厚生労働科学研究費補助金

がん臨床研究事業

オピオイド治療効果に対する実測可能な薬理学的効果予測システムORPSの開発

平成22年度　総括研究報告書

研究代表者　中　川　和　彦

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別紙１

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総合研究報告書

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研究代表者　中川　和彦
近畿大学医学部内科学腫瘍内科部門　教授

研究要旨　本研究は、がん性疼痛へのオピオイド治療効果に対する実測可能な薬理学的効果予測システムの開発を目的とする。平成22年度は予定通り順調に進行している。前向き臨床試験は50例の症例登録と計150サンプルの検体採取が終了した。各種測定系は最適化が終了し、測定に移行している。マイクロアレイは20サンプルの測定が終了した。モルヒネ関連遺伝子の遺伝子多型については、Chipを用いて225遺伝子上の1,936個の主要薬物代謝マーカーの解析を実施して20例測定が終了した。

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A．研究目的

がん性疼痛へのオピオイド治療に対して、治療効果の指標およびモニタリングできる実測可能な薬理学的バイオマーカーの開発し、実測可能な薬理学的効果予測システムORPS（Opioid treatment Response Prediction System)の開発を通じて、がん性疼痛の定量化システムに相補的に寄与することを目的とする。　前向き臨床試験においてがん性疼痛へのオピオイド治療を受ける患者を対象に臨床的・定量的エンドポイントと各種薬理学的バイオマーカー候補分子との相関を統合的に検討し、実測可能な薬理学的バイオマーカーを得る。

B．研究方法

[研究計画]

＜対象＞文書による同意が得られているがん性疼痛を有するモルヒネ治療の対象となる症例。

＜検体の種類＞末梢血10ml（３ポイント）からDNA、RNA、血漿を試料とする。

＜目標症例数＞100例。

＜方法＞前向き臨床試験においてがん性疼痛スケールNRS・心理テストおよびQOL評価尺度を臨床的・定量的エンドポイントにして、各種バイオマーカー候補を測定し、両者の相関を統合的に検討する。モルヒネ治療前・後1日目、8日目にNRS（Numeric Rating Scale)、心理テストおよびQOL評価尺度および採血を施行する。オピオイド治療は通常の治療指針に従って行う。薬理学的バイオマーカーとしては①マイクロアレイを用いた薬力学的効果関連遺伝子の特定、②モルヒネ関連代謝酵素の遺伝子多型、③モルヒネ血中濃度、④血中サイトカイン濃度、⑤血中糖鎖解析を実施する。治療は通常の治療指針に従って行う。本研究のため変更することはない。

〈薬理学的バイオマーカー〉

モルヒネ関連遺伝子の遺伝子多型（OPRM1、UGT 2B7、MDR1、COMT等）は薬剤効果に関連することが近年明らかになりつつあるため検討する。ゲノムDNAを試料として測定する。通常使用されるdirect sequence法を用い、PCR法で当該遺伝子の任意のDNA配列を増幅し、シークエンス機器で塩基配列を同定する。マイクロアレイは、末梢血白血球を対象にオピオイド治療前後で変動する遺伝子および、治療抵抗性に関連する遺伝子を特定する際に用いる。Affymetrix社製のGeneChip HG-U133 Plus2.0 array を用いる。血中サイトカイン測定はBioplex systemを用いて、血漿中の以下のサイトカイン（IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, IFN-γ, TNF-α, G-CSF, GM-CSF, MCP-1,MIP-1βなど）を測定する。解析は生物統計家の協力を得て、特定したバイオマーカーを利用してオピオイド治療効果予測システム等を構築する。

[研究体制]

研究代表者は研究の統括・計画・測定・解析を実施する。研究分担者は近畿大学医学部腫瘍内科・近畿大学医学部堺病院の2施設において症例集積を行う。バイオマーカーの測定は近畿大学医学部ゲノム生物学教室で測定する。近畿大学総合社会学部心理学科は、心理テストについての評価を行う。大阪府立大学看護学部は、QOL調査票についての評価を行う。統計解析は九州がんセンター腫瘍統計学部門が行う。研究実施環境については研究施設・研究資料・研究フィールド・現在の研究環境の状況等整備されており問題はない。

[年次計画]

平成22年度は前向き臨床試験の実施（プロトコール作成済・倫理委員会承認済）、測定系を構築および

最適化作業などを行う。平成23年度は、臨床試験試験の続行および臨床検体の測定を行なう。①マイクロアレイを用いた薬力学的効果関連遺伝子の特定、②モルヒネ関連代謝酵素の遺伝子多型、③モルヒネ血中濃度、④血中サイトカイン濃度の測定を実施する。半数の50例症例を測定する。平成24年度は残りの臨床検体の測定および解析を実施する。
測定結果を組み合わせ、実測可能な薬理学的効果予測システムORPS (Opioid treatment Response Prediction System)の開発を行なう。

（倫理面への配慮）
本研究による身体的な危険性は採血のみでありきわめて少ない。本研究に用いるゲノムDNA遺伝子多型の検出はモルヒネの代謝および薬理作用に関連した遺伝子に制限して解析を行う。本研究では、検体提供者に登録前に同意説明文書・同意書に基づき、本研究の意義、目的、方法、予測される結果や不利益について説明し、文書により自由意思による検体提供者の同意を得る。原則的に各施設は倫理委員会への承認を必要とする。個人情報は個人情報管理者により連結可能匿名化され、厳重に管理される。連結した遺伝子情報が第三者に渡ることはない。本研究では、３省合同「ヒトゲノム・遺伝子解析研究に関する倫理指針」を遵守する。各臨床試験の実施にあたっては「ヘルシンキ宣言」「臨床試験に関する倫理指針」「個人情報保護法」「ヒトゲノム・遺伝子解析研究に関する倫理指針」など関連の指針や法律・省令・告示等に従う。

C．研究結果
【前向き臨床試験】本研究のプロトコール「研究名：がん性疼痛へのモルヒネ治療に対する治療効果および薬力学的効果に関する探索的研究」を近畿大学医学部・近畿大学医学部堺病院の２施設の倫理委員会に提出し、承認を得た（平成21年6月5日承認）。本研究の臨床試験はH21年6月6日から近畿大学医学部腫瘍内科・近畿大学医学部堺病院の２施設において開始された。現在50例症例の登録と、50例x3ポイントの検体採取が終了した。

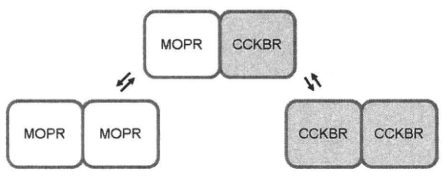
【マイクロアレイ測定】末梢血白血球を代替組織としてマイクロアレイ測定用の質の高いRNA抽出方法を確立した。具体的には、2.5 ml 全血をPAXgene RNA採血管を用いて採取・保存した。一方、未成熟な赤血球には、Globin mRNAが多く含まれ（最大70%）、全血からのTotal RNAで発現解析を行った場合に感度が低下するため、GLOBINclear™ Kitを用いてGlobin mRNAの除去を検討した。吸光度測定による濃度測定、純度検定（NanoDrop2000 Spectrophotometer）を行い、確実にGlobin除去がされていること、マイクロアレイ解析に供するに当たりRNAの質が十分確保されていることを確認した。Affymetrix社GenechipU133plus2.0を使用して20サンプルの測定が終了した。現在発現データの解析中である。Preliminaryなデータであるが、モルヒネ治療のバイオマーカーとして有望な遺伝子をいくつか特定している（図1）。

【モルヒネ関連遺伝子の遺伝子多型】モルヒネ関連遺伝子の遺伝子多型については、Affymetrix社DMETchipを用いて、225遺伝子上の1,936個の主要薬物代謝マーカーの解析を実施している。現在DMETchipは、20症例の測定が終了した。オピオイド受容体シグナルに関連する遺伝子に対して、既知のＳＮＰとしてOPRM1、UGT 2B7、MDR1、COMT等を検討する。ゲノムDNAを試料として測定する。通常使用されるdirect sequence法を用い、PCR法で当該遺伝子の任意のDNA配列を増幅し、シークエンス機器で塩基配列を同定する。最適化は終了している。

【血中サイトカイン測定】Bioplex systemを用いた臨床検体の測定は、すでに3報報告しており、最適化は終了している。26種類の主要なサイトカインの測定を予定している。

【血中モルヒネ濃度】
血中モルヒネ濃度は、HPLCを用いて慶應義塾大学医学部臨床薬剤学教室において測定する。治療後1週間の血漿をモルヒネのトラフ値として測定する。

図1　マイクロアレイ解析



モルヒネ治療前後で遺伝子発現変動が特定された*CCKBR*は、Opioid 受容体と結合することが示されており、モルヒネ治療に特異的なバイオマーカーになり得ると考えられる。

E．結論
平成22年度は前向き臨床試験については50例の症例登録・検体採取が終了し、順調に進行中である。各種測定は、最適化が終了し、測定に移行した。マイクロアレイ測定は20サンプル終了した。モルヒネ関連遺伝子の遺伝子多型については、Chipを用いて225遺伝子上の1,936個の主要薬物代謝マーカーの解析を実施している。質の高い前向き臨床試験の中でこれらのマーカーを組み合わせた研究はこれまでに存在せず、モルヒネ疼痛緩和効果の向上への貢献が期待できる。

F．研究発表

論文発表

1. ○Kaneda H, Arao T, Tanaka K, Tamura D, Aomastu K, Kudo K, De Velasco M A, Mastumoto K, Fujita Y, Yamada Y, Tsurutani J, Okamoto I, Nakagawa K, Nishio K..FOXQ1 is overexpressed in colorectal cancer and enhances tumorigenicity and tumor growth. Cancer Research. 70(5):2053-63, 2010.

2. Takeda M, Okamoto I, Fukuoka M, Nakagawa K. Successful treatment with erlotinib after gefitinib-related severe hepatotoxicity. J Clin Oncol. 28(17):e273-4, 2010.

3. Tanizaki J, Okamoto I, Takezawa K, Tsukioka S, Uchida J, Kiniwa M, Fukuoka M, Nakagawa K. Synergistic antitumor effect of S-1 and HER2-targeting agents in gastric cancer with HER2 amplification. Mol Cancer Ther. 9(5):1198-207, 2010.

4. Yamamoto N, Nakagawa K, Nishimura Y, Tsujino K, Satouchi M, Kudo S, Hida T, Kawahara M, Takeda K, Katakami N, Sawa T, Yokota S, Seto T, Imamura F, Saka H, Iwamoto Y, Semba H, Chiba Y, Uejima H, Fukuoka M. Phase III Study Comparing Second- and Third-Generation Regimens With Concurrent Thoracic Radiotherapy in Patients With Unresectable Stage III Non-Small-Cell Lung Cancer: West Japan Thoracic Oncology Group WJTOG0105. J Clin Oncol. 28(23):3739-4, 2010.

5. Okamoto I, Kaneda H, Satoh T, Okamoto W, Miyazaki M, Morinaga R, Ueda S, Terashima M, Tsuya A, Sarashina A, Konishi K, Arao T, Nishio K, Kaiser R, Nakagawa K. Phase I safety, pharmacokinetic, and biomarker study of BIBF 1120, an oral triple tyrosine kinase inhibitor in patients with advanced solid tumors. Mol Cancer Ther. 9(10):2825-33, 2010.

6. Okamoto I, Yoshioka H, Morita S, Ando M, Takeda K, Seto T, Yamamoto N, Saka H, Asami K, Hirashima T, Kudoh S, Satouchi M, Ikeda N, Iwamoto Y, Sawa T, Miyazaki M, Tamura K, Kurata T, Fukuoka M, Nakagawa K. Phase III Trial Comparing Oral S-1 Plus Carboplatin With Paclitaxel Plus Carboplatin in Chemotherapy-Naive Patients With Advanced Non-Small-Cell Lung Cancer: Results of a West Japan Oncology Group Study. J Clin Oncol. 28(36):5240-6, 2010.

7. Okamoto K, Okamoto I, Okamoto W, Tanaka K, Takezawa K, Kuwata K, Yamaguchi H, Nishio K, Nakagawa K. Role of survivin in EGFR inhibitor-induced apoptosis in non-small cell lung cancers positive for EGFR mutations. Cancer Res. 70(24):10402-10, 2010.

8. ○Yamada, Y., Arao, T., Matsumoto, K., Gupta, V., Tan, Woei, Fedynyshyn, J., Nakajima, T., Shimada, Y., Hamaguchi, T., Kato, K., Taniguchi, H., Saito, Y., Matsuda, T., Moriya, Y., Akasu, T., Fujita, S., Yamamoto, S., Nishio, K. Plasma concentrations of VCAM-1 and PAI-1: A predictive biomarker for post-operative recurrence in colorectal cancer. Cancer Sci. 101 (8) :1886-90, 2010.

9. ○Tominaga, E., Tsuda, H., Arao, T., Nishimura, S., Takano, M., Kataoka, F., Nomura, H., Hirasawa, A., Aoki, D., Nishio, K. Amplification of GNAS may be an independent, qualotative, and reproducible biomarker to progression-free survival in epithelial ovarian cancer. Gynecol Oncol. 118(2):160-6, 2010.

10. Kidera Y, Satoh T, Ueda S, Okamoto W, Okamoto I, Fumita S, Yonesaka K, Hayashi H, Makimura C, Okamoto K, Kiyota H, Tsurutani J, Miyazaki M, Yoshinaga M, Fujiwara K, Yamazoe Y, Moriyama K, Tsubaki M, Chiba Y, Nishida S, Nakagawa K. High-dose dexamethasone plus antihistamine prevents colorectal cancer patients treated with modified FOLFOX6 from hypersensitivity reactions induced by oxaliplatin. Int J Clin Oncol. 2011 in press.

11. Takezawa K, Okamoto I , Nishio K, Janne P, Nakagawa K Role of ERK-BIM and STAT3-survivin signaling pathways in ALK inhibitor-induced apoptosis in EML4-ALK-positive lung cancer. Clinical Cancer Research 2011 in press

12. Tanizaki J, Okamoto I, Fumita S, Okamoto W, Nishio K, Nakagawa K. Roles of BIM induction and survivin down-regulation in lapatinib-induced apoptosis in breast cancer cel

ls with HER2 amplification. Oncogene2011 in press

13. ○Kim, HK., Choi, IJ., Kim, CG., Kim, HS., Oshima, A., Yamada, Y., Arao, T., Nishio, K., Michalowski, A., Green, JE. Three-gene predictor of clinical outcome for gastric cancer patients treated with chemotherapy. The Pharmacogenomics J. E-pub:, 2011.

G．知的財産権の出願・登録状況

1. 特許取得
なし
2. 実用新案登録
なし
3. その他
なし

別紙４

研究成果の刊行に関する一覧表レイアウト

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kaneda H, Arao T, Tanaka K,Tamura D, Aomastu K, Kudo K, De Velasco M A, Mastumoto K, Fujita Y, Yamada Y, Tsurutani J, Okamoto I, Nakagawa K, Nishio K.	FOXQ1 is overexpressed in colorectal cancer and enhances tumorigenicity and tumor growth.	Cancer Research	70(5)	2053-63	2010
Takeda M, Okamoto I, Fukuoka M, Nakagawa K.	Successful treatment with erlotinib after gefitinib-related severe hepatotoxicity.	J Clin Oncol	28(17)	e273-4	2010
Tanizaki J, Okamoto I, Takezawa K, Tsukioka S, Uchida J, Kiniwa M, Fukuoka M, Nakagawa K.	Synergistic antitumor effect of S-1 and HER2-targeting agents in gastric cancer with HER2 amplification.	Mol Cancer Ther	9(5)	1198-207	2010
Yamamoto N, Nakagawa K, Nishimura Y, Tsujino K, Satouchi M, Kudo S, Hida T, Kawahara M, Takeda K, Katakami N, Sawa T, Yokota S, Seto T, Imamura F, Saka H, Iwamoto Y, Semba H, Chiba Y, Uejima H, Fukuoka M.	M.Phase III Study Comparing Second- and Third-Generation Regimens With Concurrent Thoracic Radiotherapy in Patients With Unresectable Stage III Non-Small-Cell Lung Cancer: West Japan Thoracic Oncology Group WJTOG0105.	J Clin Oncol	28(23)	Apr-39	2010
Okamoto I, Kaneda H, Satoh T, Okamoto W, Miyazaki M, Morinaga R, Ueda S, Terashima M, Tsuya A, Sarashina A, Konishi K, Arao T, Nishio K, Kaiser R, Nakagawa K.	Phase I safety, pharmacokinetic, and biomarker study of BIBF 1120, an oral triple tyrosine kinase inhibitor in patients with advanced solid tumors.	Mol Cancer Ther	9(10)	2825-33	2010
Okamoto I, Yoshioka H, Morita S, Ando M, Takeda K, Seto T, Yamamoto N, Saka H, Asami K, Hirashima T, Kudoh S, Satouchi M, Ikeda N, Iwamoto Y, Sawa T, Miyazaki M, Tamura K, Kurata T, Fukuoka M, Nakagawa K.	Phase III Trial Comparing Oral S-1 Plus Carboplatin With Paclitaxel Plus Carboplatin in Chemotherapy-Naive Patients With Advanced Non-Small-Cell Lung Cancer: Results of a West Japan Oncology Group Study.	J Clin Oncol	28(36)	Jun-40	2010
Okamoto K, Okamoto I, Okamoto W, Tanaka K, Takezawa K, Kuwata K, Yamaguchi H, Nishio K, Nakagawa K.	Role of survivin in EGFR inhibitor-induced apoptosis in non-small cell lung cancers positive for EGFR mutations.Cancer	Cancer Res	70(24)	10402-10	2010
Yamada, Y., Arao, T., Matsumoto, K., Gupta, V., Tan, Woei, Fedynyshyn, J., Nakajima, T., Shimada, Y., Hamaguchi, T., Kato, K., Taniguchi, H., Saito, Y., Matsuda, T., Moriya, Y., Akasu, T., Fujita, S., Yamamoto, S., Nishio, K.	Plasma concentrations of VCAM-1 and PAI-1: A predictive biomarker for post-operative recurrence in colorectal cancer.	Cancer Sci	101(8)	1886-90	2010
Tominaga, E., Tsuda, H., Arao, T., Nishimura, S., Takano, M., Kataoka, F., Nomura, H., Hirasawa, A., Aoki, D., Nishio, K.	Amplification of GNAS may be an independent, qualotative, and reproducible biomarker to progression-free survival in epithelial ovarian cancer.	Gynecol Oncol	118(2)	160-6	2010
Kidera Y, Satoh T, Ueda S, Okamoto W, Okamoto I, Fumita S, Yonesaka K, Hayashi H, Makimura C, Okamoto K, Kiyota H, Tsurutani J, Miyazaki M, Yoshinaga M, Fujiwara K, Yamazoe Y, Moriyama K, Tsubaki M, Chiba Y, Nishida S, Nakagawa K.	High-dose dexamethasone plus antihistamine prevents colorectal cancer patients treated with modified FOLFOX6 from hypersensitivity reactions induced by oxaliplatin.	Int J Clin Oncol		in press.	2011
Takezawa K, Okamoto I , Nishio K, Janne P, Nakagawa K	Role of ERK-BIM and STAT3-survivin signaling pathways in ALK inhibitor-induced apoptosis in EML4-ALK-positive lung cancer.	Clinical Cancer Research		in press.	2011
Tanizaki J, Okamoto I, Fumita S, Okamoto W, Nishio K, Nakagawa K.	Roles of BIM induction and survivin down-regulation in lapatinib-induced apoptosis in breast cancer cells with HER2 amplification	Oncogene		in press.	2011
Kim, HK., Choi, IJ., Kim, CG., Kim, HS., Oshima, A., Yamada, Y., Arao, T., Nishio, K., Michalowski, A., Green, JE.	Three-gene predictor of clinical outcome for gastric cancer patients treated with chemotherapy.	The Pharmacogenomics J	E-pub		2011

Tumor and Stem Cell Biology

Cancer Research

FOXQ1 Is Overexpressed in Colorectal Cancer and Enhances Tumorigenicity and Tumor Growth

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Abstract

Forkhead box Q1 (FOXQ1) is a member of the forkhead transcription factor family, and it has recently been proposed to participate in gastric acid secretion and mucin gene expression in mice. However, the role of FOXQ1 in humans and especially in cancer cells remains unknown. We found that *FOXQ1* mRNA is overexpressed in clinical specimens of colorectal cancer (CRC; 28-fold/colonic mucosa). A microarray analysis revealed that the knockdown of *FOXQ1* using small interfering RNA resulted in a decrease in $p21^{CIP1/WAF1}$ expression, and a reporter assay and a chromatin immunoprecipitation assay showed that *p21* was one of the target genes of FOXQ1. Stable FOXQ1-overexpressing cells (H1299/FOXQ1) exhibited elevated levels of p21 expression and inhibition of apoptosis induced by doxorubicin or camptothecin. Although cellular proliferation was decreased in H1299/FOXQ1 cells *in vitro*, H1299/FOXQ1 cells significantly increased tumorigenicity [enhanced green fluorescent protein (EGFP): 2/15, FOXQ1: 7/15] and enhanced tumor growth (437 ± 301 versus 1735 ± 769 mm^3, $P < 0.001$) *in vivo*. Meanwhile, stable p21 knockdown of H1299/FOXQ1 cells increased tumor growth, suggesting that FOXQ1 promotes tumor growth independent of p21. Microarray analysis of H1299/EGFP and H1299/FOXQ1 revealed that FOXQ1 overexpression upregulated several genes that have positive roles for tumor growth, including *VEGFA, WNT3A, RSPO2*, and *BCL11A*. CD31 and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining of the tumor specimens showed that FOXQ1 overexpression mediated the angiogenic and antiapoptotic effect *in vivo*. In conclusion, FOXQ1 is overexpressed in CRC and enhances tumorigenicity and tumor growth presumably through its angiogenic and antiapoptotic effects. Our findings show that FOXQ1 is a new member of the cancer-related FOX family. *Cancer Res; 70(5); 2053–63. ©2010 AACR.*

Introduction

The forkhead box (*Fox*) gene family is a large and diverse group of transcription factors that share certain characteristics of a conserved, ~100 amino acid DNA-binding motif known as the forkhead or winged helix domain; over 100 proteins with forkhead domains have been identified, comprising at least 17 subclasses to date (1). The Fox gene family plays various important roles, not only in biological processes including development, metabolism, immunology, and senescence but also in cancer development (2, 3).

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Forkhead box Q1 (*FOXQ1*, also known as HFH1) is a member of the FOX gene family and contains the core DNA binding domain, whereas the flanking wings of FOXQ1 contribute to its sequence specificity (4). As a transcription factor, FOXQ1 is known to repress the promoter activity of smooth muscle–specific genes, such as telokin and SM22α, in A10 vascular muscle cells (5), and FOXQ1 expression is regulated by Hoxa1 in embryonic stem cells (6). The biological function of *Foxq1* has been clearly identified in hair follicle differentiation in satin (sa) homozygous mice (7); interestingly, satin mice also exhibit suppressed natural killer cell function and T-cell function, suggesting a relation with immunology. Satin mice have provided evidence that Hoxc13 regulates foxq1 expression and that "cross-talk" occurs between Homeobox and Fox (8). Foxq1 mRNA is widely expressed in murine tissues, with particularly high expression levels in the stomach and bladder (5). Recently, two important findings have been reported regarding its involvement in stomach surface cells. Foxq1-deficient mice exhibit a lack of gastric acid secretion in response to various secretagogue stimuli (9). On the other hand, Foxq1 regulates gastric MUC5AC synthesis, providing clues as to the lineage-specific cell differentiation in gastric surface epithelia (10). Despite accumulating evidence supporting the biological function of the murine foxq1 gene in hair follicle

morphogenesis and gastric epithelial cells, no data regarding the cellular and biological functions of human *FOXQ1*, especially in cancer cells, are available.

$p21^{CIP1/WAF1}$ (hereafter called p21) is a member of the cip/kip family of cyclin kinase inhibitors, and initial reports have shown that p21 functions as a G_1 cyclin kinase inhibitor (11, 12) and a downstream molecule of p53 (13). p21 possesses a variety of cellular functions, including the negative modulation of cell cycle progression (14), cellular differentiation (15), and the regulation of p53-dependent antiapoptosis (reviewed in ref. 16). The expression of p21 is regulated by both p53-dependent and p53-independent mechanisms at the transcriptional level. Other regulatory mechanisms of p21 expression involve proteasome-mediated degradation, mRNA stability, alterations in the epigenetic silencing of the p21 promoter, and secondary decreases resulting from viral activity targeting p53, such as the activities of human papilloma virus and hepatitis C virus (17). However, its expression is considered to be regulated mainly at the transcriptional level (18). Accumulating data indicate that many molecules from diverse signaling pathways can activate or repress the p21 promoter, including p53, transforming growth factor-β (TGF-β), c-jun, Myc, Sp1/Sp3, signal transducers and activators of transcriptions, CAAT/enhancer binding protein-α (C/EBP-α), C/EBP-β, basic helix-loop-helix proteins, and myogenic differentiation 1 (reviewed in ref. 19). Thus, p21 is integrally involved in both cell cycle and apoptosis; therefore, identifying its regulatory molecules is of great importance.

We performed a microarray analysis of clinical samples of paired colorectal cancer (CRC) specimens and normal colonic mucosa specimens to identify genes that were overexpressed in CRC. Our results revealed that *FOXQ1* gene expression was ~28-fold higher in CRC than in normal colonic mucosa, and we hypothesized that FOXQ1 may play a role in CRC. In the present study, we investigated the biological function of FOXQ1.

Materials and Methods

Antibodies. The following antibodies were used: anti-p21, anti-p53, anti-cdk2, anti-cdk4, anti-cyclin D, anti-phosphorylated Rb, anti-poly(ADP-ribose) polymerase (PARP), anti-cleaved PARP, anti-caspase-3, anti-cleaved caspase-3, secondary antibodies, and Myc-tag mouse antibody (Cell Signaling), as well as anti-β-actin (Santa Cruz Biotechnology). A mouse anti-CD31 monoclonal antibody was purchased from BD Biosciences.

Cell lines and cultures. The DLD-1, MKN74, H1299, SBC3, and U251 cell lines were cultured in RPMI 1640 (Sigma). The WiDr, CoLo320DM, and human embryonic kidney cell line 293 (HEK293) cell lines were cultured in DMEM (Sigma), and the LoVo cell line was cultured in Ham/F12 medium [Life Technologies Bethesda Research Laboratories (BRL)]. All media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies BRL), and the cell lines were maintained in a 5% CO_2-humidified atmosphere at 37°C.

Patients and samples. Paired CRC and noncancerous colonic mucosa samples were evaluated using a microarray analysis in the first consecutive 10 patients. These samples and another 36 CRC samples were analyzed using real-time reverse transcription–PCR (RT-PCR). The RNA extraction method and the quality check protocol have been previously described (20). This study was approved by the institutional review board of the National Cancer Center Hospital, and written informed consent was obtained from all the patients.

Plasmid construction, viral production, and stable transfectants. The cDNA fragment encoding human full-length FOXQ1 was isolated using PCR and Prime STAR HS DNA polymerase (TaKaRa) with 5′-GGG AAT TCG CGG CCA TGA AGT TGG AGG TCT TCG TC-3′ and 5′-CCC TCG AGC GCT ACT CAG GCT AGG AGC GTC TCC AC-3′ sense and antisense primers, respectively. The methods used in this section have been previously described (21). Short hairpin RNA (shRNA) targeting p21 was constructed using oligonucleotides encoding small interfering RNA (siRNA) directed against p21 and a nonspecific target as follows: 5′-CTA AGA GTG CTG GGC ATT TTT-3′ for p21 shRNA and 5′-TGT TCG CAG TAC GGT AAT GTT-3′ for control shRNA. They were cloned into an RNAi-Ready pSIREN-RetroQZsGreen vector (Clontech) according to manufacturer's protocol. The stable transfectants expressing enhanced green fluorescent protein (EGFP) or FOXQ1 or FOXQ1 with shRNA targeting p21 for each cell line were designated as HEK293/EGFP, HEK293/FOXQ1, CoLo320/EGFP, CoLo320/FOXQ1, H1299/EGFP, H1299/FOXQ1, H1299/FOXQ1/sh-control, and H1299/FOXQ1/sh-p21. The FOXQ1 human cDNA was tagged at the NH_2 terminus with the myc epitope using the pCMV-Myc vector (Clontech) for chromatin immunoprecipitation (ChIP) assay.

SiRNA transfection. Two different sequences of siRNA targeting human FOXQ1 and negative control siRNA were purchased from QIAGEN. The sequences of FOXQ1 and control siRNA were as follows: FOXQ1#1 sense, 5′-CCA UCA AAC GUG CCU UAA A-3′ and antisense, 5′-UUU AAG GCA CGU UUG AUG G-3′; FOXQ1#4 sense, 5′-CGC GGA CUU UGC ACU UUG A-3′ and antisense, 5′-UCA AAG UGC AAA GUC CGC G-3′; control siRNA (scramble) sense, 5′-UUC UCC GAA CGU GUC ACG U-3′ and antisense, 5′-ACG UGA CAC GUU CGG AGA A-3′; control siRNA (GFP) sense, 5′-GCA AGC UGA CCC UGA AGU UCA U-3′ and antisense, 5′-GAA CUU CAG GGU CAG CUU GCC G-3′. The methods of transfection have been previously described (22).

Real-time RT-PCR and Western blot analysis. The methods used in this section have been previously described (21). The primers used for real-time RT-PCR were purchased from Takara as follows: FOXQ1 forward, 5′-CGC GGA CTT TGC ACT TTG AA-3′ and reverse, 5′-AGC TTT AAG GCA CGT TTG ATG GAG-3′; p21 forward, 5′-TCC AGC GAC CTT CCT CAT CCA C-3′ and reverse, 5′-TCC ATA GCC TCT ACT GCC ACC ATC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPD) forward, 5′-GCA CCG TCA AGG CTG AGA AC-3′ and reverse, 5′-ATG GTG GTG AAG ACG CCA GT-3′. The experiment was performed in triplicate.

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 *KpnI* and *XhoI* 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).

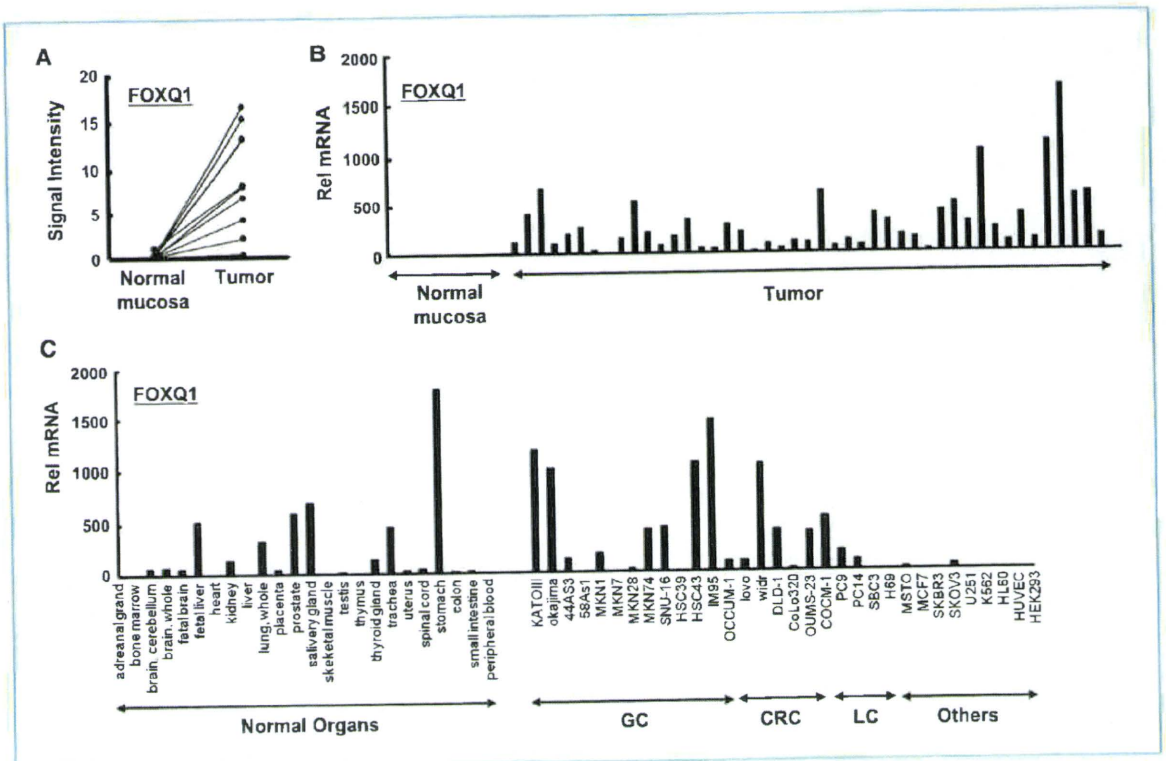

Figure 1. *FOXQ1* expression in CRC. A, mRNA expression of *FOXQ1* obtained from a microarray analysis of 10 CRC and paired normal mucosa specimens. The values indicate the normalized signal intensity. B, the mRNA expression levels of *FOXQ1* were determined using real-time RT-PCR for 10 paired and an 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).

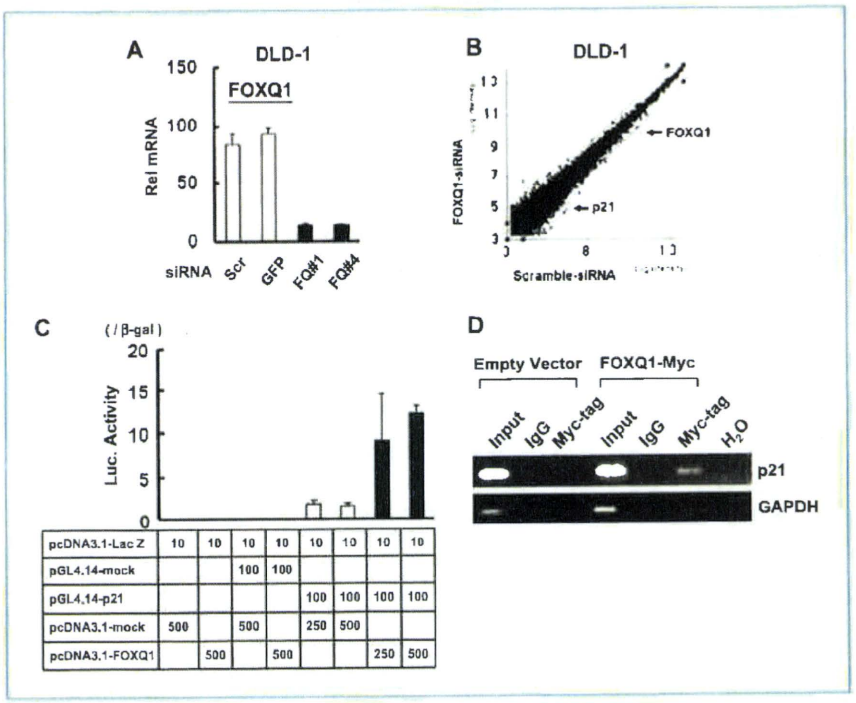

Figure 2. FOXQ directly regulates *p21* transcription. A, FOXQ1-targeting siRNA (FQ#1 and FQ#4) suppressed FOXQ1 expression in DLD-1 cells. The mRNA expression levels of *FOXQ1* were determined using real-time RT-PCR. B, microarray analysis of DLD-1 cells transfected with control-siRNA or FOXQ1-siRNA. 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

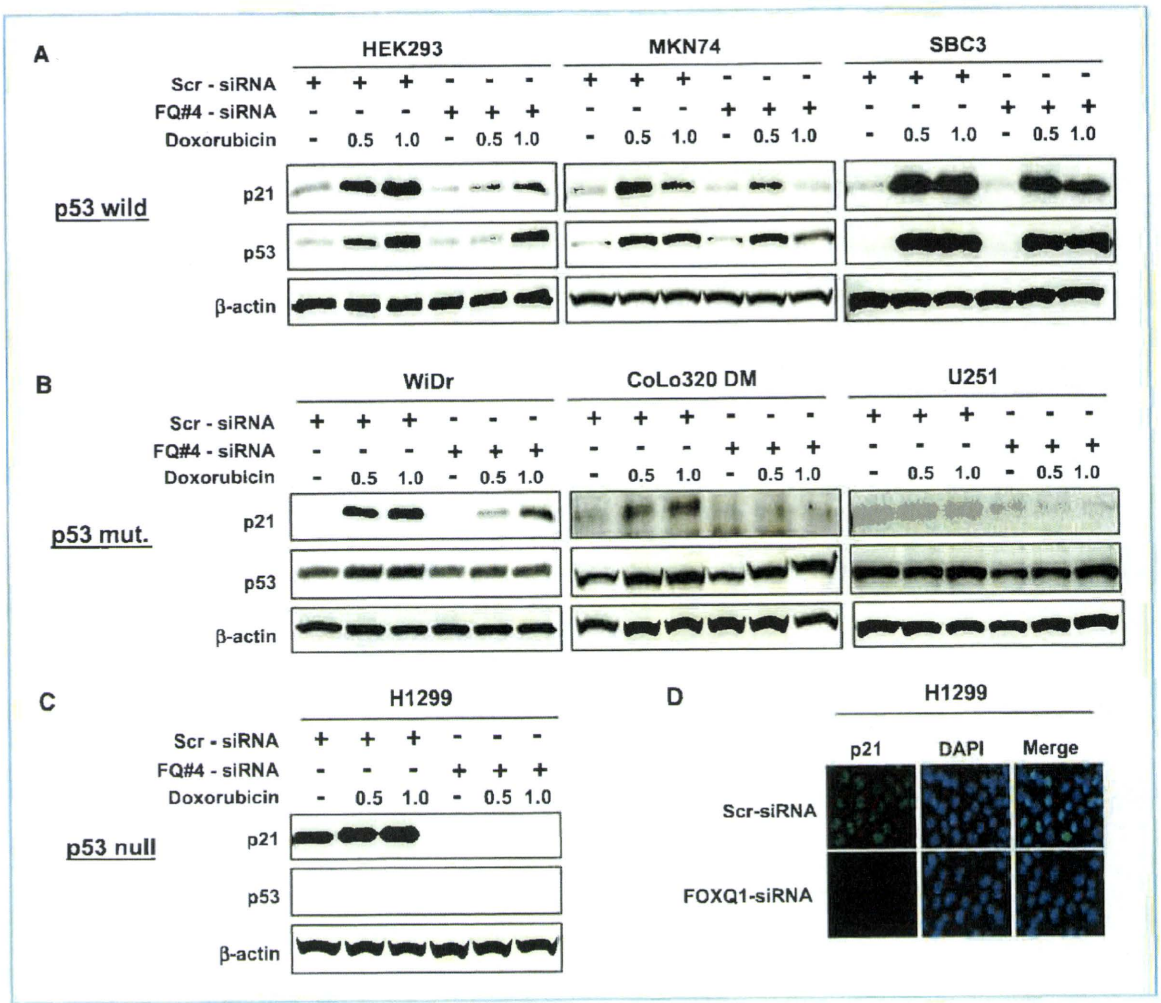

Figure 3. p21 induction by FOXQ1 and p53 status in cancer cells. The seven cell lines were transfected with control-siRNA or FOXQ1-siRNA for 24 h, and the cells were exposed to doxorubicin at a final concentration of 0.5 or 1 μmol/L for a further 24 h to enhance p21 induction. Western blot analyses for 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.

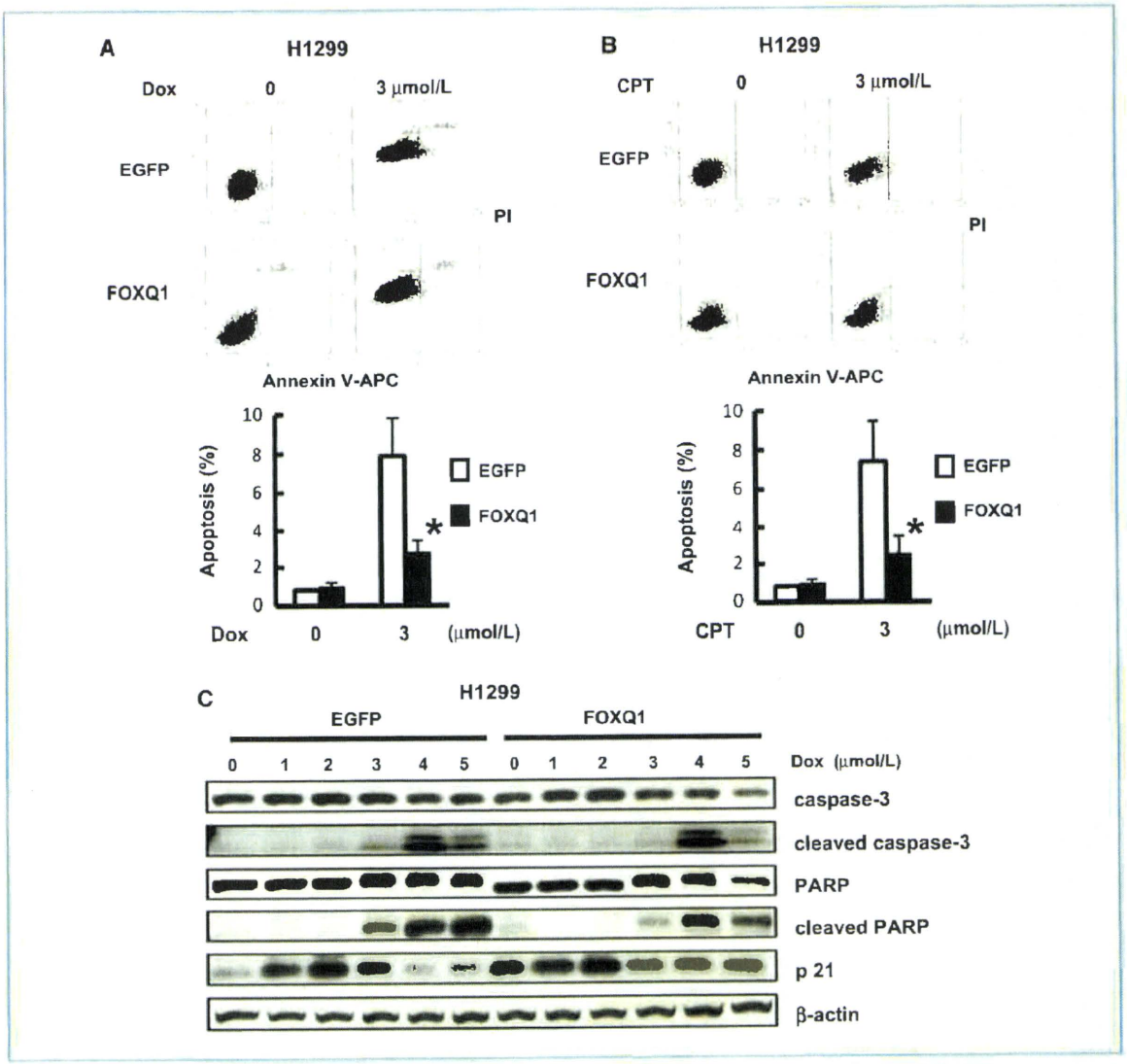

Figure 4. Overexpression of FOXQ1 promotes an antiapoptotic effect. Stable H1299 cell lines expressing EGFP or FOXQ1 (H1299/EGFP, H1299/FOXQ1) were exposed to doxorubicin (A) or camptothecin (B) at a final concentration of 3 μmol/L. Apoptotic cells were detected by Annexin V and propidium iodide (PI) 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

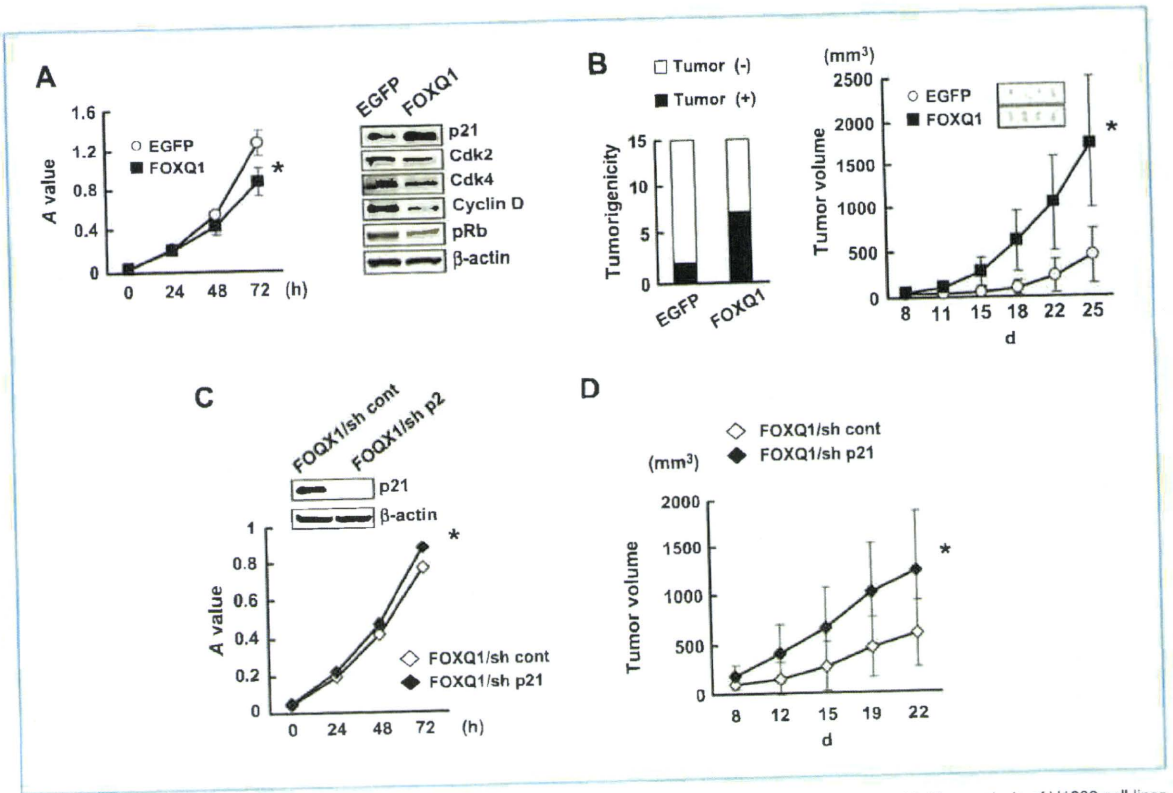

Figure 5. Overexpression of FOXQ1 enhances tumorigenicity and tumor growth *in vivo*. A, cellular growth and immunoblotting analysis of H1299 cell lines stably expressing EGFP or FOXQ1 (H1299/EGFP, H1299/FOXQ1). A total of 2×10^3 cells of each cell line were seeded in 96-well plates and evaluated after 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 6×10^5 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 ($n = 10$). *, $P < 0.05$.

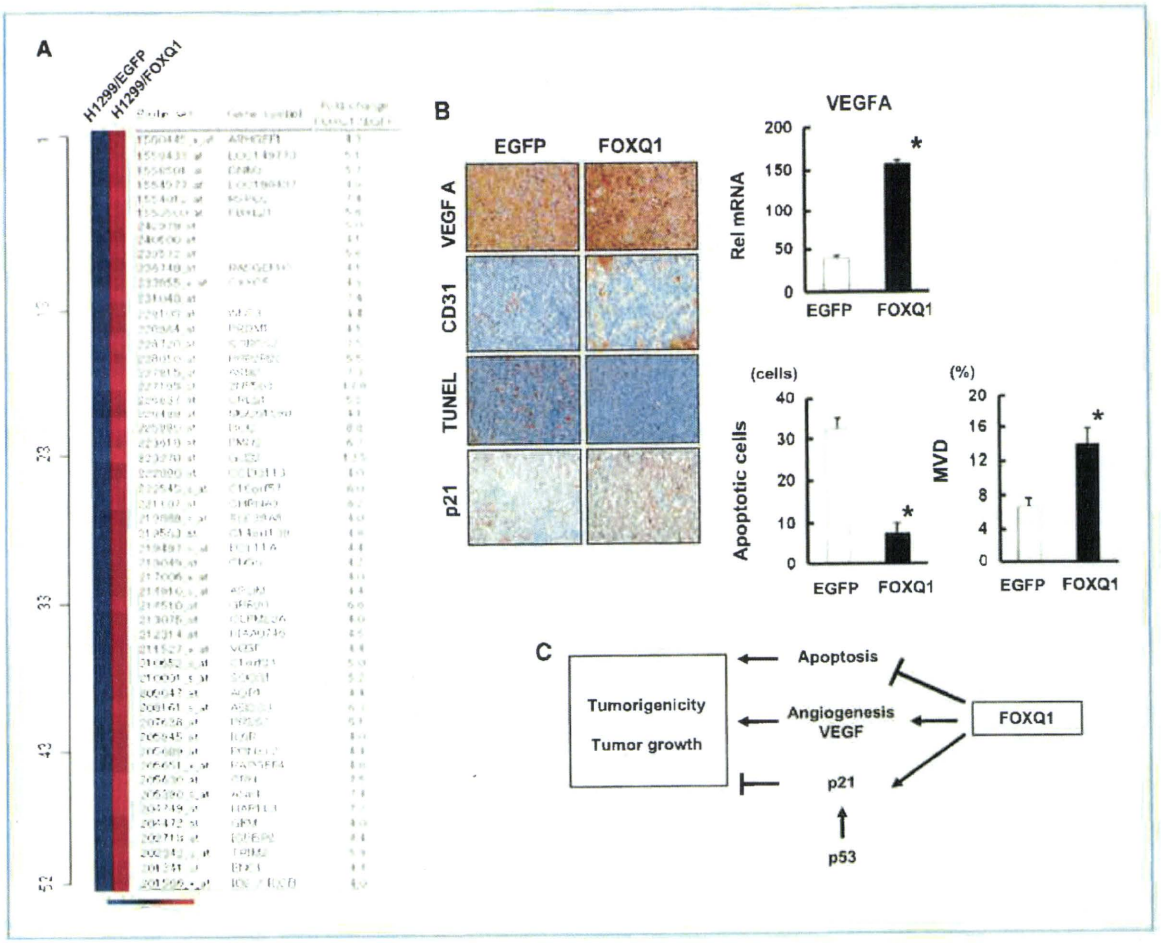

Figure 6. FOXQ1 promotes angiogenic and antiapoptotic effects *in vivo*. A, microarray analysis for H1299/EGFP or H1299/FOXQ1 cells. The upregulated genes over 4-fold by FOXQ1 were shown in the list. B, the mRNA expression levels of *VEGFA* were determined using a real-time RT-PCR analysis. Rel mRNA, normalized mRNA expression levels (*VEGFA/GAPD* × 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.

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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 I 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.

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Successful Treatment With Erlotinib After Gefitinib-Related Severe Hepatotoxicity

A 66-year-old nonsmoking woman presented with enlarged left supraclavicular lymph nodes. She had no history of liver disease, alcohol intake, or hepatitis. Baseline blood tests showed cell counts, electrolytes, as well as renal and liver function to be normal. She had not previously received medication for her condition. A chest x-ray revealed a nodular shadow in the right upper lung field. A computed tomography scan of the chest confirmed a solitary spiculated lesion in the right upper lung lobe, disseminated nodules in the interlobar fissures, and multiple pulmonary nodules. Core biopsy of left supraclavicular lymph nodes revealed adenocarcinoma, consistent with metastasis from the primary non–small-cell lung carcinoma. Mutation analysis of lung cancer specimens obtained before first-line chemotherapy showed the presence of an exon 19 deletion of the epidermal growth factor receptor gene, and gefitinib was administered orally at a dose of 250 mg once daily. Eight weeks after the initiation of treatment, computed tomography revealed marked tumor shrinkage, which was categorized as a partial response. After 13 weeks of gefitinib treatment, laboratory investigations showed a substantial increase in serum transaminase levels (AST of 84 U/L, compared with a normal range of lower than 40; ALT of 181 U/L, compared with a normal range of lower than 35; Fig 1). Initiation of treatment with ursodeoxycholic acid and ammonium glycyrrhizate resulted in a gradual decrease in transaminase levels (to values of 31 U/L and 35 U/L for AST and ALT, respectively; Fig 1). Thirty-six weeks after the initiation of daily gefitinib administration, the transaminase levels of the proband had begun to increase again, reaching a pronounced high of 599 U/L for AST and 1,011 U/L for ALT at 37 weeks (Fig 1). Gefitinib treatment was discontinued at 36 weeks. The patient had taken no other medications or supplements, and an abdominal ultrasound revealed a normal liver with no other substantial abnormalities. A drug lymphocyte stimulation test yielded a strong positive result for gefitinib, suggesting that the hepatitis of the proband was attributable to drug allergy rather than to dose-dependent toxicity. We therefore concluded that gefitinib should not be administered further at any schedule in this patient. In the 7 weeks after gefitinib withdrawal, the patient's liver function normalized but her lung cancer progressed slightly. We initiated treatment with erlotinib accompanied by careful monitoring of liver function, and the patient has continued daily oral erlotinib (150 mg) for 15 weeks with no evidence of increased hepatic toxicity or disease progression.

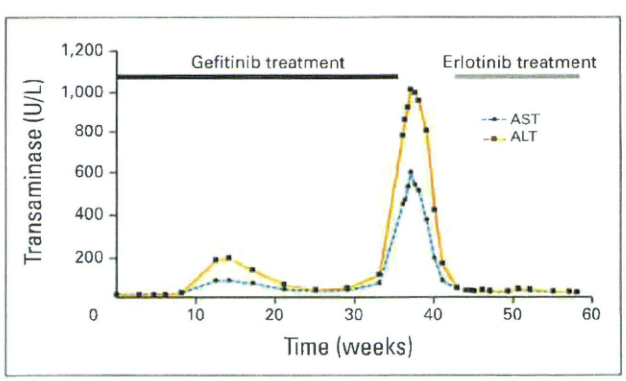

Fig 1.

Gefitinib-induced hepatitis has received little attention to date, even though phase I trials revealed hepatotoxicity as a dose-limiting toxicity of the drug and the Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL 1) trial showed that 2% of patients receiving gefitinib alone at a dose of 250 mg per day developed elevations of hepatic enzymes of grade 3 or 4 that necessitated cessation of treatment.[1] Exploration of new strategies for management of gefitinib-induced severe hepatotoxicity is thus warranted. Resumption of gefitinib treatment after its discontinuation as a result of the development of drug-induced hepatitis has been reported in three cases. However, gefitinib was again discontinued because of repeated elevation of serum transaminase levels in two of three cases[2,3]; the other case showed that an intermittent schedule of gefitinib administration (250 mg/d every 5 days) reduced hepatotoxicity, although the response had been maintained for only 8 weeks at the time of report submission.[4] These findings prompted us not to recommend resumption of gefitinib treatment after the development of severe hepatotoxicity in this patient. Erlotinib acts in a manner similar to that of gefitinib and has been shown to provide clinical benefit in patients with tumors positive for epidermal growth factor receptor gene mutations. We thus treated the proband of this study with erlotinib (150 mg once daily) as an alternative to gefitinib after discontinuation of the latter drug.

With regard to the toxicity profiles of gefitinib and erlotinib, it is important to clarify the mechanism responsible for drug-induced hepatotoxicity. Gefitinib and erlotinib share a common chemical backbone structure and exhibit similar disposition characteristics in humans after oral administration. They manifest similar oral bioavailabilities and both undergo extensive metabolism primarily by cytochrome P450 3A4, with more than 80% of the administered dose being found in feces.[5,6] Administration of erlotinib at the maximum-tolerated dose and approved dose of 150 mg once daily resulted in a steady-state plasma trough concentration that was approximately 3.5 times that for gefitinib administered at the recommended dose (approximately one third of the maximum tolerated dose) of 250 mg once daily.[7,8] This patient received no medications that influence the pharmacokinetics of gefitinib or erlotinib, suggesting that the plasma concentration of gefitinib per se did not give rise to the drug-induced hepatotoxicity, although the toxicity of gefitinib has not been directly compared with that of erlotinib alone. Instead, the positive result of the drug lymphocyte stimulation test supports a diagnosis of gefitinib-induced allergic hepatitis. Erlotinib and gefitinib share a