Luciferase reporter assay. The human p21 promoter reporter vector was constructed according to a previously described method (13). The p21 promoter fragment was cut between the *Kpn*I and *Xho*I restriction sites and was transferred into the luciferase reporter vector pGL4.14 (Promega). All sequences were verified using DNA sequencing. The empty and p21 promoter-containing reporter vectors were designated as pGL4.14-mock and pGL4.14-p21, respectively. All the samples were examined in triplicate.

ChIP. ChIP was carried out using the ChIP-IT Express Enzymatic kit (Active Motif) according to manufacturer's protocol. HEK293 cells were transfected with empty vector (Myc) or Myc-tagged FOXQ1 vector. The putative region of the p21 promoter (−2264 to −1971) was amplified with the following primers: 5′-TTG AGC TCT GGC ATA GAA GA-3′ (forward) and 5′-TAC CCA GAC ACA CTC TAA GG-3′ (reverse). As a negative control, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) second intron promoter was amplified with the following primers: 5′-AAT GAA TGG GCA GCC GTT AG-3′ (forward) and 5′-AGC TAG CCT CGC TCC ACCTGA C-3′ (reverse).

Xenograft studies. Two separate xenograft studies were performed independently. Nude mice (*BALB/c nu/nu*; 6-week-old females; CLEA Japan, Inc.) were used for the *in vivo* studies and were cared for in accordance with the recommendations for the Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animals Experiments, Kinki University. The ethical procedures followed and met the requirements of the United Kingdom Coordinating Committee on Cancer Research guidelines (23). To assess tumorigenicity, suspensions of 1×10^6 H1299/EGFP or H1299/FOXQ1 cells (in 0.1 mL PBS) were s.c. injected into the left or right flanks of nude mice (n = 15), respectively. To evaluate tumor growth, a suspension of 6×10^6 H1299/EGFP, H1299/FOXQ1, H1299/FOXQ1/sh-control, and H1299/FOXQ1/sh-p21 cells (in 0.1 mL PBS) were s.c. inoculated (n = 10) into nude mice. The tumor volume was calculated as length × width^2 × 0.5. The tumor formation was assessed every 2 to 3 d. At the end of the experiment, the mice were sacrificed and the xenografts were resected, fixed in 10% buffered formalin for 6 to 10 h, and processed for histologic analysis.

Immunohistochemical and immunofluorescence staining. The methods used in this section have been previously described (24, 25).

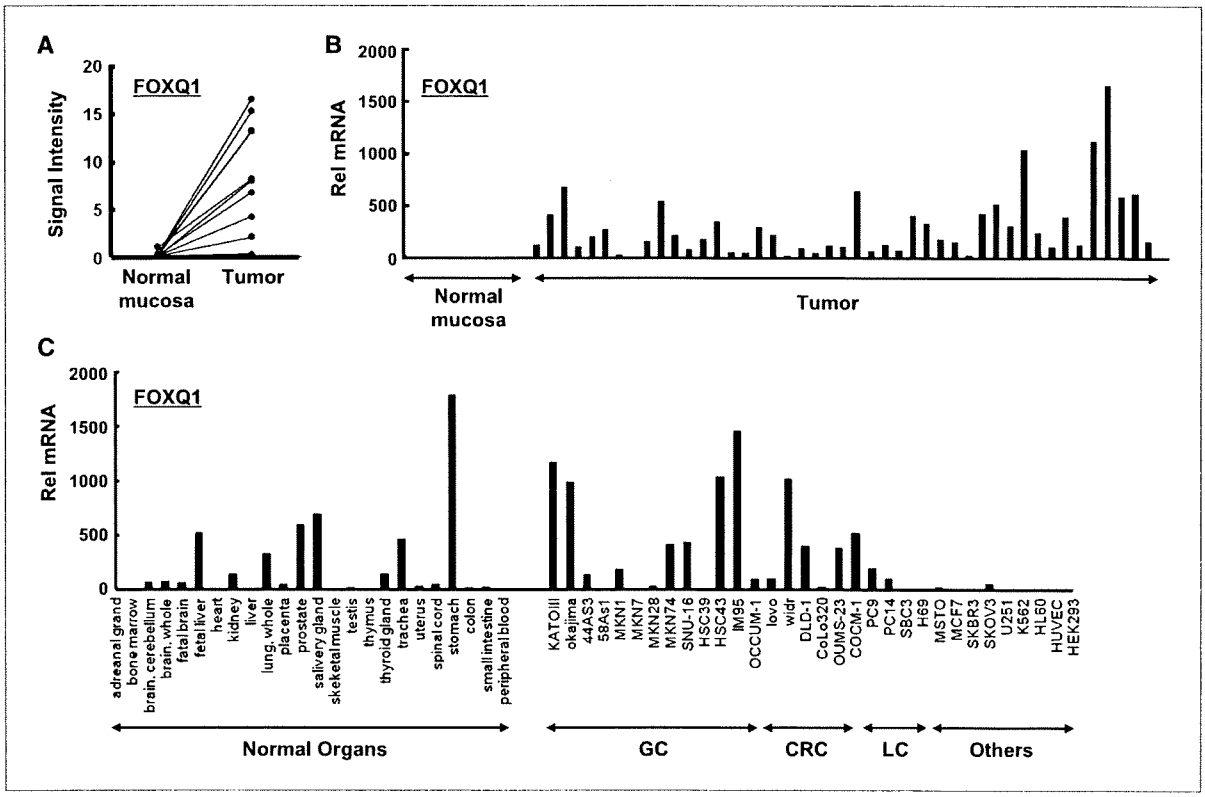
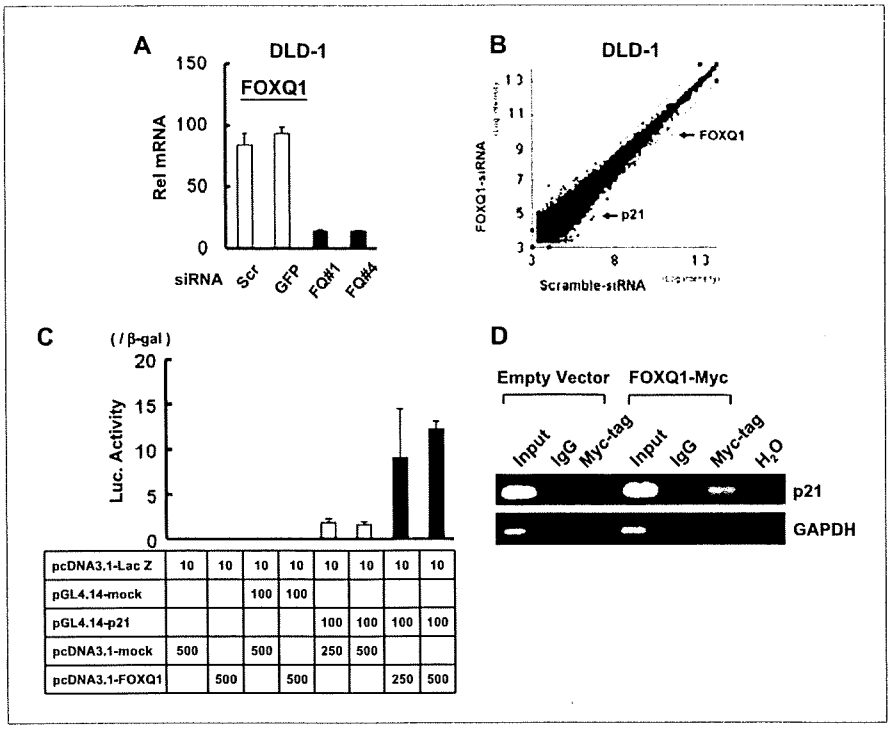


Figure 1. *FOXQ1* expression in CRC. A, mRNA expression of *FOXQ1* obtained from a microarray analysis of 10 CRC and paired normal mucosa specimens. The values indicate the normalized signal intensity. B, the mRNA expression levels of *FOXQ1* were determined using real-time RT-PCR for 10 paired and an additional 36 CRC samples. C, the mRNA expression levels of *FOXQ1* were determined using a real-time RT-PCR analysis of human normal tissue (left) and 30 human cancer cell lines, HEK293, and human umbilical vascular endothelial cell (HUVEC) cell lines (right). GC, gastric cancer; LC, lung cancer; Rel mRNA, normalized mRNA expression levels (*FOXQ1/GAPD* × 10^4).



pcDNA3.1-Lac Z	10	10	10	10	10	10	10	10
pGL4.14-mock			100	100				
pGL4.14-p21					100	100	100	100
pcDNA3.1-mock	500		500		250	500		
pcDNA3.1-FOXQ1		500		500			250	500

Figure 2. FOXQ directly regulates *p21* transcription. A, FOXQ1-targeting siRNA (FQ#1 and FQ#4) suppressed FOXQ1 expression in DLD-1 cells. The mRNA expression levels of *FOXQ1* were determined using real-time RT-PCR. B, microarray analysis of DLD-1 cells transfected with control-siRNA or FOXQ1-siRNA. The longitudinal axis indicates the mRNA expression of FOXQ1-siRNA transfected cells and the horizontal axis indicates that of control-siRNA. Arrow, FOXQ1 or p21 expression. Each point indicates the normalized and log base 2 transformed microarray data. C, induction of p21 promoter activity by FOXQ1. Luciferase vectors with either an empty or p21 promoter (pGL4.14-mock or pGL4.14-p21) were transiently cotransfected with a mock or FOXQ1 expression plasmid (pcDNA3.1-mock or pcDNA3.1-FOXQ1) expressing β-galactosidase as an internal control. The results were normalized to β-galactosidase activity and are representative of at least three independent experiments. D, ChIP of FOXQ1 on the promoter of *p21*. HEK293 cells were transfected with empty vector (Myc) or Myc-tagged FOXQ1 vector. Agarose gel shows PCR amplification (35 cycles) of the *p21* promoter using inputs (1% of chromatin used for ChIP) or ChIPs as templates. Primers to the *GAPDH* promoter were used as the negative control.

Microarray analysis. The microarray procedure and analysis were performed according to the Affymetrix protocols and BRB Array Tools software, Ver. 3.3.0,[4] developed by Dr. Richard Simon and Dr. Amy Peng, as reported previously (21, 26).

Statistical analysis. The statistical analyses were performed using Microsoft Excel (Microsoft) to calculate the SD and to test for statistically significant differences between the samples using a Student's *t* test. A *P* value of <0.05 was considered statistically significant.

Results

FOXQ1 mRNA was overexpressed in CRCs. A microarray analysis for 10 paired CRC samples identified 30 genes as being significantly upregulated by >10-fold in CRC ($P < 0.001$; Supplementary Table S1). *FOXQ1*, an uncharacterized transcription factor, was upregulated by 28-fold in the CRC specimens (Fig. 1A), exhibiting the fourth highest level of upregulation [after interleukin-8, matrix metalloproteinase-1 (MMP), and MMP-3]. Real-time RT-PCR for the 10 paired samples and an additional 36 CRC samples showed that *FOXQ1* mRNA was markedly overexpressed in the CRC samples but was only expressed at a very low level in noncancerous colonic mucosa ($P < 0.001$; Fig. 1B). The average levels of *FOXQ1* expression were 299 ± 326 and 4.0 ± 5.0 ($\times 10^4$/GAPD), respectively.

FOXQ1 expression in normal tissues and cancer cell lines. To investigate the expression of *FOXQ1*, we analyzed the mRNA expression levels of *FOXQ1* in panels of human normal tissues and cancer cell lines using real-time RT-PCR. High levels of *FOXQ1* expression were observed in the stomach, salivary gland, prostate, trachea, and fetal liver among the 24 normal tissues that were examined (Fig. 1C, left). Relatively weak expression levels were detected in brain-derived tissues, kidney, lung, placenta, and thyroid gland. These results were consistent with those of a previous report (27).

[4] http://linus.nci.nih.gov/BRB-ArrayTools.html

In the cancer cell line panel, the mRNA expression levels of *FOXQ1* were higher in gastric cancer, CRC, and lung cancer cell lines than in the other cancer cell lines, indicating that the expression of *FOXQ1* varies among specific cancers (Fig. 1C, right). Interestingly, the overexpression of *FOXQ1* in CRC arose from normal colonic mucosa with very low expression levels during carcinogenesis.

p21 is a target gene of FOXQ1. To examine the function of FOXQ1 as a transcription factor and to explore its target genes, we performed a microarray analysis using a CRC cell line, DLD-1, transfected with FOXQ1-targeting siRNA or control siRNA. Two sequences of FOXQ1-siRNA, FQ#1 and FQ#4, were used to exclude the off-target effect of siRNA. Real-time RT-PCR showed that both sequences of FOXQ1-siRNA suppressed *FOXQ1* mRNA expression by ~80% in DLD-1 cells (Fig. 2A); thus, FQ#4 was used as the FOXQ1-siRNA in the following experiments. A microarray analysis showed that 19 genes were downregulated by FOXQ1-siRNA (Fig. 2B; Supplementary Table S2); *p21* was the fifth most-downregulated gene. Because p21 is a key regulator of cell cycle and apoptosis, we focused on p21 as a target molecule of FOXQ1.

To confirm the microarray data, p21 downregulation by FOXQ1-siRNA was examined using real-time RT-PCR and a

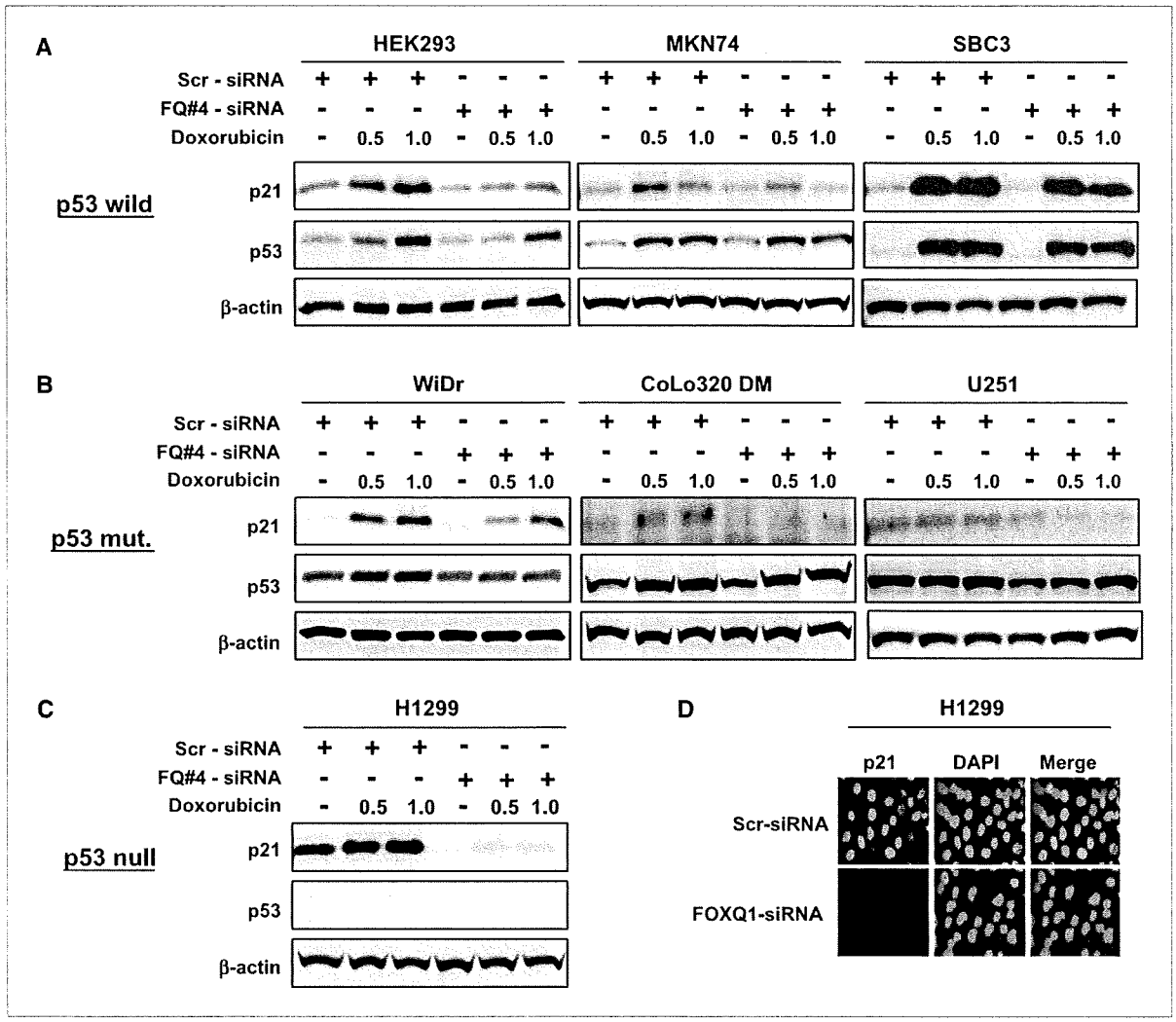


Figure 3. p21 induction by FOXQ1 and p53 status in cancer cells. The seven cell lines were transfected with control-siRNA or FOXQ1-siRNA for 24 h, and the cells were exposed to doxorubicin at a final concentration of 0.5 or 1 μmol/L for a further 24 h to enhance p21 induction. Western blot analyses for p21 and p53 were performed in three p53–wild type cell lines (A), three p53-mutant cell lines (B), and one p53-null cell line (C). The experiment was performed in duplicate. D, immunofluorescence p21 staining and 4',6-diamidino-2-phenylindole (DAPI) staining for H1299 cells transfected with control-siRNA (top) or FOXQ1-siRNA (bottom) for 48 h. Scr, scramble-siRNA (control); FQ#4, FOXQ1-targeting siRNA. β-Actin was used as an internal control.

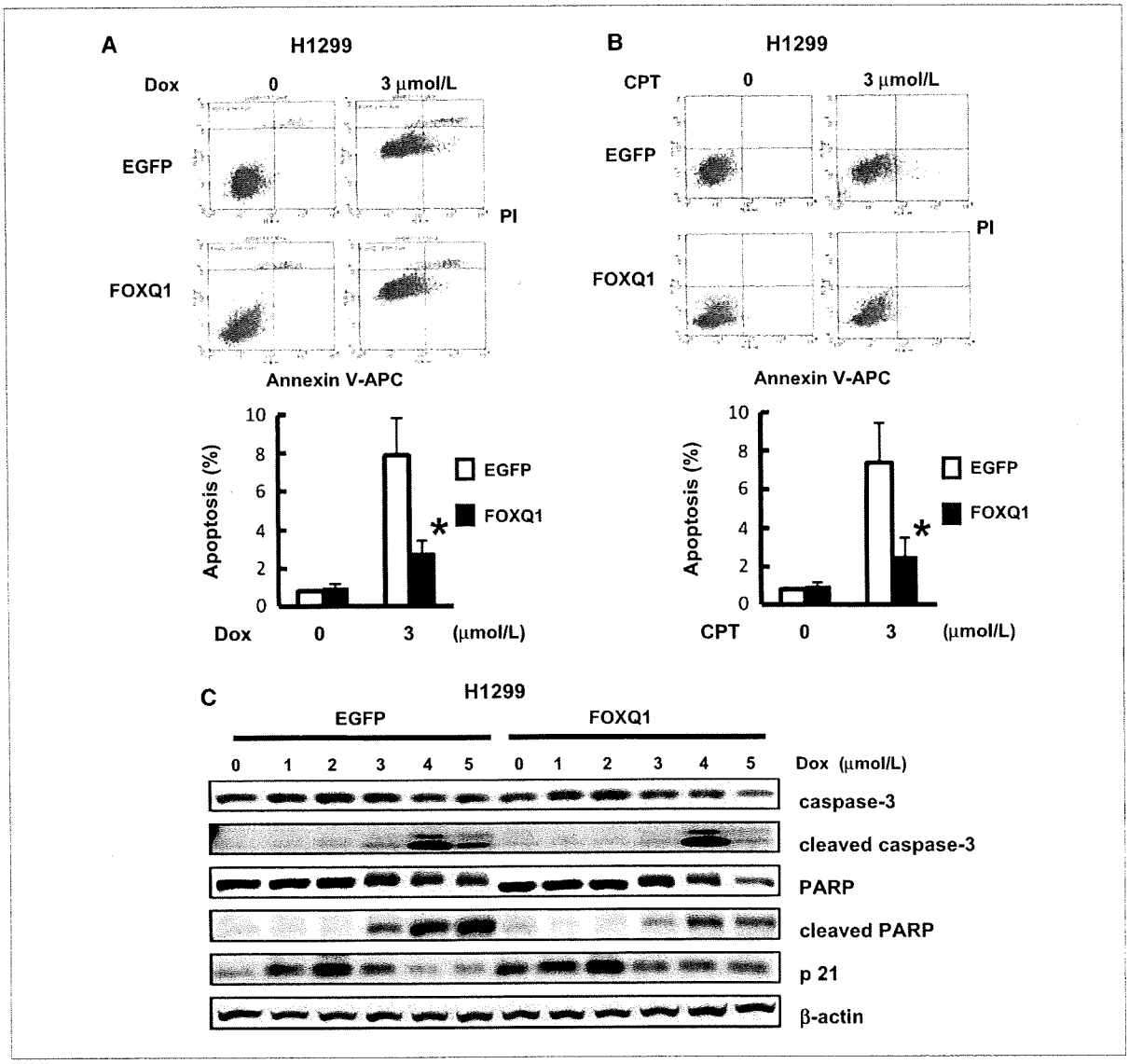


Figure 4. Overexpression of FOXQ1 promotes an antiapoptotic effect. Stable H1299 cell lines expressing EGFP or FOXQ1 (H1299/EGFP, H1299/FOXQ1) were exposed to doxorubicin (A) or camptothecin (B) at a final concentration of 3 μmol/L. Apoptotic cells were detected by Annexin V and propidium iodide (PI) using flow cytometry. C, Western blot analysis for apoptosis-related molecules. EGFP- or FOXQ1-expressing cells were exposed to doxorubicin at the indicated doses (0–5 μmol/L) for 24 h. β-Actin was used as an internal control. Dox, doxorubicin; CPT, camptothecin; EGFP, H1299/EGFP; FOXQ1, H1299/FOXQ1. *, $P < 0.05$.

Western blot analysis in DLD-1 cells. The results indicated that both sequences of FOXQ1-siRNA (FQ#1 and FQ#4) downregulated p21 expression at both the mRNA and protein levels. In addition, we confirmed the downregulation of p21 by FOXQ1-siRNA in other cell lines (WiDr and HEK293), obtaining similar results (Supplementary Fig. S1).

FOXQ1 directly increases the transcription activity of p21. We performed a luciferase reporter assay to determine whether FOXQ1 regulates p21 expression at the transcriptional level. A 2.4-kb section of the p21 promoter region was subcloned into a luciferase vector according to a previously described method (13, 28). The p21 promoter activity was increased by >8-fold when cotransfected with a FOXQ1 expression vector, compared with an empty vector (Fig. 2C). To determine whether FOXQ1 directly binds to p21 promoter, we transfected Myc or Myc-tagged FOXQ1 vectors into HEK293 cells and then conducted ChIP experiments. A segment of the *p21* promoter containing putative FOXQ1 binding site (−2264 to −1971) is precipitated with specific antibody, only if, FOXQ1 was induced (Fig. 2D).

The result indicates that FOXQ1 binds to the *p21* promoter and upregulates p21 transcriptional activity.

p53-independent p21 induction by FOXQ1 in cancer cells. Because p53 is the most important regulatory molecule of p21, we examined the downregulation of p21 by FOXQ1-siRNA in several cell lines with p53-wild type, p53-mutant, or p53-null statuses. These cell lines were transfected with control-siRNA or FOXQ1-siRNA, and p21 induction was enhanced by doxorubicin (29–31). The experiments were performed using three p53-wild type cell lines, three p53-mutation cell lines, and one p53-null cell line (Fig. 3A–C). Without doxorubicin exposure, all seven cell lines showed that p21 expression was downregulated by FOXQ1-siRNA. Notably, with doxorubicin exposure, considerable p21 downregulation by FOXQ1-siRNA was observed in the p53-mutation and p53-null cell lines, compared with in the p53-wild type cell lines. In the p53-null H1299 cell line, FOXQ1-siRNA completely suppressed p21 expression. These results suggest that p21 induction by FOXQ1 is p53 independent. An immunofluorescence study of p21 in H1299 cells also showed that p21 was completely downregulated by FOXQ1-siRNA (Fig. 3D).

Overexpression of FOXQ1 increases p21 expression and exhibits an antiapoptotic effect in cancer cells. Next, we established a stable *FOXQ1*-overexpressing cell line to confirm the induction of p21 expression by FOXQ1 and to detect any changes in the cellular phenotype of the cancer cells. FOXQ1 overexpression induced p21 expression (both mRNA and protein) in HEK293 and CoLo320 cells (Supplementary Fig. S1). Notably, p21 protein expression was markedly induced by >10-fold in the H1299/FOXQ1 cells (Supplementary Fig. S1). These results indicated that FOXQ1 robustly induces p21 expression, consistent with the findings of the siRNA study.

p21 induces an antiapoptotic effect and exerts a protective role against apoptosis induced by DNA damage. To

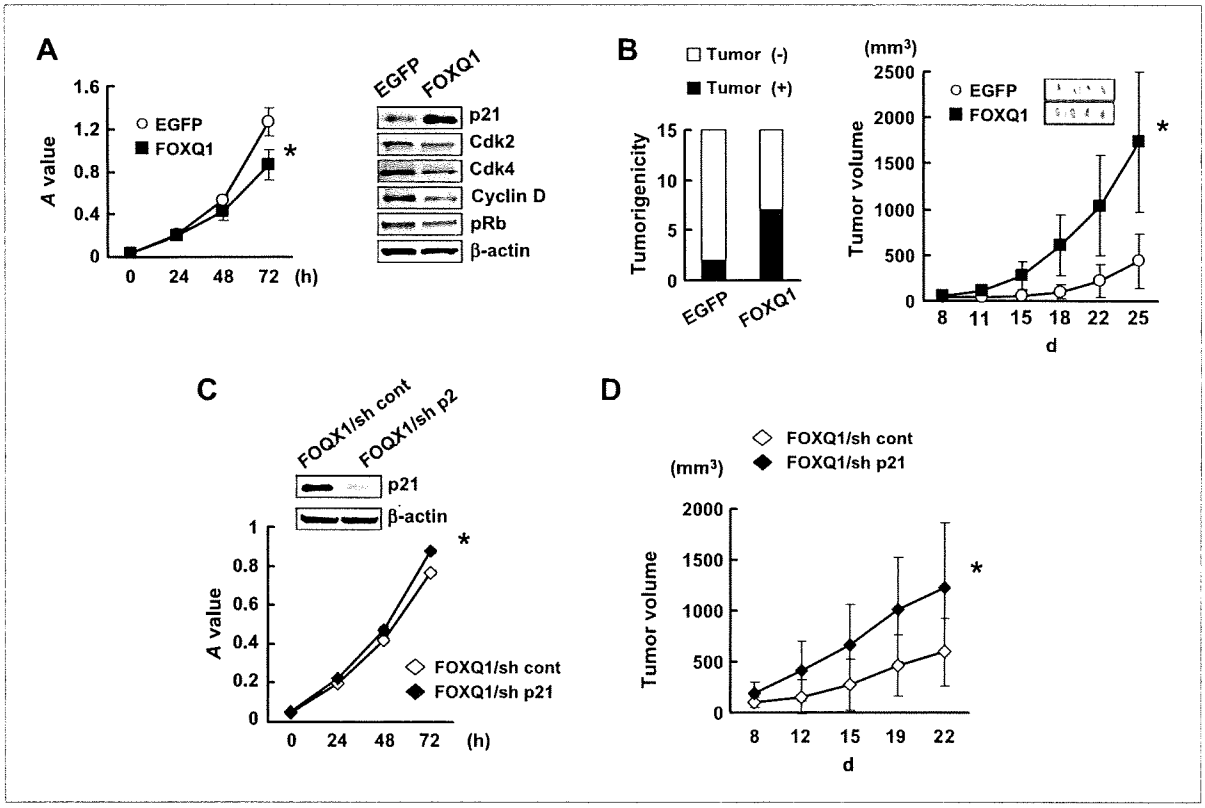


Figure 5. Overexpression of FOXQ1 enhances tumorigenicity and tumor growth *in vivo*. A, cellular growth and immunoblotting analysis of H1299 cell lines stably expressing EGFP or FOXQ1 (H1299/EGFP, H1299/FOXQ1). A total of 2×10^3 cells of each cell line were seeded in 96-well plates and evaluated after 0, 24, 48, and 72 h using MTT assay. Error bars, SD. Protein levels of H1299/EGFP and H1299/FOXQ1 cells were examined by Western blotting using specific antibody to p21, Cdk2, Cdk4, cyclin D, and phosphorylated Rb (pRb) protein. β-Actin was used as an internal control. EGFP, stable EGFP-overexpressing cells; FOXQ1, stable FOXQ1-overexpressing cells. B, H1299/EGFP and H1299/FOXQ1 cells were evaluated for their tumorigenicity *in vivo*. Mice ($n = 15$) were s.c. inoculated with a total of 1×10^6 cells. The numerical data indicate the number of mice. A total of 6×10^6 H1299/EGFP or H1299/FOXQ1 cells were s.c. inoculated into the right flank of each mouse to evaluate the tumor growth *in vivo* ($n = 12$). Representative H&E staining of tumor specimens was also shown. C, stable p21 knockdown or control cells obtained from H1299/FOXQ1 cells (H1299/FOXQ1/sh-control and H1299/FOXQ1/sh-p21) were evaluated for cellular growth and immunoblotting analysis. D, a total of 6×10^6 H1299/FOXQ1/sh-control or H1299/FOXQ1/sh-p21 cells were s.c. inoculated into the right flank of each mouse to evaluate the tumor growth ($n = 10$). *, $P < 0.05$.

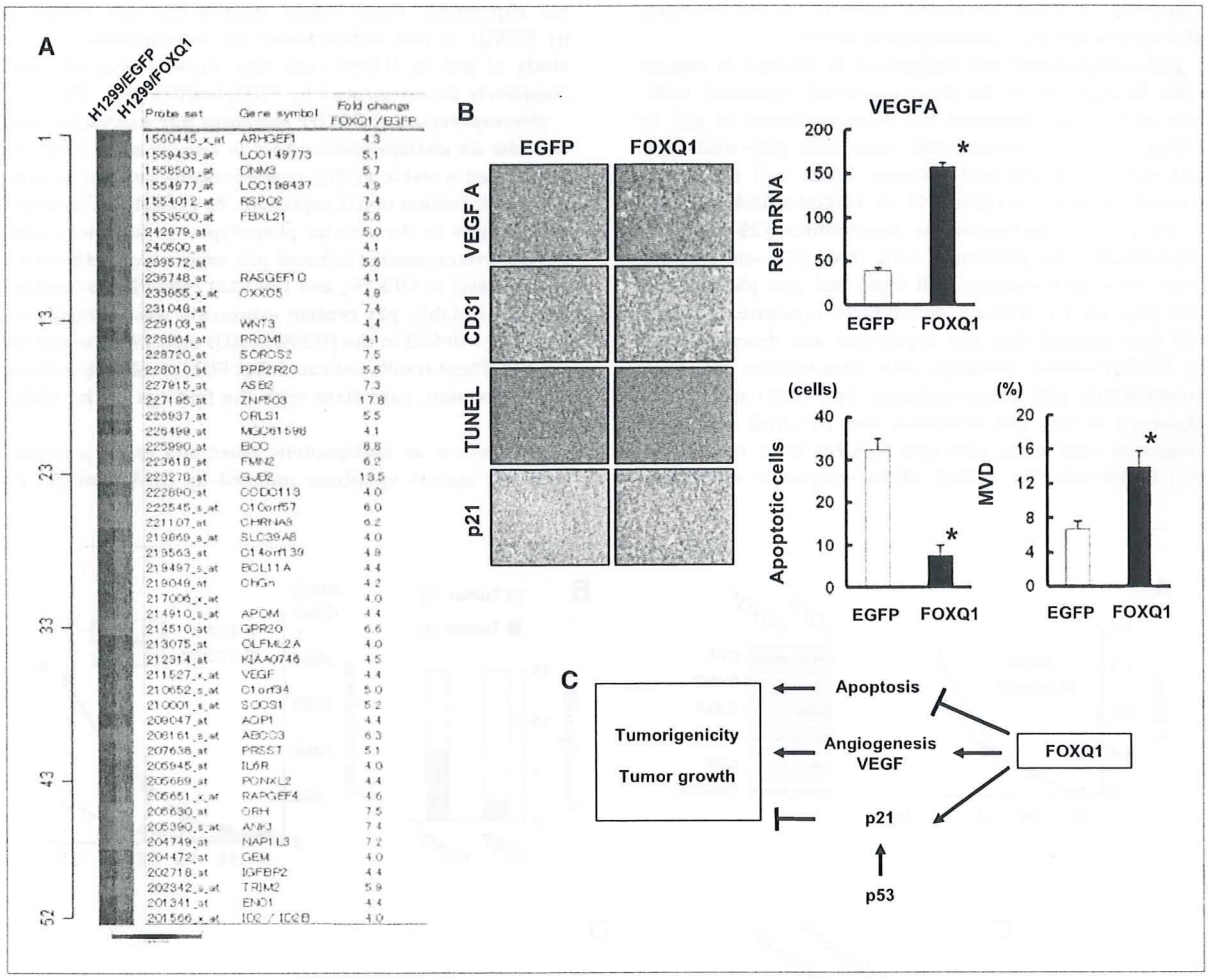


Figure 6. FOXQ1 promotes angiogenic and antiapoptotic effects *in vivo*. A, microarray analysis for H1299/EGFP or H1299/FOXQ1 cells. The upregulated genes over 4-fold by FOXQ1 were shown in the list. B, the mRNA expression levels of *VEGFA* were determined using a real-time RT-PCR analysis. Rel mRNA, normalized mRNA expression levels (*VEGFA/GAPD* x 10^4). VEGF, CD31, TUNEL, and p21 staining of tumor specimens inoculated with H1299/EGFP or H1299/FOXQ1 cells. Microvessel density (MVD) was determined by CD31-positive endothelial cells in tumor specimens using computer-assisted image analysis (Image J software package). C, diagram of a proposed mechanism of FOXQ1 for tumorigenicity and tumor growth. *, $P < 0.05$.

elucidate the role of apoptosis induced by FOXQ1 in cancer cells, we examined the apoptotic effect in H1299/EGFP and H1299/FOXQ1 cells using anticancer drugs. The overexpression of FOXQ1 inhibited the apoptosis induced by doxorubicin (H1299/EGFP: 7.9 ± 1.9%, H1299/FOXQ1: 2.7 ± 0.7%; Fig. 4A). Similarly, camptothecin-induced apoptosis was also inhibited in FOXQ1-overexpressing cells (H1299/EGFP: 7.4 ± 2.1%, H1299/FOXQ1: 2.5 ± 1.0%; Fig. 4B). Western blotting revealed that FOXQ1 overexpression decreased the levels of cleaved caspase-3 and cleaved PARP induced by doxorubicin (Fig. 4C). These results are consistent with those obtained using flow cytometry.

***Overexpression of FOXQ1 decreases cellular proliferation but enhances tumorigenicity and tumor growth* in vivo.** Stable H1299/FOXQ1 cells showed decreased cellular proliferation compared with control cells *in vitro* (Fig. 5A). Expressions of Cdk4, cyclin D1, and Cdk2 were decreased by FOXQ1 expression in H1299/FOXQ1 cells and resulted in a decrease of phosphorylated Rb expression (Fig. 5A). To examine the biological functions of FOXQ1 overexpression *in vivo*, we evaluated tumorigenicity and tumor growth using H1299/EGFP or H1299/FOXQ1 cells. H1299/FOXQ1 cells exhibited a significantly elevated level of tumorigenesis *in vivo* (GFP 2/15, FOXQ1 7/15, $P < 0.05$; Fig. 5B). In addition, the tumor volume was markedly larger in H1299/FOXQ1 cells than in H1299/EGFP cells (EGFP: 437 ± 301, FOXQ1: 1735 ± 769 mm^3, $P < 0.001$; Fig. 5B) on day 25.

***p21 does not contribute to FOXQ1-mediated tumor growth* in vivo.** Because emerging evidence has indicated that p21 may have dual functions with regard to tumor

progression and the suppression of cancer cells (32, 33), the shRNA targeting p21 or shRNA control viral vectors were further introduced into the H1299/FOXQ1 cells to elucidate the involvement of p21 in increased FOXQ1-mediated tumorigenicity and tumor growth *in vivo*. Stable H1299/FOXQ1/sh-p21 cells were slightly increased in cellular proliferation *in vitro* (Fig. 5C). In addition, tumor growth of H1299/FOXQ1/sh-p21 cells was increased compared with control cells *in vivo* (Fig. 5D). The results clearly indicate that p21 has negative roles for cellular proliferation and tumor growth in FOXQ1-overexpressing cells, suggesting that p21 does not contribute to FOXQ1-mediated tumor growth in FOXQ1-overexpressing cells *in vivo*.

***Overexpression of FOXQ1 promotes angiogenesis and antiapoptosis* in vivo.** To gain an insight into the mechanism by which FOXQ1 enhances tumor growth *in vivo*, we performed the microarray analysis on H1299/EGFP and H1299/FOXQ1 cells. Fifty-two genes were upregulated over 4-fold by overexpression of FOXQ1 including several genes that have positive roles for tumor growth, such as *VEGFA*, *WNT3A*, *RSPO2*, and *BCL11A* (Fig. 6A). Overexpression of FOXQ1 upregulated the *VEGFA* expression for 4.4-fold, suggesting the possibility of enhanced angiogenesis. Real-time RT-PCR for these cells and vascular endothelial growth factor (VEGF) staining of tumor specimens confirmed the result (Fig. 6B). Furthermore, CD31 staining of the tumor specimens showed that FOXQ1 overexpression significantly increased the angiogenesis *in vivo*.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and p21 immunostaining of the tumor specimens showed that p21 expression was increased and apoptosis was inhibited in H1299/FOXQ1 cells (Fig. 6B). These results strongly suggest that FOXQ1 promotes tumorigenicity and tumor growth with its angiogenic and antiapoptotic properties *in vivo* (Fig. 6C).

Discussion

FOX transcription factors are an evolutionarily conserved superfamily that control a wide spectrum of biological processes. Several Fox gene family members are involved in the etiology of cancer. Only the FOXO family has been regarded as *bona fide* tumor suppressors that promote apoptosis and cell cycle arrest at G_1 (34, 35). The loss of FOXO function observed in alveolar rhabdomyosarcoma through chromosomal translocation was first identified in relation to cancer. Many target genes of FOXO have been reported to date, including p21, cyclin D, Bim, TRAIL, and ER-α (36). On the other hand, the overexpression of FOXM is observed in head and neck cancer, breast cancer, and cervical cancer, and it enhances proliferation and tumor growth *in vitro* (37), suggesting that *FOXM* may be an oncogene. Although the available evidence is not conclusive, FOXP, FOXC, and FOXA have been linked to tumorigenesis and progression of certain cancers (36). Thus, the FOX family is thought to act as either an oncogene or a tumor suppressor. In the present study, we showed that the overexpression of FOXQ1 played a tumor-promoting role in CRC.

The p21 promoter region contains several definitive DNA regulatory elements, such as the p53-binding domain, E-box, Smad binding element, and TGF-β response elements. In the case of the other FOX family member FOXO, a recent report showed that the p21 promoter contains a consensus forkhead binding element (GGATCC) immediately upstream of the first Smad binding element and that the FOXO and Smad complexes activate p21 expression, whereas the FOXG1 protein binds to FOXO and blocks p21 induction (38). On the other hand, the consensus binding sequence (5′-NA(A/T)TGTTTA(G/T)(A/T)T-3′) has been defined for human FOXQ1 (4). The p21 promoter region contains several putative FOXQ1 binding sites according to its consensus binding sequence. Indeed, we have shown that FOXQ1 binds to a segment of the p21 promoter, indicating that FOXQ1 directly transactivates the p21 gene expression.

The initial descriptions of p21 were thought to indicate a tumor suppressor–like role, and p21 was almost solely regarded as a modulator with the principal function of inhibiting a cyclin-dependent kinase activity and, hence, cell cycle progression, because it was originally identified as a mediator of p53-induced growth arrest. However, emerging evidence has indicated that p21 may have dual functions with regard to tumor progression and the suppression of cancer cells, with examples of other genes with dual functions including TGF-β, Notch, Runx3, E2F, and p21 (32). Besides its growth inhibitory role, p21 is known to have a positive effect on cell proliferation (39–41). A more recent study on leukemic stem cells showed a p21-dependent cellular response that leads to reversible cell cycle arrest and DNA repair; such data clearly illustrate the oncogenic potential of p21 (33). We have shown that p21 has negative roles for tumor growth using FOXQ1-overexpressing cells with knockdown of p21 (Fig. 5D).

Recently, accumulating evidence has shown that FOX transcriptional factors are involved in VEGF regulation and angiogenesis. For example, forkhead has exhibited a positive role in mediating induction of VEGF (42–44). In the present study, we identified *VEGFA* as a candidate target gene of FOXQ1 by microarray analysis and showed that FOXQ1 increased angiogenesis *in vivo*. Interestingly, although overexpression of FOXQ1 decreases cellular proliferation *in vitro*, it enhances tumorigenicity and tumor growth *in vivo*. We consider that this discrepancy can be explained by these angiogenic and antiapoptotic effects of FOXQ1 contribute to enhanced tumor growth *in vivo*, although p21 negatively functions.

We showed that the overexpression of FOXQ1 inhibited doxorubicin-induced and camptothecin-induced apoptosis in p53-inactivated cancer cells. Therefore, we speculated that FOXQ1 might be a new determinant factor of resistance to drug-induced apoptosis and might represent a poor prognostic factor for CRC patients.

In conclusion, FOXQ1 is markedly overexpressed in CRC and enhances tumorigenicity and tumor growth *in vivo*. We have elucidated a biological function of FOXQ1, which directly upregulates p21 transcription and promotes angiogenesis and antiapoptosis. Our findings support FOXQ1

as a new member of the cancer-related FOX family in cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Richard Simon and Dr. Amy Peng for providing us with the BRB ArrayTools software. This free software was very useful and has been developed for user-friendly applications. We also thank Eiko Honda and Shinji Kurashimo for technical assistance.

Grant Support

Third-Term Comprehensive 2nd term of the 10-Year Strategy for Cancer Control, the program for the promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan grant-in-aid, and Research Resident Fellowship from the Foundation of Promotion of Cancer Research in Japan (H. Kaneda).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 06/18/2009; revised 10/28/2009; accepted 12/01/2009; published OnlineFirst 02/09/2010.

References

1. Jonsson H, Peng SL. Forkhead transcription factors in immunology. Cell Mol Life Sci 2005;62:397–409.
2. Carlsson P, Mahlapuu M. Forkhead transcription factors: key players in development and metabolism. Dev Biol 2002;250:1–23.
3. Tran H, Brunet A, Griffith EC, Greenberg ME. The many forks in FOXO's road. Sci STKE 2003;2003:RE5.
4. Overdier DG, Porcella A, Costa RH. The DNA-binding specificity of the hepatocyte nuclear factor 3/forkhead domain is influenced by amino-acid residues adjacent to the recognition helix. Mol Cell Biol 1994;14:2755–66.
5. Hoggatt AM, Kriegel AM, Smith AF, Herring BP. Hepatocyte nuclear factor-3 homologue 1 (HFH-1) represses transcription of smooth muscle-specific genes. J Biol Chem 2000;275:31162–70.
6. Martinez-Ceballos E, Chambon P, Gudas LJ. Differences in gene expression between wild type and Hoxa1 knockout embryonic stem cells after retinoic acid treatment or leukemia inhibitory factor (LIF) removal. J Biol Chem 2005;280:16484–98.
7. Hong HK, Noveroske JK, Headon DJ, et al. The winged helix/forkhead transcription factor Foxq1 regulates differentiation of hair in satin mice. Genesis 2001;29:163–71.
8. Potter CS, Peterson RL, Barth JL, et al. Evidence that the satin hair mutant gene Foxq1 is among multiple and functionally diverse regulatory targets for Hoxc13 during hair follicle differentiation. J Biol Chem 2006;281:29245–55.
9. Goering W, Adham IM, Pasche B, et al. Impairment of gastric acid secretion and increase of embryonic lethality in Foxq1-deficient mice. Cytogenet Genome Res 2008;121:88–95.
10. Verzi MP, Khan AH, Ito S, Shivdasani RA. Transcription factor foxq1 controls mucin gene expression and granule content in mouse stomach surface mucous cells. Gastroenterology 2008; 135:591–600.
11. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G_1 cyclin-dependent kinases. Cell 1993;75:805–16.
12. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. Nature 1993;366:701–4.
13. el-Deiry WS, Tokino T, Velculescu VE, et al. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75:817–25.
14. Brugarolas J, Moberg K, Boyd SD, Taya Y, Jacks T, Lees JA. Inhibition of cyclin-dependent kinase 2 by p21 is necessary for retinoblastoma protein-mediated G_1 arrest after γ-irradiation. Proc Natl Acad Sci U S A 1999;96:1002–7.
15. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G_1-phase progression. Genes Dev 1999;13:1501–12.
16. Garner E, Raj K. Protective mechanisms of p53-21-pRb proteins against DNA damage-induced cell death. Cell Cycle 2008;7:277–82.
17. Maki CG, Howley PM. Ubiquitination of p53 and p21 is differentially affected by ionizing and UV radiation. Mol Cell Biol 1997;17:355–63.
18. Gartel AL, Tyner AL. Transcriptional regulation of the p21((WAF1/CIP1)) gene. Exp Cell Res 1999;246:280–9.
19. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther 2002;1:639–49.
20. Yamanaka R, Arao T, Yajima N, et al. Identification of expressed genes characterizing long-term survival in malignant glioma patients. Oncogene 2006;25:5994–6002.
21. Tanaka K, Arao T, Maegawa M, et al. SRPX2 is overexpressed in gastric cancer and promotes cellular migration and adhesion. Int J Cancer 2009;124:1072–80.
22. Takeda M, Arao T, Yokote H, et al. AZD2171 shows potent antitumor activity against gastric cancer over-expressing fibroblast growth factor receptor 2/keratinocyte growth factor receptor. Clin Cancer Res 2007;13:3051–7.
23. United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR). Guidelines for the Welfare of Animals in Experimental Neoplasia (Second Edition). Br J Cancer 1998;77:1–10.
24. Iwasa T, Okamoto I, Suzuki M, et al. Inhibition of insulin-like growth factor 1 receptor by CP-751,871 radiosensitizes non-small cell lung cancer cells. Clin Cancer Res 2009;15:5117–25.
25. Shimada K, Nakamura M, Anai S, et al. A novel human AlkB homologue, ALKBH8, contributes to human bladder cancer progression. Cancer Res 2009;69:3157–64.
26. Igarashi T, Izumi H, Uchiumi T, et al. Clock and ATF4 transcription system regulates drug resistance in human cancer cell lines. Oncogene 2007;26:4749–60.
27. Bieller A, Pasche B, Frank S, et al. Isolation and characterization of the human forkhead gene FOXQ1. DNA Cell Biol 2001;20:555–61.
28. Datto MB, Yu Y, Wang XF. Functional analysis of the transforming growth factor β responsive elements in the WAF1/Cip1/p21 promoter. J Biol Chem 1995;270:28623–8.
29. Mahyar-Roemer M, Roemer K. p21 Waf1/Cip1 can protect human colon carcinoma cells against p53-dependent and p53-independent apoptosis induced by natural chemopreventive and therapeutic agents. Oncogene 2001;20:3387–98.
30. Mukherjee S, Conrad SE. c-Myc suppresses p21WAF1/CIP1 expression during estrogen signaling and antiestrogen resistance in human breast cancer cells. J Biol Chem 2005;280:17617–25.
31. Seoane J, Le HV, Massague J. Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. Nature 2002;419:729–34.
32. Rowland BD, Peeper DS. KLF4, p21 and context-dependent opposing forces in cancer. Nat Rev Cancer 2006;6:11–23.
33. Viale A, De Franco F, Orleth A, et al. Cell-cycle restriction limits DNA damage and maintains self-renewal of leukaemia stem cells. Nature 2009;457:51–6.
34. Brunet A, Bonni A, Zigmond MJ, et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 1999;96:857–68.
35. Paik JH, Kollipara R, Chu G, et al. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell 2007;128:309–23.
36. Myatt SS, Lam EW. The emerging roles of forkhead box (Fox) proteins in cancer. Nat Rev Cancer 2007;7:847–59.
37. Kalin TV, Wang IC, Ackerson TJ, et al. Increased levels of the FoxM1 transcription factor accelerate development and progression of prostate carcinomas in both TRAMP and LADY transgenic mice. Cancer Res 2006;66:1712–20.

38. Seoane J, Le HV, Shen L, Anderson SA, Massague J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell 2004;117:211–23.
39. Dong Y, Chi SL, Borowsky AD, Fan Y, Weiss RH. Cytosolic p21Waf1/Cip1 increases cell cycle transit in vascular smooth muscle cells. Cell Signal 2004;16:263–9.
40. Dupont J, Karas M, LeRoith D. The cyclin-dependent kinase inhibitor p21CIP/WAF is a positive regulator of insulin-like growth factor I-induced cell proliferation in MCF-7 human breast cancer cells. J Biol Chem 2003;278:37256–64.
41. Zhang C, Kavurma MM, Lai A, Khachigian LM. Ets-1 protects vascular smooth muscle cells from undergoing apoptosis by activating p21WAF1/Cip1: ETS-1 regulates basal and inducible p21WAF1/Cip: ETS-1 regulates basal and inducible p21WAF1/Cip1 transcription via distinct *cis*-acting elements in the p21WAF/Cip1 promoter. J Biol Chem 2003;278:27903–9.
42. Banham AH, Boddy J, Launchbury R, et al. Expression of the forkhead transcription factor FOXP1 is associated both with hypoxia inducible factors (HIFs) and the androgen receptor in prostate cancer but is not directly regulated by androgens or hypoxia. Prostate 2007;67:1091–8.
43. Furuyama T, Kitayama K, Shimoda Y, et al. Abnormal angiogenesis in Foxo1 (Fkhr)-deficient mice. J Biol Chem 2004;279:34741–9.
44. Gupta S, Joshi K, Wig JD, Arora SK. Intratumoral FOXP3 expression in infiltrating breast carcinoma: its association with clinicopathologic parameters and angiogenesis. Acta Oncol 2007;46:792–7.

Phase II Trial of Amrubicin for Second-Line Treatment of Advanced Non-small Cell Lung Cancer

Results of the West Japan Thoracic Oncology Group Trial (WJTOG0401)

Hiroyasu Kaneda, MD,† Isamu Okamoto, MD, PhD,* Hidetoshi Hayashi, MD,‡ Hiroshige Yoshioka, MD,‡ Masaki Miyazaki, MD, PhD,* Shinzoh Kudoh, MD, PhD,§ Tatsuo Kimura, MD, PhD,§ Takamune Sugiura, MD,†‖ Toshiyuki Sawa, MD, PhD,¶ Koji Takeda, MD,# Yasuo Iwamoto, MD, PhD,** Miyako Satouchi, MD, PhD,†† Kenji Akita, MD, PhD,‡‡ Hiroshi Saito, MD, PhD,§§ Isao Goto, MD, PhD,‖‖ Kazuhiko Shibata, MD, PhD,¶¶ Masahiro Fukuoka, MD, PhD,‖ and Kazuhiko Nakagawa, MD, PhD**

Background: Amrubicin is a synthetic anthracycline drug that is a potent inhibitor of topoisomerase II. We have performed a multicenter phase II trial to evaluate the efficacy and safety of amrubicin for patients with previously treated non-small cell lung cancer (NSCLC).

Methods: Patients with advanced NSCLC who experienced disease recurrence after one platinum-based chemotherapy regimen were eligible for enrollment in the study. Amrubicin was administered by intravenous injection at a dose of 40 mg/m^2 on 3 consecutive days every 3 weeks.

Results: Sixty-one enrolled patients received a total of 192 treatment cycles (median, 2; range, 1–15). Response was as follows: complete response, 0; partial response, seven (11.5%); stable disease, 20 (32.8%); and progressive disease, 34 (55.7%). Median progression-free survival was 1.8 months, whereas median overall survival was 8.5 months, and the 1-year survival rate was 32%. Hematologic toxicities of grade 3 or 4 included neutropenia (82.0%), leukopenia (73.8%), thrombocytopenia (24.6%), and anemia (27.9%). Febrile neutropenia occurred in 18 patients (29.5%). One treatment-related death due to infection was observed. Nonhematologic toxicities were mild.

Conclusions: Amrubicin is a possible alternative for second-line treatment of advanced NSCLC, although a relevant hematological toxicity is significant, especially with a febrile neutropenia.

Key Words: Amrubicin, Non-small cell lung cancer (NSCLC), Platinum refractory, Second-line chemotherapy.

(*J Thorac Oncol.* 2010;5: 105–109)

Non-small cell lung cancer (NSCLC) is the leading cause of death related to cancer worldwide.[1] The first-line platinum-based chemotherapy confers a moderate improvement in survival and quality of life in individuals with advanced NSCLC.[2,3] It has recently become generally accepted that the second-line chemotherapy also has beneficial effects on survival and quality of life in such patients.[3–5] Despite the availability of several options for the second-line treatment of NSCLC,[6] however, the life expectancy of patients with advanced disease remains short, highlighting the urgent need for new treatments.

Amrubicin is a fully synthetic anthracycline anticancer drug with a similar structure to doxorubicin and is a potent inhibitor of topoisomerase II.[7–9] Two phase II trials of amrubicin administered as a single agent yielded response rates of 18.7 to 27.9% with acceptable toxicities in chemotherapy-naive patients with advanced NSCLC,[10,11] suggestive of promising activity for such patients. However, the activity and safety of amrubicin for patients with NSCLC whose

*Department of Medical Oncology, Kinki University School of Medicine, Osaka-sayama, Osaka; †Department of Respiratory Medicine, Izumisano Municipal Hospital, Rinku General Medical Center, Izumisanoshi, Osaka; ‡Department of Respiratory Medicine, Kurashiki Central Hospital, Kurashiki, Okayama; §Department of Respiratory Medicine, Osaka City University Medical School, Abeno, Osaka; ‖Department of Medical Oncology, Sakai Hospital, Kinki University School of Medicine, Minami-ku, Sakai, Osaka; ¶Department of Respiratory Medicine and Oncology, Gifu Municipal Hospital, Gifu; #Department of Clinical Oncology, Osaka City General Hospital, Miyakojima, Osaka; **Department of Medical Oncology, Hiroshima City Hospital, Naka-ku, Hiroshima; ††Department of Thoracic Oncology, Hyogo Cancer Center, Akashi, Hyogo; ‡‡Department of Respiratory Medicine, Nagoya Central Hospital, Nakamura-ku, Nagoya, Aichi; §§Department of Respiratory Medicine, Aichi Cancer Center Aichi Hospital, Kake-machi, Okazaki, Aichi; ‖‖First Department of Internal Medicine, Division of Pulmonary Medicine, Osaka Medical College, Takatsuki, Osaka; and ¶¶Department of Medical Oncology, Kosciren Takaoka Hospital Comprehensive Cancer Center, Takaoka, Toyama, Japan.

Disclosure: The authors declare no conflicts of interest.

Address for correspondence: Isamu Okamoto, MD, PhD, Department of Medical Oncology, Kinki University School of Medicine, 377-2 Ohno-higashi, Osaka-sayama, Osaka 589-8511, Japan. E-mail: chiokamoto@dotd.med.kindai.ac.jp

ISSN: 1556-0864/10/0501-0105

disease progresses after first-line chemotherapy have not been previously described.

Therefore, we conducted a multicenter phase II trial of amrubicin in patients with NSCLC previously treated with platinum-based chemotherapy. This trial was designed to determine the antitumor activity and toxicity of amrubicin in the second-line setting.

PATIENTS AND METHODS

Patient Selection

The eligibility criteria for participation of subjects in the trial included histologic or cytologic evidence of NSCLC; stage IV or stage IIIB disease (including only patients with no indications for curative thoracic radiotherapy) at study entry; recurrent or refractory disease after one previous platinum-containing chemotherapy regimen; measurable disease; no chemotherapy or radiotherapy within the 4 weeks before study entry; an age of 20 to 74 years; an Eastern Cooperative Oncology Group performance status of 0 or 1; adequate bone marrow function (leukocyte count of $\geq$4000 and $\leq$12,000/mm^3, neutrophil count of $\geq$2000/mm^3, platelet count of $\geq$100,000/mm^3, and hemoglobin content of $\geq$9.5 g/dl); adequate other organ function (serum total bilirubin concentration of $\leq$1.5 mg/dl, serum aspartate aminotransferase and alanine aminotransferase levels of $\leq$2.5 times the upper normal limit, and normal serum creatinine concentration); partial pressure of arterial oxygen of $\geq$60 torr; no abnormality on the electrocardiogram requiring treatment; and a left ventricular ejection fraction of $\geq$60% on echocardiography. Patients were ineligible for participation in the study if they had undergone previous amrubicin therapy, a history of a cumulative doxorubicin dose >500 mg/m^2 (epirubicin >900 mg/m^2, pirarubicin >950 mg/m^2, and daunorubicin >25 mg/kg), symptomatic brain metastasis, third-space fluid collection requiring drainage, active concomitant malignancy, radiographic signs of interstitial pneumonia or pulmonary fibrosis, a serious or uncontrolled concomitant systemic disorder (active infection, active gastric or duodenal ulcer, heart disease, diabetes mellitus, or a condition requiring chronic systemic administration of corticosteroids), or a history of drug allergy, or if they were lactating or pregnant. This study was performed in accordance with the principles of the Declaration of Helsinki and the good clinical practice guidelines. Written informed consent was obtained from all patients before study entry. Trial document approval was obtained from the institutional review board of each participating institution.

Study Design and Sample Size

The study was a multicenter, open-label, single-arm, phase II study. The primary end point was the response rate for amrubicin in patients with recurrent or refractory NSCLC who experienced treatment failure with platinum-based chemotherapy, which determined the sample size based on an optimal two-stage design.[12] On the basis of the results of previous studies, the proposed regimen was to be considered worthy or not worthy for additional investigation in the selected patient population if a true response rate was obtained of $\geq$18 or $\leq$5%, respectively, with a power of 0.9 and an α error of 0.05. A total of 55 assessable patients was necessary for the study; 23 in the first stage and 32 in the second stage. Assuming a drop-out rate of 10%, we planned on enrolling 60 patients in the study.

Treatment

Amrubicin was reconstituted in 20 ml of physiological saline or 5% glucose solution and was administered intravenously for more than 5 minutes at a dose of 40 mg/m^2 per day on days 1 to 3 every 3 weeks. Patients with evidence of disease progression or who experienced unacceptable adverse events were withdrawn from the study. Other criteria for treatment discontinuation included treatment refusal by the patient, inadvertent enrollment in the study, use of excluded concomitant therapy, or a decision by the physician to stop treatment. Subsequent courses of treatment were withheld until the following criteria were satisfied: the leukocyte count was $\geq$3000/mm^3, the neutrophil count was $\geq$1500/mm^3, the platelet count was $\geq$100,000/mm^3, and the grade of any nonhematologic toxicity was $\leq$2. If these criteria were not satisfied within 43 days after the onset of the last treatment, the patient was removed from the study. The dose of amrubicin was reduced to 35 mg/m^2 per day if leukopenia or neutropenia of grade 4 for more than 4 days, febrile neutropenia, thrombocytopenia of grade 4, or nonhematologic toxicity of grade $\geq$3 (or of grade 4 for anorexia, nausea, body weight loss, or hyponatremia) occurred during the previous course. If these toxicities occurred after reduction of the amrubicin dose to 35 mg/m^2 per day, the dose was reduced further to 30 mg/m^2 per day. The third reduction of amrubicin dose was not allowed.

Evaluation

Tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors.[13] Tumors were measured by computed tomography within 4 weeks before the first cycle of treatment. The same measurement was performed every 4 weeks from the onset of treatment. A central radiologic review was performed to determine the eligibility of patients and the response to treatment. Response was confirmed at least 4 (for a complete or partial response) or 6 weeks (for stable disease) after it was first documented. Progression-free survival was defined as the time from registration until objective tumor progression or death. Patients whose disease had not progressed at the time of discontinuation of the study treatment were assessed until progression was documented. If a patient died without documentation of disease progression, the patient was considered to have had tumor progression at the time of death, unless there was sufficient documented evidence to conclude otherwise. Overall survival was defined as the time from registration until death from any cause. Progression-free and overall survival and the 1-year survival rate were estimated by the Kaplan-Meier method. Adverse events were graded according to National Cancer Institute Common Toxicity Criteria (version 3). All patients who received one dose of chemotherapy were assessable for toxicity. Clinical and laboratory assessment was performed at least once a week.

RESULTS

Patient Characteristics

Between February 2005 and March 2006, 61 patients were enrolled in the study at 12 participating institutions. All patients were eligible for the study and assessable both for the efficacy and safety of treatment and for survival. The characteristics of the study subjects are summarized in Table 1. Thirty-nine patients were men and 22 were women, and their median age was 63 years, with a range of 51 to 74 years. Histologic analysis revealed that 40 patients (65.6%) had adenocarcinoma, and 14 patients (23.0%) had squamous cell carcinoma. Forty-eight patients (78.7%) had stage IV disease, and the other 13 patients had stage IIIB disease at the time of enrollment in the study. All 61 patients had been previously treated with platinum-based chemotherapy, with eight and 22 patients having also undergone surgery or radiation therapy, respectively, before enrollment in the study.

Treatment Administered

Patients received a median of two cycles of treatment (range, 1–15), with 16 patients (26.2%) receiving at least four cycles. A total of 192 cycles of treatment was delivered overall. The mean relative dose intensity of amrubicin was 87.3%. Dose reduction of amrubicin was necessary according to the study protocol in 22 cycles (11.5% of total cycles). The major reasons for dose reduction were neutropenia or leukopenia of grade 4 (13 cycles of all cycles) and febrile neutropenia (nine cycles of all cycles). Treatment was discontinued in 14 patients after the first cycle and in 17 patients after the second cycle; the reasons for discontinuation included progressive disease (25 patients), toxicity (four patients), and patient refusal (two patients). Poststudy, 71% of patients eventually received subsequent therapies. Twenty-eight patients (46%) received docetaxel-containing chemotherapy, 18 (26%) received gefitinib or erlotinib, and 30 (49%) received other chemotherapy.

TABLE 1. Characteristics of the 61 Eligible Patients

Characteristic	No. of Patients (%)
Median age (yr)	
<70	48 (78.7)
≥70	13 (21.3)
Sex	
Male	39 (63.9)
Female	22 (36.1)
Performance status (ECOG)	
0	15 (24.6)
1	46 (75.4)
Disease stage	
III B	13 (21.3)
IV	48 (78.7)
Tumor histology	
Adenocarcinoma	40 (65.6%)
Squamous cell carcinoma	14 (23.0%)
Large cell carcinoma	3 (4.9)
NSCLC, not specified	4 (6.6)
Prior therapy	
Chemotherapy	61 (100)
Radiotherapy	22 (36.1)
Surgery	8 (13.1)
Time since last chemotherapy	
<3 mo	28 (46.0)
3–6 mo	16 (26.0)
≥6 mo	17 (28.0)
Response to prior chemotherapy	
Complete response	1 (1.6)
Partial response	36 (59.0)
Stable or progressive disease	19 (31.1)
Not evaluable	5 (8.2)

ECOG, Eastern Cooperative Oncology Group; NSCLC, non-small cell lung cancer.

Response and Survival

Among the 61 assessable patients, there were seven partial responses and no complete responses, for an overall response rate of 11.5% (95% confidence interval [CI], 4.7–22.2) (Table 2). Twenty patients (32.8%) had stable disease, yielding an overall disease control rate (complete response + partial response + stable disease) of 44.3% (95% CI, 31.5–57.6). Thirty-four patients had progressive disease as the best response. No correlation was apparent between the response rate and sex, age, tumor histology, disease stage, or smoking status.

Of the 61 subjects, 11 patients were still alive as of October 2008. The progression-free survival curve is shown in Figure 1; the median progression-free survival was 1.8 months (95% CI, 1.4–2.3). The curve for overall survival is shown in Figure 2; the median overall survival time was 8.5 months (95% CI, 7.7–10.4), and the 1-year survival rate was 32% (95% CI, 20.7–44.0).

Safety

The adverse events observed for all 61 treated patients are summarized in Table 3. The most frequent toxicity was myelosuppression, which mostly affected leukocytes. Neutropenia or leukopenia of grade ≥3 occurred in 82.0% and 73.8% of patients, respectively. Anemia and thrombocytopenia of grade ≥3 were relatively infrequent, occurring in 27.9% and 24.6% of patients, respectively. Eighteen patients (29.5%) developed febrile neutropenia. The most common

TABLE 2. Overall Response Rate for Amrubicin (Response Evaluation Criteria in Solid Tumors) as Determined by Independent Radiological Assessment

Response	No. of Patients
Complete response	0
Partial response	7 (11.5%; 95% CI, 4.7–22.2)
Overall response	7 (11.5%)
Stable disease	20 (32.8%)
Disease control	27 (44.3%; 95% CI, 31.5–57.6)
Progressive disease	34 (55.7%)

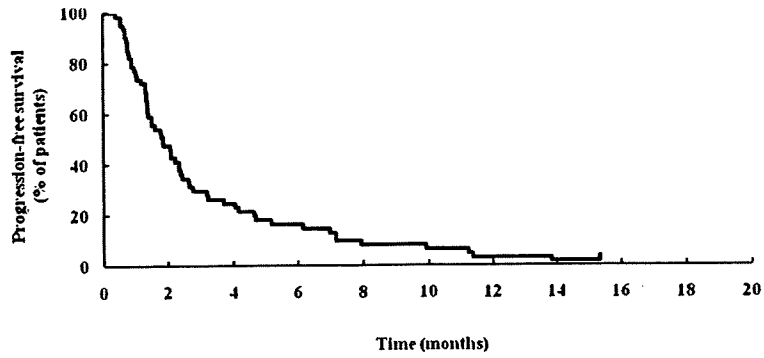


FIGURE 1. Kaplan-Meier analysis of progression-free survival for all 61 treated patients.

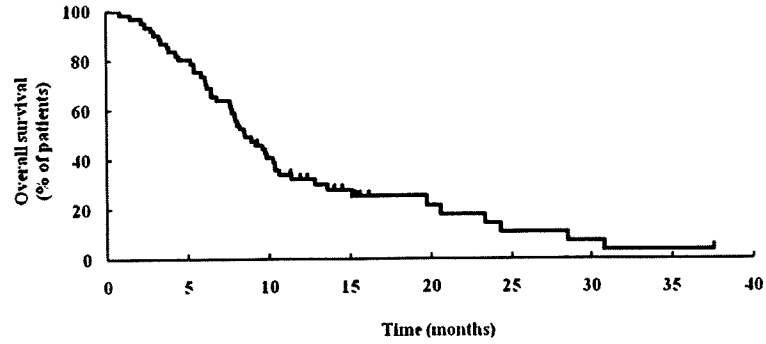


FIGURE 2. Kaplan-Meier analysis of overall survival for all 61 treated patients.

TABLE 3. Toxicity for all 61 Patients During Amrubicin Monotherapy According to the National Cancer Institute Common Toxicity Criteria (Version 3)

	Grade				Grade ≥3	
Toxicity	1	2	3	4	No.	Percentage
Leukopenia	5	8	24	21	45	73.8
Neutropenia	0	5	8	42	50	82.0
Anemia	16	27	13	4	17	27.9
Thrombocytopenia	25	7	7	8	15	24.6
Febrile neutropenia	0	0	18	0	18	29.5
Anorexia	19	9	5	1	6	9.8
Nausea	20	5	2	0	2	3.3
Vomiting	7	3	0	0	0	0
Asthenia	18	13	2	2	4	6.6
Infection	0	1	2	1	4[a]	6.6
Fever	10	6	1	0	1	1.6
Elevation of AST or ALT	15	3	1	3	4	6.6
Pneumonitis	1	0	1	0	1	1.6

[a] Includes one treatment-related death (grade 5).
AST, aspartate aminotransferase; ALT, alanine aminotransferase.

nonhematologic toxicities of grade 3 or 4 were anorexia (9.8%), asthenia (6.6%), an increase in serum alanine aminotransferase and aspartate aminotransferase levels (6.6%), and infection (6.6%), but most nonhematologic toxicities were mild. No cardiac toxicity was observed during the study. Pneumonitis of grade 3 occurred in one patient. One treatment-related death due to sepsis after febrile neutropenia occurred.

DISCUSSION

Amrubicin is a novel, fully synthetic anthracycline agent that is active against both NSCLC and small cell lung cancer (SCLC).[10,11,14–16] No prospective study evaluating the efficacy and safety of amrubicin for previously treated NSCLC has been reported. We have now demonstrated the efficacy of amrubicin monotherapy for patients with NSCLC previously treated with platinum-based chemotherapy, as shown by a response rate of 11.5%, median overall survival of 8.5 months, and 1-year survival rate of 32% in 61 patients. Previous phase III trials for second- or third-line treatment of NSCLC have shown response rates of 7.6 to 9.1%, median overall survival times of 6.7 to 8.3 months, and 1-year survival rates of 29.7 to 34%.[4,5,17–19] Amrubicin is a potent inhibitor of topoisomerase II, with its mechanism of action differing from those of currently available active agents for advanced NSCLC.[7–9] Given the encouraging results from our trial and the unique mode of action of amrubicin, this drug is a good candidate for the development of a new second-line treatment for NSCLC.

Treatment was discontinued in 14 patients after the first cycle and 17 patients after the second cycle. Of these 31 patients, 25 patients were withdrawn because of progressive disease. The study protocol required assessment of antitumor effect by computed tomography every 4 weeks. Such assessment, performed to avoid ineffective therapy, resulted in early discontinuation of treatment due to progressive disease and thereby yielded a median progression-free survival that was slightly shorter than otherwise might have been obtained.

Two recent phase II trials of amrubicin for previously treated SCLC, in which the drug was administered at the

same dose and according to the same schedule as in the present study, found that treatment was associated with a high incidence of bone marrow suppression, although drug toxicity was manageable.[20,21] Consistent with these results, the major adverse events in this study were hematologic toxicities of grade 3 or 4 including neutropenia (82.0%), leukopenia (73.8%), anemia (27.9%), and thrombocytopenia (24.6%). The incidence of these toxicities in this study was similar to that observed previously in the phase II trials for previously treated SCLC. However, the incidence of febrile neutropenia of grade 3 was higher in our study (29.5%) than in these previous trials (5–14%). One possible explanation for this difference is the frequent use of granulocyte colony-stimulating factor for treatment of SCLC, when compared with treatment for NSCLC. The incidents of significant neutropenia and febrile neutropenia were seen primarily in the first cycle. In this study, patients who experienced severe hematologic toxicities were not allowed to receive prophylactically granulocyte colony-stimulating factor in subsequent cycles. One treatment-related death due to sepsis after febrile neutropenia occurred in our study. Therefore, it is important to monitor closely leukocyte and neutrophil counts during amrubicin therapy in patients with previously treated NSCLC. Nonhematologic toxicity was manageable in this study. Another adverse event of particular concern for amrubicin is cardiac toxicity, given that the chemical structure of the drug is similar to that of doxorubicin, whose cardiac toxicity has been experimentally and clinically established. Indeed, cardiac toxicity was detected in previous trials of amrubicin, although its frequency (3.2%) was relatively low.[10,11] For safety reasons, this study allowed the enrollment only of patients with a left ventricular ejection fraction of $\geq$60% as determined by echocardiography. No cardiac toxicity was observed during our trial, even in the three patients who received more than eight cycles of amrubicin therapy.

In conclusion, in this first reported phase II study of the efficacy and safety of amrubicin monotherapy as a second-line treatment for advanced NSCLC previously treated with platinum-based chemotherapy, we obtained a response rate, overall survival, and 1-year survival rate comparable with those of other second-line treatment regimens. This activity despite a relevant hematological toxicity of amrubicin monotherapy is a possible alternative for second-line treatment of advanced NSCLC. Further evaluation of amrubicin for refractory or relapsed NSCLC in randomized phase III trials is warranted.

ACKNOWLEDGMENTS

The authors thank Yukari Hirai and Shinichiro Nakamura for data management.

REFERENCES

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin* 2007;57:43–66.
2. Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. Non-small Cell Lung Cancer Collaborative Group. *BMJ* 1995;311:899–909.
3. Pfister DG, Johnson DH, Azzoli CG, et al.; American Society of Clinical Oncology. American Society of Clinical Oncology treatment of unresectable non-small-cell lung cancer guideline: update 2003. *J Clin Oncol* 2004;22:330–353.
4. Shepherd FA, Dancey J, Ramlau R, et al. Prospective randomized trial of docetaxel versus best supportive care in patients with non-small-cell lung cancer previously treated with platinum-based chemotherapy. *J Clin Oncol* 2000;18:2095–2103.
5. Shepherd FA, Rodrigues Pereira J, Ciuleanu T, et al.; National Cancer Institute of Canada Clinical Trials Group. Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* 2005;353:123–132.
6. Noble J, Ellis PM, Mackay JA, Evans WK; Lung Cancer Disease Site Group of Cancer Care Ontario's Program in Evidence-based Care. Second-line or subsequent systemic therapy for recurrent or progressive non-small cell lung cancer: a systematic review and practice guideline. *J Thorac Oncol* 2006;1:1042–1058.
7. Hanada M, Mizuno S, Fukushima A, Saito Y, Noguchi T, Yamaoka T. A new antitumor agent amrubicin induces cell growth inhibition by stabilizing topoisomerase II-DNA complex. *Jpn J Cancer Res* 1998;89:1229–1238.
8. Yamaoka T, Hanada M, Ichii S, Morisada S, Noguchi T, Yanagi Y. Cytotoxicity of amrubicin, a novel 9-aminoanthracycline, and its active metabolite amrubicinol on human tumor cells. *Jpn J Cancer Res* 1998;89:1067–1073.
9. Noguchi T, Ichii S, Morisada S, Yamaoka T, Yanagi Y. Tumor-selective distribution of an active metabolite of the 9-aminoanthracycline amrubicin. *Jpn J Cancer Res* 1998;89:1061–1066.
10. Sawa T, Yana T, Takada M, et al. Multicenter phase II study of amrubicin, 9-amino-anthracycline, in patients with advanced non-small-cell lung cancer (Study 1): West Japan Thoracic Oncology Group (WJTOG) trial. *Invest New Drugs* 2006;24:151–158.
11. Takeda K, Takifuji N, Negoro S, et al. Phase II study of amrubicin, 9-amino-anthracycline, in patients with advanced non-small-cell lung cancer: a West Japan Thoracic Oncology Group (WJTOG) study. *Invest New Drugs* 2007;25:377–383.
12. Simon R. Optimal two-stage designs for phase II clinical trials. *Control Clin Trials* 1989;10:1–10.
13. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000;92:205–216.
14. Yana T, Negoro S, Takada M, et al.; West Japan Thoracic Oncology Group. Phase II study of amrubicin in previously untreated patients with extensive-disease small cell lung cancer: West Japan Thoracic Oncology Group (WJTOG) study. *Invest New Drugs* 2007;25:253–258.
15. Kurata T, Okamoto I, Tamura K, Fukuoka M. Amrubicin for non-small-cell lung cancer and small-cell lung cancer. *Invest New Drugs* 2007;25:499–504.
16. Okamoto I, Hamada A, Matsunaga Y, et al. Phase I and pharmacokinetic study of amrubicin, a synthetic 9-aminoanthracycline, in patients with refractory or relapsed lung cancer. *Cancer Chemother Pharmacol* 2006;57:282–288.
17. Fossella FV, DeVore R, Kerr RN, et al. Randomized phase III trial of docetaxel versus vinorelbine or ifosfamide in patients with advanced non-small-cell lung cancer previously treated with platinum-containing chemotherapy regimens. The TAX 320 Non-small Cell Lung Cancer Study Group. *J Clin Oncol* 2000;18:2354–2362.
18. Hanna N, Shepherd FA, Fossella FV, et al. Randomized phase III trial of pemetrexed versus docetaxel in patients with non-small-cell lung cancer previously treated with chemotherapy. *J Clin Oncol* 2004;22:1589–1597.
19. Kim ES, Hirsh V, Mok T, et al. Gefitinib versus docetaxel in previously treated non-small-cell lung cancer (INTEREST): a randomised phase III trial. *Lancet* 2008;372:1809–1818.
20. Inoue A, Sugawara S, Yamazaki K, et al. Randomized phase II trial comparing amrubicin with topotecan in patients with previously treated small-cell lung cancer: North Japan Lung Cancer Study Group Trial 0402. *J Clin Oncol* 2008;26:5401–5406.
21. Onoda S, Masuda N, Seto T, et al.; Thoracic Oncology Research Group Study 0301. Phase II trial of amrubicin for treatment of refractory or relapsed small-cell lung cancer: Thoracic Oncology Research Group Study 0301. *J Clin Oncol* 2006;24:5448–5453.

A Phase I Study of Topotecan Plus Carboplatin for Relapsed SCLC

WJTOG Trial

Takayasu Kurata, MD, PhD, Tatsuhiko Kashii, MD, PhD, Koji Takeda, MD, Nobuhiko Seki, MD, PhD, Masahiro Tsuboi, MD, PhD, Masashi Kobayashi, MD, Taroh Satoh, MD, Kazuhiko Nakagawa, MD, PhD, and Masahiro Fukuoka, MD, PhD

Introduction: A phase I study on relapsed small cell lung cancer (SCLC) was conducted to establish the toxicity and maximum tolerated dose of carboplatin with topotecan, and to observe the antitumor activity.

Methods: Thirty-two SCLC patients who had received one previous line of chemotherapy were enrolled. Topotecan was infused for 30 minutes on days 1 to 5, and carboplatin for 60 minutes after the topotecan infusion on day 5 every 3 weeks. Granulocyte colony-stimulating factor prophylaxis was administered from day 8 to white blood cell or neutrophil recovery.

Results: The most frequent toxicities were neutropenia and thrombocytopenia. Nonhematological toxicities were generally mild. Three of six patients experienced a dose-limiting toxicity, thrombocytopenia, at dose level 6: 0.85 mg/m^2 topotecan with area under curve 5 carboplatin (maximum tolerated dose). Of 29 evaluable patients, 5 (17.2%) had partial responses. Of 21 patients who relapsed more than 90 days after completion of first-line chemotherapy, 5 (23.8%) had partial responses. Median overall survival time and 1-year survival rate were 11.3 months and 50.0%, respectively.

Conclusions: The recommended dose for further studies is 0.75 mg/m^2 of topotecan on days 1 to 5 with area under curve 5 of carboplatin on day 5 every 3 weeks. This combination is well tolerated, and is promising for sensitive relapsed SCLC. A comparative study against single-agent topotecan for sensitive relapsed SCLC is warranted.

Key Words: Combination phase I study, Maximum tolerated dose, Topotecan, Carboplatin, SCLC.

(*J Thorac Oncol.* 2009;4: 644 -648)

Department of Medical Oncology, Kinki University, School of Medicine, Osaka-Sayama, Osaka, Japan.

Disclosure: The authors declare no conflicts of interest.

Address for correspondence: Takayasu Kurata, MD, Department of Medical Oncology, Kinki University, School of Medicine, 377-2, Ohno-Higashi, Osaka-Sayama, Osaka, 589-8511, Japan. E-mail: ctc002@poh.osaka-med.ac.jp

ISSN: 1556-0864/09/0405-0644

Small cell lung cancer (SCLC) accounts for 15 to 20% of all lung cancers. As SCLC is highly sensitive to chemotherapy and radiation, chemotherapy plays a central role in the treatment of SCLC. Although initial standard chemotherapies such as cisplatin plus etoposide[1] or cisplatin plus irinotecan[2] yield high response rates, especially in terms of complete remission, in SCLC, the majority of patients experience relapse and the 5-year survival rate is disappointing, at 20 to 25% for patients with limited disease and only 1 to 2% for those with extensive disease. Moreover, the outcome for patients who receive second-line chemotherapy after relapse is generally poor, depending on the interval from the day of the last administration of first-line chemotherapy to the day of relapse.[3,4] It seems that the response rate of second-line chemotherapy with an interval of 90 days or more for "sensitive cases" is higher than that of chemotherapy with an interval of less than 90 days for "refractory cases."

Topotecan targets DNA topoisomerase I. Recently, two randomized phase III studies on the effect of topotecan, as a single agent and second-line chemotherapy, for relapsed SCLC were performed.[5,6] The first randomized study showed that topotecan alone had an equivalent effect to the combination chemotherapy (cyclophosphamide, doxorubicin, and vincristine: CAV) in sensitive cases. In this study, topotecan achieved a response rate of 24% (CAV, 18%) and a median survival time (MST) of 25 weeks (CAV, 24.7 weeks), with significantly better symptom relief.[5] The second randomized study demonstrated that oral topotecan prolonged overall survival more significantly than best supportive care (25.9 weeks versus 13.9 weeks) in relapsed SCLC patients, who were not considered as candidates for standard intravenous chemotherapy, including refractory cases. Moreover, patients receiving oral topotecan also showed better quality of life and greater symptom control.[6] Based on these results, it would seem that topotecan alone should be the standard of care for relapsed SCLC; however, as there have been few randomized trials in the second-line setting, whether results of combination chemotherapy can exceed those of the single agent is not yet well known.

We planned combination chemotherapy using carboplatin with the present standard of care, topotecan. Cisplatin is a platinum preparation that has an important role in the

treatment of SCLC, but its cumulative toxicity such as neural and renal toxicities from first-line therapy was a matter of concern. We thus chose carboplatin rather than cisplatin. However, until now, combination chemotherapy of topotecan and carboplatin for relapsed SCLC had not been fully evaluated. Trials on ovarian cancer demonstrated that the toxicity of combination therapy for carboplatin on day 5 with topotecan on days 1 to 5 was significantly milder than that of carboplatin on day 1 with topotecan. Therefore, we chose a schedule in which carboplatin was administered on day 5 in combination with topotecan on days 1 to 5 every 3 weeks.[7] As both carboplatin and topotecan have overlapping toxicities, neutropenia and leucopenia, we decided to give granulocyte colony-stimulating factor (G-CSF) prophylaxis.

The purposes of this study were to establish the toxicity and maximum tolerated dose (MTD) of this combination, to determine the recommended dose for further studies, and to assess the antitumor activity.

PATIENTS AND METHODS

Patient Eligibility

Patients with histologic or cytologic confirmation of SCLC who had received one previous chemotherapy regimen were eligible. The eligibility criteria were as follows (1): measurable lesions (2); age 20–75 years (3); Eastern Cooperative Oncology Group performance status 0–1 (4); life expectancy of at least 3 months (5); adequate organ function (12,000/μl $\geq$ white blood cell count (WBC) $\geq$4000/μl, absolute neutrophil count $\geq$2000/μl, platelet count $\geq$100,000/μl, hemoglobin count $\geq$9.5 g/dl, serum total bilirubin $\leq$1.5 mg/dl, serum transaminase $\leq$2.5 $\times$ upper normal limits, serum creatinine $\leq$ upper normal limits, PaO_2 $\geq$60 torr). At least 4 weeks had to have passed after completion of the previous chemotherapy. Prior radiotherapy and surgery were allowed. The exclusion criteria were as follows (1): pulmonary fibrosis or interstitial pneumonitis with symptoms or apparent abnormalities on chest radiograph (2); massive pleural effusion, pericardial effusion, or ascites (3); pregnancy (4); lactation (5); fertile men with no intention of using contraception (6); symptomatic brain metastases (7); active concurrent malignancies (8); patients who had received a bone marrow transplantation or peripheral blood stem cell transplantation (9); severe drug allergies (10); severe comorbidities. Prior topotecan chemotherapy was not allowed. This study was approved by the Institutional Review Board at each institute, and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Pretreatment and Follow-Up Studies

Prior to entry, complete history-taking and physical examination were performed regarding age, height, weight, performance status, histologic diagnosis, tumor stage, details of previous treatment, and presence of complication. The pretreatment laboratory investigations included complete blood cell count, differential WBC, platelet count, serum electrolytes, total protein, albumin, total bilirubin, transaminase, alkaline phosphatase, lactate dehydrogenase, BUN, creatinine, creatinine clearance, and urinalysis. After initiation of therapy, blood count was repeated twice per week, and blood chemistry and urinalysis were repeated weekly. Lesions were measured in every second course at minimum. Toxicity was evaluated in accordance with the National Cancer Institute Common Toxicity Criteria version 2. Tumor responses were assessed using the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines.[8] Time to progression was measured from the date of registration to the date of first progression or death from any cause. Survival time was also measured from the date of registration to the date of death or latest follow-up, and was calculated using the Kaplan-Meier method.[9]

Drug Administration, Dose Escalation, and Sample Size

The treatment schedule included topotecan, diluted with 100 ml of normal saline, given intravenously as a 30-minute infusion on days 1 through 5, and carboplatin with 250 to 500 ml of normal saline, given intravenously over 60 minutes after completion of topotecan infusion on day 5 every 3 weeks. All patients were allowed to receive antiemetics with dexamethasone, metoclopramide, or 5HT3 antagonists at the treating physician's discretion. G-CSF prophylaxis was administered from day 8 until WBC recovered to more than 10,000/μl or absolute neutrophil count to more than 5000/μl. The subsequent courses were delayed if any of the following parameters were not met: WBC $\geq$3000/μl, platelet count $\geq$100,000/μl, or recovery to at least grade 1 nonhematological toxicity. If dose-limiting toxicities (DLTs) occurred, the patient was withdrawn from the study in principle, but when an antitumor effect could be expected, the dose of topotecan was decreased by one dose level and that of carboplatin was reduced to area under curve (AUC) 4. At the initial dose level, dose reduction was not permitted and when it was required the patient was withdrawn from this study.

The starting dose of topotecan was 0.50 mg/m^2 and the targeted AUC of carboplatin was 5. In the dose escalation method, the AUC of carboplatin was fixed at 5, and only the dose of topotecan was increased, by 0.10 mg/m^2 or by 0.05 mg/m^2 (topotecan: level 1, 0.50 mg/m^2; level 2, 0.60 mg/m^2; level 3, 0.65 mg/m^2; level 4, 0.70 mg/m^2; level 5, 0.75 mg/m^2; level 6, 0.85 mg/m^2). Intrapatient dose escalation was not allowed. At least three patients were treated at each dose level, and three additional patients were entered at the same dose level if DLT was observed in one or two of the first three patients. The MTD was defined as the dose level at which all three of the first three patients, or three of any six patients, experienced DLT. The definitions of DLT were as follows (1): grade 4 neutropenia for more than 4 days (2); thrombocytopenia <20,000/μl (3); grade 3 febrile neutropenia (4); grade 3 nonhematological toxicity except nausea/vomiting, appetite loss, constipation, hyponatremia, and weight loss (5); grade 2 interstitial pneumonitis or pulmonary fibrosis (6); grade 4 constipation, and hyponatremia (7); delayed administration of the subsequent course by more than 2 weeks.

TABLE 1. Patients' Characteristics

No. of patients	32
Age (yr)	
Median (range)	64 (43–74)
Sex	
Male/Female	23/9
Performance status	
0/1	15/17
Interval from the completion of 1st line (evaluable 29 patients)	
more than 90 days	21
less than 90 days	7
unknown*	1
Body weight loss	
Absent	27
Present	1
Unknown	4
Prior therapy	
Surgery	0
Radiation	14
Chemotherapy	32
CDDP/CBDCA + Etoposide	18
CPT-11-containing	12
CDDP + Amrubicin	2

*One patient was excluded from this analysis because the exact day of the last administration of 1st-line Chemotherapy was unknown.

TABLE 2. Toxicities in All Courses of Topotecan and Carboplatin (32 Patients)

	Grade			
	1	2	3	4
White blood count	5	13	11	0
Absolute neutrophil count	1	11	12	3
Hemoglobin	5	15	11	1
Platelets	6	8	17	0
Nausea	9	4	2	0
Vomiting	2	2	1	0
Fatigue	16	2	1	1
Constipation	5	7	4	0
Diarrhea	7	0	1	0
Mucositis	1	3	1	0
Pneumonitis	0	0	0	0
Neutropenic fever	0	0	0	0
Infection	0	3	1	0

RESULTS

Patients were registered from six institutions that were members of the West Japan Thoracic Oncology Group. Between October 2002 and January 2006, 32 patients (dose level- number of patients: 1–6, 2–3, 3–8, 4–3, 5–6, 6–6) were enrolled. The total and median number of courses were 90 and 2.5 (range, 1–6), respectively. The patients' characteristics are shown in Table 1. Nine patients were female. The median age was 64 (range, 43–74). The regimen most often given as first-line chemotherapy was cisplatin or carboplatin plus etoposide, which 18 patients (56.3%) received. Twelve patients (37.5%) had received a regimen including CPT-11. Fourteen patients (43.8%) had had prior radiotherapy and no patient had had prior surgery.

Toxicity

All 32 patients were evaluable for toxicity. The major toxicities following all courses are listed in Table 2. The most frequent toxicities were hematological toxicities. Grade 3 thrombocytopenia occurred in 17 of 32 patients (53.1%), and 6 patients (18.8%) received platelet transfusions (one patient at level 1, one at level 5, and 4 at level 6); however, no patient had hemorrhagic complications. Grade 3/4 leukopenia, neutropenia, and anemia occurred in 34.4/0%, 37.5/9.4%, and 34.4/3.1%, of patients, respectively. There were fewer cases with grade 3/4 leukopenia or neutropenia than expected, owing to G-CSF prophylaxis. Two patients (6.3%) received blood transfusions (one at level 1 and the other at level 6). Nonhematological toxicities were generally mild. The most frequent grade 3 nonhematological toxicities were constipation, nausea, and appetite loss. No patient experienced pneumonitis or neutropenic fever, and there was no treatment-related death. One patient at level 2 underwent dose reduction after the third course by judgment of the treating physician owing to grade 3 nausea and vomiting, and the dose for one patient at level 3 was also reduced after the second course because of delayed administration of the subsequent course by more than 2 weeks. Moreover, the dose was reduced in one patient at level 5 and 2 patients at level 6 after their first course because of thrombocytopenia. The median length of the delay before starting the subsequent course was 28 days (21–49 days). Among 90 courses, only 14 courses (15.6%) proceeded to the next course without delay, as is stipulated in the protocol.

Maximum Tolerated Dose and Dose-Limiting Toxicities

At level 1, one patient suffered from grade 3 infection, which was considered a DLT. As 5 other patients at level 1 did not experience any DLTs, the dose was escalated to the next level. At level 2, none of the patients had a DLT. At level 3, one of six evaluable patients had a DLT, grade 3 diarrhea, during administration of topotecan; however, the other patients at levels 3 and 4 did not experience any DLTs, so we escalated the dose to level 5. At level 5, one patient had grade 3 thrombocytopenia, which was considered a DLT, and received platelet transfusion. At level 6, three of six patients also experienced the DLT, grade 3 thrombocytopenia, and all 3 patients received platelet transfusions. Therefore, dose level 6, 0.85 mg/m^2 topotecan with AUC 5 carboplatin was regarded as the MTD, according to the protocol criteria. The recommended dose level for further phase II study was determined to be 0.75 mg/m^2 topotecan with AUC 5 carboplatin.

Response and Survival

A total of 29 patients were evaluable for response (Table 3). There were 5 partial responses, with an overall response rate of 17.2% (5.8–35.8%). Among 21 patients who

TABLE 3. Response Data (n = 29)

Dose Level	No. of Evaluable Patients	CR	PR	SD	PD	NE
1	6	0	0	2	4	0
2	1	0	0	0	1	0
3	7	0	2	2	2	1
4	3	0	0	3	0	0
5	6	0	0	4	2	0
6	6	0	3	1	2	0
Total	29	0	5	12	11	1

CR, complete response; PR, partial response; SD, stable disease; PD, progression disease; NE, not evaluated.

relapsed more than 90 days after completion of first-line chemotherapy, five had partial responses, with an overall response rate of 23.8%, and 8 patients had stable disease. Conversely, among seven patients who relapsed less than 90 days after completion of first-line chemotherapy, none had a complete or partial response, and four had stable disease. The median progression-free survival time was 3.2 months (Figure 1). The MST and 1-year survival rate were 11.3 months and 50.0%, respectively (Figure 2). In sensitive cases, the MST and 1-year survival rate were 10.6 months and 47.6%, respectively.

DISCUSSION

In this study, the overall response rate was 17.2%, which was significantly less than the 20% supposed to be invalid; however, the survival result in this study was excellent as compared with the results of previous phase III studies for relapsed SCLC. Moreover, the outcome of second-line chemotherapy highly depends on the interval between the day of the last administration of first-line chemotherapy and the day of recurrence. The shorter this interval, the lower the response rate. In clinical trials for relapsed SCLC, therefore, careful attention is required to evaluate the response. In the current study, the response rate for 21 patients who had disease progression more than 90 days after completion of first-line chemotherapy was 23.8%, whereas no response was observed among seven patients having disease progression less than 90 days later. The results of only sensitive cases were not necessarily disappointing, so additional studies in patients with sensitive relapsed SCLC may be warranted to evaluate the efficacy of this combination. In addition, the survival results of sensitive relapsed cases were very promising. However, from this study, this combination treatment was invalid for refractory relapsed cases.

Recently, a Japanese study showed that amrubicin, a topoisomerase II inhibitor, as a single agent, had an overall response rate of 50% in refractory cases and 52% in sensitive cases.[10] If single agents such as amrubicin are really effective for both sensitive and refractory cases, the combination of

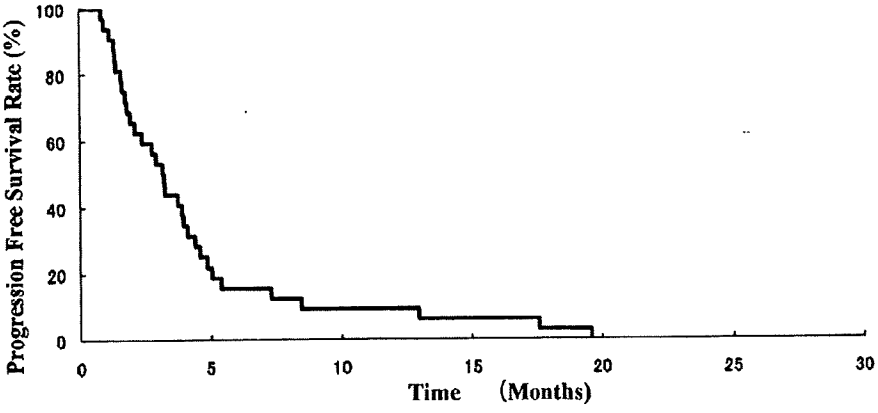


FIGURE 1. Kaplan-Meier curve for progression-free survival.

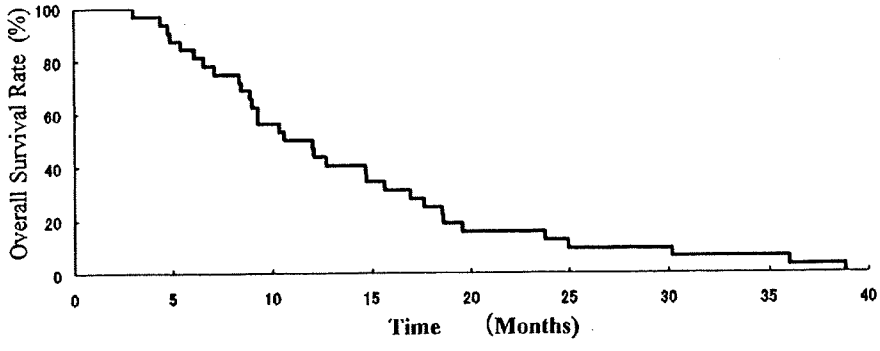


FIGURE 2. Kaplan-Meier curve for overall survival.

carboplatin and topotecan would seem unnecessary; however, no conclusion can be drawn, because no results of randomized studies that evaluated the efficacy of amrubicin have been reported as of this writing. As there have been few such controlled trials, and the standard treatment has not been established in this setting, randomized trials to determine whether combination therapy is more useful than monotherapy are warranted.

Regarding toxicity, this carboplation-topotecan combination was generally well tolerated. The most frequent toxicity was thrombocytopenia. Grade 3 thrombocytopenia occurred in 17 of 32 patients (53.1%), and 6 patients received platelet transfusions. Thrombocytopenia became the decisive factor in determination of the MTD. However, only 3 patients had grade 4 neutropenia among all courses because of G-CSF prophylaxis. In contrast, no nonhematological toxicity was associated with dose escalation. In addition, the median interval between cycles was 28 days, and most patients had a delay of 21 days, as stipulated in the protocol. There is a possibility that decreased dose intensity led to the disappointing result of low response rate.

According to the design, prophylactic G-CSF was administered from day 8. Therefore, few patients had grade 4 neutropenia and none had neutropenic fever. However, skepticism has recently risen regarding dose-intensive chemotherapy in the treatment of SCLC, especially in the second-line setting. In addition, G-CSF administration is very expensive. There is thus a risk that compliance in therapy with prophylactic G-CSF would be low. Therefore, an additional phase I study without prophylactic G-CSF may be necessary before conduct of randomized studies.

In conclusion, the combination of carboplatin and topotecan was generally well tolerated, although most patients had a delay of 21 days, as stipulated in the protocol. The main DLT was thrombocytopenia. The recommended dose for further phase II and III studies is 0.75 mg/m^2 topotecan on days 1 to 5 with AUC 5 carboplatin on day 5 with prophylactic G-CSF every 3 weeks. A phase III comparative study against single-agent topotecan for sensitive relapsed SCLC is warranted because the survival result of this combination is very promising, although the response rate was lower than expected.

REFERENCES

1. Evans WK, Shepherd FA, Feld R, et al. VP-16 and cisplatin as first-line therapy for small-cell lung cancer. *J Clin Oncol* 1985;3:1471 1477.
2. Kudoh S, Fujiwara Y, Takada Y, et al. Phase II study of irinotecan combined with cisplatin in patients with previously untreated small-cell lung cancer. *J Clin Oncol* 1998;16:1068–1074.
3. Giaccone G, Ferrati P, Donadio M, et al. Reinduction chemotherapy in small cell lung cancer. *Eur J Cancer Clin Oncol* 1987;23:1697- 1699.
4. Johnson DH, Greco FA, Strupp J, et al. Prolonged administration of oral etoposide in patients with relapsed or refractory small cell lung cancer: a phase II trial. *J Clin Oncol* 1990;8:1613 1617.
5. von Pawel J, Schiller JH, Shepherd FA, et al. Topotecan versus cyclophosphamide, doxorubicin, and vincristine for the treatment of recurrent small-cell lung cancer. *J Clin Oncol* 1999;17:658 667.
6. O'Brien ME, Ciuleanu TE, Tsekov H, et al. Phase III trial comparing supportive care alone with supportive care with oral topotecan in patients with relapsed small-cell lung cancer. *J Clin Oncol* 2006;24: 5441–5447.
7. Bowman A, Rye T, Ross G, et al. Effective dosing of topotecan with carboplatin in relapsed ovarian cancer: a phase I/II study. *J Clin Oncol* 2001;19:3255 3259.
8. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* 2000;92:205–216.
9. Kaplan EL, Meier P. Nonparametric estimation from incomplete observation. *J Am Stat Assoc* 1958;53:457 481.
10. Onoda S, Masuda N, Seto T, et al. Phase II trial of amrubicin for treatment of refractory or relapsed small-cell lung cancer: Thoracic Oncology Research Group Study 0301. *J Clin Oncol* 2006;24:5448–5453.

Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial

Tetsuya Mitsudomi, Satoshi Morita, Yasushi Yatabe, Shunichi Negoro, Isamu Okamoto, Junji Tsurutani, Takashi Seto, Miyako Satouchi, Hirohito Tada, Tomonori Hirashima, Kazuhiro Asami, Nobuyuki Katakami, Minoru Takada, Hiroshige Yoshioka, Kazuhiko Shibata, Shinzoh Kudoh, Eiji Shimizu, Hiroshi Saito, Shinichi Toyooka, Kazuhiko Nakagawa, Masahiro Fukuoka, for the West Japan Oncology Group

Summary

Background Patients with non-small-cell lung cancer harbouring mutations in the epidermal growth factor receptor (*EGFR*) gene respond well to the EGFR-specific tyrosine kinase inhibitor gefitinib. However, whether gefitinib is better than standard platinum doublet chemotherapy in patients selected by EGFR mutation is uncertain.

Methods We did an open label, phase 3 study (WJTOG3405) with recruitment between March 31, 2006, and June 22, 2009, at 36 centres in Japan. 177 chemotherapy-naive patients aged 75 years or younger and diagnosed with stage IIIB/IV non-small-cell lung cancer or postoperative recurrence harbouring *EGFR* mutations (either the exon 19 deletion or L858R point mutation) were randomly assigned, using a minimisation technique, to receive either gefitinib (250 mg/day orally; n=88) or cisplatin (80 mg/m², intravenously) plus docetaxel (60 mg/m², intravenously; n=89), administered every 21 days for three to six cycles. The primary endpoint was progression-free survival. Survival analysis was done with the modified intention-to-treat population. This study is registered with UMIN (University Hospital Medical Information Network in Japan), number 000000539.

Findings Five patients were excluded (two patients were found to have thyroid and colon cancer after randomisation, one patient had an exon 18 mutation, one patient had insufficient consent, and one patient showed acute allergic reaction to docetaxel). Thus, 172 patients (86 in each group) were included in the survival analyses. The gefitinib group had significantly longer progression-free survival compared with the cisplatin plus docetaxel group, with a median progression-free survival time of 9·2 months (95% CI 8·0–13·9) versus 6·3 months (5·8–7·8; HR 0·489, 95% CI 0·336–0·710, log-rank p<0·0001). Myelosuppression, alopecia, and fatigue were more frequent in the cisplatin plus docetaxel group, but skin toxicity, liver dysfunction, and diarrhoea were more frequent in the gefitinib group. Two patients in the gefitinib group developed interstitial lung disease (incidence 2·3%), one of whom died.

Interpretation Patients with lung cancer who are selected by EGFR mutations have longer progression-free survival if they are treated with gefitinib than if they are treated with cisplatin plus docetaxel.

Funding West Japan Oncology Group (WJOG): a non-profit organisation supported by unrestricted donations from several pharmaceutical companies.

Lancet Oncol 2010; 11: 121–28
Published Online December 21, 2009 DOI:10.1016/S1470-2045(09)70364-X

See Reflection and Reaction page 104

Department of Thoracic Surgery, Aichi Cancer Center Hospital, Nagoya, Japan (T Mitsudomi MD); Department of Biostatistics and Epidemiology, Yokohama City University Medical Center, Yokohama, Japan (Prof S Morita PhD); Department of Pathology and Molecular Genetics, Aichi Cancer Center Hospital (Y Yatabe MD); Department of Medical Oncology (S Negoro MD), and Department of Thoracic Oncology (M Satouchi MD), Hyogo Cancer Center, Akashi, Japan; Department of Medical Oncology, Kinki University School of Medicine, Osaka-sayama, Japan (I Okamoto MD, J Tsurutani MD, Prof K Nakagawa MD); Department of Thoracic Oncology, National Kyushu Cancer Center, Fukuoka, Japan (T Seto MD); Department of General Thoracic Surgery, Osaka City General Hospital, Osaka, Japan (H Tada MD); Department of Thoracic Oncology, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Habikino, Japan (T Hirashima MD); Department of Respiratory Medicine, National Hospital Organization Kinki-Chuo Chest Medical Center, Sakai, Japan (K Asami MD); Clinical Research Center, Division of Pulmonary Medicine Kobe City Medical Center General Hospital, Kobe, Japan (N Katakami MD); Department of Medical

Introduction

Lung cancer is a major cause of cancer-related mortality worldwide.[1] However, current standard platinum doublet therapy seems to have reached a therapeutic plateau,[2] although it has recently been shown that patients with non-squamous histology who are treated with pemetrexed disodium have better survival than if they are treated with older drugs.[1]

Targeted therapies are actively being developed to improve efficacy in selected patient populations.[4] Small-molecule tyrosine kinase inhibitors (TKIs) that target the epidermal growth factor receptor (EGFR), such as gefitinib and erlotinib, are the first targeted drugs to enter clinical use for the treatment of lung cancer. Subgroups of patients of east-Asian origin, female sex, adenocarcinoma, and no history of smoking have been shown to be significantly associated with a favourable response to EGFR TKIs.[5,6] In 2004, researchers noted that activating mutations of the *EGFR* gene present predominantly in patients with the above-mentioned clinical characteristics, and determine sensitivity to EGFR TKIs.[7,8] *EGFR* mutations are present in the first four exons of the tyrosine kinase domain of the *EGFR* gene, and about 90% of these *EGFR* mutations are either short in-frame deletions in exon 19, or point mutations that result in a substitution of arginine for leucine at aminoacid 858 (L858R).[7–9] Subsequent retrospective and prospective trials confirmed that the response rate to gefitinib or erlotinib in patients with *EGFR* mutations is about 70–80%.[10–13] Furthermore, patients with *EGFR* mutations have a significantly longer survival than those with wild-type *EGFR* when treated