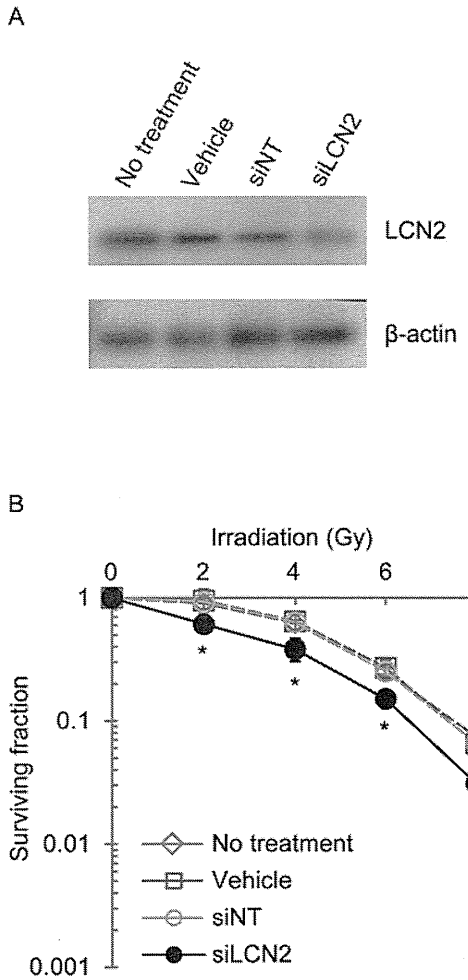

Figure 5. Functional analysis of siLCN2-transfected HSC-2 cells. (A) Decreased expression of LCN2 protein in siLCN2-transfected HSC-2 cells was validated by western blot analysis. (B) Survival of siLCN2-transfeced HSC-2 cells was significantly decreased after 2, 4, 6 and 8 Gy of radiation as compared with that of siNT-transfected cells (*P<0.01, Student's t-test).

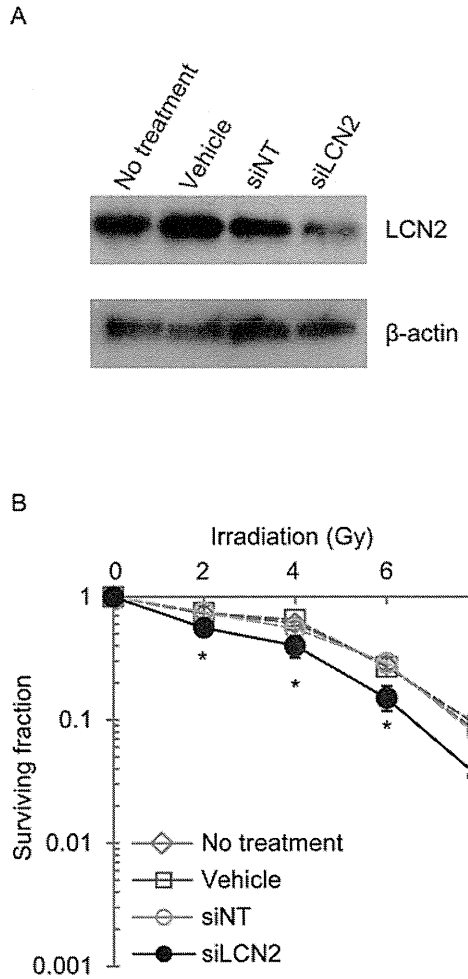

Figure 6. Functional analysis of siLCN2-transfected A549 cells. (A) Decreased expression of LCN2 protein in siLCN2-transfected A549 cells was validated by western blot analysis. (B) Survival of siLCN2-transfected A549 cells was significantly decreased after 2, 4, 6 and 8 Gy of radiation as compared with that of siNT-transfected cells (*P<0.01, Student's t-test).

the conclusive pathway regulating radioresistance in OSCC has not yet been established, suggesting that the mechanism of responsiveness for irradiation is complex and that various molecules are engaged in the process. Moreover, different subpopulations of tumor cells might have different responses to irradiation. Studies have indicated that cancer stem cells might have key roles in tumor growth, metastasis, progression and chemo-radioresistance (35). Further investigations are needed to clarify subpopulation-dependent characteristics that regulate radioresistance.

In conclusion, we identified genes that are differentially expressed in X-ray irradiated OSCC-derived cell lines. Expression of *LCN2* mRNA was significantly greater in irradiated cells than in unirradiated cells, and *LCN2* gene silencing enhanced the radiosensitivity of OSCC-derived cell lines and a lung cancer cell line. The current study indicates for the first time that LCN2 is related to radiation response. Our findings suggest that overexpression of LCN2 might contribute to radiation resistance in cancer cells and that LCN2 could be a diagnostic marker and therapeutic target for OSCC and other cancers. This information may lead to the discovery of new target genes and perhaps the development of better radiotherapy strategies for the treatment of cancer.

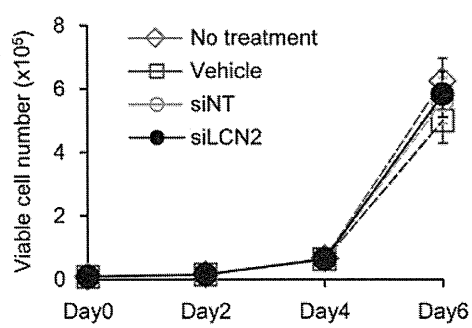

Figure 7. The effect of *LCN2* gene silencing on cell proliferation. A cellular proliferation assay was performed to investigate the effect of diminished LCN2 expression on Ca9-22 cells. There was no significant difference in the proliferation rate between siLCN2-transfected cells and control cells.

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ORIGINAL ARTICLE

Detection of chronic obstructive pulmonary disease in community-based annual lung cancer screening: Chiba Chronic Obstructive Pulmonary Disease Lung Cancer Screening Study Group

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ABSTRACT

Background and objective: **Detection of chronic obstructive pulmonary disease (COPD) is crucial in the management of COPD. The aim of this study was to establish the utility of a community-based lung cancer screening for detecting COPD.**

Methods: **In Japan, community-based lung cancer screening for residents who are 40 years or older using chest radiography is well established. A screening system in Chiba City, Japan, was used to detect COPD. The criteria to consider COPD at screening included age of 60 years or older, a smoking history and chronic respiratory symptoms. Participants fulfilling these criteria were referred for diagnostic evaluation consisting of pulmonary function testing (PFT) and chest computed tomography (CT).**

Results: **Of 89 100 Chiba City residents who underwent lung cancer screening, 72 653 residents were 60 years or older. Among them, 878 (1.0%) were identified with suspected COPD and referred for further evaluation. Of those identified, a total of 567 residents (64.6%, 567/878) underwent further evaluations, and 161 (28.4%) were reported to have COPD, with 38.5% of them requiring COPD treatment. To verify the diagnoses from the secondary evaluation centres, PFT and CT data were collected from 228 study participants, and 24.9% were diagnosed with COPD. CT findings classified according to the Goddard classification revealed that 20.1% of these participants had moderate to severe emphysema.**

Conclusions: **COPD screening added to a community-based lung cancer screening programme may be effective in the detection of patients with COPD.**

SUMMARY AT A GLANCE

We investigated the feasibility of detecting COPD using a community-based lung cancer screening programme. Approximately 30% of residents 60 years or older with smoking history and respiratory symptoms were diagnosed with COPD. Half of them had moderate or severe COPD.

Key words: chronic obstructive pulmonary disease, computed tomography, lung cancer, pulmonary function testing, screening.

Abbreviations: COPD, chronic obstructive pulmonary disease; CT, computed tomography; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; PFT, pulmonary function testing.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a rapidly growing worldwide health problem. In 2008, the World Health Organization listed COPD as the fourth most common cause of death, with 3.28 million deaths annually (5.8% of all deaths).[1] COPD is a very costly disease, with estimated direct medical costs of $14.7 billion in the United States in 1993.[2] COPD is not only a pulmonary disease, but also a systemic inflammatory disease leading to various comorbidities, such as cardiovascular disease and lung cancer,[3] and has been reported to be a risk factor of lung cancer independent from cigarette smoking.[4,5] Mannino and Braman suggested that better public health and early medical interventions targeting the risk factors for COPD may decrease the growing public health impact of COPD.[6] Therefore, encouraging smoking cessation and early detection of COPD are crucial for disease prevention and lung cancer screening.[7]

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COPD is substantially underdiagnosed[8,9] and frequently misdiagnosed.[10] Soriano *et al.* reported that 72–93% of COPD patients were not diagnosed with their condition.[9] However, although pulmonary function testing (PFT) has been well recognized as an appropriate tool for detecting COPD,[11] effective methods for early detection of COPD have not been identified. The aim of this clinical study was to evaluate the usefulness of a community-based lung cancer screening programme for detecting COPD.

METHODS

Study design

Community-based lung cancer screening using chest radiography is well established in Japan.[12,13] Japanese residents who are 40 years or older can undergo annual chest radiography. This screening programme has been supported by the national government under the Health and Medical Services Law for the Aged since 1987. Participants who are suspected to have lung cancer by chest X-ray or sputum cytology are offered further examinations to explore the diagnosis during a secondary evaluation.

For the purpose of this study, this initial lung cancer screening system was utilized to also detect COPD. While lung cancer screening utilizes only chest radiography, COPD screening utilized questionnaires on smoking status and daily symptoms. The following criteria were used to identify residents at risk for COPD at the primary screening: aged 60 years or older; a smoking history; and chronic respiratory symptoms, including cough, cough producing sputum, frequent respiratory infections and dyspnoea on effort. The reason for setting this age criterion was based on the report by Fukuchi *et al.* that the prevalence of COPD steeply increased at age 60 years and older (5.8% at 50–59 years, 15.7% at 60–69 years and 24.7% at 70 years and older).[14] During the primary screening, residents were asked about their lifestyle, smoking status and past medical history. If they fulfilled all three criteria, the residents were offered a secondary evaluation to undergo additional examinations for COPD. The additional examinations consisted of PFT and computed tomography (CT) to detect COPD and radiological emphysema.

The diagnosis was recorded by the examining physicians at the secondary centres. The primary endpoints of this study were the detection rate of COPD and identifying the distribution of COPD severity. The study protocol was approved by the committee of the Chiba COPD Lung Cancer Screening Study Group, and each participating hospital and clinic.

Participants

The screening system of Chiba City, Japan, the 13th largest city with approximately 960 000 inhabitants, was used for the study. The number of residents eligible for screening in Chiba City was 258 478 in the 2010 fiscal year. From April 2010 to March 2011, 89 100 residents (34.5%) underwent chest radiography at 257 clinics and hospitals. Of these, 72 653 residents were 60 years of age or older.

Chiba City has 39 secondary evaluation centres certified to diagnose pulmonary diseases. The diagnostic categories included the following: no abnormality, lung cancer (including suggestive findings), metastatic lung tumour, mediastinal tumour, tuberculosis, COPD/emphysema and others. The diagnosis of COPD was based on PFT, and the diagnosis of emphysema was based on CT findings. During the secondary evaluation, the evaluating physicians at the secondary centres determined the severity of COPD and made the following recommendations: no treatment and no follow-up, annual follow-up without treatment, and treatment.

To verify the reliability of the diagnoses reported from the secondary evaluation centres, the clinical data (PFT and/or CT) of 228 residents who had provided written informed consent were collected from the secondary centres and evaluated by the authors, and the authors' assessments were compared with the diagnoses of the evaluating physicians. A total of 173 participants underwent PFT and 185 underwent CT. The PFT diagnosis of COPD was based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD) staging.[15] The functional criterion for COPD was the following: forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) less than 70%. The severity of radiological emphysema was visually assessed by two independent pulmonologists according to the modified Goddard scoring system.[16,17] Six images of three lung slices (the right and left lungs were evaluated separately) were analyzed for each participant. Each image was classified and scored as follows: normal (score 0), ≤ 5% affected (score 0.5), ≤ 25% affected (score 1), ≤ 50% affected (score 2), ≤ 75% affected (score 3) and >75% affected (score 4), and the mean score of six images was considered to be representative of the severity of emphysema. The participants were then classified into three groups based on the severity of emphysema: (i) no/mild emphysema (emphysema score < 1, per cent low attenuation area in the assessed lung < 12.5% on average); (ii) moderate emphysema (emphysema score 1 to < 2.5, per cent low attenuation area in the assessed lung < 50% on average); and (iii) severe emphysema (emphysema score ≥ 2.5, per cent low attenuation area in the assessed lung ≥ 50% on average).

Statistical analysis

Data were analyzed using SPSS 20 (IBM, New York, NY, USA). Chi-square test was used to assess the correlation between the clinical diagnosis of COPD reported by the secondary examination centres and the results of PFT. The relationship between two quantitative variables was examined using Spearman tests. Possible predictor variables, including gender, age, smoking status and smoking index, were assessed as risk factors for developing airway obstruction (FEV_1/FVC < 0.7) and emphysema (emphysema score ≥ 1) using multiple regression analysis. In all statistical analyses, a significance level of 5% was adopted, and the significance of predictor variables was tested by the likelihood ratio test.

RESULTS

During the community-based lung cancer screening, 19 screened residents were found to have primary lung cancer, of these 12 had metastatic lung cancer. The lung cancer detection rate was 0.02% (19/89 100). Furthermore, 142 screened residents (0.16%) had findings suggestive of lung cancer and were recommended for continuous follow-up.

Figure 1 shows the flow chart for this study. Of the 72 653 eligible residents who were aged 60 years or older and underwent the initial screening, including chest radiography, 878 (1.0%) were suspected to have COPD because of their self-reported screening questionnaires and were referred for further examination. Of those referred, 567 (64.6%; 482 males and 85 females) underwent secondary diagnostic evaluations, including PFT and/or chest CT. Of these, 369 (65.1%, 369/567) underwent PFT and 480 (84.7%, 480/567) underwent CT.

Table 1 shows the final diagnoses and treatment recommendations from the secondary evaluation centres for the 567 screened residents suspected to have COPD. COPD/emphysema was diagnosed in 161 (28.4%), and of these 62 (38.5%) required medical treatment. The COPD/emphysema detection rate was 0.18% (161/89 100). Seven residents were thought to have lung cancer necessary for further follow-up, and four were diagnosed with lung cancer (detection rate 0.7%, 4/567).

Table 2 shows the final diagnoses reported for the 369 screened residents who underwent PFT. Similar to the results shown in Tables 1 and 102 (27.6%) were diagnosed with COPD/emphysema, and 47 of these (46.1%) required medical treatment. One resident was diagnosed with lung cancer.

Of the 228 participants providing informed consent who were found to have COPD at the secondary evaluation, 84 (36.8%) were current smokers and 144 (63.2%) were ex-smokers. The smoking index was

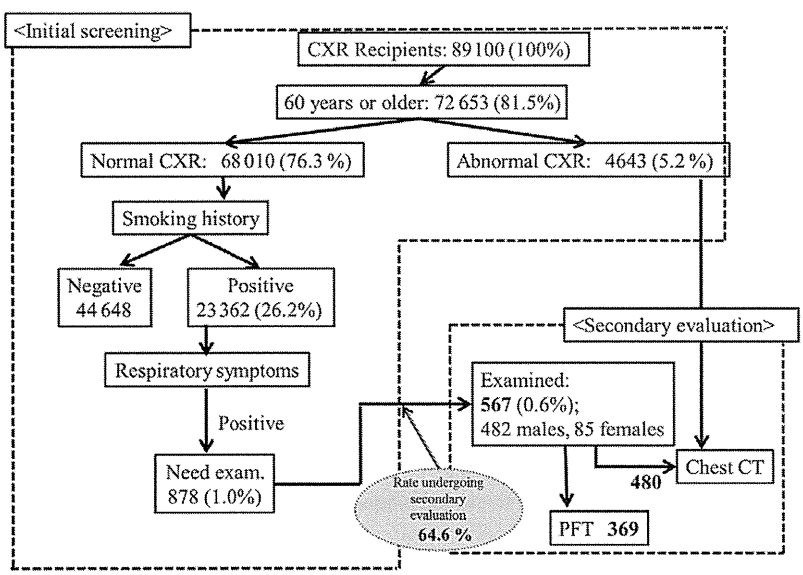

Figure 1 Flow chart from the initial screening performed as part of the lung cancer screening programme of fiscal year 2010 in Chiba City to the secondary evaluation. CT, chest x-ray; PFT, pulmonary function testing.

Table 1 Final diagnoses of residents suspected to have chronic obstructive pulmonary disease (COPD) at the first screening as reported from the secondary evaluation centres

	Treatment decision				
Diagnosis	Required annual follow-up	Treatment unnecessary	Required treatment	Others	Total
Metastatic lung tumour	1	0	0	0	1
Suspected lung cancer	7	0	4	0	11
COPD/emphysema	51 (31.7%)	28 (17.4%)	62 (38.5%)	20 (12.4%)	161
Mediastinal tumour	0	0	1	0	1
Old tuberculosis	1	10	0	0	11
No abnormality	10	225	0	6	241
Others	49	73	10	9	141
Total	119 (21.0%)	336 (59.3%)	77 (13.6%)	35 (6.2%)	567

Table 2 Final diagnoses reported for screened residents who underwent pulmonary function testing at the secondary evaluation centres

	Treatment decision				
Diagnosis	Required follow-up	Treatment unnecessary	Required treatment	Others	Total
Metastatic lung tumour	1	0	0	0	1
Known lung cancer	0	0	0	0	0
Lung cancer suspected	5	0	1	1	7
Chronic obstructive pulmonary disease/emphysema	33 (32.3%)	14 (13.7%)	47 (46.1%)	8 (7.8%)	102
Old tuberculosis	0	3	0	0	3
No abnormality	5	160	0	3	168
Others	27	51	6	4	88
Total	71 (19.2%)	228 (61.8%)	54 (14.6%)	16 (4.3%)	369

Table 3 COPD GOLD stages of 173 participants who underwent pulmonary function testing

GOLD stage	*n*	FVC (L)	%FVC	FEV_1 (L)	$\%FEV_1$	FEV_1/FVC
Non-COPD	130 (75.1%)	3.07 ± 0.68	97.0 ± 17.8	2.44 ± 0.54	102.4 ± 20.6	80.0 ± 7.1
Stage I	15 (8.7%)	3.41 ± 0.69	107.8 ± 18.2	2.28 ± 0.47	98.9 ± 13.4	66.9 ± 7.0
Stage II	21 (12.1%)	2.66 ± 0.43	82.7 ± 10.0	1.62 ± 0.28	67.4 ± 9.7	62.7 ± 6.8
Stage III/IV	7 (4.1%)	2.19 ± 0.76	68.7 ± 20.7	0.92 ± 0.18	39.3 ± 8.0	45.0 ± 12.5
Total	173	3.02 ± 0.70	95.0 ± 18.9	2.27 ± 0.63	95.3 ± 24.7	75.4 ± 11.5

COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; GOLD, GOLD, Global Initiative for Chronic Obstructive Lung Disease.

Table 4 Accuracy of COPD diagnosis reported from the secondary evaluation centres compared with PFT data

		PFT data	
		COPD	Non-COPD
Reports from the second examination centres	COPD	35	10
	Non-COPD	8	120

$P < 0.0001$ by Fisher exact test.
COPD, chronic obstructive pulmonary disease; PFT, pulmonary function test.

Table 5 CT findings of 185 participants undergoing secondary evaluation

No abnormality	117 (63.2%)
Pulmonary fibrosis	11 (5.9)
Old tuberculosis with calcification	19 (10.3)
Inflammatory nodule	3 (1.6)
Bronchiectasis	2 (1.1)
Pleural thickness/plaque	6 (3.2)
GGO	6 (3.2)
Lung cancer	1 (0.5)
Diffuse granular shadow	1 (0.5)
Giant bullae	3 (1.6)
Emphysema (moderate to severe)	38 (20.1)
Pneumothorax	1 (0.5)
Total	205

CT, computed tomography; GGO, ground glass opacity.

35.0 ± 24.9 pack years. Table 3 shows the GOLD classifications of 173 participants who underwent PFT. COPD was diagnosed in 43 (24.9%), and there were 28 (65.1%, 28/43) with stage II or higher.

We verified the reliability of COPD diagnosis ($FEV_1/FVC < 0.7$) reported from the secondary evaluation centres in 173 participants who underwent PFT (Table 4). The sensitivity, specificity, positive predictive value and negative predictive value were 0.81, 0.92, 0.78 and 0.94, respectively ($P < 0.0001$).

CT data were collected from 185 participants with 205 findings (Table 5). Although 117 had no abnormal findings, 38 (20.1%) were diagnosed with moderate to severe emphysema. The numbers of no/mild, moderate and severe emphysema were 147, 31 and 7 participants, respectively, according to the modified Goddard classification. Figure 2 shows the relationship between FEV1/FVC and emphysema score in 139 participants who underwent both PFT and CT. The emphysema score was significantly correlated with FEV_1/FVC ($P < 0.0001$). Multiple logistic regression analysis identified age as a risk factor for $FEV_1/FVC < 0.7$, and the smoking index as a risk factor for moderate to severe emphysema (Table 6).

Table 6 Distribution of $FEV_1/FVC < 0.7$ and Goddard classification ≥ 9 stratified by gender, smoking status, smoking index and age

Risk factors	*n* (%) $FEV_1/FVC < 0.7$	*n* (%) $FEV_1/FVC \geq 0.7$	95% CI for OR	*P*-value
Gender			0.101–2.436	0.387
Male	39 (25.7)	113 (74.3)		
Female	2 (9.5)	19 (90.5)		
Age			0.878–0.994	0.032
60–69	11 (15.5)	60 (84.5)		
70–79	23 (29.1)	56 (60.9)		
80–	7 (30.4)	16 (69.6)		
Smoking status			0.217–1.013	0.054
Ex-smoker	21 (19.3)	88 (80.7)		
Current smoker	20 (31.3)	44 (68.7)		
Smoking index (pack years)			0.999–1.000	0.120
–20	8 (13.1)	53 (86.9)		
21–40	15 (27.3)	40 (72.7)		
41–60	12 (30.0)	28 (70.0)		
61–	6 (35.3)	11 (64.7)		
Risk factors	Moderate/severe emphysema	No/mild emphysema	95% CI for OR	*P*-value
Gender			0.123–3.134	0.565
Male	36 (21.8)	129 (78.2)		
Female	2 (10.0)	18 (90.0)		
Age			0.932–1.066	0.920
60–69	16 (22.5)	55 (77.5)		
70–79	17 (19.1)	72 (80.9)		
80–	5 (20.0)	20 (80.0)		
Smoking status			0.338–1.613	0.447
Ex-smoker	20 (17.5)	94 (82.5)		
Current smoker	18 (25.4)	53 (74.6)		
Smoking index			0.998–1.000	0.007
–20	5 (7.8)	59 (92.2)		
21–40	14 (26.4)	39 (73.6)		
41–60	10 (21.7)	36 (88.3)		
61–	9 (40.9)	13 (59.1)		

CI, confidence interval; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; OR, odds ratio.

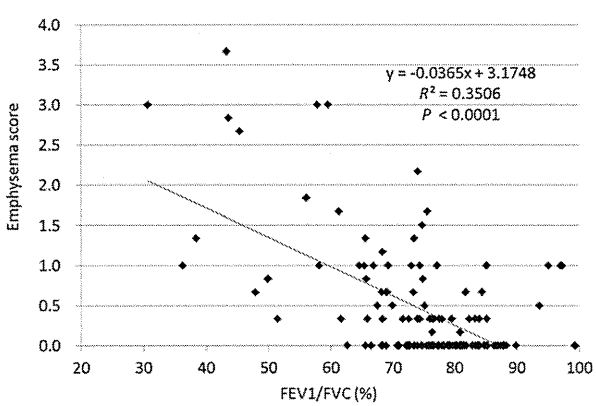

Figure 2 Correlation between forced expiratory volume in 1 s/forced vital capacity (FEV_1/FVC) and emphysema score in 139 participants who underwent both pulmonary function testing and computed tomography examinations. $R^2 = 0.3506$, $P < 0.0001$ by Spearman rank correlation test.

DISCUSSION

This is the first report of the utilization of a community-based lung cancer screening programme to screen for COPD. From our results, approximately 1.0% of eligible residents undergoing screening were suspected to have COPD, approximately 30% of them were diagnosed with COPD/emphysema and 40% of them required medical treatment. Previously we reviewed the association between COPD and lung cancer, and proposed that early detection of COPD is important for lung cancer surveillance.[7]

Using data from lung cancer screening in Chiba City performed in 2006 and 2007, we determined that approximately 2–4% of screened residents fulfilled the criteria suggestive of COPD (data not shown). COPD screening in Chiba City began in 2008. Initially, few patients were referred to the secondary evaluation centres because of inadequate and hard-to-understand questionnaires and lack of information

among physicians about the COPD screening system. Because of the improved and ongoing medical education of physicians, the number of patients identified as suspected to have COPD has since been increasing.

Early diagnosis of COPD has been attempted for high-risk patients,[18,19] and spirometry was proven to play an important role in COPD screening and early diagnosis. Our study results were similar to those of other investigators. Buffels *et al.* reported that spirometry confirmed airflow obstruction in 18% of individuals with respiratory symptoms and in 4% of those without symptoms.[18] van Schayck *et al.* investigated the effectiveness of cross-sectional case findings for patients with a history of cigarette smoking who were at risk for developing COPD using a standardized questionnaire and spirometry.[19] They reported that 18% of participants had airway obstruction, and that when smokers were preselected based on respiratory symptoms, such as chronic cough, the percentage of patients with airway obstruction increased to 48% among those older than 60 years of age. Ulrik *et al.* reported that 34.8% of general practice patients who were older than 35 years of age, with no previous diagnosis of COPD, and at least one of the following symptoms, cough, dyspnoea, wheezing, sputum or recurrent respiratory infection, had airway obstruction (FEV_1/FVC ratio < 0.7).[20]

Recently, the COPD criteria of both a simple fixed FEV_1/ FVC ratio (GOLD definition) and the use of a threshold for the per cent of predicted FEV_1 values to define lung function impairment (recommended by the National Institute for Health and Clinical Excellence) have been shown to overestimate COPD with increasing age, particularly among men, compared with statistical approaches, such as the lower limit of normal.[21] However, Güder *et al.* reported that GOLD criteria resulted in more overdiagnosed COPD, while lower limit of normal definitions resulted in more underdiagnosed COPD in elderly patients as compared with an expert panel diagnosis.[22] They suggested that incorporating FEV1 and RV/TLC into the GOLD-COPD or lower limit of normal-based definition would make both definitions closer to the expert panel diagnosis of COPD. However, GOLD criteria are clear and easy to understand for identifying COPD during screening. During this mass screening, the age distribution was very wide, from 40 to over 80 years of age. Therefore, we thought that an FEV_1/FVC of <70% still had value to classify COPD and applied this criterion for this screening.

Our study and screening system have several advantages. Screening for COPD can be performed on a large population with minimal additional expense because it can be added to an already established screening system for lung cancer. Pulmonary specialists can diagnose COPD at the secondary evaluation. Once COPD is diagnosed, the patient can be established on appropriate treatment and followed up. The reasons for the low COPD detection rate may be the lack of knowledge and understanding about the seriousness of COPD in the general population and the late appearance of symptoms.

This study had several limitations. First, COPD screening was additionally performed during lung cancer screening. Primary physicians and the participants did not always follow the recommendation for further evaluation, resulting in only 567 of 878 (64.6%) attending the secondary evaluations. Second, this was not a comparative study. However, population screening with or without a targeted approach based on risk factors or symptoms has been reported to yield acceptable diagnostic rates of around 20%.[8,23] In our study, approximately 30% of screened individuals were diagnosed with COPD/emphysema. The reliability of COPD diagnosis reported from secondary centres was uncertain. However, we verified the accuracy of these reports from the collected data. Last, at present, this system may only be established in a country that has a lung cancer screening system. However, once this screening system has been validated, it can be widened to general practice.

Future work includes validating this screening system and establishing the treatment and follow-up modalities for patients diagnosed with COPD.

In conclusion, community-based lung cancer screening may be utilized to diagnose COPD/ emphysema. The rate of detection can be increased by educating general practitioners on the importance of COPD screening. The cost-effectiveness, risks of this screening system and the appropriateness of the current COPD criteria need further evaluation.

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Perspective

Clinical Classification of Targeted Agents Used for Anticancer Treatment

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The introduction of targeted agents has resulted in a breakthrough in advanced cancer treatment. We propose a new classification for these agents to evaluate them in appropriate clinical trials according to agent class. Class I agents that inhibit driver oncogene activities result in massive and rapid tumor shrinkage, with response rates as high as 70% when administered to patients with appropriate targets. These agents can be evaluated in single-arm phase II trials with response rate as the primary endpoint. Class II agents inhibit one oncogene that is partially responsible for accelerating tumor cell proliferation. Their clinical features include synergism with cytotoxic agents and moderate single-agent activity, as shown by response rates of between 10% and 30%. Randomized phase II trials in patients with over-expressed targets are appropriate for the evaluation of these agents. Class III agents inhibit proliferation regulators that are not always oncogenic. Their clinical activity is unique, as they confer a survival benefit on patients with a minimum tumor shrinkage effect. Class IV agents target environmental molecules that act on normal cells surrounding tumor cells, such as the endothelial cells that form vessels. Placebo-controlled randomized phase II trials are required to identify the clinical activities of both class III and IV agents. Class V agents act by enhancing anti-tumor immunity. Immune-related response criteria should aid the evaluation of these agents. We believe that this classification for targeted agents should facilitate their further clinical development.

Keywords: clinical trial design; driver mutations; targeted agents; oncogene addiction; targeted cancer therapy
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Introduction

Cancer is a major health problem worldwide. About 12.7 million people were diagnosed with cancer and 7.6 million died of this disease globally in the year 2008 (Jemal et al. 2011). During the last century, many cytotoxic anticancer agents were developed, but the survival benefits in patients with distant metastases have been limited. A phase III trial for patients with advanced non-Hodgkin's lymphoma demonstrated that dose-intensive chemotherapy did not show any survival benefit over conventional chemotherapy, despite the initial promise demonstrated in phase II trials (Fisher et al. 1993). A breakthrough in the prognosis of non-Hodgkin's lymphoma was achieved by introducing rituximab, a monoclonal antibody against the cluster of differentiation (CD) 20, which is expressed on the surface of B-cell lymphomas (Coiffier et al. 2002; Habermann et al. 2006; Coiffier et al. 2010). Similarly, the introduction of tyrosine kinase inhibitors improved the efficacy of several cancer treatments in the 2000s.

These targeted agents were initially considered to be highly effective against cancers without causing severe toxicity in normal tissues. However, fatal drug-induced lung injury was observed during the clinical development of gefitinib, an epidermal growth factor receptor (EGFR) inhibitor (Inoue et al. 2003). In addition, the efficacy of gefitinib for the treatment of advanced non-small cell lung cancer was difficult to establish in randomized trials. Two large randomized phase III trials of platinum-doublet chemotherapy with or without gefitinib involving more than 1000 advanced non-small cell lung cancer patients failed to demonstrate a synergistic effect of gefitinib, compared with standard chemotherapy (Giaccone et al. 2004; Herbst et al. 2004). Another phase III trial of gefitinib monotherapy versus a placebo in nearly 1700 patients showed no survival benefit of gefitinib over the placebo in the second-line setting (Thatcher et al. 2005).

The situation took a turn for the better once activating EGFR mutations were identified as the real target of gefitinib in 2004 (Lynch et al. 2004; Paez et al. 2004). Two small, but crucial randomized phase III trials in patients with EGFR mutation-positive advanced non-small cell lung

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cancer showed that the progression-free survival, the primary endpoint of these trials, was much better for gefitinib than for platinum-based chemotherapy (Maemondo et al. 2010; Mitsudomi et al. 2010). Therefore, the clinical development of novel targeted agents for cancer therapy is likely to be impeded without appropriate consideration of the clinical trial design (Saijo 2004).

Classification of targeted agents for clinical development

Targeted agents that inhibit specific molecules implicated in tumor cell growth have been classified according to (1) the site of action (tumor-specific and tumor-environment-specific), (2) the mechanism of action (the target pathways associated with tumor growth and survival), and (3) pharmaceutical formulations (small molecular compounds and macromolecules such as antibodies) (Saijo 2004). These classifications, however, are derived from the viewpoint of pharmaceutical preclinical development.

From a general survey of clinical trials of targeted agents, we noticed that most of these agents could be categorized into 1 of 5 classes with a few exceptions (Table 1). One of the distinct clinical features of class I agents is the massive and rapid tumor shrinkage that occurs when the agent is administered to patients who have the appropriate target (Fig. 1). For example, the response rate of gefitinib monotherapy in patients with EGFR mutation-positive lung adenocarcinoma has reached as high as 70% (Maemondo et al. 2010). The mechanism of this clinical observation is well explained by the concept of oncogene addiction and its disruption. Oncogene addiction describes the acquired dependence of tumor cells on a single activated oncogene for their sustained proliferation and survival; without the oncogenic activity, the tumor cells undergo rapid apoptosis (Sharma and Settleman 2007).

Several phase III trials involving the combination of a class I agent and standard cytotoxic chemotherapy have failed to show synergistic effects on survival, although target selection was not performed for the patients enrolled in these trials (Giaccone et al. 2004; Herbst et al. 2004). The study population for clinical trials must be limited to patients who have tumors with the drug target, typically a mutated driver oncogene. Cancers arising from different organs may share the same target gene, and study populations could be defined not by the site of the cancer, but by the target itself (Mano 2012). Because this type of agent has an obvious tumor shrinkage effect, the clinical activity can be evaluated in single-arm phase II trials in previously treated patients, using the response rate as the primary endpoint. Whether placebo-controlled phase III trials are needed is controversial (Sharma and Schilsky 2012). Some agents, including imatinib for gastrointestinal stromal tumors and crizotinib for non-small cell lung cancer with an anaplastic lymphoma kinase-fusion protein, have been approved by the U.S. Food and Drug Administration without undergoing phase III trials (Dagher et al. 2002; Scagliotti et al. 2012).

Class II agents inhibit one oncogene that is partially responsible for accelerating tumor cell proliferation and survival (Fig. 1). The clinical features of class II agents include moderate monotherapy activity, with response rates of between 10%-30%, and synergism with cytotoxic agents. One of the best examples is trastuzumab, a humanized monoclonal antibody against the human epidermal growth factor receptor 2 (HER2) oncoprotein, which is overexpressed in 20%-30% of human breast cancers. Trastuzumab acts either by enhancing receptor downregulation, by inhibiting extracellular domain cleavage and the generation of

Table 1. Classification of molecular targeted agents.

Class	Target	Mechanisms of action	RR (%) in monotherapy	Synergy with chemotherapy	Trial design	Target population	Clinical examples
I	Driver oncogene	Disruption of oncogene addiction	60-90	Not demonstrated	Single-arm phase II	defined	Imatinib to CML, GIST; gefitinib to NSCLC with mutated EGFR
II	Actionable oncogene	Signaling inhibition, ADCC, CDC	10-30	Yes	Randomized phase II	defined	Trastuzumab to MBC; cetuximab to CRC; rituximab to B-cell lymphoma
III	Proliferation regulators	Signaling inhibition	< 30	Not demonstrated	Randomized phase II	not defined	Sorafenib to RCC, HCC; sunitinib to RCC
IV	Environmental molecules	Enhancing effects on chemotherapy	< 5	Yes	Randomized phase II	not defined	Bevacizumab to solid tumors
V	Immune regulators	Release from anergy against tumors	~10	Yes	Randomized phase II	not defined	Ipilimumab to melanoma

ADCC, Antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; CML, chronic myelogenous leukemia; CRC, colorectal cancer; EGFR, epidermal growth factor receptor; GIST, gastrointestinal stromal tumor; HCC, hepatocellular carcinoma; MBC, metastatic breast cancer; NSCLC, Non-Small Cell Lung Cancer; RCC, renal cell carcinoma.

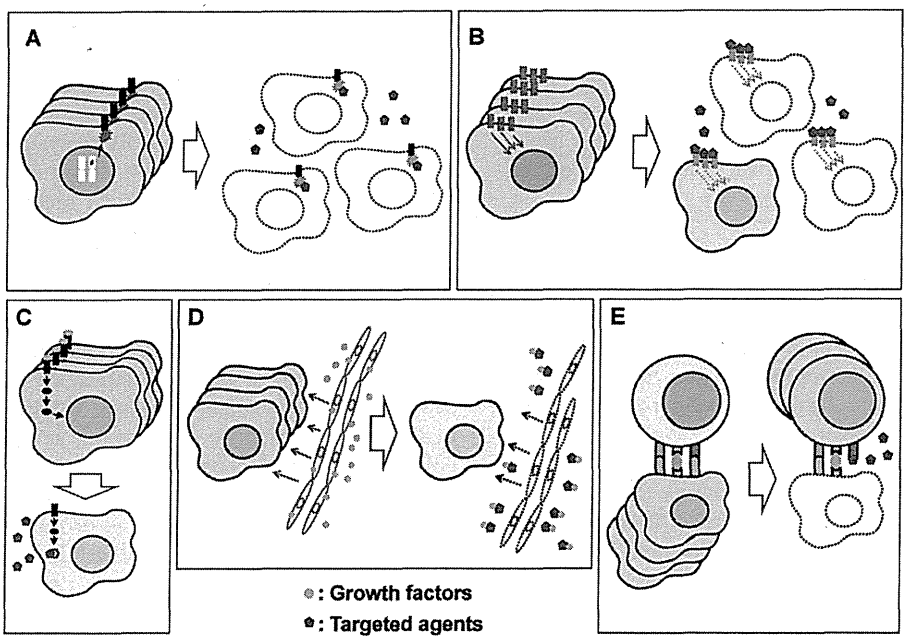

Fig. 1. Examples of targeted agents and their molecular targets according to the agent classes.
A. An example of class I agents. Tumor cells dependent on the activity of a single oncogenic driver (oncogene addiction) undergo apoptosis rapidly when a class I agent blocks the driver activity.
B. Class II agents inhibit one oncogene that is partially responsible for accelerating tumor cell proliferation and survival. This class of agents may induce apoptosis or growth suspension in tumor cells. The illustrated figure shows the case for trastuzumab, a humanized monoclonal antibody against the human epidermal growth factor receptor 2 oncoprotein.
C. The targets of class III agents are molecules that act in a specific signaling pathway involved in cell proliferation but are not always oncogenic. With class III agents, tumor cells stop proliferating, but rarely die.
D. Class IV agents target environmental molecules that support tumor growth. This figure illustrates the case for bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor.
E. Tumor cells stimulate negative immunological checkpoints, such as cytotoxic T lymphocyte antigen 4, inhibiting the proliferative drive as well as the activation of T cells (the round cell on the left side). Class V agents block these inhibitory signals, leading to the activation of cytotoxic T cells (the round cell on the right side) and the augmentation of antitumor immunity.

phosphorylated p95, by blocking receptor dimerization, or by recruiting immune system processes such as antibody-dependent cellular cytotoxicity (Hudis 2007). The rapid and successful clinical development of this agent has been attributed to its early clinical trials, which included only patients with HER2-overexpressing metastatic breast cancer with overexpression levels evaluated as 2+ or 3+ using immunohistochemistry (Cobleigh et al. 1999; Vogel et al. 2002). These phase II trials showed that when used as a single agent, trastuzumab yielded a response rate of 18%-35% in patients with HER2-overexpressing tumors at the 3+ level, while the response rate was $\leq$ 6% in patients with HER2-overexpressing tumors at the 2+ level. In addition, long periods (> 6 months) of disease stabilization were observed in a substantial number of patients who did not achieve an objective response. Furthermore, trastuzumab demonstrated a synergistic effect on the response rate and survival benefit when used in combination with conventional chemotherapy (Slamon et al. 2001).

Several discussions of trial designs are required to evaluate class II agents. The success of clinical trials largely depends on whether the study patient population can be defined clearly according to the target status. In contrast to class I agents, the response rate of class II agents is moderate and varied; therefore, single-arm phase II trials with a threshold response rate may fail to detect the clinical activity of a new agent. Randomized phase II trials are required in many cases to identify clinical activity.

Class III agents target proliferation regulators that are involved in cell proliferation but that are not always oncogenic (Fig. 1). One of the distinct clinical features of class III agents is their unique single agent activity, providing an obvious survival benefit but exerting a minimum tumor shrinkage effect. One of the best examples is sorafenib, a multikinase inhibitor of Ras/Raf, vascular endothelial growth factor receptor-1 to 3, platelet-derived growth factor receptor-b, c-kit, and Flt-3. A phase III trial of sorafenib versus a placebo in patients with advanced renal cell carcinoma resistant to standard therapy showed that the median overall survival time was significantly prolonged in the sorafenib arm (19.3 months versus 15.9 months; hazard ratio, 0.77; 95% confidence interval, 0.63-0.95) with a

response rate as low as 10% (Escudier et al. 2007). Similarly, sorafenib had a significant survival benefit over a placebo in patients with advanced hepatocellular carcinoma, with a response rate of only 2% (Llovet et al. 2008). Thus, randomized phase II trials with a placebo-controlled arm are required to identify the clinical activity of class III agents. Compared with class II agents, it may be difficult to define patient populations for clinical trials of class III agents according to the target status, partly because these agents have multiple action sites. To enrich the target population, randomized discontinuation trials may be suitable (Ratain et al. 2006). No combinations of class III agents and cytotoxic agents have been evaluated in phase III trials because no cytotoxic agents have shown activity against renal cell or hepatocellular carcinoma.

Class IV agents target environmental molecules that support tumor growth such as regulators of angiogenesis (Fig. 1). An example of this class of agents is bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor. This agent showed a significant antitumor effect in patients with advanced colorectal cancer, non-small cell lung cancer, and metastatic breast cancer when combined with standard cytotoxic chemotherapy, but was not effective as a monotherapy (Kazazi-Hyseni et al. 2010). Although its mechanisms of action have not been fully defined and may vary among different tumor types, one of the potent mechanisms of bevacizumab is the normalization of the tumor vasculature and the improvement of chemotherapy delivery to the tumor (Ellis and Hicklin 2008). This mechanism explains the clinical characteristics of bevacizumab and its antitumor activity against a broad spectrum of tumor types. Extensive studies have failed to identify patients who are likely to receive the maximum benefit from bevacizumab-containing chemotherapy.

Class V agents regulate immunomodulatory molecules so as to enhance anti-tumor immunity (Fig. 1). Ipilimumab is a fully human monoclonal antibody that binds to cytotoxic T lymphocyte antigen 4 (CTLA-4). Because CTLA-4 provokes the inhibitory signal of cytotoxic T cell activity, blocking this molecule results in T cell activation against tumors. A phase III trial of dacarbazine versus dacarbazine plus ipilimumab in treatment-naive patients with metastatic melanoma showed that overall survival improved with ipilimumab (9.1 versus 11.2 months, respectively $P < 0.001$) (Robert et al. 2011). The clinical features of class V agents include variable patterns of response and durable objective responses and stable disease in a small percentage of patients. Thus, immune-related response criteria could be helpful for evaluating these agents (Wolchok et al. 2009).

The classification presented here is tentative, and its revision may be required once larger bodies of evidence and knowledge on cancer biology have been accumulated. Class I and II agents are highly distinct from each other with regard to their response rate, which we think can be explained by the role of the target oncogene in tumor cells, that is, whether the tumor cells are entirely addicted to the oncogene or not. The distinction in the response rates of the two classes, however, may be due to suboptimal target inhibition by the class II agents. In this case, the targets of class I and II agents are essentially not different.

Strategies to overcome resistance to class I agents can be illustrated systematically, since their mechanisms of resistance are known. Secondary mutations in the target gene are a common mechanism of resistance to class I agents, which have been identified in a large proportion of resistant tumors. Another mechanism of resistance is the bypass of the original oncogene signaling by the activation of other oncogenes (compensatory signaling). Class III agents that have multiple target sites may be useful for suppressing the occurrence of this type of resistance. Resistance to class IV agents seems unlikely to be acquired easily, since the targets of these agents are located within normal cells that do not develop spontaneous mutations as frequently as tumor cells, although physiological adaptation can be provoked without mutations.

In conclusion, we have proposed a classification for targeted agents for anticancer treatment according to their clinical features to facilitate the clinical development of these new types of agents.

Conflict of Interest

The authors have no conflict of interest to declare.

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Original Article

Incidence, Risk Factors and Treatment Outcomes of Extravasation of Cytotoxic Agents in an Outpatient Chemotherapy Clinic

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Objective: Extravasation, the accidental leakage of an anticancer agent from a vessel into the surrounding tissues, can lead to irreversible local injuries and severe disability. Despite its considerable clinical importance, evidence-based information on extravasation in chemotherapy is lacking. This study characterized the clinical features of extravasation and identified issues to be resolved in current cancer chemotherapy performed in outpatient settings.
Methods: We retrospectively reviewed the medical charts of patients who received chemotherapy and sustained extravasation in our Outpatient Chemotherapy Clinic from April 2007 to August 2012. Chemotherapy administration and extravasation management procedures were standardized using the in-house chemotherapy guideline.
Results: Among 43 557 patients who received chemotherapy, 35 (0.08%) experienced extravasation. The duration between the start of infusion and extravasation was >2 h in 28 (80.0%) patients. The severity of extravasation was Grades 1, 2 and 3 in 28, 2 and 5 patients, respectively—three of whom were associated with port trouble. The contributing factor for extravasation was walking in 11 (31.4%) patients. All extravasations were cured without surgical intervention by management according to our guidelines.
Conclusions: The incidence of extravasation is as low as 0.08%, using our in-house chemotherapy guideline. Extravasation from implanted ports tends to be severe.

Key words: chemotherapy – cytotoxic agents – extravasation – outpatient

INTRODUCTION

Systemic chemotherapy plays a pivotal role in curative therapy for patients with hematological neoplasms and several types of advanced solid tumors. Although several oral molecular-targeted agents have been developed recently, most anticancer agents are administered intravenously. Extravasation, the accidental leakage of an anticancer agent from a vessel into the surrounding tissues, is an unwanted and distressing complication that can lead to irreversible local injuries and severe disability (1). The incidence of extravasation in adults is estimated to be in the range from 0.01 to 6.9% (1), but few studies report the incidence on the basis of firm data with a total number of patients who received chemotherapy (2). The symptoms of extravasation can range from self-limited localized tissue inflammation to full-thickness necrosis, ulceration and sloughing of the skin and underlying structures. The extent of symptoms depends on the type of anticancer agent that seeps into the tissue, which can be categorized as vesicant, irritant or non-irritant (3).

In addition, the risk and severity of extravasation can be influenced by the infusion site, tissue condition, concentration and volume of the agent and treatment applied. Although these factors represent expert opinion, there are no data that are referred to while managing extravasation in anticancer agents.

Therefore, this study characterized the clinical features of extravasation and identified issues to be solved in current cancer chemotherapy performed in outpatient settings.

PATIENTS AND METHODS

All chemotherapy treatments for outpatients in the Chiba University Hospital have been integrated in the Outpatient Chemotherapy Clinic since 2007; patients referred by oncologists from the specialized outpatient clinics of this hospital are attended to between 9:00 and 17:00 h. Informed consent for chemotherapy is documented by an oncologist prior to chemotherapy. All chemotherapy regimens are submitted to and approved by the Institutional Chemotherapy Committee. A chemotherapy regimen is prescribed before the day of the chemotherapy, and pharmacists independently verify each order for chemotherapy before preparation. Principally, patients visit the Outpatient Chemotherapy Clinic before the day of chemotherapy, and an expert nurse explains chemotherapy in the clinic, especially calls patient's attention to extravasation, the management of adverse events at home and the costs of chemotherapy. The agents are prepared by pharmacists in safety cabinets. Chemotherapy administration and extravasation management procedures are standardized according to the in-house chemotherapy guideline, which deals with duties and operations of stuffs, rules and engagements of the Outpatient Chemotherapy Clinic, safety management and the response to emergency. Peripheral veins are assessed carefully, and needle insertion into an antecubital vein is avoided if possible. Butterfly needles are not allowed. A transparent dressing is used to secure the insertion site. Vesicant agents are administered by drip infusion without any pump devices. A nurse visits patients for monitoring every 30 min and when a patient returns from the toilet, observes at the site of infusion and confirms a backflow of the blood, and records the findings of the monitoring on the check sheet. When an extravasation occurs or is suspected, the infusion is stopped immediately, and the affected area is inspected and recorded in digital photographs. The amount of extravasation was estimated by the area of a bulge at the site of extravasation multiplied by the height of the bulge. In the case of no apparent bulge was visible, the amount was estimated to be <10 ml. Local warming is applied for extravasations involving vinca alkaloids and oxaliplatin, and local cooling for other vesicant and irritant anticancer agents. No extravasation antidote is applied topically or systemically, because none is approved in Japan.

The purpose of this study is to characterize the clinical features of extravasation in the Outpatient Chemotherapy Clinic. Considering the retrospective nature of this study, the primary endpoint and sample size of patients were not defined. All consecutive patients receiving chemotherapy from April 2007 to August 2012 were included in this study. Data on extravasation were recorded prospectively on the extravasation sheet and aggregated weekly. For this study we retrospectively reviewed the extravasation sheet and medical charts of patients who sustained extravasation. The study was conducted in accordance with the ethical guideline for epidemiological research in Japan and was approved by the Chiba University Ethics Committee (no. 1702).

RESULTS

From April 2007 to August 2012, a total of 43 557 patients received chemotherapy in the Outpatient Chemotherapy Clinic. Of these, 35 (0.08%) patients experienced extravasation. The incidence decreased with each year: 0.11% in 2007 and 2008, 0.1% in 2009, 0.08 in 2010, 0.07 in 2011 and 0.01 in 2012. Sixteen patients complained of pain, burning sensation and/or discomfort at the site of infusion, which led to the detection of extravasation in these patients. An infusion pump alarm was another lead to find extravasation in three patients. In the remaining 16 patients, a nurse detected extravasation during her round of visits. Characteristics of these patients included 20 women and 15 men with a median age of 67 years (range, 37–80 years). Tumor types were eight breast cancers, seven colorectal cancers, six ovarian cancers, four lung cancers, two gastric cancers, two uterine cancers, two cholangiocarcinomas and four others. The blood access site was a peripheral vein in 29 (82.9%) patients (forearm in 18, cubital fossa in seven, dorsum of hand in two, upper arm in one and dorsum of foot in one patient), followed by a central venous port in four (11.4%) and arterial infusion port in two (Table 1). The interval between the start of infusion and extravasation was >2 h in 28 (80.0%) patients. The median (range) number of chemotherapy administrations was 9 (1–70). Extravasated agents included vesicant in three patients (vinorelbine in two and vincristine in one patient), and irritant in 21 patients (paclitaxel in 12, docetaxel in 3, irinotecan in 3 and cyclophosphamide in 3 patients. In the three patients receiving a vesicant agent, the infusion site was the cubital fossa, forearm and dorsum of foot in one patient each. Extravasation was detected at the end of infusion in one and during infusion in two patients. No contributing factor was specified in these patients. The severity of extravasation was Grades 1, 2 and 3 in 28, 2 and 5 patients (three of whom were associated with port trouble), respectively (Table 1). The grades of extravasation of vesicant agents were Grades 1 and 2 in two and one patient, respectively. The contributing factor for extravasation was walking in 11 (31.4%) patients and not specified in 10 (28.6%) (Table 2). All extravasations were cured without surgical intervention as a result of management according to our guidelines.

Table 1. Characteristics of extravasation

Characteristics	*n* (%)
Blood access sites	
Peripheral vein	29 (82.9)
Central venous port	4 (11.4)
Arterial infusion port	2 (5.7)
Interval between the start of infusion and extravasation (h)	
<0.5	2 (5.7)
0.5–2.0	5 (14.3)
2.1–5.0	21 (60.0)
>5.0	7 (20.0)
Estimated amount of extravasated agents	
<10 ml	27 (84.4)
11–20 ml	4 (12.5)
21–50 ml	1 (3.1)
Not estimated	3
Extravasated agents	
Vesicant	3 (8.6)
Irritant	21 (60.0)
Non-irritant	11 (31.4)
Grade (CTCAE 3.0)	
1	28 (80.0)
2	2 (5.7)
3	5[a] (14.3)
Patients' symptoms	
Yes	16 (45.7)
No	19 (54.3)

[a]Three of the four cases of Grade 3 extravasation were associated with port trouble.

Table 2. Situation at the occurrence and contributing factor

Blood access sites	*n* (%)
Peripheral vein	
After walking	11 (31.4)
During a sleep	3 (8.6)
At the end of infusion	3 (8.6)
Infusion pump alarm	3 (8.6)
Not specified	10 (28.6)
Central venous port	
Port trouble (migration or breakage)	3 (8.6)
After walking	1 (2.9)
Arterial infusion port	
Inadequate anchorage	1 (2.9)
Thrombosis	1 (2.9)

DISCUSSION

Although extravasation in association with anticancer chemotherapy is well recognized, there are few reports on its incidence based on the exact number of patients. Langstein et al. (4) report 44 cases of extravasation among 240 000–360 000 individual doses of chemotherapy during a 6-year study period, suggesting the number of incidence to range from ~0.012 to 0.018%. In another report, 216 of 35 475 (0.61%) patients sustained extravasation (2). In contrast, small case series show that the incidence of extravasation is as high as 6% (5,6). This inconsistency among reports may be attributable to differences in the monitoring and notification systems for extravasation between hospitals. The incidence of extravasation in the present study among ~44 000 patients was 0.08% which is close to the lower limits of previous reports. The results of this study suggest our integrated multidisciplinary team in the Outpatient Chemotherapy Clinic has successfully standardized the procedure through chemotherapy preparation, administration and monitoring following our chemotherapy guideline.

In this study, extravasation was associated with a long infusion time of >2 h in 80% of cases. Furthermore, it occurred in one-third of patients just after walking to the toilet. This suggests that a small problem in the needle-insertion procedure exists. Thus, securing the needles and instructing patients not to move while an anticancer agent is being administered could be improved. Patient movement is a well-described risk factor for extravasation. The European Oncology Nursing Society extravasation guideline recommends that patients should be instructed not to leave the clinical area while a vesicant is being administered (7).

In the present study, only five patients sustained Grade 3 extravasation, and none required surgical intervention, mostly because there were only three vesicant extravasations. In addition, these were attributable to the early detection of extravasation, which led to only a small amount of anticancer agents leaking into the perivenous tissues. Since less than half of all patients complained of symptoms associated with extravasation, monitored by nurses is considered to have worked well. Among the three cases of vesicant extravasations, two and one were Grades 1 and 2, respectively, suggesting that our chemotherapy guideline and staff training work well and that the entire process from chemotherapy administration to the monitoring and management of extravasation is appropriate.

Although the exact incidence of extravasation associated with totally implantable venous access ports remains unknown, the risk of extravasation is believed to be lower when anticancer agents are infused through ports than temporary intravenous needles (8). In this study, three of four extravasations from implanted ports progressed to Grade 3 while the other progressed to Grade 1. This suggests that extravasation from implanted ports tends to cause severe tissue injury once it occurs. Because there are few specifically recommended procedures for the management of extravasation from

implanted ports, there is room for further development of specific guidelines on its management.

In conclusion, the incidence of extravasation among chemotherapy patients is as low as 0.08% with a well-trained multidisciplinary team using the in-house chemotherapy guideline in the Outpatient Chemotherapy Clinic. Extravasation from implanted ports tends to be severe and should be studied further.

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Conflict of interest statement

None declared.

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PLOS ONE

Metformin, a Diabetes Drug, Eliminates Tumor-Initiating Hepatocellular Carcinoma Cells

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Abstract

Metformin has been widely used as an oral drug for diabetes mellitus for approximately 60 years. Interestingly, recent reports showed that metformin exhibited an anti-tumor action in a wide range of malignancies including hepatocellular carcinoma (HCC). In the present study, we investigated its impact on tumor-initiating HCC cells. Metformin suppressed cell growth and induced apoptosis in a dose-dependent manner. Flow cytometric analysis showed that metformin treatment markedly reduced the number of tumor-initiating epithelial cell adhesion molecule $(EpCAM)^+$ HCC cells. Non-adherent sphere formation assays of $EpCAM^+$ cells showed that metformin impaired not only their sphere-forming ability, but also their self-renewal capability. Consistent with this, immunostaining of spheres revealed that metformin significantly decreased the number of component cells positive for hepatic stem cell markers such as EpCAM and α-fetoprotein. In a xenograft transplantation model using non-obese diabetic/severe combined immunodeficient mice, metformin and/or sorafenib treatment suppressed the growth of tumors derived from transplanted HCC cells. Notably, the administration of metformin but not sorafenib decreased the number of $EpCAM^+$ cells and impaired their self-renewal capability. As reported, metformin activated AMP-activated protein kinase (AMPK) through phosphorylation; however its inhibitory effect on the mammalian target of rapamycin (mTOR) pathway did not necessarily correlate with its anti-tumor activity toward $EpCAM^+$ tumor-initiating HCC cells. These results indicate that metformin is a promising therapeutic agent for the elimination of tumor-initiating HCC cells and suggest as-yet-unknown functions other than its inhibitory effect on the AMPK/mTOR pathway.

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Introduction

Cancer stem cells (CSCs) or tumor-initiating cells (TICs) are a minor population of tumor cells with prominent tumorigenicity [1]. These cells are characterized by self-renewal capability and differentiation ability similar to those of normal stem/progenitor cells. Therefore, it has been believed that TICs play an important role in carcinogenesis, tumor growth, metastasis, and cancer recurrence. Recent progress in stem cell biology has enabled the identification and characterization of TICs in various cancers including hepatocellular carcinoma (HCC) [2]. Subsequently, the molecular machinery and signaling pathways involved in maintaining TICs have been vigorously explored [3]. Although the inhibitors of these molecules and signaling pathways are considered promising as TIC-targeting drugs, an effective therapy targeting TICs has yet to be developed.

Metformin is an oral drug that lowers blood glucose concentrations and has been widely used to treat type 2 diabetes mellitus [4]. The anti-diabetic action of metformin depends on the activation of AMP-activated protein kinase (AMPK), which contributes to a reduction in hepatic gluconeogenesis and an increase in glucose uptake in skeletal muscles [5]. Of interest, previous large case-control studies revealed that diabetic patients treated with metformin had a lower incidence of cancers than those treated with other diabetic drugs [6,7]. Various explanations for the efficacy of metformin have been proposed, such as the activation of AMPK, inhibition of insulin-like growth factor signaling, and the mTOR pathway [8]. Diabetes is known to be associated with an increase in the risk of developing HCC [9]. Indeed, the risk of HCC was significantly lower with metformin treatment than with sulphonylureas or insulin in chronic liver disease [10]. Furthermore, metformin reduced the risk of

recurrence of HCC after local ablation therapy [11]. Taken together, it is possible that metformin has direct effects on tumor-initiating HCC cells.

In the present study, we examined the effect of metformin on tumor-initiating HCC cells *in vitro*. We showed that metformin abolished their self-renewal capability and induced apoptosis in part through the activation of AMPK and subsequent inhibition of the mTOR pathway. Furthermore, xenograft transplantation experiments using nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice demonstrated that metformin but not sorafenib decreased the number of TICs and impaired their self-renewal capability.

Results

Metformin Inhibited Cell Growth and Induced Apoptosis

To investigate the effect of metformin on HCC cells, we first examined cell growth and the frequency of apoptotic cells. Metformin treatment inhibited cell growth both time-dependently and dose-dependently in HCC cells and normal hepatocytes (Fig. 1A). Immunostaining of active caspase 3 (CASP3) showed that metformin induced apoptosis in a dose-dependent manner (Fig. 1B). The percentage of apoptotic cells in Huh1 cells, Huh7 cells, and normal hepatocytes treated with metformin (5 mM) was approximately five-fold, ten-fold, and seven-fold higher than that in control cells, respectively (Fig. 1C). Consistent with this result, flow cytometric analysis by staining with Annexin V and propidium iodide (PI) revealed that metformin caused apoptosis in a dose-dependent manner (Fig. 2).

Impact of Metformin Treatment on Tumor-initiating HCC Cells

The epithelial cell adhesion molecule $(EpCAM)^+$ fraction as well as the $CD133^+$ fraction was shown to include TICs in HCC [12,13]. We examined the expression of EpCAM and CD133 using flow cytometry to analyze the effect of metformin on tumor-initiating HCC cells. Metformin treatment (10 mM) decreased the $EpCAM^{high}$ fraction from 35.2% to 17.9% in Huh1 cells and from 33.0% to 12.2% in Huh7 cells (Fig. 3A). The $EpCAM^{high}$ fraction also decreased from 18.9% to 12.0% in normal hepatocytes after metformin exposure (Fig. 3A). Likewise, the $CD133^{high}$ fraction in Huh7 cells decreased from 40.5% to 26.1% (Fig. 3B), while the $CD133^+$ fraction was not detected in Huh1 cells or normal hepatocytes with or without metformin treatment. Taking into consideration the decrease in the total cell number, metformin appears to directly act on tumor-initiating HCC cells.

Sphere Assays of HCC Cells and Normal Hepatocytes Treated with Metformin

We then performed a non-adherent sphere formation assay of $EpCAM^+$ HCC cells and normal hepatocytes sorted by flow cytometry. EpCAM expression was markedly higher in the $EpCAM^+$ fraction than in the $EpCAM^-$ fraction by Western blot analysis (Fig. 4A). Unlike $EpCAM^+$ HCC cells, $EpCAM^+$ normal hepatocytes failed to form large spheres. Metformin treatment significantly impaired the formation of large spheres dose-dependently (Fig. 4B and 4C) and also the formation of secondary spheres after the replating of primary spheres (Fig. 4D). Together, these results indicate that metformin impaired the tumorigenicity of tumor-initiating HCC cells by inhibiting their self-renewal. To confirm the inhibitory effect of metformin on the self-renewal of tumor-initiating HCC cells, we conducted immunocytochemical analyses of the expression of EpCAM and α-fetoprotein (AFP), hepatic stem/progenitor cell markers, in the resultant spheres. A marked reduction in cells positive for EpCAM was observed in spheres generated from $EpCAM^+$ Huh1 cells (Fig. 5A). Because Huh1 cells only modestly produce AFP, no remarkable change in the number of cells positive for AFP was observed after metformin treatment. In contrast, metformin decreased both AFP^+ and $EpCAM^+$ cells in spheres generated from Huh7 cells treated with metformin (Fig. 5B). These results indicate that metformin impairs the self-renewal capability of TICs and accelerates their differentiation.

Impact of Metformin on Apoptosis, Cell Growth, and Cell Cycle

To examine the effect of metformin on cell proliferation, we conducted Western blotting of $EpCAM^+$ HCC cells and normal hepatocytes (Fig. 6A). As expected, cleaved poly (ADP-ribosyl) polymerase (PARP), a marker of apoptosis, was clearly detected in $EpCAM^+$ cells treated with metformin. The levels of proliferating cell nuclear antigen (PCNA) in $EpCAM^+$ cells treated with metformin decreased in a dose-dependent manner. The alteration in cyclin D1 and p21 expression levels caused by metformin differed between $EpCAM^+$ HCC cells and $EpCAM^+$ normal hepatocytes. Although metformin treatment increased the level of cyclin D1 in $EpCAM^+$ HCC cells, no marked changes in cyclin D1 expression were observed in $EpCAM^+$ normal hepatocytes. In addition, metformin treatment decreased the level of p21 in $EpCAM^+$ HCC cells, but conversely increased p21 expression level in $EpCAM^+$ normal hepatocytes.

AMPK/mTOR Pathway Following Metformin Exposure in $EpCAM^+$ HCC Cells and Normal Hepatocytes

Previous reports demonstrated that metformin suppressed mTOR signaling by activating AMPK in various cancer cells including HCC cells [14–16]. To examine whether this machinery also operated in $EpCAM^+$ HCC cells and normal hepatocytes, cells were subjected to Western blotting (Fig. 6B). In $EpCAM^+$ Huh7 cells and normal hepatocytes, the levels of phosphorylated AMPK increased after metformin exposure in a dose-dependent manner. Conversely, the levels of phosphorylated mTOR and phosphorylated S6 kinase decreased. However, $EpCAM^+$ Huh1 cells showed no change in the mTOR pathway, while the level of AMPK increased with exposure to metformin. These results raise the possibility that metformin impaired the self-renewal capability of tumor-initiating HCC cells in part by affecting the AMPK/mTOR pathway.

Anti-tumor Effects of Metformin and/or Sorafenib in Xenograft Transplantation Model

Sorafenib is an oral multikinase inhibitor with anti-angiogenic activity and has been approved for the treatment of advanced HCC [17]. To compare the anti-tumor effects of metformin and sorafenib, we conducted xenograft transplantation using NOD/SCID mice and administered metformin and/or sorafenib to recipient mice. Metformin and/or sorafenib were administered daily after the transplantation of 2×10^6 Huh7 cells into NOD/SCID mice. Both tumor initiation and growth were apparently suppressed by the metformin and sorafanib treatment (Fig. 7A and 7B). Tumor growth was further inhibited by the co-administration of metformin and sorafenib than by a single administration. Immunohistochemical staining of subcutaneous tumors for Ki-67 and CASP3 revealed that metformin and/or sorafanib treatment inhibited cell growth and induced apoptosis (Fig. 7C). Interestingly and importantly, the frequency of $EpCAM^+$ cells was markedly decreased in tumors treated with metformin, but not with