teractions of treatment with covariates were used to identify predictive factors by assessing whether there was a significant difference in the treatment effect for progression-free survival (hazard ratio for progression or death) between subgroups.

Overall survival was analyzed with the use of methods that were similar to those used for the analysis of progression-free survival. The results of an early analysis are presented; follow-up with respect to overall survival is ongoing. The objective response rate (in the intention-to-treat population) and quality of life and rates of symptom reduction (among all patients with a baseline and at least one post-baseline quality-of-life assessment that could be evaluated) were assessed with the use of a logistic-regression model with the same covariates as those considered for progression-free survival to calculate odds ratios and 95% confidence intervals. Planned subgroup analyses of the objective response rate were performed with the use of methods that were similar to those used for the analysis of progression-free survival.

Adverse events were summarized for all patients who received at least one dose of the assigned study treatment. The incidence rates of 10 specified safety events (5 that were possibly associated with each study treatment) were compared with the use of Fisher's exact test; adjustment for multiple comparisons was performed with the use of the method of Westfall and Young.[24]

RESULTS

PATIENTS AND TREATMENT

From March 2006 through October 2007, a total of 1217 patients from 87 centers in Hong Kong, elsewhere in China, Indonesia, Japan, Malaysia, the Philippines, Singapore, Taiwan, and Thailand were randomly assigned to a study group (Fig. 1). The two groups were well balanced with respect to demographic and baseline characteristics (Table 1). The mean duration of treatment was 6.4 months (median, 5.6; range, 0.1 to 22.8) for gefitinib and 3.4 months (median, 4.1; range, 0.7 to 5.8) for carboplatin–paclitaxel. The median number of treatment cycles in the carboplatin–paclitaxel group was six. At the cutoff date for collection of data (April 14, 2008), a total of 24.5% of the patients in the gefitinib group were continuing to receive the study treatment; all patients in the carboplatin–paclitaxel group had discontinued the drugs. After discontinuation of the assigned treatment at any time during the study, 38.9% of the patients in the gefitinib group received carboplatin–paclitaxel, and 39.5% of the patients in the carboplatin–paclitaxel group received an EGFR tyrosine kinase inhibitor; 10.5% of the patients in the gefitinib group and 14.0% of those in the carboplatin–paclitaxel group received other anticancer treatments.

EFFICACY

The median follow-up period for the analysis of progression-free survival was 5.6 months. The median progression-free survival was 5.7 months in the gefitinib group and 5.8 months in the carboplatin–paclitaxel group, approximately coinciding with crossing of the Kaplan–Meier curves. The 12-month rates of progression-free survival were 24.9% with gefitinib and 6.7% with carboplatin–paclitaxel; a total of 950 patients had progression of disease. The study met its primary objective of demonstrating noninferiority and showed the superiority of gefitinib as compared with carboplatin–paclitaxel for progression-free survival (hazard ratio for progression or death, 0.74; 95% confidence interval [CI], 0.65 to 0.85; $P<0.001$). The probability that a patient would be free of disease progression was greater with carboplatin–paclitaxel in the first 6 months and greater with gefitinib in the following 16 months (Fig. 2A). Progression-free survival was longer in the gefitinib group than in the carboplatin–paclitaxel group in all clinical subgroups; the only clinical factor that affected progression-free survival was age (<65 years: hazard ratio, 0.81; 95% CI, 0.70 to 0.95; $P=0.007$; ≥65 years: hazard ratio, 0.58; 95% CI, 0.45 to 0.76; $P<0.001$; $P=0.03$ for the interaction of treatment with age) (Fig. 1 in the Supplementary Appendix).

A total of 1038 patients (85.3%) gave their consent for biomarker analyses, and 683 patients (56.1%) provided samples. *EGFR* mutation data for 437 patients (35.9%) could be evaluated. Patients with a tissue sample that could be evaluated had demographic characteristics that were similar to those of the overall population (Table 1 in the Supplementary Appendix). Of the 437 samples, 261 (59.7%) were positive for a mutation. Of these 261 samples, 140 (53.6%) had exon 19 deletions, 111 (42.5%) had a mutation at exon 21 (L858R), 11 (4.2%) had a mutation at exon 20 (T790M), and 10 (3.8%) had other mutations; 11 patients had multiple mutations. The proportions of mutations

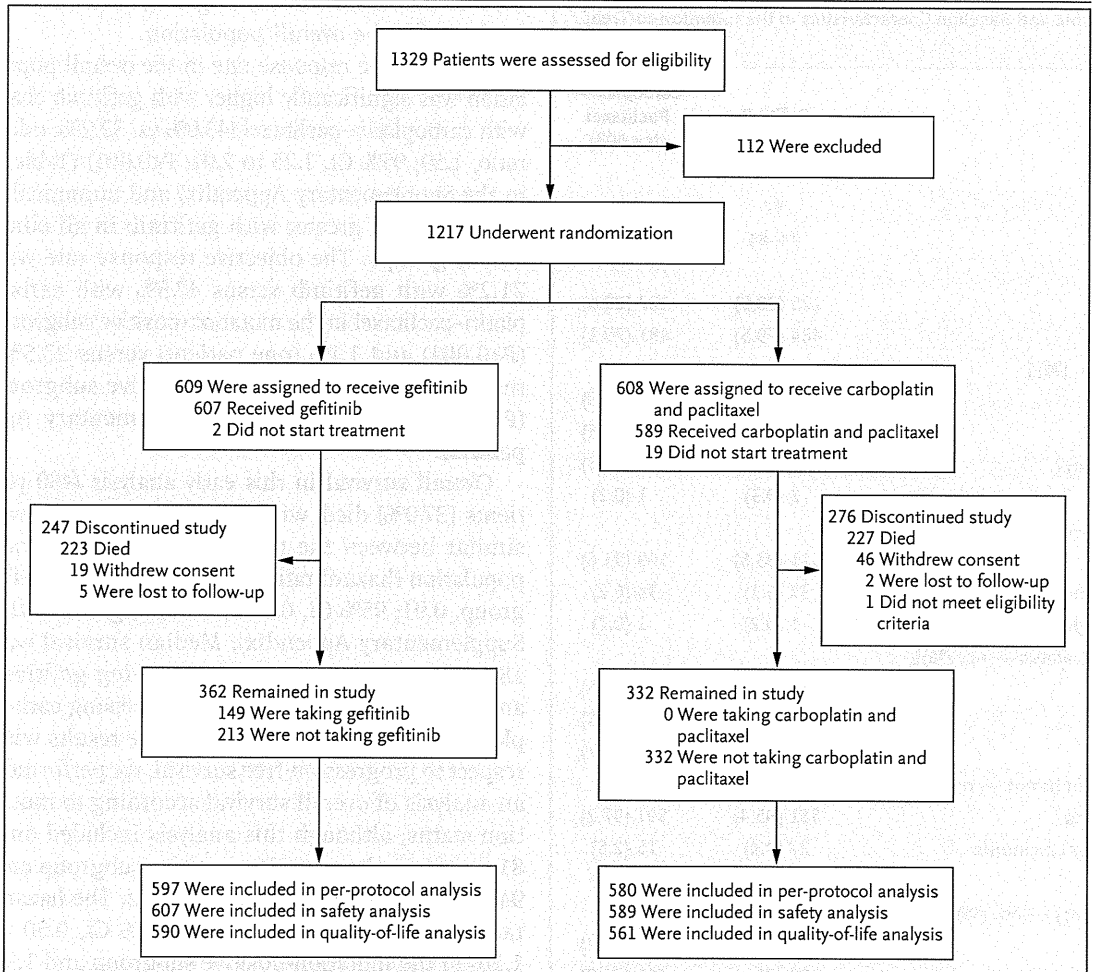

Figure 1. **Screening, Group Assignment, and Inclusion in Analyses.**
All patients who were randomly assigned to a study group were included in the intention-to-treat analysis; all patients with a baseline and at least one post-baseline quality-of-life assessment that could be evaluated were included in the quality-of-life analysis; patients who did not deviate substantially from the inclusion and exclusion criteria at entry or from the protocol were included in the per-protocol analysis; and all patients who received at least one dose of study treatment were included in the safety analysis. Among the 112 patients who were assessed for eligibility but were not assigned to a study group, the main reasons for exclusion were a serum creatinine level that was higher than 1.5 times the upper limit of the reference range or a creatinine clearance of 60 ml per minute or less; newly diagnosed central nervous system metastases that had not yet been definitively treated with surgery or radiation; or an absolute neutrophil count of less than 2.0×10^9 per liter, a platelet count of less than 100×10^9 per liter, or a hemoglobin level of less than 10 g per deciliter. A total of 63 patients who were treated with gefitinib continued to receive gefitinib after disease progression, and 1 patient who was treated with carboplatin–paclitaxel continued to receive carboplatin–paclitaxel after disease progression because the investigator believed that the treatment was providing a benefit.

were well balanced between the two groups (Table 2 in the Supplementary Appendix).

There was a significant interaction between treatment and *EGFR* mutation with respect to progression-free survival (P<0.001). Progression-free survival was significantly longer among patients receiving gefitinib than among those receiving carboplatin–paclitaxel in the mutation-positive subgroup (hazard ratio for progression, 0.48; 95% CI, 0.36 to 0.64; P<0.001) (Fig. 2B) and significantly shorter among patients receiving gefitinib than among those receiving carboplatin–paclitaxel in the mutation-negative subgroup (hazard ratio, 2.85; 95% CI, 2.05 to 3.98; P<0.001) (Fig. 2C). Results in the subgroup with unknown *EGFR*-mutation status (hazard ratio with gefitinib, 0.68; 95%

Table 1. **Demographic and Baseline Characteristics in the Intention-to-Treat Population.***

Characteristic	Gefitinib (N=609)	Carboplatin–Paclitaxel (N=608)
Age — yr		
Median	57	57
Range	24–84	25–84
Sex — no. (%)		
Male	125 (20.5)	127 (20.9)
Female	484 (79.5)	481 (79.1)
Ethnic group — no. (%)†		
Chinese	314 (51.6)	304 (50.0)
Japanese	114 (18.7)	119 (19.6)
Other East Asian‡	179 (29.4)	184 (30.3)
Other	2 (0.3)	1 (0.2)
Smoking history — no. (%)		
Never smoked	571 (93.8)	569 (93.6)
Former light smoker	37 (6.1)	38 (6.2)
Former non–light smoker	1 (0.2)	1 (0.2)
WHO performance status — no. (%)§		
0	157 (25.8)	161 (26.5)
1	391 (64.2)	382 (62.8)
2	61 (10.0)	65 (10.7)
Histologic feature of tumor — no. (%)		
Adenocarcinoma	581 (95.4)	591 (97.2)
Bronchoalveolar carcinoma	27 (4.4)	15 (2.5)
Unknown	1 (0.2)	2 (0.3)
Disease stage at entry — no. (%)		
IIIB	150 (24.6)	144 (23.7)
IV	459 (75.4)	463 (76.2)
Unknown	0	1 (0.2)
Time from diagnosis to randomization — no. (%)		
<6 mo	582 (95.6)	573 (94.2)
≥6 mo	27 (4.4)	34 (5.6)
Unknown	0	1 (0.2)
Disease stage at diagnosis — no. (%)¶		
IA	7 (1.1)	12 (2.0)
IB	2 (0.3)	9 (1.5)
IIA	2 (0.3)	1 (0.2)
IIB	1 (0.2)	6 (1.0)
IIIA	6 (1.0)	3 (0.5)
IIIB	166 (27.3)	163 (26.8)
IV	424 (69.6)	413 (67.9)
Unknown	1 (0.2)	1 (0.2)

* Percentages may not sum to 100 because of rounding.
† Ethnic group was self-reported.
‡ Other East Asian refers to patients who belong to East Asian ethnic groups other than Chinese and Japanese.
§ The World Health Organization (WHO) performance status measures level of activity and is assessed on a scale of 0 to 4, with lower numbers indicating a higher degree of activity.
¶ All patients had Stage IIIB or IV disease at entry.

CI, 0.58 to 0.81; P<0.001) (Fig. 2D) were similar to those for the overall population.

The objective response rate in the overall population was significantly higher with gefitinib than with carboplatin–paclitaxel (43.0% vs. 32.2%; odds ratio, 1.59; 95% CI, 1.25 to 2.01; P<0.001) (Table 3 in the Supplementary Appendix) and numerically or statistically greater with gefitinib in all clinical subgroups. The objective response rate was 71.2% with gefitinib versus 47.3% with carboplatin–paclitaxel in the mutation-positive subgroup (P<0.001) and 1.1% (one patient) versus 23.5%, respectively, in the mutation-negative subgroup (P=0.001) (Table 3 in the Supplementary Appendix).

Overall survival in this early analysis (450 patients [37.0%] died, with follow-up ongoing) was similar between the two groups in the overall population (hazard ratio for death in the gefitinib group, 0.91; 95% CI, 0.76 to 1.10) (Fig. 2A in the Supplementary Appendix). Median survival was 18.6 months among patients receiving gefitinib and 17.3 months among patients receiving carboplatin–paclitaxel. After observing the results with respect to progression-free survival, we performed an analysis of overall survival according to mutation status, although this analysis included only 81 deaths in the mutation-positive subgroup and 94 in the mutation-negative subgroup. The hazard ratios with gefitinib were 0.78 (95% CI, 0.50 to 1.20) in the mutation-positive subgroup and 1.38 (95% CI, 0.92 to 2.09) in the mutation-negative subgroup (Fig. 2B and 2C in the Supplementary Appendix).

Significantly more patients in the gefitinib group than in the carboplatin–paclitaxel group had a clinically relevant improvement in quality of life, as assessed by scores on the FACT-L questionnaire (odds ratio, 1.34; 95% CI, 1.06 to 1.69; P=0.01) and by scores on the TOI (odds ratio, 1.78; 95% CI, 1.40 to 2.26; P<0.001) (Fig. 3). Rates of reduction in symptoms, as assessed on the basis of the LCS scores, were similar between patients who received gefitinib and those who received carboplatin–paclitaxel (odds ratio with gefitinib, 1.13; 95% CI, 0.90 to 1.42; P=0.30) (Fig. 3). Results according to mutation status are provided in Figure 3 in the Supplementary Appendix.

SAFETY AND ADVERSE-EVENT PROFILE

Table 2 lists the most common adverse events. Gefitinib, as compared with carboplatin–paclitaxel, was associated with a lower rate of grade 3 or 4

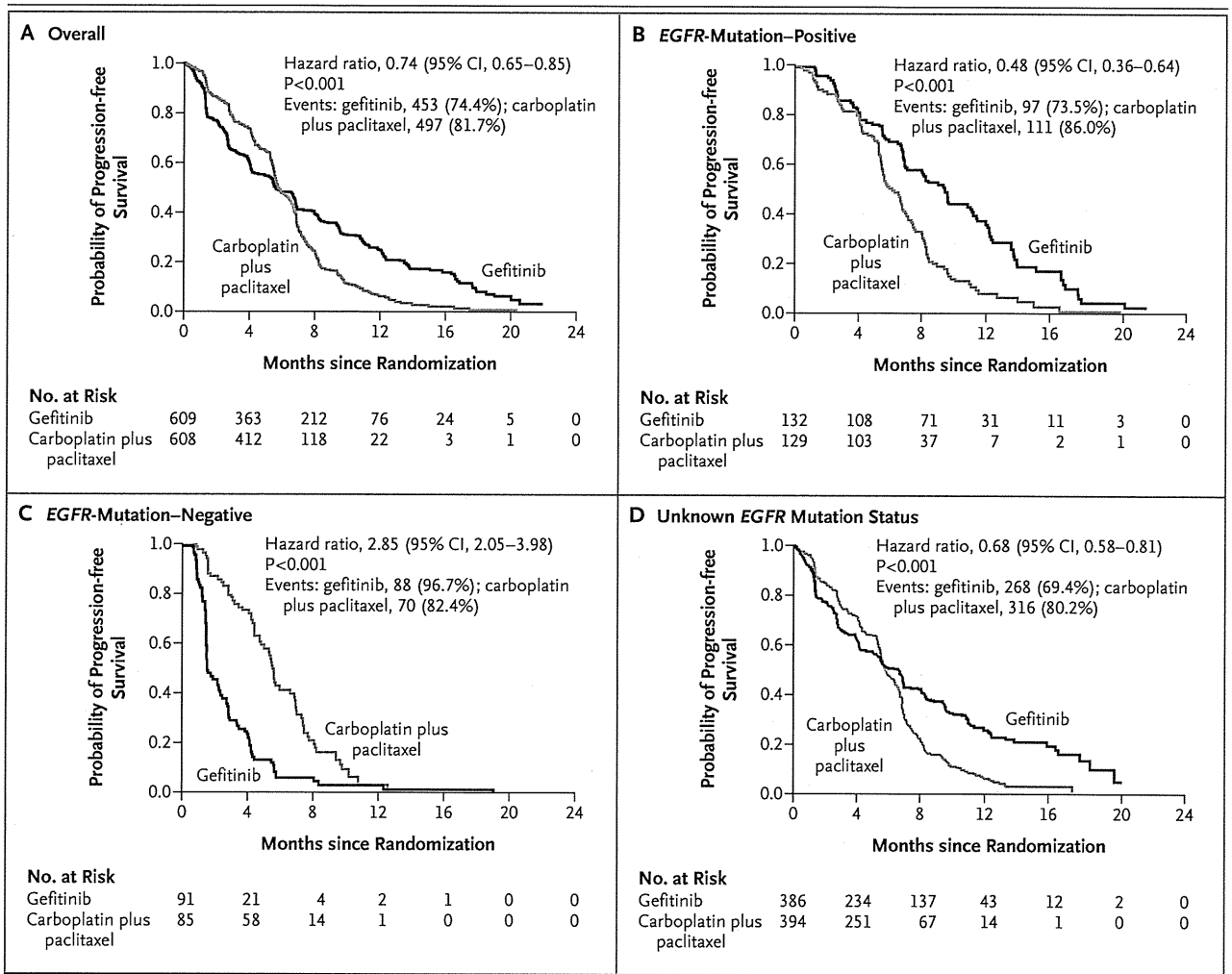

Figure 2. **Kaplan–Meier Curves for Progression-free Survival.**

Kaplan–Meier curves for progression-free survival are shown for the overall population (Panel A), patients who were positive for the *EGFR* mutation (Panel B), patients who were negative for the *EGFR* mutation (Panel C), and patients with unknown *EGFR* mutation status (Panel D). Analyses were performed on the basis of the intention-to-treat population. With respect to the overall population, results of the supportive secondary analyses (including a log-rank test, which is valid under the null hypothesis even when hazards are not proportional, and analysis in the per-protocol population) were consistent with the result of the primary analysis. Hazard ratios were calculated with the use of a Cox proportional-hazards model, with the WHO performance status (0 or 1, or 2), smoking history (nonsmoker or former light smoker), and sex as covariates. EGFR denotes epidermal growth factor receptor.

adverse events, as defined according to the Common Terminology Criteria for Adverse Events (28.7% vs. 61.0%), a lower rate of adverse events leading to discontinuation of the drug (6.9% vs. 13.6%), and a lower rate of dose modification due to toxic effects (16.1% vs. 35.2% for carboplatin and 37.5% for paclitaxel). Adverse events leading to death occurred in 3.8% of the patients treated with gefitinib and in 2.7% of the patients treated with paclitaxel–carboplatin; serious adverse events, including death, occurred in 16.3% and 15.6% of patients in the two groups, respectively; and serious adverse events leading to hospitalization occurred in 13.8% and 13.1% of patients in the two groups, respectively. The incidences of rash or acne, diarrhea, and elevated liver aminotransferase levels were significantly higher with gefitinib than with carboplatin–paclitaxel, whereas the incidences of neurotoxic effects, nausea and vomiting, and hematologic toxic effects were significantly higher with carboplatin–paclitaxel (Table 4 in the Supplementary Appendix). Interstitial-lung-disease events (i.e., the acute respiratory distress syndrome, interstitial lung disease, pneumonitis, or radiation

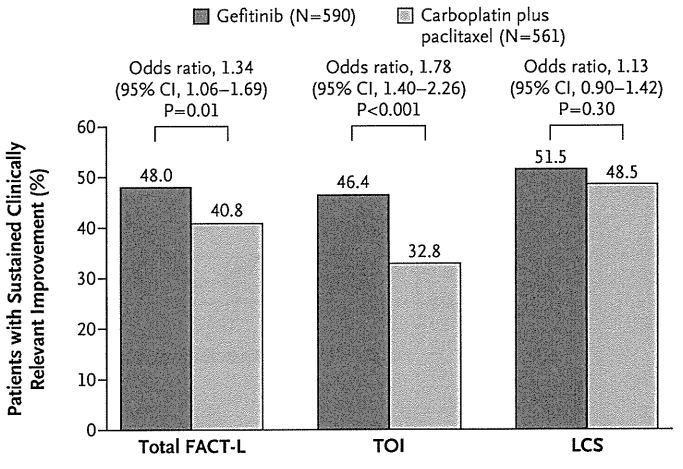

Figure 3. **Rates of Improvement in Scores for Quality for Life and Symptoms.** Calculations were performed on the basis of all patients with a baseline and at least one post-baseline quality-of-life assessment that could be evaluated. P values were calculated with the use of logistic regression, with the WHO performance status (0 or 1, or 2), smoking history (nonsmoker or former light smoker), and sex as covariates. Clinically relevant improvement was predefined as an improvement of six points or more in scores on the Functional Assessment of Cancer Therapy–Lung (FACT–L, in which scores range from 0 to 136, with higher scores indicating better quality of life) and Trial Outcome Index (TOI, in which scores range from 0 to 84, with higher scores indicating better quality of life) or an improvement of two points or more in scores on the lung-cancer subscale (LCS) of the FACT–L (in which scores range from 0 to 28, with higher scores indicating fewer symptoms), with the higher scores maintained for at least 21 days.

pneumonitis) occurred in 16 patients treated with gefitinib (2.6%), 3 of whom died, and in 8 patients treated with carboplatin-paclitaxel (1.4%), 1 of whom died.

DISCUSSION

Platinum-based combination chemotherapy, such as carboplatin–paclitaxel, is the standard first-line therapy for advanced non–small-cell lung cancer.[25,26] The results of this trial showed that gefitinib by itself is superior to carboplatin–paclitaxel in a selected population of East Asian patients.

As initial treatment of non–small-cell lung cancer in East Asian nonsmokers or former light smokers with pulmonary adenocarcinoma, gefitinib, as compared with carboplatin–paclitaxel, prolonged progression-free survival, increased the objective response rate, reduced toxic effects, and improved quality of life. The overall benefit was driven primarily by the subgroup of patients with *EGFR* mutations; in this subgroup, patients treated with gefitinib, as compared with those treated with carboplatin–paclitaxel, had a remarkably high objective response rate (71.2%) and prolonged progression-free survival (hazard ratio for progression or death, 0.48; 95% CI, 0.36 to 0.64; P<0.001). In the subgroup of patients without *EGFR* mutations, the objective response rate with gefitinib was 1.1%, and progression-free survival favored chemotherapy (hazard ratio with gefitinib, 2.85; 95% CI, 2.05 to 3.98; P<0.001). These contrasting outcomes probably explain the change over time in treatment effect for progression-free survival in the overall population. The initial superiority of carboplatin–paclitaxel was attributed to the benefit that the *EGFR*-mutation–negative subgroup received from chemotherapy but not from gefitinib, whereas prolonged progression-free survival in the *EGFR*-mutation–positive subgroup explained the subsequent improvement favoring gefitinib. Crossing of the Kaplan–Meier curves did not occur in the mutation-positive subgroup or the mutation-negative subgroup.

Lynch et al. found specific *EGFR* mutations that correlated with tumor response to gefitinib.[7] In the Iressa Survival Evaluation in Lung Cancer trial (ISEL; ClinicalTrials.gov number, NCT00242801), the objective response rate for gefitinib-treated patients was 37.5% among the 16 patients with a tumor bearing an *EGFR* mutation as compared with 2.6% among the 116 patients without a mutation.[27] Our trial confirms the predictive value of *EGFR* mutations for the responsiveness of pulmonary adenocarcinoma to gefitinib as compared with carboplatin–paclitaxel. The difference in the rates of objective response between gefitinib-treated patients with an *EGFR* mutation and those without an *EGFR* mutation (71.2% vs. 1.1%) was remarkable. The rate of an objective response to first-line gefitinib in our study is similar to rates reported in other studies in which patients were selected according to *EGFR*-mutation status, including patients in Western countries.[10,12,28] Sequist et al. screened patients (who were selected on the basis of clinical characteristics) for an *EGFR* mutation and reported an objective response rate of 54.8% among 31 gefitinib-treated patients who were positive for an *EGFR* mutation, only 2 of whom were Asian.[12] However, in our study, objective response rates among patients without an *EGFR* mutation were lower than expected, given the results of previous studies.[16,29] One possible explanation is our use of ARMS, a more sensitive technique for detecting *EGFR* mutations.[21,22] When Zhu et al. used ARMS to reanalyze 148 samples

Table 2. **Adverse Events.***

Adverse Event	Gefitinib (N=607)		Carboplatin–Paclitaxel (N=589)	
	All Adverse Events	CTC Grade 3, 4, or 5	All Adverse Events	CTC Grade 3, 4, or 5
	number (percent)			
Rash or acne†	402 (66.2)	19 (3.1)	132 (22.4)	5 (0.8)
Diarrhea	283 (46.6)	23 (3.8)	128 (21.7)	8 (1.4)
Dry skin	145 (23.9)	0	17 (2.9)	0
Anorexia†	133 (21.9)	9 (1.5)	251 (42.6)	16 (2.7)
Pruritus†	118 (19.4)	4 (0.7)	74 (12.6)	1 (0.2)
Stomatitis†	103 (17.0)	1 (0.2)	51 (8.7)	1 (0.2)
Asthenic conditions†	102 (16.8)	2 (0.3)	259 (44.0)	11 (1.9)
Nausea	101 (16.6)	2 (0.3)	261 (44.3)	9 (1.5)
Paronychia	82 (13.5)	2 (0.3)	0	0
Vomiting	78 (12.9)	1 (0.2)	196 (33.3)	16 (2.7)
Constipation	73 (12.0)	0	173 (29.4)	1 (0.2)
Alopecia	67 (11.0)	0	344 (58.4)	0
Neurotoxic effects†	66 (10.9)	2 (0.3)	412 (69.9)	29 (4.9)
Myalgia	47 (7.7)	3 (0.5)	186 (31.6)	10 (1.7)
Arthralgia	39 (6.4)	1 (0.2)	113 (19.2)	6 (1.0)
Neutropenia‡				
Any	NA	22 (3.7)	NA	387 (67.1)
Febrile	1 (0.2)	1 (0.2)	17 (2.9)	17 (2.9)
Anemia‡	NA	13 (2.2)	NA	61 (10.6)
Leukopenia‡	NA	9 (1.5)	NA	202 (35.0)

* Calculations were based on 1196 patients who received at least one dose of the study treatment. The Common Terminology Criteria (CTC) grade is defined on the basis of the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0. Events are included if they occurred in at least 10% of patients in either treatment group, either while the patients were receiving treatment or during the 28-day follow-up, and if there was at least a 5% difference between groups. There were other adverse events that occurred in few patients and that may or may not have been related to the study drug. NA denotes not available.

† This is a group term (sum of high-level and preferred terms, according to the definitions in the *Medical Dictionary for Regulatory Activities*).

‡ Data are from the laboratory reports of 599 patients who were taking gefitinib and 577 who were taking carboplatin–paclitaxel. Events were included if there was a worsening in the laboratory value (absolute neutrophil count in the case of neutropenia, hemoglobin in the case of anemia, and white-cell count in the case of leukopenia) from baseline to CTC grade 3 or 4.

that had previously been classified as negative for an *EGFR* mutation, they found 11 new samples with exon 19 mutations.[30] Another possible explanation is that studies that showed higher response rates among mutation-negative patients were not always conducted in previously untreated patients. Mutation-negative status that is determined in a diagnostic sample obtained at the time of the initial presentation may change during subsequent tumor progression or during the course of chemotherapy.[31]

Our findings suggest that, whenever possible, *EGFR*-mutation status should be determined before the initial treatment of pulmonary adenocarcinoma. Ethnic origin, smoking status, and histologic findings help to identify patients who have a high likelihood of having an *EGFR* mutation; in this study, 59.7% of the tumors in a clinically selected population had *EGFR* mutations, as compared with 12.1% and 14.8% in the unselected populations in the ISEL and Iressa in NSCLC Trial Evaluating Response and Survival versus Taxotere (INTEREST; NCT00076388) studies, respectively.[2,27]

The efficacy of gefitinib seen in this study was coupled with lower incidences of alopecia, nausea, vomiting, neurotoxic symptoms, and myelosuppression than those seen with carboplatin–paclitaxel. Among 607 patients who received gefitinib

and who were included in the safety analysis, interstitial-lung-disease events developed in only 16 (2.6%), 3 of whom (0.5%) died.

In summary, this study shows that first-line therapy with gefitinib as compared with carboplatin–paclitaxel prolongs progression-free survival, increases the objective response rate, and improves quality of life among clinically selected patients with non–small-cell lung cancer. The presence of an *EGFR* mutation was a robust predictor of improved progression-free survival with gefitinib, as compared with carboplatin–paclitaxel, and of the benefit of gefitinib with respect to the objective response rate, indicating that patients in whom an *EGFR* mutation has been identified will benefit most from first-line therapy with gefitinib.

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APPENDIX

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SNP Communication

Genetic Polymorphisms of Copper- and Platinum Drug-efflux Transporters *ATP7A* and *ATP7B* in Japanese Cancer Patients

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Summary: ATP7A and ATP7B are involved in cellular resistance to platinum compounds such as cisplatin. By sequencing *ATP7A*, 38 genetic variations, including 30 novel ones were detected from 203 Japanese cancer patients. Of these, seven nonsynonymous variations were found: novel 1030A>G (R344G), 2111A>G (Q704R), 2200C>A (Q734K), 2948C>T (T983M) and 3112G>A (V1038I) at 0.004 frequencies and known 2299G>C (V767L) and 4390A>G (I1464V) at 0.351 and 0.075 frequencies, respectively. Regarding *ATP7B*, 28 novel and 33 known genetic variations were detected including 13 nonsynonymous ones: novel 1258A>G (M420V), 1426G>A (A476T), and 2401A>C (T801P) were found at 0.002, 0.005, and 0.002, respectively and known 1216G>T (A406S), 1366G>C (V456L), 2495A>G (K832R), 2785A>G (I929V), 2855G>A (R952K), 2871delC (P957PfsX9), 3419T>C (V1140A), 3836A>G (D1279G), 3886G>A (D1296N) and 3889G>A (V1297I) at 0.483, 0.463, 0.387, 0.005, 0.384, 0.005, 0.387, 0.002, 0.012, and 0.015 frequencies, respectively. Linkage disequilibrium between detected variations was also analyzed. Our results would provide fundamental and useful information for genotyping *ATP7A* and *ATP7B* in the Japanese and probably other Asian populations.

Keywords: ATP7A; ATP7B; genetic variation; amino acid alteration; linkage disequilibrium

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On Aug. 19, 2009, these variations were not found on the homepage of the Japanese Single Nucleotide Polymorphisms (JSNP) (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB (http://www.pharmgkb.org/do/) database.
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Introduction

ATP7A and ATP7B are copper transporters that sequester copper from the cytosol into the trans-Golgi network for loading onto copper-requiring enzymes.[1] ATP7A is expressed in the majority of tissues except for the liver, while ATP7B expression is found mainly in the liver, but also in the kidney and placenta.[1-4] Under elevated copper levels in polarized cells, ATP7A relocates toward the basolateral plasma membranes, while ATP7B travels to the apical side of the membrane to export the metal from the cell. Both proteins are predicted to have 8 transmembrane domains (TMD).[1,4,5] Several functionally important motifs facing the cytoplasm have been found: 6 repeated metal binding motifs (GMxCxxCxxIE) in the N-terminal domain; the transduction motif (TGExxP) in the loop between TMDs 4 and 5; ATP binding (GDGxNDxD) and phosphorylation motifs (DKTGTLT) in the loop between TMDs 6 and 7 and the endocytic signal LL in the C-terminal.[5] Certain mutations in *ATP7A* and *ATP7B* abrogate protein function and cause Menkes and Wilson diseases, respectively.[1-3,5] The *ATP7A* gene located on q13.2-q13.3 of the X chromosome consists of 23 exons spanning approximately 140 kb. The *ATP7B* gene spanning *ca.*79 kb is comprised of 21 exons and located on chromosome 13q14.3. The two transporter proteins share ~65% amino acid sequence similarity.

Recent studies demonstrate that ATP7A and ATP7B are involved in cellular resistance to platinum compounds such as cisplatin.[5,6] Regarding ATP7A, the resistance to cisplatin, carboplatin and oxaliplatin has been observed through sequestration of the drugs into intracellular vesicles in an ATP7A-transfected cell line.[7] Oxaliplatin exposure to HT29 cells enhances ATP7A expression.[8] As for ATP7B, Komatsu *et al.* showed that overexpression of ATP7B conferred cisplatin resistance to a human epidermal carcinoma cell line through ATP-dependent decrease of drug accumulation.[9] Similar resistance to carboplatin due to increased expression of ATP7B has been reported,[10] while oxaliplatin resistance is controversial depending on the cell line used.[11] It has been reported that tumor tissues show higher expression levels of ATP7A[12] and ATP7B[13,14] proteins than corresponding normal tissues and that this higher expression is associated with shorter survival times in cisplatin or carboplatin-based chemotherapy. Higher ATP7B expression levels in tumors are also associated with shorter time to progression in colorectal cancer patients treated with oxaliplatin-based chemotherapy.[15] The polymorphisms of *ATP7A* and *ATP7B* may thus possibly affect the efficacy or toxicity of platinum drugs. In this study, we sequenced the *ATP7A* and *ATP7B* genes of 203 Japanese subjects to survey novel variations of these genes.

Materials and Methods

Human genomic DNA samples: A total of 203 Japanese cancer patients administered paclitaxel/carboplatin (90 non-small cell lung and 6 other cancer patients) or oxaliplatin/5-fluorouracil/leucovorin (107 colorectal cancer patients) participated in this study. The ethical review boards of the National Cancer Center, the Aichi Cancer Center and the National Institute of Health Sciences approved this study. Written informed consent was obtained from all participating patients. Genomic DNA for sequencing was extracted from blood leukocytes.

PCR conditions for sequencing *ATP7A* The reference sequences (GenBank), NT_011651.17 (genomic) and NM_000052.4 (mRNA) were used for assignment of nucleotide positions and primer design. For sequencing *ATP7A*, two sets of long-range PCRs were made to amplify all 23 exons from 50 ng of genomic DNA using multiple primers (1 μM) and 0.02 units/μl of Z-Taq (Takara Bio Inc., Shiga, Japan). In the first set, 5 pairs of primers amplified the regions from the promoter region to exon 2 and from exons 7 to 18; in the second set, 2 pairs of primers amplified from exons 3 to 6 and from exons 19 to 23. The primers were designed in the promoter or intronic regions as listed in "1st PCR" of **Table 1**. The conditions for the 1st round PCR were 30 cycles of 98°C for 5 sec, 55°C for 10 sec and 72°C for 190 sec. Next, in the 2nd round PCR, the promoter region and exonic regions, except for exon 1, were separately amplified using the 1st PCR products as templates by Ex-Taq (0.02 units/μl, Takara Bio Inc.) with the primers (0.2 μM) listed in "2nd PCR" of **Table 1**. Because of a high GC content, exon 1 was amplified using 0.05 units/μl of LA-Taq (Takara Bio Inc.) in GC buffer I with 0.5 μM of the primers shown in **Table 1**. The 2nd round PCR conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min and then a final extension at 72°C for 7 min. Thereafter, the PCR products were treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and directly sequenced on both strands using an ABI BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the primers listed in "Sequencing" of **Table 1**. Excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany). The eluates were analyzed on an ABI Prism 3730XL DNA Analyzer (Applied Biosystems). All detected rare variations were confirmed by repeating the PCR from the genomic DNA and sequencing newly generated PCR products.

PCR conditions for sequencing *ATP7B*: The following sequences obtained from GenBank were used as reference sequences of *ATP7B*: NT_024524.14 (genomic) and NM_000053.2 (mRNA). First, multiplex long-range PCR was performed to amplify the promoter region and

Table 1. Primers used for sequencing *ATP7A*

		Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified length (bp)
1st PCR	Mix 1	Promoter to Exon 1	GAGCCTCTCCCTCTTTTTACTGTTA	GTGTCAAAGATAAGATGCCACAGGG	1,755
		Exon 2	TCTTGGAAGTCACACCTTGTCGCTT	TAGTGAGACCCCCATCGCTACAAAA	2,373
		Exon 7 to Exon 12	ATTTGTGGTATGCCCTTTGGTCAAT	GCGGTTTCCCCTATGCTGTTGTCAT	8,077
		Exon 13 to Exon 14	TTTTCCTGTCTTTTTCTGAGCCCTC	CACAGTCCAGTTCTGCTTTACCACT	3,073
		Exon 15 to Exon 18	CCTCCTGCCTTAGCCTCCAAAAGTA	GAGAGAGACAAAATGGGCACTTTAT	11,619
	Mix 2	Exon 3 to Exon 6	TAAATCTTCTGACTCCCAACCCAGT	GAGCCACCACACCCAGCCTACATTT	17,069
		Exon 19 to Exon 23	ACGGAGTTTCTCTCTTGTTGCCCAA	AAACCTCACCTTCAAAAGCCTTGCC	11,876
2nd PCR		Promoter	AGAGACTGTAACACTTTTGC	CCACGGGAAAGAGAGCGACT	774
		Exon 1[a]	ACACAGTCTACGGGAAGCAAGTTA	TCACTAAGCAAAGACCCCAGTCCA	1,116
		Exon 2	CAGGAAGAATGCTTACCATA	GTTCAGTATGAGATTCAGAG	615
		Exon 3	CCATTAGATTGAGTTGTCTC	ACCTCAATGATACAGCAAGC	727
		Exon 4	TGATGACAAGAATGAGAGAG	CCACGAGTTATTGTTTCCAG	1,055
		Exon 5	TGCGGAGGAAAGTGTAGAGA	GGTTGTCCCACACATTACTG	509
		Exon 6	GTTTGGGGTCAAGACTGGTA	GCTTGAAGAGTACCATTAGA	488
		Exon 7	AAGAATCACTTGAACCTGGA	CCTTTGCCTAACTTTTCCTG	541
		Exon 8 to Exon 9	GTATTCCCCAGAGTGACTTG	TGAACTCTTTCTTAGGGGTT	825
		Exon 10	TCTCCCTTTAGTGTTTATGG	AGCAAACTGATGTGACAGACTTAG	864
		Exon 11	TTGTGTACTTCGTCTTTCTG	CTGGGAGACAGATTATGTGA	425
		Exon 12	GTTCACTAACAGTAAGCAAG	AGCCACAAAGTAAATCTGAG	461
		Exon 13	GGTTTTTCCAGTTCAAGGTT	GAACTTAGGAGGTCAAGGGT	564
		Exon 14	TTTATAGAAACAGGGTCTCC	TTGACAGTAAATGACAGAGC	709
		Exon 15	TTCTGGAATCTCAGTATGTC	CCTACCTCAAATCTCTGGAT	544
		Exon 16	TCCCGAAGACCATCAGTTTT	AGTCTTTTTAGCCTCATACC	459
		Exon 17	CAAAATCCACTGTCAAGTAG	CATAGGGTATTGACTTGAGG	487
		Exon 18	CACTGTTGGAGGCTATGTTC	GAATAACCCTCATAGTTCAG	376
		Exon 19	AAGTCTGTGTGGGCTTAGAG	AGGAACCAGATAGGACTACT	421
		Exon 20	CCACATCCTTGCTATCACTA	ATGACTTCCCATAATCCCAC	503
		Exon 21	AAAGTGTTTTCAGAACCCTG	CACCATACCAGTAGGCTACA	444
		Exon 22	ATACCCCACAGAAACTCTCA	TAGTAGACATAGGGTTTCAC	576
		Exon 23	ACTAAGTGTGGATGAGCAAA	AAAGATGGGAGGCAGGGAAC	1,134
			GTGCTTTTTTAGATGCTCCA	CTGGTAATGGGAACAAAATG	1,182
			AGTTAGTGTGGTTGGCAAAT	GCAGTATTTTTGATTCCCTC	1,070
			ACAGGAGAAAGAGGTGATTA	GTGCTCTATCTGGTTACTCA	960
Sequencing[b]		Promoter	AGAGACTGTAACACTTTTGC	CCACGGGAAAGAGAGCGACT	
		Exon 1	GGACTCGTACCCTAACAAAG	GTTAGGGGAGGTAAAACATA	
		Exon 4	TGATGACAAGAATGAGAGAG	GAAACTACTATGCTGCTTAC	
			GTAAGCAGCATAGTAGTTTC	CCACGAGTTATTGTTTCCAG	
		Exon 5	GAGGAAAGTGTAGAGATAAC	GAGAACAAAAAAGATGGAGC	
		Exon 7	AAAAAAAGTGGTAACTCAT	GAAGTGTTCAAAGGAGTTAG	
		Exon 8 to Exon 9	GTATTCCCCAGAGTGACTTG	CATTGTGACCATTTCATCCA	
			CTGGATGAAATGGTCACAAT	TGAACTCTTTCTTAGGGGTT	
		Exon 10	TCTCCCTTTAGTGTTTATGG	AGACATACTGTACTATCTAC	
				TATTTCTCATTTGTCTCTCT	
		Exon 14	AAAGTGTTGGGATTACAGGT	CTCTCCCACTCCAAACCTTT	
		Exon 22	TCTACCACCAAGAGGATAAA	ATGGTTTGGGCTTATCATTG	
		Exon 23	ACTAAGTGTGGATGAGCAAA	GCAGCAGTTCAGCAATCTCT	
			GCCCAAGAAGAAGAAAATGA	CAATGAAAAACCACCTAAAC	
			GTGCTTTTTTAGATGCTCCA	CGAAACCCCGTCTCTACTGA	
			TATTTTTCAGTAGAGACGGG	CTGGTAATGGGAACAAAATG	
			AGTTAGTGTGGTTGGCAAAT	CATTGGTCTAAAAAAAGGGC	
			AAGGCAAACCCATTTCACTG	GCAGTATTTTTGATTCCCTC	
			ACAGGAGAAAGAGGTGATTA	ATGACACACCATACATCTTG	
			GTAGTCTCAAGATGTATGGT	GTGCTCTATCTGGTTACTCA	

[a] LA-Taq with GC buffer I was used for amplification because of its high GC content.
[b] Exons not listed were sequenced using 2nd PCR primers.

Table 2. Primers used for sequencing *ATP7B*

	Amplified or sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplified length (bp)
1st PCR	Promoter to Exon 1	GGTAGCATTCCTGGGGTTTTTTCCT	ACCAGGCTCTGAGTAACTTCTCCAG	2,148
	Exon 2 to Exon 4	GTGTGTAAGTGACTCTATGATGGTC	ATGAACAATGTCACCTGTACTCGGA	9,526
	Exon 5 to Exon 9	TCCCACTCCTGATGCTGAACCAATG	CTAACCCCAAGGAAATACAGAAGCC	10,283
	Exon 10 to Exon 16	TCACCAGTATTTCCCCCTTGTCTGT	TGTACTCTGTGCGACACCAGTCTGT	11,551
	Exon 17 to Exon 21	GCTCAGATTCTATCCTGGGCTTTAC	TCGTAAGTGGGAGATGAACAATGAG	8,565
2nd PCR	Promoter to Exon 1[a]	GCCTTCCAGCCAATAGAATA	TTTCTCCCACGCCAAGACAT	1,145
	Exon 2	GTTGTGTGAGAACGACATTT	AGAAGGCTCTCACCAGATGT	1,825
	Exon 3	GAGGGACAAGGTAGTTACTG	AATGCCAGTTATACAAGGAC	573
	Exon 4	GAGACCAGACATCGTGATTG	CATTGTTGTCGGCTTCAAAG	517
	Exon 5	AGGGAAAGGCTCTTGGCTGC	CTTTCTCTTACCCATTCACT	480
	Exon 6	GAGGCACTTTTAGATTCACT	GAGGGTTCACATTACAAGGG	334
	Exon 7	ATGTGACAAAGGCAGGTCTT	GCCCTTAGTAGTCCCCCACA	496
	Exon 8	CATAAACGCCCATCACAGAG	TAAGTCTGTCTCTATGCTGT	492
	Exon 9	AGAGCCTTTTATCGTGCCGT	TGCCCACACTCACAAGGTCT	335
	Exon 10 to Exon 12	AACAGTGCCTGGTATTCAGC	GGCTTAGATTTTGCTGTCAA	1,061
	Exon 13	ATGGCAGAGCAGTGTGGAAT	TCAGGCTTTTCTCTCAATGT	428
	Exon 14	AACCCTGAGATTGAACGACA	CTTTGTGATAACCTGGAACT	532
	Exon 15	AGTTCCCGCTTTCCGCTGCT	CCCAAGAACATAAGAGAAAC	458
	Exon 16	AGAGGTGCTTACAAGGTTAC	ACAATCTTCTGGAAAACAGG	419
	Exon 17	TGCTTCCAGACTTTTGTGTA	AGAGAAAAGCATCCAGCAAG	460
	Exon 18 to Exon 19	CAACATCACTGACTGGACCC	AAACAGCCTTTCTAAAACGC	644
	Exon 20	TGGGAACATCAGGGCGAGTGGAA	TTGAGGAGCAGAGTAAGGGC	574
	Exon 21	CTCTTGAGGTTTTGATACTG	AGCAAAGACCACAAGGACAT	1,010
		TGTGCTTGTCAGTGGGGACC	AGTGAAACTAACCATCCAAG	1,162
		GCACTTGATTCAGGAGGTCA	ATCCTCCTCTGCCCCCTAAA	550
Sequencing[b]	Promoter to Exon 1	GCCTTCCAGCCAATAGAATA	TGAGAGCGTGAGGGGAGAGT	
		ACTCTCCCCTCACGCTCTCA	TTTCTCCCACGCCAAGACAT	
	Exon 2	GTTGTGTGAGAACGACATTT	GGACCTTGCCTTCAATGGAG	
		TGCCATCGGTTGTGTGCCTG	ACTGGGCTGGTACAAGAAGG	
		CTTGGAGAACAAAACTGCCC	AGAAGGCTCTCACCAGATGT	
	Exon 10 to Exon 12	AACAGTGCCTGGTATTCAGC	CCCAGAACTCTTCACATAAT	
		TAACTTCATCTTTCTCGTTTTAG	GGCTTAGATTTTGCTGTCAA	
	Exon 20	TCAGGGCGAGTGGAAGAGAG	GTGAATGGGCAGCAGTGAAT	
	Exon 21	TAGAATGGCTCAGATGCTGT	GGGCAGGATGACTGGACATA	
		TATGTCCAGTCATCCTGCCC	AGCAAAGACCACAAGGACAT	
		TGTGCTTGTCAGTGGGGACC	CTCCTTTTCTGAAGCCCCTG	
		TGTGTGGCTTGGAGGAAATG	AGTGAAACTAACCATCCAAG	
		GCACTTGATTCAGGAGGTCA	ATCCTCCTCTGCCCCCTAAA	

[a] LA-Taq with GC buffer I was used for amplification because of its high GC content.
[b] Exons not listed were sequenced using 2nd PCR primers.

all 21 exons of *ATP7B* from 50 ng of genomic DNA with 0.025 units/μl of Z-Taq and five sets of primers (in "1st PCR" of **Table 2**, 1 μM) designed in the promoter or intronic regions. The 1st round PCR conditions were 30 cycles of 98°C for 5 sec, 55°C for 10 sec, and 72°C for 190 sec. Next, exonic regions, except for promoter to exon 1 region, were amplified separately in the 2nd round PCR using the 1st PCR products as templates by Ex-Taq (0.02 units/μl) with the primers (0.2 μM) listed in "2nd PCR" of **Table 2**. Because of its high GC content the promoter to exon 1 region was amplified using 0.05 units/μl of LA-Taq in GC buffer I with 0.5 μM of the primers listed in **Table 2**. The 2nd round PCR conditions, purification of the PCR products and sequencing with the primers listed in "Sequencing" of **Table 2** were performed as described in the above *ATP7A* section. All rare variations were confirmed by repeating PCR from the genomic DNA and sequencing newly generated PCR

Table 3. Summary of *ATP7A* variations detected in this study

SNP ID		Location	Position		Nucleotide change	Amino acid change	Frequency	
This Study	dbSNP (NCBI)		NT_011651.17	From the translational initiation site or from the end of the nearest exon				95% Confidence interval
MPJ6_A7A001[a]		5′-Flanking	462076_462077	−61371_−61370 (−586_−585)[b]	TTACATCTTGGC/ins 98bp/AGTTAACACAGT		0.004	0.000–0.010
MPJ6_A7A002	rs17174131	5′-Flanking	462154	−61293 (−508)[b]	GACTTATAAGGAT>GCTTTTATGTTAC		0.086	0.059–0.113
MPJ6_A7A003[a]		5′-Flanking	462472	−60975 (−190)[b]	GCCGCCGCGGCGG>TGGGGTGGGAAAA		0.004	0.000–0.010
MPJ6_A7A004[a]		5′-UTR, Exon 1	462520	−60927 (−142)[b]	GCTGCCGCCGCCG>ACAGCCGCAGCTA		0.004	0.000–0.010
MPJ6_A7A005[a]		Intron 2	523760	IVS2+194	GATATATTTTCAA>GTTTAAAAACATC		0.183	0.145–0.220
MPJ6_A7A006[a]		Intron 2	523829	IVS2+263	TATTTTATAAGTA>GTATGAGTATTTA		0.004	0.000–0.010
MPJ6_A7A007[a]		Intron 3	541000	IVS3–37	AAGTAGCCCAGGA>GATAACTGAATTA		0.004	0.000–0.010
MPJ6_A7A008[a]		Exon 4	541456	1030[c]	CCGGGGCTATATA>GGAGTTAGTATCA	Arg344Gly	0.004	0.000–0.010
MPJ6_A7A009[a]		Intron 4	541816	IVS4+54	CTTCCATTTTGCT>CGCTTCTTTTGGC		0.037	0.019–0.056
MPJ6_A7A010[a]		Intron 5	550575_550576	IVS5+86_87	TGTAACTATGTT/insT/ATGATTCTTGGT		0.343	0.297–0.389
MPJ6_A7A011[a]		Exon 9	563418	2111[c]	TCCTGGAGCGCCA>GGATTCTTCCAGG	Gln704Arg	0.004	0.000–0.010
MPJ6_A7A012[a]		Intron 9	563491_563492	IVS9+12_+13	GCAAGTGAATTG/insAATTG/CAAATATATTTG		0.019	0.005–0.032
MPJ6_A7A013[a]		Exon 10	564711	2200[c]	TACTTCTACATTC>AAGGCTTATAAAG	Gln734Lys	0.004	0.000–0.010
MPJ6_A7A014	rs2227291	Exon 10	564810	2299[c]	ATTATTCTTCTAG>CTTGCAATGTATG	Val767Leu	0.351	0.304–0.397
MPJ6_A7A015[a]		Intron 10	565122	IVS10+205	ATAGTACAGTATG>ATCTGTTTATTTT		0.004	0.000–0.010
MPJ6_A7A016	rs5959964	Intron 10	566283	IVS10–184	AAACATTTTCTAG>TTGAAACATTTTG		0.295	0.250–0.339
MPJ6_A7A017	rs7053543	Intron 13	572344	IVS13+141	TTTTGAGATAGGG>ATCTCACTCTGTT		0.351	0.304–0.397
MPJ6_A7A018[a]		Intron 13	572721	IVS13−29	ATGCTTCTTCTTC>ATTATTATTGTTG		0.351	0.304–0.397
MPJ6_A7A019[a]		Exon 15	581086	2948[c]	CCCGAACAGAAAC>TGATAATACGATT	Thr983Met	0.004	0.000–0.010
MPJ6_A7A020[a]		Exon 16	583206	3112[c]	ATTTTTTTACAGG>ATAAAGGTAGTGG	Val1038Ile	0.004	0.000–0.010
MPJ6_A7A021[a]		Intron 18	590825	IVS18+37	TAACTCAATGTTT>GTGTTATTGTTTT		0.004	0.000–0.010
MPJ6_A7A022[a]		Intron 21	597158	IVS21−117	AATCTCTACCAC/delC/AAGAGGATAAAT		0.004	0.000–0.010
MPJ6_A7A023	rs2234938	Exon 23	598262	4390[c]	AGCAGAGCCTCTA>GTAAACTCACTAC	Ile1464Val	0.075	0.049–0.100
MPJ6_A7A024[a]		3′-UTR	598480	4608[c] (*105)[d]	TTTTCTCATGCTC>TTTATATTAGGGA		0.004	0.000–0.010
MPJ6_A7A025[a]		3′-UTR	598705	4833[c] (*330)[d]	CAAAAAAAAAAAG>CGCCCAAGAAGAA		0.004	0.000–0.010
MPJ6_A7A026[a]		3′-UTR	598947	5075[c] (*572)[d]	CTGCATCCTTGTC>TCTTGCAGGTGCT		0.004	0.000–0.010
MPJ6_A7A027[a]		3′-UTR	599056	5184[c] (*681)[d]	CTGACAACTGTTC>GTAATATTTTGCT		0.004	0.000–0.010
MPJ6_A7A028[a]		3′-UTR	599309	5437[c] (*934)[d]	CAAAGATTAAAAC>TTATTATACATAT		0.056	0.034–0.078
MPJ6_A7A029[a]		3′-UTR	599390_599392	5518_5520[c] (*1015_*1017)[d]	TTGTTGTTGTTG/delTTG/AGACAGAGTCTT		0.011	0.001–0.021
MPJ6_A7A030[a]		3′-UTR	599466	5594[c] (*1091)[d]	ACCTCTGCCTACC>TGGATTCAAGGAA		0.004	0.000–0.010
MPJ6_A7A031[a]		3′-UTR	599855	5983[c] (*1480)[d]	ACTAAAATTTCCC>TTAGGTTATGACG		0.343	0.297–0.389
MPJ6_A7A032	rs1062471	3′-UTR	600286	6414[c] (*1911)[d]	GTAGGGGATGGAG>CTTCTTCCTTTCC		0.325	0.279–0.370
MPJ6_A7A033	rs1062472	3′-UTR	600335	6463[c] (*1960)[d]	CATATATACACAT>CGCAAAGTTTACA		0.422	0.374–0.470
MPJ6_A7A034[a]		3′-UTR	600567	6695[c] (*2192)[d]	TATTTATTATTTT>AAATTCCAGTGGC		0.004	0.000–0.010
MPJ6_A7A035	rs17139614	3′-UTR	600616	6744[c] (*2241)[d]	TTCTAGAAGACAG>CAGCTGATAGGGT		0.078	0.052–0.104
MPJ6_A7A036[a]		3′-UTR	600837	6965[c] (*2462)[d]	ACAGAAAACATGC>ATAATTAGAAAAA		0.004	0.000–0.010
MPJ6_A7A037[a]		3′-UTR	600904	7032[c] (*2529)[d]	CACAAGTCTTTTT>CTGCAATCTTGAA		0.004	0.000–0.010
MPJ6_A7A038[a]		3′-UTR	601497	7625[c] (*3122)[d]	TTTTTTAAAAAGT>CATTCTTTATTCA		0.004	0.000–0.010

[a] Novel variations detected in this study.
[b] Positions in parenthesis are calculated by skipping the intron 1.
[c] Positions in cDNA (NM_000052.4).
[d] Numbered from termination codon TAA.

products.

Linkage disequilibrium (LD) analysis: Hardy-Weinberg equilibrium and LD analysis were performed by SNPAlyze software (Dynacom Co., Chiba, Japan) and pairwise LD between variations with minor allele frequency (MAF) greater than 0.03 was analyzed using r^2 values.

Results and Discussion

For *ATP7A*, the 5′-flanking region (up to 872 bases upstream of exon 1), all 23 exons and their flanking introns were sequenced for 203 Japanese subjects. Thirty-eight genetic variations, including 30 novel ones were detected (see **Table 3**): 3 were in the 5′-flanking region, 1 in the 5′-untranslated region (UTR), 7 in the coding exons (7 nonsynonymous variations), 12 in the introns and 15 in the 3′-UTR. Since we did not find any significant differences in the frequencies of these variations between the 96 patients with carboplatin- and 107 patients with oxaliplatin-based chemotherapies (by Fisher's exact test, $P>0.13$), the data for all subjects were analyzed as one group. Since this gene resides on the X-chromosome, allele frequencies were also compared between 138 males and 65 females and no significant differences were found (by Fisher's exact test, $P>0.24$). In the female patients (with two X chromosomes), detected variations were in Hardy-Weinberg equilibrium ($P\geqq 0.10$). Five novel nonsynonymous variations, 1030A>G (R344G), 2111A>G (Q704R), 2200C>A (Q734K), 2948C>T (T983M) and 3112G>A (V1038I), were found as heterozygotes in single patients at 0.004 frequencies (**Table 3**). Among these, Q734 is presumed to be the first amino acid following TMD2 and is conserved between ATP7A and ATP7B.[4] Using the PolyPhen program (http://genetics.bwh.harvard.edu/pph/) to predict functional effects of amino acid substitutions, Q734K was expected to probably alter the protein function based on the PSIC (position specific independent count) profile score differences derived from multiple alignments. R344G and Q704R substitutions were predicted to have possible functional alterations. The effects of T983M and V1038I were predicted as benign. Functional analysis for these variations is warranted. Moreover, it is necessary to evaluate real frequencies of very rare variations found in only one subject (frequency: 0.004). We also detected the previously published variations 2299G>C (V767L) and 4390A>G (I1464V) at 0.351 and 0.075 frequencies, respectively.

Regarding *ATP7B*, 61 genetic variations including 28 novel ones, were detected by sequencing the 5′-flanking regions (up to 768 bases upstream of exon 1), all 21 exons and their flanking introns of 203 Japanese subjects: 9 were in the 5′-flanking region, 2 in the 5′-UTR, 19 in the coding exons (13 nonsynonymous and 6 synonymous ones), 25 in the introns, 5 in the 3′-UTR and 1 in the 3′-flanking region (see **Table 4**). Just as with *ATP7A*, no significant differences were found in the frequencies of these variations between patients with carboplatin- and patients with oxaliplatin-based chemotherapies (by Fisher's exact test, $P>0.20$) and the data for all subjects were analyzed as one group. Detected variations were in Hardy-Weinberg equilibrium ($P>0.05$), except for −408T>C and IVS13−129C>T. The deviations were probably caused by an unexpected occurrence of one extra homozygote in these low-frequency variations. Three novel nonsynonymous variations, 1258A>G (M420V), 1426G>A (A476T) and 2401A>C (T801P) were found at 0.002, 0.005 and 0.002, respectively. The PolyPhen program predicted that M420V and T801P, located within conserved regions between ATP7A and ATP7B, probably had damaging effects on protein function. Functional analysis should be conducted for these variations. Moreover, it is necessary to evaluate real frequencies of very rare variations found in only one subject (frequency: 0.002). We also detected 10 known nonsynonymous variations, 1216G>T (A406S), 1366G>C (V456L), 2495A>G (K832R), 2785A>G (I929V), 2855G>A (R952K), 2871delC (P957PfsX9), 3419T>C (V1140A), 3836A>G (D1279G), 3886G>A (D1296N) and 3889G>A (V1297I) at 0.483, 0.463, 0.387, 0.005, 0.384, 0.005, 0.387, 0.002, 0.012 and 0.015 frequencies, respectively. Of these, 2871delC (P957PfsX9), the most frequent causative variation for Wilson disease in Japanese,[16] causes a frame-shift downstream of codon 957, resulting in an early stop codon at codon 966. This variation most probably results in a non-functional protein without 34% of the protein at the C-terminus, including TMDs 6–8 and the large cytoplasmic loop containing the ATP binding site.[3] Compared to Chinese healthy individuals, MAFs in this study are lower for V456L (0.463 in Japanese *vs.* 0.609 in Chinese) and comparable for K832R and V1140A (0.387 *vs.* 0.42 for both variations), respectively.[17] Functional changes were not observed for K832R, I929V and R952K when assessed by growth of recombinant yeast in the presence of copper cations.[18]. Known variations −119_−118insCGCCG and −75A>C were detected at 0.488 and 0.468 frequencies, these values being higher than those in Chinese volunteers (0.218 for −119_−118insCGCCG and 0.372 for −75A>C).[17]

Using the detected variations at >0.03 frequencies, linkage disequilibrium (LD) was analyzed. For *ATP7A*, using 14 variations, strong linkages ($r^2>0.8$) were observed between −61293T>G and 6744 (*2241) G>C, and among IVS5+86_87insT, 2299G>C (V767L), IVS13+141G>A, IVS13−29C>A, and 5983 (*1480)C>T.

As for the 22 common variations (MAF>0.03) of *ATP7B*, strong linkages ($r^2>0.8$) were observed among −520C>T, −119_−118insCGCCG and −75A>C; between 1216G>T (A406S) and IVS2+287A>G;

Table 4. Summary of *ATP7B* variations detected in this study

SNP ID		Reference	Location	Position		Nucleotide change	Amino acid change	Frequency	
This Study	dbSNP (NCBI)			NT_024524.14	From the translational initiation site or from the end of the nearest exon				95% Confidence interval
MPJ6_A7B001[a]			5′-Flanking	33566377	−904	GTAGACTAGTGTT>ACGGCGTGGCGCA		0.005	0.000–0.012
MPJ6_A7B002[a]			5′-Flanking	33566130	−657	TCTTGCCGCGGT/delT/GCTTCCTTTGGG		0.002	0.000–0.007
MPJ6_A7B003	rs28362533		5′-Flanking	33566061	−588	AGCGCAGAGCGGA>CCCCGACGCGGCG		0.017	0.005–0.030
MPJ6_A7B004[a]			5′-Flanking	33566055	−582	GAGCGGACCCGAC>TGCGGCGCCGCCG		0.005	0.000–0.012
MPJ6_A7B005	rs9563084		5′-Flanking	33565993	−520	CTGAGTCTGCGGC>TCCGGCTCTGCGC		0.488	0.439–0.536
MPJ6_A7B006	rs28362532		5′-Flanking	33565881	−408	GGAGGACAGGCCT>CCCGCCCTGCGGC		0.039	0.020–0.058
MPJ6_A7B007[a]			5′-Flanking	33565841	−368	GACATTGTGGCAC>GTGGCACGGCAGA		0.002	0.000–0.007
MPJ6_A7B008[a]			5′-Flanking	33565835	−362	GTGGCACTGGCAC>GGGCAGAGAACAC		0.002	0.000–0.007
MPJ6_A7B009[a]			5′-Flanking	33565751	−278	GCGAGGGTCCGAG>TGCCCACTCTCCC		0.002	0.000–0.007
MPJ6_A7B010	rs28362531	19)	5′-UTR	33565592_33565591	−119_−118	CGAGCCGCGCCG/insCGCCG/ATGCCCTCACAC		0.488	0.439–0.536
MPJ6_A7B011	rs2277448	19)	5′-UTR	33565548	−75	GACTTTAACACCA>CCGCTCTCCTCCA		0.468	0.419–0.517
MPJ6_A7B012[a]			Exon 2	33528876	480[b]	CTGTGTCAGCTCC>AATTGAAGGCAAG	Ser160Ser	0.002	0.000–0.007
MPJ6_A7B013		16)	Exon 2	33528234	1122[b]	TGCATCCTGTGTC>GCATTCCATTGAA	Val374Val	0.002	0.000–0.007
MPJ6_A7B014	rs1801243	19)	Exon 2	33528140	1216[b]	CTTTATAATCCCG>TCTGTAATTAGCC	Ala406Ser	0.483	0.434–0.532
MPJ6_A7B015[a]			Exon 2	33528098	1258[b]	GCTATAGAAGACA>GTGGGATTTGAGG	Met420Val	0.002	0.000–0.007
MPJ6_A7B016	rs1951922		Intron 2	33527784	IVS2+287	GATATGGAATTTA>GTTTCTTATAGTT		0.483	0.434–0.532
MPJ6_A7B017	rs3742288		Intron 2	33524978	IVS2−93	GGGAGCCGGGACA>CATGAACCCTCAC		0.463	0.415–0.512
MPJ6_A7B018	rs1801244	19)	Exon 3	33524805	1366[b]	ACACCTACATCTG>CTGCAGGAAGTGG	Val456Leu	0.463	0.415–0.512
MPJ6_A7B019[a]			Exon 3	33524745	1426[b]	CCGGACATCTTGG>ACAAAGTCCCCAC	Ala476Thr	0.005	0.000–0.012
MPJ6_A7B020[a]			Intron 3	33524588	IVS3+40	TAGGAATGCTGCG>ATATAGACCTCGT		0.002	0.000–0.007
MPJ6_A7B021[a]			Intron 3	33522913	IVS3−170	ATCGTGATTGTCG>AAAGGCTTTCCAA		0.025	0.010–0.040
MPJ6_A7B022	rs2147363		Intron 3	33522796	IVS3−53	TTGACTGTGTCAA>CCCTAGAGGCCCT		0.463	0.415–0.512
MPJ6_A7B023	rs9535809		Intron 5	33516114	IVS5−65	AAAGTGCTTTCTG>ACCAATGCATATT		0.037	0.019–0.055
MPJ6_A7B024[a]			Exon 6	33516023	1896[b]	TGCTTCCCTGGCC>ACAGAGAAACCCC	Ala632Ala	0.002	0.000–0.007
MPJ6_A7B025[a]			Intron 6	33515876	IVS6+97	TTCCCATGGTGCC>TTTCCTCCTGGAT		0.002	0.000–0.007
MPJ6_A7B026[a]			Intron 6	33514462	IVS6−4	TGCATTTGCTTTC>TCAGGTGGAAGAA		0.020	0.006–0.033
MPJ6_A7B027[a]			Exon 9	33511698	2401[b]	TCTCTCCAAGCCA>CCAGAAGCCACCG	Thr801Pro	0.002	0.000–0.007
MPJ6_A7B028[a]			Intron 9	33511612	IVS9+40	TGGTTGGTATCTA>GTCAATCTGTGTG		0.005	0.000–0.012
MPJ6_A7B029	rs9526811		Intron 9	33504560	IVS9−25	GAGTGGCCATGTG>AAGTGATAAGTGG		0.350	0.303–0.396
MPJ6_A7B030	rs1061472	19)	Exon 10	33504488	2495[b]	GCGATATCGTCAA>GGGTGGTCCCTGG	Lys832Arg	0.387	0.339–0.434
MPJ6_A7B031	rs2281814		Intron 10	33504327	IVS10−30	ATGGGGCTGAGCA>GAGTGACAGTTGT		0.010	0.000–0.019
MPJ6_A7B032		18)	Exon 12	33503878	2785[b]	GTCCCATTTATCA>GTCATCATGTCAA	Ile929Val	0.005	0.000–0.012
MPJ6_A7B033	rs732774	19)	Exon 12	33503808	2855[b]	GTGTTGTTCAGAG>AATACTTTCCTGT	Arg952Lys	0.384	0.337–0.431
MPJ6_A7B034	rs2296246		Intron 12	33500704	IVS12−90	ACGTTGTGTCCAG>TTGCCCCCCTGAA		0.345	0.299–0.391
MPJ6_A7B035	rs7325983		Intron 12	33500627	IVS12−13	GCCTCTGACTCTG>CTCCTGTTTTCAG		0.030	0.013–0.046

Table 4. (Continued)

SNP ID		Reference	Location	Position		Nucleotide change	Amino acid change	Frequency	
This Study	dbSNP (NCBI)			NT_024524.14	From the translational initiation site or from the end of the nearest exon				95% Confidence interval
MPJ6_A7B036		16)	Exon 13	33500609	2871[b]	TTTTCAGAACCC/ delC /AACAAGCACATC	Pro957ProfsX9	0.005	0.000–0.012
MPJ6_A7B037	rs1801247	19)	Exon 13	33500471	3009[b]	CGGGGTGGCCGC G>A CAGAACGGCATC	Ala1003Ala	0.007	0.000–0.016
MPJ6_A7B038	rs17076121		Intron 13	33498556	IVS13–129	GACAGAGGATCA C>T GTTAGGAAGCTG		0.017	0.005–0.030
MPJ6_A7B039	rs17076116		Intron 14	33498207	IVS14+38	CCCTCCCCGCCA A>G TGCTCTTTTATT		0.002	0.000–0.007
MPJ6_A7B040[a]			Intron 14	33498125	IVS14+120	AAAACCACTTAG A>G GGGCCCTTCTGC		0.005	0.000–0.012
MPJ6_A7B041[a]			Intron 14	33498080	IVS14+165	TCACAGTCAGCC/ delC /TTGCCACAGTTC		0.007	0.000–0.016
MPJ6_A7B042[a]			Intron 15	33496515	IVS15+7	AAAAAGGTATTG C>T TGGCTTTTGTCT		0.002	0.000–0.007
MPJ6_A7B043	rs1801249	19)	Exon 16	33495354	3419[b]	GAATAGATGCAG T>C CCCCCAGACCTT	Val1140Ala	0.387	0.339–0.434
MPJ6_A7B044[a]			Intron 16	33495135	IVS16+82	GTCCTCCTTTAT A>G AAAGAAAAGAAG		0.002	0.000–0.007
MPJ6_A7B045[a]			Exon 17	33493319	3567[b]	AGGTGTGCTCTG T>C GGGATGATCGCA	Cys1189Cys	0.002	0.000–0.007
MPJ6_A7B046		21)	Exon 18	33491679	3836[b]	TGGCCCAGGCAG A>G CATGGGTGTGGC	Asp1279Gly	0.002	0.000–0.007
MPJ6_A7B047		22)	Exon 18	33491629	3886[b]	ATCGAGGCAGCC G>A ACGTCGTCCTTA	Asp1296Asn	0.012	0.002–0.023
MPJ6_A7B048		20)	Exon 18	33491626	3889[b]	GAGGCAGCCGAC G>A TCGTCCTTATCA	Val1297Ile	0.015	0.003–0.027
MPJ6_A7B049	rs2282057	19)	Intron 18	33491606	IVS18+6	TATCAGAGTGAG C>T GTGGCTGCAGCC		0.397	0.349–0.444
MPJ6_A7B050[a]			Exon 19	33491443	3990[b]	CCTGGCACTGAT T>C TATAACCTGGTT	Ile1330Ile	0.002	0.000–0.007
MPJ6_A7B051	rs9535795		Intron 19	33491362	IVS19+50	AGAAAGGCTTCT G>C TCTCCCAGGTTC		0.394	0.347–0.442
MPJ6_A7B052[a]			Intron 19	33490036	IVS19–205	GAGAGCCAGGCC C>T ACTCAACAGCAT		0.007	0.000–0.016
MPJ6_A7B053	rs2282059		Intron 19	33489990	IVS19–159	AGCCTCACTTTG G>C GGGGGGCCTGTG		0.037	0.019–0.055
MPJ6_A7B054[a]			Intron 19	33489990	IVS19–159	AGCCTCACTTTG G>T GGGGGGCCTGTG		0.002	0.000–0.007
MPJ6_A7B055[a]			Intron 20	33489547	IVS20+182	CATGAGCAGGCA A>G TTCACTGCTGCC		0.002	0.000–0.007
MPJ6_A7B056[a]			3′-UTR	33487929	5361b (*963)[c]	AGCCTCCCTGCA C>T GGCCCAAGGGGC		0.005	0.000–0.012
MPJ6_A7B057[a]			3′-UTR	33487764	5526b (*1128)[c]	ACGCTGCCCAGG G>A GCTTCAGAAAAG		0.002	0.000–0.007
MPJ6_A7B058	rs1051332		3′-UTR	33487720	5570b (*1172)[c]	AAGGGAGCATCT G>A TTTACCTGGCAG		0.350	0.303–0.396
MPJ6_A7B059[a]			3′-UTR	33487483	5807b (*1409)[c]	CAACCAACCAGC A>C GGGTAGCTATTA		0.007	0.000–0.016
MPJ6_A7B060	rs928169		3′-UTR	33487110	6180b (*1782)[c]	TTTCAGCCCCCC C>G ACTCCAGCCCGC		0.384	0.337–0.432
MPJ6_A7B061	rs9535793		3′-Flanking	33486762	6485+43[d] (*2087+43)[c]	GCCAGTGCCGTC T>C TGTCTTCACGAG		0.384	0.337–0.432

[a] Novel variations detected in this study.

[b] Positions in cDNA (NM_000053.2).

[c] Positions are shown as * and bases from the translational termination codon TGA.

[d] Positions are shown as 6485 (*2087) (final base of exon 21) + bases from the end of exon 21.

among IVS2−93A>C, 1366G>C (V456L) and IVS3−53A>C; between IVS5−65G>A and IVS19−159G>C; and among IVS9−25G>A, 2495A>G (K832R), 2855G>A (R952K), IVS12−90G>T, 3419T>C (V1140A), IVS18+6C>T, IVS19+50G>C, 5570 (*1172)G>A, 6180 (*1782)C>G and 6485+43 (*2087+43)T>C.

We analyzed colorectal and mostly non-small cell lung cancer patients treated with oxaliplatin/5-fluorouracil/leucovorin and paclitaxel/carboplatin, respectively. In these tissues in normal, ATP7A but not ATP7B is reported to be expressed mainly. However, ATP7B levels are up-regulated in colorectal and lung cancer tissues with varying degrees.[15,23] In addition to *ATP7A* polymorphisms, some *ATP7B* polymorphisms found in the promoter region may affect the expression levels of ATP7B in the tumor tissues to thus possibly influence the efficacy of oxaliplatin and carboplatin treatment by changing the drug concentrations within tumor cells. As for adverse effects of these platinum drugs, bone marrow toxicities and neuropathies (especially in oxaliplatin-administered patients) were frequently observed in our patients. Since ATP7A is expressed in the majority of normal tissues except for liver, the detected polymorphisms in the *ATP7A* possibly influence the onset of these toxicities. We are planning to conduct association analysis between the polymorphisms of both genes and efficacy and adverse reactions caused by these drugs after increase in patient number.

In conclusion, 38 and 61 genetic variations, including 30 and 28 novel ones, were detected in *ATP7A* and *ATP7B*, respectively, in a Japanese population. Our results would provide fundamental and useful information for genotyping the platinum drug transporters *ATP7A* and *ATP7B* in the Japanese and probably other Asian populations.

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Individuals susceptible to lung adenocarcinoma defined by combined *HLA-DQA1* and *TERT* genotypes

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Adenocarcinoma (ADC) is the commonest histological type of lung cancer, and its weak association with smoking indicates the necessity to identify high risk individuals for targeted screening and/or prevention. By a genome-wide association study (GWAS), we identified an association of polymorphisms in the 6p21.31 locus containing four HLA (human leukocyte antigen)-class II genes with lung ADC risk. DQA1*03 of the *HLA-DQA1* gene was defined as a risk allele with odds ratio (OR) of 1.36 (95%CI=1.21–1.54, P=5.3x10^{-7}) by analysis of 1,656 ADC cases and 1,173 controls. DQA1*03 and the minor allele for a polymorphism, rs2736100, in *TERT*, another lung cancer susceptibility locus identified in recent GWASs on Europeans and Americans, were indicated to independently contribute to ADC risk with per allele OR of 1.43 (95%CI=1.31–1.56, P=7.8x10^{-16}). Individuals homozygous both for the *DQA1*03* and minor *TERT* alleles were defined as high-risk individuals with an OR of 4.76 (95%CI=2.53–9.47, P=4.2x10^{-7}). The present results indicated that individuals susceptible to lung ADC can be defined by combined genotypes of *HLA-DQA1* and *TERT*.