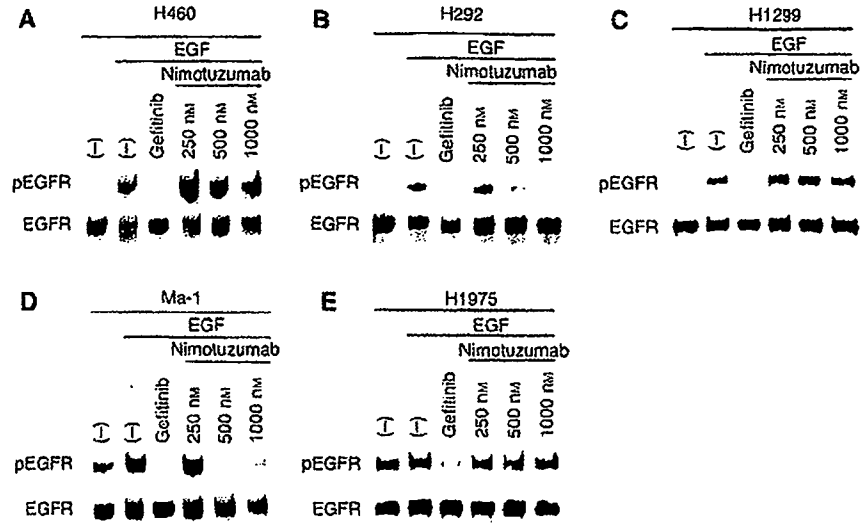

Figure 2 Effect of nimotuzumab on EGFR phosphorylation in NSCLC cells. H460 (**A**), H292 (**B**), H1299 (**C**), Ma-1 (**D**), and H1975 (**E**) cells were deprived of serum overnight and then incubated first for 15 min in the absence or presence of the indicated concentrations of nimotuzumab or gefitinib (10 μM) and then for an additional 15 min in the additional absence or presence of EGF (100 ng ml^{-1}). Cell lysates were then subjected to immunoblot analysis with antibodies to the Tyr1068-phosphorylated form of EGFR (pEGFR) as well as with those to total EGFR.

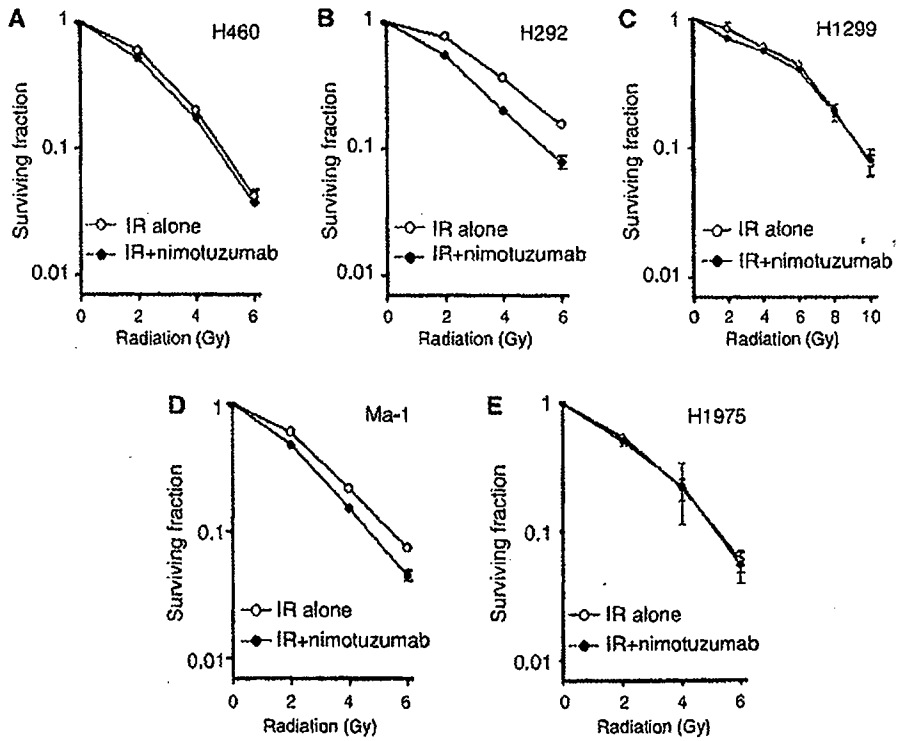

Figure 3 Effect of nimotuzumab on the response of NSCLC cells to radiation *in vitro*. H460 (**A**), H292 (**B**), H1299 (**C**), Ma-1 (**D**), and H1975 (**E**) cells were incubated with or without 700 nM nimotuzumab in medium supplemented with 1% fetal bovine serum for 24 h, exposed to the indicated doses of γ-radiation, and then incubated in drug-free medium supplemented with 10% serum for 10–14 days for determination of colony-forming ability. Survival curves were generated after correction of colony formation observed for combined treatment with ionising radiation (IR) and nimotuzumab by that apparent for treatment with nimotuzumab alone. Data are means ± s.d. of triplicates from a representative experiment.

tumour growth increased to a value similar to that seen in control animals. Combined treatment with radiation and nimotuzumab resulted in a substantial delay in tumour growth and subsequent inhibition of the growth rate of H292 and Ma-1 xenografts. The growth delay after treatment with nimotuzumab alone, radiation alone, or both nimotuzumab and radiation was thus 27.2, 19.6, and 53.6 days, respectively, for H292 cells and 26.7, 13.0, and 78.3 days, respectively, for Ma-1 cells (Table 2). The enhancement factor for the effect of nimotuzumab on the efficacy of radiation was 1.3 for H292 cells and 4.0 for Ma-1 cells, revealing the effect to be more

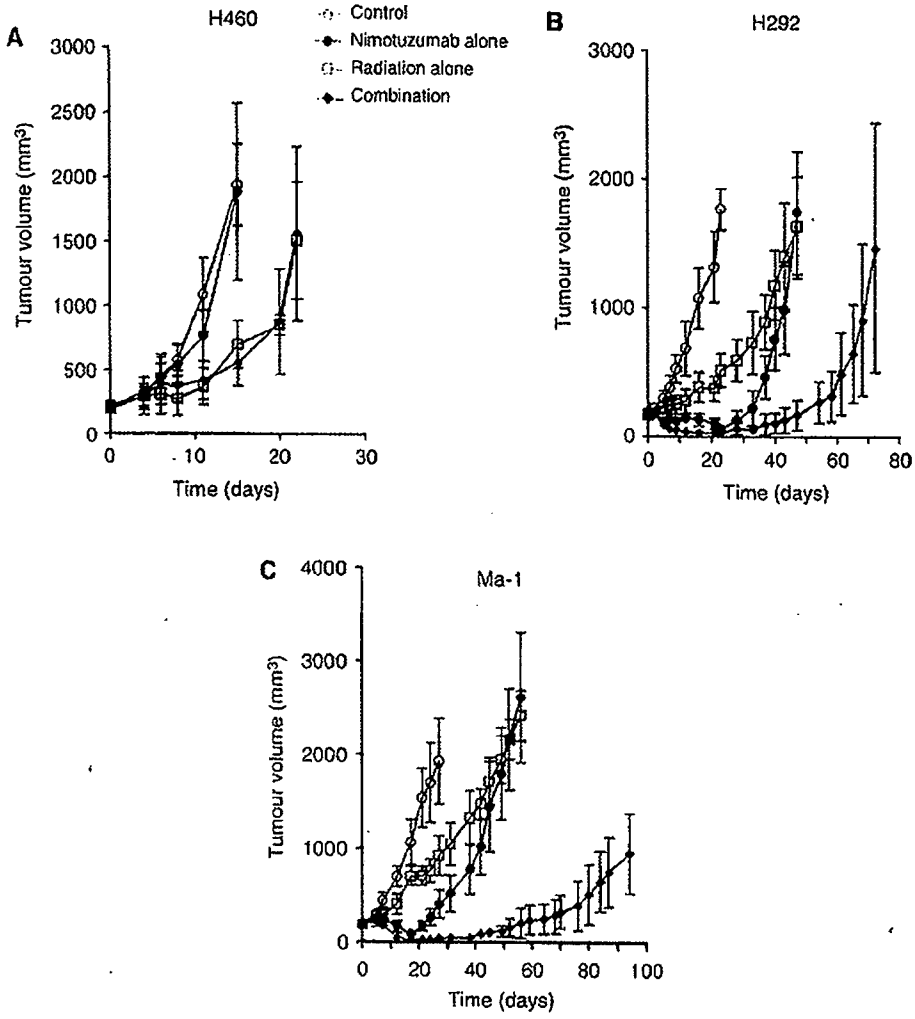

Figure 4 Effect of nimotuzumab on the response of NSCLC cells to radiation *in vivo*. H460 (**A**), H292 (**B**), or Ma-1 (**C**) cells were injected subcutaneously in athymic nude mice. Treatment was initiated when tumours in each group achieved an average volume of approximately 170–200 mm^3. Mice were treated with a single dose of nimotuzumab (1.0 mg per mouse) intraperitoneally, a single dose of γ-radiation (10 Gy), or neither (control) or both modalities, and tumour volume was determined at the indicated time points thereafter. Data are means ± s.d. for seven to eight mice per group.

Table 2 Tumour growth delay in nude mice treated with nimotuzumab, radiation, or both modalities

	H460		H292		Ma-1	
Treatment	**Days[a]**	**GD[b]**	**Days**	**GD**	**Days**	**GD**
Control	10.4		13.2		15.1	
Nimotuzumab alone	11.8	1.4	40.4	27.2	41.8	26.7
Radiation alone	20.4	10.0	32.8	19.6	28.1	13.0
Nimotuzumab+radiation	20.5	10.1	66.8	53.6	93.4	78.3
Enhancement factor	0.86		1.3		4.0	

GD = growth delay. [a]Time required for xenografts in each group to achieve a fivefold increase in volume. [b]The additional time (days) required for xenografts in each treatment group to achieve a fivefold increase in volume relative to the corresponding time for xenografts in the control group.

than additive. No pronounced tissue damage or toxicities such as diarrhoea or a decrease in body weight of >10% were observed in mice in any of the four treatment groups. These results thus suggested that nimotuzumab potentiated the antitumor activity of radiation in H292 and Ma-1 cells *in vivo* as well as *in vitro*.

DISCUSSION

Somatic mutations in the EGFR kinase domain and *EGFR* amplification have been associated with a better response to EGFR-TKIs, such as gefitinib and erlotinib, in patients with NSCLC (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004; Cappuzzo *et al*, 2005; Mitsudomi *et al*, 2005; Takano *et al*, 2005). Given that little is known of the relation between such *EGFR* alterations and the response to treatment with anti-EGFR mAbs, we investigated the antitumor effect of combined treatment with the anti-EGFR mAb nimotuzumab and radiation in NSCLC cell lines of differing *EGFR* status.

The antitumor effect of EGFR-specific mAbs has been thought to result from inhibition of ligand binding to EGFR and consequent inhibition of EGFR activation (Li *et al*, 2005; Marshall, 2006). We, therefore, examined the effect of nimotuzumab on EGF-dependent EGFR signalling. Nimotuzumab inhibited the EGF-induced or constitutive phosphorylation of EGFR in H292 and Ma-1 cells (with high and moderate levels of surface EGFR expression, respectively), consistent with the mode of action of this antibody. However, nimotuzumab did not block EGF-induced or constitutive EGFR phosphorylation in H460, H1299, or H1975 cells (all with a

low level of surface EGFR expression). These observations suggest that the inhibitory effect of nimotuzumab on EGFR signalling depends on the expression level of EGFR on the cell surface. A clonogenic cell survival assay revealed that nimotuzumab enhanced the cytotoxic effect of radiation in H292 and Ma-1 cells, but not that in H460, H1299, or H1975 cells. These findings support the notion that the inhibition of EGFR signalling by nimotuzumab is responsible, at least in part, for the enhancement of the cytotoxic effect of radiation by this antibody. Irradiation of tumour cells has been shown to activate EGFR via ligand-independent and ligand-dependent mechanisms, possibly accounting for radiation-induced acceleration of tumour cell repopulation and the development of radioresistance (Schmidt-Ullrich *et al*, 1997, 2003; Dent *et al*, 2003). Such radiation-induced activation of EGFR-dependent processes may represent a rationale for combined treatment with radiation and EGFR inhibitors. It remains to be determined whether nimotuzumab is able to block radiation-induced activation of EGFR.

Consistent with our *in vitro* results, we found that nimotuzumab enhanced the antitumor effect of radiation on H292 or Ma-1 cells in nude mice. Such enhancement was not apparent for tumours formed by H460 cells. Nimotuzumab alone also manifested a substantial antitumor effect for xenografts formed by H292 or Ma-1 cells but not for those formed by H460 cells. Together these results suggest that the efficacy of nimotuzumab monotherapy is a prerequisite for augmentation of radioresponse by this mAb. Nimotuzumab was previously shown to induce the regression of A431 tumour xenografts *in vivo* as a result of inhibition of both tumour cell proliferation and tumour angiogenesis (Crombet-Ramos *et al*, 2002). Immunohistochemical analysis of tumour specimens from head and neck cancer patients treated with the combination of nimotuzumab and radiation also showed evidence of antiproliferative and antiangiogenic effects (Crombet *et al*, 2004). These observations suggest that effects of nimotuzumab on both NSCLC cell proliferation and tumour angiogenesis might contribute to the enhancement of the antitumor efficacy of radiation by this antibody observed in the present study. Enhancement of the anticancer effect of radiation by the anti-EGFR mAb cetuximab was previously shown to be increased by transfection of cells to upregulate the level of EGFR expression, suggesting that potentiation of the antitumor efficacy of radiation by anti-EGFR mAbs is related to the absolute level of EGFR expression (Liang *et al*, 2003; Bonner *et al*, 2004). This finding is consistent with our present results showing that potentiation of the antitumor activity of radiation by nimotuzumab was related to the level of surface EGFR expression. The nimotuzumab-resistant cell line H460 harbours a mutant form of *KRAS* (Balko *et al*, 2006) that has been associated with resistance to cetuximab (Lievre *et al*, 2006). However, we found that nimotuzumab also failed to inhibit EGF-induced EGFR phosphorylation and to enhance the cytotoxic effect of radiation in H1299 cells, which harbour wild-type *KRAS* (Coldren *et al*, 2006). These observations thus support the notion that a low level of EGFR expression at the cell surface is related to resistance to combined treatment with nimotuzumab and radiation, irrespective of *KRAS* status.

We demonstrated that nimotuzumab inhibited EGFR phosphorylation and enhanced the antitumor effect of radiation in *EGFR* mutant Ma-1 cells (with a moderate level of surface EGFR expression) but not in *EGFR*-mutant H1975 cells (with a low level of surface EGFR expression). Nimotuzumab also potentiated the cytotoxic effect of radiation in H292 cells, which harbour wild-type *EGFR* alleles and have a high level of surface EGFR expression. These findings support the notion that *EGFR* mutation is not the major determining factor for enhancement of the antitumor effect of radiation by nimotuzumab, consistent with previous observations with cetuximab (Barber *et al*, 2004; Tsuchihashi *et al*, 2005). However, the mechanisms underlying such enhancement of the antitumor effect of radiation may differ between NSCLC cells harbouring wild-type or mutant *EGFR* alleles. We and others have previously shown that mutations in the tyrosine kinase domain of EGFR are associated with increased ligand-independent tyrosine kinase activity of EGFR (Lynch *et al*, 2004) and aberrant EGFR signalling (Amann *et al*, 2005; Okabe *et al*, 2007). Given that cell-cycle checkpoints activated by ionising radiation are defective in *EGFR*-mutant NSCLC cell lines (Das *et al*, 2006), the constitutive activity of EGFR in such cells may result in unchecked DNA synthesis and in apoptosis on exposure to ionising radiation. It is possible that these defects in *EGFR*-mutant cells affect the enhancement of the antitumor efficacy of radiation by nimotuzumab.

In summary, we have shown that nimotuzumab enhanced the antitumor efficacy of radiation *in vitro* and *in vivo*, providing a rationale for future clinical investigations of the therapeutic efficacy of nimotuzumab in combination with radiotherapy. Our data suggest that potentiation of the antitumor activity of radiation by nimotuzumab may be related to the level of EGFR expression at the cell surface rather than to *EGFR* mutation. The preselection of patients on the basis of genetic factors that predict treatment sensitivity or resistance may thus be required for the combination therapy with nimotuzumab and radiation.

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Evaluation of epidermal growth factor receptor mutation status in serum DNA as a predictor of response to gefitinib (IRESSA)

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The aim of this study was to evaluate the usefulness of *EGFR* mutation status in serum DNA as a means of predicting a benefit from gefitinib (IRESSA) therapy in Japanese patients with non-small cell lung cancer (NSCLC). We obtained pairs of tumour and serum samples from 42 patients treated with gefitinib. *EGFR* mutation status was determined by a direct sequencing method and by Scorpion Amplification Refractory Mutation System (ARMS) technology. *EGFR* mutations were detected in the tumour samples of eight patients and in the serum samples of seven patients. *EGFR* mutation status in the tumours and serum samples was consistent in 39 (92.9%) of the 42 pairs. *EGFR* mutations were strong correlations between both *EGFR* mutation status in the tumour samples and serum samples and objective response to gefitinib ($P<0.001$). Median progression-free survival time was significantly longer in the patients with *EGFR* mutations than in the patients without *EGFR* mutations (194 *vs* 55 days, $P=0.016$, in tumour samples; 174 *vs* 58 days, $P=0.044$, in serum samples). The results suggest that it is feasible to use serum DNA to detect *EGFR* mutation, and that it's potential as a predictor of response to, and survival on gefitinib is worthy of further evaluation.
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Lung cancer is a major cause of cancer-related mortality worldwide and is expected to remain a major health problem for the foreseeable future (Parkin *et al*, 2005). Most patients have advanced disease at the time of diagnosis. Initial therapy for advanced non-small cell lung cancer (NSCLC) is typically systemic chemotherapy with a two-drug combination regimen, which often includes a platinum agent, but the median survival of patients treated with such regimens has ranged from only 8 to 10 months (Breathnach *et al*, 2001; Kelly *et al*, 2001; Schiller *et al*, 2002). Little improvement in the efficacy of chemotherapy has been made in the last 20 years. A recent report shows that the addition of bevacizumab, a monoclonal antibody against vascular endothelial growth factor, to paclitaxel plus carboplatin in patients with advanced NSCLC has a significant survival benefit, and the median survival was 12.3 months, as compared with 10.3 months in the chemotherapy-alone group (Sandler *et al*, 2006).

Targeting epidermal growth factor receptor (EGFR) is an appealing strategy for the treatment of NSCLC, because EGFR has been found to be expressed, sometimes strongly, in NSCLC (Franklin *et al*, 2002). Gefitinib ('Iressa', AstraZeneca) is a small molecule and selective EGFR tyrosine kinase inhibitor (EGFR-TKI) that has shown antitumour activity in NSCLC patients as a single agent in phase II and III trials (Fukuoka *et al*, 2003; Thatcher *et al*, 2005). An association between mutations in *EGFR* tyrosine kinase sites in NSCLC patients and hyper-responsiveness to gefitinib has recently been reported (Lynch *et al*, 2004; Paez *et al*, 2004). The mutations consisted of small in-frame deletions or substitutions clustered around the ATP-binding site in exons 18–21 of *EGFR*. Some investigators subsequently found that *EGFR* mutations are one of the strong determinants of tumour response to EGFR tyrosine kinase inhibitors (Pao *et al*, 2004; Han *et al*, 2005; Shigematsu *et al*, 2005). The mutation status could be evaluated stably in studies that used surgical tissues to detect the *EGFR* mutations, but most patients who require gefitinib therapy already have advanced disease at the time of diagnosis and therefore are not operated on. It is difficult to obtain sufficient tumour DNA from non-surgical tissue samples, for example, those derived from bronchoscopy that allow detection of *EGFR* mutations by direct sequencing. Actually, translational research in patients with advanced NSCLC in whom gefitinib therapy recommended has been limited by the scarcity of available tumour biopsy tissue, and tumour samples for genetic research were only available for 12.7 and 44.5%, respectively, of patients enrolled in two large phase III clinical studies with EGFR-TKIs (Tsao *et al*, 2005; Hirsch *et al*, 2006). It is therefore important to have sensitive methods for detecting *EGFR* mutations from DNA derived from non-surgical tissue specimens.

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It is well known that the concentration of circulating DNA in plasma or serum has been found to be higher in cancer patients than in cancer-free control subjects, and that significantly higher DNA levels are found in the serum of patients with metastatic disease (Leon *et al*, 1977; Jahr *et al*, 2001; Sozzi *et al*, 2003). The tumour-derived DNA in serum may have been released by a tumour mass that has undergone cell necrosis or tumour cells lysis, or by circulating tumour cells, resulting in a very elevated serum DNA concentration. Some investigators have shown that testing for DNA alterations in peripheral blood has great potential, especially for early detection and diagnosis and for monitoring for a relapse during follow-up (Chen *et al*, 1996; Nawroz *et al*, 1996; Sozzi *et al*, 1999, 2001; Cuda *et al*, 2000; Nunes *et al*, 2001). The same alterations which mean mutations, methylation, and loss of heterozygosity, in genomic DNA have been observed in DNA from both tumour cells in resected and biopsy specimens, and from serum samples in patients with various types of tumours, including NSCLC (Sanchez-Cespedes *et al*, 1998; Esteller *et al*, 1999). Some studies have even reported that genetic aberrations in serum DNA modulate survival in NSCLC patients treated with chemotherapy. Their authors have proposed that the assay used in their studies may obviate the need for tumour tissue analysis (Ramirez *et al*, 2005; de las Penas *et al*, 2006). Serum samples can be obtained safely, with the option of repeat sampling from all NSCLC patients regardless of patient characteristics. The detection of *EGFR* mutations in serum provides a unique and potentially valuable tumour marker for prediction of response and prognosis.

We have previously reported the feasibility of detecting *EGFR* mutations in serum DNA using the Scorpion Amplification Refractory Mutation System (ARMS) method (Kimura *et al*, 2006). The Scorpion ARMS method is one of the most sensitive and fastest methods for specific detection of mutations in DNA (Newton *et al*, 1989; Whitcombe *et al*, 1999). Although *EGFR* mutations were detectable by both PCR direct sequencing, which has generally been used to detect the mutations and the Scorpion ARMS method, mutation status determined with Scorpion ARMS predicted response to gefitinib in our study (Kimura *et al*, 2006). Since the previous study did not clarify the feasibility of using serum DNA as a practical source for detection of *EGFR* mutations, in the present study, we sought to demonstrate that *EGFR* mutation status determined in serum DNA is the same as in actual tumour samples.

The aim of this study was (1) to determine whether the *EGFR* mutations in tumour tissue and serum samples from advanced NSCLC patients are the same, and (2) to identify whether there is a correlation between *EGFR* mutation status detected in serum DNA and both response to gefitinib and survival benefit from gefitinib.

PATIENTS AND METHODS

Patients

The subjects were patients with advanced NSCLC in whom gefitinib therapy was started between July 2002 and February 2006. All patients were treated with gefitinib alone, and 14 patients were treated with gefitinib as initial therapy. The others were treated with gefitinib as second- or third-line therapy. The diagnosis of NSCLC was based on the histological or cytological findings, and the histological type was determined according to WHO criteria (Travis *et al*, 1999). Patients' records consisted of age, gender, smoking habit, and histological tumour type. Patients were divided into three groups according to their smoking status: never-smokers (<100 cigarettes per lifetime), former smokers (≥100 cigarettes per lifetime, but quit 1 year before diagnosis), and current smokers (≥100 cigarettes per lifetime). The response to gefitinib was evaluated in accordance with the 'Response Evaluation Criteria in Solid Tumours (RECIST)' guidelines (Therasse *et al*, 2000). This study was approved by the Institutional Review Board of Kanazawa University Hospital. Written informed consent was obtained from all participants. No research results were entered into the patient's records or released to the patient or the patient's physician.

Tissue preparation and DNA extraction

Tumour specimens were obtained at diagnosis and analysed retrospectively. Twenty-eight tumour samples were collected from the primary cancer (19 via transbronchial lung biopsy, 2 via percutaneous lung biopsy, and 7 surgical specimens). Fourteen tumour samples were from metastatic sites (three from bone, eight lymph nodes, one brain, and one small bowel). All specimens were examined histologically to confirm the diagnosis of NSCLC. The tumour specimens obtained were fixed in formalin and embedded in paraffin wax. Serial sections containing representative malignant cells were deparaffinised in xylene washes and dehydrated in 100% ethanol. DNA was extracted from five serial 10-μm thick sections by using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the protocol described in the manufacturer's instructions. The DNA obtained was eluted in 50 μl of buffer AE, and the concentration and purity of the extracted DNA were assessed by spectrophotometry. The extracted DNA was stored at −20°C until used.

Blood sample collection and DNA extraction

Blood samples were collected before the start of gefitinib therapy. The volume of each blood sample was 4 ml. Serum was separated within 2 h from the sample collection and stored at −80°C until used. Serum DNA was extracted and purified by using a Qiamp Blood Kit (Qiagen), with the following protocol modifications. One column was used repeatedly until the whole sample had been processed. The resulting DNA was eluted in 50 μl of sterile bi-distilled buffer. The concentration and purity of the extracted DNA were determined by spectrophotometry. The extracted DNA was stored at −20°C until used.

Direct sequencing for detection of *EGFR* mutations

EGFR mutations in exons 18, 19, and 21 were detected by PCR-based direct sequencing. PCR amplification was performed in 10 ng of genomic DNA using the TaKaRa Ex Taq™ Hot Start Version kit (TaKaRa, Tokyo, Japan). The primers (forward and reverse) were: exon 18 (5′-CCTTGTCTCTGTGTTCTTGT-3′ and 5′-CTGCGGCCCAGCCCAGAGGC-3′), exon 19 (5′-CATGTGGCAC CATCTCACA-3′ and 5′-CCACACAGCAAAGCAGAA AC-3′), and exon 21 (5′-CAGGGTCTTCTCTGTTTCAG-3′ and 5′-TAAAGC CACCTCCTTACTTT-3′). DNA was amplified for 35 cycles at 95°C for 30 s, 61°C for 30 s, and 72°C for 60 s followed by 7 min of extension at 72°C. Sequencing was performed with a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and the results were analysed with Sequencer 3.11 software (Applied Biosystems) to compare variations. The sequences were compared with the GenBank human sequence for *EGFR* (accession number AF288738).

Scorpion ARMS for detection of E746_A750del and L858R

An EGFR Scorpion Kit (DxS Ltd, Manchester, UK), which combines two technologies, namely ARMS and Scorpion was to detect mutations in real-time PCR as described previously (Kimura *et al*, 2006). Four scorpion primers for detection of E746_A750del, L858R, and the wild type in both exons 19 and 21 were designed and synthesised by DxS Ltd. All reactions were performed in 25 μl volumes using 1 μl of template DNA, 7.5 μl of reaction buffer mix, 0.6 μl of Primer mix and 0.1 μl of Taq polymerase. All reagents are

included in the kit. Real-time PCR was carried out by using SmartCycler® II (Cepheid, Sunnyvale, CA, USA) under the following conditions: initial denaturation at 95°C for 10 min, 50 cycles of 95°C for 30 s, 62°C for 60 s with fluorescence reading (set to FAM, which allows optical excitation at 480 nm and measurement at 520 nm) at the end of each cycle. Data analysis was performed with Cepheid SmartCycler software (version 1.2b). The cycle threshold (C_t) was defined as the cycle at the highest peak of the second-derivative curve, which represented the point of maximum curvature of the growth curve. Both C_t and maximum fluorescence (F_l) were used to interpret the results. Positive results were defined as follows: $C_t \leqslant 45$ and $F_l \geqslant 50$. These analyses were performed in duplicate for each sample and reviewed by two investigators blinded to any clinical information.

Statistical analyses

Patient characteristics, including gender, tumour histology, smoking habit, and response to gefitinib, were tabulated according to mutation status. Fisher's exact test was used to test for associations between the presence of *EGFR* mutations and the patients' characteristics. Overall survival (OS) and progression-free survival (PFS) according to *EGFR* mutation status were estimated by the Kaplan-Meier method, and compared using the two-sided log-rank test. Overall survival was defined as the interval between the start of gefitinib therapy and death from any cause; patients known to be still alive at the time of the analysis were censored at the time of their last follow-up. Progression-free survival was defined as the interval between the start of gefitinib therapy and the first manifestation of progressive disease (PD) or death from any cause; patients known to be alive and without PD at the time of analysis were censored at the time of their last follow-up.

RESULTS

Patient's characteristics

Forty-two patients were enrolled in this study (Table 1). This study covered a long period. There are two reasons why it took 4 years to assemble the 42 patients enrolled. One is that this study was carried out in Kanazawa University Hospital alone, and was not a multicentre study. The other is that not all patients with NSCLC at the hospital during that period were enrolled in this study, because some were enrolled in other trials or the patients refused. Their median age was 58 years (range, 40–81 years), and there were 14 females (33.3%) and 14 never-smokers (33.3%). The histological and/or cytological diagnosis was adenocarcinoma in 31 patients (73.8%), squamous cell carcinoma in 7 (16.7%), and large-cell carcinoma in 4 (9.5%). The results for response to gefitinib showed that 10 patients (23.8%) had a partial response (PR) and 14 (33.3%) had stable disease (SD). The other 18 patients (42.9%) had PD. Serum DNA was extracted in all 42 samples at a median concentration of 62.0 ng ml^{-1} (range, 0–342.8). The concentrations in 10 samples were below the minimum concentration detectable.

Table 1 Patient characteristics and *EGFR* mutation status

	(*n*)
No. of patients	42
Age (years)	
Median	58
Range	40–81
Gender	
Male	28 (66.7%)
Female	14 (33.3%)
Smoking habit	
Current	20 (47.6%)
Former	8 (19.1%)
Never	14 (33.3%)
Histology	
Adenocarcinoma	31 (73.8%)
Squamous cell carcinoma	7 (16.7%)
Large-cell carcinoma	4 (9.5%)
Response to gefitinib	
Partial response	10 (23.8%)
Stable disease	14 (33.3%)
Progressive disease	18 (42.9%)

EGFR mutation status detected

Direct sequencing of PCR products from tumour tissues of all patients allowed their mutation status to be determined. Both direct sequencing and Scorpion ARMS allowed mutation status to be determined in the serum samples of all patients. As summarised in Table 2, mutations were identified in 9 (21.4%) of the 42 patients. Mutations in eight patients were detected in tumour samples and seven in serum samples. Five mutations were deletion mutations located in exon 19 (E746_A750del in four and L747_T751del in one). Four mutations were substitution mutations located in exon 21 (L858R), and one was a substitution mutation located in exon 18 (V689L). One patient had double substitution mutations (V689L and L858R). The E746_A750 deletion and L858R substitution mutation were the most common (8 out of 9, 88.9%), and both are well-known hot spot mutations described previously (Kosaka *et al*, 2004; Han *et al*, 2005). There were no T790M mutations identified by direct sequencing on tumour samples or serum samples. Of the nine patients with mutations, six (66.7%) were never-smokers, and five (55.6%) were female patients. Almost all of the patients with mutations had adenocarcinoma (8 out of 9, 88.9%).

Sensitivity and specificity of detection in serum DNA

In six of the patients, the same *EGFR* mutation was detected in both the tumour sample and the serum sample. There were no *EGFR* mutations detected in either the tumour sample or serum sample from 33 of the patients. *EGFR* mutation status was consistent in 39 (92.9%) of the 42 of the pairs (Table 3). In two patients the tumour samples was positive for an *EGFR* mutation and the serum sample was negative. The concentrations of serum DNA in the two patients were below the minimum level of detection by spectrophotometry. In one patient, the serum sample was positive for an *EGFR* mutation and the tumour sample was negative. The tumour sample that contained no mutations from the patient whose serum was positive for a mutation was collected by transbronchial lung biopsy.

Correlation between *EGFR* mutation status and patient characteristics

Detection of *EGFR* mutations occurred significantly more frequently in the serum DNA from the never-smokers (never-smokers 5 out of 14 (35.7%); current/former smokers 2 out of 28 (7.1%); $P=0.031$) (Table 4). Mutations were more frequently detected in the DNA from tumour samples of never-smokers than of current/former smokers (never-smokers 5 out of 14 (35.7%); current/former smokers 3 out of 28 (10.7%); $P=0.092$), but the difference was not statistically significant. Mutations were detected more frequently in the samples from females (tumour: females 5 out of 14 (35.7%), males 3 out of 28 (10.7%); serum: females 3 out

Table 2 Patients with *EGFR* mutation

Age	Gender	Histology	Stage	Smoking	Response	EGFR mutation status: Tumour tissue	EGFR mutation status: Serum
44	M	Ad	Re	Never	PR	E746_A750del	E746_A750del
79	M	Ad	IV	Former	PR	L858R	L858R
53	M	Ad	IV	Never	PR		V689L, L858R*
59	M	La	IV	Current	PD	E746_A750del	E746_A750del
63	F	Ad	IIIB	Never	PR	L858R	
62	F	Ad	IV	Never	PR	E746_A750del	E746_A750del
56	F	Ad	IV	Never	PR	E746_A750del	E746_A750del
57	F	Ad	IIIB	Former	SD	E746_T751del	
62	F	Ad	IV	Never	PR	L858R	L858R

Ad = adenocarcinoma; del = deletion; EGFR = epidermal growth factor receptor; F = female; La = large-cell carcinoma; M = male; PD = progressive disease; PR = partial response; Re = recurrence after surgery; SD = stable disease. The numbering of the mutation sites was based on NP_005219.2 (amino acid). *L858R was detected both by Scorpion ARMS and direct sequencing. V689L was detected by direct sequencing. All samples detected in serum DNA but the samples (*) were detected by Scorpion ARMS alone.

Table 3 Sensitivity for detection of *EGFR* mutations in serum samples

		Serum: +	Serum: −
Tumour tissue	+	6	2
	−	1	33

EGFR = epidermal growth factor receptor; + = mutation positive; − = mutation negative.

Table 4 Frequency of *EGFR* mutations

	Tumour tissue: +	Tumour tissue: −		Serum: +	Serum: −	
(A) Gender and EGFR mutation status						
Female	5	9		3	11	
Male	3	25	$P=0.092$	4	24	$P=0.669$
(B) Histology and EGFR mutation status						
Ad	7	24		6	25	
Non-Ad	1	10	$P=0.657$	1	10	$P=0.654$
(C) Smoking habit and EGFR mutation status						
Never	5	9		5	9	
Current/former	3	25	$P=0.092$	2	26	$P=0.031$
(D) Response to gefitinib						
PR	6	4		6	4	
SD/PD	2	30	$P<0.001$	1	31	$P<0.001$

Ad = adenocarcinoma; EGFR = epidermal growth factor receptor; PD = progressive disease; PR = partial response; SD = stable disease; + = mutation positive; − = mutation negative. *P*-value: Fisher's exact test.

of 14 (27.2%), males 4 out of 28 (14.3%)) and from patients with adenocarcinoma (tumour: adenocarcinoma 7 out of 31 (22.6%), non-adenocarcinoma 1 out of 11 (9.1%); serum: adenocarcinoma 6 out of 31 (19.4%), non-adenocarcinoma 1 out of 11 (9.1%)), but the differences were not statistically significant. There were no statistically significant differences in demographic characteristics between the patients with *EGFR* deletion mutations and patients with *EGFR* substitution mutations (data not shown).

Correlation between *EGFR* mutation status and response to gefitinib

EGFR mutations were detected significantly more frequently in responders to gefitinib. Seven of the nine patients with mutations had a PR to gefitinib. Comparison between *EGFR* mutation status and response to gefitinib showed that *EGFR* mutation was more frequent in patients with a PR than in patients with SD/PD (Table 4D).

EGFR mutations are associated with increased survival

The median PFS and OS of the patients treated with gefitinib was 60 days (95% CI, 52–68) and 228 days (95% CI, 150–306), respectively. Patients with *EGFR* mutations in both tumour samples and serum samples had a significantly longer median PFS than the patients without *EGFR* mutations (194 *vs* 55 days, $P=0.016$, in tumour samples; 174 *vs* 58 days, $P=0.044$, in serum samples; Figure 1A). The patients with *EGFR* mutations had a longer median OS than the patients without *EGFR* mutations, but the difference was not statistically significant (716 *vs* 193 days, $P=0.070$, in tumour samples; 387 *vs* 228 days, $P=0.489$, in serum samples; Figure 1B). These results suggest that the patients who were serum *EGFR*-mutation-positive had better outcomes of gefitinib therapy in terms of PFS, OS, and response, than patients who were *EGFR*-mutation-negative. In addition smoking status (never-smoker *vs* former/current smoker) was found to be an independent predictor of longer PFS ($P=0.002$) and longer OS ($P=0.035$). Progression-free survival and OS were longer in female patients and patients with adenocarcinoma than in male patients and non-adenocarcinoma patients, respectively, but the differences were not statistically significant.

DISCUSSION

We previously reported detecting *EGFR* mutations in serum DNA by Scorpion ARMS method and that mutation status is useful for predicting response to gefitinib (Kimura *et al*, 2006). The two major findings in the present study provide additional support for the use of serum DNA as an alternative to tumour samples for detection of *EGFR* mutations in patients with advanced NSCLC. First, these results demonstrate that *EGFR* mutation status in serum DNA was the same as in tumour samples in almost every patient. In addition, mutation status in serum DNA predicted for a significantly greater response and time to progression with gefitinib, as well as showing a trend towards increased OS in patients treated with gefitinib. The results confirm the clinical

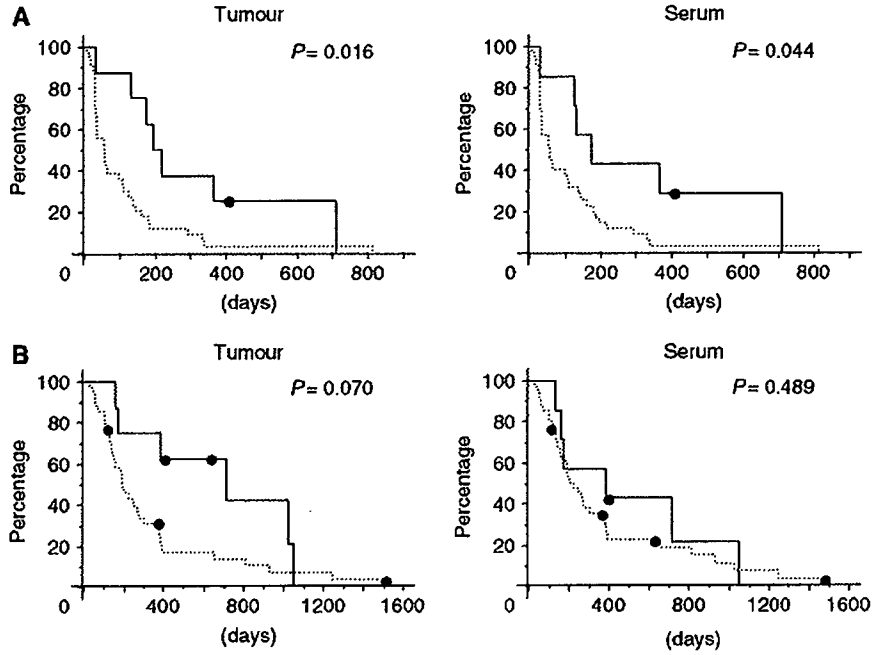

Figure 1 Kaplan–Meier probability of progression-free survival (**A**) and overall survival (**B**) with respect to the EGFR mutation status of NSCLC. *P*-values were calculated by the log-rank test.

reliability of *EGFR* mutation detection in serum DNA as a predictive marker of response to gefitinib.

The sites of the *EGFR* mutations detected in this study are identical to those reported in previous studies (Kosaka *et al*, 2004; Pao *et al*, 2004). The majority mutations were in-frame deletions in exon 19 and the missense mutation L858R in exon 21. The comparison between mutation status and clinical manifestations in this study confirmed the finding in previous studies that *EGFR* mutations are frequently present in small subgroups of NSCLC patients, including females, never-smokers, and patients with adenocarcinoma histology, although these findings were not statistically significant.

EGFR mutations were detected in only 1.0 ml serum samples. The amount of DNA extracted was minute, and its concentration in roughly one-third of patients was below the minimum concentration detectable by spectrophotometry. Moreover, lung cancers are very heterogeneous, and patients' serum also contains DNA derived from normal cells. Direct sequencing seems unable to provide satisfactory results for detection of *EGFR* mutations in samples containing a mixture of mutated and wild-type DNA. Although direct sequencing has generally been used to detect *EGFR* mutations, detection by direct sequencing requires at least 30% of the DNA in the sample to be mutated (Bosari *et al*, 1995; Fan *et al*, 2001). Small amounts and low percentages of mutated DNA in serum can be missed by direct sequencings. When serum is used as the material for detection of *EGFR* mutations, patients with *EGFR* mutations may be diagnosed as having wild-type *EGFR* because of the two limitations described above. In this study, the mutation was detected by direct sequencing in only one patient. The mutation status detected by Scorpion ARMS in serum samples was nearly identical to that in tumour samples. The concentrations of serum DNA in two of seven patients with *EGFR* mutations in serum samples were below the minimum concentration detectable. The high-sensitive method, Scorpion ARMS, completely resolved the problem.

The mutation status in the pairs of samples from three patients (3 out of 42, 7.1%) did not match. The results in the serum DNA of two patients were mutation-negative, whereas mutations were detected in actual tumour samples. The amount of tumour-specific DNA may have been below the threshold of detection with the Scorpion ARMS Kit in the patient with L858R. Little tumour-specific DNA may be circulating in patients, and the quality of the DNA is also a determinant of successful detection. Prolonged storage of serum samples has been reported to result in a decrease in the amount of DNA extracted (Sozzi *et al*, 2005). The other patient had an E746_T751del, and the mutation was not detected with the Scorpion ARMS in the patients. Although we have showed the usefulness of Scorpion ARMS for detection of *EGFR* mutation in serum samples (Kimura *et al*, 2006), Scorpion ARMS is only able to detect mutations targeted by the Scorpion primers designed in advance and in this study was capable of detecting the specific mutation of E746_A750del in exon 19 and L858R in exon 21. E747_P753del insS and L747_T751del are minor variations of deletional mutations in exon 19 and were not detected by this method in a preliminary experiment (data not shown). We do not think that E746_T751del can be detected with Scorpion ARMS. Mutation status in serum DNA was positive (V689L and L858R) in one patient in whom no mutations were detected in actual tumour samples. V689L and L858R are somatic mutations. We concluded that the direct sequencing of DNA from the tumour sample yielded the wrong result. Low rate of tumour-derived DNA in total DNA or impure DNA extracted from tumour samples may have prevented a detection of the mutation by direct sequencing.

On the basis of the results of this study, we conclude that it is feasible to use serum DNA to detect *EGFR* mutation status and evaluate its potential as a predictor of response to EGFR-TKI. The serum assay to detect *EGFR* mutations circumvents the need for tumour tissue and merits further validation of the use of serum DNA to detect *EGFR* mutations as a predictor of response to, and survival on gefitinib in prospective studies.

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Evaluation of the Recommended Dose and Efficacy of Amrubicin as Second- and Third-Line Chemotherapy for Small Cell Lung Cancer

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Introduction: This study was conducted to evaluate the recommended dose and activity of amrubicin (AMR) as second- or third-line chemotherapy for small-cell lung cancer (SCLC).

Methods: Small-cell lung cancer patients with measurable disease who had previously been treated with at least one platinum-based chemotherapy regimen and had an Eastern Cooperative Oncology Group performance status of 0–2 were eligible. Two groups of patients were selected: (1) a group to be treated with second-line chemotherapy and (2) a group to be treated with third-line chemotherapy. AMR was administered to both groups as a 5-minute daily intravenous injection at a dose of 40 or 35 mg/m^2 for three consecutive days every 3 weeks.

Results: Between March 2003 and June 2006, 27 patients (second-line, 40 mg/m^2: 13 patients; third-line, 40 mg/m^2: seven patients; and 35 mg/m^2: seven patients) were enrolled. Although the 40-mg/m^2 dose of AMR was feasible (one of 13 patients developed febrile neutropenia and four of 13 patients had grade 4 neutropenia) and effective (six of 13 patients had a partial response) in the second-line group, it produced unacceptable toxicity in a third-line setting (three of seven patients with grade 3 nonhematologic toxicities [febrile neutropenia in two patients and fatigue in one patient] and four of seven patients with grade 4 neutropenia). The 35-mg/m^2 dose of AMR had acceptable toxicity in the third-line group (one of seven patients with febrile neutropenia and one of seven had grade 4 neutropenia) and moderate efficacy (one of seven patients had a partial response and two of seven had stable disease).

Conclusions: AMR exhibits significant activity as second-line or third-line chemotherapy for small-cell lung cancer. The recommended dose is 40 mg/m^2 in a second-line setting and 35 mg/m^2 in a third-line setting.

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Approximately 15% of lung cancer patients have small-cell lung cancer (SCLC). Unlike other types of lung cancer, SCLC is one of the most chemosensitive solid tumors.[1] However, after remarkably successful induction therapy, most patients have a relapse within 2 years as a result of the emergence of drug-resistant tumor cells. Thus, long-term survival is extremely uncommon, and less than 25% of patients with limited-stage disease and less than 2% of patients with extensive-stage disease remain alive at 5 years.[2–4] The results of second-line chemotherapy against SCLC are quite disappointing, with relatively low response rates, brief remissions, and a short survival time. A new effective agent is needed to achieve better treatment results in patients with recurring or refractory SCLC.

Amrubicin hydrochloride is a totally synthetic 9-aminoanthracycline that is metabolically activated to amrubicinol by a liver enzyme. Amrubicin and amrubicinol inhibit DNA topoisomerase II and exert a cytotoxic effect by stabilizing a topoisomerase II–mediated cleavable complex. They are approximately one tenth weaker than doxorubicin as a DNA intercalator.[5,6] The catatonic activity of amrubicinol in vitro is 18 to 220 times more potent than that of its parent compound, amrubicin.[7]

Amrubicin has been reported to have shown more potent antitumor activity than the representative anthracycline doxorubicin in several human tumor xenografts implanted in nude mice and to have produced almost no cardiotoxicity.[8,9] Amrubicin at 45 mg/m^2 on days 1 to 3 every 3 weeks has been shown to be active against previously untreated SCLC, with an overall response rate of 78.8% and a median survival time (MST) of 11.0 months.[10] Onoda et al.[11] found that amrubicin 40 mg/m^2 had significant activity and acceptable toxicity in previously treated patients. However, Kato et al.[12] reported finding that amrubicin at 45 mg/m^2 not only had promising activity but severe and unacceptable toxicity in patients, similar to those in the Onoda et al. study. Most patients enrolled in both studies received amrubicin as second-line treatment.

Accordingly, the results of the previous studies are of value in considering amrubicin as a key agent for the treatment of SCLC, not only untreated cases but in previously treated cases. However, the recommended dose for previ-

ously treated patients has not been determined, especially in third-line or greater settings.

The purpose of this study was to evaluate the toxicity and efficacy of amrubicin as second- and third-line chemotherapy in SCLC patients in a clinical setting to determine the recommended dose.

PATIENTS AND METHODS

Patient Selection

The subjects of this study were 27 patients with previously treated SCLC between March 2003 and June 2006 at Shizuoka Cancer Center. The recruitment criteria were (1) history of at least one platinum-based chemotherapy regimen and confirmation of refractory or recurrent SCLC based on the results of the following examinations: chest radiograph, computed tomography of the chest and abdomen, and other procedures as indicated, including magnetic resonance imaging of the head and positron emission tomography or combined positron emission tomography/computed tomography; (2) age 75 years or younger; (3) performance status of 2 or less according to the Eastern Cooperative Oncology Group scale; (4) adequate bone marrow, hepatic, and renal function; (5) no other serious disease; (6) written informed consent.

Treatment Methods

The treatment schedule comprised a 5-minute intravenous infusion of amrubicin in 50 ml normal saline on days 1 to 3 every 3 weeks. Patients receiving second-line chemotherapy were treated with a dose of 40 mg/m^2. The first seven consecutive patients in the third-line group were treated with a dose of 40 mg/m^2, and the next seven patients were treated with a dose of 35 mg/m^2. Before the start of treatment, the patient had to have an absolute neutrophil count of 1500/mm^3 or more, a platelet count of 100,000/mm^3 or more, aspartate aminotransferase and alanine aminotransferase values less than three times the maximum value in the normal range, and total bilirubin and creatinine values less than 1.5 times the maximum value in the normal range. Treatment with granulocyte colony-stimulating factor was permitted as a therapeutic intervention but was not mandatory as a prophylactic agent against neutropenia as hematologic toxicity. Subsequent doses were modified based on hematologic and nonhematologic toxicities at the discretion of the physician in charge. Complete blood count and biochemistry examinations were repeated at least once per week after the initial evaluation.

Evaluation of Response and Toxicity

Adverse events were recorded and graded using the National Cancer Institute Common Toxicity Criteria, Version 3.0 grading system. Tumor response was classified in accordance with the Response Evaluation Criteria in Solid Tumors. Patients were evaluated to determine the stage of their disease before treatment and when their disease progressed or recurred by taking a complete medical history and performing a physical examination, chest radiography, computed tomography of the chest and abdomen, and other staging procedures, such as magnetic resonance imaging of the head, and positron emission tomography.

Limited disease was defined as disease confined to one hemithorax, including bilateral mediastinal and bilateral supraclavicular nodes, and extensive disease (ED) was defined as any involvement beyond these confines. Primary refractory disease was defined as recurrence during the first-line chemotherapy regimen or less than 8 weeks after completing the initial chemotherapy regimen, and sensitive disease was defined as recurrence more than 8 weeks after completion of the first-line chemotherapy.

Definition of Recommended Dose

The recommended dose of amrubicin was defined as the dose at which severe toxicity occurred in less than 33% of the patients treated. At least six patients were treated at each dose level. Severe toxicity was defined as grade 4 hematologic toxicity and grade 3 or higher nonhematologic toxicity including febrile neutropenia.

Statistical Methods

Kaplan-Meier plots were prepared for overall survival, and median values were calculated. Overall survival was measured from the first day of amrubicin treatment to the day of death or the day last seen alive (cutoff).

RESULTS

Patient Characteristics

Between March 2003 and June 2006, 27 patients with recurring or refractory disease were enrolled in this study. The characteristics of the patients are listed in Table 1. Four patients were women and 23 were men, and the patients' median age was 64 years (range, 53–74 years). At the start of the treatment one patient had limited disease and 26 patients had ED. All 27 patients had been treated with some form of chemotherapeutic regimen: 13 had received one previous regimen, 14 had received two previous regimens. All patients had been previously treated with some form of cisplatin- (23 patients) or carboplatin-based combination chemotherapy; 19 patients had received an irinotecan-containing regimen and one patient had received a topotecan-containing regimen.

TABLE 1. Patient Characteristics

Characteristic	
Age, yr, median (range)	64 (53–74)
Gender: male/female	23/4
PS: 0/1/2	2/21/4
Stage: limited disease/extended disease	1/26
No. of previous chemotherapy regimens: 1/2	13/14
Amrubicin dose, days 1–3	
Second-line: 40 mg/m^2	13
Third-line: 40/35 mg/m^2	7/7
Refractory/sensitive	8/19

PS, performance status.

TABLE 2. Toxicity by Dose

		ANC		Hb		PLT		FN	Other
	Pts	G3	G4	G3	G4	G4	G3	G3	G3
Second-line: 40 mg/m^2	13	2	4	0	0	2	0	1	1 Dyspnea
Third-line: 40 mg/m^2	7	0	4	1	0	2	1	2	1 Fatigue anorexia
Third-line: 35 mg/m^2	7	5	1	1	0	3	0	1	0

Pts, patients; ANC, absolute neutrophil count; Hb, hemoglobin; PLT, ●●●; FN, febrile neutropenia; G, grade.

Toxicity

The toxicity of the regimen is summarized in Table 2. Table 2 shows the worst toxicity level in each patient. Grade 4 neutropenia was observed in four (31%) of the 13 patients receiving second-line chemotherapy with 40 mg/m^2 of amrubicin, and four cases of grade 4 neutropenia (57%) and one case of grade 3 (14%) were observed among the seven patients receiving third-line chemotherapy. However, only one of the seven third-line patients who received the reduced dose of 35 mg/m^2 developed grade 4 neutropenia (14%). Febrile neutropenia was observed in one patient (8%) in the second-line group who received 40 mg/m^2, two patients (28%) in the third-line group who received 40 mg/m^2, and one patient (14%) in the third-line group who received 35 mg/m^2. All other hematologic toxicities were mild. Two cases (28%) of grade 3 nonhematologic toxicities other than febrile neutropenia were observed one in the second-line group and the other in third-line group.

Accordingly, the recommended dose of amrubicin for second-line treatment and third-line treatment was concluded to be 40 mg/m^2/day and 35 mg/m^2/day, respectively.

Response and Survival

No complete responses and eight partial responses were observed among the 27 patients (29%). A comparatively high response rate was achieved in the second-line chemotherapy group, with a response rate of 46% (six of 13 patients) in the group who received the 40-mg/m^2 dose. The patients in the third-line group who received the 40-mg/m^2 dose and the 35-mg/m^2 dose had a similar response rate: 14% (one of seven patients). The sensitive cases and refractory recurrence cases had response rates of 42% (3/7) and 25% (5/20), respectively. The overall median survival time (MST) and 1-year survival rate were 9.2 months and 33.3%, respectively (Figures 1 and 2).

DISCUSSION

The outlook for SCLC patients who receive second-line chemotherapy has been poor, and few single agents have been capable of producing a high response rate among patients with early recurrence or disease progression during treatment. The new agents that have been most extensively evaluated in SCLC are the topoisomerase I inhibitors irinotecan and topotecan. A recent randomized phase III trial demonstrated that single-agent topotecan was at least as effective as the three-drug combination of cyclophosphamide, doxorubicin, and vincristine in patients with disease progression at least 60 days after initial therapy. Topotecan yielded a response rate of 24.3% versus 18.3% for cyclophosphamide, doxorubicin, and vincristine, with an MST of 25.0 versus 24.7 weeks and improved symptom control.[13] Two studies of irinotecan in patients with refractory SCLC have been reported in Japan, and the response rate in both studies was high: 50% in 16 patients and 47% in 15 patients.[14,15] Irinotecan and topotecan have been recognized as key drugs in the second-line treatment of SCLC. A recent phase III study that compared irinotecan plus cisplatin with etoposide and cisplatin in patients with ED-SCLC revealed a superior median survival rate and a superior 2-year survival rate for the irinotecan plus cisplatin combination.[16] As a result, irinotecan plus cisplatin was established as one of the standard first-line regimens for SCLC in Japan. Thus, it has been necessary to search for effective drugs other than the topoisomerase I inhibitors for previously treated SCLC.

A response rate of 79% has been reported for amrubicin at a dose of 45 mg/m^2 on days 1 to 3 in chemotherapy-naive ED-SCLC patients in an intent-to-treat analysis.[10] Because of its very high activity as a single agent in untreated ED-SCLC patients, amrubicin has since been approved for SCLC in Japan in April 2002. Amrubicin has mainly been used for previously treated SCLC in clinical practice because of the higher response rate of untreated SCLC and an antitumor mechanism that differs from that of platinum and topoisomerase I inhibitors.

However, the recommended dose of amrubicin for previously treated SCLC was unknown.

The dose approved by the Japanese Ministry of Labor, Health, and Welfare is 45 mg/m^2 on days 1 to 3. Kato et al.[12] conducted a phase II study of amrubicin 45 mg/m^2 in previ-

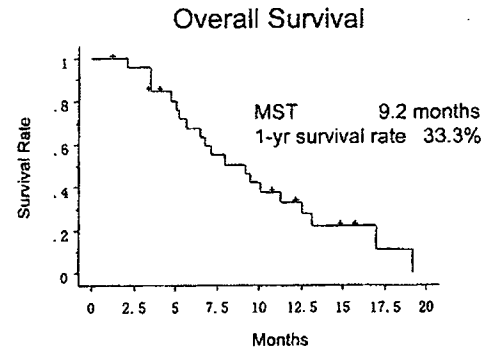

FIGURE 1. Kaplan-Meier plot of overall survival in all patients.

TABLE 3. Response by Dose

	No. of Patients	PR	SD	PD	RR (%)
Overall	27	8	8	11	29
Second-line: 40 mg/m^2	13	6	3	4	46
Third-line: 40 mg/m^2	7	1	3	3	14
Third-line: 35 mg/m^2	7	1	2	4	14
Sensitive	7	3	3	1	42
Refractory	20	5	5	10	25

PR, partial response; SD, stable disease; PD, progressive disease; RR, relative risk.

ously treated SCLC patients, mostly second-line chemotherapy patients, and reported severe hematologic toxicity and a high incident of febrile neutropenia.

The incidence of severe amrubicin toxicity at the 40-mg/m^2 dose as second-line chemotherapy was 31% (four of 13: grade 4 neutropenia in two, grade 4 neutropenia and febrile neutropenia in one, grade 4 neutropenia and grade 3 dyspnea in one), and this dose was acceptable. These results are similar to those reported in another study.[11] However, amrubicin 40 mg/m^2 induced severe toxicity in 57% of the third-line chemotherapy patients (4/7: grade 4 neutropenia in 2, grade 4 neutropenia and febrile neutropenia in 1, grade 4 neutropenia, febrile neutropenia and grade 3 fatigue in one). However, the rate of severe toxicity (14%: one of seven patients with grade 4 neutropenia and febrile neutropenia) at the lower dose (35 mg/m^2) of amrubicin was acceptable (Table 3).

The MST and 1-year survival rate in this study were 9.2 months and 33.3%, respectively. The results were much better than in a recent phase II study that evaluated the activity of topotecan against recurrent SCLC.[17] In addition, the results of our study were comparable with those of a phase II study that evaluated the activity of amrubicin against refractory or recurring SCLC.[11] In conclusion, amrubicin is an active agent for previously treated SCLC, and the recommended doses in second-line and third-line settings are 40 mg/m^2 on days 1 to 3 and 35 mg/m^2 on days 1 to 3, respectively. Because of the greater activity of the single-agent amrubicin, further studies on amrubicin either as a single agent or in combination with other agents, such as cytotoxic or target-based agents, are warranted in previously treated SCLC patients.

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Functional Analysis of Human *MLH1* Variants Using Yeast and *In vitro* Mismatch Repair Assays

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Abstract

The functional characterization of nonsynonymous single nucleotide polymorphisms in human mismatch repair (MMR) genes has been critical to evaluate their pathogenicity for hereditary nonpolyposis colorectal cancer. We previously established an assay for detecting loss-of-function mutations in the *MLH1* gene using a dominant mutator effect of human MLH1 expressed in *Saccharomyces cerevisiae*. The purpose of this study is to extend the functional analyses of nonsynonymous single nucleotide polymorphisms in the *MLH1* gene both in quality and in quantity, and integrate the results to evaluate the variants for pathogenic significance. The 101 MLH1 variants, which covered most of the reported *MLH1* nonsynonymous single nucleotide polymorphisms and consisted of one 3-bp deletion, 1 nonsense and 99 missense variants, were examined for the dominant mutator effect by three yeast assays and for the ability of the variant to repair a heteroduplex DNA with mismatch bases by *in vitro* MMR assay. There was diversity in the dominant mutator effects and the *in vitro* MMR activities among the variants. The majority of functionally inactive variants were located around the putative ATP-binding pocket of the NH_2-terminal domain or the whole region of the COOH-terminal domain. Integrated functional evaluations contribute to a better prediction of the cancer risk in individuals or families carrying *MLH1* variants and provide insights into the function-structure relationships in *MLH1*. [Cancer Res 2007;67(10):4595–604]

Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC) is one of the most common familial cancer syndromes caused by mainly germ-line mutations in DNA mismatch repair (MMR) genes, such as *MLH1*, *MSH2*, *MSH6*, and *PMS2*. MMR contributes to genome integrity by correcting replication errors, particularly mismatch base pairs or slippages in simple repeat sequences. The MMR system is well conserved from *Escherichia coli* to mammals, and the *E. coli* MMR system, where MutS, MutL, and MutH complexes function, has been well analyzed. In mammalian cells, heterodimers of MutS homologues (MSH2-MSH6 and MSH2-MSH3) recognize replication errors, and the heterodimer of the MutL homologue (MLH1-PMS2) interacts with MutS homologues and recruits further repair proteins.

In the two MMR genes, *MLH1* and *MSH2*, which account for the majority of HNPCC kindred mutations, 31.6% of the *MLH1* mutations and 19.4% of the *MSH2* mutations are missense (nonsynonymous single nucleotide polymorphisms) according to the InSiGHT database.[4] Pathogenic significances of nonsynonymous single nucleotide polymorphisms are not easily evaluated without a functional assay. Generally, this makes genetic diagnosis more difficult, especially when phenotype-genotype segregation analysis is limited because of an ethical issue, the small number of family members, or other reasons. Therefore, functional analysis has been needed to interpret the pathogenicity of *MLH1* variants in genetic diagnoses of HNPCC.

Several groups, including ours, have attempted to resolve this issue by analyzing the functional significance of MLH1 variants by various methods (1–8). We found a dominant mutator effect (DME) of wild-type MLH1 on interference in the yeast MMR system and evaluated the pathogenicity of 20 MLH1 missense variants using this effect for a yeast-based functional assay (1). One of the other strategies was focusing on the well-defined functions of MLH1, ATPase activity in the NH_2 terminus, and the binding abilities with PMS2 in the COOH terminus (2, 4, 7). Another strategy was based on the assessment of total MMR activity such as *in vitro* MMR assay, monitoring the MMR rate of cell extracts for heteroduplex DNA-containing mismatch bases (4, 5), or yeast system measuring the replication error rate resulting from the expressed yeast-human hybrid proteins or equivalent yeast variants (3). An alternative approach was to analyze the effects of an MLH1 variant for the expression of MLH1 mRNA and protein and the proliferation rate of cells when an MLH1 variant was introduced into the cells (6). Recently, MLH1 variants were characterized for the multiple functional properties of wild-type MLH1, including protein expression, subcellular localization, MLH1-PMS2 interaction, and MMR efficiency (8). However, it still seems to be important to establish the database on the functional effects of as many variants as possible by various methods and integrate the accumulated data for better understanding of *MLH1* variants.

Beyond functional analyses, crystal structure analyses of *E. coli* MutL were used to predict the structural alterations of MLH1 variants (9, 10). Besides the NH_2 terminus, the crystal structure of the COOH terminus of *E. coli* MutL was identified recently (11). Consideration of the protein structure permits more comprehensive analysis of MLH1 and is thought to provide useful information for interpreting the pathogenesis of *MLH1* variants in genetic diagnoses.

In this study, we examined 101 *MLH1* variants in yeast assays and an *in vitro* MMR assay, to compare these two assays and to develop the functional database for a large number of variations.

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[4] http://www.insight-group.org/

Table 1. Summary of *MLH1* variants

Variant	Nucleotide change	Dominant mutator effect			*In vitro* MMR activity (%)	Relative MLH1 expression (%)	SIFT score	AC+/All families*	Reference
		LacZ	GFP	ADE2					
Missense									
E23D†	69A>T	+	+	+	25.2	>75	0.00	NI	I
I25F†	73A>T	+	+	+	36.4	>75	0.00	NI‡	I
I25T†	74T>C	+	+	+	67.2	25–75	0.00	1/1	I
P28L	83C>T	–	–	+	9.2	>75	0.00	1/3	I, S, 8
A29S	85G>T	+	+	+	81.7	>75	0.26	1/1	I, 8
M35R	104T>G	–	–	–	23.4	25–75	0.00	1/1	I, S
N38D†	112A>G	–	+	+	0.0	25–75	0.00	0/1	15
S44A†	130T>G	+	+	+	65.9	>75	1.00	P	16
S44F	131C>T	–	–	–	23.1	<25	0.00	1/2	I, S
G54E†	161G>A	–	–	+	47.9	>75	0.00	So	S
N64S†	191A>G	–	–	+	36.6	>75	0.02	1/1	I, S
G67R	199G>A	–	–	–	5.9	<25	0.00	2/4	I, S, 16, 17
G67W†	199G>T	–	–	–	7.3	25–75	0.00	1/1	S
I68V†	202A>G	+	+	+	79.8	>75	0.02	P	16
I68N	203T>A	–	–	–	20.8	25–75	0.00	1/1	I, S
R69K	206G>A	+	+	+	75.0	>75	0.91	0/1	S
C77Y	230G>A	–	+	+	11.2	25–75	0.15	0/1	I, S
F80V	238T>G	–	–	+	23.7	>75	0.07	1/1	S, 8
T82I†	245C>T	–	+	+	27.2	>75	0.00	1/1	18
K84E	250A>G	–	+	+	22.5	>75	0.00	0/1	I, S
S93G	277A>G	+	+	+	80.3	>75	0.16	1/2	I, S, 8
R100P†	299G>C	–	+	+	0.0	<25	0.00	NI	I
E102K	304G>A	+	+	+	44.4	>75	0.00	1/1	I
E102D†	306G>T	+	+	+	56.1	>75	0.00	NI	I
I107R	320T>G	–	–	–	39.5	25–75	0.00	5/7	I, S, 8, 19
A111V†	332C>T	–	–	–	25.5	25–75	0.01	2/2	S, 20
T117M	350C>T	–	–	–	34.8	<25	0.00	12/14	I, S, 15, 21, 22
T117R	350C>G	–	–	–	25.2	25–75	0.00	1/1	I, S
A128P	382G>C	–	–	–	24.4	25–75	0.01	1/1	I, S
D132H	394G>C	+	+	+	63.0	25–75	0.02	0/1	S, 23
A160V†	479C>T	+	+	+	80.9	>75	0.01	NI	I
R182G†	544A>G	+	+	+	74.3	25–75	0.00	0/1	I, S
V185G	554T>G	–	–	–	8.5	25–75	0.00	2/3	I, S, 8, 15
S193P†	577T>C	–	–	–	0.0	>75	0.10	1/1	I, S, 24
E199Q†	595G>C	+	+	+	67.7	>75	0.15	0/1	I
V213M	637G>A	+	+	+	85.0	>75	0.11	P	I, S
R217C	649C>T	+	–	+	64.8	>75	0.11	1/2	I, S
I219L	655A>C	+	+	+	85.2	>75	0.52	P	I
I219V	655A>G	+	+	+	60.7	25–75	0.46	P	I, S
R226L†	677G>T	–	–	+	39.2	25–75	0.04	2/2	I, S
G244D	731G>A	–	–	–	19.4	>75	0.00	1/1	I, S
G244V†	731G>T	–	–	–	18.8	>75	0.00	So	S, 25
H264R†	791A>G	+	+	+	68.7	>75	0.07	1/1	I
R265C	793C>T	–	+	+	55.0	25–75	0.00	3/4	I
R265H	794G>A	+	+	+	61.1	>75	0.00	NI‡	I, S
E268G†	803A>G	+	–	+	78.9	25–75	0.05	NI‡	I, S
L272V†	814T>G	+	+	+	90.2	>75	0.10	NI	I
A281V†	842C>T	+	+	+	88.6	25–75	0.27	NI	I
K286Q†	856A>C	+	–	+	78.6	25–75	0.39	1/1	26
S295G†	883A>G	–	+	+	75.5	25–75	0.46	1/1	I, S
D304V†	911A>T	–	–	+	0.0	>75	0.00	1/1	27
V326A	977T>C	+	+	+	26.9	25–75	0.00	3/5	I, S, 21, 28
H329P	986A>C	–	–	+	25.7	>75	0.21	2/2	I, S, 8
V384D†	1151T>A	+	+	+	64.8	>75	0.07	P	I, S
R389Q†	1166G>A	+	+	+	83.6	>75	0.31	NI	I

(*Continued on the following page*)

Table 1. Summary of *MLH1* variants (Cont'd)

Variant	Nucleotide change	Dominant mutator effect			*In vitro* MMR activity (%)	Relative MLH1 expression (%)	SIFT score	AC+/All families*	Reference
		LacZ	GFP	ADE2					
S406N	1217G>A	+	+	+	73.5	>75	0.62	P	I, S
T413I†	1238C>T	+	+	+	84.0	>75	0.34	NI	Unpublished
S420C†	1259C>G	+	+	+	70.7	>75	0.13	NI	23
A441T†	1321G>A	+	+	+	71.1	25–75	0.56	1/3	I, S
R474Q†	1421G>A	+	+	+	81.1	>75	0.60	0/1	I
D485E†	1455T>A	+	+	+	48.3	25–75	0.95	1/1	17
A492T	1474G>A	—	+	+	65.3	>75	0.51	0/1	I, S
P496L†	1487C>T	+	+	+	65.7	>75	0.36	NI	Unpublished
V506A	1517T>C	—	+	+	67.6	25–75	0.04	1/1‡	I, S, 18
E523D†	1569G>T	+	+	+	76.6	>75	0.24	So	I, 29
Q542L	1625A>T	+	+	+	13.3	>75	0.00	1/1	I, S
L549P†	1646T>C	—	—	—	31.0	25–75	0.00	1/1	I, S
N551T†	1652A>C	—	—	—	78.9	25–75	0.00	1/1	I, S
I565F†	1693A>T	—	—	—	52.5	>75	0.08	NI	I, S
L574P	1721T>C	—	—	—	2.9	>75	0.02	1/1	I, S
E578G	1733A>G	—	+	+	51.2	25–75	0.12	0/2	I, S
L582V	1744C>G	+	+	+	65.6	>75	0.02	1/1	I, S
A586P†	1756G>C	—	—	—	28.0	25–75	0.31	1/1	I, S
L588P†	1763T>C	+	—	—	68.3	>75	0.10	1/1	S
P603R†	1808C>G	+	+	+	82.5	>75	0.32	0/1	S
L607H†	1820T>A	+	+	+	88.8	>75	0.04	2/2	S, 30
K618T	1853A>C	—	—	—	48.7	25–75	0.41	2/4‡	I, S, 28, 30
L622H†	1865T>A	—	—	—	69.2	25–75	0.00	1/1	S
P640T†	1918C>A	—	—	—	53.6	>75	0.00	NI	Unpublished
P648L	1943C>T	—	—	—	39.2	25–75	0.02	0/1	S
L653R†	1958T>G	—	—	—	12.9	25–75	0.00	0/1	I
P654L†	1961C>T	—	—	—	49.1	25–75	0.00	1/5	I, 8
I655V†	1963A>G	+	+	+	70.6	>75	0.49	NI	I
I655T†	1964T>C	+	+	+	73.8	>75	0.35	NI	25
R659P	1976G>C	—	—	—	24.9	<25	0.06	3/3	I, S
R659Q	1976G>A	—	+	+	79.7	25–75	0.18	1/1	8
T662P†	1984A>C	—	—	—	64.0	25–75	0.20	1/1	I, S
E663G†	1988A>G	+	+	+	69.7	>75	0.16	1/1	I
E663D†	1989G>T	—	+	+	68.5	>75	0.31	1/1	I
L676R†	2027T>G	—	—	—	39.8	25–75	0.00	NI	I
A681T	2041G>A	—	—	—	69.8	>75	0.00	3/4	I, S, 8
R687W†	2059C>T	—	—	—	57.2	25–75	0.02	1/1	S, 20
Q689R†	2066A>G	+	+	+	68.0	25–75	0.52	0/1	S
V716M	2146G>A	—	—	+	75.1	25–75	0.22	6/11‡	I, S, 15, 20, 21, 22
H718Y	2152C>T	—	+	+	84.5	25–75	0.00	P	I, S
L729V	2185C>G	+	+	+	80.3	>75	0.70	NI	S
K751R†	2252A>G	+	+	+	66.6	>75	0.59	1/2	S, 15
R755S†	2265G>C	+	+	+	7.9	>75	0.00	1/1	18
K618A	1852-3AA>GC	—	—	+	82.7	25–75	0.44	5/19‡	I, S, 8, 22
In-frame deletion									
K618del	1846–48del	—	—	—	38.9	<25		14/19	I, S, 8
Nonsense									
W714X	2141G>A	—	—	—	0.0	25–75		6/6	I, 17

NOTE: Data on functions in yeast and *in vitro* MMR assays and on MLH1 protein levels when transiently expressed in HCT116 cells are from this study. SIFT score was calculated by an online program, SIFT, that uses sequence homology to predict whether a substitution affects protein function. If the value is <0.05, the amino acid substitution is predicted to affect protein function. Information on families is from databases or references.

Abbreviations: AC, Amsterdam criteria; NI, not informative; So, found as somatic mutation in a patient with sporadic colorectal cancer; P, putative polymorphisms; I, the InSiGHT online database; S, the SWISS-PROT online database.

*The number in the left side is the number of families fulfilling the Amsterdam criteria; the number in the right side is the total number of families whose familial information was available from the databases or the various reports.

† The variant has never been evaluated for pathogenicity in functional assays.

‡ The variant was found also in a family or families carrying the other second mutation in *MLH1* or *MSH2* gene. The family or families was excluded in this table.

We also analyzed the distribution of loss-of-function variants on the secondary MLH1 structure or the crystal structure, to predict the pathogenicity of the *MLH1* variants, and to get the structural basis for defects of MLH1 function.

Materials and Methods

Yeast strains and plasmids. The *Saccharomyces cerevisiae* haploid strains used in this study were as follows: YPH499 from Stratagene, and yCA23 (leu2, his3, trp1, can1, pCYC1-28C::ADE2) and yCA25 [leu2, his3, trp1, can1, pCYC1-$(CA)_{15}$::ADE2]. yCA23 and yCA25 were made by integrating plasmids pCA2 and pCA3 into the URA3 locus of a haploid derivative of ASZ3 (12). pCA2 and pCA3 were derived from pLS210 (13) and contain out of frame C_{28} and $(CA)_{15}$ repeats at the amino terminus of ADE2 expressed by the CYC1 promoter. Constructs of the low-copy *MLH1* expression plasmid, pCI-ML10, the gap repair vectors, pCI-ML22 and pCI-ML24, and the reporter LacZ plasmid, pCIZ1, were described previously (1). The mutation-reporter green fluorescent protein (GFP) plasmid, pCGFP2μ(URA3), was constructed by inserting an enhanced GFP (EGFP) coding sequence with a poly C (C_{22}) tract instead of the *β-galactosidase* gene, including the poly-GT tract of pCIZ1. The wild-type *MLH1* human expression plasmid was constructed by inserting wild-type *MLH1* cDNA into the Bam*HI* site of a pCMV-Neo-Bam (14), and each variant *MLH1* expression plasmid is identical to the wild-type MLH1 expression plasmid but contains each mutagenized *MLH1* cDNA.

Site-directed mutagenesis. One hundred one *MLH1* variants consisted of 99 missense variants, one nonsense variant (W714X), and one 3-bp in-frame deletion (K618del), and were constructed by site-directed mutagenesis (Table 1; ref. 1). The information on the majority of *MLH1* variants were based on the InSiGHT and SWISS-PROT[5] databases, whereas others were based on various publications (15–30). Each of the *MLH1* sequences mutated specifically was confirmed by DNA sequencing. These *MLH1* variants included 90 germ-line variants found in HNPCC patients fulfilling the Amsterdam criteria (AC+) or individuals not fulfilling the Amsterdam criteria (AC−), three somatic variants found in individuals with sporadic cancer and eight major or putative polymorphisms.

Yeast-based assays. For the LacZ and GFP assays, competent yeast cells YPH499 were transformed with an *MLH1* expression plasmid and a reporter plasmid [pCIZ1 for LacZ assay, pCGFP2μ(URA3) for GFP assay], as described previously (1). In the LacZ assay, the MMR ability of each yeast transformant could be monitored by the appearance of a blue color on the Xgal plate after 5 days incubation at 37°C (1). In the GFP assay, the MMR ability could be monitored by the appearance of green-fluorescent colonies observed under fluorescent microscopy after 3 days incubation at 30°C. In the ADE2 assay, yCA23 or yCA25 cells were transformed with an *MLH1* expression plasmid, and the MMR ability could be monitored by the appearance of a red color on a low-adenine plate after 2 or 3 days incubation at 30°C (31).

***In vitro* MMR assay and Western blot analysis.** Human colon cancer HCT116 cells were cultured in DMEM with 10% fetal bovine serum at 37°C. HCT116 cells (3.0×10^6) were transfected with 5 μg of each MLH1 expression plasmid, 5 μg of a PMS2 expression plasmid, and 1.2 μg of pEGFP-C3 (BD Biosciences) using FuGENE6 (Roche). After 24-h incubation, cytoplasmic extracts were prepared and MMR activity in human cell extracts containing heteroduplex DNA was measured *in vitro* as described previously (32). M13mp2 heteroduplex DNA with the T-G mismatch was used in a MMR reaction with HCT116 cell extracts transfected with each *MLH1* expression plasmid. Each experiment was done twice or more and *in vitro* MMR activity (%) is shown as the mean ± SD. The extract was also examined in Western blot analyses using an anti-MLH1 antibody (G168-728, BD Biosciences), an anti-PMS2 antibody (A16-4, BD Biosciences), and an anti-GFP antibody (MMS-118P, Covance). Bands of MLH1 and GFP were quantified by densitometry.

Prediction of protein activity and structure. The Sorting Intolerant from Tolerant (SIFT) algorithm was adopted to predict the effects of amino acid substitutions on protein functions (33). Based on the crystal structure of the *E. coli* NH_2 terminus of MutL (9, 10), the MLH1 structure was predicted by a homology modeling method (34) using software ICM-Molsoft (Molsoft L.L.C.). The secondary structure alignment of the MutL homologues was based on reports (11, 35) and predicted using the PSIPRED server (36).

[5] http://kr.expasy.org/sprot/

Results

DME was detected in the three yeast assays. We previously developed a yeast-based MMR assay using a LacZ reporter plasmid with dinucleotide (GT) repeats (Fig. 1*A*, *left*; ref. 1). To establish simpler methods to detect yeast MMR deficiency, we also developed three distinct assays: a GFP assay using mononucleotide (C) repeats (Fig. 1*A*, *center*) and two ADE2 assays using mononucleotide (C) repeats and dinucleotide (CA) repeats (Fig. 1*A*, *right*). In each assay, replication errors of mononucleotide or dinucleotide repeats were observed in yeast colonies transformed with wild-type MLH1 due to the DME and expressed reporter genes, whereas those were not observed in yeast colonies transformed with an empty vector (Fig. 1*B*). The results of the two ADE2 assays coincided in most of the MLH1 variants and, therefore, the result of the ADE2 assay using mononucleotide (C) repeats was adopted as the representative ADE2 assay. The 101 *MLH1* variants were classified into one of the following four categories depending on the results of the LacZ, GFP, and ADE2 assays: (*a*) DME positive in all three assays (DME3+) contained 43 variants, (*b*) DME positive in two assays (DME2+) contained 16 variants, (*c*) DME positive in one assay (DME1+) contained 10 variants, and (*d*) DME negative in all three assays (DME−) contained 32 variants. The results of the DME phenotypes of all variants are summarized in Table 1.

***In vitro* MMR activity of human cell extracts expressing MLH1 variants.** Although the dominant mutator assays are simple and quick methods to detect functional alterations of *MLH1* variants, DME deficiency may not be directly linked to pathogenicity on HNPCC onset. To measure the MMR activities of *MLH1* variants, we adopted a well-established *in vitro* MMR assay to analyze the ability of cell extracts to repair DNA substrates with mismatched bases (4, 5). In the assay, cytoplasmic extracts of HCT116 cells had no MMR activity (0%) in the *in vitro* MMR assay because the cells were defective in MLH1 and PMS2 expressions. The transient expression of wild-type MLH1 and PMS2 in the cells complemented the defect and the MMR activity (79.7 ± 7.8%) was comparable with that of MMR-proficient HCT116+Ch3 cells (71.3 ± 11.7%). The results indicated that exogenous MLH1 was able to restore the MMR function of MLH1-deficient cells as also described in other reports (4, 5). The MMR activities of the 101 MLH1 variants expressed in HCT116 cells were then evaluated. The results indicated that there was diversity (0–90.2%) in the ability to repair mismatch DNA among the MLH1 variants (Fig. 2*A*). All of the eight putative polymorphisms had >60% of *in vitro* MMR activity. Therefore, 51 variants that showed 60% or more of MMR activity were classified into "MMR+," and 50 showing <60% of MMR activity were classified into "MMR−," in this study.

Protein expression levels among the majority of MLH1 variants. In many previous studies on MLH1 protein, nonsynonymous single nucleotide polymorphisms have been shown to affect protein stability (5, 8, 30, 37). To evaluate the level of expressed MLH1 protein, the 101 variants were transiently coexpressed with PMS2 in HCT116 colon cancer cells. The two MMR proteins were detected by Western blot analyses and the transfection efficiency was normalized by the cotransfected GFP level. The representative results of 11 variants for the MLH1 protein

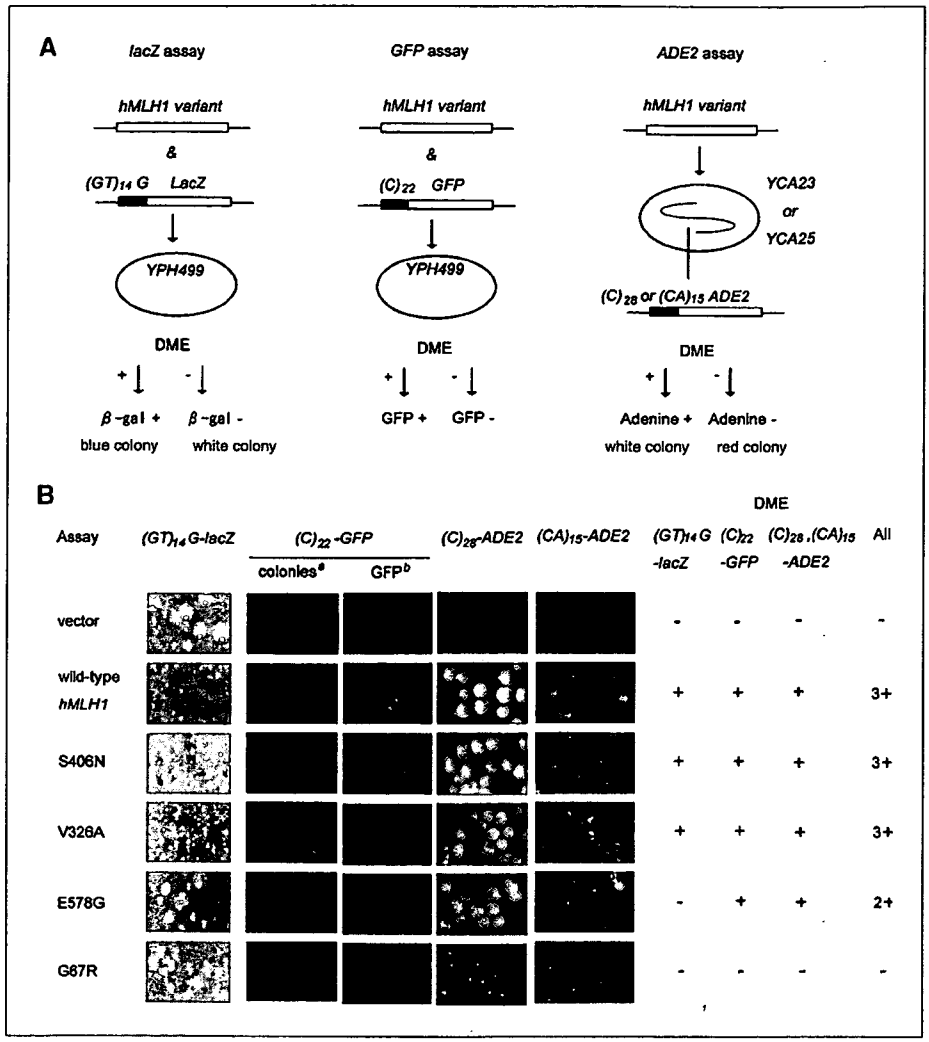

Figure 1. Functional analysis of *MLH1* variants in *S. cerevisiae*. *A*, schematic diagram of yeast assays. *B*, appearance of yeast colonies on indicator media for each functional assay of *MLH1* variants. The evaluation of each assay was summarized on the right of the panel.

levels are shown in Fig. 2*B*. Among these, the protein levels of R100P, G67R, T117M, K618del, and R659P variants were lower than those of the wild-type. The level of coexpressed PMS2 was also reduced in the four variants except for R100P. In S193P and E23D, PMS2 expressions were reduced although MLH1 expressions were comparable with wild-type MLH1. Comparing *in vitro* MMR activities with MLH1 expression levels, some variants, such as R100P, G67R, T117M, K618del, and R659P, showed MMR− with low MLH1 and/or PMS2 expression levels, whereas other variants, such as E102D, showed MMR− despite the retained expression levels of the two proteins (Fig. 2*B*). In all, only a slight correlation ($R = 0.225$, $P = 0.024$) between *in vitro* MMR activities and MLH1 expression levels was observed (Fig. 2*C*), suggesting that the MMR activities of MLH1 variants may not solely depend on MLH1 expression levels.

Correlations between DME phenotype and *in vitro* MMR activities. To examine whether the functions of the DME of *MLH1* variants correlate with the ability to repair mismatched DNA, we compared the results of yeast assays with the *in vitro* MMR assay. The *in vitro* MMR activities were higher in the DME-positive variants than in the DME-negative variants in any yeast assay ($P < 0.01$ by two-sided Mann-Whitney's U test; Fig. 3*A*). Moreover, in the three yeast assays, more DME-positive results correlated with higher *in vitro* MMR activity (Fig. 3*A*). The scatter diagram in which 101 variations are plotted by *in vitro* MMR activities and DME showed also correlation between these two assays (Fig. 3*B*). The majority of MMR+ variants showed DME3+ (35 of 51) and MMR− variants showed DME−, DME1+, or DME2+ (42 of 50; Table 1; Fig. 3*B*).

Relationship between the function and structure of MLH1. To elucidate the function-structure relationship of *MLH1* variants, we first compared the *in vitro* MMR activities with the SIFT score of the *MLH1* variants. SIFT is a program that predicts the effect of amino acid substitutions on protein function, based on sequence conservation during evolution and the nature of amino acids substituted in a gene of interest (33). As shown in Table 1, a majority of variants sorted as tolerant by SIFT showed MMR+ (38 of 48;79%) and those sorted as intolerant showed MMR− (38 of 51;75%), suggesting that SIFT had a good accuracy for predicting functions of variants and there was correlation between amino acids conservations and functions. Second, we focused on the relationships between MLH1 functions and the functional domain. As shown in Table 1, a large number of *MLH1* variants showing DME negative in each yeast assay and/or MMR− were located within either of the two functional domains, the NH_2-terminal ATPase domain or the COOH-terminal PMS2-interactive domain, whereas those showing DME+ or MMR+ were distributed throughout the whole gene product. Finally, we constructed a