from 14.6% in 1991 to 11.7% in 1999 (Mohammad et al., 2001). Based on WHO report in the Global Tobacco Epidemic in 2009 (WHO, 2011b), the prevalence of current smoking was 11.8% (22.1% among male and 1.3% among female) for age group 15-64 years in Iran.

Over the past 50 years, the results of cohort studies showed that middle-aged people with two or more major risk factors had radically higher risks for cardiovascular death, myocardial infarction and stroke across their life. The study reported that decrease in smoking and cholesterol levels have influenced to decline in cardiovascular disease rates over the past several decades and it reflects changes in the prevalence of the risk factors rather than access to and effects of treatment. Researchers also found similar trends across all races and age groups (Berry et al., 2012). It is deemed necessary to have overview of present situation among this age group to prevent tobacco-related risks. The aim of this study was to determine the prevalence of smoking among people aged 40-64 years in the population of Shahroud, Iran. Shahroud is representative for urban population of Iran.

Materials and Methods

This cross-sectional population-based study is the first phase of Shahroud Eye Cohort Study. Shahroud has stable population and good Primary Health Care (PHC) system for running the prospective study. According to the 2006 census; the population of Shahroud was 133,835. Out of them, about 28,000 people were aged 40-64.

Target population in this study was Shahroud inhabitants between the ages of 40 and 64 years. A multistage stratified cluster sampling method was used to select randomly the survey sample from the Shahroud population. Since the population of Shahroud is covered by 9 health centers in the Iranian Primary Health Care system, each center was considered as a stratum and the number of the clusters was calculated proportionate to the population of each center. The electronic databases of the health centers provided the sampling frame (complete listing of all households) for each stratum. Three hundred clusters were selected, each cluster consisting of sufficient number of households to provide a total of at least 20 eligible persons. The households were visited based on a determined plan. The index households for each cluster were selected randomly. After identification of the index household in each cluster, the enumeration of the neighboring households continued from the right side of that household in the cluster until at least 20 eligible individuals were found.

The questionnaire was developed and approved for use by experts. The questionnaire consisted of two parts; the first part covered the demographic characteristics of surveyed households (date of birth, gender, marital status, education and occupation). The second part interviewed about smoking (status, frequency, type, duration and the age of starting smoking). The Information about smoking habit was obtained by a face-to-face interview. This study data were collected on two types of smoking cigarette and water-pipe. The protocol of this study which is published previously (Fotouhi et al., 2013) has been approved by the ethics committee of the Shahroud University of Medical Sciences. Written informed consent was obtained from all participants.

Data were analyzed using Epi-info, version 7 and SPSS, version 19.0. Smoking rates are showed with 95% confidence intervals (CIs) for current smokers; the cluster sampling design was applied. The means of variables were presented with standard deviation (SD). Univariate and multivariate logistic regression were performed to see the association between smoking and background variables. Odds ratios (ORs) with 95% confidence intervals (CIs) were used for comparison, $P<0.05$ was considered statistically significant.

Results

A total of 6,311 people aged 40-64 years were sampled in 2009. Among them 5,190 people participated in the study with a response-rate of 82.24%. The mean age of the studied population was 50.92 (±6.25) years. Of the total participants, 26.78% were 45-49 years; 57.36% were female; 50.00% had primary education; 51.48% were housekeepers; 92.41% were married.

Table 1 summarizes the current use of cigarette and water-pipe among interviewed participants. The overall smoking rate was 11.33% (95%CI: 10.45-12.27), the rate of prevalence was higher in male 25.70% (95% CI: 23.74-27.66) than in female 0.71% (95%CI: 0.40-1.01), ($P<0.001$). The peaked prevalence of smoking was observed among unemployed people (41.51%). The prevalence of current cigarette smoking was 10.79% (95%CI: 9.90-11.68), 24.90% (95%CI: 22.97-26.86) for male and 0.30% (95%CI: 0.11-0.50) among female. The highest prevalence of current cigarette smoking was in unemployed respondents (37.74%). The prevalence of smoking increased from 5.39% in illiterate people to 14.23% in college educated people. In total, the prevalence of current water-pipe users was 0.67% (95%CI: 0.44-0.90), male had higher rates of current water-pipe smoking than female 1.04% (95%CI: 0.63-1.45) versus 0.40% (95%CI: 0.16-0.63), ($P=0.006$).

The prevalence of past smokers was 1.75%. It was significantly higher in male than in female ($P<0.001$) and increased with age from 0.52% in the age group of 40-44 to 2.66% in the age group of 60-64 ($P=0.005$).

The overall mean age of starting to smoke was 25.06 (±10.57) years, 25.84 (±9.25) years in male and 29.33 (±10.63) years in female. Between the ages 40-64 years, smoking was initiated at similar ages. The mean number of cigarettes was smoked in a day among current smokers 11.89 (±8.38). The highest number of cigarettes was smoked by participants aged 55-64, 13.16 (±8.84).

Multivariate logistic regression analysis was used to determine the association between smoking and sex, age, education, occupation and marital status. Smoking was considered as dependent variable. The odds of smoking among the unemployed were 2.66 times (95%CI 1.38-5.14) greater than employed peoples. People with college education had a more than two-fold increase of the odds of smoking compared to illiterate people (OR=2.40, 95%CI 52-3.76) in univariate logistic regression model,

Table 1. The Prevalence of Current Smoking According to Different Demographic Characteristics in Shahroud, Iran; 2009

Variables		No.	Current cigarette smoking (%) Prevalence (95% CI*)	Current water- pipe smoking (%) Prevalence (95% CI)	Overall current smoking (%) Prevalence (95% CI)
Age	40-44	960	7.81 (5.96-9.67)	0.52 (0.07-0.97)	8.23 (6.31-10.15)
	45-49	1390	10.36 (8.61- 12.12)	0.58 (0.18-0.97)	10.82 (9.05-12.6)
	50-54	1285	13.23 (11.37-15.09)	0.62 (0.20-1.05)	13.79 (11.9-15.67)
	55-59	954	11.85 (9.68-14.01)	0.84 (0.26-1.41)	12.68 (10.47-14.90)
	60-64	601	9.65 (7.33-11.97)	1.00 (0.21-1.78)	10.33 (7.95-12.72)
Gender	Female	2977	0.30 (0.11-0..50)	0.40 (0.16-0.65)	0.71 (0.40-1.01)
	Male	2213	24.90 (22.97-26.82)	1.04 (0.63-1.45)	25.70 (23.74-27.66)
Education	Illiterate	427	5.39 (3.36-7.42)	1.17 (0.004-3.25)	6.57 (4.30-8.84)
	Primary school	2595	10.02 (8.71-11.33)	0.81 (0.46-1.16)	10.72 (9.39-12.05)
	Guidance	481	13.51 (10.51-16.51)	0.21 (0.001-0.014)	13.72 (10.70-16.75)
	High school	1146	11.78 (9.93-13.63)	0.44 (0.06-0.81)	12.16 (10.33-14.00)
	College	541	14.23 (11.29-17.17)	0.56 (0.002-0.017)	14.42 (11.44-17.40)
Job	Employed	1114	19.57 (17.06-22.08)	0.72 (0.22-1.21)	20.14 (17.61-22.68)
	Retired	853	20.75 (17.92-23.58)	0.35 (0.11-0.83)	21.00 (18.13-23.84)
	Unemployed	53	37.74 (24.00-51.47)	3.77 (0.95-13.80)	41.51 (29.04-55.98)
	Disabled	45	20.00 (7.61-32.39)	0	20.00 (7.61-32.39)
	House keeper	2672	0.26 (0.02-0.43)	0.45 (0.18-0.72)	0.67 (0.34-1.01)
	Others	453	28.70 (24.33-33.06)	2.21 (0.91-3.50)	30.38 (25.97-34.78)
Marital Status	Single	67	8.96 (1.64-16.28)	0	8.96 (1.64-16.28)
	Married	4796	11.45 (10.51-12.39)	0.63 (0.40-0.85)	11.96 (11.00-12.92)
	Widow	291	1.03 (0.34-3.11)	1.72 (0.24-3.19)	2.75 (0.92-4.58)
	Divorced	36	5.57 (0.014-0.194)	0	5.56 (0.014-0.194)
Total		5190	10.79 (9.90-11.68)	0.67 (0.44-0.91)	11.33 (10.45-12.27)

*CI=Confidence Interval

Table 2. Predictors of Smoking Among Middle Aged Population of Shahroud, Iran; 2009

Variables	Univariate Logistic Regression Odds ratio (95% CI*)	P value	Multivariate Logistic Regression Odds ratio (95% CI*)	P value
Sex				
Female	1		1	
Male	48.64 (31.05-76.20)	<0.001	16.22 (9.50-27.69)	<0.001
Age				
40-44	1		1	
45-49	1.35 (1.02 -1.80)	0.038	1.13 (0.83-1.54)	0.435
50-54	1.78 (1.34-2.36)	<0.001	1.38 (1.02-1.86)	0.040
55-59	1.62 (1.20-2.18)	0.002	1.07 (0.76-1.51)	0.702
60-64	1.29 (0.91-1.82)	0.159	0.79 (0.52-1.19)	0.252
Education				
Illiterate	1		1	
Primary school	1.71 (1.14-2.55)	0.009	1.05 (0.66-1.69)	0.829
Guidance	2.26 (1.42-3.59)	0.001	1.09 (0.63-1.89)	0.761
High school	1.97 (1.29-3.00)	0.002	0.94 (0.57-1.54)	0.799
College	2.40 (1.52-3.76)	<0.001	0.71 (0.42-1.22)	0.217
Job				
Employed	1		1	
Retired	1.05 (0.84-1.31)	0.647	1.18 (0.90-1.55)	0.223
Unemployed	2.81 (1.60-4.95)	<0.001	2.66 (1.38-5.14)	0.004
Disabled	0.99 (0.47-2.09)	0.981	1.26 (0.54-2.90)	0.592
House keeper	0.03 (0.02-0.04)	<0.001	0.19 (0.10-0.36)	<0.001
Others	1.73 (1.35-2.22)	<0.001	1.62 (1.24-2.12)	0.001
Marital Status				
Single	1		1	
Married	1.38 (0.60-3.21)	0.453	1.77 (0.69-4.51)	0.232
Widow	0.29 (0.10-0.86)	0.025	5.25 (1.53-17.98)	0.009
Divorced	0.60 (0.11-3.13)	0.542	2.36 (0.36-15.50)	0.370

*CI= Confidence Interval

but in multivariate model this association was not significant(Table 2).

Male gender, Housekeeper job and being widow were the other important variables which had significant association to smoking.

Table 3. Comparison of The Prevalence of Smoking with some Developed and Developing Countries.

	Age (years)	Male	Female	Total	Year of survey
Country					
Iraq	≥12	26.5	2.9	14.8	2007
Bahrain	20-64	33.4	7	19.9	2007
Kuwait	20-64	42.3	4.4	23.6	2006
Egypt	≥15	37.6	0.5	19.4	2009
UAE	≥18	28.1	2.4	20.5	2003
Pakistan	≥18	32.4	5.7	19.1	2002-2003
Turkey	≥15	47.9	15.2	31.2	2009
China	≥15	52.9	2.4	28.1	2009
India	≥15	24.3	2.9	14	2009
Thailand	≥15	45.6	3.1	23.7	2009
Malaysia	≥18	46.4	1.6	21.5	2006
Japan	≥20	38.2	10.9	23.4	2009
Australia	≥14	18	15.2	16.6	2007
USA	≥15	31.2	23	27	2009
New Zealand	≥15	21.1	18.8	19.9	2007
Italy	≥11	29.5	17	23	2009
Germany	≥15	30.5	21.2	25.7	2009
France	12-75	33.3	26.5	29.9	2005
Canada	≥15	23	16	19.5	2009
Iran	15-64	22.1	1.3	11.8	2009

Discussion

According to the result of our survey, the prevalence of current cigarette smoking in Shahroud was 10.8% (24.9% male and 0.3% female). In 2003, Fotouhi et al. (2009) in Tehran reported prevalence of smoking among people aged over 15 years was 11.9% (20.6% male and

2.9% female); while according to another study among people aged 15-69, it was 14.6% (27.2% male and 3.4% female) in 1991 and 11.7% (24.0% male and 1.5% female) in 1999 (Mohammad et al., 2001). In 2007, Meyasamie et al. (2010) found smoking prevalence of 12.5% (23.4% male and 1.4% female) among population aged 15-64 years. Most of previous studies in Iran found higher results than our findings. We assume our lower end of overall prevalence contributed by low cigarette smoking rate among female. Table 3 compares the prevalence of smoking in Iran with other countries according to their prevalence in sex, age groups, location and year of study (WHO, 2011b).

The prevalence of cigarette smoking among female in our study is the lowest among the Eastern Mediterranean countries (Youssef et al., 2002; Khattab et al., 2012). In many countries around the world, the ratio of cigarette smoking among people aged 15 years old and older for female compared to male is higher than one to ten (Eriksen et al., 2012). Similar ratio has been reported from age group 16-69 in Iran (Mohammad et al., 2001). However, the ratio in our study was one to eighty. Previous study of Egyptian smoking aged 18 year and older reported similar finding (WHO, 2011b). This ratio seems low compared to other countries where in female to male ratio were one to 7.14 (Pakistan), one to 1.25 (United State), one to 1.17 (Australia), one to 1.04 (United Kingdom) (Nasir and Rehan, 2001). This low prevalence of smoking may due to social reasons or religion binding that discourage smoking in female. Similar finding concerning gender gap in the prevalence of smoking was reported in the Eastern Mediterranean countries (Nasir and Rehan, 2001; Youssef et al, 2002; Al Riyami and Afifi, 2004; WHO, 2011b; Khattab et al., 2012). This scenario was proven by a study carried out in Iran, whereby self-reported cigarette prevalence in male and female aged 19 to 49 years old was 19.4% and 1.5%, respectively. In the same sample population, the prevalence increased to 21.2% and 6.7%, respectively, when tested by serum continine level as indicator of nicotine metabolism among smokers (Sarraf-Zadegan et al., 2004).

The highest ranked smoking rate was in the age group of 50-54 years old and the second highest rate was among the age group of 55-59 years old. This could be explained by relevant studies that found these specific ages representing the previous generations, who started their smoking habit 25-34 years ago during the beginning of smoking epidemic in developing world where there was insufficient public awareness regarding harmful effects of smoking (Youssef et al., 2002).

In this study, education had no role on cigarette smoking. However, majority of studies in developing countries reported tobacco use was the highest among those with lowest levels of education (Nasir and Rehan, 2001; Rani et al., 2003; Al Riyami and Afifi, 2004; Al Turki et al., 2010; Hosseinpoor et al., 2011).

Unemployed people had highest smoking rate. De Vogli et al. (2005) revealed that jobless has negative direct and indirect effect to smoking habit. Another study also reported smoking is a way to relieve life-related stresses, such as jobless (Jarvis and Wardle, 2005).

The prevalence of past smokers was increased by age. Those who smoke for many years often start to experience physical symptoms of smoking-related diseases at the age of our studied group. In many occasions, smokers from the higher age group are forced to quit due to adverse effect of smoking on their health (Lundqvist et al., 2007). Cumulative quitting among the older adults over time will result in a lower prevalence of smoking and a higher prevalence of cessation.

The main strength of our study is the use of a large sample size what makes the results more representative. It is a population-based study with appropriate sampling and good interview process with supervision. The limitations of this study are the fact that the sample did not include young people. Also this study did not cover rural area.

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ORIGINAL ARTICLE

Combination chemotherapy with S-1 plus cisplatin for gastric cancer that recurs after adjuvant chemotherapy with S-1: multi-institutional retrospective analysis

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Abstract

Background It is unclear whether S-1 plus cisplatin is effective for patients with recurrent gastric cancer after adjuvant S-1 chemotherapy.

Methods We retrospectively evaluated the efficacy of S-1 plus cisplatin in patients whose gastric cancer recurred after adjuvant S-1 chemotherapy.

Results In the 52 patients evaluated, the median duration of adjuvant S-1 chemotherapy was 8.1 months, and the median recurrence-free interval (RFI) since the last administration of adjuvant S-1 was 6.4 months. Among the 36 patients with measurable lesions, 7 achieved a complete or partial response, and 13 were evaluated as having stable

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disease, for an overall response rate of 19.4% and a disease control rate of 55.6%. For all patients, the median progression-free survival (PFS) was 4.8 months, and the median overall survival (OS) was 12.2 months. Compared with patients with an RFI of <6 months ($n = 25$), patients with an RFI of ≥6 months ($n = 27$) had a significantly higher response rate (5.0 vs. 37.5%, respectively), longer PFS (2.3 vs. 6.2 months, respectively), and longer overall survival (7.3 vs. 16.6 months, respectively). According to a multivariate Cox model including performance status (PS) and reason for discontinuation of adjuvant S-1, an RFI of 6 months was still significantly associated with PFS and OS. *Conclusions* S-1 plus cisplatin is effective for patients with gastric cancer that recurs after adjuvant S-1 chemotherapy, especially for those with an RFI of ≥6 months.

Keywords Adjuvant chemotherapy · Gastric cancer · Recurrence · S-1

Introduction

Gastric cancer is the fourth most common malignancy in the world (988,602 cases in 2008, 7.8% of total malignancy cases) and the second leading cause of cancer death (737,419 deaths, 9.7% of total) [1]. The prognosis of patients with advanced or recurrent gastric cancer remains poor; chemotherapy confers only a minimal survival advantage, with a median survival of approximately 1 year. The most commonly used regimens are combination chemotherapy consisting of a fluoropyrimidine [5-fluorouracil (5-FU) or oral fluoropyrimidine] plus a platinum agent with or without docetaxel or anthracyclines [2–6].

S-1 is an oral anticancer drug composed of the 5-FU prodrug tegafur and two 5-FU modulators; it has achieved high response rates in patients with gastric cancer in phase II studies [7, 8]. In the Japan Clinical Oncology Group (JCOG) 9912 trial, which compared S-1, cisplatin plus irinotecan, and 5-FU, S-1 demonstrated non-inferiority compared to 5-FU [9]. In another phase III trial that compared S-1 alone to S-1 plus cisplatin (SPIRITS trial), S-1 plus cisplatin showed a significantly higher response rate (54 vs. 31%), longer progression-free survival (PFS; 6.0 vs. 4.0 months), and longer overall survival (OS; 13 vs. 11 months) [4]. Also, in a large, non-Japanese, phase III trial (the First-Line Advanced Gastric Cancer Study; FLAGS trial), S-1 plus cisplatin was associated with fewer toxic effects and demonstrated non-inferiority compared with 5-FU plus cisplatin by exploratory analysis [6]. Therefore, S-1 plus cisplatin is now considered to be one of the standard regimens for metastatic or recurrent gastric cancer.

In addition, the ACTS-GC trial has demonstrated that S-1 is also effective as adjuvant chemotherapy for Japanese patients who have undergone curative gastrectomy for locally advanced gastric cancer [10]. However, approximately 30% of patients still develop recurrence after curative resection followed by adjuvant S-1 [10]. As few patients who received adjuvant chemotherapy were included in the phase III trials described above [4, 7, 9], it is unclear whether patients who develop recurrence after adjuvant S-1 could achieve efficacy with S-1 plus cisplatin similar to that achieved in patients without adjuvant chemotherapy. To address this issue, we conducted the following multi-institutional retrospective analysis.

Patients and methods

Patients

This retrospective study was designed to evaluate the efficacy of first-line chemotherapy with S-1 plus cisplatin for recurrence in patients with gastric cancer who had undergone curative gastrectomy followed by adjuvant S-1 chemotherapy. Patients with histopathologically proven recurrent gastric adenocarcinoma after gastrectomy and lymph node dissection with no residual tumor were eligible for analysis. Additional eligibility criteria were: (1) previous adjuvant S-1 chemotherapy at a planned standard dose and schedule (80 mg/m^2 for 28 consecutive days followed by a 14-day rest; 42-day cycles to be repeated for 1 year); (2) Eastern Cooperative Oncology Group performance status (ECOG PS) 0–2; (3) adequate bone marrow, hepatic, and renal function to be treated with S-1 plus cisplatin; (4) evaluable lesions according to Response Evaluation Criteria in Solid Tumors (RECIST ver. 1.1); and (5) treated with a standard regimen of S-1 plus cisplatin (S-1 80 mg/m^2 for 21 consecutive days followed by a 14-day rest; cisplatin 60 mg/m^2 intravenous infusion on day 8; 35-day cycles to be repeated) [4]. Written informed consent for treatment was obtained from each patient prior to treatment initiation. The Institutional Review Board of each participating center approved the study.

Evaluation of treatment and statistical analysis

The tumor response was assessed objectively according to RECIST ver. 1.1, and the best overall response was recorded as the antitumor effect for that patient. The disease control rate (DCR) represented the percentage of patients with a complete response (CR), partial response (PR), or stable disease (SD). PFS was measured from the date of initiation of S-1 plus cisplatin to the date of progressive disease or death from any cause. Time to treatment failure

(TTF) was measured from the date of initiation of S-1 plus cisplatin to the date of last administration of S-1. OS was estimated from the date of initiation of S-1 plus cisplatin to the date of death or last follow-up visit, using the Kaplan–Meier method. The interval from the last administration of adjuvant S-1 to recurrence was defined as the recurrence-free interval (RFI).

The Cox proportional hazards model was used to estimate the impact of the RFI on TTF, PFS, and OS, with adjustment for other factors that were shown to be significant with a univariate log-rank test. *P* values for testing differences between proportions and response rates were calculated with χ^2 tests for homogeneity or for trend, or with Fisher's exact test. Results were considered to be statistically significant when the *P* value was <0.05. All reported *P* values are two-sided. In particular, we compared the response rate, DCR, time to progression (TTP), PFS, and OS between patients with RFIs of ≥6 and <6 months, because several clinical trials in the first-line setting set this interval of ≥6 months as an inclusion criterion [5, 9, 11].

Results

Patient characteristics

A total of 406 patients with recurrent gastric cancer after adjuvant S-1 chemotherapy had received chemotherapy at 18 institutions until October 2010. Among them, 57 patients (14.0%) had received S-1 plus cisplatin as first-line chemotherapy for recurrence. After the exclusion of 5 patients (1 patient with a non-evaluable lesion and 4 patients with insufficient data), 52 patients were included in the final

Table 1 Patient characteristics

Characteristic	All ($n = 52$)	RFI <6 months ($n = 25$)	RFI ≥6 months ($n = 27$)	*P* value
Age, years				
Median (range)	61 (32–77)	59 (32–77)	62 (32–77)	
Gender, *n* (%)				
Male	30 (58)	15 (60)	15 (56)	0.75
Female	22 (42)	10 (40)	12 (44)	
ECOG PS at recurrence, *n* (%)				
0	32 (62)	11 (44)	21 (78)	*0.012*
1	20 (38)	14 (56)	6 (22)	
Histological type[a], *n* (%)				
wel or mod	27 (52)	10 (40)	17 (63)	0.1
por or sig	24 (46)	15 (60)	9 (33)	
Other	1 (2)	–	1 (4)	
Pathological stage[a], *n* (%)				
Stage I or II	8 (15)	4 (16)	4 (15)	0.57
Stage IIIA	17 (33)	6 (24)	11 (41)	
Stage IIIB	15 (29)	8 (32)	7 (26)	
Stage IV	12 (23)	7 (28)	5 (19)	
Site of recurrence, *n* (%)				
Peritoneum	21 (40)	7 (28)	14 (52)	0.08
Lymph node	25 (48)	13 (52)	12 (44)	0.59
Liver	14 (27)	10 (40)	4 (15)	*0.041*
Lung	4 (8)	3 (12)	1 (4)	0.262
Bone	6 (12)	1 (4)	5 (19)	0.102
Local	2 (4)	1 (4)	1 (4)	0.96
Number of recurrence sites, *n* (%)				
1	38 (73)	18 (72)	20 (74)	0.87
2 or more	14 (27)	7 (28)	7 (26)	

P values shown in italics indicate significant differences

RFI Recurrence-free interval, *PS* performance status, *ECOG* Eastern Cooperative Oncology Group, *wel* well-differentiated adenocarcinoma, *mod* moderately differentiated adenocarcinoma, *por* poorly differentiated adenocarcinoma, *sig* signet-ring-cell-like carcinoma

[a] According to the Japanese classification

analysis (Table 1). The median duration of adjuvant S-1 chemotherapy was 8.1 months (range 0.7–37.4 months), and the median RFI since the last administration of adjuvant S-1 was 6.4 months (range 0–81.3 months). Thirty of the 52 patients (57.7%) completed the planned duration of adjuvant S-1 therapy. In contrast, 14 patients discontinued S-1 due to disease recurrence, and 8 patients stopped therapy due to toxicity or patient refusal. Other than PS and liver metastasis, characteristics did not differ significantly between patients with an RFI of ≥6 months ($n = 27$) and those with an RFI of <6 months ($n = 25$) (Table 1).

Treatment results and efficacy

The median TTF was 4.1 months (95% confidence interval [CI] 2.5–5.1 months), with a median duration of follow-up of 32 months. Forty-four patients discontinued S-1 plus cisplatin due to disease progression ($n = 40$, 90.9%) or toxicity ($n = 4$, 9.1%). Of the 36 patients with measurable lesions, 7 achieved a CR ($n = 3$) or a PR ($n = 4$), and 13 were evaluated as having SD, for an overall response rate of 19.4% (95% CI 7.0–37.0%) and a DCR of 55.6% (95% CI 38.1–72.1%). The median PFS was 4.8 months (95% CI 3.9–6.2 months), and the median OS of all patients was 12.2 months (95% CI 10.2–16.6 months) (Fig. 1). Of the 44 patients who had discontinued S-1 plus cisplatin, 31 (70.4%) received second-line or third-line chemotherapy, including taxanes ($n = 25$) or irinotecan ($n = 17$).

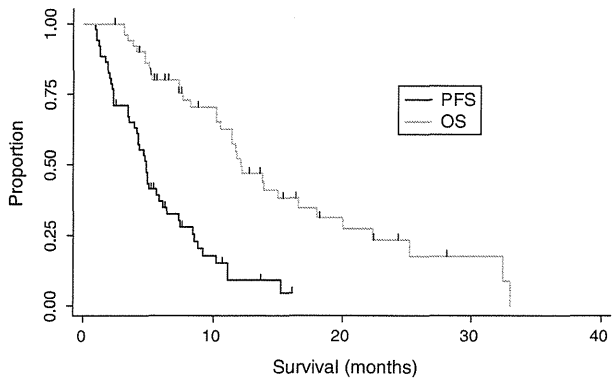

Fig. 1 Progression-free survival (*PFS*) and overall survival (*OS*) in all patients. The median PFS was 4.8 months (95% confidence interval [CI] 3.9–6.2 months), and the median OS was 12.2 months (95% CI 10.2–16.6 months). *PFS* progression-free survival, *OS* overall survival

Significance of the RFI

The response rate was significantly better in patients with an RFI of ≥6 months (37.5%; 95% CI 14–61%) than that in patients with an RFI of <6 months (5.0%; 95% CI 0–15%, $P = 0.014$, Table 2). In addition, compared with patients with an RFI of <6 months, patients with an RFI of ≥6 months had a significantly longer TTF (2.5 vs. 5.1 months, respectively, $P = 0.025$), longer PFS (2.3 vs. 6.2 months, respectively, $P < 0.001$, Fig. 2), and longer OS (7.3 vs. 16.6 months, respectively, $P = 0.003$, Fig. 2). According to a multivariate Cox model including PS and reason for discontinuation of adjuvant S-1, an RFI of 6 months was still significantly associated with PFS (hazard ratio [HR] 0.35, 95% CI 0.16–0.77, $P = 0.009$) and OS (HR 0.21, 95% CI 0.08–0.54, $P = 0.001$), although the association with TTF was not significant (HR 0.55, 95% CI 0.27–1.12, $P = 0.1$). When we divided the patients into two groups based on an RFI of 12 months, no significant difference between the groups was found in response rate, TTP, PFS, or OS.

Discussion

In the ACTS-GC study, adjuvant S-1 chemotherapy significantly improved the survival of patients who had undergone curative gastrectomy for locally advanced gastric cancer [10]. On the other hand, several small studies have suggested that patients with recurrence after adjuvant S-1 were refractory to S-1-containing regimens or had a worse prognosis compared with that of patients without adjuvant chemotherapy [12–14]. Although these reports never precluded the use of adjuvant S-1 chemotherapy, they raised the issue of how to treat recurrent disease after adjuvant S-1.

In the present retrospective study, we evaluated the efficacy of S-1 plus cisplatin in patients whose gastric cancer recurred after adjuvant chemotherapy with S-1. The response rate of 19.4% and PFS of 4.8 months were

Table 2 Objective response rates in patients with measurable lesions

	n	CR	PR	SD	PD	NE	ORR (%)	95% CI (%)
All	36	3	4	13	14	2	18.8	7–32
RFI <6 months	20	0	1	6	13	0	5.0	0–15
RFI ≥6 months	16	3	3	7	1	2	37.5	14–61

CR Complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *NE* not evaluable, *ORR* objective response rate, *CI* confidence interval

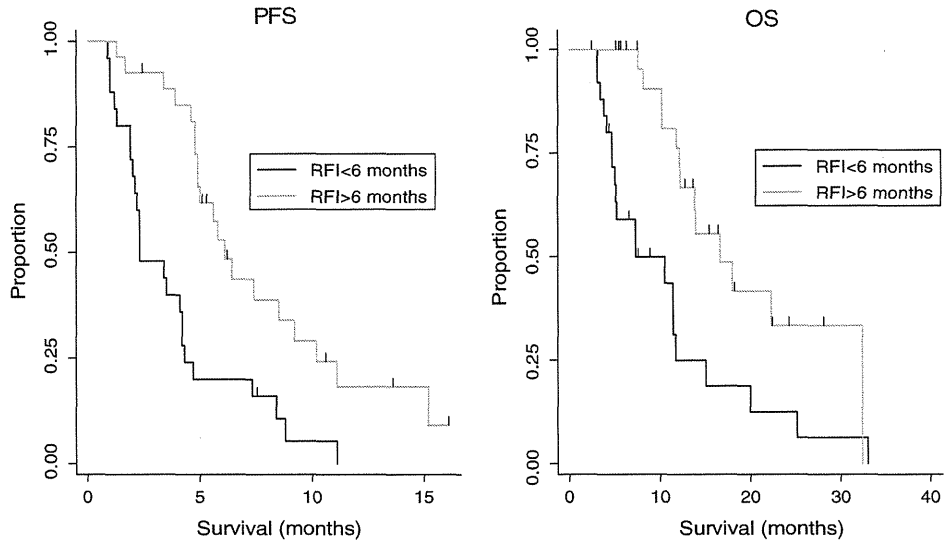

Fig. 2 Progression-free survival (*PFS*) and overall survival (*OS*) according to the length of the recurrence-free interval (*RFI*). Patients with an RFI of ≥6 months had a significantly longer median PFS (6.2 vs. 2.3 months, $P < 0.001$) and OS (16.6 vs. 7.3 months, $P = 0.003$) than patients with an RFI of <6 months. *RFI* recurrence-free interval, PFS progression-free survival, OS overall survival

relatively worse compared with those in the SPIRITS study [4]. However, our results also suggested that patients with an RFI of ≥6 months who received S-1 plus cisplatin had a significantly better response rate, longer PFS, and longer OS compared to patients with an RFI of <6 months. The efficacy of S-1 plus cisplatin for patients with an RFI of ≥6 months in this study was almost compatible with that of patients in the SPIRITS trial in terms of PFS and OS, although these results should be interpreted cautiously due to the heterogeneity of the characteristics of the patients in the two studies. Although no prospective study has evaluated any chemotherapy specifically for patients who have failed adjuvant S-1, Kang and colleagues [15] conducted a phase II study of capecitabine plus cisplatin for 32 patients with gastric cancer that recurred after adjuvant chemotherapy with doxifluridine or 5-FU-containing regimens. They reported a response rate of 28% and a median TTP of 5.8 months, and concluded that capecitabine plus cisplatin was effective as first-line treatment in patients with recurrent gastric cancer after fluoropyrimidine-based adjuvant chemotherapy. In their report, the response rates (21 vs. 39%, $P = 0.427$), TTF (8.3 vs. 5.4 months, $P = 0.072$), and OS (14.1 vs. 9.3 months, $P = 0.075$) tended to be better in patients with an RFI of >6 months ($n = 13$) than in patients with an RFI of ≤6 months ($n = 19$), although the differences did not reach statistical significance [15]. These results were also consistent with those of previous studies in patients with other types of cancer, which suggested the importance of the RFI or treatment-free interval as a predictive marker of responsiveness to similar types of chemotherapy after recurrence [16–18]. Additionally, in the present study, the RFI cut-off value of 6 months was better than that of 12 months for predicting better outcomes and this finding may support the use of the conventional exclusion criteria in clinical trials in the first-line setting, which excluded patients who experienced disease recurrence within 6 months after the last adjuvant chemotherapy [5, 9, 11]. Therefore, selected patients with an RFI of ≥6 months with sufficient organ function may be adequately treated as chemo-naïve patients with standard chemotherapies such as S-1 plus cisplatin.

In contrast to the results for patients with an RFI of ≥6 months, the response rate in patients with an RFI of <6 months in the present study seemed to be worse than that of commonly used second-line chemotherapy regimens such as irinotecan and taxane combinations, which have a reported response rate of approximately 20% for patients with gastric cancer who received prior chemotherapy with fluoropyrimidines alone [18–23]. Based on these results, it may be suggested that the evaluation of chemotherapy regimens other than S-1 plus cisplatin might be warranted for the initial treatment of gastric cancer recurrence after adjuvant S-1. The response rate of 5.0% in our subset of patients with an RFI of <6 months was also lower than that reported previously by Kang et al. for capecitabine plus cisplatin after adjuvant chemotherapy (21%) [15]. The exact reasons for this difference are unknown. One possible reason is that Kang and colleagues did not use the same fluoropyrimidine (capecitabine after doxifluridine or 5-FU), and this choice might have contributed to a higher response in regard to early recurrence, although rechallenge with different types of fluoropyrimidine after the failure of another drug is still controversial in several types of cancer [24–28]. Second, the planned dose intensity of cisplatin as another key drug for gastric cancer was higher in their capecitabine plus cisplatin regimen (60 mg/m^2 every 3 weeks) [15] than that in the S-1 plus cisplatin regimen (60 mg/m^2 every 5 weeks). The efficacy of capecitabine plus cisplatin compared with other

chemotherapy (irinotecan, taxane or irinotecan plus cisplatin) for recurrence after adjuvant S-1 should be evaluated in future clinical trials.

It is important to note the limitations of the present study. First, it was retrospective, and treatment after recurrence was selected by each physician individually. Considering the low proportion of patients who received S-1 plus cisplatin after recurrence (14.0%), the selected population may have been biased toward patients with good performance status (PS) and low tumor burden. Second, toxicity was not evaluated in this study, although the proportion of patients who discontinued S-1 plus cisplatin due to toxicity was low. Third, human epidermal growth factor receptor 2 (HER2) status was not evaluated. Trastuzumab, a humanized monoclonal antibody against HER2, has recently been shown to improve the prognosis of HER2-positive advanced gastric cancer [29], and the HER2 status of all gastric cancer types should be evaluated, even in this setting of recurrent disease. Fourth, the moderate sample size in a single-country study is another limitation; therefore, it would be better to validate the significance of the RFI after adjuvant failure on the PFS in other cohorts as well.

In conclusion, this is the first report to have evaluated the efficacy of chemotherapy with S-1 plus cisplatin in patients with gastric cancer that recurred after adjuvant chemotherapy with S-1. S-1 plus cisplatin was effective in such patients, especially in those with an RFI of $\geq$6 months. Further well-defined, prospective trials in this important patient population are required to identify optimal treatment regimens.

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Conflict of interest None of the authors have financial or personal conflicts of interest to disclose.

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CLINICAL TRIAL

High expression of ATP-binding cassette transporter ABCC11 in breast tumors is associated with aggressive subtypes and low disease-free survival

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Abstract ATP-binding cassette (ABC) transporters are membrane proteins that efflux various compounds from cells, including chemotherapeutic agents, and are known to affect multidrug resistance. Recent reports disagree on whether ABCC11 is a risk factor for breast tumorigenesis, but its expression in breast cancer is poorly investigated. We hypothesized that both frequency and expression levels of ABC transporters in breast tumors would vary by cancer subtype, and be associated with prognosis. Here, we constructed a tissue microarray breast tumor samples from 281 patients, and analyzed expressions of ABCB1, ABCC1, ABCC11, and ABCG2 immunohistochemically. Breast cancer subtypes were determined by immunohistochemistry of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2). Protein expression was correlated to clinicopathological characteristics, clinical follow-up, and pathological complete response to neoadjuvant chemotherapy. The tissue microarray comprised 191 luminal A (68.0 %), 17 luminal B (6.0 %), 27 HER2 (9.6 %), and 46 triple-negative (16.4 %) samples. ABCC1 and ABCC11 expressions were associated with significantly shorter disease-free survival ($P = 0.027$ and $P = 0.003$, respectively). ABCC1, ABCC11, and ABCG2, but not ABCB1, were expressed significantly more, and more frequently, in aggressive subtypes. Patients with HER2+ and triple-negative tumor subtypes that expressed high levels of ABCC11 had significantly worse disease-free survival ($P = 0.017$ and $P < 0.001$, respectively). We have shown, for the first time, that ABCC1, ABCC11, and ABCG2 are highly expressed in aggressive breast cancer subtypes, and that tumor ABCC11 expression is associated with poor prognosis.

Keywords Breast cancer · ATP-binding cassette transporters · ABCC11 · Tissue microarray · Subtype

Introduction

Breast cancer is a heterogeneous disease [1]. DNA microarray profiling studies on breast cancer have identified distinct subtypes: luminal A, luminal B, human epidermal

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growth factor receptor 2 (HER2)-enriched, and triple-negative (which is sometimes further subdivided into the core-basal and five-negative subtypes) [2]. These subtypes are reportedly associated with differences in resistance to chemotherapy [3–5] and subsequent outcomes [6, 7]. Several mechanisms affect how cancer cells become resistant to cytotoxic drugs, which include efflux of the drug compound from cancer cells, and others such as mutation, overexpression of the drug's targets, and drug inactivation [8].

The ATP-binding cassette (ABC) transporters are transmembrane proteins that use ATP to transport various molecules across extra- and intra-cellular membranes. This function is thought to have evolved as a xenobiotic protective mechanism [9]. Of the 49 human ABC transporters so far identified (which have been classified into seven subfamilies), ABCA2, ABCB1, ABCC1–6, ABCC11, and ABCG2 have been associated with chemoresistance in breast cancer [8]. Unfortunately, all clinical trials that have targeted ABC transporters failed to improve outcomes [10]. One explanation for this is that they all targeted ABCB1 [also known as MDR1, permeability glycoprotein 1 (P-glycoprotein or Pgp), and cluster of differentiation 243 (CD243)]. This led us to hypothesize that other ABC transporters may be more important for drug resistance.

ABCC11 is a member of the ABCC1 (also known as MDR-associated protein) sub-family. A single nucleotide polymorphism (SNP) in the *ABCC11* gene was shown to be responsible for "wet earwax" in humans [11]. Reports as to whether *ABCC11* is a risk factor for breast tumorigenesis conflict; although this gene was originally shown to be a risk factor for development of breast cancer among Japanese women [12], it is reportedly not the case in Caucasian women [13, 14]. There has been no investigation of ABCC11 protein expression levels in breast tumors or their association with cancer subtype and prognosis. We hypothesized that both frequency and expression levels of ABC transporters (ABCB1, ABCC1, ABCC11, and ABCG2) in breast tumors would differ by cancer subtype and be associated with prognosis. Here, utilizing a tissue microarray newly constructed from 281 breast cancer samples, we analyzed the expression of these transporters in light of breast cancer subtype and prognosis, as well as investigating the effects of neoadjuvant chemotherapy.

Methods

Tissue sources and clinical characteristics

Tissues for this study were obtained from 281 patients treated in Yokohama City Medical Center, Japan, between 2006 and 2008, involving all stages of breast cancer. This study was approved by the Institutional Review Board of Yokohama City University, Kanagawa, Japan, and the patients gave their informed consent before their inclusion in the study. Core biopsy samples taken prior to treatment were obtained from 50 patients who received neoadjuvant chemotherapy (35 patients received anthracycline followed by taxane; 14 received anthracycline alone; and one received taxane alone). One hundred and eight patients received adjuvant chemotherapy after surgery (45 received anthracycline followed by taxane; 38 received anthracycline alone; 15 received taxane alone; and 10 received other regimens) and 208 patients received adjuvant hormonal therapy (tamoxifen and luteinizing hormone-releasing hormone-agonist for 61 premenopausal patients; tamoxifen or aromatase inhibitor for 147 postmenopausal patients). None of the tissues described here was obtained after any treatment. All the patients were followed up at least every 3 months after surgery. The mean observation period was 49 months (range: 28–60 months). The clinical characteristics are presented in Table 1.

Table 1 Patients' characteristics

	N	%
Age		
<65	197	70.1
65≦	80	28.5
	4	1.4
Menstruation states		
Pre menopause	87	31.0
Post menopause	154	54.8
NA	40	14.2
Estrogen receptor		
Positive	210	74.8
Negative	71	25.2
NA	0	0.0
Progesterone receptor		
Positive	162	42.7
Negative	119	57.3
NA	0	0.0
HER2 overexpression		
Present	44	15.7
Absent	237	84.3
NA	0	0.0
Basal markers		
Basal	34	12.1
Non basal	235	83.4
NA	12	4.5
Subtype		
Luminal A	191	68.0
Luminal B	17	6.0
HER2	27	9.6

Table 1 continued

	N	%
Triple negative	46	16.4
Core basal	26	9.3
Five-negative	20	7.1
Tumor stage		
T1	123	43.8
T2	122	43.4
T3	11	3.9
T4	19	6.8
NA	6	2.1
Node		
N0	150	53.4
N1	83	29.5
N2	23	8.2
N3	11	3.9
NA	14	5.0
Metastases		
M0	259	92.2
M1	6	2.1
NA	16	5.7
TNM stage		
1	106	37.8
2	122	43.4
3	31	11.0
4	6	2.1
NA	16	5.7
Observation time (days)	1458 ± 509[a]	

[a] Expressed as mean ± standard deviation

Tissue microarray

The tissue microarray was constructed by taking 3.0-mm cores from representative areas of surgical specimens from patients using a KIN-2 tissue arrayer (Azumaya, Tokyo, Japan), and re-embedding these cores into a gridded paraffin block. Tissue cores were excluded from the tissue microarray if they fail to adhere to the glass slide, did not include invasive carcinoma, or were a non-interpretable specimen.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks were sliced into 5-μm sections. The sections were baked at 60 °C, deparaffinized in xylene, and gradually rehydrated in ethanol. Sections were boiled in antigen retrieval solution (Funakoshi, Japan) for 30 min. Activity of endogenous peroxidase was blocked by 20 min of quenching in 0.3 % H_2O_2 and methanol; the sections were then incubated in 5 % rabbit serum for ABCB1 and ABCC1, or goat serum for ABCC11 and ABCG2. Immunohistochemical reactions were performed overnight at 4 °C using monoclonal mouse antibodies against ABCB1 (C219; 1:100; Abcam, UK), monoclonal rat antibodies against ABCC1 (MRPr1; 1:40; Monosan, The Netherlands), polyclonal rabbit antibodies against ABCC11 (1:500) [15], or monoclonal mouse antibodies against ABCG2 (BXP-21; 1:100; Abcam). For the triple-negative subtype, cytokeratin 5/6 (D5/16 B4; Dako, Denmark) and epidermal growth factor receptor (EGFR; Roche Diagnostics K.K, Japan,) were used for subdivision into the core-basal or non-basal (five-negative) subtypes. After washing, the slides were incubated with biotinylated antibodies (15 min, room temperature) and streptavidin-biotinylated peroxidase complex (5 min, room temperature). 3,3′-diaminobenzidine (Dako Japan, Tokyo, Japan) was used as the chromogen. All sections were counterstained with Meyer's hematoxylin.

Evaluation of staining

Staining results were assessed by two pathologists independently, using a 4-point scoring system as shown in Fig. 1: 0 = invasive tumor cells present in the tissue core with no staining; 1 = invasive tumor cells present with weak staining intensity; 2 = invasive tumor cells present with strong staining intensity and <30 % of tumor cells stained or intermediate staining intensity in ≥30 % of tumor cells; and 3 = invasive tumor cells present with strong staining in ≥30 % of tumor cells. To evaluate positivity, both membranous and/or cytoplasmic staining scoring 2 or above was considered positive (high expression). CK5/6 and EGFR were considered positive when cytoplasmic and/or membranous staining of invasive carcinoma cells was observed, regardless of intensity.

Genotyping

Genotyping of ABCC11 by the SmartAmp method was performed as previously reported [12].

Statistical analysis

Statistical analysis used SPSS 19.0 for Windows software (SPSS Inc., Chicago, IL). Correlations among the clinicopathologic parameters and each transporter were evaluated by the Pearson χ^2 test, the Fisher exact test, and the Mann–Whitney test. Tukey-type multiple comparison analyses with the χ^2 test and Mantel test were carried out to compare expression of each transporter among the subtypes. Patient outcomes were assessed by disease-free survival. Survival distributions were estimated by the Kaplan–Meier method; differences were compared using the log-rank test. The multivariate Cox proportional hazard regression method

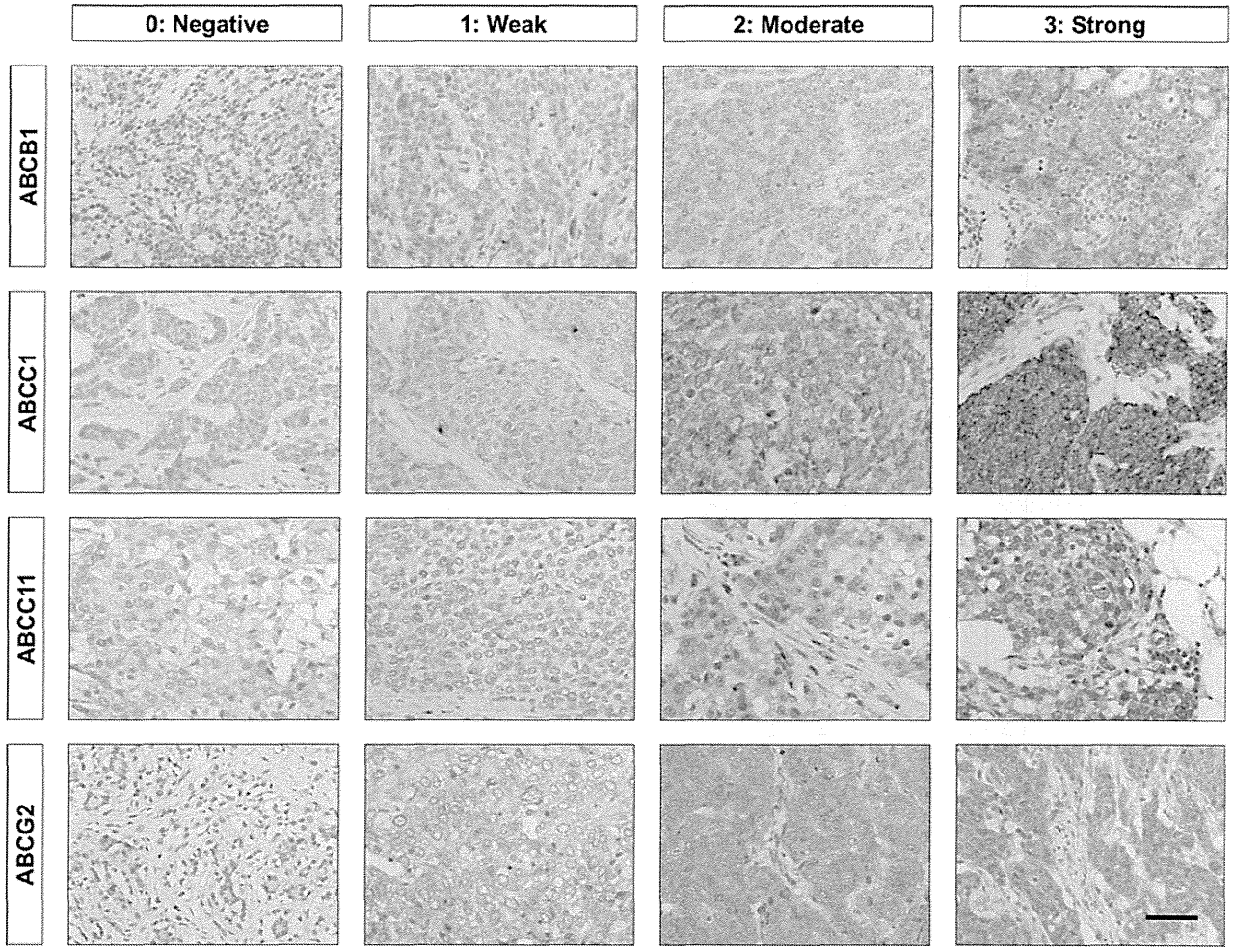


Fig. 1 4-Point scoring system for ABCB1, ABCC1, ABCC11, and ABCG2 protein expression. Our tissue microarray contained 281 breast tumor tissues, and was stained with antibodies against ABCB1 (1:100), ABCC1 (1:40), ABCC11 (1:500), and ABCG2 (1:100). Stain intensity was graded as negative (0), weak (1), moderate (2), or strong (3). Representative images are shown under high magnification. *Scale bar*: 50 μm

was used to determine the independent prognostic value. $P < 0.05$ was considered statistically significant.

Results

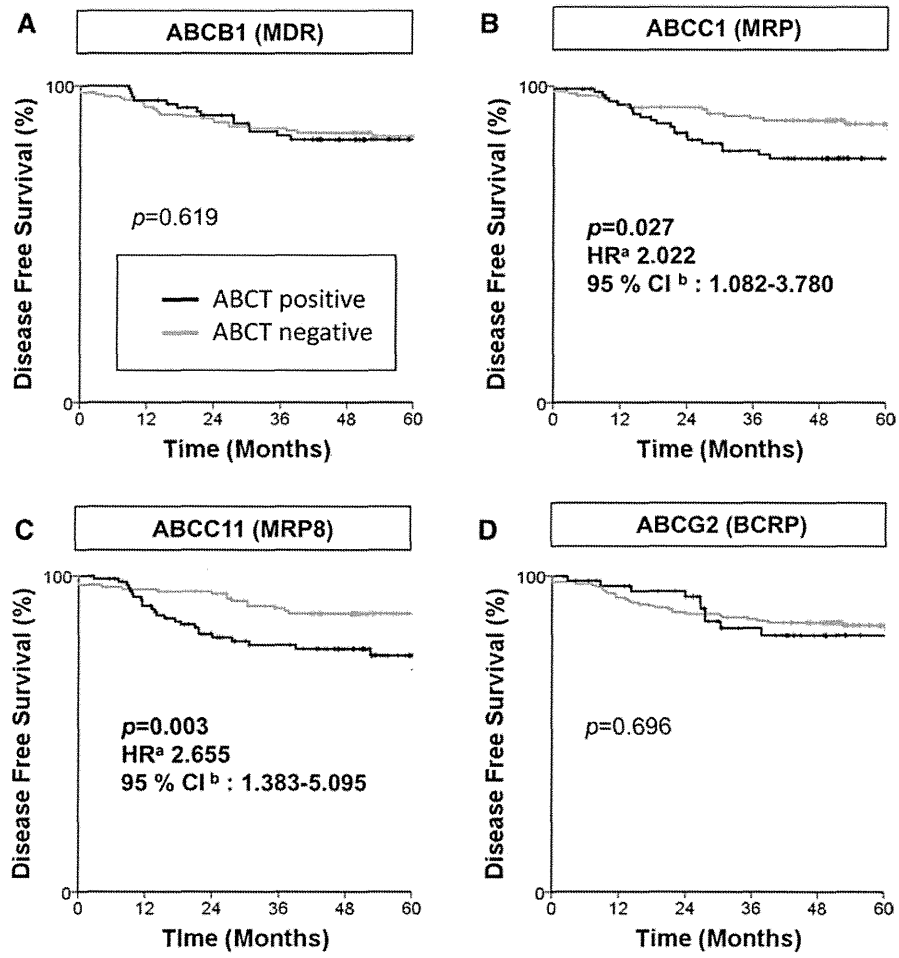
Characteristics of samples used for the tissue microarray

Subtypes of the 281 samples on the tissue microarray were determined using immunohistochemistry for the estrogen receptor (ER), progesterone receptor (PgR), and HER2, as previously reported [5, 16]. Patients' and tumor characteristics used for the tissue microarray are summarized in Table 1. The numbers of cases of the respective subtypes were: luminal A (ER+ and HER2−): 191 (68.0 %); luminal B (ER+ and HER2+): 17 (6.0 %); HER2 (ER− and HER2+): 27 (9.6 %); and triple-negative (ER− and HER2−): 46 (16.4 %). Triple-negative tumors were further sub-divided into two groups, core-basal (CK5/6+ and/or EGFR+) and five-negative (CK5/6− and EGFR−). The core-basal subtype constituted 56.5 % (26/46) of triple-negative tumors.

Associations between ABC transporter expression and clinical features of the tumors are shown in Table 2. ABCB1 was detected in 32.4 % (91/277) of the tumors, ABCC1 in 39.1 % (110/279), ABCC11 in 40.2 % (113/259), and ABCG2 in 24.2 % (68/278). There was no association between ABCB1 expression and any clinical features. ABCG2 was more frequently highly expressed in young premenopausal patients. High expressions of ABCC1 and ABCG2 were significantly more frequent in ER− tumors than in ER + ones ($P = 0.001$ and $P = 0.006$, respectively). There was no association between HER2 expression and ABC transporter expression.

Table 2 The expression of ABC transporters and clinical features

	ABCB1			*p* Value	ABCC1			*p* Value	ABCC11			*p* Value	ABCG2			*p* Value
	Negative	Positive	NA		Negative	Positive	NA		Negative	Positive	NA		Negative	Positive	NA	
N (%)	186 (66.2 %)	91 (32.4 %)	4 (1.4 %)		169 (60.2 %)	110 (39.1 %)	2 (0.7 %)		146 (52.0 %)	113 (40.2 %)	22 (7.8 %)		210 (74.7 %)	68 (24.2 %)	3 (1.1 %)	
Age																
<65	125 (63.5 %)	68 (34.5 %)	4 (2 %)	0.14	119 (60.4 %)	76 (38.6 %)	2 (1 %)	0.54	97 (49.2 %)	83 (42.1 %)	17 (8.7 %)	0.13	138 (70.0 %)	56 (28.4 %)	3 (1.6 %)	<0.01
65≦	58 (72.5 %)	22 (27.5 %)	0		49 (61.2 %)	31 (38.8 %)	0		47 (58.8 %)	28 (35.0 %)	5 (6.2 %)		69 (86.3 %)	11 (13.7 %)	0	
Menstruation status																
Pre menopause	55 (63.2 %)	29 (33.3 %)	3 (3.5 %)	0.40	51 (58.6 %)	36 (41.4 %)	0	0.32	41 (47.2 %)	37 (42.5 %)	9 (10.3 %)	0.35	54 (62.1 %)	31 (35.6 %)	2 (2.3 %)	<0.01
Post menopause	104 (67.5 %)	49 (31.8 %)	1 (0.7 %)		95 (61.7 %)	57 (37.0 %)			81 (52.6 %)	63 (40.9 %)	10 (6.5 %)		81 (52.6 %)	63 (40.9 %)	10 (6.5 %)	
Estrogen receptor																
Negative	50 (70.4 %)	21 (29.6 %)	0	0.61	30 (42.3 %)	39 (54.9 %)	2 (2.8 %)	<0.01	37 (52.1 %	27 (38.0 %)	7 (9.9 %)	0.65	43 (60.6 %)	27 (38.0 %)	1 (1.4 %)	<0.01
positive	135 (64.5 %)	70 (33.5 %)	4 (2.0 %)		139 (66.5 %)	70 (33.5 %)	0		108 (51.7 %)	86 (41.1 %)	15 (7.2 %)		166 (79.4 %)	41 (19.6 %)	2 (1.0 %)	
Progesterone receptor																
Negative	78 (65.5 %)	38 (31.8 %)	3 (2.7 %)	0.78	63 (52.9 %)	54 (45.4 %)	2 (1.7 %)	0.06	57 (47.9 %)	49 (41.2 %)	13 (10.9 %)	0.55	81 (68.0 %)	35 (29.4 %)	3 (2.6 %)	0.15
Positive	107 (66.5 %)	53 (32.9 %)	1 (0.6 %)		106 (65.8 %)	55 (34.2 %)	0		88 (54.7 %)	64 (40.0 %)	9 (5.3 %)		128 (79.5 %)	33 (20.5 %)	0	
HER2 expression																
Absent	155 (66.0 %)	77 (32.8 %)	3 (1.2 %)	1.00	146 (62.1 %)	88 (37.4 %)	1 (0.5 %)	0.18	123 (52.4 %)	97 (41.2 %)	15 (6.4 %)	1.00	179 (76.1 %)	55 (23.4 %)	1 (0.5 %)	0.33
Present	179 (76.1 %)	55 (23.4 %)	1 (0.5 %)		22 (50.0 %)	21 (47.7 %)	1 (2.3 %)		21 (47.7 %)	16 (36.4 %)	7 (15.9 %)		29 (65.9 %)	13 (29.5 %)	2 (4.6 %)	



[a] *HR* hazard ratio, [b] *CI* confidence interval

Fig. 2 Kaplan–Meier disease-free survival curves according to expression of ABCB1 (**a**), ABCC1 (**b**), ABCC11 (**c**), and ABCG2 (**d**). The *thick bold line* indicates positivity; and the *light gray line* indicates negativity, for the respective transporters. Only the ABCC1+ and ABCC11+ groups showed significantly improved survival ($P = 0.027$ and $P = 0.003$, respectively)

Expression of ABCC1 and ABCC11 is associated with poor patient survival

We compared expression of each transporter and patient disease-free survival (Fig. 2). In the entire study group, patients with ABCC1+ or ABCC11+ tumors had significantly shorter disease-free survival compared to patients with corresponding ABCC1− or ABCC11− tumors ($P = 0.027$ or $P = 0.003$, respectively).

ABC transporters are more frequently highly expressed in aggressive subtypes of breast cancer

Because breast cancer subtypes are associated with different clinical behaviors [2], we further analyzed clinical outcomes according to cancer subtype and ABC transporter expression. Expression of each transporter according to breast cancer subtype is shown in Fig. 3. The percentage of patients whose tumors expressed ABCB1 did not differ among the subtypes. ABCC1 and ABCG2 were more frequently highly expressed in triple-negative subtype, especially in the core-basal subtype, compared with the luminal A subtype, whereas highly expressed ABCC11 was more common in HER2-enriched, core-basal, and luminal A subtypes. Although core-basal tumors tended to express ABC transporters more often than five-negative tumors did, only ABCC11 showed significantly more frequent high expression in the core-basal subtype. Semi-quantification of ABC transporters expression is shown in Fig. 3b. ABCC1, ABCC11, and ABCG2 were more highly expressed in HER2-enriched and/or the core-basal subtypes, which is consistent with frequency data shown in Fig. 3a.

Patients whose tumors expressed high levels of ABCC11 tended towards decreased pathological complete responses to neoadjuvant chemotherapy

We next investigated whether there was any association between the "wet earwax" genotypes and ABCC11 expression. Figure 4a and b show the relationship between *ABCC11* genotypes and ABCC11 expression in breast

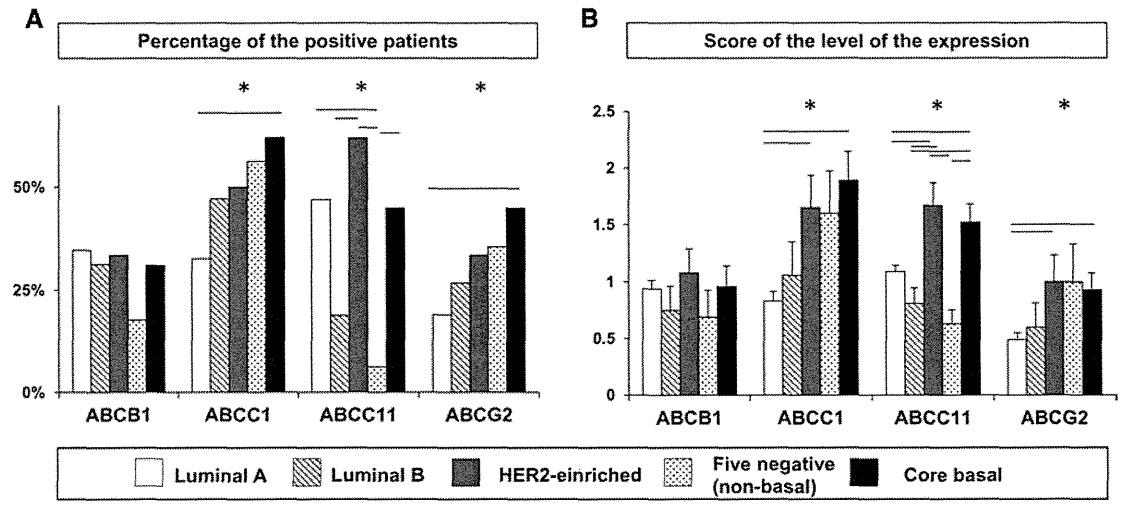


Fig. 3 Frequency (**a**) and intensity (**b**) of high ABC transporter expression classified by subtype, including luminal A (*open columns*), luminal B (*hatched columns*), HER2-enriched (*gray columns*), five-negative (*dotted columns*), and core-basal (*filled columns*). **a** Percentage of patients who showed high expression of each transporter. **b** Semi-quantification of expression level of each transporter, using a 4-point scoring system

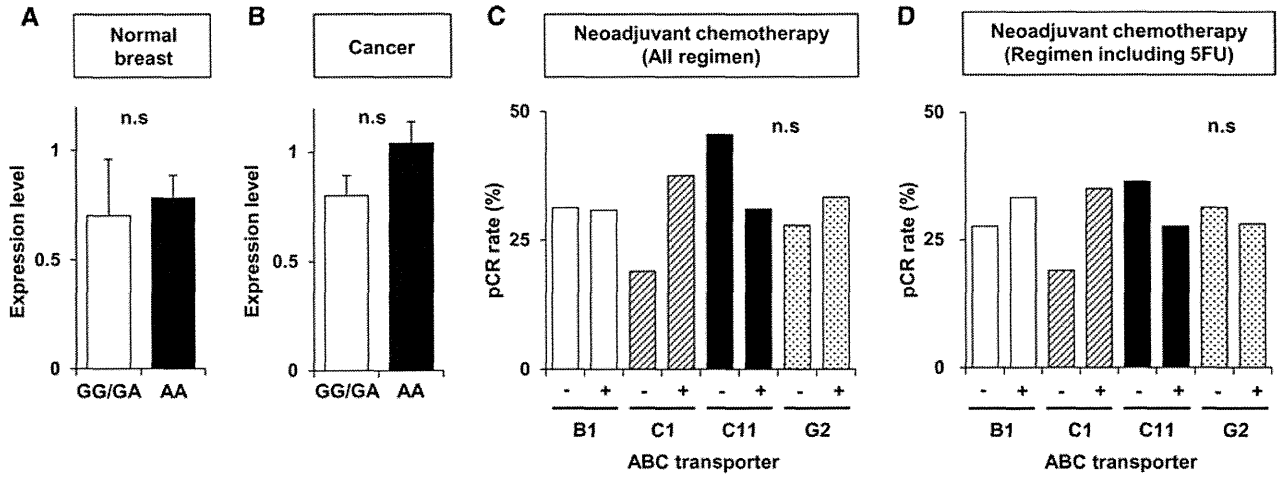


Fig. 4 Semi-quantification of ABCC11 expression levels in normal breast tissue (**a**) and cancer tissue (**b**) in patients carrying 538G/G, 538G/A (white open column, GG/GA, wet earwax phenotype), and 538A/A alleles (*black filled column* AA, dry earwax phenotype). **c**, **d** Pathological complete response ratios to neoadjuvant chemotherapy of all regimens (**c**) and regimens including 5-FU (**d**). *Bars* indicate ABCB1 (*white columns*), ABCC1 (*hatched columns*), ABCC11 (*black columns*), and ABCG2 (*dotted columns*)

cancer tissues. ABCC11 expression did not differ among the wet earwax genotype (538G/G + 538G/A) and the dry earwax genotypes (538A/A), in either normal breast tissues or breast cancer tissues.

As ABCC11 is known to efflux fluoropyrimidines (5-FU) in vitro [17], assessment of responses of ABCC11+ tumors to 5-FU-based regimens could be particularly valuable. Analysis of the association between ABC transporter expression and pathological complete response to neoadjuvant chemotherapy showed no statistically significant differences, regardless of regimen, but patients whose cancers expressed high levels of ABCC11 tended to have decreased pathological complete responses to neoadjuvant chemotherapy (Fig. 4c, d).

ABCC11+ tumors show worse prognoses among aggressive breast cancer subtypes

Because patients with ABCC1+ or ABCC11+ tumors tend to have poor prognoses, we investigated prognosis according to subtype. Patients with ABCC1+ tumors ended to have worse prognoses for luminal A tumors, but not significantly so ($P = 0.096$). Interestingly, patients with ABCC11+ tumors had significantly worse prognoses than did patients

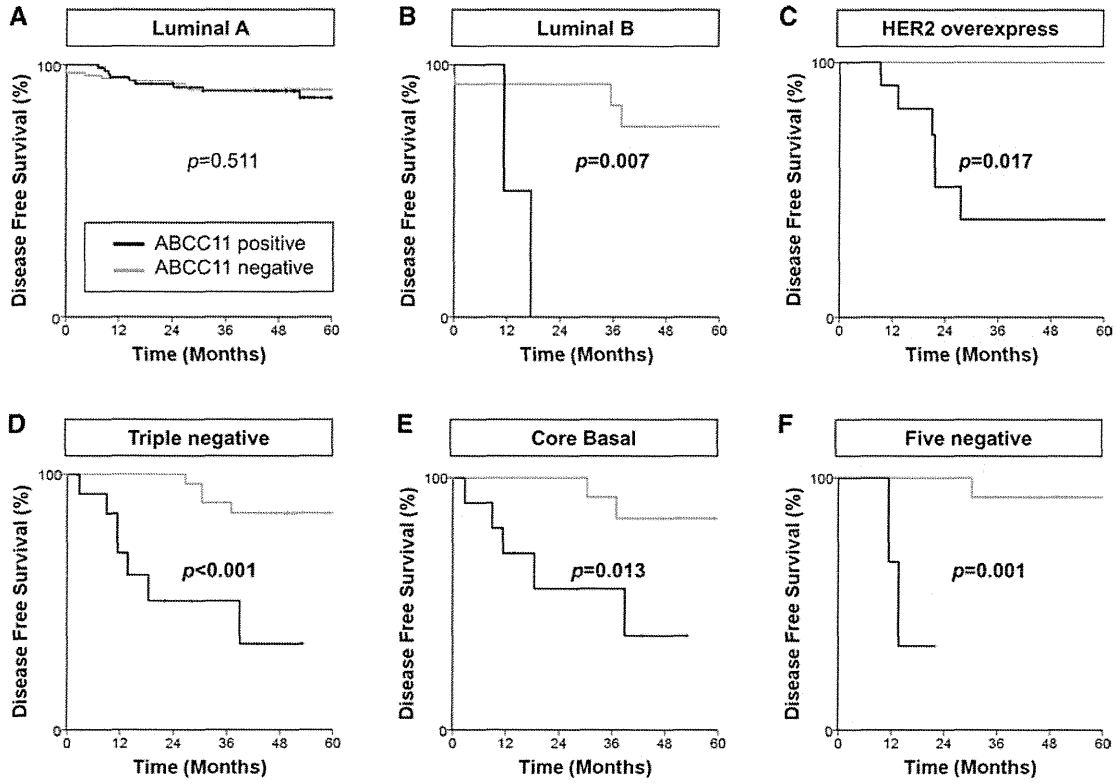


Fig. 5 Kaplan–Meier disease-free survival curve according to the subtype of breast cancer: luminal A (**a**), luminal B(B), HER2-enriched (**c**), triple-negative (**d**), core-basal (**e**), and five-negative (**f**). The *thick bold line* indicates ABCC11+, and the *light gray line* indicates ABCC11−

with ABCC11− tumors, except for the luminal A subtype, which is known to have a better prognosis than the other subtypes (Fig. 5a–f).

Discussion

Different subtypes of breast cancer have different biological behaviors, including responses to systemic and local therapies [3–5] and subsequent clinical outcomes [6, 7]. The two hormone receptor-negative subtypes, triple-negative and HER2-enriched, have poor outcomes compared with the luminal subtypes. Among the triple-negative subtypes, the core-basal subtype, which responds poorly to cytotoxic chemotherapy, has the worst prognosis. Thus, there is a particular need to elucidate drug resistance mechanisms for this subtype.

Expression of ABC transporters is reportedly related to chemoresistance [9]. Some ABC transporters, namely ABCB1, ABCC1, and ABCG2, have been identified as MDR proteins in breast cancer, which contribute to drug resistance via ATP-dependent drug efflux pumps [8]. Because ABCB1 effluxes drugs important for breast cancer—anthracyclines (doxorubicin, epirubicin, and daunomycin) and taxanes (paclitaxel, docetaxel)—ABC transporter inhibitors were the subjects of several widely anticipated clinical trials. Unfortunately, these agents proved disappointing [8, 18]. The vast majority of clinical trials targeting ABC transporters focused on ABCB1 (the most investigated ABC transporter) but data that associates patients' clinicopathological factors with ABCB1 expression tends to conflict [10]. This led us to investigate expression of multiple ABC transporters that are associated with MDR, in the context of different breast cancer subtypes. We felt that this information would be particularly relevant for the triple-negative subtype.

Patient characteristics and our tissue microarray staining data generally agree with previous reports [10, 19, 20]. The proportion of breast cancer subtypes may differ among different races or geographic populations; e.g., prevalence of the luminal A subtype may be higher, and the triple-negative subtype may be lower, in Asian women than in Western women [19]. The demographics of our tissue microarray are consistent with the prevalence among Japanese women. Leonessa et al. [10] reported that the detection rate of ABCB1 and ABCC1 in untreated tumors by immunohistochemistry was 40 % (range: 0–100 %) and 49 % (range: 20–100 %), respectively, with no clear association between ABCB1 and hormone receptors. In