results found by Derogatis LR[15], Miyagawa[16] and Ogawa[17] is shown in Fig 1. Derogatis[15] examined 215 randomly accessed cancer patients in three cancer centers and showed that 47% of them received a DSM-III diagnosis. Approximately 68% of the psychiatric diagnosis consisted of adjustment disorders. Miyagawa[16] assessed terminally ill cancer patients and 50(53.7%) of them met the DSM-III-R criteria for a psychiatric disorder. Delirium was observed in 26 patients (28%) as the most common disorder. It seemed that the rate of delirium increased according to the deterioration of physical conditions. Ogawa[17] analyzed 2000 cancer patients referred to the palliative care team at the National Cancer Center Hospital East, Japan and consultation-liaison psychiatrists provided medical care to 80% of all referrals. The main diagnosis were delirium (28%), adjustment disorders (18%), major depressive disorder (7.6%) and dementia (6.6%).

Psycho-oncological intervention

The aim of psycho-oncological intervention is to alleviate the mental distress of cancer patients and their family members and to support their cancer treatment. Firstly, most patients received brief individual supportive sessions by PSM doctors. For patients who did not respond to basic brief supportive psychotherapy alone, anxiolytics were used as drugs for the first step. For patients who manifested considerable depressive symptoms, antidepressants such as mirtazapine and duloxetine hydrochloride were prescribed. Antidepressants and anxiolytics were chosen referring to the results of SDS/STAI. When main physical doctors consulted us to control delirium in two hospitalized cases, we advised them to prescribe risperidone. In addition, for patients who mainly complained of cancer-related pain, a consultation was set up with the palliative care doctor. For patients manifesting anxiety, combination therapy of pharmacotherapy with anxiolytics such as clotiazepam and bromazepam ; psychotherapy such as autogenic training for relaxation ; and counseling by clinical psychologists was conducted. Problems related to the patient's relationship with family members, job and lifestyle, and spiritual and existential issues related to the meaning of life were mainly dealt with in counseling sessions. Environmental adjustment was also a very important aspect of psycho-oncological intervention as some patients had grievances against their main doctors, medical staff and/or other patients, and their family members. In addition to basic brief individual supportive sessions, transactional analysis psychotherapy and group discussion with family members or medical staff were used. We also arranged consultations with appropriate professionals, such as palliative care doctors or medical social workers, in order to fulfill their request for information of palliative care, hospice or home medical care. Thus, PSM doctors played the role of director in this multidisciplinary team.

Psycho-oncological intervention also acted as a type of consultation-liaison service, when main physical doctors asked us for advice for controlling delirium or other psychiatric/psychological problems. Our advice included the prescription of medicine and participating in meetings to discuss the cases.

Moreover, patients' family care and bereaved family care were conducted. Several family members visited our outpatient service, manifesting anxiety and depression at the same level as cancer patients. One man had excess anxiety after his wife was diagnosed with advanced pancreatic cancer. He had several symptoms, such as palpitations, abdominal pain and insomnia and seemed confused at how to support his wife. He visited our outpatient service of his own accord. Another case was a housewife who suffered from depression after her husband was diagnosed with liver cancer. She was exhausted from years of nursing her husband and complained of general fatigue, insomnia and appetite loss. Her husband's main doctor recommended her to consult our department and we prescribed antidepressant drugs for her.

Discussion

Recent research indicates that about half of all cancer patients have psychiatric/psychological disorders as shown by Derogatis[15] and Miyagawa[16] in Fig. 1. They examined all cancer patients including those who had no psychiatric/psychological symptoms. Compared with those patients, our patients had a higher prevalence of psychiatric diagnosis since they visited our psycho-oncological outpatient service with psychiatric/psychological/physical symptoms. In Ogawa's report,[17] 20% of referrals did not need psychiatric consultation, and among the rest of the patients no psychiatric diagnosis was given

to 142(7.1%) of them. In our study, no psychiatric diagnosis was given to 2(3.4%) and this is comparable with Ogawa's report. In addition, approximately 80% of patients visited our outpatient service, so they seemed to be in better physical condition and the delirium rate was lower than in Miyagawa and Ogawa's report.

The significance of a specific outpatient service for psycho-oncology should be discussed. With a specific psycho-oncology outpatient service, it is easier to accept cancer patients from different departments in our hospital. This system is also useful for accepting patients who do not need to be hospitalized. Moreover, if the patients' condition permits, we can see hospitalized patients at the specific outpatient service intensively. This method makes it easier to evaluate and intervene in the relationship between patients and their main doctors/co-medical staff, as an outsider, independent of the human relationship in the wards. In addition, in this specific outpatient service, it is easier to accept cancer patients from other hospitals, not only those in our hospital.

Two family members of cancer patients visited our specific outpatient service. They also needed psycho-oncological intervention. The care for a cancer patient's family is a very important issue.[18–20] After we set up the specific outpatient service, it opened the door to family members of cancer patients.

Next, the advantages of PSM doctors should be discussed. PSM doctors are basically physicians and are adept at dealing with physical symptoms. In this study, 43.1% of patients complained of physical symptoms. In addition, PSM doctors are familiar with controlling symptoms derived from depression and anxiety. Physical and psychiatric symptoms sometimes combine and relate to depression and anxiety, so these symptoms need a psychosomatic approach from the viewpoint of holistic medicine. This concept coincides with psycho-oncology.

Some patients came to our specific outpatient service complaining of conflict between family members, grievances against their main doctors, medical staff and/or other patients. Physician-patient communication is related to patient satisfaction.[21,22] PSM doctors are adept at psychotherapies such as transactional analysis and cognitive behavior therapy used to improve the relationship between patients and the people they have grievances with. PSM doctors also have a lot of communication skill training, which is very useful and effective in a crucial cancer treatment process, for example, explaining bad news about a cancer diagnosis, relapse and shifting curative treatment to palliative care.[23–26]

Moreover, a multidisciplinary team approach is essential in the cancer medical field and a PSM doctor can act as a director of the team. There is a team dynamic and complicated human relationships among medical professionals, such as doctors, nurses, pharmacists, dieticians, medical social workers and others. A director is necessary to arrange the medical team to make it work significantly and PSM doctors have the skill to do this. Overall, 17.2% of patients were consulting the Department of Palliative Care at the same time, mainly to control cancer pain ; therefore, cooperation with a palliative care doctor in the same hospital is beneficial for cancer patients. When PSM doctors control the psychological, social and spiritual distress of cancer patients in association with a palliative care doctor controlling cancer pain, the "total pain" of cancer patients can be alleviated from the viewpoint of holistic medicine. PSM doctors also cooperate with medical social workers and social resources, such as primary care doctors, home care nurses and care managers, when cancer patients want to discharge themselves from the hospital and receive care at home.

However, there are several problems with this system. First of all, the new outpatient service for cancer patients is understaffed, including a lack of certified nurses. It is somewhat difficult to respond adequately to patient requests. Second, there is not enough economic support for this psycho-oncology service. This activity is time-consuming but is not counted towards the special points program under Japan's health insurance system. Third, it is very important to work together with main physical doctors and psycho-oncology doctors ; however, there are sometimes discordant opinions derived from different viewpoints. For example, when we try to prescribe anxiolytics for cancer patients, their main physical doctors sometimes oppose this idea as they are afraid of the respiratory depressant effect of anxiolytics. In addition, it is sometimes difficult to cooperate with main physical doctors belonging to other hospitals. Fourth, this specific psycho-oncological outpatient service began only one year ago. Some doctors and patients are unaware of our service

and appropriate support might not reach cancer patients in need. Posters and brochures are necessary to gain recognition for our specific outpatient service. Although there are several problems with this new system, a specific psycho-oncological outpatient service is useful and effective to support cancer patients with mental distress. JPOS surveyed the present situation of psycho-oncological outpatient services in 2010, and 233 hospitals returned answers out of 287 (response rate=81.2%). It showed that 176 hospitals had an outpatient service for cancer patients (176/233=75.6%), however only 25 out of 176 (14.2%) hospitals have a specific psycho-oncological outpatient service run by PSM doctors. Further improvements and promotion by PSM doctors are needed in this field.

The present study has several limitations. First, this study was based on consultation cases only in our hospital. There were 841[27] PSM doctors in Japan in 2006 and the number of doctors certified by the Japanese Society of Psychosomatic Internal Medicine was 469[28] in 2010. The number of members of the JPOS was 1,123 in 2010, and the number of PSM doctors was only 54. Only a small number of PSM doctors are therefore in charge of psycho-oncology activities. Further studies are needed to investigate the present psycho-oncology activities by PSM doctors all over Japan to discuss the significance of our practice. Second, this study was performed by extracting all the items from the patients' medical charts and assessments by PSM doctors; therefore, a possibility of assessment bias exists. The evaluation of satisfaction of patients and main physical doctors, and the effectiveness of psycho-oncological intervention by PSM doctors should be examined in the future. Third, in Fig. 1, Derogatis diagnosed his samples by DSM-III, Miyagawa by DSM-III-R, Ogawa by DSM-IV while we diagnosed patients by DSM-IV-TR. This difference should be considered when these data are compared.

Although our study has several limitations, some highly suggestive results were seen as helpful information for clinical psycho-oncology practice and for suggesting future studies. In order to elucidate the significance of a specific outpatient service for cancer patients and to promote psycho-oncology activities by PSM doctors, further research addressing the present study's limitations is necessary.

In conclusion, PSM doctors can play an important role in the field of cancer treatment through psycho-oncological activities. The advantages of a specific outpatient service are that it can open the door to patients and their families who belong to other departments/hospitals and it can support cancer patients intensively and efficiently. However, there are several problems, such as the lack of trained staff and economic support, which clearly indicate that further improvements and studies are needed.

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Expression changes in arrestin β 1 and genetic variation in catechol-O-methyltransferase are biomarkers for the response to morphine treatment in cancer patients

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Abstract. Genetic differences in individuals with regard to opioid-receptor signaling create clinical difficulties for opioid treatment; consequently, useful pharmacodynamic and predictive biomarkers are needed. In this prospective study, we studied gene expression changes in peripheral blood leukocytes using a microarray and real-time RT-PCR analysis to identify pharmacodynamic biomarkers for monitoring the effect of morphine in a cohort of opioid-treatment-naïve cancer patients. We also examined genetic variations in opioid receptor mu 1 (*OPRM1*, 118A→G) and catechol-O-methyltransferase (*COMT*, 472G→A) to evaluate predictive biomarkers of the treatment outcome of morphine. The plasma concentration of morphine was measured using a liquid chromatography-tandem mass spectrometry method. Microarray analysis revealed that the mRNA expression levels of arrestin β 1 (*ARRB1*) were significantly down-regulated by morphine treatment. Real-time RT-PCR analysis against independent samples confirmed the results (P=0.003) and changes during treatment were negatively correlated with the plasma morphine concentration (R=-0.42). No correlation was observed between the genotype of *OPRM1* and morphine treatment; however, the plasma concentration of morphine and the required dose of morphine were significantly lower for the A/A genotype of *COMT* (vs. A/G+G/G, P=0.008 and 0.03). We found that changes in the expression of *ARRB1* may be a novel pharmacodynamic biomarker and the *COMT* 472G→A genotype may be a predictive biomarker of the response to morphine treatment.

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Key words: arrestin, catechol-O-methyltransferase, morphine, microarray, opioid receptor μ 1

Introduction

Pharmacogenetic, pharmacokinetic and pharmacodynamic variations among individuals result in a wide variety of responses to pain sensation and to analgesics; therefore, intensive investigations of biomarkers for opioid treatment have been performed to improve the effectiveness of morphine treatment (1).

The opioid receptors are G-protein coupled receptors (GPCRs), and three types of receptors μ, δ and κ-opioid receptors (*OPRM1*, *OPRD1* and *OPRK1*) are known to serve as receptors for morphine (2). Among them, *OPRM1* generated the main analgesic effect induced by morphine in a knock-out study performed in mice (3). Agonists for opioid receptors induce the activation of GPCRs, triggering the activation of various downstream molecules (2). A regulator of the G-protein signaling (RGS)-protein family negatively regulates opioid-receptor signaling by accelerating the deactivation of G proteins, and the regulators RGS2 and RGS9 are thought to be involved in resistance to morphine (4-6). In addition, G-protein coupled receptor kinase (GRK) phosphorylates the opioid receptors, leading to the binding of arrestin β 1 and 2 (ARRB1 and 2) to the opioid receptors (7). Thus, GRKs and ARRBs negatively regulate opioid-receptor signaling and are thought to be involved in resistance to morphine (8,9). To identify pharmacodynamic biomarkers that are capable

of monitoring the drug effect, we examined the gene expression changes in opioid signaling-related molecules using a microarray and real-time RT-PCR analysis in peripheral blood leucocytes (PBLs).

Meanwhile, genetic variants associated with varying pain sensitivity and responses to morphine are thought to be potential biomarkers for predicting the outcome of morphine treatment (1,10). In this study, we also evaluated two functional genetic variants, *OPRM1* 118A→G and catecholamine-O-methyltransferase gene (*COMT*) 472G→A (also known as Val158Met). The 118A→G variant of *OPRM1* leads to a change in amino acids at position 40, affecting a putative glycosylation site of the receptor and biologically altering receptor activity (1). The enzyme activity of *COMT* is genetically defined as high in G/G, intermediate in G/A and low in A/A, and its genotype is thought to be associated with the effect of opioid-signaling (11).

In this prospective study, we examined gene expression to explore possible pharmacodynamic biomarkers and to evaluate the use of functional genetic variants as predictive biomarkers of the response to morphine treatment in a cohort of opioid-treatment-naïve cancer patients.

Materials and methods

Patients and samples. This prospective study was conducted between 2009 and 2011 at the Kinki University Faculty of Medicine and Sakai Hospital, Kinki University Faculty of Medicine. Clinicopathological features including age, gender, ECOG performance status (PS) and type of primary malignant neoplasm were recorded. Morphine treatment was performed according to the standard method including titration (NCCN Guidelines™, Adult Cancer Pain). The required doses of morphine on Day 1 and on Day 8 are thought to be associated with the results of titration and the dose in the stationary phase, respectively.

PBL samples were obtained at baseline (pretreatment) and on Day 1 for the gene expression analyses. PBL samples for DNA were obtained at baseline. To measure the plasma concentrations of morphine, blood samples were collected on Days 1 and 8. The separated plasma were stocked at -80°C until use. The present study was approved by the institutional review boards of both centers and written informed consent was obtained from all the patients.

RNA extraction. Each 2.5-ml whole blood sample was stored in a PAX gene Blood RNA tube (Qiagen, Hilden, Germany). RNA was extracted according to the manufacturer's protocol (Qiagen). Then, massively containing globin mRNA was removed using a biotinylated Globin-capture oligonucleotides-based method and the GLOBINclear™ kit (Ambion, Austin, TX). The quality and quantity of RNA obtained from these samples were verified using a NanoDrop2000 spectrophotometer (Cole-Parmer, Vernon Hills, IL).

Real-time reverse transcription PCR. The methods used in this section have been previously described (12). *GAPD* was used to normalize the expression levels in the subsequent quantitative analyses. The primers used for real-time RT-PCR were purchased from Takara (Otsu, Japan) as follows: *OPRM1* forward, 5'-TCA ATG TCT GCA ACT GGA TCC TC-3' and reverse, 5'-CAC TGG CAT AAT GAA GGC GAA G-3'; *OPRD1* forward, 5'-CTG GGC AAC GTG CTT GTC A-3' and reverse, 5'-CAT CAG GTA CTT GGC ACT CTG GAA-3'; *OPRK1* forward, 5'-CAC TTC ACG TGC TCT TAC AGC GTT A-3' and reverse, 5'-CCC TTG TGG GCA CAT ACA GCT AC-3'; *ARRB1* forward, 5'-GAG AAC GAG ACG CCA GTA GAT ACC A-3' and reverse, 5'-GGC GAG CAA AGT CCT CAA ATA CA-3'; *ARRB2* forward, 5'-ACC AAC CTG GCT TCC AGC A-3' and reverse, 5'-AAA GGC AGC TCC ACA GAG ACA TC-3'; *GRK5* forward, 5'-GGA GCT GAA CGT GTT TGG ACC TA-3' and reverse, 5'-AGC TGG GCG AAC TCT TGG AA-3'; *RGS9* forward, 5'-GCA CAA ACC CAC ATT TAC ATG CTC-3' and reverse, 5'-GCT TTG GCC AGC ATG TCC TTA-3'; *GAPD* forward, 5'-GCA CCG TCA AGG CTG AGA AC-3' and reverse, 5'-ATG GTG GTG AAG ACG CCA GT-3'.

Microarray analysis. The microarray procedure was performed according to the Affymetrix protocols (Santa Clara, CA), as described previously (13). Briefly, cRNA was synthesized using the GeneChip® 3'-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix). The labeled cRNAs were then purified and used for the construction of the probes. Hybridization was performed using the Affymetrix Gene Chip HG-U133 Plus 2.0 array for 16 h at 45°C. The signal intensities were measured using a GeneChip® Scanner 3000 (Affymetrix) and converted to numerical data using GeneChip Operating Software, Ver. 1 (Affymetrix).

Genotyping. The genotype was evaluated for *OPRM1* 118A→G (rs1799971, p.Asn40Asp) and *COMT* 472G→A (rs4680, p.Val158Met). Genomic DNA isolated from blood samples using a QIAamp® DNA Blood Mini Kit (Qiagen) were amplified with the following primers: for *OPRM1* forward, 5'-AAG TCT CGG TGC TCC TGG CTA CC-3' and reverse, 5'-GTT TCC GAA GAG CCC CAC CAC GC-3'; and for *COMT* forward, 5'-GAT TCA GGA GCA CCA GCC CTC C-3' and reverse (intronic), 5'-CAC TGA GGG GCC TGG TGA TAG TG-3'. Each PCR reaction was performed in a 20-μl volume containing 20 ng of template, 0.5 μM of each primer, Ampdirect Plus (Shimadzu Corp., Kyoto, Japan) and 0.5 units of NovaTaq™ DNA Polymerase (Merck, Darmstadt, Germany). The amplification was performed for 35 cycles (95°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec). The resulting PCR fragments consisting of 320 bp (*OPRM1*) and 210 bp (*COMT*) were directly sequenced with the corresponding forward and reverse primers, respectively.

Measurement of plasma concentration of morphine. The plasma concentration of morphine was measured using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. Morphine was purchased from Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Imipramine, an internal standard (IS), was obtained from Sigma-Aldrich (St. Louis, MO, USA). Pretreatment of the plasma samples was performed using protein precipitation. Briefly, 100 μl of plasma was mixed with 250 μl of IS solution (1 ng/ml imipramine in methanol). After vortexing (30 sec) and centrifugation (13,000 rpm, 5 min), the supernatant was directly analyzed using an autosampler. An

Table I. Clinical characteristics of study patients.

Characteristics	No. of patients
Age (years)	
<65	14
≥65	34
Gender	
Male	25
Female	23
PS	
0-2	32
3-4	16
Tumor types	
Lung cancer	20
CRC	8
Gastric cancer	5
Others	15
Required dose of morphine	
Day 1	
20 mg	3
30 mg	36
60 mg	6
90 mg	2
NE	1
Day 8	
20 mg	5
30 mg	20
40 mg	1
60 mg	9
90 mg	1
120 mg	1
NE	11

PS, performance status; CRC, colorectal cancer; Others, other primary tumor types; NE, not evaluated.

LC-MS/MS device was equipped with an Acquity UPLC (Ultra Performance LC) system and a Xevo TQ MS (Waters, Milford, MA, USA). Chromatographic separations were obtained under gradient conditions using an ACQUITY UPLC BEH C18 Column (100 mm x 2.1 mm ID, 1.7-μm particle size; Waters). The mobile phase consisted of eluent A (10 mmol/l ammonium formate) and eluent B (methanol). The flow rate was 0.5 ml/min and the gradient was from 2 to 60% B in 3.5 min, then an increase to 98% B in 0.5 min, holding at 98% B for 1 min and resetting to the initial conditions. The total run time was 8.5 min per sample. The column temperature was 45°C, the sample temperature was 10°C and the injection volume was 5 μl. The retention times of morphine and imipramine (IS) were 1.69 and 4.31 min, respectively. The mass spectrometer was operated in a positive electrospray mode. The capillary voltage was 0.5 kV and the desolvation temperature was 500°C. The multiple reaction monitoring mode detected morphine and imipramine (IS) as follows: transitions, 286.4→152.3 and 281.2→86.0; cone voltages, 42 and 28 V; collision energies, 48 and 16 V, respectively. The chromatographic data were acquired and analyzed using MassLynx software, equipped with QuanLynx (Waters). Standard curves were prepared for a concentration range of 0.5-50 ng/ml for morphine. The inter- and intra-day variabilities in precision (expressed as the coefficient of variation) for morphine ranged from 4.2 to 7.7% and from 4.4 to 4.7%, respectively. The average accuracies for morphine were between 100.4 and 106.1%.

Statistical analysis. Differences between groups were analyzed using the Student's t-test or the Fisher's exact test. A P-value of <0.05 was considered statistically significant. All analyses were performed using JMP (SAS Institute, Cary, NC). A microarray analysis was performed using BRB-Array Tools software, Ver. 3.6.0 (http://linus.nci.nih.gov/BRB-ArrayTools.html), as described previously (13,14).

Results

Patient results. A total of 48 patients with opioid-treatment naïve and histologically confirmed malignant neoplasms who were scheduled to undergo opioid treatment were evaluated in this study (Fig. 1A). All 48 patients and a total of 96 samples (baseline and Day 1) were evaluated in the gene expression analysis. Forty-one patients and samples were evaluated in the genotype analysis because the DNA samples were insufficient in 7 cases. The plasma concentration of morphine was determined for 47 samples on Day 1 and for 43 samples on Day 8.

The patient characteristics are summarized in Table I. The median age was 69 years (40-85 years); 25 patients were men and 23 patients were women. Sixty-seven percent of the patients had a PS of 0-2 and 42% had advanced lung cancer. The other primary tumors were 8 colorectal cancers, 5 gastric cancers, 4 unknown primary cancers, 2 pancreas cancers, 2 breast cancers, 2 gallbladder cancers, 1 renal cell carcinoma, 1 bladder cancer, 1 malignant lymphoma, 1 malignant pheochromocytoma and 1 skin cancer. The median required dose of morphine on Day 1 was 30 mg (20-90 mg), while that on Day 8 was 30 mg (20-120 mg).

Down-regulation of ARRB1 mRNA expression and morphine treatment. To identify pharmacodynamic biomarkers for monitoring the effect of morphine, we examined changes in gene expression during morphine treatment (baseline vs. Day 1) using a microarray analysis for 20 samples from 10 cases and validated the results using real-time RT-PCR for 76 samples from 38 cases, focusing on opioid receptor signaling. A schema for opioid receptor signaling is shown (Fig. 1B). The microarray analysis revealed that the mRNA expression levels of *ARRB1* and *GRK5* were significantly down-regulated by morphine treatment (P=0.01 and 0.001, Table II). Interestingly, down-regulated genes including *ARRB1*, *GRK5* and *RGS9* (P=0.054) are known as negative regulators of opioid receptor signaling. The gene expressions of the opioid receptors were not changed. To confirm these results, we examined the gene expressions of these genes including *OPRM1*, *OPRD1*,

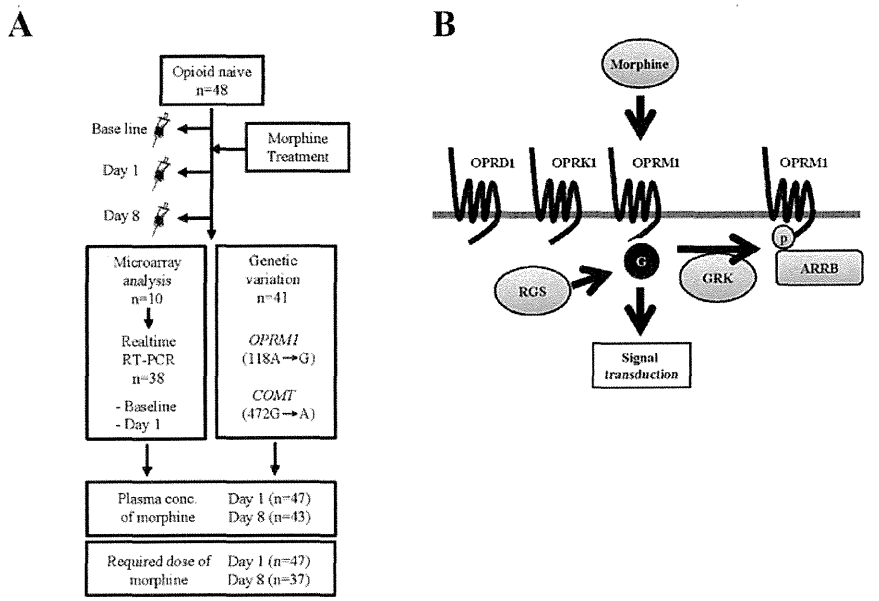

Figure 1. Schemas of study design and opioid receptor signaling. (A) Flow diagram of study. (B) Schema of opioid receptor signaling. OPRM1, D1 and K1 represent opioid receptor μ 1, δ 1 and κ 1, respectively; ARRB1 and 2 represent arrestin β 1 and 2; GRK, G protein-coupled receptor kinase; RGS, regulator of G-protein signaling; G, G protein; p, phosphorylation.

Table II. Gene expression changes in opioid signal-related molecules during morphine treatment.

		Microarray (n=10)			Real-time RT-PCR (n=38)		
Symbol	Name	Base line	Day 1	P-value[a]	Base line	Day 1	P-value[a]
OPRM1	Opioid receptor, μ 1	10.8±1.6	10.9±1.4	0.83	0.3±0.9	1.4±7.9	0.40
OPRD1	Opioid receptor, δ 1	11.3±4.2	10.0±0.0	0.37	0.2±0.4	0.3±1.4	0.46
OPRK1	Opioid receptor, κ 1	13.7±4.4	12.6±3.5	0.31	0.2±0.5	0.2±0.3	0.68
ARRB1	Arrestin β 1	123.7±40.2	101.0±30.9	0.01[b]	48.6±18.1	41.8±17.0	0.003[b]
ARRB2	Arrestin β 2	1193.5±476.5	1158.3±317.6	0.77	416.6±177.4	430.9±164.3	0.56
GRK1	G protein-coupled receptor kinase 1	10.3±1.3	11.3±2.7	0.36	ND	ND	ND
GRK4	G protein-coupled receptor kinase 4	11.2±3.6	10.9±2.5	0.82	ND	ND	ND
GRK5	G protein-coupled receptor kinase 5	419.6±121.1	346.7±137.4	0.001[b]	14.9±6.9	13.8±6.1	0.12
GRK6	G protein-coupled receptor kinase 6	464.4±87.5	457.3±120.8	0.87	ND	ND	ND
RGS2	Regulator of G-protein signaling 2	5776.5±1845.2	5872.8±1847.0	0.83	ND	ND	ND
RGS9	Regulator of G-protein signaling 9	26.7±16.5	17.6±7.9	0.05	2.8±2.4	2.5±1.7	0.17

Gene expression changes were examined using microarray and real-time RT-PCR. Peripheral blood leukocytes sampled during morphine treatment at baseline (pretreatment) and Day 1 (after treatment) were used for the analysis. [a]Comparisons between baseline vs. Day 1. The P-values were calculated using a t-test. [b]$P<0.05$. Data are shown as the average ± standard deviation.

OPRK1, *ARRB1*, *ARRB2*, *GRK5* and *RGS9* using real-time RT-PCR in 38 independent cases. The mRNA expression level of *ARRB1* was significantly and reproducibly down-regulated by morphine treatment (P=0.003, Table II and Fig. 2A). This result strongly suggests that *ARRB1* may be a promising and pharmacodynamic biomarker of morphine.

Next, we evaluated whether the down-regulation of *ARRB1* was correlated with the plasma concentration of morphine or the required dose. A moderate and weak inversed correlation was observed between the down-regulation of *ARRB1* and the plasma concentration of morphine (R=-0.42, Fig. 2B) or the required dose of morphine (R=-0.19, Fig. 2C). The results suggest that a higher plasma concentration or a higher dose of morphine induces the significant down-regulation of *ARRB1* and the change in *ARRB1* expression may be useful as a monitoring marker for morphine, although further studies are necessary.

COMT genotype is involved in outcome of morphine treatment. To find predictive biomarkers of the treatment outcome of morphine, we performed a functional genotype analysis

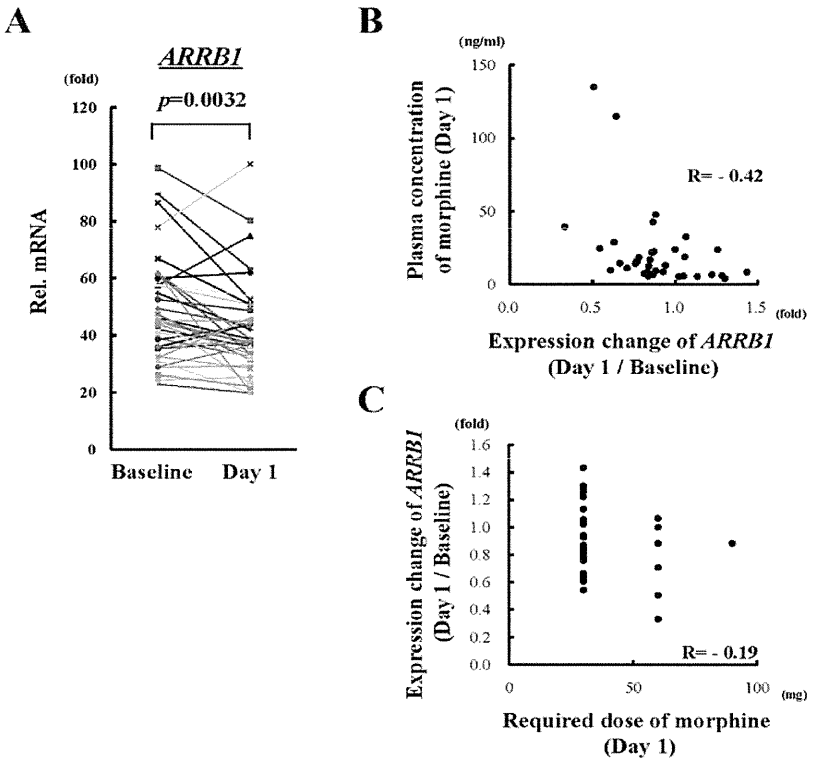

Figure 2. Down-regulation of arrestin β 1 (*ARRB1*) and outcome of morphine treatment. (A) A real-time RT-PCR analysis of peripheral blood leukocytes obtained at baseline (pretreatment) and on Day 1 showed that the mRNA level of *ARRB1* was significantly down-regulated during morphine treatment. Rel mRNA, normalized mRNA expression levels (*ARRB1*/*GAPD*x10^6). (B) Expression changes in *ARRB1* and plasma concentration of morphine treatment on Day 1. R, correlation coefficient. (C) Expression changes of *ARRB1* and required dose of morphine on Day 1.

Table III. Genotypes and treatment outcome of morphine.

		OPRM1 118A→G				*COMT* 472G→A			
Symbol	Days	A/A	A/G	G/G	P-value[a]	G/G	G/A	A/A	P-value[b]
Plasma concentration	D1	26.1±36.1	21.0±23.2	15.1±18.3	0.45	34.1±35.7	11.9±6.2	8.7±4.0	0.008[c]
of morphine (ng/ml)	D8	28.0±20.0	29.6±25.2	28.1±22.2	0.94	33.0±21.2	23.1±23.2	36.7±26.3	0.56
Required dose of	D1	43.1±23.9	32.2±9.0	34.0±15.2	0.78	43.7±21.4	28.9±3.2	30.0±0.0	0.03[c]
morphine (mg)	D8	38.9±16.2	37.1±15.4	36.0±15.2	0.82	40.0±15.4	34.6±15.1	40.0±17.3	0.81

Genotypes were evaluated for *OPRM1* 118A→G (rs1799971, p.Asn40Asp) and *COMT* 472G→A (rs4680, p.Val158Met). The treatment outcome of morphine was examined using the plasma concentration of morphine (Days 1 and 8) and the required dose of morphine (Days 1 and 8). [a]Comparisons between G/G vs. A/A+A/G of the *OPRM1*. [b]Comparisons between A/A vs. G/G+G/A of *COMT*. The P-values were calculated using a t-test. [c]P<0.05. Data are shown as the average ± standard deviation.

of *OPRM1* 118A→G (rs1799971, p.Asn40Asp) and *COMT* 472G→A (rs4680, p.Val158Met). The treatment outcome of morphine was examined based on the plasma concentration of morphine (Days 1 and 8) and the required dose (Days 1 and 8) according to genotype. No correlation was observed between the *OPRM1* 118A→G genotype and the plasma concentration or the required dose of morphine (Table III). However, the plasma morphine concentration on Day 1 was significantly lower in patients with the A/A genotype of COMT, compared with those with the A/G+G/G genotypes (A/A: n=4, 8.7±4.0 ng/ml; G/A: n=18, 11.9±6.2 ng/ml; G/G: n=19, 34.1±35.7 ng/ml; P=0.008, Fig. 3A). In addition, the required dose of morphine on Day 1 was also significantly lower for the A/A genotype of *COMT*, compared with the A/G+G/G genotypes (A/A, 30.0±0.0 mg; G/A, 28.9±3.2 mg; G/G, 43.7±21.4 mg; P=0.03, Fig. 3B). On the other hand, the genotype was not correlated with the treatment outcome of morphine on Day 8. Collectively, our results indicate that the *COMT* genotype is involved in the outcome of morphine treatment, suggesting that it may be useful as a predictive biomarker for morphine treatment.

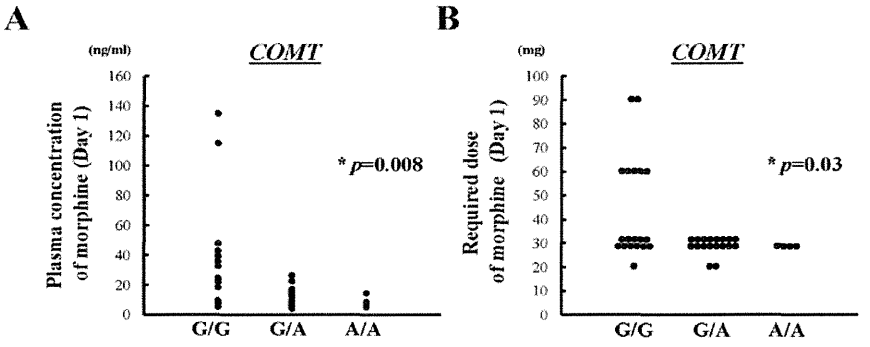

Figure 3. Catechol-O-methyltransferase genotypes (*COMT* 472G→A, rs4680, p.Val158Met) and outcome of morphine treatment. (A) Genotypes and plasma concentration of morphine treatment on Day 1. *Comparisons between A/A vs. G/G+G/A of *COMT*. (B) Genotypes and required dose of morphine on Day 1.

Discussion

Morphine activates opioid receptor signaling in the cells of the central nervous system (CNS). Unlike easily available tissues, such as blood cells, these CNS cells cannot be sampled and used for analysis; therefore, clinically useful pharmacodynamic biomarkers of morphine have remained largely unclear to date. We recently described an approach examining PBLs as surrogate tissues to evaluate drug response and found that it is a feasible, non-invasive and repeatable pharmacodynamic approach in clinical settings (15). In this study, we found that *ARRB1* mRNA expression is a reproducible and useful biomarker for monitoring the effects of morphine treatment using PBLs as surrogate tissues.

ARRB1 regulates the desensitization of numerous GPCRs including OPRM1, D1 and D2 dopamine receptors and emerging evidence has demonstrated that ARRB1 functions as a scaffold protein that links GPCRs to intracellular signaling, such as MAPK and as a transcription factor that translocates to the nucleus (16,17). A recent study showed that chronic morphine treatment blocked the agonist-induced redistribution of ARRB1 in stably *OPRM1*-transfected HEK293 cells through the persistent stimulation of MAPK activity and the authors concluded that chronic morphine treatment produces adaptational changes at the *ARRB1* level (18). These observations and our findings suggest that the drug response of PBLs to morphine mediates the down-regulation of *ARRB1* expression during morphine treatment and reflects the overall cellular response to opioid signaling in an individual.

COMT is one of the enzymes that inactivate catecholamines; therefore, it is regarded as key regulator of adrenergic, noradrenergic and dopaminergic signaling (19). Various diseases are thought to be involved in COMT function including mental disorders, suicidal behavior and personality traits, and tardive dyskinesia (11,21-23). Regarding the COMT genotype as it relates to cancer pain, individuals with a A/A (Met/Met) genotype had a lower regional opioid signal response to pain and a higher sensitivity to pain, compared with heterozygous individuals (24). On the other hand, several clinical studies have demonstrated that the required dose of morphine was lower in subjects with an A/A genotype of *COMT*, compared with others (25-27). These results are consistent with our result. The question why the required dose of morphine is lower in patients with an A/A genotype, even though they are more sensitive to pain, can be explained by a possibly elevated density of OPRM1 in patients with the A/A genotype (28). Our results indicate that *COMT* 472G→A may be a predictive biomarker, although further studies are necessary.

Taken together, our results may provide novel insights into the relations between morphine treatment and *ARRB1* expression and the *COMT* 472G→A genotype.

Acknowledgements

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Clinical Investigation

A Phase I Study of Chemoradiotherapy with Use of Involved-Field Conformal Radiotherapy and Accelerated Hyperfractionation for Stage III Non–Small-Cell Lung Cancer: WJTOG 3305

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Summary

This phase I study of chemo-radiotherapy used involved-field conformal radiotherapy with accelerated Twice-daily hyperfractionation in patients with stage III non-small cell lung cancer. Although the dose of radiation was escalated to 72 Gy in 48 fractions, the maximum tolerated dose was not reached.

Purpose: A Phase I study to determine a recommended dose of thoracic radiotherapy using accelerated hyperfractionation for unresectable non–small-cell lung cancer was conducted.
Methods and Materials: Patients with unresectable Stage III non–small-cell lung cancer were treated intravenously with carboplatin (area under the concentration curve 2) and paclitaxel (40 mg/m^2) on Days 1, 8, 15, and 22 with concurrent twice-daily thoracic radiotherapy (1.5 Gy per fraction) beginning on Day 1 followed by two cycles of consolidation chemotherapy using carboplatin (area under the concentration curve 5) and paclitaxel (200 mg/m^2). Total doses were 54 Gy in 36 fractions, 60 Gy in 40 fractions, 66 Gy in 44 fractions, and 72 Gy in 48 fractions at Levels 1 to 4. The dose-limiting toxicity, defined as Grade $\geq$4 esophagitis and neutropenic fever and Grade $\geq$3 other nonhematologic toxicities, was monitored for 90 days.
Results: Of 26 patients enrolled, 22 patients were assessable for response and toxicity. When 4 patients entered Level 4, enrollment was closed to avoid severe late toxicities. Dose-limiting toxicities occurred in 3 patients. They were Grade 3 neuropathy at Level 1 and Level 3 and Grade 3 infection at Level 1. However, the maximum tolerated dose was not reached. The median survival time was 28.6 months for all patients.

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Conclusions: The maximum tolerated dose was not reached, although the dose of radiation was escalated to 72 Gy in 48 fractions. However, a dose of 66 Gy in 44 fractions was adopted for this study because late toxicity data were insufficient. © 2011 Elsevier Inc.

Keywords: Non–small-cell lung cancer, Chemoradiation, Three-dimensional conformal radiotherapy, Accelerated hyperfractionation, Dose escalation

Introduction

For the treatment of locally advanced inoperable non–small-cell lung cancer (NSCLC), concurrent chemoradiotherapy has shown significantly better survival than sequential therapy (1–4). Even in concurrent chemoradiotherapy, however, locoregional control is unsatisfactory at a standard dose of 56 to 60 Gy (3, 5–7). To improve locoregional control, several dose escalation trials have been performed using three-dimensional (3D) planning techniques, and it has been suggested that 74 Gy is tolerable with concurrent or sequential chemotherapy (8–11).

In conventional fractionation, the benefits of dose escalation are considered limited. Irradiation at a dose of 74 Gy in conventional fractionation requires more than 7 weeks. Even at standard doses, accelerated repopulation is induced during the later part of radiation therapy and is a cause of radiation failure (12). When the treatment time is prolonged, the influence of accelerated repopulation becomes more evident.

Therefore, dose escalation without prolonged treatment time is supposed to bring better outcome, and accelerated hyperfractionation seems to be an effective strategy for shortening treatment time. As the first step to verify the hypothesis, the West Japan Thoracic Oncology Group designed a Phase I trial to define the maximum tolerated dose (MTD) of 3D conformal radiotherapy (CRT) using accelerated hyperfractionation in NSCLC patients.

Methods and Materials

Eligibility

The patient eligibility was as follows: histologic or cytologic diagnosis of NSCLC, unresectable Stage IIIA or IIIB disease, age less than 75 years, Eastern Cooperative Oncology Group performance score of 0 to 1, and function as shown by laboratory determinations including leukocyte count of at least 4,000/mm^3, hemoglobin concentration of at least 9.5 g/dL, platelet count of at least 100,000/mm^3, aspartate aminotransferase and alanine aminotransferase of 2.0 times the upper limit of normal range or less, serum total bilirubin of 1.5 mg/dL or less, serum creatinine of 1.5 mg/dL or less, and PaO_2 at rest of at least 70 mm Hg.

The patients were ineligible if they met any of the following criteria: supraclavicular nodal metastases, interstitial pneumonitis or pulmonary fibrosis, prior thoracic radiation therapy, malignant pleural effusion or malignant pericardial effusion, active concomitant malignancy or recent (<3 years) history of any malignancy, or other serious concomitant medical conditions. The study protocol was approved by each institutional review board for clinical use. All patients gave written informed consent before enrollment.

Patient assessment

All patients underwent a complete medical history and physical examination. Imaging studies, including chest X-ray, computed tomography of the chest and upper abdomen, computed tomography or magnetic resonance imaging of the brain, and positron emission tomography, were required.

Treatment schedule

The patients received concurrent chemoradiotherapy using accelerated hyperfractionation. On Days 1, 8, 15, and 22, carboplatin (area under the concentration curve 2 using the Calvert equation) and paclitaxel (40 mg/m^2) were administered intravenously.

After the concurrent chemoradiotherapy, the patients received two cycles of consolidation chemotherapy consisting of carboplatin (area under the concentration curve 5) and paclitaxel (200 mg/m^2) with an interval of 3 weeks. The first cycle of consolidation chemotherapy was begun 4 weeks after the concurrent chemoradiotherapy, if leukocyte count was at least 4,000/mm^3, platelet count at least 100,000/mm^3, aspartate aminotransferase and alanine aminotransferase 2.0 times the upper limit of normal range or less, serum total bilirubin 1.5 mg/dL or less, serum creatinine 1.5 mg/dL or less, and Eastern Cooperative Oncology Group performance score of 0 to 2. The subsequent cycle of consolidation chemotherapy was repeated if leukocyte count was at least 3,000/mm^3, neutrophil count at least 1,500/mm^3, platelet count at least 100,000/mm^3, serum creatinine 1.5 mg/dL or less, and body temperature not exceeding 38°C.

The 3D CRT began on Day 1. Irradiation was performed with 4-MV or higher photons from a linear accelerator. Patients received 1.5 Gy per fraction twice daily with at least a 6-hour interval between each fraction.

Target volume definitions

Elective nodal irradiation was not performed. The gross tumor volume (GTV) was defined as the volume occupied by visible disease. The GTV included the primary tumor and involved lymph nodes measuring larger than 1.0 cm (short axis measurement) or lymph nodes with a diameter of 5 mm or more as shown by positron emission tomography. The clinical target volume (CTV) was defined as the GTV of the primary tumor plus a margin of 5 mm for all borders and GTV of the lymph nodes without a margin. The planning target volume (PTV) was the CTV plus an adequate margin added to compensate for variability in treatment setup, breathing, or motion during treatment. In

general, the PTV included the CTV plus 1.0 cm of expansion at all borders.

Tissue inhomogeneity corrections were used. The volume of both lungs that received more than 20 Gy should not exceed 35% of the total lung, and the maximum dose to the spinal cord could not exceed 45 Gy. It was desirable but not required that the PTV receive more than 93% but less than 107% of its prescribed dose. After the dose of 36 Gy was reached, the PTV could be reduced after shrinkage of the GTV.

Dose escalation

The MTD was defined as the dose at which 3 or more of 6 patients experienced a dose-limiting toxicity (DLT). The DLT was defined as Grade 4 or more esophagitis, neutropenic fever, dermatitis, or nausea/vomiting and other Grade 3 or more nonhematologic toxicity. Furthermore, interruption of irradiation for more than 2 weeks was also defined as a DLT. The DLT was monitored for 90 days.

Irradiation was performed for 5 days per week. The prescribed doses were 54 Gy in 36 fractions over 3.6 weeks, 60 Gy in 40 fractions over 4.0 weeks, 66 Gy in 44 fractions over 4.4 weeks, and 72 Gy in 48 fractions over 4.8 weeks (Levels 1–4). When the DLT was observed in 0 of 4 patients, in $\leq$1 of 5 patients, or in $\leq$2 of 6 patients at each level, the radiation dose was to be escalated.

Evaluation

The Response Evaluation Criteria in Solid Tumors were used for response assessment (13). Toxicity was evaluated according to the National Cancer Institute Common Toxicity Criteria (version 3.0). An extramural review was conducted to validate the eligibility of the patients and staging.

The duration of survival was counted from the day of entry to the study, and the overall survival was calculated according to the Kaplan-Meier method (14).

Results

Patients' characteristics

Between April 2006 and April 2008, 26 patients were enrolled in this study. Four patients were excluded because of allergic reactions to paclitaxel on Day 1 (n = 1), cerebral infarction on Day 2 (n = 1), and supraclavicular nodal metastases (n = 2). The remaining 22 patients were included in the analysis. They were 6, 7, 5, and 4 patients at Levels 1 through 4, respectively. Although, as a rule, 4 to 6 patients were enrolled in each level, 1 patient was increased at Level 2 because the sixth and seventh patients enrolled at the same time. The baseline characteristics of the 22 patients are summarized in Table 1.

When 4 patients entered Level 4, enrollment was closed to avoid severe late toxicities in the esophagus and the bronchia.

Treatment administration

All patients received full doses of radiation therapy, and interruption of radiation therapy was required in only 4 patients.

Table 1 Patient characteristics (n = 22)

Characteristics	n	%
Age (y)		
Median		63
Range		45–70
Sex		
M	19	86
F	3	14
ECOG performance status		
0	7	32
1	15	68
Histology		
Squamous cell carcinoma	10	45
Adeno carcinoma	10	45
Large cell carcinoma	0	0
Others	2	10
Stage		
IIIA	11	50
IIIB	11	50

Abbreviation: ECOG = Eastern Cooperative Oncology Group.

Interruptions ranged from 1 day to 7 days. All patients received four cycles of concurrent chemotherapy, and 19 patients (86%) received two cycles of consolidation chemotherapy.

Toxicity

The major toxicities are summarized in Table 2.

The DLTs occurred in 3 patients. Two cases of Grade 3 neuropathy were observed, one at Level 1 and the other at Level 3, and one case of Grade 3 infection occurred at Level 1. Furthermore, Grade 5 radiation pneumonitis was observed at Level 1; however, it was not treated as a DLT because the event occurred after the observation period of 90 days. Grade 3 esophagitis was observed in 3 patients, 1 at Level 3 and the others at Level 4. Grade 3 esophagitis and nausea were not defined as DLTs.

At Level 4, no DLT occurred in the 4 patients. Therefore, the MTD was not reached in the present study.

Response and survival

The figure shows the overall survival. The median survival time was 28.6 months for all patients and 30.2 months for patients who received more than 60 Gy. The response rate was 77% for all patients.

Patterns of relapse

Table 3 shows the first sites of relapse. Of 11 patients with locoregional relapse, 1 had upper mediastinal lymph node metastasis, which was located out of the radiation portal. Of 5 patients with distant metastasis, 3 had lung metastasis.

Discussion

With the use of 3D planning techniques, several dose escalation trials have been performed. Kong *et al.* reported that doses of CRT

Table 2 Major toxicities (n = 22)

	Grade 3		Grade 4		Grade 5	
Toxicity	n	%	n	%	n	%
Hematologic						
Leukopenia	16	73	1	5		
Neutropenia	5	23	12	55		
Anemia	2	9	0	0		
Thrombocytopenia	0	0	0	0		
Nonhematologic						
Neuropathy	2	9	0	0	0	0
Infection	1	5	0	0	0	0
Pneumonitis	0	0	0	0	1	5
Esophagitis	3	14	0	0	0	0
Nausea	1	5	0	0	0	0

could be escalated up to 103 Gy for smaller tumors (15). However, the 5-year local control rates were only 49% even at 92 to 103 Gy and 35% at 74 to 84 Gy. The insufficient local control indicated limitation of dose escalation in conventional fractionation and warranted further exploration for different strategies.

A risk of severe late toxicities, such as esophageal stenosis and bronchial occlusion, was predicted from the beginning of the study. After that, experience with Levels 1 through 3 indicated that prescription of high doses in the esophagus or the main bronchi was inevitable in most patients. Therefore, enrollment was closed in the middle of Level 4 to avoid severe late toxicities. Emami *et al.* reported that in treatment of the esophagus, the tolerance dose that would result in a 50% probability of complications within 5 years of treatment was 72 Gy (16). However, data on tolerance doses by accelerated hyperfractionation are lacking. Therefore, careful long-term follow-up of the present study is required. Recently, Atsumi *et al.* reported that the severity and frequency of esophageal stenosis after radiation therapy were greater in patients with esophageal cancer with full circumference involvement and increased with esophageal wall thickness (17). The tolerance dose for the esophagus might be higher in patients without esophageal cancer than in those with esophageal cancer.

In radiation therapy using accelerated hyperfractionation, acute esophagitis is a toxicity of particular concern. In the present study, 3 patients experienced Grade 3 esophagitis: 2 of 4 patients at Level 4, but only 1 patient at Levels 1 through 3 (n = 18). The

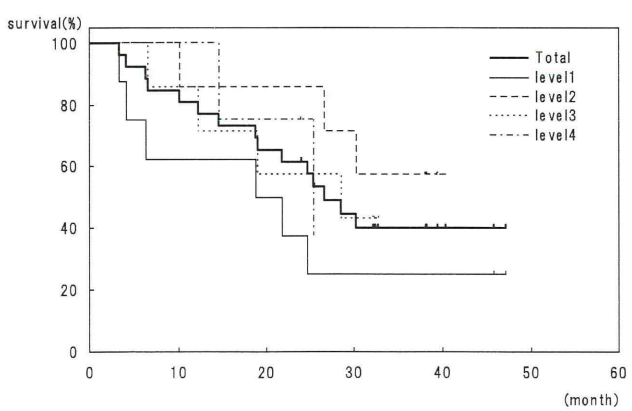

Fig. Kaplan-Meier survival curves for all patients and for patients in Levels 1–4. The median survival time was 28.6 months for all patients.

Table 3 Site of first failure (n = 22)

Site	n	%
Progression free	9	41
Locoregional alone	8	36
Locoregional and distant	3	14
Distant*	5	23
Lung	3	14
Brain	1	5
Small intestine	1	5

* Distant includes locoregional and distant, and distant alone.

low frequency of esophagitis has often been observed in other Japanese trials (18, 19). The causes of this phenomenon are not well known. One possible explanation is differences in ethnic background. Twice-daily CRT with a dose of 1.3 to 1.45 Gy per fraction could be recommended in patients with other ethnic backgrounds, if this regimen is shown to bring a better outcome.

Another toxicity of concern is radiation pneumonitis. In the present study, Grade 5 radiation pneumonitis was observed in 1 patient. In other patients, however, Grade 3 or more radiation pneumonitis was not observed. Some radiation pneumonitis is inevitable to some degree, and the frequency of Grade 3 or more pneumonitis was rather low in the present study. Tsujino *et al.* reported a decreased incidence of radiation pneumonitis by accelerated hyperfractionation in the treatment of limited-stage small-cell lung cancer, although initially they expected that accelerated hyperfractionation would increase the incidence and severity of radiation pneumonitis (20). The results in the present study are consistent with those reported by Tsujino *et al.*

The DLTs observed in the current study were Grade 3 neuropathy and Grade 3 infection. They were mainly caused by chemotherapy. By contrast, Grade 5 radiation pneumonitis was not treated as a DLT because it occurred after the observation period of 90 days. The definition of DLT used in this study was probably inadequate for a radiation dose escalation study. Chemotherapy-induced toxicities should be given less consideration, and those caused by radiation therapy should be more strictly weighed.

The cutoff of 90 days for the observation period of DLTs was considered not sufficient. However, the observation period could not be more prolonged because the Phase I study had to be completed within a suitable time. Toxicities were evaluated after the observation period. In recent Phase I studies, the observation period was defined similarly (21–23).

Although the number of patients was relatively small in the present study, the method of assigning 6 patients to each dose bin is an option in a Phase I study (21). However, the data about survival were not reliable because of the small sample size. The data are to be verified in the following Phase II study.

In the present study, the dose of CRT was escalated to 72 Gy in 48 fractions, and MTD was not reached. In principle, 72 Gy should be the recommended dose. However, late toxicity data are insufficient, and enrollment was closed in the middle of Level 4. Furthermore, on the basis of our experience with the treatment of small-cell lung cancer, CRT with a dose of 66 Gy in accelerated hyperfractionation brings better local control than 74 Gy in conventional fractionation, which was defined as a recommended dose in several trials. The favorable median survival time of 30.2 months for patients who received 60 to 72 Gy in the present study is consistent with our experience. Therefore, a dose of 66 Gy in 44 fractions was adopted in the present study. On the basis

of the results presented here, we are currently preparing a Phase II study.

In conclusion, the MTD was not reached in the present study, although the dose of radiation was escalated to 72 Gy in 48 fractions. Acute toxicities were relatively mild. However, a dose of 66 Gy in 44 fractions was adopted for the present study because late toxicity data were insufficient.

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ORIGINAL ARTICLE

Phase I study of continuous afatinib (BIBW 2992) in patients with advanced non-small cell lung cancer after prior chemotherapy/erlotinib/gefitinib (LUX-Lung 4)

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Abstract
Purpose This Phase I study determined the maximum-tolerated dose (MTD) of afatinib (Afatinib is an investigational compound and its safety and efficacy have not yet been established) (BIBW 2992; trade name not yet approved by FDA), an irreversible inhibitor of epidermal growth factor receptor (EGFR)/human epidermal growth factor receptor (HER)1 and 2, up to a dose of 50 mg/day in advanced non-small cell lung cancer (NSCLC), to establish the recommended dose for Phase II.

Methods Patients with advanced NSCLC who had received prior platinum-doublet chemotherapy and/or erlotinib/gefitinib therapy, or who were ineligible for, or not amenable to, treatment with established therapies, received oral afatinib once daily. The MTD was determined based on dose-limiting toxicities (DLTs); other assessments included safety, pharmacokinetic profile, antitumour activity according to response evaluation criteria in solid tumours and EGFR/HER1 mutation analysis where possible.
Results Twelve evaluable patients were treated at doses of 20–50 mg/day. One DLT was observed at 50 mg/day in Course 1 (Grade 3 mucositis). The most frequent drug-related adverse events were diarrhoea, dry skin, stomatitis, rash, paronychia and anorexia; most were Grade 1 or 2. Six out of 12 patients had tumour size reductions; durable stable disease was achieved in three patients including one with EGFR/HER1 exon 19 and T790 M mutations. Peak plasma concentrations of afatinib were reached 3–4 h after administration and declined with a half-life of 30–40 h. Afatinib 50 mg/day was well tolerated with an acceptable safety profile during Phase I.
Conclusion Recommended dose for Phase II was defined as 50 mg/day for Japanese patients; the same as for non-Japanese patients.

Keywords Phase I · Afatinib · BIBW 2992 · Epidermal growth factor receptor · Tyrosine kinase inhibitor · Non-small cell lung cancer

All authors have contributed equally to the development of the manuscript, and all authors are in agreement with the content of the manuscript.

Clinical trial registration: NCT00711594 (http://clinicaltrials.gov).

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Introduction

Despite the availability of a variety of conventional anti-cancer agents, non-small cell lung cancer (NSCLC)

remains a leading cause of cancer death worldwide. However, increased understanding of the mechanisms underlying cancer development has led to rational approaches to drug development and new treatment agents designed to specifically target these mechanistic pathways [1]. The epidermal growth factor receptor (EGFR or ErbB) tyrosine kinase family is one of the most extensively studied signal transduction networks and is known to promote cancer cell proliferation and tumour invasion [2]. The ErbB receptor family consists of four receptor tyrosine kinases, which includes EGFR (also known as ErbB1 or human epidermal growth factor receptor [HER]1), HER2 (neu/ErbB2), HER3 (ErbB3) and HER4 (ErbB4) [2, 3]. Hyperactivation of the ErbB signalling network has been observed in a variety of malignancies [2, 4] and represents an attractive option for targeted therapy in patients with NSCLC, as overexpression of EGFR/HER1 has been detected in 40–80% of NSCLC tumours [5, 6]. Indeed, the small molecule, reversible, EGFR/HER1 tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib, demonstrate selectivity for EGFR/HER1 and are associated with antitumour activity in NSCLC [7–10]. Unfortunately, resistance to reversible TKIs such as gefitinib and erlotinib develops in all patients. This has been attributed to clonal selection of tumour cells, which exhibit resistance mechanisms such as additional mutations in EGFR/HER1, for example T790 M, that renders gefitinib and erlotinib ineffective inhibitors of EGFR/HER1 kinase activity, or by amplification of the hepatocyte growth factor receptor (MET) oncogene, another receptor tyrosine kinase [11–13]. Thus, there is a need for improved targeted therapies that can overcome the mechanisms associated with resistance.

Afatinib (BIBW 2992) is a novel, next-generation, irreversible TKI that selectively targets EGFR/HER1 (half-maximal inhibitory concentration [IC_{50}] 0.5 nM) and HER2 (IC_{50} 14 nM) [14]. Irreversible binding of afatinib to the target receptor is an attractive feature and may help to overcome the issue of resistance. Furthermore, afatinib is thought to inhibit all cancer-relevant EGFR/HER1- and HER2-containing dimers [14]. In vitro studies have shown that afatinib inhibits the anchorage-independent proliferation of NSCLC cell lines irrespective of the EGFR/HER1 mutational status [14] and has demonstrated antitumour activity in NSCLC models in vivo [14]. Afatinib has also shown superior activity to gefitinib and erlotinib in T790 M models in vivo [14].

Data from Phase I/II trials have demonstrated the efficacy of afatinib in patients with NSCLC harbouring EGFR/HER1-activating mutations [15, 16]. This small-scale, open-label, uncontrolled Phase I/II trial was planned to specifically estimate the efficacy of afatinib in patients with advanced NSCLC. An assessment of overall safety data from four previous Phase I trials in non-Japanese patients [17–20] established a recommended Phase II dose of 50 mg/day for continuous daily dosing of afatinib [20]. Based on this experience, treatment groups receiving higher than 50 mg were not included in this study to ensure the safety of Japanese patients. The Phase I step of this study was, therefore, performed to determine the maximum-tolerated dose (MTD) at dose levels of up to 50 mg/day (i.e. recommended Phase II dose in non-Japanese patients) and to determine the recommended dose for the Phase II step in Japanese patients. Here, we report the Phase I findings.

Materials and methods

Study design

This was a Phase I/II, open-label, multicentre trial conducted in Japan. Here, we report the findings from the Phase I part of this trial, which followed a dose-escalation design. The primary endpoint of this study was to assess the safety of afatinib based on the incidence of dose-limiting toxicities (DLTs) and the incidence and intensity of adverse events (AEs). This study was conducted according to the Declaration of Helsinki and in accordance with the Guideline for Good Clinical Practice. Written informed consent was obtained from all participants.

Study population

Eligible patients were adults ($\geq$20 and $\leq$74 years) with pathological confirmation of NSCLC with tissue or cytological diagnosis who had previously received platinum-doublet chemotherapy and/or erlotinib/gefitinib therapy or who were ineligible for, or not amenable to, treatment with established therapies. Patients were required to have a life expectancy of at least 3 months and an Eastern Cooperative Oncology Group (ECOG) performance score of 0 or 1. Patients were also required to have fully recovered from all therapy-related toxicities (except for alopecia) from previous chemo-, hormone-, immuno- or radiotherapies to Common Terminology Criteria for Adverse Events (CTCAE) Grade $\leq$1 and from previous surgery. All patients must have terminated prior chemo-, hormone-, immuno- or radiotherapy 4 weeks before enrolment. Patients with significant gastrointestinal disorders with diarrhoea as a major symptom, e.g. Crohn's disease, malabsorption or CTCAE Grade >2 diarrhoea of any aetiology at the time of enrolment, were excluded from study entry. Additional exclusion criteria included current or previous history of distinct/suspected pulmonary fibrosis or interstitial lung disease determined by the chest radiographic findings, brain tumour and/or brain metastases, active double cancer, a history of

uncontrolled cardiac disease, coelomic fluid retention requiring treatment or uncontrolled concomitant disease (such as diabetes mellitus and hypertension). Sexually active patients unwilling to use a medically acceptable method of contraception during the trial, and pregnant or breast-feeding women, were also excluded.

Dose escalation

Afatinib was administered orally in continuous daily dosing based on a 28-day treatment course and was continued until disease progression or intolerable toxicity. Dose escalation followed a 3 + 3 escalation scheme; a minimum of three patients were treated per dose level with expansion to six patients if a DLT was observed in Course 1. If no patient experienced a DLT, dose escalation was continued. The starting dose was 20 mg/day with escalation to 40 mg/day and then 50 mg/day. Dose escalation was discontinued when the upper limit (50 mg) was reached or MTD was determined.

Concomitant medications

Anti-diarrhoeal drugs, such as loperamide, were permitted to manage diarrhoea, and patients with CTCAE Grade $\geq$2 nausea and/or vomiting were permitted anti-emetic therapy. Patients who experienced CTCAE Grade $\geq$2 diarrhoea, nausea and/or vomiting for 7 days or more, despite supportive care, were required to stop afatinib treatment until recovery (CTCAE Grade $\leq$1).

Study assessments

The safety and tolerability of afatinib was assessed by changes in the incidence and severity of AEs according to CTCAE version 3.0 and by physical examination, vital signs (including twelve-lead electrocardiogram) and laboratory parameters.

All toxicities were graded using CTCAE version 3.0. A DLT was defined as a drug-related CTCAE Grade 3 or 4 non-haematological toxicity (except for transient electrolyte abnormality), or Grade 4 drug-related haematological toxicity. Additional DLTs included: CTCAE Grade $\geq$2 decrease in cardiac left ventricular function; CTCAE Grade $\geq$3 nausea and/or vomiting or persistent CTCAE Grade $\geq$2 nausea and/or vomiting for $\geq$7 days, despite anti-emetic medication; and CTCAE Grade $\geq$3 diarrhoea or persistent CTCAE Grade $\geq$2 diarrhoea for $\geq$7 days, despite anti-diarrhoeal medication, such as loperamide. The MTD was defined on the basis of DLTs observed during the first treatment course (4 weeks) and was a dose $\leq$50 mg once daily, at which no more than 33% of patients experienced a DLT.

Tumour response was assessed according to response evaluation criteria in solid tumours. Target lesions were defined as either having a complete response, partial response, stable disease or progressive disease. Patients were assessed at screening and then at the end of every treatment course.

EGFR/HER1 mutation analysis was performed where possible. Tumour biopsies and surgical material from prior biopsy sampling or surgery were collected from respective sites (pathology departments) during the screening period. Initial diagnostic tumour specimens and/or tissue material obtained at disease recurrence after initial EGFR/HER1 TKI treatment was considered adequate. If multiple biopsies were available for individual patients, the most recent and/or the most appropriate biopsy material was requested. Tumour material and a single serum sample collected during the screening period were centrally analysed for EGFR/HER1 mutations using the Scorpion Amplified Refractory Mutation System™ and Direct Sequencing. The genome DNA solution was analysed using a DxS EGFR/HER1 Mutation Test Kit. A Sequence Detection System (Applied Biosystems, ABI PRISM 7700) was used to detect mutations in EGFR/HER1. A Genetic Analyzer (Applied Biosystems, ABI PRISM 3100) was used to analyse the nucleic acid sequence of the EGFR/HER1 gene.

Pharmacokinetic sampling and data analysis in Course 1

Blood samples for the evaluation of pharmacokinetic (PK) parameters were collected at pre-dose, 0.5, 1, 2, 3, 4, 5, 7 and 9 h, and 24 h after dosing on Days 1, 28 and 48, and 72 h after dosing on Day 28. Pre-dose blood samples to determine trough plasma concentrations were collected on Days 8, 15 and 22 before drug administration. Afatinib plasma concentrations were determined by validated high-performance liquid chromatography coupled to tandem mass spectrometry. Non-compartmental PK parameters were determined using WinNonlin®.

Statistical analyses

All patients who received at least one dose of afatinib (treated set) were included in the efficacy and safety analyses. Safety, efficacy and PK characteristics were analysed in an exploratory and descriptive manner.

Results

Patient population

In total, 13 patients were enrolled in this study, and 12 patients were treated (five men and seven women); three

patients were treated with afatinib 20 mg/day, three patients were treated with afatinib 40 mg/day and six patients were treated with afatinib 50 mg/day. The median (range) duration of afatinib exposure was 68.5 (28–370) days. Patient demographics and clinical characteristics are summarized in Table 1. Patients were heavily pre-treated with an average of three and a half previous chemotherapy regimens. All patients eventually discontinued treatment. Ten patients discontinued study medication because of disease progression, one patient discontinued owing to an AE not related to afatinib and one patient withdrew consent. EGFR/HER1 mutation analysis was conducted for 11 patients: five patients were found to have EGFR/HER1 mutations, of which four had double mutations including the T790 M mutation (see Table 1).

Table 1 Patient demographic characteristics

Characteristic	Afatinib
Age (years): median (range)	62.5 (39–67)
Men/Women	5/7
ECOG score:	
0	8
1	4
Smoking history:	
Non-smoker	7
Ex-smoker	5
EGFR/HER1 mutation status:	
Del 19 + T790 M (tissue)/NA (serum)	1
Del 19 (tissue)/Del 19 + T790 M (serum)	1
NA (tissue)/Del 19 + T790 M (serum)	1
NA (tissue)/L858R + T790 M (serum)	1
NA (tissue)/S768I (serum)	1
Wild (tissue)/Wild (serum)	1
NA (tissue)/Wild (serum)	5
NA (tissue)/NA (serum)	1
Tumour histology	
Adenocarcinoma	10
Squamous cell carcinoma	1
Squamous/adenocarcinoma	1
Number of metastasis sites: median (range)	4.0 (0–11)
Number of prior chemotherapy (range)	3.5 (1–8)
Prior therapies:	
Surgery	3
Radiotherapy	5
Prior erlotinib and/or gefitinib	8
Clinical stage at screening:	
IIIB	1
IV	11
Starting dose of afatinib (mg):	
20	3
40	3
50	6

ECOG Eastern Cooperative Oncology Group, *EGFR* Epidermal growth factor receptor, *HER* Human epidermal growth factor, *NA* not applicable

Safety

All twelve treated patients were evaluable for safety, and all experienced at least one drug-related AE (Table 2). The most frequent drug-related AEs observed in more than 40% of the patients were diarrhoea, dry skin, stomatitis, rash, paronychia and anorexia. Only one patient (afatinib 20 mg/day group) experienced an AE leading to discontinuation of study medication. This patient developed bile duct cancer (active second primary cancer; serious AE), which occurred in Course 1, and was considered unrelated to the study medication. Two additional patients experienced serious AEs of mucosal inflammation and enteritis, respectively; both were experienced in patients receiving afatinib 50 mg/day, were considered related to the study medication and were resolved following study drug discontinuation.

Four patients required a one-step dose reduction of afatinib related to AEs (one patient in the 40 mg/day dose group and three patients in the 50 mg/day dose group). Adverse events necessitating dose reduction included rash, paronychia, mucosal inflammation, diarrhoea and enteritis.

Only one DLT was reported during Course 1; Grade 3 mucosal inflammation in a patient receiving afatinib 50 mg/day that resolved following dose interruption followed by reduction. Two further DLTs were reported after Course 1 in patients receiving afatinib 50 mg/day; one patient experienced Grade 3 enteritis in Course 4 and one patient experienced Grade 3 diarrhoea in Course 2. Both events resolved following dose reduction.

No clinically significant changes were noted in clinical laboratory parameters, vital signs, electrocardiograms and left ventricular function.

Efficacy

All twelve treated patients were evaluable for response. No complete responses or partial responses were reported. Six out of twelve patients had tumour size reductions; details of the patients experiencing tumour reduction are shown in Table 3 and Fig. 1, which illustrate the maximum tumour size reduction of individual patients by mutation status. Nine patients reported a best overall response as stable disease, with three achieving prolonged stable disease. One patient, a 64-year-old woman with an adenocarcinoma of the lung diagnosed 2.9 years ago and resistant to gefitinib and erlotinib, was progression-free for 310 days and had a maximum tumour size reduction of −7.7% (stable disease), despite the presence of T790 M resistance mutations.