will always support you, I promise.'	
Patient: 'Thank you. Your support is very helpful and meaningful to me.'	
(6) *S: Strategy and summary*	39 (95)
Ex. Nurse: 'Let me say this again. We're going to get through this together. Here is what I noted down today. Check it out. You can always ask me anything. I am your nurse.'	

cancer diagnosis only when patients wished to confirm it, and provided emotional support only when patients requested it. When patients expressed strong emotions, the nurses tended to avoid reacting to them, because they did not know how to deal with them. All four nurses in this group communicated with patients in their own way but had no confidence because they never had a chance to learn the strategy.

Effect of CS training program on intercorrelations between patient's self-reported distress, nurse's ratings of patient's distress, and number of CS used by nurses

For the patients who were supported by the nurses in the experimental group, the nurse's ratings of patient's distress correlated significantly with patient's self-reported distress at each of the three assessment times (T1, $P \leqslant 0.001$; T2, $P \leqslant 0.01$; T3, $P \leqslant 0.001$) (Table 3). For those patients, the nurse's ratings of patient's distress also significantly correlated with the number of CS used in the interview at two assessment times (T1 and T2; T1, $P \leqslant 0.01$; T2, $P \leqslant 0.01$). Also, patient's self-reported distress was significantly correlated with the number of CS used in the interview at T2 ($P \leqslant 0.001$). Meanwhile, for the patients supported by the nurses in the control group, none of these variables were significantly correlated.

Table 3. Intercorrelations between patient's self-reported distress (HADS), nurse's ratings of patient's distress (VAS), and number of CS used by nurses

Characteristic	Experimental group ($n = 41$)			Control group ($n = 45$)		
	HADS	VAS	No. of CS	HADS	VAS	No. of CS
T1: 1 week after cancer diagnosis (T1)						
HADS	1.00	0.57***	0.21	1.00	0.12	—
VAS	N	1.00	0.44**	N	1.00	—
No. of CS[a]	N	N	1.00	N	N	—
T2: 1 month after cancer diagnosis (T2)						
HADS	1.00	0.46**	0.59***	1.00	0.05	—
VAS	N	1.00	0.45**	N	1.00	—
No. of CS	N	N	1.00	N	N	—
T3: 3 months after cancer diagnosis (T3)						
HADS	1.00	0.62***	—	1.00	0.20	—
VAS	N	1.00	—	N	1.00	—
No. of CS[b]	—	—	—	—	—	—

*$P<0.05$; **$P<0.01$; ***$P<0.001$. CS, communication skills.
[a]Number of CS used within 6-step SPIKES through nurse's interview, which is evaluated through the interview transcription by investigators (range: 0–6).
[b]No data available because nurse's interview was not conducted at T3.

Effects of CS training on nurse's ability to detect patient's distress

The MANOVA revealed that the change of patient's self-reported distress was significant in group-by-time ($F = 3.51$, $P = 0.03$) (Table 1). Meanwhile, neither the change of nurse's ratings of patient's distress nor that of the nurse's ability to detect patient's distress changed significantly in time or between groups (Table 1).

Factors associated with nurse's ability to detect patient's distress

Patient's age ($P = 0.004$), time of nurse's interview after physician's consultation ($P = 0.07$), nurse's self-reported evaluation for CS during the interview ($P < 0.001$), nurse's ratings of patient's distress ($P = 0.07$), and patient's self-reported distress ($P < 0.001$) were identified as possible predictors and were retained in the multivariate model. Other demographic and clinical characteristics of patients, nurses' and physicians' characteristics, and

DOI. 10.1002/pon

Table 4. Mixed-effects model for nurse's ability to detect patient's distress using MANOVA (fixed effects)

Variable	Estimates	Standard error	t-Value	P
Nurse's ability to detect patient's distress (intercept)[a]	117.41	11.69	10.04	<0.001
Group (Experimental/Control)	6.84	4.25	1.61	0.11
Time (T1/T3)	2.49	2.25	1.11	0.27
Time (T2/T3)	0.95	2.26	0.42	0.68
Group × Time (Experimental/Control × T1/T3)	−3.44	2.99	−1.15	0.25
Group × Time (Experimental/Control × T2/T3)	−0.82	3.14	−0.26	0.79
Patient's age	−0.37	0.13	2.72	<0.001
Time of nurse's interview after physician's consultation	0.61	0.20	−3.09	0.003
Nurse's self-reported evaluation for CS during interview (VAS)	0.60	0.13	−4.73	<0.001
Nurse's ratings of patient's distress (VAS)	2.36	0.73	−3.25	0.002
Patient's self-reported distress (HADS)	1.70	0.18	9.27	<0.001

T1, 1 week after diagnosis; T2, 1 month after diagnosis; T3, 3 months after diagnosis.
[a]Computed from difference between nurse's ratings of patient's distress (VAS) and patient's self-reported distress (HADS).

interview characteristics did not satisfy the inclusion criteria (i.e. $P<0.10$).

The mixed-effects model showed that the nurse's ability to detect patient's distress was negatively associated with patient's age ($P<0.001$) and positively associated with time of nurse's interview ($P=0.003$), nurse's self-reported evaluation for CS during the interview ($P<0.001$), nurse's ratings of patient's distress ($P=0.002$), and patient's self-reported distress ($P<0.001$) (Table 4). No significant difference was found with the nurse's ability to detect patient's distress between the experimental and control groups ($P=0.11$).

Discussion

The most important finding of this study was that the nurse's ratings of patient's distress were significantly correlated with patient's self-reported distress in the experimental group in all three assessment times, whereas no such correlation was found in the control group. Although previous studies indicated that health professionals often failed to detect their patients' distress accurately [5,23], the nurses who had attended a CS training program properly recognized patients' distress. As shown in the results of nurse–patient interviews (Table 2), the nurses were considered to have learned how to detect and handle patient's distress through a CS training program. Previous studies showed that CS training programs need to be longer and more intensive for health professionals to acquire and improve their skills [10,15,24]. Thus, we conducted a 3-month CS training program in which nurses were trained 2 times at the start and end of this period. During the 3-month period, the nurses had chances to practice the CS, and found their own questions and problems with the technique. These points were discussed and solved at the second training. We assume that having this 'practice period' was very helpful for the nurses in the experimental group, which could lead to the positive findings in this study.

Another important finding of this study was that the nurses in the experimental group used significantly more CS from 6-step SPIKES during interviews when they perceived their patients as more distressed. This result showed that those nurses use their CS effectively according to the level of distress they perceive in their patients. We chose and used the model of CS training developed by Baile *et al.* [18] because it is highly structured, systematic, and brief, and its effectiveness for Japanese physicians was confirmed [25]. Studies also reported that a short-term and basic training program failed to give health professionals the needed CS [10,22]. Based on these findings, in the present study, an intensive program was held (2 times during the 3 months), which successfully transmitted the CS to the nurses.

The nurse's ability to detect patient's distress did not change significantly during the 3-month study period. Although we hypothesized that an improvement in nurses' CS and increase in knowledge of distress in cancer patients would lead to an improvement in nurses' ability to detect patient's distress, that hypothesis was not supported. This was consistent with the results of a study on physicians [1]. One possible reason was that the CS training program in this study might have failed to provide the nurses enough knowledge and skills to detect patient's distress. Another reason may be the cultural attitude of Japanese patients. Some studies have shown that Japanese patients do not seek professional assistance for psychosocial problems created by cancer experience [26] and that Japanese cancer patients usually repress their emotions about having cancer [27]. As Japanese people are accustomed to these attitudes and their attitude may be rooted more in habits, it may be difficult for nurses to improve their ability to detect patient's distress and it may require a specific training module. The study design might be another possible reason for the negative finding. We

DOI. 10.1002/pon

assessed patient's distress using HADS 3 times: 1 week, 1 month, and 3 months after cancer diagnosis. Patients diagnosed with cancer at a cancer screening center usually are hospitalized for treatment as soon as possible. Thus, it is likely that most patients who participated in the present study were hospitalized during the 3-month study period [8]. Therefore, patient's distress at later assessment times might have been influenced by their experience of hospitalization, treatments, and so on. Such experiences of the patients could have some influence on the nurses' assessment and detection of patients' distress.

The mixed-effects modeling also showed that the patient's age was significantly correlated with a nurse's ability to detect patient's distress. A previous study revealed that the distress in older patients is more difficult to detect, because elderly patients tend to show less overt symptoms of distress and are often more reluctant to talk explicitly about problems with emotional functioning [28]. This finding would suggest that health professionals must be careful to provide support for the elderly after having been informed of a cancer diagnosis.

The generalizability of our findings is uncertain. First, the subjects of our study were limited to a small number of nurses and patients in one cancer screening institution. If the study had been performed on a larger number of patients in various institutions, it would have been possible to observe the improvements in health professionals' ability to detect distress. Second, we used a new variable to assess the nurse's ability to detect patient's distress, which was made by subtracting the HADS scores from the VAS. Although we assume that the variable represents how precisely the nurse recognized the distress of the patients, its reliability has not been established. Future studies are needed to develop a better scale to evaluate nurses' ability in this regard. Third, since we performed transcript analysis of the interviews only in the experimental group, it was not possible to compare the actual CS between the groups. Moreover, although the results from the two investigators were highly consistent, an inter-rater reliability on the transcript analysis was not confirmed. Further studies are needed to address such limitations.

To our knowledge, this is the first study to use a randomized design to assess the impact of a CS training program on nurse's detection of distress in patients who have just been given a cancer diagnosis. Although no significant change was observed in this study in the nurse's ability to detect patient's distress, such programs provide nurses with the capacity to communicate with patients efficiently. Our findings are encouraging for health professionals in oncology practice in that the risk of unrecognized distress in cancer patients can be reduced by training in CS.

Acknowledgements

This work was supported by a Grant-in-Aid for Exploratory Research from the Japan Society for the Promotion of Science and a Grant from the Pfizer Health Research Foundation. We thank the Tokyo Metropolitan Cancer Screening Center and its health professionals for their generous cooperation in this study. We are also grateful to Ms Chiaki Kusano, Ms Masayo Tokuhiro, Ms Kazuko Tanaka, and Ms Asako Tamura for their research assistance.

References

1. Merckaert I, Libert Y, Delvaux N *et al.* Factors that influence physicians' detection of distress in patients with cancer: can a communication skills training program improve physicians' detection? *Cancer* 2005; **104**:411–421.
2. Northouse LL, Mood DW, Montie JE *et al.* Living with prostate cancer: patients' and spouses' psychosocial status and quality of life. *J Clin Oncol* 2007;**25**: 4171–4177.
3. Neron S, Correa JA, Dajczman E *et al.* Screening for depressive symptoms in patients with unresectable lung cancer. *Support Care Cancer* 2007;**15**:1207–1212.
4. Valdimarsdottir U, Helgason AR, Fürst CJ *et al.* The unrecognised cost of cancer patients' unrelieved symptoms: a nationwide follow-up of their surviving partners. *Br J Cancer* 2002;**86**:1540–1545.
5. Fallowfield L, Ratcliffe D, Jenkins V, Saul J. Psychiatric morbidity and its recognition by doctors in patients with cancer. *Br J Cancer* 2001;**84**:1011–1015.
6. Detmar SB, Aaronson NK, Wever LD, Muller M, Schornagel JH. How are you feeling? Who wants to know? Patients' and oncologists' preferences for discussing health-related quality-of-life issues. *J Clin Oncol* 2000;**18**:3295–3301.
7. Gustafsson O, Theorell T, Norming U *et al.* Psychological reactions in men screened for prostate cancer. *Br J Urol* 1995;**75**:631–636.
8. Fukui S, Ozawa H. Relation of psychological distress after diagnosis of gastric cancer at a cancer screening center with psychological support from public health nurses and family members. *Nippon Koshu Eisei Zasshi* 2003;**50**:583–593 (in Japanese).
9. Boehmke MM, Dickerson SS. The diagnosis of breast cancer: transition from health to illness. *Oncol Nurs Forum* 2006;**33**:1121–1127.
10. Razavi D, Merckaert I, Marchal S *et al.* How to optimize physicians' communication skills in cancer care: results of a randomized study assessing the usefulness of posttraining consolidation workshops. *J Clin Oncol* 2003;**21**:3141–3149.
11. Roter DL, Hall JA, Kern DE, Barker LR, Cole KA, Roca RP. Improving physicians' interviewing skills and reducing patients' emotional distress. A randomized clinical trial. *Arch Intern Med* 1995;**155**:1877–1884.
12. Passik SD, Donaghy KB, Theobald DE, Lundberg JC, Holtsclaw E, Dugan WM. Oncology staff recognition of depressive symptoms on videotaped interviews of depressed cancer patients: implications for designing a training program. *J Pain Symptom Manage* 2000; **19**:329–338.
13. Grassi L, Travado L, Gil F, Campos R, Lluch P, Baile W. A communication intervention for training southern European oncologists to recognize psychosocial mor-

bidity in cancer. I—development of the model and preliminary results on physicians' satisfaction. *J Cancer Educ* 2005;**20**(2):79–84.
14. Sheldon LK. Communication in oncology care: the effectiveness of skills training workshops for healthcare providers. *Clin J Oncol Nurs* 2005;**9**:305–312.
15. Merckaert I, Libert Y, Razavi D. Communication skills training in cancer care: where are we and where are we going? *Curr Opin Oncol* 2005;**17**:319–330.
16. Fallowfield L, Jenkins V. Current concepts of communication skills training in oncology. *Recent Results Cancer Res* 2006;**168**:105–112.
17. Moynihan C, Bliss JM, Davidson J, Burchell L, Horwich A. Evaluation of adjuvant psychological therapy in patients with testicular cancer: randomised controlled trial. *Br Med J* 1998;**316**:429–435.
18. Baile WF, Buckman R, Renzi R *et al.* SPIKES—a six-step protocol for delivering bad news: application to the patient with cancer. *Oncologist* 2000;**5**:302–311.
19. Radziewicz R, Baile WF. Communication skills: breaking bad news in the clinical setting. *Oncol Nurs Forum* 2001;**28**(6):951–953.
20. Zigmond AS, Snaith RP. The hospital anxiety and depression scale. *Acta Psychiatr Scand* 1983;**67**:361–370.
21. Kugaya A, Akechi T, Okuyama T *et al.* Screening for psychological distress in Japanese cancer patients. *Jpn J Clin Oncol* 1998;**28**:333–338.
22. Delvaux N, Razavi D, Marchal S *et al.* Effects of a 105 hours psychological training on attitudes, communication skills and occupational stress in oncology: a randomized study. *Br J Cancer* 1994;**90**:106–114.
23. Passik SD, Dugan W, McDonald MV, Rosenfeld B, Theobald DE, Edgerton S. Oncologists' recognition of depression in their patients with cancer. *J Clin Oncol* 1998;**16**:1594–1600.
24. Fallowfield L, Jenkins V, Farewell V, Saul J, Duffy A, Eves R. Efficacy of a cancer research UK communication skills training model for oncologists: a randomised controlled trial. *Lancet* 2002;**359**:650–656.
25. Fujimori M, Oba A, Koike M *et al.* Communication skills training for Japanese oncologists on how to break bad news. *J Cancer Educ* 2006;**18**:194–201.
26. Singer MK, Wellish DK, Durvasula R. Impact of breast cancer on Asian American and Anglo American women. *Cult Med Psychiatry* 1997;**21**: 449–480.
27. Uchitomi Y, Sugihara J, Fukue M *et al.* Psychiatric liaison issues in cancer care in Japan. *J Pain Symptom Manage* 1994;**9**:319–324.
28. Watts SC, Bhutani GE, Stout IH *et al.* Mental health in older adult recipients of primary care services: is depression the key issue? Identification, treatment and the general practitioner. *Int J Geriatr Psychiatry* 2002;**17**:427–437.

DOI. 10.1002/pon

New Sequence Variants in HLA Class II/III Region Associated with Susceptibility to Knee Osteoarthritis Identified by Genome-Wide Association Study

Masahiro Nakajima[1], Atsushi Takahashi[2], Ikuyo Kou[1], Cristina Rodriguez-Fontenla[3], Juan J. Gomez-Reino[3,4], Tatsuya Furuichi[1], Jin Dai[1,5], Akihiro Sudo[6], Atsumasa Uchida[6], Naoshi Fukui[7], Michiaki Kubo[8], Naoyuki Kamatani[2], Tatsuhiko Tsunoda[9], Konstantinos N. Malizos[10,11], Aspasia Tsezou[12], Antonio Gonzalez[3], Yusuke Nakamura[13,14], Shiro Ikegawa[1]*

1 Laboratory for Bone and Joint Diseases, Center for Genomic Medicine, RIKEN, Tokyo, Japan, **2** Laboratory for Statistical Analysis, Center for Genomic Medicine, RIKEN, Tokyo, Japan, **3** Laboratorio Investigacion 10 and Rheumatology Unit, Hospital Clinico Universitario de Santiago, Santiago de Compostela, Spain, **4** Department of Medicine, University of Santiago de Compostela, Santiago de Compostela, Spain, **5** The Center of Diagnosis and Treatment for Joint Disease, Drum Tower Hospital Affiliated to Medical School of Nanjing University, Nanjing, China, **6** Department of Orthopaedic Surgery, Mie University Faculty of Medicine, Mie, Japan, **7** Department of Pathomechanisms, Clinical Research Center for Rheumatology and Allergy, National Hospital Organization Sagamihara National Hospital, Kanagawa, Japan, **8** Laboratory for Genotyping Development, Center for Genomic Medicine, RIKEN, Kanagawa, Japan, **9** Laboratory for Medical Informatics, Center for Genomic Medicine, RIKEN, Kanagawa, Japan, **10** Department of Orthopaedics University of Thessaly, Larissa, Greece, **11** Institute for Biomedical Research and Technology, Larissa, Greece, **12** Department of Biology, University of Thessaly Medical School, Larissa, Greece, **13** Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan, **14** Center for Genomic Medicine, RIKEN, Kanagawa, Japan

Abstract

Osteoarthritis (OA) is a common disease that has a definite genetic component. Only a few OA susceptibility genes that have definite functional evidence and replication of association have been reported, however. Through a genome-wide association study and a replication using a total of ~4,800 Japanese subjects, we identified two single nucleotide polymorphisms (SNPs) (rs7775228 and rs10947262) associated with susceptibility to knee OA. The two SNPs were in a region containing HLA class II/III genes and their association reached genome-wide significance (combined $P=2.43\times10^{-8}$ for rs7775228 and 6.73×10^{-8} for rs10947262). Our results suggest that immunologic mechanism is implicated in the etiology of OA.

Citation: Nakajima M, Takahashi A, Kou I, Rodriguez-Fontenla C, Gomez-Reino JJ, et al. (2010) New Sequence Variants in HLA Class II/III Region Associated with Susceptibility to Knee Osteoarthritis Identified by Genome-Wide Association Study. PLoS ONE 5(3): e9723. doi:10.1371/journal.pone.0009723

Editor: Sudha Agarwal, Ohio State University, United States of America

Received December 28, 2009; **Accepted** February 26, 2010; **Published** March 18, 2010

Funding: The Japanese study is supported by the Leading Project for Personalized Medicine in the Ministry of Education, Culture, Sports, Science and Technology. The European study is supported by Instituto de Salud Carlos III from the Spanish Health Ministry with participation of FEDER (Fondo Europeo para el Desarrollo Regional), the Foundation Mutua Madrile (Madrid). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: sikegawa@ims.u-tokyo.ac.jp

Introduction

We are living in the "Bone and Joint Decade" (http://www.boneandjointdecade.org/). As the WHO initiative shows, bone and joint diseases are serious problems all over the world, putting us under severe medical, economical and social burden. Osteoarthritis (OA; MIM 165720) is one of the most common diseases among them. OA affects synovial joints of all over the body, mainly knee, hip, hand and spine. OA is characterized by progressive loss of articular cartilage and, often, proliferation of synovium and bone, which lead to pain, loss of joint function and disability. More than tens of millions patients in the world are suffering from this non-lethal, but intractable disease, and the number is relentlessly increasing; however, its etiological picture remains unclear and we have no fundamental treatment for it.

OA is a polygenic disease. Both environmental and genetic factors contribute to its etiology and pathogenesis [1]. To understand its genetic factor, identification of its susceptibility gene(s) must be the first step. Many OA susceptibility genes identified by candidate-gene association studies have been reported, but only a few have supporting functional evidence and replication of the results in different populations [1,2]. Large-scale association studies including the genome-wide association study (GWAS) using high-density single nucleotide polymorphisms (SNPs) have been reported by a few groups in Asia and Europe [3–6], but only a gene fulfilled genome-wide significance level [2]. The genetic basis of OA susceptibility remains largely uncharacterized. To identify OA susceptibility gene(s), we conducted a GWAS for knee OA and identified two SNPs with genome-wide significance level.

Methods

Samples

Characteristics of each cohort group are shown in Table 1. Case samples of GWAS for the Japanese population were obtained from

Table 1. Basal characteristics of the subjects.

Cohort	Source	Platform	Number of samples	Nationality	Female (%)	Age (mean +/− sd)	BMI (mean +/− sd)	Severity[a] (% severe OA)
GWAS								
knee OA	RIKEN	Illumina HumanHap550	899	Japanese	759 (84.4)	71.6+/−7.6	24.9+/−3.6	76.5
control	ORC+BioBank Japan	Illumina HumanHap550	3,396	Japanese	1,491 (43.9)	52.5+/−15.2	22.5+/−3.7	–
Replication								
Japanese								
knee OA	RIKEN	Invader assay	167	Japanese	124 (74.3)	73.8+/−6.1	24.5+/−3.3	48.5
control	RIKEN	Invader assay	347	Japanese	223 (64.3)	65.9+/−8.7	22.3+/−2.7	–
European Caucasian								
knee OA	Santiago de Compostela	SNaPshot	243	Spanish	197 (81.1)	68.0+/−5.7	32.8+/−4.8	ND[b]
control	Santiago de Compostela	SNaPshot	426	Spanish	165 (38.7)	68.4+/−9.1	28.3+/−3.8	–
knee OA	University of Thessaly	SNaPshot	570	Greek	468 (82.1)	65.8+/−8.7	29.1+/−3.3	77.1
control	University of Thessaly	SNaPshot	645	Greek	417 (64.6)	59.5+/−11.6	25.4+/−3.7	–

OA: osteoarthritis, ORC: Osaka-Midosuji Rotary Club.
[a]Kellegren-Laurence grade ≥3 was considered as severe OA.
[b]All cases underwent TKR (total knee replacement) surgery.
doi:10.1371/journal.pone.0009723.t001

several medical institutes in Japan, as previously described [5,7]. Knee OA was diagnosed on the basis of clinical and radiographic findings using previously described criteria [5,7]. Rheumatoid arthritis (RA) and polyarthritis associated with autoimmune diseases were excluded, as were secondary OA due to crystal deposition (gout and pseudogout), posttraumatic OA and infection-induced OA. Patients who had clinical and radiographic findings suggestive of skeletal dysplasias, including overt short stature, multiple symmetric involvements of epiphyses and a definitely positive Mendelian family history were also excluded from the study. The control groups consisted of 3,396 individuals that were registered in the Leading Project for Personalized Medicine in the Ministry of Education, Culture, Sports, Science and Technology, Japan as the subjects with diseases unrelated to OA and the volunteers in the Osaka-Midosuji Rotary Club, Osaka, Japan [8]. For replication study, we recruited population-based cohorts from inhabitants of Odai and Minami-ise town (previously Miyagawa village and Nansei town, respectively in the Mie prefecture in Japan) [9]. The Spanish and Greek knee OA and control populations were recruited as described previously from the Hospital Clinico de Santiago, the Departments of Biology and Genetics and of Orthopaedics, University of Thessaly and the Institute of Musculoskeletal Sciences [10]. All the participants provided written informed consent. This research project was approved by the ethical committees at Center for Genomic Medicine (formerly, SNP Research Center), RIKEN and the participating institutions.

SNP genotyping

For the GWAS, we genotyped 906 patients with OA and 3,396 controls using Illumina HumanHap550v3 Genotyping BeadChip. After excluding seven cases with call rate of <0.98, we applied SNP QC (call rate of ≥0.99 in both cases and controls and P value of Hardy-Weinberg equilibrium test of $\geq 1.0\times10^{-6}$ in controls). Finally, 459,393 SNPs on autosomal chromosomes passed the QC filters and were further analyzed. Among the SNPs analyzed in the GWAS, we selected top 15 SNPs showing the smallest P values ($P<1\times10^{-5}$) for the replication study using an independent 514 Japanese subjects from a resident cohort. SNPs with minor allele frequency of ≤0.1 in both case and control samples were excluded from the further analysis. In the replication analysis, we genotyped SNPs using the multiplex PCR-based invader assay (Third Wave Technologies) or by direct sequencing of PCR products using ABI 3700 DNA analyzers (Applied Biosystems), or by SNaPshot Multiplex System (Applied Biosystems) according to manufacturers' protocols.

Statistical analysis

In the GWAS and replication analyses, we applied Fisher's exact test to two-by-two contingency table in three genetic models: an allele frequency model, a dominant-effect model, and a recessive-effect model. We conducted the meta-analysis using the Mantel-Haenszel method. We examined heterogeneity among studies by using the Breslow-Day test. Significance levels after the Bonferroni correction for multiple testing were $P=1.09\times10^{-7}$ (0.05/459,393). Age, gender- and BMI-adjusted odds ratios were obtained by logistic regression analysis [11]. Odds ratios and confidence intervals were calculated using the risk allele as a reference. We analyzed the haplotype association using Haploview software [12]. We conducted a principal component analysis to detect population stratification [13].

Software

For general statistical analysis, we used R statistical environment version 2.6.1 or Microsoft Excel. Drawing the LD map, estimation of haplotype frequencies and analysis of haplotype association were performed by Haploview software.

Results

To identify genetic variants that determine OA susceptibility, we conducted a GWAS in Japanese knee OA. We examined 906 individuals with knee OA and 3,396 control individuals (Table 1) using Illumina HumanHap550v3 Genotyping BeadChip. After confirming the data quality, we compared the results of 459,393 SNPs between cases and controls by Fisher's exact test for three genetic models: allelic, dominant or recessive (Figure 1). Fifteen

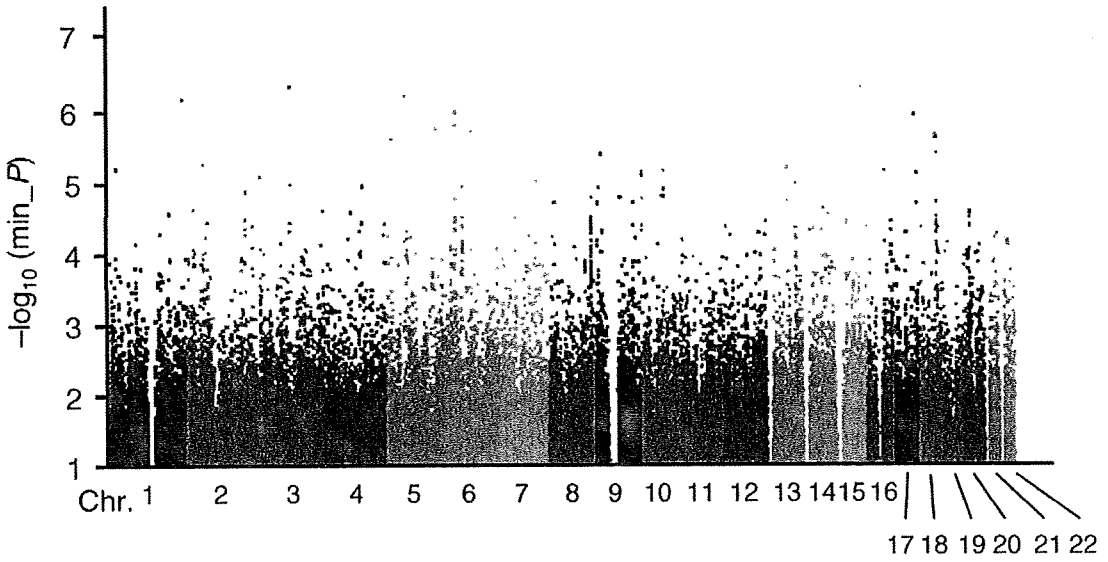


Figure 1. Results of a genome-wide association study (–$\log_{10}$ *P* value plot). Each *P* value is the minimum of Fisher's exact tests for three models: dominant, recessive and allele frequency model.
doi:10.1371/journal.pone.0009723.g001

SNPs selected for the replication study that had the smallest *P* values (minimum $P<1\times10^{-5}$) were next genotyped in an independent set of 167 Japanese knee OA individuals and 347 Japanese controls from a resident cohort study. Through these studies, only two SNPs, rs7775228 (combined $P=2.43\times10^{-8}$; OR = 1.34; 95% CI = 1.21–1.49) and rs10947262 (combined

Table 2. Association of rs7775228 and rs10947262 with knee osteoarthritis.

SNP (Nearest gene)	Allele	Population	Minor allele Frequency				
			Case	Control	OR (95% CI)[a]	*P*[b]	P_{het}[c]
rs7775228	C/T	Japanese					
(*HLA-DQB1*)		GWAS	0.318	0.379	1.31 (1.18–1.47)	1.38 E-06	
		Replication	0.290	0.385	1.53 (1.15–2.02)	3.07 E-03	
		Combined[d]			1.34 (1.21–1.49)	2.43 E-08	0.33
		European Caucasian					
		Spanish	0.194	0.209	1.10 (0.83–1.45)	0.521	
		Greek	0.094	0.075	0.78 (0.58–1.03)	0.084	
		European combined[e]			0.93 (0.76–1.13)	0.178	0.09
		All combined[f]			–	–	0.003
rs10947262	C/T	Japanese					
(*BTNL2*)		GWAS	0.358	0.419	1.30 (1.16–1.44)	2.45 E-06	
		Replication	0.332	0.422	1.47 (1.12–1.93)	5.74 E-03	
		Combined[d]			1.32 (1.19–1.46)	6.73 E-08	0.40
		European Caucasian					
		Spanish	0.122	0.136	1.13 (0.81–1.59)	0.465	
		Greek	0.068	0.094	1.43 (1.06–1.92)	0.019	
		European combined[e]			1.29 (1.03–1.61)	0.025	0.32
		All combined[f]			1.31 (1.20–1.44)	5.10 E-09	0.63

Odds ratios (ORs) and *P* values for the independence test were calculated by the Mantel-Haenszel method.
[a]OR of the risk allele from the two-by-two allele frequency table.
[b]P values of the Pearson's χ^2 test for the allele model.
[c]Results of the Breslow-Day test.
[d]Meta-analysis of Japanese studies.
[e]Meta-analysis of European studies.
[f]Meta-analysis of all studies.
doi:10.1371/journal.pone.0009723.t002