

Fig. 4 Associations of p16 immunoreactivity and aberrant methylation status in tumor samples. Data are means \pm 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 heterogeneous 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.

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Conflict of Interest Statement None

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

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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.

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

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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

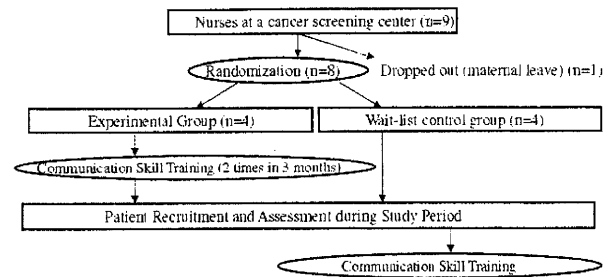


Figure 1. Nurses randomization procedure

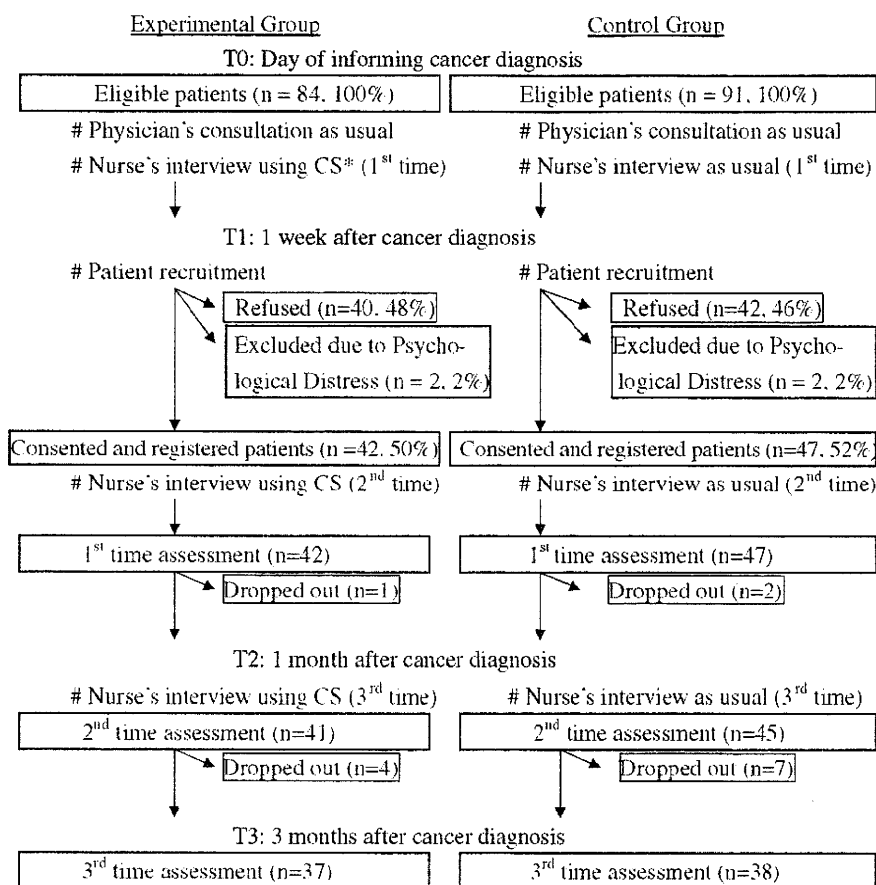
in the experimental group attended a 1-day CS training program 2 times during a 3-month period (October and December 2005). Nurses assigned to the wait-listed control group were invited to participate in the CS training program after the study period.

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.



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

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).

Table 1. Comparison of patient variables between groups

	Experimental group n = 41 (100%)	Control group n = 45 (100%)	P-value
<i>(a) Patient demographic characteristics</i>			
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)	
<i>(b) Patient clinical characteristics</i>			
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)	
<i>(c) Interview characteristics</i>			
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.

^aEvaluation of communication skills of the nurse through the interview transcription by investigators.

^bRepeated measures analysis of variance (MANOVA) was done.

^cA significant change in group-by-time was shown.

^dThis 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

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 Ex. Before the interview, nurses carefully checked the patient status for presence of mind, possible disturbance, etc.	38 (93)
(2) P: Assessing the patient's perception of the illness 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?'	31 (76)
(3) I: Obtaining an invitation by the patient to disclose information 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.)'	29 (71)
(4) K: Giving knowledge and information to the patient 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.'	39 (95)
(5) E: Addressing the patient's emotions with empathic responses 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.'	32 (78)
(6) S: Strategy and summary 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.'	39 (95)

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

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
<i>T1: 1 week after cancer diagnosis (T1)</i>						
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	—
<i>T2: 1 month after cancer diagnosis (T2)</i>						
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	—
<i>T3: 3 months after cancer diagnosis (T3)</i>						
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.

^aNumber of CS used within 6-step SPIKES through nurse's interview, which is evaluated through the interview transcription by investigators (range: 0–6).

^bNo data available because nurse's interview was not conducted at T3.

assessment times (T1, $P \leq 0.001$; T2, $P \leq 0.01$; T3, $P \leq 0.001$) (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, $P \leq 0.01$; T2, $P \leq 0.01$). Also, patient's self-reported distress was significantly correlated with the number of CS used in the interview at T2 ($P \leq 0.001$). Meanwhile, for the patients supported by the nurses in the control group, none of these variables were significantly correlated.

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

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.

^aComputed 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

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.

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

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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 $P = 2.43 \times 10^{-8}$ for rs7775228 and 6.73×10^{-8} for rs10947262). Our results suggest that immunologic mechanism is implicated in the etiology of OA.

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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.

^aKellegren-Laurence grade ≥ 3 was considered as severe OA.

^bAll cases underwent TKR (total knee replacement) surgery.

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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 $\geq 1.0 \times 10^{-6}$ 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 ($P < 1 \times 10^{-5}$) 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 $P = 1.09 \times 10^{-7}$ (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

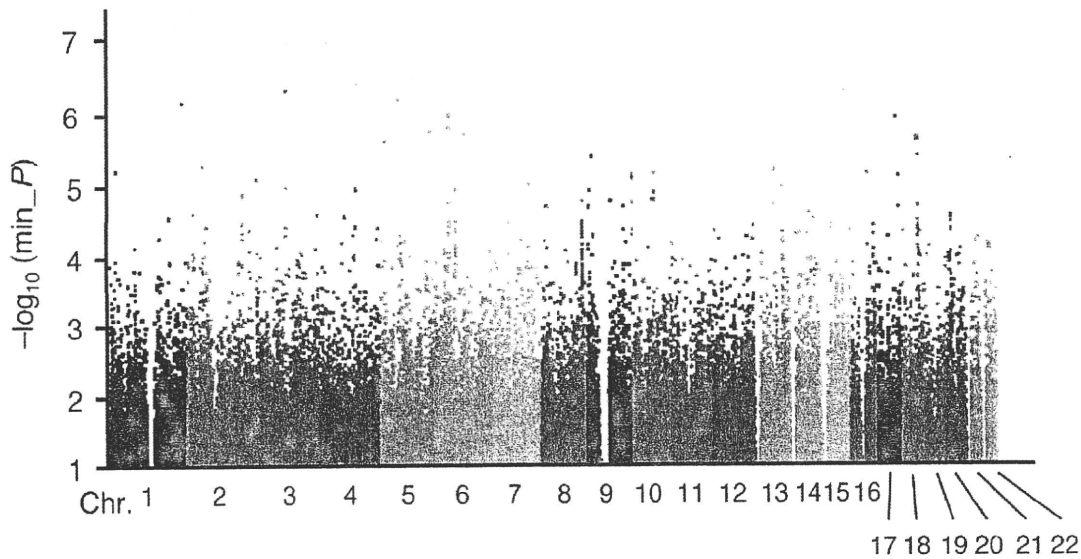


Figure 1. Results of a genome-wide association study ($-\log_{10} P$ value plot). Each P value is the minimum of Fisher's exact tests for three models: dominant, recessive and allele frequency model.
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SNPs selected for the replication study that had the smallest P values (minimum $P < 1 \times 10^{-5}$) 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 $P = 2.43 \times 10^{-8}$; 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		OR (95% CI) ^a	P^b	P_{het}^c
			Case	Control			
rs7775228 (HLA-DQB1)	C/T	Japanese					
		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 (BTNL2)	C/T	Japanese					
		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.

^aOR 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.

^cResults of the Breslow-Day test.

^dMeta-analysis of Japanese studies.

^eMeta-analysis of European studies.

^fMeta-analysis of all studies.

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$P = 6.73 \times 10^{-8}$; OR = 1.32; 95% CI = 1.19–1.46) were significant even after the Bonferroni correction for multiple testing ($P = 1.09 \times 10^{-7}$) (Table 2). The two SNPs showing significant associations are located within a 340-kb region within the HLA locus, including *BTNL2*, *HLA-DRA*, *HLA-DRB5*, *HLA-DRB1*, *HLA-DQA1* and *HLA-DQB1* (Figure 2). Although the HLA region is known to show extensive linkage disequilibrium (LD) spanning over 7 Mb, only SNPs in the 340-kb region showed strong associations with OA (Figure 2), and SNPs outside of this region did not have significant association.

Application of the Cochran-Armitage test to all the tested SNPs indicated that the genetic inflation factor lambda was 1.08 for GWAS (Figure 3), implying a low possibility of false positive associations due to population stratification. We also carried out age, gender- and BMI-adjusted analysis using a logistic regression model, and confirmed similar association after adjustment (data not shown). The principal component analysis [13] revealed that there was no evidence for population stratification between the two control groups used for the GWAS (Figure S1).

To check the association of rs7775228 and rs10947262 in different ethnic populations, we examined the association of the SNPs with knee OA in two European Caucasian populations from Greece and Spain. We genotyped a total of 813 OA and 1,071 control subjects (Table 1). We conducted the meta-analysis using the Mantel-Haenszel method. The combined European results for

rs7775228 were not significant with OR (95%CI) of 0.93 (0.76–1.13) (Table 2), while those for rs10947262 were supportive with OR (95%CI) of 1.29 (1.03–1.61). rs10947262 showed replication in the Greek population and the same trend in the Spanish population (Table 2). A meta-analysis of the Japanese and two European studies gave more significant association (combined $P = 5.10 \times 10^{-9}$).

We estimated the pairwise LD indexes (D' and r^2) between rs7775228 and rs10947262 using the genotype data of Japanese populations (GWAS and the replication study), and found that they were in strong LD with each other ($D' = 0.82$, $r^2 = 0.56$). They formed two frequent haplotypes (Haplotype I and II; Table 3) accounting for about 90% of all observed chromosomes. The haplotypes were also significantly associated with knee OA; Haplotype I, the most frequent haplotype was a risk haplotype ($P = 1.48 \times 10^{-8}$; OR = 1.33; 95% CI = 1.20–1.46).

Discussion

We performed a GWAS followed by a replication in an independent population using a total of ~4,800 Japanese subjects, and identified two SNPs (rs7775228 and rs10947262) in the HLA class II/III locus associated with susceptibility to knee OA. To our knowledge, this study represents the first GWAS of OA with extensive coverage (~550,000 markers) and definite genome-wide significance even after Bonferroni's correction, which is very

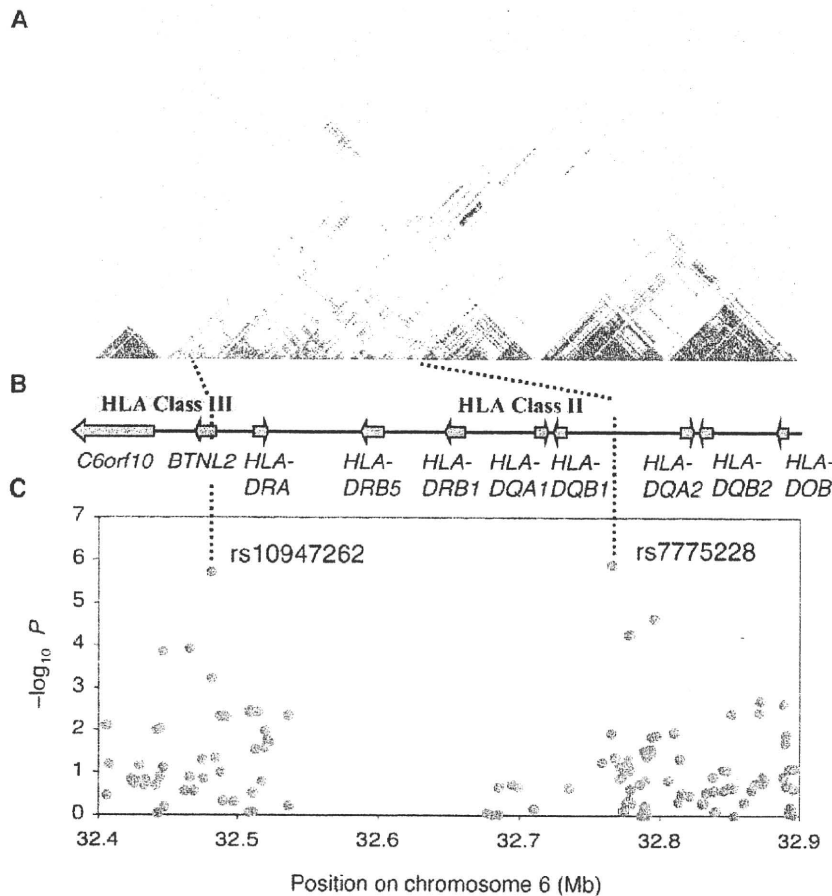


Figure 2. Case-control association analysis and linkage disequilibrium (LD) map of the HLA class II/III region of chromosome 6. (A) The LD map based on D' was drawn using HapMap data release 24 for the JPT population. (B) Genomic structure within the extended HLA-II/III region. (C) Results of GWAS for osteoarthritis in Japanese population. The \log_{10} -transformed P values are plotted on the y axis. doi:10.1371/journal.pone.0009723.g002

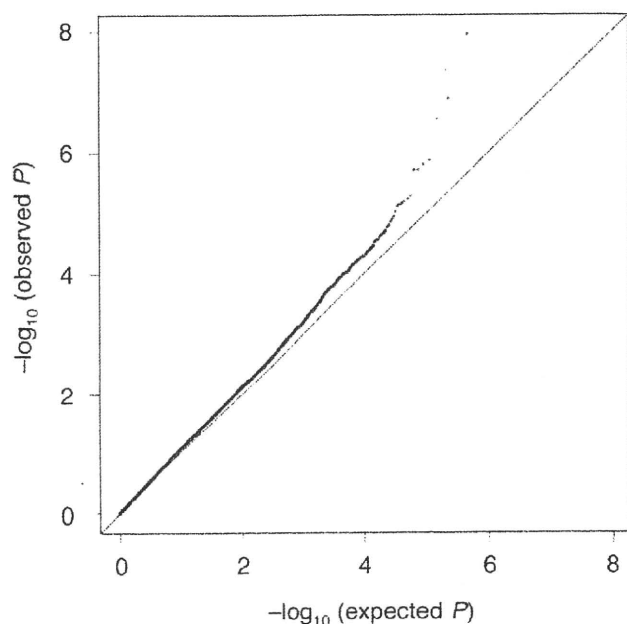


Figure 3. GWAS of knee osteoarthritis. Q-Q plot with Cochran-Armitage trend P in the GWAS. Horizontal and vertical lines represent expected P values under a null distribution and observed P values, respectively. The genetic inflation factor λ is 1.08.
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conservative. There were no effects of population stratification and confounding factors. Since two groups of controls were used in the GWAS, we evaluated the possibility of genetic heterogeneity between the two groups by a principal component analysis and found it unlikely (Figure S1). Although there was large age difference between the case and control groups of GWAS (Table 1), significant association was observed after the age adjustment.

In the NCBI genome database, rs7775228 and rs10947262 located between upstream region of *HLA-DQA2* and *HLA-DQB1*, and within the intron 1 of *BTNL2*, respectively (Figure 2). *HLA-DQA2* and *HLA-DQB1* encode HLA-DQ α and β chains, which belong to the HLA class II molecules. HLA class II molecules are expressed in antigen presenting cells (B lymphocytes, dendritic cells and macrophages) and play a central role in the immune system by presenting peptides derived from extracellular proteins [14]. The HLA-DQA2 protein is expressed, but at a very low level in comparison with the HLA-DQA1 protein [15,16]. Moreover,

the HLA-DQA2 α chain does not dimerize with class II β chains [16]. *BTNL2* encodes butyrophilin-like 2, a member of butyrophilin family that shares sequence homology with the B7 costimulatory molecules. *BTNL2* regulates T-cell activation through unknown receptor, distinct from CD28 and CTLA-4 [17]. In Japanese population, the haplotype association was more significant than those of respective SNPs (Tables 2 and 3). Therefore, there may be hidden SNP(s) with a lower P value than rs7775228 and rs10947262, or the haplotype may be implicated in the OA susceptibility. An association of sarcoidosis with rs2076530, a coding SNP on exon 5 of the *BTNL2* gene has been reported [18], but the SNP was not in LD with rs10947262 ($D' = 0.11$, $r^2 = 0$).

The 340-kb region of HLA locus, where the two SNPs are located also includes *HLA-DRA*, *HLA-DRB1*, *HLA-DRB3*, *HLA-DRB4*, *HLA-DRB5* and *HLA-DQA1*. *HLA-DRA*, *HLA-DRB1/3/4/5* and *HLA-DQA1* encode HLA-DR α , β and HLA-DQ α chains, which could also belong to the HLA class II molecules. *HLA-DRB1* is present in all individuals. Allelic variants of *HLA-DRB1* are linked with either none or one of the genes *HLA-DRB3*, *HLA-DRB4* and *HLA-DRB5* [19]. Among these genes, *HLA-DRB1* is strongly associated with RA. Some subtypes of HLA-DRB1 alleles, such as *0101, *0401, and *0405, is associated with RA [20], but not with generalized OA [21].

Although OA has generally been considered a non-inflammatory disease, accumulating evidences suggest that this is not the case. Inflammation involving activated T cells in the synovial membrane of OA patients is well documented [22]. Recently, we identified a genetic variant of *EDG2* gene encoding lysophosphatidic acid receptor associated with knee OA [23]. A GWAS has identified a genetic variant of the *PTGS2* gene encoding cyclooxygenase-2 involved in risk for knee OA [6]. These genetic associations of genes such as *EDG2* and *PTGS2* underscore the potential role of inflammatory pathways in the pathogenesis of knee OA.

Several studies have suggested associations of OA with HLA class I and class II alleles. Study on generalized OA revealed association with HLA A1-B8 in Caucasian [24] and with HLA-Cw4 in Japanese [21]. An association of the HLA-DRB1*02 alleles with knee and hip OA was identified in a cohort of 106 patients [25]. Interestingly, chondrocyte, which are normally HLA-DP, DQ and DR-negative, become positive for them in OA [26,27], suggesting their function as antigen-presenting cells. Cartilage fragments are mechanically shaved from the joint surface and frequently found in the synovial membrane of OA patients [28]. So, physical interaction between chondrocytes and T cells is conceivable. Peripheral blood T cells from OA patients show significantly higher proliferative responses to autologous chondrocytes [27]. Our results further support the concept that OA is an immunologic disorder.

Table 3. Haplotype association analysis for knee osteoarthritis susceptibility SNPs, rs7775228 and rs10947262.

Haplotype ^a	TC	Frequency		Haplotype effect ^b	
		Case	Control	OR (95% CI)	P value
I	TC	0.610	0.541	1.33 (1.20–1.46)	1.48 E-8
II	CT	0.277	0.340	0.74 (0.67–0.83)	5.07 E-8
III	CC	0.077	0.080	0.96 (0.80–1.15)	0.653
IV	TT	0.037	0.040	0.91 (0.71–1.18)	0.475

OR: odds ratio, CI: confidence interval.

^aAlleles of rs7775228 (C/T) and rs10947262 (C/T) with this order.

^bA haplotype vs. all others.

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Supporting Information

Figure S1 Principal component analysis of GWAS samples. Samples in the GWAS and in HapMap database are analyzed by a program of Smartpca [12], and plotted for the first (X axis) and the second (Y axis) principal component (PC), respectively. Found at: doi:10.1371/journal.pone.0009723.s001 (0.16 MB TIF)

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Author Contributions

Wrote the paper: MN SI. Planned and supervised the whole project: SI. Performed the Japanese association study: MN IK TF JD. Managed the European association study: A. Tsezou AG. Helped with statistic analysis:

A. Takahashi NK TT. Managed DNA sample and/or clinical information: CR-F JJG-R AS AU NF KNM A. Tsezou AG YN. Oversaw a genotyping of GWAS: MK YN.

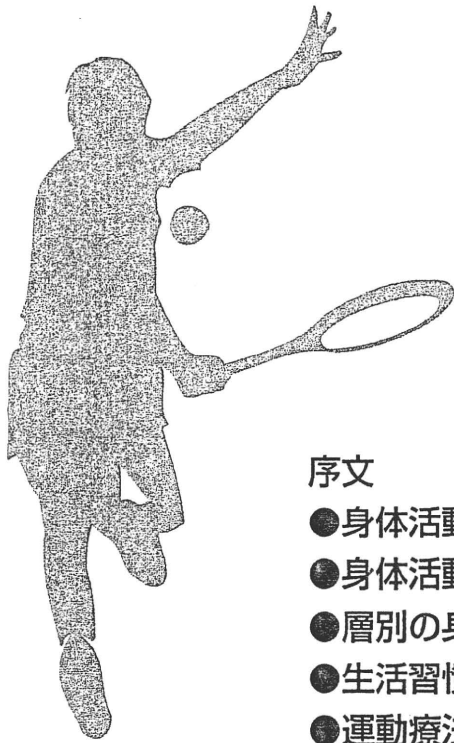
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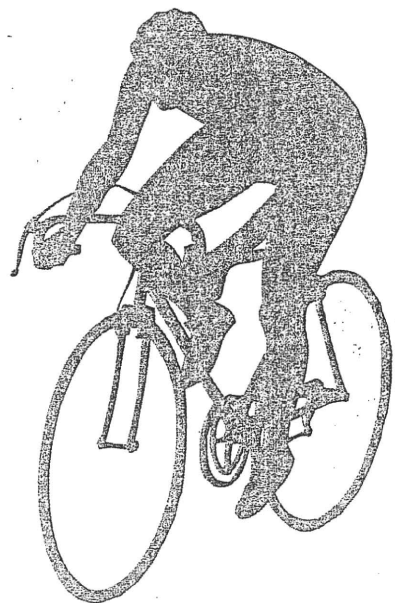
身体活動・運動と生活習慣病

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