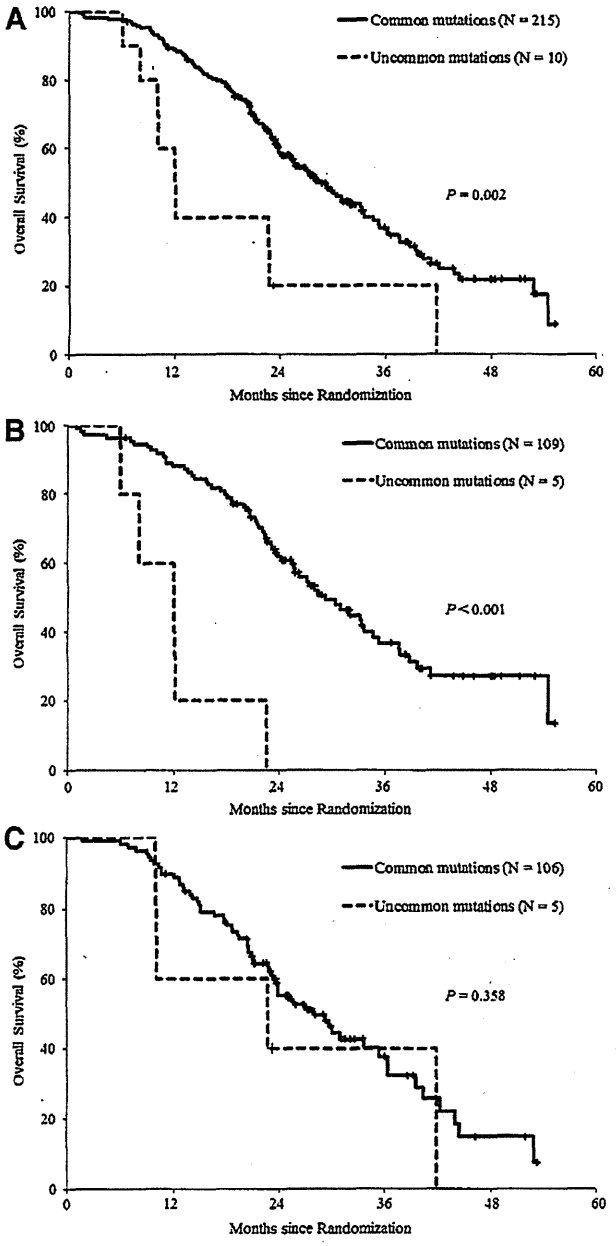

FIGURE 2. The overall survival curves of patients with common mutations and uncommon mutations in the entire population (*A*), the gefitinib group (*B*), and the carboplatin-paclitaxel group (*C*).

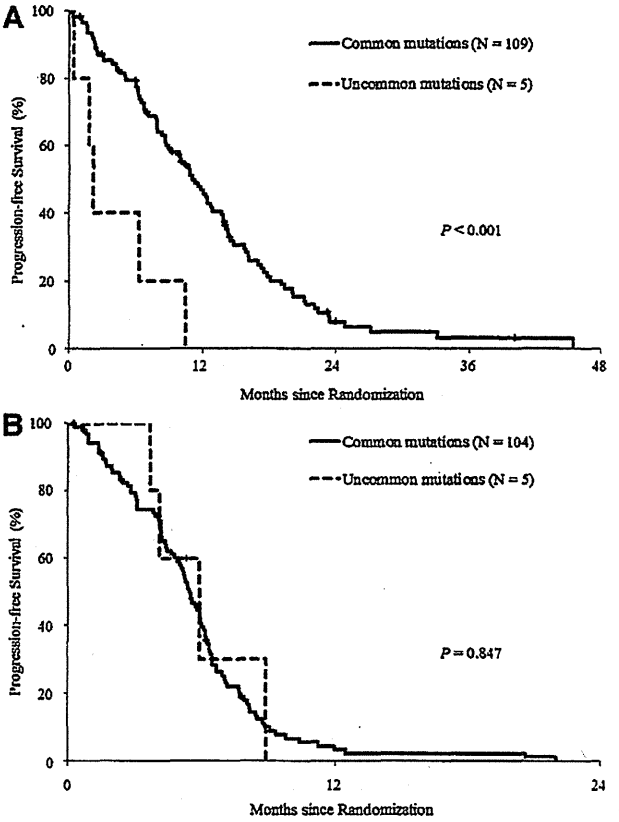

FIGURE 3. Progression-free survival curves in the gefitinib group (*A*) and the carboplatin-paclitaxel group (*B*) according to the type of epidermal growth factor receptor mutation.

lower concentration than those with wild-type EGFR. Indeed, Sequist et al.[28] reported that the effectiveness of an irreversible pan-ErbB receptor TKI, neratinib, on NSCLC patients with G719X. Niratinib induced partial responses in three of four patients with G719X and the fourth had durable stable disease for 40 weeks. It may be beneficial to evaluate erlotinib as a treatment for NSCLCs with G719X and irreversible EGFR-TKIs as treatments for NSCLCs with G719X and L861Q. Because previous phase 3 trials that investigated erlotinib or irreversible TKIs for NSCLC with EGFR mutations did not include uncommon EGFR mutations, further clinical studies may need to be performed.[7,8,29]

Another possible strategy for the treatment of uncommon EGFR mutations is the combination of EGFR-TKIs and cytotoxic agents. Our group has undertaken a randomized phase 3 trial to compare gefitinib plus carboplatin plus pemetrexed with gefitinib monotherapy for patients with NSCLC with an exon 19 deletion or an L858R, G719X, or L861Q EGFR mutation (NEJ009; University Hospital Medical Information Network Clinical Trials Registry [UMIN-CTR] number, UMIN000006340). The data from this study will advance the treatment of NSCLC with uncommon EGFR mutations.

In conclusion, our post-hoc analysis clearly demonstrated shorter survival of TKI-treated patients with uncommon EGFR mutations compared with survival of those with common EGFR mutations. Furthermore, the data suggest that the first-line chemotherapy may be relatively effective for NSCLC with uncommon EGFR mutations.

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Risk Factors for Drug-Resistant Pathogens in Community-acquired and Healthcare-associated Pneumonia

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Rationale: **Identification of patients with drug-resistant pathogens at initial diagnosis is essential for treatment of pneumonia.**
Objectives: **To elucidate clinical features of community-acquired pneumonia (CAP) and healthcare-associated pneumonia (HCAP), and to clarify risk factors for drug-resistant pathogens in patients with CAP and HCAP.**
Methods: **A prospective observational study was conducted in hospitalized patients with pneumonia at 10 institutions in Japan. Pathogens identified as not susceptible to ceftriaxone, ampicillin-sulbactam, macrolides, and respiratory fluoroquinolones were defined as CAP drug-resistant pathogens (CAP-DRPs).**
Measurements and Main Results: **In total, 1,413 patients (887 CAP and 526 HCAP) were analyzed. CAP-DRPs were more frequently found in patients with HCAP (26.6%) than in patients with CAP (8.6%). Independent risk factors for CAP-DRPs were almost identical in patients with CAP and HCAP. These included prior hospitalization (adjusted odds ratio [AOR], 2.06; 95% confidence interval [CI], 1.23–3.43), immunosuppression (AOR, 2.31; 95% CI, 1.05–5.11), previous antibiotic use (AOR, 2.45; 95% CI, 1.51–3.98), use of gastric acid–suppressive agents (AOR, 2.22; 95% CI, 1.39–3.57), tube feeding (AOR, 2.43; 95% CI, 1.18–5.00), and nonambulatory status (AOR, 2.45; 95% CI, 1.40–4.30) in the combined patients with CAP and HCAP. The area under the receiver operating characteristic curve for counting the number of risk factors was 0.79 (95% CI, 0.74–0.84).**
Conclusions: **The clinical profile of HCAP was different from that of CAP. However, physicians can predict drug resistance in patients with either CAP or HCAP by taking account of the cumulative number of the risk factors.**
Clinical trial registered with https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=brows&action=brows&type=summary&recptno=R000004001&language=E; number UMIN000003306.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The optimal prediction method of the occurrence of drug-resistant pathogens at diagnosis of pneumonia needs to be developed. From this perspective, the necessity of distinguishing community-acquired pneumonia (CAP) and healthcare-associated pneumonia (HCAP) has been debated, and multicenter studies that clarify the risk factors for drug-resistant pathogens are needed.

What This Study Adds to the Field

This multicenter prospective study elucidated six independent risk factors for resistance to commonly used antibiotics for pneumonia, and revealed the risk factors were similar in patients with CAP and HCAP. We suggest that a simple clinical prediction rule comprised of counting the number of risk factors for drug resistance may be used by physicians to predict risk of drug-resistant pathogens in patients with either CAP or HCAP.

Keywords: antibacterial agents; microbial sensitivity tests; decision support techniques; respiratory tract infections; nosocomial infections

Pneumonia is a common disease and one of the world's leading causes of death (1). To achieve appropriate initial antibiotic

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treatment, accurate assessment and classification of patients with pneumonia at initial diagnosis is essential. The optimal method of achieving this goal has been greatly debated (2–6).

The 2005 and 2007 guidelines for the management of pneumonia provided by the American Thoracic Society and the Infectious Diseases Society of America recommend that pneumonia should be classified into one of three categories at diagnosis: (*1*) community-acquired pneumonia (CAP), (*2*) healthcare-associated pneumonia (HCAP), and (*3*) hospital-acquired pneumonia (HAP) (7, 8). These three types of pneumonia have different clinical features (7, 8). In the last decade, several studies have argued that HCAP should be distinguished from CAP because of the higher prevalence of drug-resistant pathogens (DRPs), such as *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA), in patients with HCAP (9–12). Other studies showed that patients with HCAP received inappropriate initial antibiotic treatment (IIAT) more often than patients with CAP (11–14). However, administration of a broad-spectrum multidrug antibiotic regimen is not necessary in all patients with HCAP because of the wide regional variation of the frequency of multidrug-resistant pathogens in this type of pneumonia (2). The necessity of distinguishing HCAP and CAP to predict the risk of drug resistance has also been debated (3, 15, 16).

IIAT has been clearly associated with poor outcomes (12, 17). To ensure that appropriate initial antibiotic treatment is administered, more accurate information is needed regarding risk factors for drug resistance and an improved method of quantifying those factors (2, 18–20). Recently, two single-center studies proposed two separate scoring systems to predict drug resistance in pneumonia arising in communities (21, 22). Shorr and colleagues (22) proposed a prediction model using the following weighted point assignments: 4, recent hospitalization; 3, nursing home; 2, chronic hemodialysis; and 1, critically ill. However, simpler indicators for drug resistance would be helpful for physicians who prescribe antibiotics in clinical settings.

Therefore, a multicenter, prospective, observational study including hospitalized adult patients with pneumonia was conducted. The objectives of this study were to identify the clinical and microbiologic features of CAP and HCAP, both of which occur in communities, and to clarify the risk factors for drug resistance to common antibiotics.

Some of the results of this study have been previously reported in the form of an abstract (23), and the revised version was distributed to meeting attendees.

METHODS

Supplemental information on methods is provided in the online supplement.

Study Design and Setting

This observational study was performed prospectively from March 15, 2010 through December 22, 2010 at 10 medical institutions (a 1,000-bed university hospital and nine major community hospitals, each equipped with more than 500 beds), all of which are members of the Central Japan Lung Study Group. This study was approved by the institutional review boards of these institutions. The protocol in this study adhered to the Japanese Ethical Guidelines for Epidemiological Studies. This study is registered with University Hospital Medical Information Network in Japan (number UMIN000003306).

Participants and Categories of Pneumonia

All adult patients (age ≥ 20 yr) in whom pneumonia had developed during daily community living and to whom in-hospital treatment was subsequently administered in the participating institutions were included in the study. Pneumonia was diagnosed according to previously published international guidelines (7, 8). The details of diagnostic criteria and exclusion criteria are provided in the online supplement.

Further details associated with the different categories of pneumonia are as follows (7, 8, 12):

1. HAP: pneumonia occurring 48 hours or more after hospital admission, including ventilator-associated pneumonia
2. HCAP: pneumonia co-occurring with any of the following conditions:
   a. Hospitalization for 2 days or more during the preceding 90 days
   b. Residence in a nursing home or extended care facility
   c. Home intravenous therapy (including antibiotics and chemotherapy)
   d. Chronic dialysis (including hemodialysis and peritoneal dialysis) during the preceding 30 days
   e. Home wound care during the preceding 30 days
3. CAP: pneumonia not matching the criteria for HAP and HCAP

In this study, patients with CAP and HCAP were enrolled, and those with HAP were not included in the current analysis because the data on HAP were collected in limited two institutions.

Procedure and Data Collection

The procedure of this study is provided in the online supplement. The following data were collected at diagnosis (Day 0): demographic information, including past medical history and living conditions; comorbidities; use of antibiotics within the previous 90 days; use of gastric acid–suppressive agents (histamine H_2-receptor blockers or proton pump inhibitors) at the time of diagnosis; tube feeding, functional status, and positive MRSA history within the previous 90 days; symptoms; physical, laboratory, and radiologic findings; indexes of disease severity (including Pneumonia Severity Index and the age, dehydration, respiratory failure, orientation disturbance, and low blood pressure [A-DROP] score) (24, 25); microbiologic characteristics; and initial empirical antibiotic therapy. Additional details of the collected data and definitions of comorbidities are provided in the online supplement. Information regarding outcomes was obtained after Day 30.

Microbiologic Evaluation

Microbiologic laboratories in all study institutions provided possible causative pathogens, which were cultured in a semiquantitative manner from samples of sputum, tracheobronchial aspirates, bronchoalveolar lavage fluid, pleural fluid, and blood. Serologic tests were performed to detect antibodies against *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* (26, 27). *Legionella pneumophila* serogroup 1 antigen in urine was tested by immunochromatography. Microbiologic test results were independently reviewed by two investigators (Y. Shindo and I.Y.). Pathogens provided by the 10 institutions were recultured and antibiotic susceptibility tests were performed at a central laboratory (SRL, Inc., Tokyo, Japan). Viruses, acid-fast bacilli, fungus, and anaerobes were not recultured. The susceptibility tests focused on antibiotics frequently prescribed or recommended for the treatment of pneumonia (7, 8). Additional details including susceptibility tests are provided in the online supplement.

Endpoints

In this observational study, we set several clinical and microbiologic endpoints. In those, we focused on the following endpoints: (*1*) the drug resistance of identified pathogens, (*2*) the IIAT, (*3*) 30-day mortality and in-hospital mortality, and (*4*) receiving mechanical ventilation from Day 0 through Day 30.

The definition of multidrug-resistant pathogens from a recent international consensus statement was adopted to facilitate international comparison regarding the epidemiology of DRPs (28). In the initial empirical antibiotic treatment of CAP, two regimens (combination therapy with nonantipseudomonal β-lactams and macrolides or monotherapy with fluoroquinolones) have been recommended in the international

guidelines (8). Therefore, identified pathogens that were not susceptible to β-lactams (ceftriaxone or ampicillin-sulbactam), macrolides (azithromycin or clarithromycin), and fluoroquinolones (moxifloxacin, levofloxacin, or garenoxacin) were defined as CAP-DRPs.

Statistical Analysis

Statistical analyses were performed using PASW Statistics 18 (SPSS Inc., Chicago, IL). All tests were two-tailed and a P value less than 0.05 was considered statistically significant. Demographic, clinical, and microbiologic characteristics, and antibiotic use, were described. Here categorical data were summarized as frequencies in percentage and continuous data as median with interquartile range. Pearson chi-square test or Mantel extension test for trend was used for analyzing discrete variables, and the Wilcoxon rank sum test for continuous variables.

Variables were further examined for association with CAP drug resistance by univariable and multivariable logistic regression analysis. Odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated. For the analysis of risk factors for CAP drug resistance, candidate factors were determined *a priori* referring to those published in previous reports (7, 8, 12, 29–31). At least five patients with CAP-DRPs per risk factor were needed for it to be included in the analysis (32). Based on the logistic regression findings of these risk factors, a predictive index was created by assigning risk scores based on the regression coefficients of the significant variables (33). Traditional 2 × 2 tables were used to calculate sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the predictive rule, the HCAP definition, and two previous prediction models (21, 22). The validity of the prediction rule was evaluated using the receiver operating characteristic (ROC) curve, compared with two previous prediction models (21, 22). Calculation procedures of these previous prediction rules are provided in the online supplement.

Subanalyses were performed after CAP-DRPs were classified into the following two groups: MRSA and CAP-DRPs other than MRSA (e.g., *P. aeruginosa* and extended-spectrum β-lactamase-producing Enterobacteriaceae). The risk factors for them were evaluated separately.

RESULTS

Participants and Baseline Characteristics

A total of 1,742 patients with pneumonia were assessed for eligibility, and 1,413 of whom (887 with CAP and 526 with HCAP) were included in the study (Figure 1). The baseline characteristics of patients with CAP and HCAP are described in Table 1. Advanced age, neoplastic diseases, congestive heart failure, central nervous system disorders, and severe pneumonia were more frequent in patients with HCAP than in those with CAP. Frequency of hypoalbuminemia, previous use of antibiotics, use of gastric acid suppressive–agents, tube feeding, nonambulatory status, and positive MRSA history was higher in patients with HCAP than in those with CAP.

Identified Pathogens

Pathogens were identified in 475 (53.6%) of 887 patients with CAP and 320 (60.8%) of 526 patients with HCAP. Pathogen distribution according to type of pneumonia is shown in Table 2, and additional descriptions are shown in the online supplement. In patients with CAP, *S. pneumoniae* (17.1%) and *Haemophilus influenzae* (10.4%) were the two most frequently isolated pathogens. In patients with HCAP, *Klebsiella pneumoniae* (15.6%) was isolated most frequently, followed by *S. pneumoniae* (12.7%), MRSA (10.8%), methicillin-susceptible *S. aureus* (9.9%), and *P. aeruginosa* (8.7%).

Initial Antibiotics

Initially prescribed antibiotics are shown in Table 3. Patients with HCAP received monotherapy more frequently than patients with CAP. Antipseudomonal antibiotics were given to 22.4% of patients with CAP and 31.2% of patients with HCAP as initial empirical therapy. However, only 0.2 and 1.3% of patients with CAP and HCAP, respectively, received anti-MRSA antibiotics, although MRSA was detected in 2.3 and 10.8% of patients with CAP and HCAP, respectively.

Drug-Resistant Pathogens, IIAT, and Mortality

Microbiologic and clinical outcomes are shown in Table 4. Among patients with identified pathogens, CAP-DRPs were more frequently isolated in patients with HCAP (26.6%) than in those with CAP (8.6%). Regarding the relationship between IIAT and the occurrence of CAP-DRPs, IIAT was administered in 71.1% (27 of 38) and 10.2% (41 of 403) of patients with CAP with and without CAP-DRPs, respectively. In patients with HCAP with and without CAP-DRPs, IIAT was administered in 85.0% (68 of 80) and 13.0% (29 of 223), respectively. The proportion of patients receiving mechanical ventilation was similar between patients with CAP and HCAP. Thirty-day mortality was higher in patients with HCAP (20.3%) than in those with CAP (7.0%), and in-hospital mortality was also higher in HCAP (24.9%) than in CAP (10.0%). In patients with and without CAP-DRPs, the 30-day mortality was 21.0% (25 of 119) and 10.2% (64 of 627), respectively.

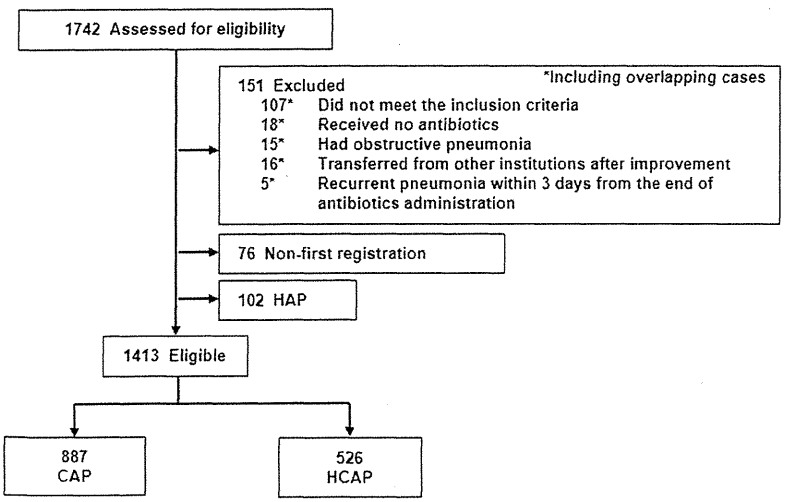


Figure 1. Patient flow. CAP = community-acquired pneumonia; HAP = hospital-acquired pneumonia; HCAP = healthcare-associated pneumonia.

TABLE 1. BASELINE CHARACTERISTICS OF THE STUDY PATIENTS

Variables	CAP (*n* = 887)	HCAP (*n* = 526)	*P* Value
Male, n (%)	580 (65.4)	335 (63.7)	0.518
Age, yr, median (IQR)	75 (66–83)	79 (70–85)	<0.001
Hospitalization for 2 days or more during the preceding 90 d, n (%)	—	246 (46.8)	—
Residence in a nursing home or extended care facility, n (%)	—	224 (42.6)	—
Home intravenous therapy (including antibiotics and chemotherapy), n (%)	—	137 (26.0)	—
Chronic dialysis during the preceding 30 d, n (%)	—	21 (4.0)	—
Home wound care during the preceding 30 d, n (%)	—	35 (6.7)	—
Comorbidities, n (%)			
Neoplastic diseases	111 (12.5)	97 (18.4)	0.002
Chronic lung diseases	309 (34.8)	161 (30.6)	0.103
Congestive heart failure	98 (11.0)	85 (16.2)	0.006
Chronic renal diseases	64 (7.2)	49 (9.3)	0.159
Chronic liver diseases	35 (3.9)	18 (3.4)	0.616
CNS disorders	139 (15.7)	165 (31.4)	<0.001
Diabetes	160 (18.0)	98 (18.6)	0.780
Immunosuppression*	58 (6.5)	40 (7.6)	0.446
Physical findings, n (%)			
Orientation disturbance (confusion)	121 (13.6)	153 (29.1)	<0.001
Systolic blood pressure < 90 mm Hg	37 (4.2)	44 (8.4)	0.001
Pulse rate ⩾ 125/min	73 (8.2)	67 (12.7)	0.006
Respiration rate ⩾ 30/min†	182 (21.1)	132 (25.6)	0.054
Laboratory findings			
BUN, mg/dl, median (IQR)	19.0 (13.3–27.0)	21.3 (14.5–31.2)	<0.001
Pao_2/Fi_{O2},‡ median (IQR)	291 (231–347)	256 (181–319)	<0.001
Hematocrit, %, median (IQR)	36.7 (33.1–40.1)	34.9 (31.0–38.3)	<0.001
C-reactive protein, mg/dl, median (IQR)	12.0 (6.2–19.1)	10.5 (4.8–16.2)	0.001
Albumin < 3.0 mg/dl, n (%)	225 (25.5)	253 (48.3)	<0.001
Radiographic findings, n (%)			
Bilateral lung involvement	374 (42.2)	275 (52.3)	<0.001
Use of antibiotics within the previous 90 d, n (%)	246 (27.7)	292 (55.5)	<0.001
Use of gastric acid suppressive agents (H_2-blockers or proton pump inhibitors), n (%)	199 (22.4)	169 (32.1)	<0.001
Tube feeding, n (%)	7 (0.8)	54 (10.3)	<0.001
Nonambulatory status,§ n (%)	89 (10.0)	249 (47.3)	<0.001
Positive MRSA history within the previous 90 d, n (%)	1 (0.1)	22 (4.2)	<0.001
PSI class,‖ n (%)			<0.001¶
I–III	358 (42.4)	83 (16.5)	
IV	320 (37.9)	214 (42.6)	
V	167 (19.8)	205 (40.8)	

Definition of abbreviations: BUN = blood urea nitrogen; CAP = community-acquired pneumonia; CNS = central nervous system; H_2-blockers = histamine H_2-receptor blocker; HCAP = healthcare-associated pneumonia; IQR = interquartile range; MRSA = methicillin-resistant *Staphylococcus aureus*; PSI = Pneumonia Severity Index.

*Immunosuppression included any immunosuppressive diseases, such as congenital or acquired immunodeficiency, hematologic diseases, and neutropenia (<1,000/mm^3), treatment with immunosuppressive drugs within the previous 30 days, or corticosteroids in daily doses of at least 10 mg/day of a prednisone equivalent for more than 2 weeks.

† Respiration rate was evaluated in 863 patients with CAP and 516 patients with HCAP.

‡ Arterial blood gas analysis was performed in 866 patients with CAP and 508 patients with HCAP. In cases where arterial blood gas analyses were not performed, Pao_2 was estimated from Spo_2.

§ Nonambulatory status was defined as being bedridden or using a wheelchair because of difficulty walking.

‖ The PSI was evaluated in 845 patients with CAP and 502 patients with HCAP.

¶ Trend test.

Risk Factors for CAP Drug-Resistant Pathogens

In the provisional analysis (*see* Table E1 in the online supplement), the significant risk factors for CAP-DRPs in patients with CAP included previous use of antibiotics; use of gastric acid–suppressive agents (histamine H_2-receptor blockers or proton pump inhibitors); tube feeding; and nonambulatory status. Similarly, the significant risk factors for CAP-DRPs in patients with HCAP were previous use of antibiotics, use of gastric acid–suppressive agents, tube feeding, and nonambulatory status. Therefore, assessment of risk factors was performed combining data for patients with CAP and HCAP, and using the definitional components of HCAP (Table 5). The independent risk factors for CAP-DRPs were as follows: hospitalization for 2 days or more during the preceding 90 days (adjusted OR [AOR], 2.06; 95% CI, 1.23–3.43); immunosuppression (AOR, 2.31; 95% CI, 1.05–5.11); use of antibiotics within the previous 90 days (AOR, 2.45; 95% CI, 1.51–3.98); use of gastric acid–suppressive agents (AOR, 2.22; 95% CI, 1.39–3.57); tube feeding (AOR, 2.43; 95% CI, 1.18–5.00); and nonambulatory status (AOR, 2.45; 95% CI, 1.40–4.30). These results were almost unchanged when the severity of illness (Pneumonia Severity

TABLE 2. IDENTIFIED PATHOGENS ACCORDING TO TYPE OF PNEUMONIA*

Microbes	CAP (*n* = 887)	HCAP (*n* = 526)
Identified	475 (53.6)	320 (60.8)
Gram-positive pathogens		
Streptococcus pneumoniae	152 (17.1)	67 (12.7)
Methicillin-susceptible *Staphylococcus aureus*	68 (7.7)	52 (9.9)
Methicillin-resistant *S. aureus*	20 (2.3)	57 (10.8)
Streptococci other than *S. pneumoniae*	23 (2.6)	31 (5.9)
Enterococcus sp.	0	3 (0.6)
Gram-negative pathogens		
Haemophilus influenzae	92 (10.4)	26 (4.9)
Klebsiella pneumoniae	77 (8.7)	82 (15.6)
ESBL+	2 (0.2)	1 (0.2)
Pseudomonas aeruginosa	33 (3.7)	46 (8.7)
Moraxella catarrhalis	32 (3.6)	12 (2.3)
Escherichia coli	26 (2.9)	22 (4.2)
ESBL+	4 (0.5)	5 (1.0)
Enterobacter sp.	15 (1.7)	12 (2.3)
Klebsiella oxytoca	7 (0.8)	9 (1.7)
Serratia marcescens	4 (0.5)	5 (1.0)
Citrobacter sp.	4 (0.5)	1 (0.2)
Acinetobacter sp.	4 (0.5)	8 (1.5)
Stenotrophomonas maltophilia	4 (0.5)	2 (0.4)
Other Enterobacteriaceae	4 (0.5)	3 (0.6)
Other nonfermenting gram-negative bacteria	3 (0.3)	1 (0.2)
Proteus group	2 (0.2)	8 (1.5)
ESBL+	0	2 (0.4)
Other gram-negative pathogens	3 (0.3)	2 (0.4)
Atypical pathogens	48 (5.4)	26 (4.9)
Mycoplasma pneumoniae†	11 (1.2)	4 (0.8)
Chlamydophila pneumoniae‡	31 (3.5)	21 (4.0)
Legionella pneumoniae	7 (0.8)	2 (0.4)
Others	4 (0.5)	5 (1.0)
Unidentified	412 (46.4)	206 (39.2)

Definition of abbreviations: CAP = community-acquired pneumonia; ESBL = extended-spectrum β-lactamase-producing; HCAP = healthcare-associated pneumonia.

* Data are presented as n (%).

† Serologic tests for *Mycoplasma pneumoniae* were performed in 307 patients with CAP and 123 patients with HCAP, and positive test results were obtained in 11 and 4, respectively.

‡ Serologic tests for *Chlamydophila pneumoniae* were performed in 260 patients with CAP and 94 patients with HCAP, and positive test results were obtained in 31 and 21, respectively.

TABLE 3. INITIALLY PRESCRIBED ANTIBIOTICS ACCORDING TO TYPE OF PNEUMONIA*

Antibiotics	CAP (*n* = 887)	HCAP (*n* = 526)
Monotherapy	442 (49.8)	356 (67.7)
β-Lactams	427 (48.1)	352 (66.9)
Quinolones	10 (1.1)	3 (0.6)
Other	5 (0.6)	1 (0.2)
Combination therapy	445 (50.2)	170 (32.3)
β -Lactams + macrolides	312 (35.2)	81 (15.4)
β -Lactams + minocycline	11 (1.2)	5 (1.0)
β -Lactams + quinolones	71 (8.0)	38 (7.2)
β -Lactams + aminoglycosides	1 (0.1)	2 (0.4)
β -Lactams + clindamycin	27 (3.0)	28 (5.3)
β -Lactams + anti-MRSA antibiotics†	1 (0.1)	4 (0.8)
β -Lactams + quinolones + anti-MRSA antibiotics†	1 (0.1)	2 (0.4)
Other combinations	21 (2.4)	10 (1.9)
Antipseudomonal antibiotics used‡	199 (22.4)	164 (31.2)
Anti-MRSA antibiotics used†	3 (0.2)	7 (1.3)

Definition of abbreviations: CAP = community-acquired pneumonia; HCAP = healthcare-associated pneumonia; MRSA = methicillin-resistant *Staphylococcus aureus*.

*Data are presented as n (%).

† Vancomycin, linezolid, teicoplanin, and arbekacin were defined as anti-MRSA antibiotics.

‡ Piperacillin-tazobactam, piperacillin, ceftazidime, cefepime, cefozopran, cefoperazone-sulbactam, aztreonam, imipenem-cilastatin, meropenem, doripenem, biapenem, ciprofloxacin, pazufloxacin, tobramycin, isepamycin, amikacin, and arbekacin were defined as antipseudomonal antibiotics.

TABLE 4. OUTCOMES ACCORDING TO TYPE OF PNEUMONIA*

Microbiologic and clinical outcomes	CAP (*n* = 887)	HCAP (*n* = 526)	*P* Value
Multidrug-resistant pathogens	45/475 (9.5)	74/320 (23.1)	<0.001
CAP drug-resistant pathogens†, ‡	38/442 (8.6)	81/304 (26.6)	<0.001
Inappropriate initial antibiotic treatment‡, §	69/442 (15.6)	99/305 (32.5)	<0.001
Mechanical ventilation‖	87 (9.8)	44 (8.4)	0.366
30-d mortality¶	62 (7.0)	107 (20.3)	<0.001
In-hospital mortality	89 (10.0)	131 (24.9)	<0.001

Definition of abbreviations: CAP = community-acquired pneumonia; HCAP = healthcare-associated pneumonia.

*Data are presented as n (%).

† Identified pathogens that were not susceptible to β-lactams (ceftriaxone or ampicillin-sulbactam), macrolides (azithromycin or clarithromycin), and fluoroquinolones (moxifloxacin, levofloxacin, or garenoxacin) were defined as CAP drug-resistant pathogens. Major CAP drug-resistant pathogens in CAP included methicillin-resistant *Staphylococcus aureus* (47.6% [20 of 42]), *Pseudomonas aeruginosa* (23.8% [10 of 42]), and extended-spectrum β-lactamase-producing Enterobacteriaceae (11.9% [5 of 42]); and those in HCAP included methicillin-resistant *S. aureus* (61.3% [57 of 93]), *P. aeruginosa* (20.4% [19 of 93]), and extended-spectrum β-lactamase-producing Enterobacteriaceae (6.5% [6 of 93]).

‡ CAP drug resistance and appropriateness of initial antibiotics was assessed in patients with the results of susceptibility testing of identified pathogens.

§ Antibiotic treatment was classified as inappropriate when the identified pathogens were not susceptible to the initially prescribed antibiotics, on the basis of *in vitro* susceptibility testing.

‖ Noninvasive positive-pressure ventilation was included.

¶ Patients who were discharged or transferred to other hospitals within 30 days with improvement of pneumonia were considered alive.

Index class V or A-DROP scores ≥ 3) was included as a factor (24, 25).

Prediction Rule for CAP Drug-Resistant Pathogens

ORs of individual risk factors were 2.0–2.5. Therefore, a prediction rule for the CAP-DRP occurrence was constructed using a simple counting of the number of risk factors (Figure 2). As shown in Figure 2A, no risk factors or only one risk factor was identified in 86.4% of patients with CAP, two risk factors were identified in 10.9% of these patients, and three or more risk factors were identified in 2.7% of these patients. However, no risk factors or only one risk factor was observed in 35.9% of patients with HCAP, two risk factors were counted in 30.9% of these patients, and three or more risk factors were identified in 33.2% of these patients. Compared with patients with CAP, therefore, multiple risk factors for CAP-DRPs were present in patients with HCAP. When data for patients with CAP and HCAP were combined, the probability of the CAP-DRP occurrence was 3.5, 9.2, 21.8, 42.7, 53.8, and 83.3% in patients with zero, one, two, three, four, and five to six risk factors, respectively (Figure 2B). The diagnostic performance of this simple counting of the number of risk factors and the HCAP definition were as follows: sensitivity of 73.1% and specificity of 73.2%, with values of PPV of 34.1% and NPV of 93.5% of two or more risk factors; sensitivity of 47.1% and specificity of 90.9%, with values of PPV of 49.6% and NPV of 90.0% of three or more risk factors; and sensitivity of 68.1% and specificity of 64.4%, with values of PPV of 26.6% and NPV of 91.4% of the HCAP definition, respectively (*see* Table E2). Figure 3 shows the ROC curves for our counting method of the number of risk factors and for the two previous prediction rules. The area under the ROC curve (AU-ROC) for our method was 0.79 (95% CI, 0.74–0.84), and it was greater than 0.71 (95% CI, 0.66–0.77) of Shorr's scoring, and 0.66 (95% CI, 0.61–0.71) of Aliberti's scoring. When a predictive index based on the log-transformed ORs of the six risk factors was calculated for individuals, the AU-ROC was 0.79 (95% CI, 0.74–0.84). Additional results regarding the relationship between the number of risk factors and disease severity is shown in the online supplement.

Subanalyses of Risk Factors for MRSA and CAP Drug-Resistant Pathogens Other than MRSA

Risk factors for MRSA and CAP-DRPs other than MRSA were separately evaluated among combined patients with CAP and HCAP. The details of the results are provided in the online supplement. Comparing the risk factors for all CAP-DRPs with those for MRSA, the risk factors for MRSA included chronic dialysis during the preceding 30 days, positive MRSA history within the previous 90 days, and congestive heart failure, in addition to hospitalization for 2 days or more during the preceding 90 days, use of antibiotics within the previous 90 days, and use of gastric acid–suppressive agents. Regarding the risk factors for CAP-DRPs other than MRSA, the following five factors that were included in the risks for all CAP-DRPs were significant: (*1*) immunosuppression, (*2*) use of antibiotics within the previous 90 days, (*3*) use of gastric acid–suppressive agents, (*4*) tube feeding, and (*5*) nonambulatory status.

When counting the number of risk factors for all CAP-DRPs, the probabilities of both MRSA and CAP-DRPs other than MRSA were similar to that of all CAP-DRPs. Specifically, the probabilities of these two groups were low (<5%) in patients with no or one risk factor, and were high (28.3%) in patients with three or more risk factors (Table 6). There was a difference in the probabilities in patients with two risk factors between those two groups, that is, 17.6% for MRSA and 6.3% for CAP-DRPs other than MRSA. The AU-ROC of counting the number of risk factors for all CAP-DRPs was 0.76 (95% CI, 0.70–0.81) and 0.82 (95% CI, 0.75–0.88) for MRSA and CAP-DRPs other than MRSA, respectively. The probability of MRSA was increased in patients with two or more risk factors for all CAP-DRPs when considering any one of specific risk factors for MRSA (Table 6).

TABLE 5. RISK FACTORS FOR CAP DRUG RESISTANCE* IN PATIENTS WITH CAP AND HCAP COMBINED

Variables	Resistance		Univariable Analysis OR (95% CI)	Multivariable Analysis OR (95% CI)
	Yes	No		
Hospitalization for ≥2 d during the preceding 90 d				
No (n = 604)	67	537	1 (ref)	1 (ref)
Yes (n = 142)	52	90	4.63 (3.03–7.09)	2.06 (1.23–3.43)
Residence in a nursing home				
No (n = 599)	78	521	1 (ref)	1 (ref)
Yes (n = 147)	41	106	2.58 (1.68–3.98)	1.13 (0.63–2.02)
Home intravenous therapy (including antibiotics and chemotherapy)				
No (n = 679)	107	572	1 (ref)	1 (ref)
Yes (n = 67)	12	55	1.17 (0.60–2.25)	0.84 (0.40–1.80)
Chronic dialysis during the preceding 30 d				
No (n = 734)	116	618	1 (ref)	1 (ref)
Yes (n = 12)	3	9	1.78 (0.47–6.66)	2.23 (0.51–9.69)
Home wound care during the preceding 30 d				
No (n = 726)	112	614	1 (ref)	1 (ref)
Yes (n = 20)	7	13	2.95 (1.15–7.56)	1.44 (0.47–4.39)
Immunosuppression				
No (n = 699)	104	595	1 (ref)	1 (ref)
Yes (n = 47)	15	32	2.68 (1.40–5.13)	2.31 (1.05–5.11)
Use of antibiotics within the previous 90 d				
No (n = 481)	46	435	1 (ref)	1 (ref)
Yes (n = 265)	73	192	3.60 (2.40–5.40)	2.45 (1.51–3.98)
Chronic lung disease				
No (n = 511)	77	434	1 (ref)	1 (ref)
Yes (n = 235)	42	193	1.23 (0.81–1.85)	1.13 (0.68–1.89)
Congestive heart failure				
No (n = 656)	97	559	1 (ref)	1 (ref)
Yes (n = 90)	22	68	1.86 (1.10–3.16)	1.68 (0.92–3.08)
CNS disorder				
No (n = 554)	73	481	1 (ref)	1 (ref)
Yes (n = 192)	46	146	2.08 (1.37–3.14)	1.36 (0.80–2.29)
Albumin < 3.0 mg/dl				
No (n = 468)	53	415	1 (ref)	1 (ref)
Yes (n = 274)	65	209	2.44 (1.63–3.63)	1.30 (0.81–2.09)
Use of gastric acid suppressive agents (H_2-blocker or PPI)				
No (n = 543)	64	479	1 (ref)	1 (ref)
Yes (n = 203)	55	148	2.78 (1.86–4.17)	2.22 (1.39–3.57)
Tube feeding				
No (n = 695)	94	601	1 (ref)	1 (ref)
Yes (n = 51)	25	26	6.15 (3.41–11.10)	2.43 (1.18–5.00)
Nonambulatory status				
No (n = 518)	51	467	1 (ref)	1 (ref)
Yes (n = 228)	68	160	3.89 (2.60–5.84)	2.45 (1.40–4.30)
Positive MRSA history within the previous 90 d				
No (n = 727)	109	618	1 (ref)	1 (ref)
Yes (n = 19)	10	9	6.30 (2.50–15.86)	2.47 (0.86–7.09)

Definition of abbreviations: CAP = community-acquired pneumonia; CI = confidence interval; CNS = central nervous system; H_2-blocker = histamine H_2-receptor blocker; HCAP = healthcare-associated pneumonia; MRSA = methicillin-resistant *Staphylococcus aureus*; OR = odds ratio; PPI = proton pump inhibitor; ref = reference.

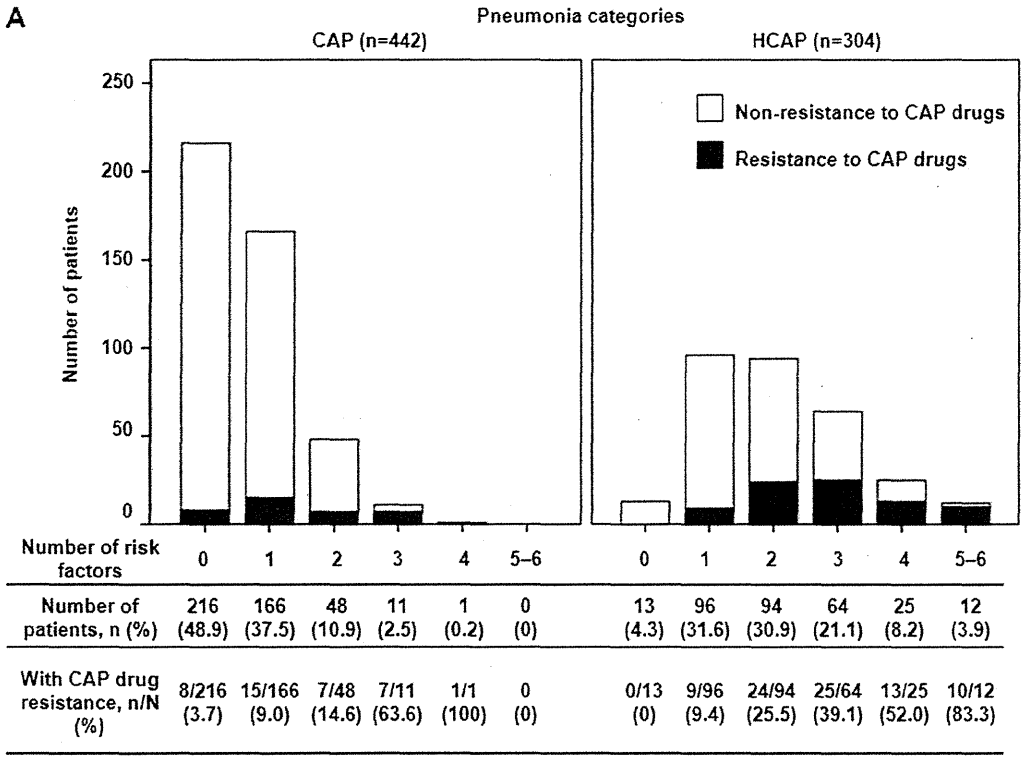
*Identified pathogens that were not susceptible to β-lactams (ceftriaxone or ampicillin-sulbactam), macrolides (azithromycin or clarithromycin), and fluoroquinolones (moxifloxacin, levofloxacin, or garenoxacin) were defined as CAP drug-resistant pathogens.

Administered Antibiotics and Clinical Outcome According to the Number of Risk Factors for CAP Drug-Resistant Pathogens

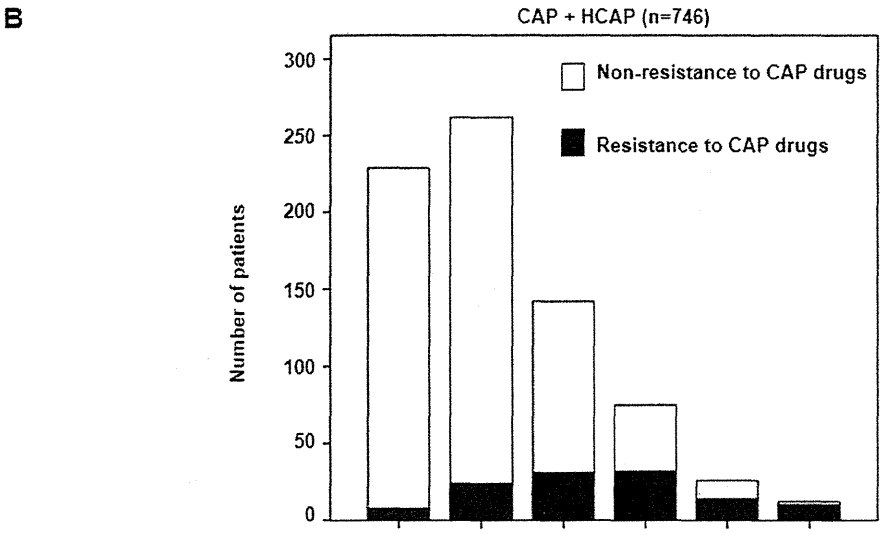
The relationships of the number of risk factors for CAP-DRPs to IIAT, administered antibiotics, and the 30-day mortality among patients who received their antibiotic treatment are shown in Table 6 and the additional descriptions are provided in the online supplement. Among patients with identified pathogens, IIAT was given in 14.7, 31.0, and 43.8% of patients with less than or equal to one, two, and three or more risk factors for CAP-DRPs, respectively. The 30-day mortality in patients who received IIAT in these three risk classes was 9.7% (7 of 72), 15.9% (7 of 44), and 28.6% (14 of 49), respectively. In these three risk classes, traditional antibiotic regimens of CAP drugs were administered in 155, 23, and 7 of patients with identified pathogens, respectively; and in 129, 24, and 6 of those without, respectively. The 30-day mortality in patients with less than or equal to one risk factor who received traditional regimens of CAP drugs was 1.3% (2 of 155) and 3.1% (4 of 129) in patients with and without identified pathogens, respectively. These 30-day mortality proportions were lower than those in patients who received monotherapy with nonantipseudomonal β-lactams, that is, 10.8% (22 of 203) in patients with identified pathogens and 9.6% (17 of 177) in those without, respectively.

DISCUSSION

In this multicenter, prospective, observational study, the clinical profile of HCAP was different from that of CAP concerning DRP identification. However, the risk factors for CAP drug resistance were almost identical in patients with CAP and HCAP.



Number of risk factors	0	1	2	3	4	5–6	0	1	2	3	4	5–6
Number of patients, n (%)	216 (48.9)	166 (37.5)	48 (10.9)	11 (2.5)	1 (0.2)	0 (0)	13 (4.3)	96 (31.6)	94 (30.9)	64 (21.1)	25 (8.2)	12 (3.9)
With CAP drug resistance, n/N (%)	8/216 (3.7)	15/166 (9.0)	7/48 (14.6)	7/11 (63.6)	1/1 (100)	0 (0)	0/13 (0)	9/96 (9.4)	24/94 (25.5)	25/64 (39.1)	13/25 (52.0)	10/12 (83.3)

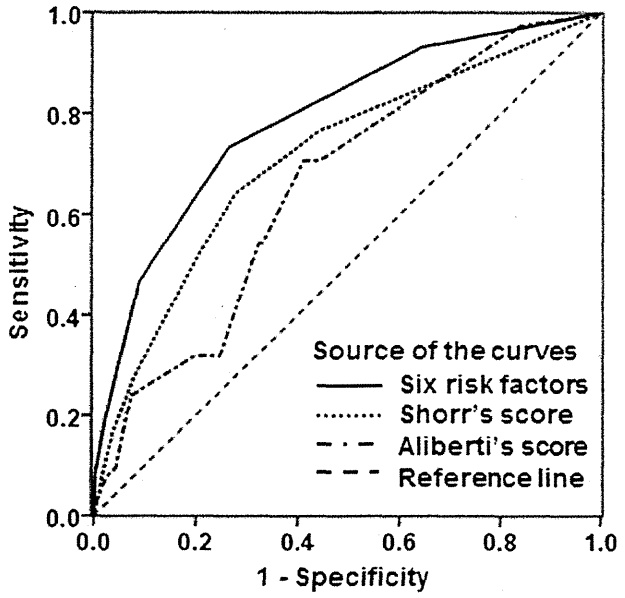


Number of risk factors	0	1	2	3	4	5–6
Number of patients, n (%)	229 (30.7)	262 (35.1)	142 (19.0)	75 (10.1)	26 (3.5)	12 (1.6)
With CAP drug resistance, n/N (%)	8/229 (3.5)	24/262 (9.2)	31/142 (21.8)	32/75 (42.7)	14/26 (53.8)	10/12 (83.3)

Figure 2. Number of risk factors for CAP drug resistance. Patients without identified pathogens were not included. CAP = community-acquired pneumonia; HCAP = healthcare-associated pneumonia.

As a result of this finding, a simple estimation of drug resistance was proposed using the counting of the number of risk factors (prior hospitalization, immunosuppression, previous use of antibiotics, use of gastric acid–suppressive agents, tube feeding, and nonambulatory status) irrespective of pneumonia category. An example of how this estimation system may be used is as follows. When no risk factors or only one risk factor is observed in a pneumonia patient, CAP-DRPs are lower (<10% in this study). For these patients (86% of patients with CAP and 36% of patients with HCAP in the current study), administration of broad-spectrum antibiotics should be curtailed, and CAP drugs should be given instead. When three or more risk factors are present, physicians should consider prescribing broad-spectrum antibiotics.

In this study, 30-day mortality and in-hospital mortality were higher in patients with HCAP than in those with CAP, as previously reported (10, 12, 13). More serious underlying conditions and treatment with monotherapy were more frequently observed in patients with HCAP than in those with CAP. The



Area under the curve

Test result variable(s)	Area	95% CI
Six risk factors	0.79	0.74–0.84
Shorr's score	0.71	0.66–0.77
Aliberti's score	0.66	0.61–0.71

Figure 3. The receiver operating characteristic (ROC) curves for prediction of community-acquired pneumonia drug resistance. CI = confidence interval. The six risk factors were as follows: prior hospitalization, immunosuppression, previous use of antibiotics, use of gastric acid–suppressive agents, tube feeding, and nonambulatory status. Shorr's score (range, 0–10) was calculated as the sum of the following weighted point assignments: 4, recent hospitalization; 3, nursing home; 2, chronic hemodialysis; and 1, critically ill (Pneumonia Severity Index class V). Aliberti's score (range, 0–12.5) was calculated as the sum of the following weighted point assignments: 5, chronic renal failure; 4, hospitalization for greater than or equal to 2 days or more in the preceding 90 days; 3, residence in a nursing home; and 0.5, one or more of cerebrovascular disease, diabetes, chronic lung disease (substitute for chronic obstructive pulmonary disease), antimicrobial therapy in preceding 90 days, immunosuppression, home wound care, and home infusion therapy.

frequency of receiving mechanical ventilation in patients with HCAP was similar to that in those with CAP, despite the fact that patients with HCAP had more severe disease than patients with CAP. These results suggest that differences in mortality between patients with these two types of pneumonia may be attributable to differences in personal characteristics and background and the resulting treatment restrictions, as suggested by Ewig and colleagues (3).

The spectrum of pathogens identified in patients with HCAP was different from that in patients with CAP. The pathogens in HCAP included those frequently found in both CAP and HAP (i.e., *S. pneumoniae*, *K. pneumoniae*, methicillin-susceptible *S. aureus*, MRSA, and *P. aeruginosa*) (9–12, 15, 34, 35). This finding was consistent with that of some previous studies (9, 11), but not with those of other studies (15, 16). The spectrum of pathogens may vary because of the wide range of clinical situations in which HCAP develops.

Although CAP-DRPs were more frequently found in patients with HCAP, the proportion was 26.6% at most. Thus, broad-spectrum antibiotic administration is not appropriate for treatment of all patients with HCAP, as suggested by Brito and Niederman (2). However, CAP-DRPs were found in 8.6% of patients with CAP. Thus, the type of pneumonia (CAP or HCAP) may not determine the presence or absence of CAP-DRPs. Other clinical factors may be at work. In this study, 22.4% of patients with CAP received antipseudomonal antibiotics, which may indicate an overuse of broad-spectrum antibiotics for patients with CAP. Furthermore, the discrepancy between the proportion of MRSA identification and that of initial administration of anti-MRSA antibiotics may suggest undertreatment for patients with MRSA. Because CAP-DRPs were strongly associated with IIAT in this study, identification of the risk factors associated with CAP-DRPs is crucial to ensure appropriate initial antibiotic treatment.

Here, six independent risk factors for CAP-DRPs were revealed in patients with CAP and HCAP. Because these risk factors were identical in CAP and HCAP, a prediction rule was developed combining the data for patients with these two types of pneumonia. Among the variables included in the HCAP definition, only hospitalization for 2 days or more during the preceding 90 days was statistically significant. Previous studies have proved the HCAP definition to be less accurate in predicting the occurrence of DRPs in patients with pneumonia (18, 19, 22). This study elucidated the importance of five other factors not included in the HCAP definition (i.e., use of antibiotics within the previous 90 d, immunosuppression, use of gastric acid–suppressive agents, tube feeding, and nonambulatory status). Although there was variation of the risk factors for drug resistance among studies, differences between our results and findings of previous studies may be attributable to the fact that some of the previously mentioned five factors were not available in previous studies (21, 36–39). Use of gastric acid–suppressive agents, which is known as a risk factor for the occurrence of CAP and HAP (40, 41), was newly identified to be a risk factor for drug resistance. Although increased pH levels in gastric juice have been associated with proliferation of bacteria (42), the connection between drug resistance acquisition and use of gastric acid–suppressive agents is a topic for future investigation.

This study indicated a difference in CAP drug resistance between patients with CAP and those with HCAP. This difference can be easily quantified by the cumulative risk factors for CAP-DRPs. These factors are common to both patients with CAP and HCAP. Therefore, a unified strategy of initial antibiotic selection for treatment of CAP and HCAP may be used.

Prediction of the presence or absence of DRPs at diagnosis is crucial in the treatment of pneumonia (20, 43). Recently, two research groups have developed scoring systems to predict drug resistance; these systems assign various weights to the respective risk factors (21, 22). However, a simpler method is preferable because of the high prevalence of this disease and the need for rapid decision-making about the most appropriate antibiotic regimen. Fortunately, the ORs of all independent risk factors included in this study were similar (2.0–2.5). Therefore, the proposed prediction rule for CAP drug resistance, which consisted of counting the number of risk factors observed in a given pneumonia patient, is feasible. In comparing the simple counting of the number of risk factors with the scoring system using their different weight based on the logistic regression findings in this study, the AU-ROC of these two methods were similar. Furthermore, the AU-ROC using this proposed method (0.79) was not inferior to 0.71 of Shorr's scoring and 0.79 of Aliberti's scoring that were published in their original reports (21, 22).

TABLE 6. ADMINISTERED ANTIBIOTICS AND CLINICAL OUTCOME IN EACH RISK GROUP OF CAP DRUG-RESISTANT PATHOGENS*

	Number of Risk Factors for CAP-DRPs†		
	≤1	2	≥3
Patients with identified pathogens‡, n	491	142	113
Drug-resistant pathogens			
All CAP-DRPs	32/491 (6.5)	31/142 (21.8)	56/113 (49.6)
CAP-DRPs other than MRSA	12/491 (2.4)	9/142 (6.3)	32/113 (28.3)
MRSA	20/491 (4.1)	25/142 (17.6)	32/113 (28.3)
MRSA in patients who had any one of specific risk factors for MRSA§	5/56 (8.9)	12/33 (36.4)	12/28 (42.9)
Inappropriate initial antibiotic treatment	72/490 (14.7)	44/142 (31.0)	49/112 (43.8)
Administered initial antibiotics			
Traditional regimens of CAP drugs‖	155/491 (31.6)	23/142 (16.2)	7/113 (6.2)
Monotherapy with nonantipseudomonal β-lactams¶	203/491 (41.3)	67/142 (47.2)	50/113 (44.2)
Antipseudomonal antibiotics	114/491 (23.2)	39/142 (27.5)	48/113 (42.5)
Anti-MRSA antibiotics	3/491 (0.6)	1/142 (0.7)	3/113 (2.7)
30-d mortality			
Overall	42/491 (8.6)	21/142 (14.8)	26/113 (23.0)
Inappropriate initial antibiotic treatment	7/72 (9.7)	7/44 (15.9)	14/49 (28.6)
Traditional regimens of CAP drugs**	2/155 (1.3)	3/23 (13.0)	0/7 (0)
Monotherapy with nonantipseudomonal β-lactams††	22/203 (10.8)	11/67 (16.4)	11/50 (22.0)
Patients without identified pathogens, n	439	122	57
Administered initial antibiotics			
Traditional regimens of CAP drugs‖	129/439 (29.4)	24/122 (19.7)	6/57 (10.5)
Monotherapy with nonantipseudomonal β-lactams¶	177/439 (40.3)	52/122 (42.6)	28/57 (49.1)
Antipseudomonal antibiotics	93/439 (21.2)	40/122 (32.8)	20/57 (35.1)
Anti-MRSA antibiotics	0/439 (0)	2/122 (1.6)	1/57 (1.8)
30-d mortality			
Overall	38/439 (8.7)	22/122 (18.0)	13/57 (22.8)
Traditional regimens of CAP drugs**	4/129 (3.1)	1/24 (4.2)	1/6 (16.7)
Monotherapy with nonantipseudomonal β-lactams††	17/177 (9.6)	14/52 (26.9)	7/28 (25.0)

Definition of abbreviations: CAP = community-acquired pneumonia; CAP-DRP = CAP drug-resistant pathogen; MRSA = methicillin-resistant *Staphylococcus aureus*.

* Data are presented as n (%) unless indicated otherwise.

† Risk factors for CAP-DRPs include prior hospitalization, immunosuppression, previous use of antibiotics, use of gastric acid–suppressive agents, tube feeding, and nonambulatory status.

‡ Patients in whom susceptibilities of pathogens to CAP drugs could not be assessed were not included.

§ Specific risk factors for MRSA include chronic dialysis, positive MRSA history, and congestive heart failure.

‖ Traditional regimens of CAP drugs include the following regimens: combination therapy with β-lactams (ceftriaxone or ampicillin-sulbactam) plus macrolides (azithromycin, clarithromycin, or erythromycin) or monotherapy with fluoroquinolones (moxifloxacin, levofloxacin, or garenoxacin).

¶ Nonantipseudomonal β-lactams include the following antibiotics: ampicillin, ampicillin-sulbactam, ceftriaxone, and cefotaxime.

** β-Lactams (ceftriaxone or ampicillin-sulbactam) plus macrolides (azithromycin, clarithromycin, or erythromycin) were administered to all of 185 patients with identified pathogens. In 159 patients without identified pathogens, β-lactams plus macrolides and monotherapy with a fluoroquinolone (levofloxacin) were administered to 156 and 3 of them, respectively.

†† Ampicillin-sulbactam, ceftriaxone, and cefotaxime were administered to 178, 141, and 1 patient with identified pathogens, respectively. Ceftriaxone, ampicillin-sulbactam, and ampicillin were administered to 136, 120, and 1 patient without identified pathogens, respectively.

Therefore, the proposed simple prediction rule is a useful addition in clinical settings. Validation studies are awaited.

In 86% of patients with CAP and 36% of patients with HCAP in this study, no risk factors or only one risk factor were identified. Administration of CAP drugs to these patients would be acceptable because the risk of resistance to these drugs was low (<10%). Therefore, administration of broad-spectrum antibiotics should be refrained for patients of this low-risk group. In fact, 30-day mortality was low (≤3.1%) in patients who received traditional regimens of CAP drugs including combination therapy with β-lactams plus macrolides. Regarding administration of CAP drugs, monotherapy with nonantipseudomonal β-lactams may not be suitable as reported previously (44–46). However, for patients with CAP and HCAP with three or more risk factors, the risk of resistance to CAP drugs was high (>40%). Broad-spectrum antibiotics should be considered for these patients. Physicians should take into account the fact that the frequency of IIAT and the 30-day mortality in patients who received IIAT increased as the risks for CAP-DRPs rose in this study. Patients with two risk factors were at intermediate risk (~20%). In this group, the probabilities of MRSA and CAP-DRPs other than MRSA were 17.6% and 6.3%, respectively. Therefore, in patients with two or more risk factors, administration of anti-MRSA antibiotics should be considered for patients with the specific risk factors for MRSA (i.e., chronic dialysis, positive MRSA history, and congestive heart failure). Administration of antipseudomonal antibiotics should be curtailed in patients with two or less risk factors, and should be limited to those with three or more risk factors. The effectiveness of initial antibiotics in each risk group should be validated in future interventional studies.

This study has some limitations. First, patients enrolled in this study were all hospitalized. Therefore, the results of this study should not be applied in a straightforward manner to outpatients. Second, the pathogens identified in this study may not have been the cause of pneumonia. Laboratory samples were obtained from only sputa in as many as about 80% of patients with CAP and HCAP. Furthermore, the cultures were performed semiquantitatively rather than quantitatively. However, avoiding invasive procedures to obtain samples from lower respiratory tracts and semiquantitative culturing are common in clinical settings; thus, the results obtained in this study would be clinically relevant. A methodology for determining causative pathogens semiquantitatively and using sputa must be developed in future studies. Third, the period of patient enrollment did not include

the influenza season because a sufficient number of patients with pneumonia were registered by 2010 early winter. Finally, to deal with potential colinearity of the risk factors for CAP-DRPs, alternative statistical analysis, such as a regression tree method, might give better discrimination and be worthy of exploration. Despite these limitations, we believe that the associations between patient profile and drug resistance identified in this study are robust.

In conclusion, this multicenter, prospective, observational study examined the clinical and microbiologic features of hospitalized patients with CAP and HCAP. Risk factors for CAP-DRPs were identical in patients with CAP and HCAP. A new prediction rule for drug resistance was proposed that is applicable to patients in these two groups. This simple and feasible prediction rule involves the simple counting of the number of risk factors to determine appropriate initial antibiotic treatment for patients with pneumonia.

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TIMELESS is overexpressed in lung cancer and its expression correlates with poor patient survival

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***TIMELESS* (*TIM*) is a mammalian homolog of a Drosophila circadian rhythm gene, but its circadian properties in mammals have yet to be determined. *TIM* appears to be essential for replication protection and genomic stability. Recently, the involvement of *TIM* in human malignancies has been reported; therefore, we investigated the role of *TIM* in lung cancer. Microarray expression analysis of lung cancer cell lines showed that *TIM* expression was elevated 3.7-fold ($P < 0.001$) in non-small cell lung cancer cell lines ($n = 116$) compared to normal lung controls ($n = 59$). In addition, small cell lung cancer cell lines ($n = 29$) expressed *TIM* at levels 2.2-fold ($P < 0.001$) higher than non-small cell lung cancer. Western blot analysis of 22 lung cancer cell lines revealed that all of them expressed TIM protein and that 20 cell lines (91%) expressed TIM protein at higher levels than a normal control line. Remarkably, immunohistochemistry of 30 surgically resected lung cancer specimens showed that all lung cancer specimens but no matched normal lung tissues were positive for TIM expression. Moreover, immunohistochemistry of surgically resected specimens from 88 consecutive patients showed that high TIM protein levels correlated with poor overall survival ($P = 0.013$). Mutation analysis for *TIM* in 23 lung cancer cell lines revealed no mutation. *TIM* knockdown suppressed proliferation and clonogenic growth, and induced apoptosis in H157 and H460 cells. Taken together, our findings suggest that *TIM* could be useful as a diagnostic and prognostic marker for lung cancer and targeting it would be of high therapeutic value for this disease. (*Cancer Sci* 2013; 104: 171–177)**

Circadian rhythms of approximately 24-h periodicity are commonly observed in a variety of physiological functions of organisms from bacteria to humans.[1] In *Drosophila*, *period* (*per*) and *timeless* (*tim*) are two main clock genes that participate in an intracellular transcriptional/translational feedback loop and create 24-h rhythmicity.[2,3] In mammals, homologs of several of the fly clock genes have been identified and characterized. Mammalian *Timeless* (*mTim*) was cloned as a mammalian homolog of *Drosophila tim*, but its role in mammalian circadian clock systems has not been fully clarified.[4] TIM knockdown in the rat suprachiasmatic nucleus (SCN) disrupted SCN neuronal activity rhythms and caused a reduction of PER1, PER2 and PER3 and an increase in CRYPTOCHROME (CRY)1and CRY2.[5] Furthermore, full-length TIM protein exhibited a 24-h oscillation, whereas a truncated isoform was constitutively expressed,[5] indicating the active role of mTIM in the circadian clock system. By contrast, several reports suggest that mTIM does not function as a clock protein.[6,7] Database mining and phylogenetic sequence analysis showed that mTIM may not be a true ortholog of a Drosophila circadian rhythm gene, because it shares even greater sequence similarities with other TIM-related genes that do not function as clock genes, including *Drosophila* TIMEOUT, *Bombyx mori* TIMEOUT, and *Caenorhabditis elegans* TIM-1.[7] In addition, a heterozygous *Tim* mutant mouse did not show a change in circadian phenotype despite reduced TIM protein levels.[7] Thus, it is still unclear whether TIM functions as a clock protein.

Previous reports show the involvement of *TIM* in human cancers. First, screens of human breast cancers have identified *TIM* mutations.[8] Second, a study has reported that *TIM* depletion sensitizes HCT116 colon cancer cells to doxorubicin-induced cytotoxicity.[9] The authors conclude that this effect might be due to the requirement for *TIM* in the ataxia telangiectasia mutated-dependent Chk2 DNA damage-response pathway that causes cells to arrest at the G2/M phase.[9] Third, Fu *et al.* provide data suggesting that TIM may contribute to the carcinogenesis of breast cancer. They report an association between breast cancer risk and *single-nucleotide polymorphism* (SNP) in *TIM* and increased levels of TIM in tumor tissues compared to controls.[10] These findings suggest that *TIM* plays an important role in the development of human cancer. However, the role of *TIM* in lung cancer, one of the deadliest cancers,[11] has not been investigated. Therefore, in the current study we investigate the role of *TIM* in lung cancer. We evaluate the expression level and mutational state of *TIM* in lung cancers and examine the effects of RNA interference (RNAi)-mediated *TIM* knockdown on growth, apoptosis, and sensitivity to doxorubicin and cisplatin using the lung cancer cell lines H157and H460. No mutation was found in the cell lines studied, but TIM was overexpressed in both lung cancer cell lines and clinical lung cancer specimens. A high TIM protein level correlated with poor overall survival in lung cancer patients. In addition, *TIM* knockdown suppressed growth and induced apoptosis in lung cancer cells. These findings suggest the potential of TIM as a prognostic marker and a therapeutic target for lung cancer.

Materials and Methods

Cell culture. Cell lines used in this study were purchased from American Type Culture Colllection (Manassas, VA, USA) or obtained from the Hamon Center collection (University of Texas Southwestern Medical Center, Dallas, TX, USA). These cell lines included H820, H1975, HCC44, HCC2279, H838, PC9, H3255, HCC4011, HCC2935, A549, H1650, HCC4006, HCC827, H1666, H358, H1299, H1155, H460, H157, H146, H526, H82, H740 and the *cdk4/hTERT*-immortalized normal human bronchial epithelial cell line HBEC4.[12] Lung cancer cell lines were cultured in

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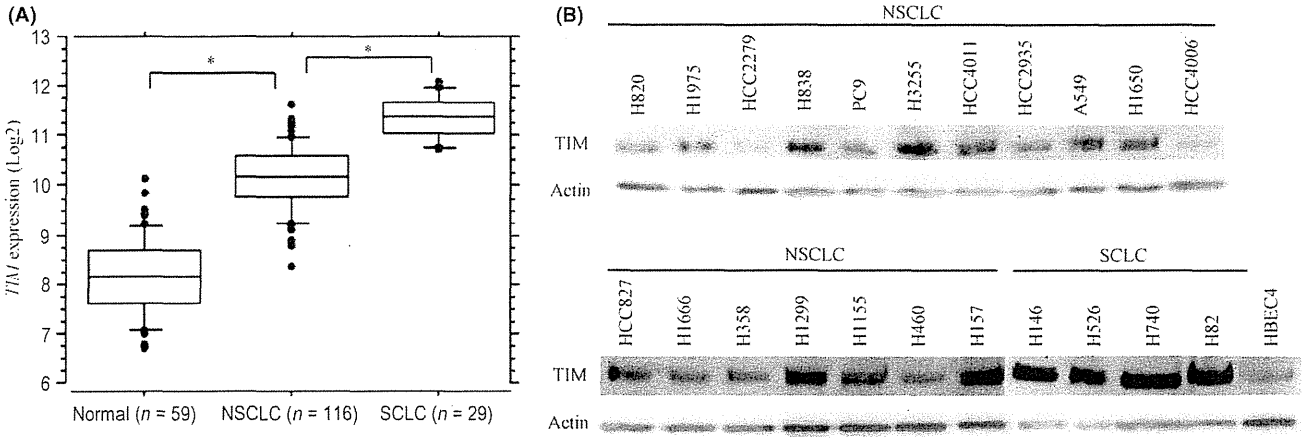

Fig. 1. TIM expression in lung cancer cell lines. (A) Microarray analysis of *TIM* mRNA. The bottom and top of the box are the lower and upper quartiles, respectively, and the band near the middle of the box is the median. The whiskers represent the 10th and 90th percentile. *$P < 0.001$ (Mann–Whitney *U*-test). (B) Western blot of TIM in lung cancer cell lines. Twenty-two lung cancer cell lines (18 non-small cell lung cancer [NSCLC] and 4 small cell lung cancer [SCLC]) and the HBEC4 cell line as a control were studied.

RPMI-1640 (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% FBS, and HBEC4 was cultured in keratinocyte serum-free medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 50 ng/mL bovine pituitary extract and 5 ng/mL epidermal growth factor.

RNA isolation. Total RNA was isolated using Trizol (Life Technologies). For mRNA analysis, 5 μg of total RNA was reverse transcribed using a Super Script III First-Strand Synthesis System using a Random primer system (Life Technologies).

Microarray analysis. RNA quality and concentration were checked using the Experion Bioanalyzer (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Total RNA (500 ng) from each sample was used to label the cRNA probes with the Illumina TotalPrep RNA Amplification kit (Cat# IL1791; Ambion, Austin, TX, USA). Amplified and labeled cRNA probes (1.5 μg) were hybridized to Illumina Human WG-6 v3.0 Expression BeadChip (Cat# BD-101-0203; Ambion) overnight at 58°C, then washed, blocked, and detected by streptavidin-Cy3 following the manufacturer's protocol. After drying, the chips were scanned using an Illumina iScan system (Ambion). Bead-level data were obtained and pre-processed using the R package Model-Based Background Correction (MBCB) for background correction and probe summarization. Pre-processed data were then quantile-normalized and log-transformed.

Direct sequencing. The sequences of primers used for reverse transcriptase PCR and for direct sequencing were: *TIM*-S101, GGA GCG GGC CTC ATC ATT TC; *TIM*-AS941, TGG CGC AAC ACC TCC AGT TC; *TIM*-S790, TCG TCT GCT GAG GAG CAA TG; *TIM*-AS 1652, GGC TGG CAT CTC TCA TCA AAC; *TIM*-S1509, GGC AAC AGT GAA TGA GAT GGA C; *TIM*-AS2442, AGG GTC ACT AAG CAG ACG ATT G; *TIM*-S 2293, CTG CTA CTA AGG AGC TAC CAG C; *TIM*-AS3175, TTG AAA GGA CTA AGC TAC CCT G; *TIM*-S3022, GCA TCC TCC ATC TTG CCA AAT G; and *TIM*-AS3929, TCC GTG AAA GAG CCT GGG ATT C. The reaction was performed in a 20 μL mixture containing primers (final concentrations 0.25 μM), HotStar Taq Master Mix (Qiagen, Tokyo, Japan; 10 μL) and cDNA (0.5 μL). Amplification was carried out in a TaKaRa PCR Thermal Cycler Dice (Takara Bio, Otsu, Japan). Cycling conditions were one cycle at 95°C for 5 min, followed by 35 cycles at 94°C for 30 s, 60°C (except for *TIM*-S2293 and *TIM*-AS3175) or 56°C (*TIM*-S2293 and *TIM*-AS3175) for 30 s and 72°C for 1 min. The final extension was at 72°C for 10 min. PCR products were purified using ExoSAP IT (GE Healthcare, Buckinghamshire, UK). PCR products were sequenced with the same primers as the PCR reaction or with internal primers using a BigDye Terminator v1.1 Cycle Sequencing

Table 1. Clinical features of 30 patients with lung cancer

Pt number	Age (Y)	Sex	Histopathological subtype	TIM immunostain	Post-operative staging
1	75	M	Ad	1+	3A
2	63	M	Sq	2+	3A
3	70	F	Ad	1+	3A
4	53	M	Ad	3+	3A
5	82	M	Sq	1+	3A
6	78	F	Sq	3+	3A
7	75	M	Ad	1+	3A
8	71	M	Sq	2+	3A
9	60	F	Ad	3+	3A
10	70	M	Sq	3+	3A
11	59	M	Ad	1+	2B
12	74	M	Ad	1+	2B
13	77	M	Sq	2+	2B
14	71	F	Ad	1+	2B
15	81	M	Sq	1+	2B
16	65	M	Sq	2+	2B
17	79	F	Ad	1+	2B
18	71	M	Ad	1+	2B
19	77	M	Sq	2+	2B
20	79	F	Sq	2+	2B
21	54	F	Ad	1+	2A
22	85	F	Ad	1+	2A
23	74	F	Ad	1+	2A
24	75	M	Sq	2+	2A
25	64	M	Sq	1+	2A
26	65	M	Sq	2+	2A
27	68	M	Ad	1+	2A
28	45	M	Ad	2+	2A
29	59	M	Sq	1+	2A
30	60	M	Sq	1+	2A

1+, 2+ and 3+ indicate that <33%, 33–66% and >66%, respectively, of tumor cells were positively stained. Ad, adenocarcinoma; Pt, patient; Sq, squamous cell carcinoma.

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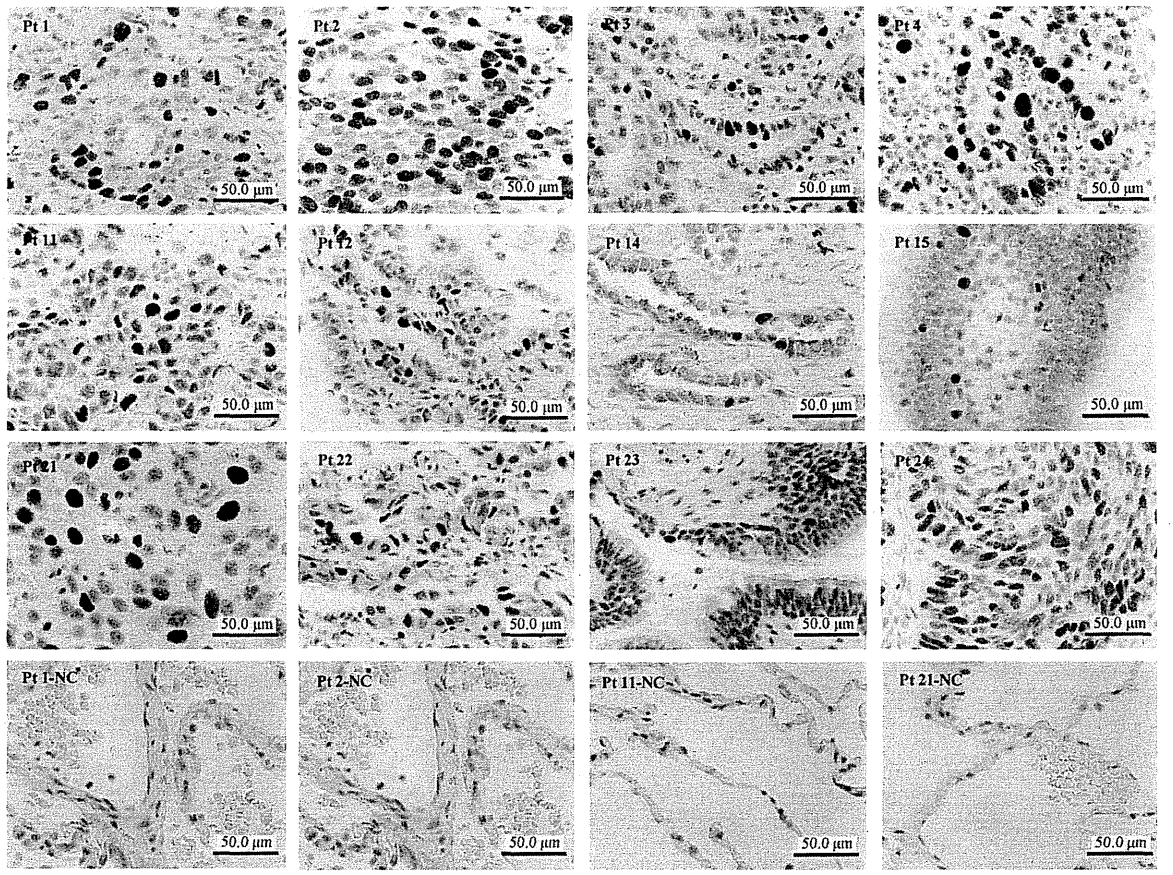

Fig. 2. Immunohistochemical staining of TIM in surgically resected lung cancer and matched normal lung specimens. Scale bars are 50 μm. Pt, patient; NC, normal lung control.

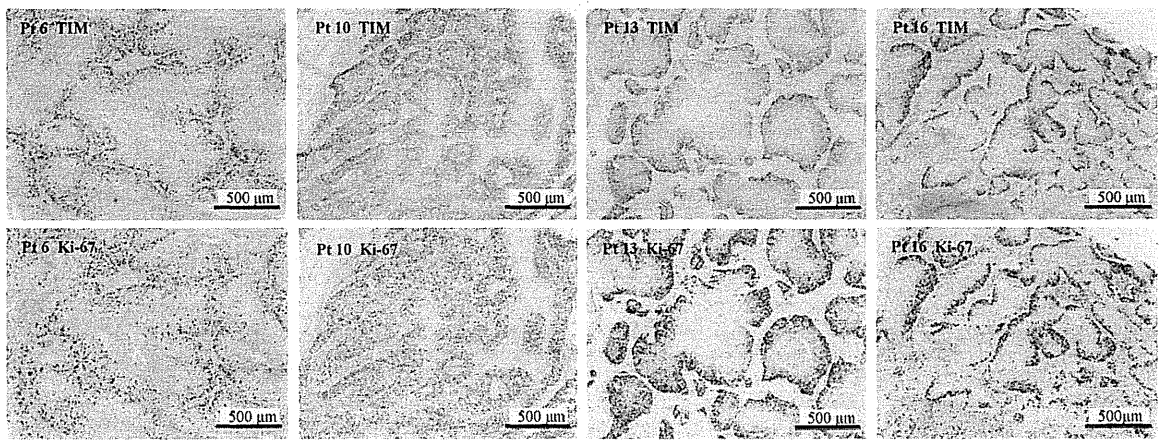

Fig. 3. Serial sections stained for TIM and Ki-67. Scale bars are 500 μm.

Kit (Life Technologies). The sequences of the internal primers were: *TIM*-S470, CTA AGG AGC CCA GCT TTC GG; *TIM*-AS658, TGA CCA GCA GTA GGA TCC GTT C; *TIM* S1902, GGA CTC CGT GGT TCC CTT TG; *TIM*-AS2060, ACA TCT CCT TCA GGC CAC ACC; *TIM*-S 2638, CCC GAA GAA GAG GCT CAT C; *TIM*-AS2800, CCT TGA CAC TGT CAG CCA GTC C; *TIM*-S3295, CCA TTG GTG CCA CTC ACA GAG; and *TIM*-AS3558, TCG GTG CTC TTT ACA GTG CTC C. Samples were electrophoresed on an ABI Prism 310 Genetic Analyzer (Life Technologies) and analyzed using Sequencing Analysis Software Version 5.1 (Life Technologies).

Western blot analysis. Western blot analysis was performed as described previously using whole cell lysates.[13] Primary antibodies used were rabbit polyclonal anti-actin (Sigma–Aldrich), rabbit polyclonal anti-cleaved caspase3 (Cell Signaling Technology, Boston, MA, USA), and rabbit monoclonal anti-timeless (Abcam, Cambridge, MA, USA). Actin protein levels were measured as a control for equality of protein loading. Anti-rabbit

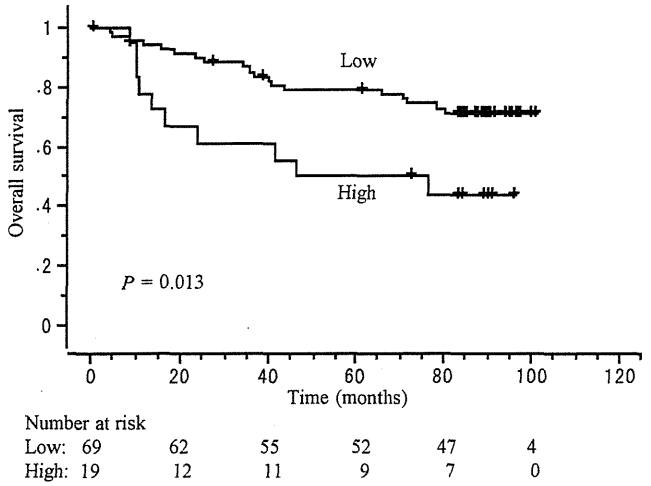

Fig. 4. Overall survival of patients with lung cancer treated with surgery according to TIM expression level. The *P*-value was calculated by log-rank test. High and Low: ≥50% and <50% of tumor cells were positive for TIM, respectively.

antibody (GE Healthcare) was used at 1:2000 dilution as a secondary antibody.

Immunohistochemistry. Surgically resected lung cancer samples were obtained from patients at Nagoya University Hospital. Before tissue samples were collected, ethical approval and fully informed written consent were obtained from all patients (ethical approval number: 285-4).

Tissue sections (4-μm thick) were cut, deparaffinized in xylene, rehydrated in graded alcohols, and blocked with 5% skim milk for 10 min. For the first set of 30 paired normal and tumor tissues, BenchMark XT (Ventana Medical Systems, Tucson, AZ, USA) was used. The antigens were retrieved by heating 60 min at 100°C in EDTA (pH 8.5). Sections were incubated with anti-timeless antibody (Abcam) at 37°C for 32 min or anti-Ki-67 antibody (Roche, Tokyo, Japan) at 37°C for 16 min, and then biotinylated using a secondary antibody at 37°C for 8 min and streptavidin-peroxidase conjugate at 37°C for 8 min. For the second set of tumor tissues that were consecutively obtained from 88 patients, immunohistochemistry (IHC) was performed manually. The antigens were retrieved by heating for 60 min at 98°C in citrate buffer (pH 6). Sections were incubated with anti-timeless antibody at room temperature for 60 min, and then biotinylated using a secondary antibody at room temperature for 30 min and streptavidin-peroxidase conjugate at room temperature for 30 min. Diaminobenzidine (DAB) substrate was used for color development. Cells with strongly stained nuclei were defined as positive. For the first set of 30 paired tissues, positive cells were divided into three categories: 1+, 2+ and 3+, indicating that <33%, 33–66% and >66%, respectively, of tumor cells were positive. For the second set of 88 tumor tissues, High and Low indicate that ≥50% and <50%, respectively, of tumor cells were positive.

Transfection of short interfering RNA. H157 and H460 cells (4×10^5) were plated in 6-well plates. The following day, cells were transiently transfected with either 10 nM predesigned short interfering RNA (siRNA) (Stealth Select RNAi, Life Technologies) targeting *TIM* or control siRNA (Life Technologies) using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's protocol. After 48 h, the transfected cells were harvested for further analyses or plated for cell growth assays.

Cell growth assays. A colorimetric proliferation assay was performed using a WST-1 assay kit (Roche) according to the manufacturer's instructions. Liquid and soft agar colony formation assays were done as described previously.[14]

Drug sensitivity assay. H157 and H460 cells were transfected with *TIM* RNAi or control oligos. Forty-eight hours after transfection, cells were seeded in 96-well plates at a density of 2×10^4 cells/mL (50 μL/well) and incubated for 24 h. Then the cells were treated with various doses of doxorubicin (Wako, Osaka, Japan) or cisplatin (Sigma–Aldrich) for 5 days, and cell viability was measured using a WST-1 assay.

Statistical analysis. Stat View version 5.0 (SAS Institute, Cary, NC, USA) was used for all statistical analyses in this study. The Mann–Whitney *U*-test was used for analyzing differences between two groups. Overall survival (OS; OS event, death resulting from any cause) were measured from the date of surgery and estimated using the Kaplan–Meier method. Differences were assessed using the log-rank test. Pearson's χ^2-test and Fisher's exact test were used to evaluate the independency between TIM expression and disease stage or tumor histology, respectively.

Results

TIMELESS is overexpressed in human lung cancers. We examined *TIM* mRNA levels using microarray expression analysis in a large panel of non-small cell lung cancer (NSCLC; $n = 116$), small cell lung cancer (SCLC; $n = 29$) and normal lung controls (normal human lung cultures and immortalized normal human bronchial epithelial cell lines; $n = 59$; Fig. 1A). *TIM* expression was elevated 3.7-fold (Mann–Whitney *U*-test, $P < 0.001$) in NSCLC cell lines compared to normal lung controls. SCLC expressed *TIM* more abundantly than NSCLC 2.2-fold (Mann–Whitney *U*-test, $P < 0.001$).

To evaluate TIM expression at the protein level, we performed western blot analysis for TIM in 18 NSCLC cell lines and 4 SCLC cell lines. All 22 cell lines expressed TIM, and 20 cell lines (91%) expressed TIM at higher levels than the normal control line HBEC4 (Fig. 1B). TIM expression levels of SCLC were higher than those of NSCLC. We obtained tumor specimens and matched normal lung tissues from 30 patients with lung cancer who underwent surgery and performed IHC of TIM on them (Table 1, Fig. 2). All the lung cancer specimens studied were positively stained, but all the matched normal lung tissues were negative for TIM staining. These results demonstrated that TIM was overexpressed in lung cancer.

Cells positively stained for TIM were distributed mainly in the invasive front of the tumor in several specimens (Fig. 3, Pt6, Pt13, Pt16, upper panels). We performed IHC on serial sections for TIM and Ki-67, an established marker for cellular proliferation. A correspondence was observed between the distributions of cells stained for TIM and Ki-67 (Fig. 3). This suggests the possible role of TIM as a proliferation marker.

TIMELESS expression correlates with poor overall survival in non-small cell lung cancer. Immunohistochemistry of TIM was performed on surgically resected NSCLC tissues from 88 consecutive patients. The operations were performed from January 2004 to April 2005, and the median follow-up period was 84 months. Patients whose tumor expressed higher levels of TIM protein had significantly shorter overall survival ($P = 0.013$; Fig. 4). Five-year survival rates for high TIM and low TIM expression groups were 79.2% and 50.2%, respectively. TIM expression did not correlate with disease stage or histological subtype ($P = 0.87$, $P = 0.75$, respectively).

No *TIMELESS* mutation was found in lung cancer cell lines. We searched for a mutation in *TIM* by direct sequencing. We analyzed the whole coding sequence of *TIM*. A total of 19 NSCLC cell lines (H820, H1975, HCC44, HCC2279, H838, PC9, H3255, HCC4011, HCC2935, A549, H1650, HCC4006, HCC827, H1666, H358, H1299, H1155, H460 and H157) and

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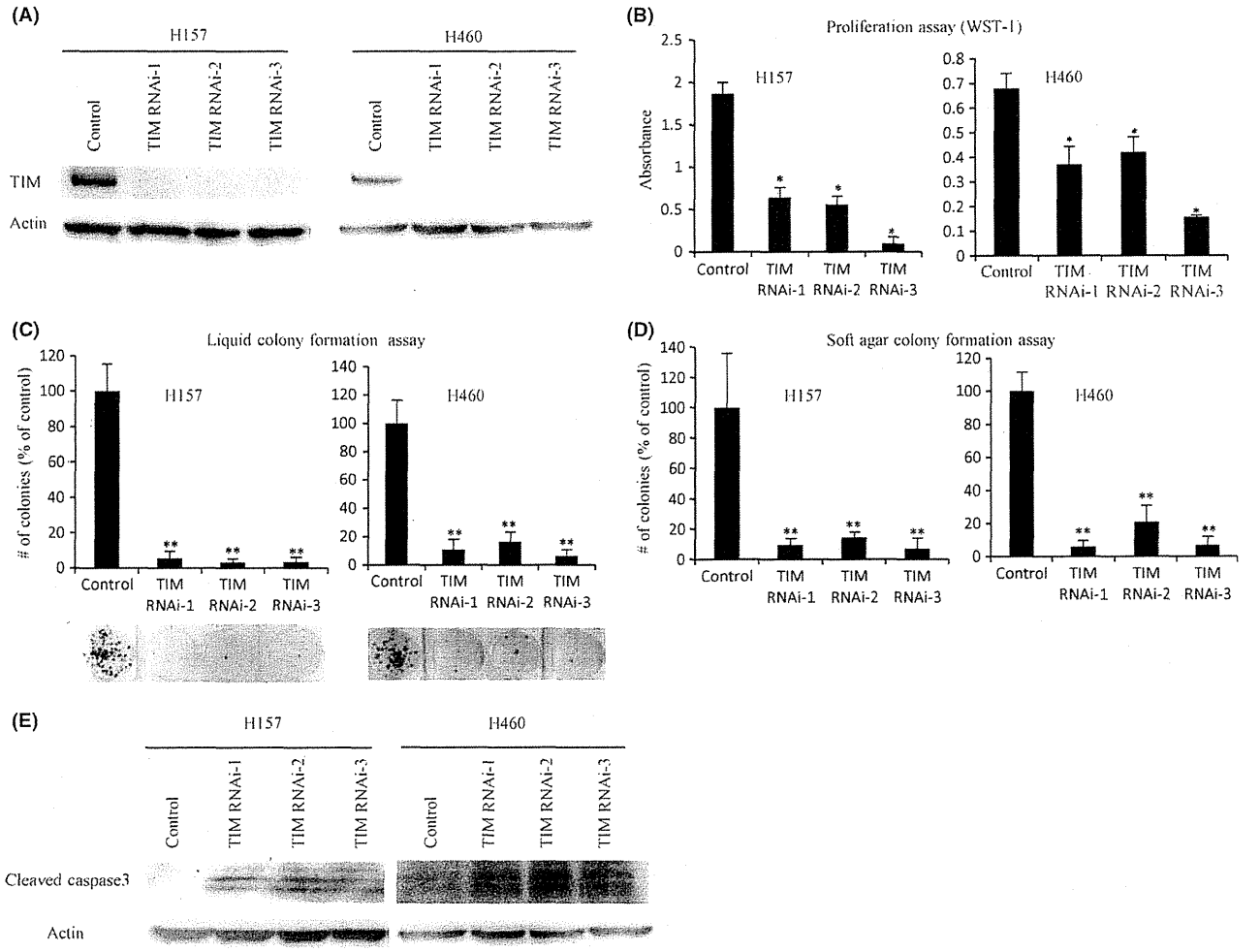

Fig. 5. RNAi-mediated knockdown of *TIM* in H157 and H460 lung cancer cells suppressed cell proliferation, liquid colony formation, soft agar colony formation, and induced apoptosis. (A) Western blot analysis of TIM in H157 and H460 cells transfected with *TIM* RNAi or control oligos. (B) Cell proliferation (WST-1) assay of H157 and H460 cells transfected with *TIM* RNAi or control oligos. Forty-eight hours after transfection, cells were seeded in 96-well plates at a density of 1×10^4 cells/mL (100 μL/well) and incubated for 6 days for H157 cells and 5 days for H460 cells. Cell viability was measured by a WST-1 assay. (C) Liquid colony formation assay of H157 and H460 cells transfected with *TIM* RNAi or control oligos. Forty-eight hours after transfection, 500 cells were plated in 6-well plates in triplicate, cultured 9 days for H157 cells and 15 days for H460 cells, and stained with methylene blue. Colonies >2 mm were counted. Colony numbers of cells transfected with control oligos were set as 100%. (D) Soft agar colony formation assay of H157 and H460 cells transfected with *TIM* RNAi or control oligos. Forty-eight hours after transfection, 1000 cells were suspended in 0.37% SeaKem GTG Agarose (Lonza, Rockland, ME, USA), plated in 12-well plates in triplicate, and H157 cells were incubated for 11 days and H460 cells for 15 days. Colonies (>32 cells) were counted. Colony numbers of cells transfected with control oligos were set as 100%. (E) Western blot of cleaved caspase 3 in H157 and H460 cells transfected with *TIM* RNAi or control oligos. Results are from three independent experiments and shown as mean ± SD (B–D). $^*P < 0.05$ and $^{**}P < 0.001$ (Mann–Whitney *U*-test), respectively.

Table 2. IC_{50} **for doxorubicin and cisplatin after** *TIM* **knockdown**

Cell line	RNAi	Doxorubicin (IC_{50} nM)	Cisplatin (IC_{50} μM)
H157	Control	107.3	4.9
	TIM RNAi-1	23.1	1.7
	TIM RNAi-2	42.3	0.91
H460	Control	27.6	0.74
	TIM RNAi-1	61.8	3.8
	TIM RNAi-2	47.1	1.1

4 SCLC cell lines (H146, H526, H82 and H740) were analyzed, but no mutation of *TIM* was found in cDNA from these cell lines (data not shown).

***TIMELESS* knockdown suppresses growth of lung cancer cells and induces apoptosis.** To evaluate the role of TIM expression in the pathogenesis of lung cancer cells, a transient knockdown of *TIM* by RNAi was done in the H157 and H460 cell lines (Fig. 5A). To minimize the possibility of off-target effects, we used three non-overlapping synthesized oligos targeting *TIM*. To examine the effect of *TIM* knockdown on cellular proliferation, we performed WST-1 colorimetric assays and found that the *TIM* knockdown suppressed proliferation to 7–35% in H157 cells and 23–62% in H460 cells of that of the controls (Fig. 5B). The effect of *TIM* knockdown on clonal growth was measured by liquid colony formation assay. *TIM* knockdown suppressed colony formation to 3–5% in H157 cells and 6–16% in H460 cells (Fig. 5C). Next, to evaluate the effects of the *TIM* knockdown on anchorage-independent growth, we carried out a soft agar colony formation assay. *TIM* knockdown suppressed growth in soft agar to 7–15% in H157 cells and 6–21%

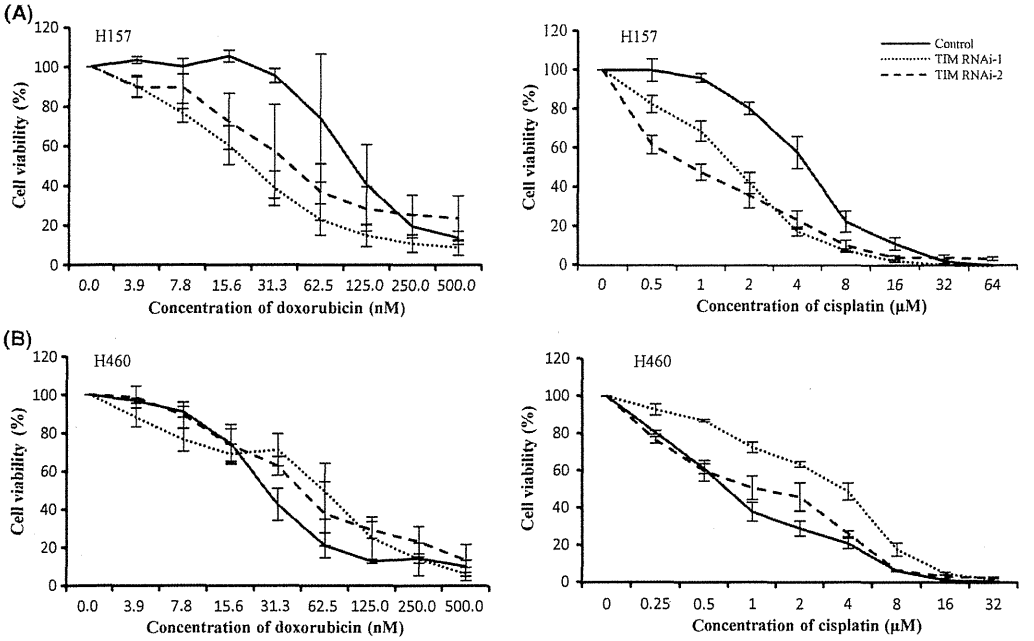

Fig. 6. *TIM* knockdown enhanced cytotoxicity of doxorubicin and cisplatin in H157 but not in H460 cells. (A) H157 and (B) H460 cells were transfected with *TIM* RNAi or control oligos. Forty-eight hours after transfection, cells were seeded in 96-well plates at a density of 1×10^4 cells/mL (100 μL/well) and incubated for 24 h. Then the cells were treated with various doses of doxorubicin or cisplatin for 5 days, and cell viability was measured by a WST-1 assay. Results are from three independent experiments and are shown as mean ± SD.

in H460 cells (Fig. 5D). Western blot analysis revealed increased levels of cleaved caspase3 after *TIM* knockdown, suggesting that apoptosis was involved in growth inhibition (Fig. 5E).

Sensitivity to doxorubicin and cisplatin is increased by *TIM* knockdown in H157 cells but not in H460 cells. Yang *et al.* (2010)[9] show that *TIM* knockdown leads to chemosensitization of HCT116 colon cancer cells to doxorubicin. Therefore, we decided to explore the sensitivity of H157 and H460 cells to doxorubicin as well as cisplatin, which is one of the most widely used chemotherapeutic drugs for the treatment of NSCLC, following *TIM* knockdown. As shown in Figure 6A and Table 2, *TIM* RNAi exhibited increased sensitivity to doxorubicin and cisplatin in H157, which is consistent with Yang *et al.* By contrast, *TIM* RNAi did not increase sensitivity to these drugs in H460 (Fig. 6B and Table 2). These results suggest that *TIM* knockdown may exert different effects on the sensitivity to cytotoxic drugs in lung cancer, possibly depending on the cellular context.

Discussion

In the present study, we found that TIM was overexpressed in a panel of human lung cancer cell lines as well as clinical lung cancer specimens compared to normal lung controls. Fu *et al.*,[10] using public databases, reported the overexpression of *TIM* in breast tumor tissue compared to adjacent normal tissue. The authors also found *TIM* promoter hypomethylation in peripheral blood samples from stage II, III and IV breast cancer patients. Although they did not analyze the methylation status of the corresponding tumor tissue, further investigation of the association between *TIM* promoter methylation status and the expression level of TIM in lung cancer may be warranted because *TIM* hypomethylation is one possible molecular mechanism accounting for TIM overexpression in lung cancer cells.

In some cases, the patterns of IHC staining for TIM and Ki-67 showed similarities, implying a possible role for TIM as a marker of cellular proliferation. Conversely, Xiao *et al.*[15] find no significant association between the patterns of proliferating cell nuclear antigen staining and TIM on IHC analysis of serial sections of mouse embryonic lungs. This might reflect a possible difference between tissues during morphogenesis and developed tissues. However, this point needs further study for clarification.

Recurrence of NSCLC is observed in 30–75% of patients who have undergone complete resection, depending on pathologic stage.[16] TIM might help stratify patients for appropriate adjuvant therapy. Our results were based on retrospective data and we observed only 29 deaths in the follow-up period. Because of the relatively small number of observed deaths, we did not perform multivariate analysis. To verify the usefulness of TIM expression as a prognostic factor, the present results need to be confirmed by an adequately designed prospective study with an appropriate multivariate analysis taking into account the classical well-defined prognostic factors for survival in lung cancer patients. While we are writing this manuscript, Schepeler *et al.*[17] reported that abundant TIM expression in non-muscle-invasive bladder cancer correlated with risk of progression to muscle-invasive disease. This result is in line with our findings and supports our notion that TIM could be used as a prognostic marker.

Sjöblom *et al.*[8] found mutations of *TIM* in two breast cancer xenografts of 35 breast cancer cell lines or xenografts during a comprehensive mutation search. However, we did not detect *TIM* mutation in the 23 lung cancer cell lines examined. Our provisional results suggest that *TIM* mutations might not contribute to the development of lung cancer. Taking into consideration the relatively small number of cell lines used in our study, mutation analysis for *TIM* in a larger panel of lung cancer samples might be necessary. An SNP in the *TIM* promoter was associated with breast cancer risk among 441 breast cancer cases and 479 cancer-free controls.[10] It would be

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interesting to examine whether such an association is also seen in lung cancer cases.

Silencing of *TIM* suppressed cell proliferation and clonogenic growth and induced apoptosis in lung cancer cell lines. Previous published studies demonstrate the critical role of *TIM* in DNA replication and intra-S checkpoint. From these findings, we speculated that the growth-suppressive effect of *TIM* knockdown may occur through inhibition of DNA replication, and, therefore, we evaluated DNA synthesis by BrdU incorporation analysis. However, we did not see significant differences in BrdU incorporation between cells treated with *TIM* knockdown oligos and control cells (data not shown), implying that the growth inhibitory effects of *TIM* knockdown may not be attributable primarily to inhibition of DNA synthesis. Instead, we assume that the apoptosis induced by *TIM* knockdown may mainly suppress growth. We speculate that *TIM* knockdown-induced apoptosis might result from an impaired intra-S checkpoint that leads to apoptosis. Replication protein A (RPA), a single-stranded DNA binding protein, might be involved in this process because RPA was shown to interact with DNA damaged by cisplatin,[18] and Gotter *et al.*[19] demonstrate that TIM-Tipin complex binds directly to RPA.

We observed different effects of *TIM* knockdown on drug sensitivity between H157 and H460 cells. *TIM* knockdown enhanced the sensitivity to doxorubicin and cisplatin in H157 cells but not in H460 cells. Cellular capacity for DNA damage response is an important determinant of drug sensitivity in cancer cells. H157 and H460 cells differ in regards to the status of p53 protein, which plays a pivotal role in DNA damage response; H157 has a p53 nonsense mutation, resulting in non-functional p53 protein, while H460 has the wild-type p53 gene.[20] Therefore, it is possible that the observed difference in the effects of *TIM* knockdown between these two cell lines was due to their different p53 statuses.

In conclusion, TIM is overexpressed in lung cancer and its expression is associated with poor patient survival. Moreover, *TIM* knockdown inhibited tumor growth, and resulted in apoptosis in cultured lung cancer cells. These results suggest that *TIM* is a promising diagnostic and prognostic marker and an attractive therapeutic target for lung cancer.

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Disclosure Statement

The authors have no conflict of interest to declare.

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