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YAP1 is involved in mesothelioma development and negatively regulated by Merlin through phosphorylation

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We previously reported the results of bacterial artificial chromosome array comprehensive genomic hybridization of malignant pleural mesotheliomas (MPMs), including two cases with high-level amplification in the 11q22 locus. In this study, we found that the *YAP1* gene encoding a transcriptional coactivator was localized in this amplified region and overexpressed in both cases, suggesting it as a candidate oncogene in this region. We analyzed the involvement of YAP1 in MPM proliferation, as well as its functional and physical interaction with Merlin encoded by the *neurofibromatosis type 2 (NF2)* tumor suppressor gene, which is frequently mutated in MPMs. YAP1-RNA interference suppressed growth of a mesothelioma cell line NCI-H290 with *NF2* homozygous deletion, probably through cell-cycle arrest and apoptosis induction, whereas YAP1 transfection promoted the growth of MeT-5A, an immortalized mesothelial cell line. We also found that the introduction of *NF2* into NCI-H290 induced phosphorylation at serine 127 of YAP1, which was accompanied by reduction of nuclear localization of YAP1, whereas nuclear localization of a YAP1 S 127A mutant was not affected. Furthermore, results of immunoprecipitation and *in vitro* pull-down assays indicated a physical interaction between Merlin and YAP1. These results suggest that YAP1 is involved in mesothelial cell growth and that the transcriptional coactivator activity of YAP1 is functionally inhibited by Merlin through the induction of phosphorylation and cytoplasmic retention of YAP1. This is the first report of negative regulatory signaling from Merlin to YAP1 in mammalian cells. Future studies of transcriptional targets of YAP1 in MPMs may shed light on the molecular mechanisms of MPM development and lead to new therapeutic strategies.

Introduction

A malignant pleural mesothelioma (MPM) is a highly lethal neoplasm that is thought to develop from pleural mesothelial cells, with exposure to asbestos playing a crucial role in tumor development (1–4). Patients with an MPM are usually diagnosed at an advanced stage and

Abbreviations: BAC, bacterial artificial chromosome; CGH, comprehensive genomic hybridization; EGFP, enhanced green fluorescent protein; GST, glutathione S-transferase; MPM, malignant pleural mesothelioma; NF2, neurofibromatosis type 2; NHERF1, Na(+)/H(+) exchanger regulatory factor 1; PCR, polymerase chain reaction; RNAi, RNA interference; SDS, sodium dodecyl sulfate; sh, short hairpin.

the tumors are refractory to conventional therapeutic modalities; thus, their prognosis is very poor, even though advancements in chemotherapeutical modalities that combine cisplatin and antifolate, such as pemetrexed or raltitrexed, have been made (5,6). The long latency period between asbestos exposure and tumor appearance implies that multiple genetic changes are required for malignant transformation of mesothelial cells (7,8). Accumulated genetic studies have identified that tumor suppressor genes are crucial for MPM development, including frequent inactivation of *p16INK4a/p14ARF* at 9p21 (9–11) and neurofibromatosis type 2 (*NF2*) at 22q12 (12–14). The *NF2* gene is responsible for NF2 syndrome (15) and encodes Merlin (also known as schwannomin), an ezrin/radixin/moesin family protein that has been shown to be involved in cytoskeletal dynamics, growth factor receptor signaling and cell adhesion (16,17).

To further elucidate the alterations of oncogenes and tumor suppressor genes responsible for MPM development, we previously carried out bacterial artificial chromosome (BAC) array comprehensive genomic hybridization (CGH) analyses of MPM specimens from a total of 22 individuals and reported several distinct chromosomal alterations including high copy amplification of 11q22 (18). In the present study, we found that the *YAP1* gene, which was originally cloned as a partner of Yes kinase (19), resides within the 11q22 amplification region and that YAP1 is involved in mesothelial cell growth. Furthermore, we found that YAP1 activity may be negatively regulated via Merlin signaling in mesothelial cells. To our knowledge, this is the first known report of the existence of negative regulatory signaling from Merlin to YAP1 in mammalian cells, which may play a crucial role in growth regulation of mesothelial cells and development of malignant mesothelioma.

Materials and methods

Array CGH analysis and quantitative polymerase chain reaction analyses of copy number and expression

Genome-wide array CGH analysis of 22 individual MPMs using microarrays with 2304 BAC and P-1 phage-derived artificial chromosome clones covering the whole human genome at a resolution of roughly 1.3 Mb was previously reported (18). To determine the precise copy numbers within the amplification, quantitative polymerase chain reaction (PCR) using custom TaqMan probes (Applied Biosystems, Foster City, CA) corresponding to the genomic sequences of seven genes (*PGR, TRPC6, ANGPTL5, YAP1, BIRC2, MMP13* and *AB082S8*) dispersed within the 3 Mb region were designed and used together with TaqMan PCR master mix (Applied Biosystems) and an ABI7500 system (Applied Biosystems), according to the manufacturer's instructions. The copy number of the leucine-rich repeat containing the *4C* gene (also called *NGL1*) localized at 11p12 was used as a control. To examine the expression of genes within each amplification, TaqMan expression probes (Applied Biosystems) for *ANGPTL5, YAP1, BIRC2* and *BIRC3* were used, and quantification was performed as above.

Construction of RNA interference vectors and expression vectors

To construct RNA interference (RNAi) vectors, short hairpin (sh) oligonucleotides were inserted into a plasmid containing the U6 promoter and a puromycin-resistant gene (20). Two sh oligonucleotides were designed for two different sequences within the YAP1 open reading frame (YAP1-sh1, GGCCATGCTGTCCCAGATGAAT and YAP1-sh2, GGAGATGGAATGAACATAGAAT). In addition, control vectors, YAP1-scr1 and green fluorescent protein (GFP)-sh, were constructed using oligonucleotides with scrambled sequences for YAP1-sh1 (GGCTGCCATTCGCGACATGAAT) and GFP open reading frame (GFP-sh, GCAAGCTGACCCTGAAGTTCA). YAP1 cDNA was purchased from OriGene (Rockville, MD) and inserted into pcDNA (Invitrogen, Carlsbad, CA) and pEGFP-C1 (Clontech, Mountain View, CA) vectors. The phosphorylation-defective mutant YAP1 was constructed by *in vitro* mutagenesis at codon 127 from serine to alanine (S 127A), as the phosphorylation of serine 127 was reported to induce an interaction between 14-3-3 and cytoplasmic retention (21). *NF2* cDNA was amplified with reverse transcription–PCR and cloned into pcDNA (Invitrogen) and the lentivirus vector pLentiLox3.7. The *Na(+)/H(+) exchanger regulatory factor 1 (NHERF1)/*ezrin/radixin/moesin-*binding phosphoprotein 50 kD* gene expression constructs

were kindly provided by Dr Maria-Magdalena Georgescu (University of Texas M. D. Anderson Cancer Center) and Dr Martha C. Nowycky (University of Medicine and Dentistry of New Jersey).

Cell culture and colorimetric and flow cytometry analyses

A malignant mesothelioma cell line (NCI-H290), a gift from Dr Adi F. Gazdar (University of Texas Southwestern Medical Center), and a non-malignant mesothelial cell line (MeT-5A), purchased from American Type Culture Collection (Rockville, MD), were cultured as described previously (18). YAP1-RNAi vectors were transfected into NCI-H290 or MeT-5A cells using Lipofectamine 2000 (Invitrogen). For cell proliferation analysis, transfected cells were treated with puromycin at 1 μg/ml for 10 days, then stained using TetracolorOne (Seikagaku, Tokyo, Japan), after which absorbance was determined at 450 nm. For analysis of the cell cycle and sub-G_1 population, transfected cells were treated with puromycin at 1 μg/ml for 24 h, after which the culture medium and dead cells were removed. Residual and viable cells were further cultured without puromycin for 24 h, then harvested and stained with propidium iodide for flow cytometry analysis, as described previously (20).

Immunoblotting analysis

For preparation of nuclear and cytoplasmic fractions, cells were incubated in hypotonic buffer with 0.5% NP-40, then the nuclei were pelleted using a brief centrifugation, as described previously (22). For immunoblotting analysis, after harvesting the cells with lysis buffer, protein concentration was determined with a DC Protein assay kit (Bio-Rad, Hercules, CA). The same amounts of protein samples were applied to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, then electrotransferred to Immobilon-P polyvinylidene difluoride membranes. Each membrane was incubated with anti-V5-tag (Invitrogen) for V5-tagged NF2, anti-YAP1 (Cell Signaling and Abnova, Taipei, Taiwan) and anti-S 127 phospho-YAP1 (Cell Signaling, Danvers, MA) antibodies, then visualized using an ECL detection kit (GE Healthcare, Amersham Place, UK).

Immunofluorescent microscopic analysis

NCI-H290 cells were transfected with expression vectors for the enhanced green fluorescent protein (EGFP)-fused YAP1 wild-type or S 127A mutant together with V5-tagged NF2 or an empty vector and cultured on cover glass slides. The transfected cells were then fixed, permeabilized and incubated with anti-V5 and Alexa Fluor568-conjugated anti-mouse antibodies and examined with a confocal microscope (LSM510, Carl Zeiss MicroImaging GmbH, Jena, Germany).

Immunoprecipitation and in vitro pull-down assays

For immunoprecipitation analyses, 293T cells were transfected with the EGFP-fused wild-type or S 127A mutant YAP1 constructs together with V5-tagged NF2 or an empty vector. Immunoprecipitates of lysates transfected with the anti-V5 antibody were subjected to SDS–polyacrylamide gel electrophoresis and immunoblotting with various antibodies.

For *in vitro* pull-down assays, human NF2 full-length cDNAs were inserted into a pGEX-KG vector (Amersham Pharmacia Biotech, Uppsala, Sweden) to express bacterial glutathione S-transferase (GST)–Merlin fusion protein. GST–Merlin or GST-alone proteins were purified from the transformed bacterial lysates by incubation with glutathione sepharose beads (GE Healthcare). The YAP1 protein expressed with an *in vitro* transcription/translation system (Promega, Madison, WI) or cell lysate of 293T transfectants were incubated with beads containing 3 μg of immobilized GST-alone or GST–Merlin fusion proteins for 3 h at 4°C, then washed four times. Proteins bound to GST proteins were eluted by boiling in SDS sample buffer, then separated by SDS–polyacrylamide gel electrophoresis and immunoblotted with various antibodies.

Results

Precise mapping of 11q22 amplification region in malignant mesotheliomas

We previously reported the results of genome-wide array CGH analyses of MPMs derived from 22 individuals, in which it was notable that two primary MPM cases (KD1033 and KD 1041) showed two discrete and significant high-level amplifications in the chromosome 1p32 and 11q22 regions (18). Since we demonstrated that the *JUN* proto-oncogene resided in the 1p32 amplification region, whose expression was shown to be induced by asbestos exposure in rat pleural mesothelial cells (23) and whose amplification was recently demonstrated in aggressive sarcomas (24), we speculated that the 11q22 amplification region may also harbor an important target gene whose overexpression is involved in MPM cell growth. To identify the target gene, we precisely determined the extent of the amplified regions

from these two cases that were overlapped and bounded by RP11-40B14 and RP11-652L13 BAC probes, with only two BAC probes (RP11-203C2 and RP11-864G5) included between them (Figure 1A). With quantitative PCR analysis using TaqMan probes, the copy numbers of seven genes (*PGR, TRPC6, ANGPTL5, YAP1, BIRC2, MMP13* and *AB08258*) which dispersed within the 3-Mb-long region were investigated. As expected, both tumors were shown to carry high copy numbers of the *ANGPTL5, YAP1, BIRC2* and *MMP13* genes, while no gains were detected in *TRPC6* and *AB08258* genes, indicating that the extent of the common amplification region was ~1 Mb in length including 14 candidate genes (Figure 1B). In addition, comparing with each gene amplification level carefully, both *ANGPTL5* and *YAP1* showed about a 2-fold greater increase in copy numbers than *BIRC2* and *MMP13* in KD1033, while each amplification level of the four genes was similar in KD1041 (Figures 1B and 2C). This result suggested that the amplification of the centromeric half region including *ANGPTL5* and *YAP1* might be more important than that of telomeric region including the *BIRC2* and *MMP* cluster during the development of those MPMs, at least in KD1033.

Overexpression of YAP1 and BIRC2 in malignant mesotheliomas

To determine which gene residing in the 1 Mb amplification region was the most responsible for the development of these MPM cases, we next studied the expression levels of each gene using real-time reverse transcription–PCR analysis. Among the four genes in the centromeric half amplification region, *ANGPTL5, YAP1* and *BIRC2* were overexpressed in KD1033, whereas *YAP1, BIRC2* and *BIRC3* in KD1041, indicating that only *YAP1* and *BIRC2*, but not *ANGPTL5* or *BIRC3*, were commonly overexpressed in these tumors (Figure 2A). These results strongly suggest that the most probable target genes in this amplification region are *YAP1* and *BIRC2*. Meanwhile, we also examined the expression levels of several *MMP* cluster genes, but did not detect any overexpression, again suggesting that amplification of the telomeric half region was not significant (data not shown). The immunohistochemical staining results also clearly demonstrated the overexpression of YAP1 in KD1033 (Figure 2B, left panel), though normal pleural mesothelial cells did not show any YAP1 signals (arrowheads in Figure 2B, right panel).

Next, to determine whether other MPMs not shown to have clear amplification in BAC/P-1 phage derived artificial chromosome array CGH analysis may also have a more confined amplification or deregulation of the *YAP1* and *BIRC2* genes, we examined the copy numbers as well as expression levels of these genes using 12 additional primary MPM cases (Figure 2C and D) and 13 MPM cell lines (data not shown). However, amplification of these genes in these MPM specimens and cell lines was not detected, except for KD1033 and KD1041, nor was there significant upregulation of the others observed (Figure 2C and D). These results suggest that even though significant overexpression of the *YAP1* and *BIRC2* genes can occur, amplification of these genes is a relatively infrequent event in MPMs.

Involvement of YAP1 in mesothelial cell proliferation

To determine cancer-promoting roles of these genes in mesothelioma cells, we first focused on the *YAP1* gene since its positive role has also been suggested in other malignancies (25,26). We synthesized two YAP1-RNAi vectors, YAP1-sh1 and YAP1-sh2, to suppress endogenous YAP1 expression, a scramble control vector, YAP1-scr1, and a GFP-RNAi vector, GFP-sh. These vectors were transfected into MPM cell line NCI-H290 cells, and expression levels of endogenous YAP1 protein were examined. Both YAP1-RNAi vectors effectively reduced the expression levels of YAP1 to 30 and 10%, respectively, whereas the control vectors (GFP-sh and YAP1-scr1) did not demonstrate any suppression (Figure 3A). We then studied the effects of YAP1-RNAi on cell proliferation of mesothelioma. Both YAP1-RNAi vectors demonstrated significant suppression of cell proliferation, with YAP1-sh2 showing complete abolition of cell proliferation (Figure 3B). Flow cytometry analysis revealed that cells transfected by YAP1-sh2 showed G_1 arrest and induction of a sub-G_1 population
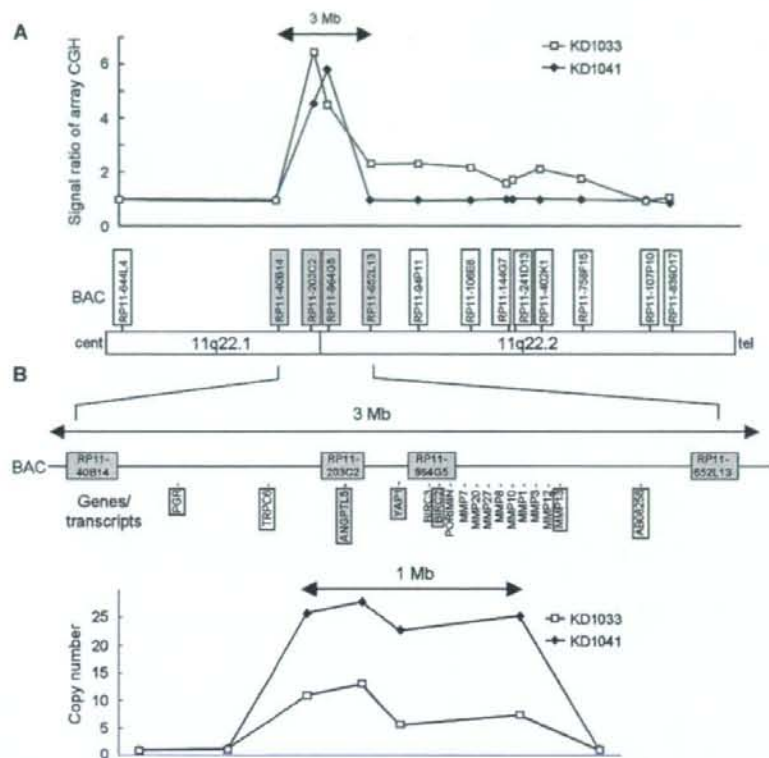
Fig. 1. Mapping of amplified region of 11q22 locus in two MPM cases. (A) Details of array-CGH results of 11q22 amplification in two MPM cases. The signal ratios from array CGH analyses of two primary MPM cases (KD1033 and KD 1041) were plotted for all BAC clones based on chromosome position, and the results indicated discrete and significant amplifications at the 11q22 region. Amplifications in both cases were similar within a 3-Mb-long region, which was bounded by RP11-40B14 and RP11-652L13, and included only two BAC probes (RP11-203C2 and RP11-864G5), represented by open squares and closed diamonds, respectively. (B) Copy number analyses using quantitative PCR with TaqMan probes. Genes and registered transcripts within the 3-Mb-long region are shown. To further determine the boundaries of the amplified regions, the copy numbers of seven genes (*PGR, TRPC6, ANGPTL5, YAP1, BIRC2, MMP13* and *AB08258*, indicated by boxes) were investigated using TaqMan probes. Four genes (*ANGPTL5, YAP1, BIRC2* and *MMP13*) showed high copy numbers in the two MPM cases, suggesting that both carried quite similar 1-Mb-long amplifications.

and those by YAP1-sh1 a moderate induction of the sub-G_1 population (Figure 3C and D). In contrast, the control vectors did not show any growth-suppressive effect.

Furthermore, we transfected a YAP1 expression vector into the immortalized mesothelial cell line MeT-5A to figure out whether YAP1 has growth-promoting activity in mesothelial cells. YAP1 overexpression moderately supported cell proliferation in a low-serum condition of 1% fetal calf serum, whereas it did not demonstrate clear promotion of cell proliferation in the usual condition (fetal calf serum 5%) (Figure 3E).

Functional interaction between YAP1 and Merlin

Since the Merlin-encoding *NF2* gene is frequently altered in MPMs, and the Merlin–Hippo–Warts pathway in *Drosophila* is known to negatively regulate Yorkie, the *Drosophila* ortholog of YAP1 through its phosphorylation (27), the results shown above strongly suggested that Merlin, as an upstream molecule, may functionally interact with and also suppress YAP1 in human mesothelial cells. In order to confirm this hypothesis, we cotransfected the *NF2* and *YAP1* expression vectors into NCI-H290 cells carrying a homozygous deletion of the *NF2* gene and studied whether exogenous Merlin has an effect on the phosphorylation status of YAP1 using the antibody against phosphorylated serine 127 (S 127) of YAP1, a critical phos-

phorylation site that has been indicated to induce inactivation of YAP1 as transcription coactivator through the induction of cytoplasmic retention (21). We found that cotransfection significantly induced the phosphorylation of YAP1 at S 127 (Figure 4A). To further demonstrate YAP1 S 127 phosphorylation by the activated form of Merlin, we also synthesized lentivirus vectors for full-length NF2 and truncated four-point-one/ezrin/radixin/moesin (FERM)-NF2 which translates 340 amino acids of the amino terminal and transfected into NCI-H290 cells. As expected, S 127 phosphorylation of YAP1 was induced by full-length NF2 in a dose-dependent manner, but not with truncated FERM-NF2 (Figure 4B).

Next, we determined whether phosphorylation of YAP1 protein induced its cytoplasmic localization, resulting in YAP1 inactivation as a transcriptional coactivator. We transfected NCI-H290 cells with EGFP-fused wild-type or S 127A mutant *YAP1*, together with *NF2* or an empty vector, and studied the subcellular localization using immunoblotting (Figure 4C) and immunofluorescence (Figure 4D). Immunoblotting of fractionated lysates, also depicted as a bar graph in the figure, clearly showed that wild-type phospho-YAP1 was scarcely detectable in the nuclear fractions, whereas the total YAP1 protein was localized in both the nucleus and cytoplasm (Figure 4C). In addition, the nuclear proportion of mutant YAP1 was higher than that of wild-type YAP1 (Figure 4C). These results suggested that

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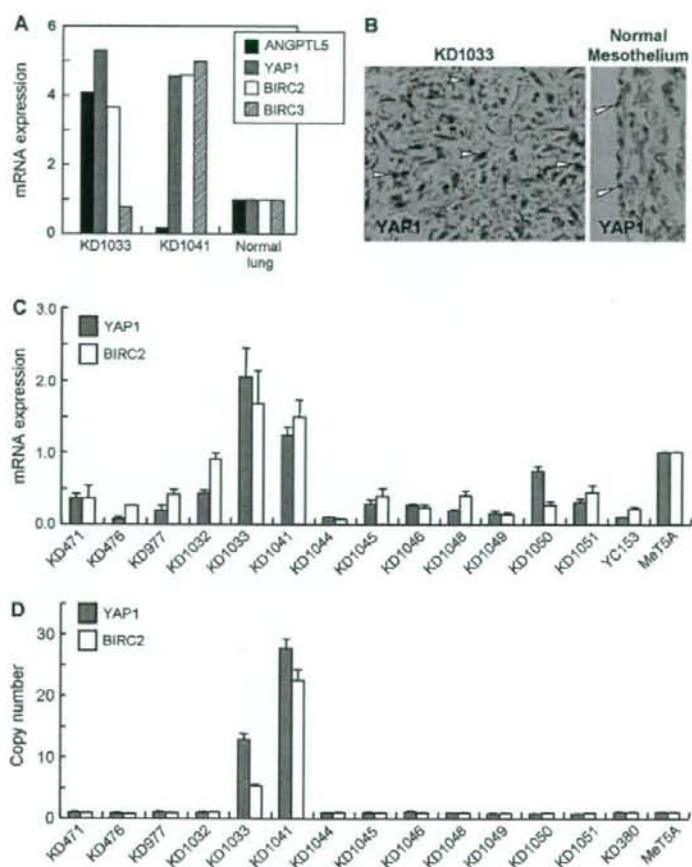
Fig. 2. Alterations of copy number and expression of *YAP1* and *BIRC2*. (**A**) Expression analyses of the *ANGPTL5*, *YAP1*, *BIRC2* and *BIRC3* genes indicated that *YAP1* and *BIRC2* were overexpressed in common in both MPM cases with amplification. (**B**) Immunohistochemical staining of YAP1. Immunohistochemical analysis clearly demonstrated overexpression and nuclear accumulation of YAP1 in KD1033 (left panel), whereas normal pleural mesothelial cells did not show any YAP1 signals (arrowheads, right panel). (**C**) Expression analysis in primary MPM cases. In an examination of 14 primary MPM cases and the normal mesothelial cell line MeT-5A, the two MPM cases showed the greatest amount of upregulated expressions of the *YAP1* and *BIRC2* genes with amplification. (**D**) Copy number analysis of primary MPM cases. In an examination of 14 MPM cases and the normal mesothelial cell line MeT-5A, only the two MPM cases demonstrated amplification of the *YAP1* and *BIRC2* genes.

phosphorylation negatively regulates nuclear localization and transcriptional activity of YAP1 protein. Although Merlin induced phosphorylation of YAP1, the nuclear proportion of wild-type total YAP1 was not significantly reduced, probably because of the existence of YAP1-alone transfectants.

Next, we performed immunofluorescence to further confirm that the subcellular localization of YAP1 protein is dependent on phosphorylation induced by Merlin. NCI-H290 cells were transfected with an EGFP-fused wild-type or S 127A mutant *YAP1* construct, together with *NF2* or an empty vector. Both the wild-type and mutant YAP1 proteins were found to be localized in both the nuclei and cytoplasm of the empty vector-cotransfected cells (Figure 4D). In contrast, cotransfection of the *NF2* vector clearly reduced nuclear localization of the wild-type YAP1 proteins, but not the mutant YAP1 protein. In addition, immunohistochemical staining of nuclear accumulation of YAP1 in the MPM case with YAP1 gene amplification (arrowheads in Figure 2B, left panel) also supported the idea

that YAP1 localization in nuclei of the tested cell lines was not due to an artificial event.

Physical interaction between YAP1 and Merlin

Since these transfection experiments indicated a functional relationship between Merlin and YAP1, we next studied whether these molecules physically interact with each other. We immunoprecipitated Merlin from the lysates of 293T cells that were cotransfected with *NF2* and/or *YAP1* expression vectors and then investigated whether YAP1 could be coprecipitated with Merlin (Figure 5A). The results clearly demonstrated the coprecipitation of YAP1 with Merlin, indicating a physical interaction between them. Interestingly, the anti-phospho-YAP1 antibody did not show any signals, while the S 127A mutant YAP1 also interacted with Merlin as did wild-type YAP1 (Figure 5A), suggesting that Merlin may also interact with unphosphorylated YAP1.
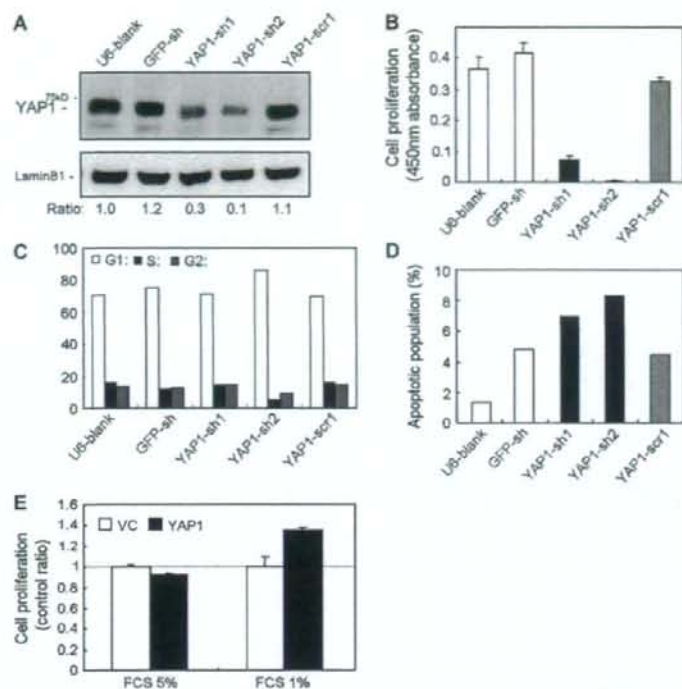
Fig. 3. Involvement of YAP1 in cell proliferation. (**A**) Knockdown of endogenous YAP1 in MPM cell line NCI-H290. Both YAP1-RNAi vectors, YAP1-sh1 and YAP1-sh2, showed effective suppression of the level of YAP1 protein, whereas the control vectors, GFP-sh and YAP1-scr1, showed no inhibition. (**B**) Inhibition of NCI-H290 proliferation by YAP1-RNAi. Colorimetric assay results demonstrated that both the YAP1-sh1 and YAP1-sh2 vectors induced significant suppression of cell proliferation. YAP1-sh2 inhibited proliferation to a greater degree as compared with YAP1-sh1, consistent with the RNAi effect. (**C**) Cell-cycle arrest by YAP1-RNAi. YAP1-sh2 clearly induced G_1 arrest of NCI-H290 cells. (**D**) Induction of sub-G_1 population by YAP1-RNAi. The sub-G_1 population of NCI-H290 cells was induced by both YAP1-RNAi vectors, though induction by YAP1-sh2 was greater. (**E**) Promotion of MeT-5A cell proliferation by YAP1 overexpression. Although YAP1 overexpression did not show a clear effect in the usual condition [fetal calf serum (FCS) 5%], YAP1 overexpression moderately promoted cell proliferation in the low-serum condition (FCS 1%).

To further confirm the physical interaction between YAP1 and Merlin, we prepared GST-alone or GST—Merlin-bound glutathione beads and then performed *in vitro* pull-down assays (Figure 5B). First, we conducted a pull-down assay using *in vitro*-translated YAP1 protein; however, no association between YAP1 and GST—Merlin was detected (Supplementary Figure 1), which suggested that YAP1 was not directly associated with Merlin. Next, we performed a pull-down assay using the lysate of 293T cells transfected with the YAP1 expression vector, as we considered that the cell lysate possibly contained endogenous molecules that could bridge YAP1 and Merlin. The pull-down assay using the 293T cell lysate clearly demonstrated that YAP1 was associated with GST–Merlin (Figure 5B, lane 2). In addition, we also studied the effects of NHERF1/ezrin/radixin/moesin-binding phosphoprotein 50 kD because it was reported to be associated with YAP1 (28) as well as with Merlin (29), and we considered that it might bridge the YAP1 and Merlin proteins. However, NHERF1 seemed unable to enhance the YAP1–Merlin association, though NHERF1 bound to GST–Merlin (Figure 5B, lane 4). These results indicate that YAP1 is indirectly associated with Merlin, probably through an endogenous bridging molecule other than NHERF1.

Discussion

In the present study, we demonstrated that the *YAP1* gene is localized in the high-level 11q22 amplification region, which we previously reported in a study of two cases with MPMs, and that *YAP1* together with *BIRC2* are overexpressed in these tumors. We also found that upregulation of YAP1 induced mesothelial cell proliferation, whereas its downregulation inhibited that proliferation. Furthermore, Merlin-dependent phosphorylation inhibits the nuclear localization of YAP1, which might result in inactivation of YAP1 transcriptional activity.

Amplifications at the 11q22 locus have been reported for several different types of human cancer (25,30–33). Amplifications at mouse chromosome 9qA1, the syntenic region of human chromosome 11q22, have also been shown in mouse mammary and liver cancers (25,26). Furthermore, during preparation of the present manuscript, MPM cell lines carrying chromosomal gain at the 11q22 locus were also reported (34). These findings, together with our previous array CGH analysis on malignant mesotheliomas, suggested a significant role for 11q22 amplification in carcinogenesis. In the present study, we demonstrated that, among the several candidate oncogenes at the 11q22 amplification region, both the *YAP1* and *BIRC2* genes were commonly overexpressed in the two MPM tissues, suggesting that *YAP1* and *BIRC2* were the most likely target genes. We focused on YAP1 primarily because previous reports have suggested its oncogenic activity of YAP1 (25,26). *In vitro* transfection assay that utilized knockdown or overexpression of YAP1 indicated that YAP1 promotes growth of mesothelial lineage cells, and cotransfection experiments strongly suggested that Merlin inhibits the transcriptional activator activity of YAP1 through induction of phosphorylation and inhibition
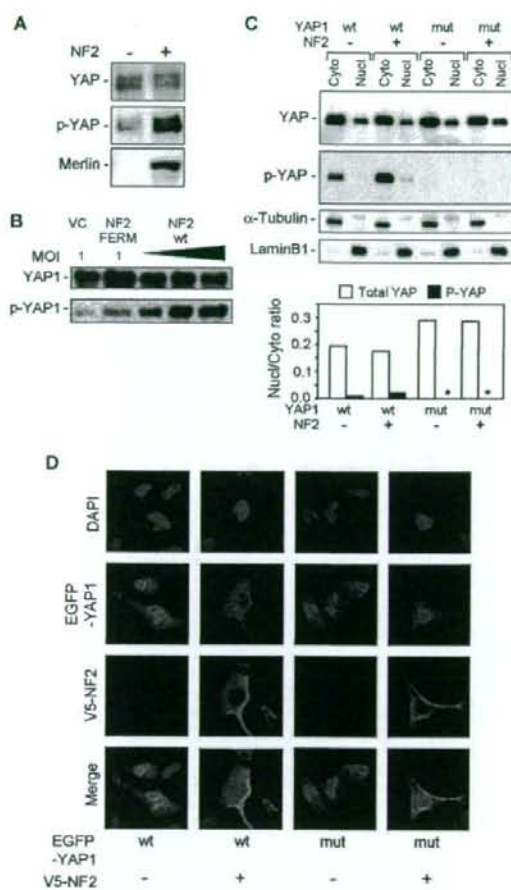
Fig. 4. Functional interaction between YAP1 and Merlin. (A and B) Induction of YAP1 phosphorylation by Merlin. (**A**) NF2-null NCI-H290 cells were transfected with YAP1 and NF2 expression vectors. Cotransfection of NF2 significantly induced phosphorylation of the YAP1 S 127 residue. (**B**) Either full-length or truncated NF2 was introduced into NCI-H290 cells with a lentivirus. Phosphorylation of YAP1 was induced by the full-length NF2 lentivirus in a dose-dependent manner, whereas the truncated NF2 FERM lentivirus did not have any clear effect. (**C**) Immunoblotting of cytoplasmic and nuclear fractions. NCI-H290 cells were transfected with wild-type (wt) or phosphorylation-defective S 127A mutant (mut) YAP1 and NF2 expression vectors. Immunoblots of nuclear and cytoplasmic fractions of transfectants clearly showed that phospho-YAP1 was mainly localized in the cytoplasm, as detected by the anti-S 127 phospho-YAP1 antibody, though total YAP1 was localized in both the nucleus and cytoplasm. In addition, the nuclear/cytoplasmic ratio of YAP1 and phospho-YAP1 proteins was measured with a densitometer and indicated with a bar graph, which clearly demonstrates the tight cytoplasmic retention of wild-type phospho-YAP1 protein. No signals were detected in mutant YAP1 transfectants with the anti-phospho-YAP1 antibody (asterisks). Results of immunoblotting with antibodies against α-tubulin and nuclear laminB1 indicated that proper fractionation occurred. (**D**) Reduction of YAP1 nuclear localization by NF2. NCI-H290 cells were transfected with expression vectors of the EGFP-fused wild-type (wt) or S 127A mutant (mut) YAP1, together with the vector control or V5-tagged NF2. Both the wild-type and mutant YAP1 proteins localize in both the nuclei and cytoplasm of H290 cells. Cotransfection of NF2 reduced nuclear localization of wild-type YAP1, whereas the localization of mutant YAP1 was not affected by NF2.
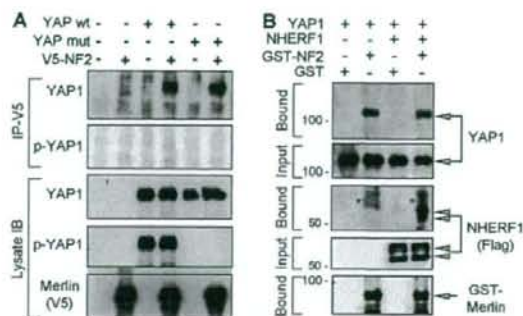
Fig. 5. Physical interaction between YAP1 and Merlin. (**A**) Immunoprecipitation: 293T cells were transfected with V5-tagged *NF2* and/or *YAP1* expression vectors, then Merlin was immunoprecipitated with the anti-V5 antibody and coprecipitation with YAP1 was studied. Both wild-type and S 127A mutant YAP1 were coprecipitated with Merlin. The anti-phospho-YAP1 antibody did not show any clear signals. (**B**) *In vitro* pull-down assay. Cell lysates of 293T cells were transfected with a *YAP1* or *NHERF1* vector, then incubated with GST-alone or GST—Merlin-bound beads. Immunoblots of bead-bound proteins (Bound) and the initial cell lysates (Input) are shown. Both YAP1 and NHERF1 proteins bound to GST–Merlin, though NHERF1 did not enhance the association of YAP1 with GST–Merlin. In the immunoblot of bound NHERF1, non-specific bands are indicated by asterisks.

of nuclear localization. To our knowledge, the present results are the first to show that YAP1 is regulated by Merlin through induction of phosphorylation, indicating that YAP1 is a downstream effector of Merlin tumor suppressor signaling in mammals. Our findings also suggest that YAP1 may play a crucial role in MPM development because Merlin tumor suppressor signaling is frequently altered in those tumors.

Recent genetic and biochemical analyses of *Drosophila* demonstrated that cell proliferation and organ size are negatively regulated by a kinase cascade of Hippo and Warts (also called Lats) and that two membrane-associated proteins, Merlin and Expanded, function upstream of this kinase cascade (35). Yorkie, the *Drosophila* ortholog of YAP1, is a critical target of the growth-inhibitory Hippo–Warts/Lats kinase cascade and a potential oncogene because its overexpression induces tissue overgrowth and apoptosis inhibition through the transactivation of cycE and diap1 expression (27,36). *Drosophila* rescue experiments also indicated evolutionary conservation of the signaling components, that is the mammalian *LATS1, MOB1, MST2* and *YAP* genes, which are orthologs of the *Drosophila Warts, Mats, Hippo* and *Yorki* genes, respectively. During the preparation of this manuscript, Zhao *et al.* (37) clearly demonstrated that the S 127 phosphorylation of YAP1 was catalyzed by LATS1 and that YAP1 inactivation plays significant roles in cell contact inhibition and tissue growth regulation. However, the signaling pathway from Merlin to YAP1 has not been clearly demonstrated in mammalian cells.

YAP1 has been reported to bind to and regulate the activities of various transcriptional regulators, including p73, RUNX2, ERBB4, and several TEA domain/transcription enhancer factor-type transcription factors, and also shown to function as an oncogene in mammals (25,26), possibly through an association with RUNX2 (38,39) and ERBB4 (19,40). YAP1 is phosphorylated at S 127, leading to its association with 14-3-3, which sequestrates YAP1 in the cytoplasm and inhibits its coactivator activity (21,41). The present results demonstrated that Merlin induces phosphorylation of YAP1 S 127 and inhibits its nuclear localization. Merlin–YAP1 signaling is conceivable, because, based on conserved *Drosophila* signaling, Merlin may activate the MST2–LATS1 kinase cascade, while it also appears to bind to unphosphorylated YAP1 protein.

YAP1 has also been reported to promote apoptosis through an association with p73 (21,42,43). In addition, a recent report demonstrated that LATS1 was activated by tumor suppressor RASSF1A to phosphorylate YAP1 and promote nuclear localization of the YAP1–p73 complex, resulting in apoptosis induction (44). Therefore, YAP1 may promote both cell growth and apoptosis, depending on the associated transcription factors. In this context, overexpression of *BIRC2* and *BIRC3* genes, which colocalize at 11q22 and encode apoptosis inhibitors, might be essential for exhibition of the oncogenic activity of YAP1 in cancers with 11q22 amplification, with our cases being consistent with this idea.

The *NF2* gene is frequently inactivated in MPMs, indicating that downregulation of Merlin signaling is essential for MPM development. The antiproliferative effect of Merlin in *NF2*-deficient mesothelioma cells has been suggested to be induced by repressing cyclin D1 expression (45), attenuating focal adhesion kinase phosphorylation (46) or interacting with NF2-associated guanosine triphosphate-binding protein (47). *Nf2(+/−)* mice exposed to asbestos exhibit accelerated formation of highly malignant mesothelial tumors (48,49). In addition, in a recent study, conditional knockout mouse models developed by inactivating *Nf2* together with *p16^{Ink4a}/p19^{arf}*, *p53* or both, developed malignant mesotheliomas at a high incidence, supporting the notion that *Nf2* inactivation is important for the pathogenesis of these tumors (50). Our results revealed that the *YAP1* gene can also be an activating target for a subset of MPMs, which was coincidentally found to be a downstream effector of the Merlin cascade. However, though YAP1 may be important in the subset of MPM with 11q22 amplification, its relevance in the vast majority of MPM cases is unknown at present. Therefore, precise analysis of tumor suppressor signaling in NF2–MST–WARTS/LATS–YAP1 is needed to shed light on the molecular mechanisms of MPM development in greater detail. Preliminary analysis of immunohistochemical staining of YAP1 revealed overexpression and nuclear localization of YAP1 in a subset of MPM cases, indicating the frequent involvement of YAP1 in MPM development. Nevertheless, the present results provide new insights into genetic alterations in MPMs and clues for development of a new molecular target therapy for patients with these tumors.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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Multicentre prospective phase II trial of gefitinib for advanced non-small cell lung cancer with epidermal growth factor receptor mutations: results of the West Japan Thoracic Oncology Group trial (WJTOG0403)

Clinical Studies

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The purpose of this study was to evaluate the efficacy of gefitinib and the feasibility of screening for epidermal growth factor receptor (EGFR) mutations among select patients with advanced non-small cell lung cancer (NSCLC). Stage IIIB/IV NSCLC, chemotherapy-naive patients or patients with recurrences after up to two prior chemotherapy regimens were eligible. Direct sequencing using DNA from tumour specimens was performed by a central laboratory to detect EGFR mutations. Patients harbouring EGFR mutations received gefitinib. The primary study objective was response; the secondary objectives were toxicity, overall survival (OS), progression-free survival (PFS), 1-year survival (1Y-S) and the disease control rate (DCR). Between March 2005 and January 2006, 118 patients were recruited from 15 institutions and were screened for EGFR mutations, which were detected in 32 patients – 28 of whom were enrolled in the present study. The overall response rate was 75%, the DCR was 96% and the median PFS was 11.5 months. The median OS has not yet been reached, and the 1Y-S was 79%. Thus, gefitinib chemotherapy in patients with advanced NSCLC harbouring EGFR mutations was highly effective. This trial documents the feasibility of performing a multicentre phase II study using a central typing laboratory, demonstrating the benefit to patients of selecting gefitinib treatment based on their EGFR mutation status.
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Gefitinib, a tyrosine kinase inhibitor (TKI), is an orally active small molecule that functions as a selective epidermal growth factor receptor (EGFR) inhibitor (Ranson et al, 2002). Two phase II trials (Fukuoka et al, 2003; Kris et al, 2003) for previously treated non-small cell lung cancer (NSCLC) (IDEAL-1 and -2, respectively) have documented favourable objective responses in 14–18% of patients. However, in a phase III

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trial (Thatcher et al, 2005), no survival benefit of gefitinib was observed when compared with best-supportive care (BSC) for previously treated NSCLC. In contrast, we have seen a significant survival benefit of erlotinib compared with BSC as a salvage therapy (BR21); erlotinib is also an EGFR-TKI and its chemical structure, which is based on quinazoline, is quite similar to that of gefitinib (Shepherd et al, 2005). Although we do not know whether differences between gefitinib and erlotinib were responsible for these different outcomes, appropriate patient selection to identify good responders is likely crucial for revealing the clinical benefits of the EGFR-TKI family.

Patient subset analyses of these randomised phase III trials or retrospective trials (Kaneda et al, 2004; Miller et al, 2004) clearly show the existence of populations that are more likely to respond to gefitinib and erlotinib, including women, patients with adenocarcinoma (especially with bronchial alveolar carcinoma (BAC)), nonsmokers and Asian patients (compared with Caucasians). Somatic mutations in specific regions of exons 18, 19 and 21 of the ATP-binding domain of EGFR have recently been shown to have strong associations with sensitivity to gefitinib or erlotinib (Lynch et al, 2004; Paez et al, 2004; Pao et al, 2004). Consistent with these findings, the frequencies of these EGFR mutations were higher in women, patients with adenocarcinoma, nonsmokers and Asians, all of whom are among the more frequent responders, as mentioned above (Shigematsu et al, 2005). There are two characteristic types of EGFR mutations. One is the presence of in-frame deletions, including the amino acids at codons 746–750 in exon 19, and the other is an amino-acid substitution at codon 858 (L858R) in exon 21. Recent analyses (Bell et al, 2005) of phase II and III trials for EGFR-TKI, in which patients were not selected based on their mutation status, have suggested that EGFR mutations are correlated with response to therapy but are not correlated with overall survival (OS). Furthermore, EGFR gene amplification/copy number (Cappuzzo et al, 2005; Hirsch et al, 2005) or overexpression (Hirsch et al, 2003) has been shown to be a more useful prognostic marker of response to gefitinib treatment. Patient selection according to EGFR mutation status may yield a superior survival rate by excluding patients who are unlikely to respond to gefitinib treatment. However, other populations that might obtain a clinical benefit from gefitinib treatment, even in the absence of EGFR mutation, may exist.

Three Japanese groups (Asahina et al, 2006; Inoue et al, 2006; Yoshida et al, 2007) have reported prospective phase II studies of gefitinib for advanced-stage NSCLC that were designed to consider the EGFR mutation status of the patients. All of these studies have reported a high response rate and extended progression-free survival (PFS) period, compared with historical controls. However, all of these studies had a relatively short observation period, making the data preliminary. Moreover, the original sample size was calculated after patient selection, and a critical consideration of the suitability of the assay used to detect the mutations (which was performed using small paraffin-embedded specimens obtained from bronchoscopic biopsies), and the estimated EGFR-positive rate were lacking. Additionally, all the trials were conducted at single institutions located in one small area of Japan. Thus, the published data may not be representative of the situation found in general clinical practice throughout Japan and therefore may not directly translate to the general feasibility of gefitinib treatment in Japan.

In view of this situation, we performed a multicentre prospective phase II trial of gefitinib for advanced NSCLC harbouring EGFR mutations. We prospectively registered patients from 15 different institutes in Japan at the beginning of EGFR mutation screening using a central database. Whether or not tissue was available from a bronchoscopic biopsy or surgery was not an inclusion criterion. All the clinical samples from the registered patients were delivered to a central laboratory that then determined the EGFR mutation status or the histological BAC features. The analysis of the survival data was based on a minimum observation period of at least 15 months from the time of entry of the last patient.

MATERIALS AND METHODS

Eligibility criteria

Eligible patients had histologically confirmed stage III NSCLC for which thoracic irradiation was not indicated or were stage IV. Chemotherapy-naive patients or those who had previously received up to two prior chemotherapy regimens, including those performed in an adjuvant setting, were eligible. Other eligibility criteria included an age $\geqslant 20$ years, measurable disease, the availability of sufficient amounts of tumour specimen for EGFR mutation analysis, an Eastern Cooperative Oncology Group performance status of 0–2, adequate organ function (WBC $\leqslant 3000\,\mu l^{-1}$, platelets $\geqslant 75\,000\,\mu l^{-1}$, AST and ALT $\leqslant 100\,IU l^{-1}$, serum creatinine $\leqslant$ twice the upper limit of the reference range; $P_{aO2} \geqslant 60$ mm Hg). The exclusion criteria included pulmonary fibrosis, the presence of symptomatic brain metastasis, active concomitant malignancy, severe heart disease, active gastrointestinal bleeding and continuous diarrhoea. All the patients signed a written informed consent form. Approval of this study and the gene analyses were obtained from the Institutional Review Board and the Ethics Committee of each hospital.

EGFR gene analysis

Tumour specimens were obtained using bronchial fiberscope or surgical procedures. The specimens were fixed with formalin and embedded in paraffin. Four slices ($4–5\,\mu m$) from the embedded block were sent to a central laboratory (Mitsubishi Chemical Safety Institute Ltd., Ibaraki, Japan) for genetic analysis. Most of the tumour specimens were available prior to the registration of this study. Genomic DNA was isolated from specimens using QIAamp Micro kits (QIAGEN KK, Tokyo, Japan). The EGFR mutations in exons 18, 19 and 21, as previously reported (Lynch et al, 2004; Paez et al, 2004), were determined using polymerase chain reaction (PCR) amplification and intron–exon boundary primers according to the published method. An EGFR registrant mutation in exon 20, which was reported by Pao et al (2005) was also examined using PCR and the previously reported primers. Polymerase chain reaction was performed using a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA), and the PCR products were confirmed using a Bioanalyzer 2100 (Agilent Technologies Inc., Santa Clara, CA, USA), then sequenced directly using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 3100 (Applied Biosystems). All sequencing reactions were performed in both forward and reverse directions and were analysed using the Basic Local Alignment Search Tool (BLAST); all the electropherograms were reanalysed by visual inspection to check for mutations. The presence of an EGFR mutation was confirmed using at least three independent PCR.

All sequence data were sent from the central laboratory to Kinki University. A principle investigator then confirmed whether or not the EGFR mutation status was positive, and the results were sent to the West Japan Thoracic Oncology Group (WJTOG) data centre. The data centre then informed each participating centre of the results of the genetic analysis and requested that the eligibility criteria of the patients be rechecked to insure that only EGFR-positive subjects were registered in the trial. Each tumour was categorised according to histology by a pulmonary pathologist (JF). The percentage of area exhibiting a BAC pattern was also examined to determine the WHO pathological category.

Treatment plan

Gefitinib ($250\,mg\,day^{-1}$) was administered once daily. Treatment was continued uninterrupted until disease progression or intolerable toxicity (grade 4 nonhaematological toxicities, any incidents of interstitial pneumonia or a treatment delay of more than 2 weeks because of adverse effects). Gefitinib administration was delayed if the patient's leukocyte and platelet counts were lower than 1500 and $5000\,\mu l^{-1}$, respectively, and was withheld until these counts had recovered. Gefitinib administration was also delayed if grade 3 or greater nonhaematological toxicities without nausea, vomiting or alopecia occurred and was withheld until recovery to grade 2.

Routine clinical and laboratory assessments and chest X-ray assessments were performed weekly or biweekly, where possible; CT examinations of the target lesion were performed every month, and magnetic resonance imaging of the whole brain and a bone scan were performed every 3 months. The objective responses of the patients were evaluated every month using the Response Evaluation Criteria in Solid Tumours (RECIST) guidelines (Therasse *et al*, 2000). Tumour response was centrally evaluated by independent reviewers at an extramural conference and was performed for the intent-to-treat population. All adverse effects that occurred during gefitinib treatment were reported, and the severity of the effects was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0.

Statistical analyses

The primary end point of this study was the response rate. A one-stage design using the binominal probability was used to determine the sample size. Assuming that a response rate of 50% would indicate potential usefulness, whereas a rate of 25% would be the lower limit of interest, and with $\alpha = 0.10$ (two side) and $\beta = 0.20$, the estimated accrual number was 23 patients. Estimating that the *EGFR*-positive rate would be about 20%, the screening number required to accrue 23 *EGFR*-positive patients was 115. After assuming an inevaluability rate of $<10\%$, the final required screening number was 125.

The secondary end points of this study were toxicity, OS, PFS, 1-year survival (1Y-S) and the disease control rate (DCR). Survival analyses were conducted on the intent-to-treat population using follow-up data available as of 30 April 2007. The survival curves were estimated using Kaplan–Meier plots.

RESULTS

Patient characteristics

Between March 2005 and January 2006, 118 patients were prospectively screened from 15 institutions; 117 of them underwent *EGFR* mutation analysis (tumour tissue was not available for one patient). The median time required for the *EGFR* mutation analysis was 12 days (range: 7–28 days). Among the 117 patients, *EGFR* mutations were detected in 32 patients (27%), 14 of whom had a deletion in or near E746-A750 (including one del E746-T751 ins A, two del L747-T751 and one del L747-T753 ins S) in exon 19. A further 17 had L858R, and one had a L861Q point mutation in exon 21 (Table 1).

Tissue samples from 17 patients (53%) were obtained by transbronchial biopsy. The *EGFR* detection rates for the surgical specimens and the bronchoscopic biopsy specimens were similar (30 *vs* 25%). The *EGFR* mutations were significantly more frequent in women ($P \leqslant 0.02$), in patients with adenocarcinoma ($P = 0.001$) and in people who had never smoked ($P < 0.001$) (Table 2). Finally, 28 patients (14 with deletions in exons 19 and 14 with point mutations in exon 21) were actually registered and received treatment with gefitinib, whereas four patients were dropped from the study as they became ineligible because of tumour progression during the time required for the mutation analysis.

Patient characteristics are listed in Table 3. In the initial screening, there were 56 female patients (48%), 97 patients (83%) with adenocarcinoma and 53 (45%) who had never smoked. The frequency of these characteristics was higher among the patients with *EGFR* mutations who were actually registered; namely, 18 patients (64%) were women, 27 (96%) had adenocarcinoma and 19 (68%) had never smoked. The median age of the 28 actually registered patients was 68 years; 24 patients (86%) had a good performance status (0–1), 22 (79%) had stage IV diseases and 17

Table 1 Type of EGFR mutations (n = 32)

Characteristics	No. of patients	%
Exon 18	0	0
Exon 19	14	44
del E746-A750	10	32
del E746-T751 ins A	1	3
del L747-T751	2	6
del L747-T753 ins S	1	3
Exon 21	18	56
L858R	17	53
L861Q	1	3

EGFR = epidermal growth factor receptor.

Table 2 Relationship between patient characteristics and EGFR mutation status

Characteristics	EGFR mutation positive (n = 32)		EGFR mutation negative (n = 85)		
	No. of Patients	%	No. of Patients	%	P
Sex					
Male	11	34	50	59	
Female	21	66	35	41	<0.02
Histology					
Adenocarcinoma	31	97	66	78	
Nonadenocarcinoma	1	3	19	22	=0.001
Smoking status					
Never	21	66	31	36	
Current/former	11	34	54	64	<0.001

EGFR = epidermal growth factor receptor.

(61%) were chemotherapy naive. Thoracic irradiation was contraindicated in one patient with stage IIIA disease because of the large irradiation field that would have been required. All five patients with stage IIIB diseases had malignant effusions. Four patients had received adjuvant therapies; five had received platinum doublets or a combination of gemcitabine and vinorelbine as their first-line therapy. Two patients had received two regimens of platinum doublets followed by docetaxel or pemetrexed. One patient had received local radiation for pain control.

Response and survival

The objective tumour responses are listed in Table 4. The overall response rate and DCR were 75% (95% CI: 57.6–91.0%) and 96% (95% CI: 87.0–96.4%), respectively. Five out of ten male patients (50%), six out of nine smokers (67%) and five out of eight male smokers with adenocarcinoma (63%) achieved a PR. One female nonsmoker with squamous cell carcinoma also achieved a PR. Among the registered patients with *EGFR* mutations, the response rate was no different between current/former smokers and those who had never smoked (67 *vs* 79%) or between chemotherapy-naive and postchemotherapy patients (77 *vs* 73%). Female and patients with a mutational deletion in exon 19 tended to have a higher response rate than male (89 *vs* 50%) and patients with a missense mutation in exon 21 (86 *vs* 64%), respectively.

The median follow-up time was 18.6 months (range: 13.8–23.4 months). The median PFS time was 11.5 months (95% CI: 7.3 months to -) (Figure 1A). The median OS has not yet been reached, and the 1Y-S was 79% (95% CI: 63.4–93.8%) (Figure 1B).

Clinical Studies

Table 3 Patient characteristics of all registered patients (n = 28)

Characteristics	No. of patients (%)
Age	
Median	68
Range	49–89
Performance status	
0	11 (39)
1	13 (47)
2	4 (14)
Sex	
Male	10 (36)
Female	18 (64)
Histology	
Adenocarcinoma	27 (96)
Squamous cell carcinoma	1 (4)
Large cell carcinoma	0 (0)
Adenosquamous carcinoma	0 (0)
Other	0 (0)
Smoking status	
Never	19 (68)
Current/former	9 (32)
Stage	
IIIA*	1 (3)
IIIB	5 (18)
IV	22 (79)
Prior cancer therapy	
Chemotherapy	
No	17 (61)
One regimen (adjuvant)	4 (14)
One regimen (not adjuvant)	5 (18)
Two regimens	2 (7)
Recurrence after surgery	11 (39)
Radiation	1 (4)

*Unresectable, no indication for thoracic radiation because of a large radiation field.

Table 4 Response rate (n = 28)

Response	No. of patients	Response rate (%)	95% CI
Complete response	1	3.6	
Partial response	20	71.4	
Stable disease	6	21.4	
Progressive disease	0	0.0	
Not evaluable*	1	3.6	
Overall response	21	75.0	57.6–91.0
Disease control rate	27	96.4	87.0–96.4

CI = confidence interval. *One patient was not evaluable because of a poor evaluation of efficacy.

Safety and toxicity

Toxicity was evaluated in all eligible patients (Table 5). The most frequent adverse events were rash, dry skin, diarrhoea, stomatitis and elevated AST/ALT levels. Two patients experienced grade 3 rash and one patient experienced grade 3 keratitis; however, these patients all achieved a PR, and the adverse effects subsided after pausing gefitinib treatment for around 2 weeks. Four patients experienced grade 3 hepatotoxicity; three of these patients had to discontinue treatment for this reason.

One patient developed interstitial lung disease (ILD) (Ando *et al*, 2006). Ground-glass opacity was detected in the right upper lobe 19 days after the start of gefitinib administration, resulting in the cessation of treatment. However, the lesion enlarged into bilateral

lung fields on day 25, and steroid therapy was initiated. Nonetheless, the patient died of respiratory failure on day 48. Two patients also experienced grade 1 ILD. They recovered without steroid administration.

Subsequent treatment after disease progression

Of the 14 patients who become refractory to gefitinib and exhibited disease progression, 10 received chemotherapy as their first treatment regimen after gefitinib (Table 6); 5 patients received platinum doublets and 1 patient received vinorelbine as a second-line treatment; and 3 received docetaxel and 1 received platinum doublet as a third-line treatment. In all, 4 out of the 10 patients (40%) had a PR. Of the nine patients who become refractory to the first treatment regimen after gefitinib, six received chemotherapy as their second regimen after gefitinib, including one who received gemcitabine, one who received docetaxel, and one who was re-treated with gefitinib as a third-line therapy; two other patients received docetaxel and one was re-treated with gefitinib as a fourth-line therapy. Two of the six patients (33%) had a PR. The two patients who received gefitinib re-treatment both had SD.

BAC features, *EGFR* amplification and T790M mutation in exon 20

A total of 110 tissue samples were available for pathological review, of which 90 were from adenocarcinoma; 33 of these specimens (37%) revealed proportional BAC components in the specimen. Among them, 15 were considered extensive and the remaining 18 were found to have minor BAC components. The 39 surgical specimens included 36 from adenocarcinomas. The *EGFR* mutations were detected in 12 out of the 36 adenocarcinoma specimens. None of the samples with a BAC component, micropapillary pattern or mucin production was associated with an *EGFR* mutation (Table 7).

Data on *EGFR* gene copy numbers were available in only 12 samples. We used the criteria for defining a high *EGFR* gene copy number (gene amplification or high polysomy, as determined using FISH) that were described in a previous report (Cappuzzo *et al*, 2005). A total of 7 out of the 12 samples had a high gene copy number (FISH positive), and 6 (3 with *EGFR* mutations) out of the 7 samples had proportional BAC components. In all, 5 out of the 12 samples were FISH negative, only 1 (with no *EGFR* mutation) of which had a BAC component. Two patients that were FISH negative, BAC negative and *EGFR* mutation positive had SD when treated with gefitinib.

Another *EGFR* mutation, T790M in exon 20, has been reported to be associated with resistance to gefitinib (Kobayashi *et al*, 2005; Pao *et al*, 2005). We checked for this mutation in six patients who did not respond to gefitinib; however, the mutation could not be identified in any of the patients.

DISCUSSION

We performed a multicentre phase II study examining the use of gefitinib for advanced NSCLC in patients with *EGFR* mutations, prospectively recruiting patients at the time of genetic screening and avoiding a selection bias. All patients were registered in a central database. All tissues were delivered from the local participants to the central facility, where they were reviewed by a pathology specialist and the *EGFR* mutation status was evaluated. The median time for the *EGFR* mutation detection analysis was 12 days, which is probably an acceptable time lag before the start of treatment for advanced NSCLC. However, a shorter period would clearly be desirable for routine clinical practice. Indeed, 4 out of the 32 *EGFR*-positive patients were dropped from the study because of disease progression before their actual registration
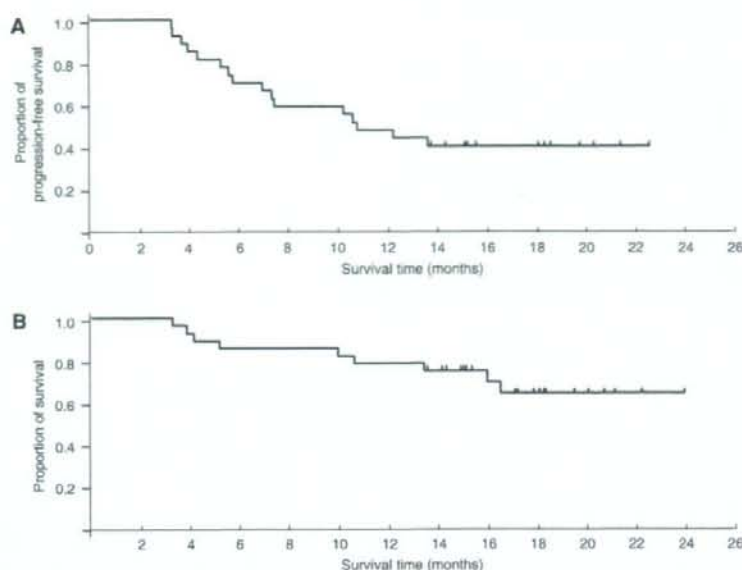
Figure I (**A**) Progression-free survival (PFS) and (**B**) overall survival (OS) of all eligible patients (*n* = 28). The median PFS was 11.5 months. The median OS has not yet been reached. The 1-year survival rate was 79%.

Table 5 Common adverse events (*n* = 28)

Adverse events	No. of patients (%)			
	Grade 1	Grade 2	Grade 3	Grade 4
Haematologic				
Anaemia	12 (43)	3 (11)	0 (0)	0 (0)
Leucopaenia	4 (14)	1 (4)	2 (7)	0 (0)
Neutropaenia	4 (14)	1 (4)	1 (4)	0 (0)
Thrombocytopaenia	3 (11)	0 (0)	0 (0)	0 (0)
Nonhaematologic				
Rash	10 (36)	11 (39)	2 (7)	0 (0)
Dry skin	9 (32)	10 (36)	0 (0)	0 (0)
Nail changes	5 (18)	2 (7)	0 (0)	0 (0)
Keratitis	0 (0)	0 (0)	1 (4)	0 (0)
Fever	0 (0)	1 (4)	0 (0)	0 (0)
Fatigue	3 (10)	3 (10)	3 (10)	0 (0)
Diarrhoea	7 (25)	1 (4)	0 (0)	0 (0)
Constipation	1 (4)	0 (0)	0 (0)	0 (0)
Stomatitis	8 (29)	1 (4)	0 (0)	0 (0)
Gastritis	1 (4)	0 (0)	0 (0)	0 (0)
Anorexia	2 (7)	1 (4)	0 (0)	0 (0)
Nausea	3 (11)	1 (4)	0 (0)	0 (0)
Vomiting	2 (7)	2 (7)	1 (4)	0 (0)
Dyspnoea	2 (7)	0 (0)	1 (4)	0 (0)
ILD	2 (7)	0 (0)	· 0 (0)	1 (4)*
Vertigo	1 (4)	1 (4)	0 (0)	0 (0)
Dysgeusia	0 (1)	1 (4)	0 (0)	0 (0)
Elevated AST/ALT	10 (36)	2 (7)	4 (14)	1 (4)*
Elevated creatinine	2 (7)	1 (4)	2 (7)	0 (0)

ALT = alanine transaminase; AST = aspartate transaminase; ILD = interstitial lung disease. *Same patient.

could occur. Yatabe *et al* (2006) has developed a rapid assay to detect *EGFR* mutations, and we have decided to use this assay in a phase III trial. The *EGFR* mutation rates in transbronchial biopsy samples were found to be the same as those in surgical specimens, suggesting that this assay can also accommodate stage IV NSCLC. We detected the two characteristic types of *EGFR* mutations (in exons 19 and 21) in 44 and 56% of the patients, respectively (Table 1); these percentages are identical to those in previous reports from Japan (Shigematsu *et al*, 2005; Asahina *et al*, 2006; Inoue *et al*, 2006; Yatabe *et al*, 2006; Yoshida *et al*, 2007). In summary, we confirmed the feasibility of using the *EGFR* detection assay in daily practice.

The overall response rate was 75%, which was comparable to those of other phase II studies of gefitinib in patients with *EGFR* mutations (Asahina *et al*, 2006; Inoue *et al*, 2006), despite our study permitting the entry of patients who had previously received up to two chemotherapy regimens. The DCR of 96% was relatively high, and the median PFS of 11.5 months and 1Y-S of 79% were also very promising. In a Korean study, Lee *et al* (2006) also reported a very promising response rate (56%) and 1Y-S (76%) for gefitinib in a prospective study of selected NSCLC patients with adenocarcinoma and never/light smokers, defined as having smoked no more than 100 cigarettes during one's lifetime. In the screening process for the present study, *EGFR* mutations were significantly more frequent in women, patients with adenocarcinoma and those who had never smoked. However, among the patients who were selected according to their *EGFR* mutation status, no differences in response were observed between never smokers and current/former smokers or between chemotherapy-naive and postchemotherapy patients. In a retrospective study, Han *et al* (2006) directly compared clinical predictors (smoking history, gender and histology) and the *EGFR* mutation status for their ability to predict response and survival. They showed that female never smokers with adenocarcinoma (three clinical predictors) had a 33% response rate, whereas patients with a positive *EGFR* mutation status had a 62% response rate. Furthermore, in a multivariate analysis, only a positive *EGFR* mutation status was associated with an improved OS, suggesting that the *EGFR* mutation status should be analysed whenever possible to optimise response predictions based on clinical

Table 6 Subsequent treatments after failure to respond to gefitinib (*n* = 28)

Gefitinib treatment	No. of Patients	1st regimen after gefitinib	No. of patients	2nd regimen after gefitinib	No. of patients
1st line	17	Plt doublet	5	Gem or Doce	2
				Gefitinib[a]	1
		VNR	1	—	-
2nd line[b]	4	Doce	2	Doce	1
		Plt doublet	1	Doce	1
2nd line	5	Doce	1	Gefitinib[a]	1
3rd line	2	—		—	—
Total	28		10		
Response			4/10		2/6

Doce = docetaxel; Gem = gemcitabine; Plt = platinum; VNR = vinorelbine. [a]Both patients had an SD response after gefitinib re-treatment. [b]First regimen as systemic chemotherapy after adjuvant treatment.

Table 7 Bronchial alveolar carcinoma (BAC) features and *EGFR* mutation status

	EGFR mutation		
	+	–	P-value
Surgically resected adenocarcinoma case	12	24	
BAC component			
Yes	8	17	1.0
No	4	7	
Micropapillary pattern			
Yes	4	12	0.48
No	8	12	
Mucin production			
Yes	1	5	1.0
No	11	19	

EGFR = epidermal growth factor receptor.

background factors. In the present study, *EGFR* mutations were detected in 16 out of 40 (40%) female never smokers with adenocarcinoma who underwent the screening process, and 14 out of these 16 patients (88%) achieved a response after undergoing gefitinib therapy. We could not compare the predictive powers of clinical predictors and the *EGFR* mutation status with regard to the clinical benefits of gefitinib in this study. Thus, the need for *EGFR* mutation testing among clinically favourable patients remains uncertain. Decisions regarding the first-line therapy of choice for patients with *EGFR* mutations or a clinically favourable profile (nonsmoker with adenocarcinoma) must also await the results of an ongoing randomised phase III study in an Asian population (IPASS: Iressa Pan-Asian Study) comparing platinum doublets with gefitinib.

In contrast, 50% of the men, 67% of the smokers and 63% of the men who were smokers achieved a PR in this study. Furthermore, one female nonsmoker with squamous cell carcinoma also responded to gefitinib. The histological type of this tumour was reassigned by a pulmonary pathologist, and the tumour was finally confirmed to be a squamous cell carcinoma. Squamous cell carcinoma harbouring an *EGFR* mutation is rarely seen but has been previously reported (Asahina *et al*, 2006). In a Japanese phase II trial of gefitinib for unselected chemotherapy-naive patients (Niho *et al*, 2006), the response rates among smokers, men, and patients with nonadenocarcinoma were 19, 13 and 10%, respectively. Thus, NSCLC patients who are either smokers, men or have a nonadenocarcinoma histology are unlikely to receive gefitinib treatment as a first-line treatment instead of standard chemotherapies (platinum doublets), which yield a response rate of about 30% (Schiller *et al*, 2002). Therefore, *EGFR* mutation screening may

have a higher impact on the selection of responders to gefitinib treatment among these kinds of Asian patient subset (for example, smokers with adenocarcinoma, and nonsmoking men or women with nonadenocarcinoma).

The benefit of chemotherapy in general among patients with *EGFR* mutations, compared with *EGFR* mutation-negative patients, remains uncertain. Previous studies (Bell *et al*, 2005) have suggested that patients with *EGFR* mutations tend to be more sensitive to chemotherapy than those with wild-type *EGFR*. In the present study, 40 and 33% of the patients responded to first- and second-line chemotherapy regimens after gefitinib, respectively. These relatively high response rates for refractory NSCLC suggest that patients with an *EGFR* mutation-positive status are generally sensitive to chemotherapy. Large-scale multivariate analyses, using pooled data from prospective phase II or III trials in which the *EGFR* mutation status was clearly confirmed, are needed to clarify this point.

The toxicities observed in the present study were mostly tolerable. Most of the common adverse events, like rash, diarrhoea or hepatotoxicity, were mild and subsided after gefitinib administration was paused for a short period. One male smoker with adenocarcinoma died of ILD. Thus, even among patients who are selected based on their *EGFR* mutation status, men or smokers may still be at risk for developing ILD; therefore, biomarkers to predict ILD are needed.

Patients with exon 19 mutations tended to have a higher response rate than those with a missense mutation in exon 21, consistent with the findings of previous reports (Jackman *et al*, 2006; Riely *et al*, 2006). The Spanish Lung Cancer Group also reported on a prospective phase II study of erlotinib in advanced NSCLC patients with *EGFR* mutations (Paz-Ares *et al*, 2006). The overall response rate was 82%. They also showed a difference in response rates between patients with mutations in exons 19 and 21 (95 and 67%, respectively). Exon 11 c-kit mutations are more closely correlated with a good prognosis in patients with gastrointestinal stromal tumour, who may benefit from lower doses of imatinib, whereas patients with exon 9 mutations may require higher doses (Debiec-Rychter *et al*, 2006). In the case of *EGFR*, functional differences between mutation types may also exist.

We found no discernible associations between the *EGFR* mutation frequency and the presence of a BAC component. Several reports, including that of Hirsch *et al* (2005) suggest that a higher *EGFR* copy number is correlated with BAC histological features. We also found an association between a high *EGFR* copy number and the presence of a BAC component, even though the number of specimens examined was relatively small. In a study on erlotinib, the presence of a BAC component was clearly associated with *EGFR* amplification. As the *EGFR* mutation rate is lower in western populations than in Asian populations, the *EGFR* gene copy number might be a more useful biomarker in western populations, especially with regard to the use of erlotinib.

In conclusion, gefitinib treatment for patients with advanced NSCLC harbouring an *EGFR* mutation demonstrated a promising activity in patients with a good performance status. Patient screening according to *EGFR* mutation status may be a useful tool in daily practice and will likely have a great impact on the selection of patients who are likely to benefit from gefitinib treatment.

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Clinical Studies

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Randomized phase II study of two different schedules of gemcitabine and oral TS-1 in chemo-naïve patients with advanced non-small cell lung cancer (NSCLC).

Sub-category: Metastatic Lung Cancer
Category: Lung Cancer--Metastatic Lung Cancer
Meeting: 2008 ASCO Annual Meeting

Abstract No: 8103
Citation: *J Clin Oncol* 26: 2008 (May 20 suppl; abstr 8103)
Author(s): M. Satouchi, Y. Kotani, N. Katakami, T. Shimada, Y. Urata, S. Yoshimura, Y. Funada, A. Hata, M. Ando, S. Negoro

Abstract: **Background:** TS-1, a novel oral fluorouracil derivative, has been shown to have anti-tumor activity with relatively mild adverse effects, and it is used in the treatment of NSCLC in Japan. The combination of gemcitabine (GEM) and 5-FU demonstrates a marked synergistic cytotoxic effect in a sequence-dependent manner in *in vitro* assay. This study was conducted in order to evaluate the efficacy and safety and to compare dosing schedules of gemcitabine combined with TS-1 in chemo-naïve NSCLC patients (pts). **Methods:** Pts with chemo-naive stage IIIB/IV NSCLC, an ECOG-PS of 0 or 1, and normal renal, liver, and bone marrow functions were randomized into 1 of 2 treatment arms. Oral TS-1 was administered daily from day 1 to 14, and GEM was given on days 1 and 8 (Arm A) or days 8 and 15 (Arm B). This cycle was repeated every 21 days. **Results:** A total of 80 pts were entered and 79 pts, treated in this trial. Randomization was well balanced across patient characteristics except for cell type (adenocarcinoma/squamous cell carcinoma = 37/4 (Arm A), 27/10 (Arm B)). Grade 3/4 hematological toxicities were neutropenia (54%), febrile neutropenia (9%), thrombocytopenia (11%) and anemia (4%). The hematological toxicity profiles did not differ very much between the two arms. Grade 3 pneumonitis was observed in 2 pts (3%). The response rate was 23.1% (95% confidence interval [CI]=11.1-39.3%) in Arm A and 30.6% (95% CI=16.3-46.1%), Arm B. Median time-to- progression (TTP) in Arm A was 4.1 months (95% CI=2.8-5.5) and Arm B, 5.4 months (95% CI=3.8-6.3) (p=0.75). Median survival time in Arm A was 15.7 months (95% CI=8.6-23.3) and Arm B, 22.4 months (95% CI=11.5-unknown) (p=0.32). **Conclusions:** The combination of GEM and TS-1 was determined to be feasible and effective for advanced NSCLC, and these results, particularly the favorable MST of Arm B, warrant further investigation of the Arm B dosing schedule for this combination for NSCLC.

Abstract Disclosures

Associated Presentation(s):

1. **Randomized phase II study of two different schedules of gemcitabine and oral TS-1 in chemo-naïve patients with advanced non-small cell lung cancer (NSCLC).**

   Meeting: 2008 ASCO Annual Meeting
   Presenter: Miyako Satouchi, MD
   Session: Lung Cancer — Metastatic (General Poster Session)

Other Abstracts in this Sub-Category

1. **FLEX: A randomized, multicenter, phase III study of cetuximab in combination with cisplatin/vinorelbine (CV) versus CV alone in the first-line treatment of patients with advanced non-small cell lung cancer (NSCLC).**

   Meeting: 2008 ASCO Annual Meeting   Abstract No: 3   First Author: R. Pirker
   Category: Lung Cancer--Metastatic Lung Cancer - Metastatic Lung Cancer

2. **Characterization of NSCLC patients responding to anti-IGF-IR therapy.**

   Meeting: 2008 ASCO Annual Meeting   Abstract No: 8000   First Author: A. Gualberto
   Category: Lung Cancer--Metastatic Lung Cancer - Metastatic Lung Cancer

3. **Molecular and clinical subgroup analyses from a phase III trial comparing gefitinib with docetaxel in previously treated non-small cell lung cancer (INTEREST).**

   Meeting: 2008 ASCO Annual Meeting   Abstract No: 8001^   First Author: J. Douillard
   Category: Lung Cancer--Metastatic Lung Cancer - Metastatic Lung Cancer

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Abstracts by M. Satouchi

1. **Randomized phase II study of two different schedules of gemcitabine and oral TS-1 in chemo-naïve patients with advanced non-small cell lung cancer (NSCLC).**

   Meeting: 2008 ASCO Annual Meeting   Abstract No: 8103   First Author: M. Satouchi
   Category: Lung Cancer--Metastatic Lung Cancer - Metastatic Lung Cancer

2. **Randomized phase III study of platinum-doublet chemotherapy followed by gefitinib versus continued platinum-doublet chemotherapy in patients (pts) with advanced non-small cell lung cancer (NSCLC): Results of West Japan Thoracic Oncology Group trial (WJTOG.**

Meeting: 2008 ASCO Annual Meeting   Abstract No: LBA8012   First Author: T. Hida
Category: Lung Cancer--Metastatic Lung Cancer - Metastatic Lung Cancer

3. Randomized, phase III study of mitomycin/vindesine/cisplatin (MVP) versus weekly irinotecan/carboplatin (IC) or weekly paclitaxel/carboplatin (PC) with concurrent thoracic radiotherapy (TRT) for unresectable stage III non-small cell lung cancer (NSCLC).

Meeting: 2007 ASCO Annual Meeting   Abstract No: 7530   First Author: T. Kimura
Category: Lung Cancer - Non-Small Cell Lung Cancer

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Presentations by M. Satouchi

1. Randomized phase II study of two different schedules of gemcitabine and oral TS-1 in chemo-naïve patients with advanced non-small cell lung cancer (NSCLC).

Meeting: 2008 ASCO Annual Meeting
Presenter: Miyako Satouchi, MD
Session: Lung Cancer — Metastatic (General Poster Session)

slides

2. Randomized Phase II Study Of Docetaxel (doc) Plus Cisplatin (cddp) Versus Doc Plus Irinotecan In Advanced Non-small Cell Lung Cancer (nsclc); A West Japan Thoracic Oncology Group (wjtog) Study

Meeting: 2001 ASCO Annual Meeting
Presenter: Miyako Satouchi, MD, PhD
Session: Small Cell and Non-Small Cell Lung Cancer (General Poster)

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Three-dimensional Conformal Radiation Therapy for In Situ or Early Invasive Central Airways Lung Cancer

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Atsushi Imai, MD, Satoaki Nakamura, MD, Osamu Suzuki, MD, Yuki Akazawa, MD,
and Kinji Nishiyama, MD

Introduction: Central airways lung cancer is occasionally discovered in early stage. Because of comorbidities, surgical resection is not always advisable for this type of lung cancer. Photodynamic therapy or endobronchial brachytherapy can produce cure for centrally located small lung cancers and is an alternative for surgery in selected patients. However, their application is limited by size and depth of invasion of the tumors or bronchoscopic access. External beam radiation can be applicable to almost all patients, when planned well. In this study, we evaluate the safety and efficacy of 3-dimensional conformal radiotherapy (3D-CRT) for in situ or early invasive central airways lung cancers.

Methods: Between November 2001 and December 2004, 8 patients with newly diagnosed or recurrent central airways lung cancer without nodal and distant metastasis were treated by 3D-CRT of 60 Gy in 3-Gy fractions. Target volume included the primary tumor but did not include regional lymph nodes. All patients were evaluated for disease control, survival, and complications.

Results: All lesions responded to the treatment. The median survival time was 36.8 months (30 to 50 mo), and the cause-specific survival time was 36.8 months (30 to 50 mo). Two-year overall, cause-specific survival, and locoregional control rate were 100%. Toxicity included pneumonitis observed in 1 patient, which resolved by conservative therapy.

Conclusions: 3D-CRT is a safe and effective treatment modality for in situ or early invasive central airways lung cancer when surgical resection or endobronchial therapy is not advisable.

Key Words: conformal radiotherapy, in situ or early invasive central airways lung cancer, local control, radical radiotherapy

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Lung cancer is occasionally detected as small centrally located tumors such as carcinoma in situ (CIS) or early invasive cancer. When lymph node and distant metastasis are not present, good clinical outcome including cure can be expected. Surgical resection, photodynamic therapy (PDT), and endobronchial brachytherapy are the modalities of treatment of choice. Although surgical resection is the standard treatment for early invasive central airways lung cancer, elderly patients or those with severe comorbidities are frequently determined medically inoperable. Additionally, as most of CIS or early invasive central airways lung cancers are smoking-related and have a tendency to be multifocal, conservative treatment is often sought. PDT is less invasive and effective for CIS or early invasive cancer, but complete remission is unlikely with large lesions and those deeper than bronchial cartilage.[1] In endobronchial brachytherapy, control of radiation dose is difficult and could lead to massive hemoptysis and exsanguination.[2]

Although external beam radiation remains an option for these patients, conventional one is associated with poor outcomes with 5-year survival rates of 25% to 30%.[3–17] Dose escalation of radiation using conventional fractionation and techniques would likely cause prohibitive toxicity. Three-dimensional conformal radiotherapy (3D-CRT) is intended to deliver higher doses of radiation, while minimizing damage to surrounding normal tissues. Because good results are reported in 3D-CRT for stage I peripheral lung cancer, 3D-CRT may have a potential to be curative for central-type lung cancers.[18] However, high-dose irradiation to hilar regions is still considered to be unsafe.[19] However, high but acceptable dose of irradiation seems to be necessary for centrally located small lung cancers.

Since 2001, we have been treating CIS and early invasive central airways lung cancer using 3D-CRT, when the lesions were inoperable or too invasive to treat with PDT. In this manuscript, we report the safety and efficacy of 3D-CRT for small centrally located lung cancers.

MATERIALS AND METHODS

Patient Characteristics

Between November 2001 and December 2004, 8 centrally located lung cancers without nodal (N0) and