● 併用療法では複数の第Ⅱ相試験でCDDPと5-FUのFP療法の奏効率が30%以上と報告され，現在の標準療法である[2]（表2）．

● 3剤併用では上記の2剤併用にアドリアシン®（保険適応なし）を加えたFAP療法，ドセタキセルを加えたDCF療法にFP療法を上回る奏効率が報告されているが（DCFは頭頸部癌の成績），エビデンスはまだ明確ではない．DCFは現在JCOGで第Ⅱ相試験が予定されている（表2）．

● 欧米ではパクリタキセル，イリノテカン（CPT-11），ゲムシタビンとCDDPの併用療法の有効性が報告されているがわが国では保険適応されていない（表2）．

表2　併用化学療法治療成績

薬　剤	化学療法剤投与量・スケジュール	症例数	奏効率	CR率	文　献
CDDP/ビンデシン	CDDP70mg/m², ビンデシン30mg/m²	31	16	–	Iizuka et al : Jpn J Clin Oncol 1991
CDDP/5-FU	CDDP70mg/m² day 1, 5-FU 700mg/m² day 1-5	39	36	–	Iizuka et al : Jpn J Clin Oncol 1992
CDDP/5-FU	CDDP20mg/m² day 1-5, 5-FU 800mg/m² day 1-5	36	33	3	Hayashi et al : Jpn J Clin Oncol 2001
CDDP/5-FU	CDDP100mg/m² day 1, 5-FU 1,000mg/m² day 1-5, 2 cycle	44	35	2	Bleiberg et al : Eur J Cancer 1997
CDDP/5-FU /ドキソルビシン	CDDP75mg/m² day 1, ドキソルビシン30mg/m² day 1, 5-FU 600mg/m² day 1, 8, every 4weeks	21	33	10	Gisselbrecht et al : Cancer 1983
CDDP or ネダプラチン /5-FU/ドキソルビシン	CDDP60mg/m² or ネダプラチン50mg/m² day 1, ドキソルビシン30mg/m² day 1, 5-FU 600mg/m² day 1-7	26	46	–	Kosugi et al : Scand J Gastroenterol 2005
CDDP /パクリタキセル	パクリタキセル200～250mg/m² day 1, CDDP75mg/m² day 2, every 3 weeks	32	44	–	Ilson et al : Cancer J 2000
CDDP/5-FU /パクリタキセル	パクリタキセル175mg/m² day 1, CDDP 20mg/m² day 1-5, 5-FU 750mg/m² day1-5	61	48	3-20	Ilson et al : J Clin Oncol 1998
CDDP/CPT-11	CDDP30mg/m², CPT-11 65mg/m²	35	57	6	Ilson et al : J Clin Oncol 1999
CDDP /ゲムシタビン	CDDP75mg/m² day1, ゲムシタビン1,250～1,000/m² day 1 and day8	32	45	9	Millar et al : Br J cancer 2005
ネダプラチン/5-FU	ネダプラチン90mg/m² day1, 5-FU 800mg/m² day 1-5	38	40	3	Muro et al : 癌の臨床　2004
ドセタキセル/CDDP	ドセタキセル75mg/m², CDDP80mg/m², every 3 weeks	16	31	–	Laack et al : Oncologie 2005
ドセタキセル/ネダプラチン	ドセタキセル30mg/m², ネダプラチン40mg/m² every 2 weeks	27	11	0	Kanai et al : Int J Clin Oncol 2007

■ 放射線療法

● 放射線単独治療の適応症例は表在癌や化学放射線療法の適応でない症例である．

● 化学療法を併用できる全身状態が良好なT1-3，N0-1，M0の切除可能例では放射線単独療法より化学放射線療法が有意に生存率を向上させることから，化学放射線療法が標準的治療である[3]．

■ 化学放射線療法

● 切除可能のT1-3 N0，1 M0，切除不能のT4 N0，1 M0，一部のM1（リンパ節）症例に適応がある．

● 手術療法との直接比較試験はないが，後ろ向き研究で手術に劣るものの差は大きくないとされている．

● 照射量は欧米ではFP＋50.4Gy（RTOG9405/INT0123）[4]が標準とされるが，わが国ではFP＋60Gyとする施設が多く，まだ標準的照射量は定まっていない．

● 化学療法剤はRTOG9405/INT0123[4]では5-FU1,000mg/m² day1-4持続及びCDDP75mg/m² day1投与，わが国では5-FU700～800mg/m² day1-4持続及びCDDP70-80mg/m² day1投与がなされる（表3）．

● 欧米ではタキサン系とCDDPの併用でFP療法と同等の成績が報告されている．

● 食道温存が利点である．

● 早期有害事象は悪心・嘔吐，骨髄抑制，食道炎を中心にグレード3以上の毒性も少なからず存在し，T4症例では食道穿孔の危険がある（表4）．

● 晩期有害事象では放射線肺臓炎，胸水貯留，心嚢液貯留などがあり時に重篤となる[5]（表5）．

● 50Gy以上照射例に対するsalvage手術では無治療例に比し手術合併症の増加が報告されており，化学放射線治療選択時に説明が不可欠である[6]．

表3　根治的化学放射線療法スケジュール

対象病期	化学療法剤投与量・スケジュール	放射線療法	症例数	MST	CR率	3年生存率	TRD	文　献
T1-3 N0-1 M0	CDDP75mg/m² day 1, 5-FU 1,000mg/m² day 1-4, 2 cycle（追加 2 cycle）	2.0Gy×25	134	—	—	26***	2	Cooper et al : JAMA 1999
T1-3 N0-1 M0	CDDP75mg/m² day 1, 5-FU 1,000mg/m² day 1-4, 2 cycle（追加 2 cycle）	2.0Gy×25	61	14M	—	27***	2	Al-Sarraf et al : J Clin Oncol 1997
T1-4 N0-1 M0	CDDP75mg/m² day 1, 5-FU 1,000mg/m²/day 1-4, 2 cycle（追加 2 cycle）	1.8Gy×28	109	18M	—	40	2	Minsky et al : J Clin Oncol 2002
	CDDP75mg/m² day 1, 5-FU 1,000mg/m²/day 1-4, 2 cycle（追加 2 cycle）	1.8Gy×36	109	13M	—	31	10	
T1 N0 M0	CDDP70mg/m² day 1, 5-FU 700mg/m²/day 1-4, 2 cycle	2.0Gy×30	72	—	96	93**	—	Kato et al : Proc ASCO 2003
T4 N0-1 M0	CDDP70mg/m² day 1, 5-FU 700mg/m²/day 1-4, 2 cycle	2.0Gy×30	60	10M	15	32**	3	Ishida et al : Jpn J Clin Oncol 2004
T4/M1 lymph	CDDP40mg/m² day 1, 8, 5-FU 400mg/m²/day 1-5, 8-12, 2 cycle（追加 2 cycle）	2.0Gy×30	54	9M	33	23	7	Otsu et al : J Clin Oncol 1999
T4 M0	CDDP10mg/body day 1, 8, 5-FU 300mg/m²/day×14（追加 2 cycle）	2.0Gy×30	28	12M*	32	27*	—	Nishimura et al : Int J Radiat Oncol Biol Phys 2002

＊：stageⅢ　＊＊：2年　＊＊＊：5年
MST：median survival time　TRD：treatment related death　CR：complete response

表4　急性毒性

急性毒性	グレード3以上の毒性(%)
白血球減少	43
貧血	23
血小板減少	18
嘔気・嘔吐	2
下痢	0
粘膜障害	4
食道炎	10
腎機能障害	3
肺炎	4

n＝139

（文献5）より）

表5　晩期毒性

晩期毒性*	グレード2以上（例数）	死　亡
心嚢水貯留	16/139（12%）	2（心不全）
心筋梗塞	2/139（ 1%）	2（心筋梗塞）
胸水貯留	15/139（11%）	3（放射線肺臓炎）
再発を認めない死亡	8/139（ 6%）	

＊90日以後の発生

（文献5）より）

術前補助療法

- 理論的根拠は原発巣を縮小し外科手術を容易とするとともに，リンパ節転移や微小転移をコントロールすることにより，手術侵襲における抗腫瘍反応低下時の転移を防止し，遠隔治療成績の向上を期待するものである．

- 切除検体の組織学的検討により化学療法および化学放射線療法の感受性試験が可能である．

- 無効例では局所進行や転移に広がりを助長すること，放射線治療例では手術合併症の増加に繋がる[7]．

- 術前化学療法では欧米の臨床試験では術前FPは手術単独に対して予後を改善する結果は得られていない（表6）．わが国では術前FPと術後FPが比較検討され，すでに症例登録が終了し，結果が待たれている（JCOG9907）．

- 術前化学放射線療法では予後を改善する結果が得られている報告は少ないが（表7），メタアナリシスでは切除可能なT1-3 N0，1 M0に対する術前同時化学放射線療法（20-45Gy）により3年以上の長期生存率を向上させるとされる[7]．

- 術前化学放射線療法と根治的化学放射線療法の比較試験が最近報告され，化学放射線療法反応症例に対しては追加の化学放射線療法に比して手術による予後向上は得られていない（表8）．

表6　手術単独 VS 術前化学療法

治療内容	化学療法剤投与量・スケジュール	症例数	MST	2年生存率	有意差	CR率	文　献
手術単独	なし	73	13M	31	0.17		Law et al. J Thorac Cardiovasc Surg 1997
術前FP	CDDP100mg/m² day 1, 5-FU 500mg/m² day 1-5, 2 cycle	74	17M	44		7	
手術単独	なし	227	16M	37	0.53		Kelsen et al. NEJM 1998
術前FP	CDDP100mg/m² day1, 5-FU 1,000mg/m² day 1-5, 2 cycle	213	15M	35		—	
手術単独	なし	47	24M	41*	0.55		Ancona et al. Cancer 2001
術前FP	CDDP100mg/m² day 1, 5-FU 1,000mg/m² day 1-5, 2 or 3 cycle	47	25M	44*		13	
手術単独	なし	402	13M	34	0.004		MRC
術前FP	CDDP80mg/m² day 1, 5-FU 1,000mg/m² day 1-4, 2 cycle	400	17M	43		—	The Lancet 2002

＊：3年生存　MST：median survival time　CR：complete response

表7　手術 VS 術前化学放射線療法

治療内容	化学療法剤投与量・スケジュール，手術内容	放射線療法	組織型・病期	症例数	MST	3年生存率	CR率	TRD	有意差	文　献
手術単独	開胸開腹食道切除および経横隔膜食道切除		腺癌/扁平上皮癌	128	19M	32		—	0.57	Burmeister et al. Lancet Oncol 2005
術前化学放射線療法	CDDP80mg/m² day1, 5-FU 800mg/m² day 1-5	35Gy		128	22M	36	16	—		
手術単独	規定なし		腺癌	55	11M	6		4	0.01	Walsh et al. N Engl J Med 1996
術前化学放射線療法	CDDP75mg/m² day 7, 5-FU 15mg/kg/body day1-5, 2 cycle	40Gy		58	16M	32	25	9		
手術単独	右開胸開腹食道切除		扁平上皮癌 stage I-II	139	19M	35		4	0.78	Bosset et al. N Engl J Med 1997
術前化学放射線療法	CDDP80mg/m² day 0-2, 2 cycle	18.5Gy×2		143	19M	37	26	12		
手術単独	経横隔膜食道切除		腺癌/扁平上皮癌	50	18M	16		4	0.15	Urba et al. J clin Oncol 2001
術前化学放射線療法	CDDP20mg/m² day 1-5, 17-21, 5-FU 300mg/m² day 1-21, ビンブラスチン1mg/m² day 1-4, 17-20	45Gy		50	17M	30	28	2		

MST：median survival time　CR：complete response　TRD：treatment related death

表8　化学放射線療法 VS 術前化学放射線療法

治療内容	化学療法剤投与量・スケジュール	放射線療法	手術	症例数	MST	2年生存率	CR率	有意差	文献
術前化学放射線療法	CDDP15mg/m² day1-5, 5-FU 800mg/m² day 1-5, 2 cycle	30-46Gy	+	129	18M	34	23	0.03	Bedenne et al :
根治的化学放射線療法	CDDP15mg/m² day1-5, 5-FU 800mg/m² day 1-5, 5 cycle	45-66Gy	−	130	19M	40			J Clin Oncol 2007
術前化学放射線療法	FLEP(CDDP50mg/m², 5-FU 500mg/m²,	40Gy	+	86	16M	40	−	なし	Stahl et al :
根治的化学放射線療法	ロイコボリン300mg/m², エトポシド100mg/m²) day 1-3 every 3 weeks	65Gy	−	86	15M	35			J Clin Oncol 2005

MST：median survival time　CR：complete response

- 術前放射線療法は治療成績を向上させない[8].

術後補助療法

- 術後化学療法ではわが国で行われた臨床試験（JCOG 9204）により術後FPにより手術単独に比して生存率では有意差は認めなかったが，リンパ節転移症例で生存率が向上し，無再発生存率も有意に向上した．リンパ節転移症例に対して術後化学療法が勧められる[9]（表9）．

- 術後放射線療法ではわが国のJCOG試験で術後照射が有意に生存率を向上させたが，欧米の臨床試験では有意差は認められていない[8].

- 術後化学放射線療法についてのエビデンスはない．

表9　手術 VS 術後化学療法

治療内容	化学療法剤投与量・スケジュール	症例数	MST	生存率(無再発率)	有意差(無再発率)	TRD	文献
手術単独	根治切除のみ	122	−	52(45)	0.13(0.037)		Ando et al :
術後化学療法	CDDP80mg/m² day1, 5-FU 800mg/m² day 1-5, 2 cycle	120	−	61(55)		1	J Clin Oncol 2003
手術単独	非根治切除を含む	68	14	<10	なし		Pouliquen et al :
術後化学療法	CDDP20mg/m² day 1-5 or 100mg/m² day1, 5-FU 100mg/m² day 1-5	52	13	<10		4	Ann Surg 1996

TRD：treatment related death　MST：median survival time

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【Ⅲ　医療での実用化を目指した応用】

食道癌化学放射線感受性予測チップの開発と実用化に向けた取り組み

嶋田　裕，辻本豪三，信正　均，藤元治朗

治療前生検による検体からマイクロアレイ解析が行える高感度DNAチップを開発し，現在多施設共同臨床試験において治療効果を予測する標的遺伝子の同定を行っている．今後，臨床試験により得られた情報からバイオインフォマティクスにより診断のアルゴリズムを作成する．最終的に生検標本の遺伝子情報に基づいて患者の治療選択を行う検証臨床試験を行って臨床導入を図る予定である．

はじめに

われわれは，治療モダリティー*1に大きな差がある治療法（化学放射線療法と手術）の治療前選択を可能とするために，マイクロアレイの臨床導入を目指している．そのために，遺伝子増幅なしに治療前生検標本での解析を可能とする高感度チップの開発を行った．このチップが完成したことから，現在は多施設共同研究により治療効果を予測する標的遺伝子の同定を行っている．本稿では，なぜマイクロアレイ解析を食道癌で導入する必要があるのかを明らかにし，その解決のためにこれまでに行ってきた実用化への取り組みについて概説する．

1．食道癌治療の問題点と化学放射線感受性予測

日本における食道外科を専門とする施設での手術単独におけるステージⅡ～Ⅳの5年生存率は50％を超えており，食道癌は治癒できる癌となってきたが[1]，外科手術のなかでも食道切除術は侵襲が最も大きく，リスクの高い治療法である．一方，食道癌は化学療法や放射線療法に反応しやすいなどの特徴をもっており，化学放射線療法が従来の手術成績に匹敵する生存成績が得られることが報告されてきた[2]．しかしながら，化学放射線療法には副作用も少なからず見受けられ，治療関連死も存在する．また，晩期後遺症も報告されている．さらには，化学放射線療法後の病変の遺残や，再発に対する外科的切除術では術後合併症発生割合が高くなる[3]．

以上のことから，食道癌治療ではモダリティーの大きく異なる手術と化学放射線療法が行われているが，治療法の選択に明確な選択規準が存在しない．そのために過大な侵襲の手術を回避できた患者や，過度な化学放射線療法への期待から根治切除の機会を逃すような患者，さらには合併症が増加してしまう患者などの選別ができていなかった．

そこで，化学放射線感受性予測を選別に応用しようとする試みを行ってきたが[4]，化学放射線療法の感受性には多様な遺伝子群が関与していると考えられており，単一遺伝子のみの解析では不十分で，多数の遺伝子を同時に解析するマイクロアレイのような解析方法による検討が不可欠であることが明らかとなってきた．

2．化学放射線療法感受性予測へのマイクロアレイの導入における問題点

治療前診断には生検標本による診断が必要であるが，従来のDNAチップでは微量検体からの検討には遺伝子増幅が不可欠であった．この遺伝子増幅では複数の遺伝子間で増幅効率が異なることが予測され，プロファイルが変化する可能性がある．また，われわれのリンパ節転移予

＊1：モダリティー
治療手段や治療内容を指す．

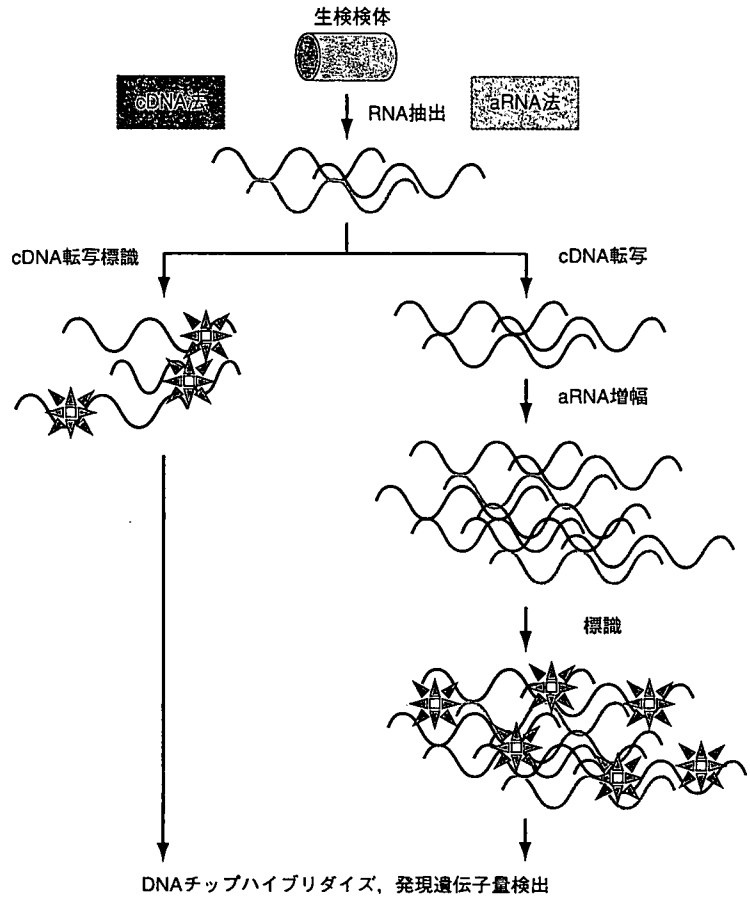

図1●DNAチップハイブリターゲットの種類

cDNA法の場合，検体から抽出したRNAを逆転写したcDNAを直接ターゲットとして利用するのに対し，aRNA法ではcDNAからさらにRNAを転写増幅することによってターゲット量を増やす．この時増幅バイアスによって検体由来のRNAの存在比が適切に反映されない可能性を否定できない

測に関係する遺伝子解析の結果から，腫瘍のみならず腫瘍に存在する間質細胞における遺伝子発現など，宿主側も含めて腫瘍全体をトータルに見る必用があることが判明している[5]．近年，マイクロダイセクションで腫瘍細胞のみを取り出した解析が試みられているが，腫瘍以外の生体側の反応を除いた解析では，重要な役割を担っている生体反応を見落とす可能性がある．なによりも実施の臨床や検査の場でマイクロダイセクションは労力の面から推奨されない．さらには，マイクロダイセクション後には遺伝子増幅が不可欠なために，前述のごとくプロファイルが変化してしまうことも避けられない（図1）．そこで，遺伝子増幅なしに網羅的遺伝子解析を行えるDNAチップの作製に取り組んできた．

3. 生検標本で遺伝子増幅なしに評価可能な高感度・高再現性マイクロアレイチップの作製

われわれはバイオ・IT融合機器開発プロジェクト〔独立行政法人新エネルギー・産業技術総合開発機構（NEDO），2003～2005年度〕の支援により，生検標本の発現遺伝子検出を遺伝子増幅することなく実施可能な高感度DNAチップを

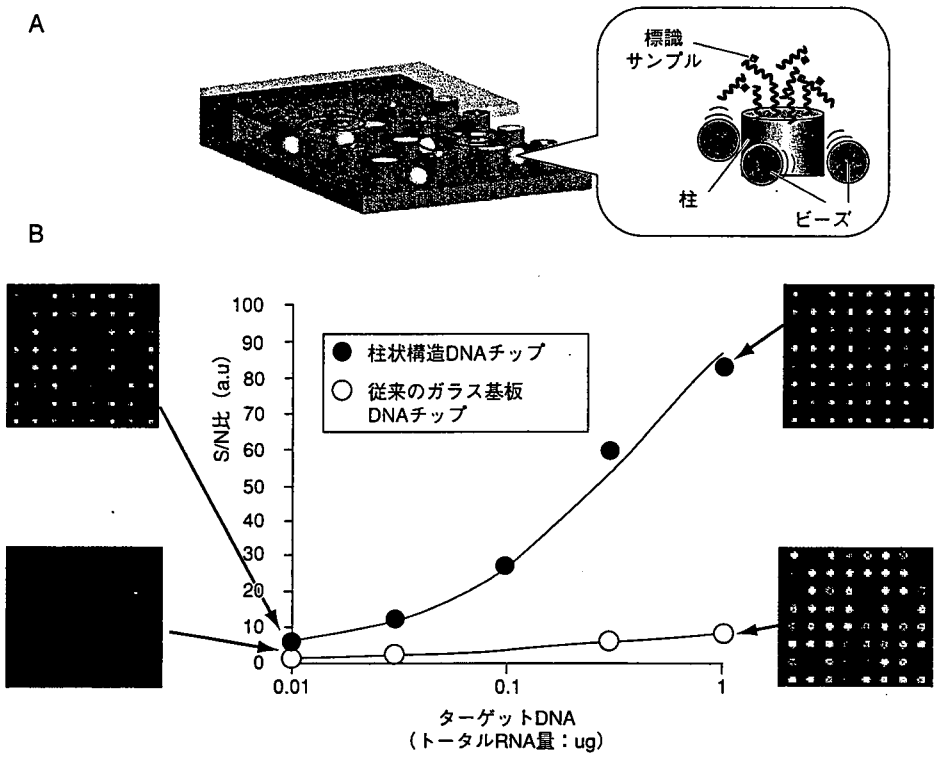

図2●高感度DNAチップ「3D-Gene」

A)「3D-Gene」のチップ構造（柱状構造）とハイブリダイゼーション時のイメージ．B)「3D-Gene」と従来のガラス基板DNAチップの感度比較

作製した[6]．この高感度DNAチップは東レ株式会社製「3D-Gene」基板を用いており，樹脂チップ基板の立体構造と表面修飾，および立体構造を利用したハイブリダイゼーション時の撹拌機構によって，従来のガラス基板による発現遺伝子検出の約100倍の感度を示す（図2）[7]．一方で，従来用いられてきたガラス基板とほぼ同一の設備，プロトコールで実験が可能であり，導入が容易である．

前述のプロジェクトによって開発したこの基板および判別アルゴリズムを用いることによって，食道癌との関係が知られていなかった約20種類の遺伝子による癌の判別が95％以上の確率で可能となったばかりでなく，癌の転移の有無についても，新たに40種類の診断用遺伝子を特定でき，85％以上の高い確率で判別，予測が期待できるようになった．本成果については，現在NEDO「食道ガン及び腎臓ガン診断用DNAチップの評価・検証及び成果普及事業」(2006年度)によって検証中である．

化学放射線療法感受性予測を行うためには改めて化学放射線療法感受性診断用遺伝子の絞り込みとアルゴリズムの構築の開発を行う必要がある．そこで今回は，前述の開発技術を応用した網羅型DNAチップ「3D-Gene」シリーズから「3D-Gene Human Digestive Cancer 9k」(東レ株式会社より市販，http://www.3d-gene.com)（図3）を用いた臨床研究を計画した．

4. 食道癌化学放射線感受性予測の臨床試験

生検標本からの網羅的遺伝子解析を行える

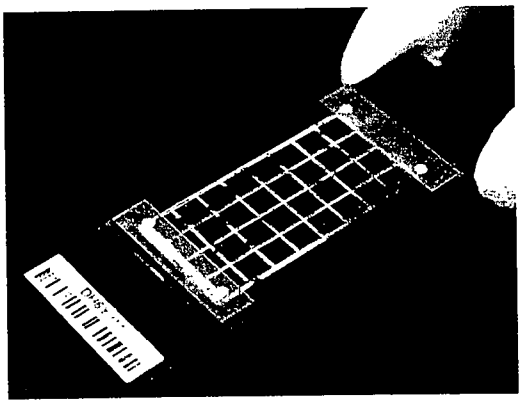

図3● 3D-Gene Human Digestive Cancer 9k
東レ株式会社より市販，http://www.3d-gene.com

＊2：前向き検証試験
過去と現在のデータを扱うのが後ろ向き研究（retrospective study）で，これから生じる現象を観察するのが前向き研究（prospective study）．後ろ向き研究ではすでに判明している事項をあつかうので研究者によるバイアスが入りやすいのに対して，前向き研究では結果がわかっていないために，バイアスがかかりにくく，より信頼のおける結果が得られるとされている．

DNAチップを開発できたことから，生検標本が採取できた患者において，化学放射線療法を行ってその効果判定を行い，その結果と生検標本における遺伝子プロファイル解析を照合し，バイオインフォマティクスにより，化学放射線療法に関与する遺伝子群を同定し，化学放射線療法感受性予測のアルゴリズムを作成する多施設共同研究（32施設）を開始している（プロトコール，図4）．すでに主任研究者施設での倫理委員会の承認，臨床データ登録機関の承認を受け，検体搬送システムの確立，最終プロトコール作成およびウェブ登録システムを2007年1月までに終了した．現在まで12施設で倫理委員会の承認が得られ，近日中に実際の登録が開始される．本臨床試験終了後は，絞り込まれた遺伝子解析にて感受性を予測するアルゴリズムを完成させ，

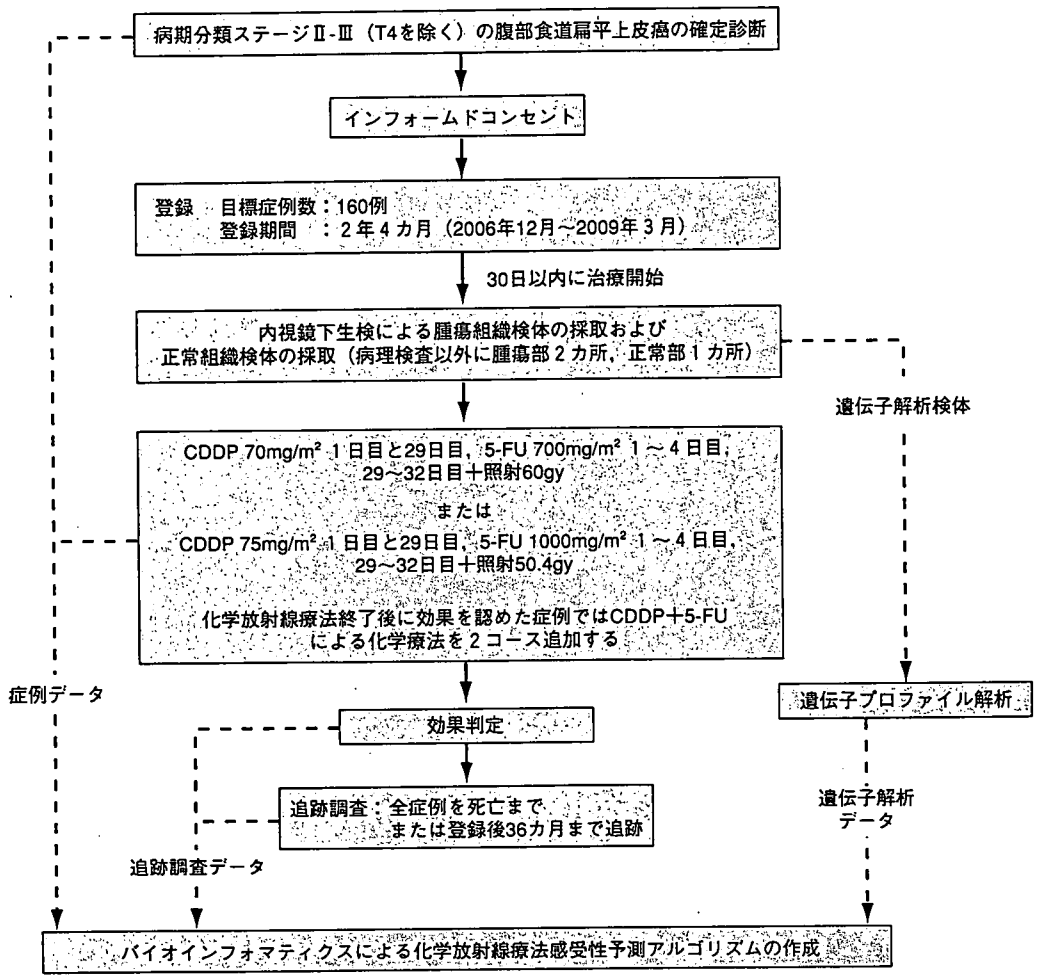

図4●食道癌化学放射線療法感受性予測における臨床試験の概要

そのアルゴリズムで患者の治療を振り分けて，治療成績を前向き検証試験*2で検証をすることにより臨床導入を予定している．この検討により化学放射線療法感受性が治療前に予測可能となれば，化学放射線療法の効果がないと予測された場合は副作用のある化学放射線療法を回避して根治切除を行い，化学放射線療法の効果が期待できる患者では侵襲の大きな手術を回避でき，テーラメード治療が可能となる（図5）．なお，本研究は，厚生労働省科学研究費による「食道癌生検標本の遺伝子発現プロファイル解析による放射線化学療法感受性予測の臨床導入を目指した基盤的研究（2006年度－ファーマコ－一般－003）」（主任研究者　嶋田　裕）にて補助を受けて行われている．

おわりに

なぜこの研究をやり始めたのか．それは患者に治療法の提示をするときにいつになっても各治療法での治る確率の話はできるが，「その治療法を選んだとして，患者が治る方に入るのか治らない方なのか，やってみないと解らない」と答えなければならなかったからである．われわれの開発してきたDNAチップが広く他疾患の診

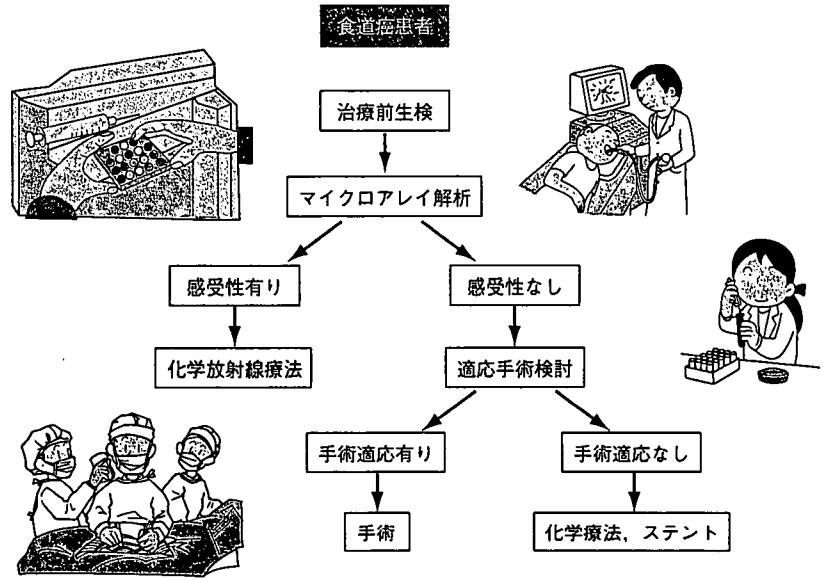

図５●食道癌のテーラーメード治療

断治療に是非応用されることを願っている．

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専門：消化器外科，細胞生物学，分子生物学．
消化器外科におけるテーラーメード治療をめざしている．現在までの多施設共同研究では多くの障害があった．個人情報保護法による遺伝子の取り扱いに対する過度な制約，各施設の倫理委員会における審査の長期化，マイクロアレイを含めた分子生物学に対する臨床医の無理解，症例の内容でなく数が幅をきかせる臨床至上主義などが本研究の進行を遅らせている．なんとかここまでこぎ着けたので是非成果を出したいと考えている．

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The Suppression of *Aurora-A/STK15/BTAK* Expression Enhances Chemosensitivity to Docetaxel in Human Esophageal Squamous Cell Carcinoma

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Abstract **Purpose:** We previously reported that the expression of Aurora-A was frequently up-regulated in human esophageal squamous cell carcinoma (ESCC) tissues as well as cell lines and the up-regulation contributed to a poor prognosis. In this study, we assessed the possibility of Aurora-A suppression as a therapeutic target for ESCC using ESCC cell lines.

Experimental Design: We established subclones using vector-based short hairpin RNA (shRNA). Then, we investigated the effect of Aurora-A suppression on proliferation and cell cycle changes *in vitro*. Next, chemosensitivity against docetaxel was investigated by tetrazolium salt–based proliferation assay (WST assay) and cell number determinations, and furthermore, the type of cell death induced by docetaxel was analyzed by flow cytometry. Finally, to examine the effect of Aurora-A shRNA on proliferation and chemosensitivity against docetaxel *in vivo*, a s.c. tumor formation assay in nude mice was done.

Results: We established two genetically different stable cell lines (510 A and 1440 A) in which levels of Aurora-A were reduced. Cell growth was inhibited by 38.7% in 510 A and by 24.3% in 1440 A *in vitro* compared with empty vector–transfected controls (510 m and 1440 m), and this growth inhibition was mediated through G_2-M arrest as confirmed by flow cytometry. Next, in a WST assay, the IC_{50} for Aurora-A shRNA–transfected cells was lower than that of empty vector–transfected cells (510 A, 2.7×10^{-7} mol/L; 510 m, 4.8×10^{-7} mol/L; 1440 A, 2.6×10^{-7} mol/L; 1440 m, 4.9×10^{-7} mol/L). In addition, 0.3 nmol/L docetaxel induced a notable level of apoptosis in Aurora-A shRNA–transfected cells compared with empty vector–transfected cells. In the assay of s.c. tumors in nude mice, tumor growth in 510 A was inhibited by 36.1% compared with that in 510 m, and in tumors treated with docetaxel, the suppression of Aurora-A resulted in 44.0% tumor growth suppression *in vivo*.

Conclusions: These results indicated that Aurora-A might play an important role in chemosensitivity to docetaxel, and the suppression of its expression might be a potential therapeutic target for ESCC.

Several proteins strictly regulate the process of cellular division. Defects in chromatid segregation cause genetic instability, a condition associated with tumorigenesis. During the proliferation of normal cells, the centrosome ensures the equal segregation of chromosomes to the postmitotic daughter cells by organizing the bipolar mitotic spindle. In contrast, in cancer cells, multipolar mitotic spindles and various centrosomal anomalies, such as supernumerary centrosomes, centrosomes of abnormal size and shape, aberrantly phosphorylated centrosomic proteins, and prematurely split centrosomes, are frequently observed (1–7).

Aurora-A, a member of the Aurora/Ipl1p family of cell cycle–regulating serine/threonine kinases, is expressed at interphase mitotic centrosomes and the spindle poles in the nucleus where it regulates segregation of chromosomes and cytokinesis. Recent studies have shown that the ectopic expression of Aurora-A in mouse NIH/3T3 cells and Rat 1 fibroblasts causes centrosome amplification and transformation *in vitro* as well as tumorigenesis *in vivo* (8, 9). Furthermore, the up-regulation of Aurora-A expression in diploid human breast epithelial cells leads to abnormal numbers of centrosomes and the induction of aneuploidy (8). A correlation between the up-regulated expression of Aurora-A and clinical aggressiveness has also been reported for several cancers (10–14). Moreover, recent reports showed that the up-regulation of Aurora-A resulted in resistance

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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to apoptosis induced by taxanes in a human cancer cell line (15, 16) and inhibition of the expression of Aurora-A resulted in potent antitumor activity and chemosensitizing activity to taxanes in pancreatic cancer (17). These findings suggest that *Aurora-A* is a critical kinase-encoding gene and a potential chemotherapeutic target.

Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies in the world, including Japan, despite the recent refinement of various therapeutic strategies, including surgery, chemotherapy, radiotherapy, and combined therapy (18, 19). Docetaxel is used as monotherapy or in combination with other agents to treat ESCC, but its activity is far from satisfactory (20, 21). Therefore, identifying and targeting genes conducive to the treatment of ESCC, such as enhancement of conventional chemotherapy, is necessary to improve the survival of patients with this type of refractory cancer.

Using comparative genomic hybridization, we previously investigated changes in the copy number of chromosomes in 29 ESCC cell lines and found that a chromosome gain of the proximal part of 20q, where the *Aurora-A* gene is located, is one of the most common sites of aberrations (19 of 29, 65.5%; ref. 22). We also reported that the expression of Aurora-A was frequently up-regulated in ESCC tissues as well as cell lines, and this contributed to a poor prognosis (14). In the present study, to further elucidate the possibility of using *Aurora-A* in the treatment of human ESCC, we analyzed the phenotypic changes of cultured ESCC cells induced by suppression of Aurora-A expression using a plasmid vector-mediated short hairpin RNA (shRNA) expression system, especially synergistic enhancement of the cytotoxicity of docetaxel.

Materials and Methods

Cell culture. All tested ESCC cell lines of the KYSE series were established in our laboratory and maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) and Ham's F12 (Nissui Pharmaceutical, Tokyo, Japan) mixed (1:1) medium containing 2% fetal bovine serum (23). HeLa cells were purchased from the American Type Culture Collection (Rockville, MD), cultured in DMEM (Life Technologies) with 10% FCS, and used as a positive control (24, 25).

Western blot analysis. Cells were lysed in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100] containing Complete Mini protease inhibitor (Roche Diagnostics, Mannheim, Germany). Cell lysates were sonicated and the protein concentration was estimated by the Bradford method using bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Cell lysates (15 μg) were electrophoresed on 2% to 15% gradient polyacrylamide gel (Daiichi Pure Chemicals, Tokyo, Japan) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) using a semidry transfer blot system (Bio-Rad, Hercules, CA). After blocking with TBS containing 1% Tween 20 and 5% skim milk for 1 h, the membranes were incubated at 4°C overnight with anti-human Aurora-A polyclonal antibody (diluted 1:100; TransGenic, Inc., Kumamoto, Japan) or anti-human β-actin monoclonal antibody (1:2,000; Sigma, Inc., St. Louis, MO). The membranes were subsequently incubated at room temperature for 1 h with secondary antibody and analyzed using enhanced chemiluminescence plus reagent (Amersham, Buckinghamshire, United Kingdom). Quantitative analysis was done on a Macintosh computer using the public domain NIH Image program version 1.61 (developed at the NIH and available on the Internet).[3]

Immunofluorescent staining. 510 A, 510 m, 1440 A, and 1440 m cells were cultured onto collagen-coated glass coverslips (BD Biosciences, Bedford, MA). Then, cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The fixed cells were incubated with 0.3% Triton X-100 solution for 15 min at room temperature, and the cells were blocked for 1 h with 2% fetal bovine serum in PBS at room temperature. Subsequently, the cells were incubated with anti-human Aurora-A polyclonal antibody (diluted 1:50; TransGenic) for 1 h at room temperature. After washing twice with PBS, the cells were incubated with goat anti-rabbit FITC-conjugated secondary antibody (Invitrogen Co., Carlsbad, CA) for 1 h at room temperature. The cells were washed and mounted in glycerol and viewed under a laser scanning microscope (Axioskop 2 plus, Carl Zeiss Co. Ltd., Jena, Germany).

Construction of an Aurora-A shRNA expression vector. To construct a vector for Aurora-A shRNA, the pSUPERIOR-puro (OligoEngine, Seattle, WA) was digested with *Bgl*II and *Hin*dIII (TaKaRa Bio, Shiga, Japan). A chemically synthesized oligonucleotide encoding an Aurora-A short hairpin small interfering RNA, including a loop motif, was inserted downstream of the H1 promoter of the plasmid using a DNA ligation kit (TaKaRa Bio) and cloned. The sequence of the oligonucleotide targeted at Aurora-A is 5′-ATGCCCTGTCTTACTGTCA-3′ for KYSE 510, corresponding to positions 853 to 871 within the Aurora-A mRNA sequence (17). To confirm the result, we designed another sequence for KYSE 1440, 5′-GCCGGTTCAGAATCAGAAG-3′, corresponding to positions 335 to 353 within the Aurora-A mRNA. For the negative control vector, an empty pSUPERIOR-puro was used. We checked the internal stabilities of each sequences using Oligo 4.0-s software (National Biosciences, Inc., Plymouth, MN) and found that the 3′-end in the sense strand was less stable than the 5′-end, which is known to cause less off-target effects according to literature (26). Furthermore, we checked each sequence using BLAST Web site software[4] and found that each sequence was specific to *Aurora-A*. Moreover, we also stably transfected each sequence to HeLa cell, and preliminary experiments were undertaken to further confirm their effects (Supplementary Data 1).

Transfections. The ESCC cell lines KYSE 510 and KYSE 1440 were stably transfected with the Aurora-A shRNA expression vector or the empty pSUPERIOR-puro vector using Lipofectin reagent (Invitrogen) as suggested by the manufacturer's instruction. Briefly, 2 μg of each plasmid DNA and 20 μL of Lipofectin reagent together with Opti-MEM I medium (Invitrogen) were used with serum-free medium for 8 h. Cells were incubated for another 48 h with normal growth medium, and subsequently, the cell clones were selected against 1.0 μg/mL puromycin (Nacalai Tesque, Kyoto, Japan) for 3 weeks, and we picked up single colonies originated from single cells and expanded to obtain stably transfected cell lines.

WST assay for sensitivity to doxetaxel. Cytotoxic activity against docetaxel was measured by the tetrazolium salt–based proliferation assay (WST-8 assay; Wako Chemicals, Osaka, Japan) following the manufacturer's instructions. Briefly, cells were cultured in 96-well microtiter plates in 90 μL of growth medium (4,000 cells per well) and incubated for 24 h for sufficient cell growth. Then, 10 μL of a graded concentration of docetaxel (10^{-5} to 10^{-10} mol/L) were added into each well and cultured for 48 h. Control cultures received normal growth medium only. After 48 h, 10 μL of WST-8 solution were added to each well and the plates were incubated at 37°C for another 3 h. Absorbance at 450 and 640 nm was measured using the Delta Soft ELISA analysis program, and cell viability was measured and compared with that of control cells. Each experiment was carried out independently and repeated at least thrice. The IC_{50} value was defined as the concentration that reduced the absorbance in each test by 50%.

Cell proliferation assay. Cells were cultured in 6-cm dishes (2×10^4 per dish) and incubated for 24 h for sufficient cell growth and then treated with medium containing 0.3, 0.6, or 1.0 nmol/L of docetaxel for

[3] http://rsb.info.nih.gov/nih-image/

[4] http://www.ddbj.nig.ac.jp/search/blast-j.html

48 h. Then, they were cultured for another 48 h with normal growth medium. Control cultures received normal growth medium only. Cells were harvested with trypsin/EDTA every 48 h for 4 days and enumerated using a cell counter (Coulter Z1, Beckman Coulter, Fullerton, CA). A comparison was made with the control culture to examine the effect of suppressing the expression of Aurora-A on cell proliferation and to investigate the effect of the suppression on chemosensitivity to docetaxel. The experiment was repeated at least thrice.

Flow cytometry for analyzing the cell cycle. A flow cytometric analysis of DNA content was done to assess the cell cycle phase distribution. Cells were harvested at the 70% confluent stage and fixed in 70% ethanol at −20°C. After being washed with PBS, the cells were treated with PBS containing RNase A (100 mg/mL) at 37°C for 30 min. After centrifugation, the cells were resuspended in PBS containing propidium iodide (50 μg/mL) and stained at room temperature for 30 min. DNA content was evaluated using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and CellQuest software (Becton Dickinson). The experiment was repeated thrice.

Flow cytometry for detection of apoptotic cells. KYSE cells transfected with empty vector or shRNA against Aurora-A were cultured in 6-cm dishes (2×10^4 per dish) and treated as already described for the proliferation experiment. Briefly, cells were incubated for 24 h and treated with medium containing 0, 0.3, 0.6, or 1.0 nmol/L of docetaxel for 48 h. Then, they were cultured for another 48 h with normal growth medium. Subsequently, floating cells in the medium and adherent cells were collected. Using an Annexin V-FITC Apoptosis Detection kit (Medical & Biological Laboratories Co. Ltd., Woburn, MA), cells were stained with Annexin V-FITC and propidium iodide according to the manufacturer's instructions. Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained) from early apoptotic cells (stained only with Annexin V) and late apoptotic or necrotic cells (stained with both Annexin V and propidium iodide). Untreated cells and cells treated with 3% formaldehyde for 30 min served, respectively, as negative and positive controls for double staining. Cells were analyzed immediately after staining by using a FACScan flow cytometer and the CellQuest software. For each measurement, >10,000 cells were counted.

Tumor formation assay in nude mice. Suspensions of 1.0×10^6 KYSE 510-derived cells (Aurora-A shRNA-transfected cells, 510 A; empty vector-transfected cells, 510 m) in PBS (50 μL) were injected s.c. into the left flanks of 5-week-old male BALB/c *nu/nu* mice (Japan SLC, Shizuoka, Japan) at day 0. The inoculation was conducted in five mice, and tumor growth was estimated from the average volume of tumors. Tumor volume was calculated by the formula $1/2 \times L^2 \times W$ (L = length and and W = width of the tumor). At 46 days after inoculation, all mice were sacrificed, and s.c. tumors were resected and fixed in 10% formaldehyde/PBS. The tumors were paraffin embedded and stained with H&E and for Aurora-A. Immunohistochemical staining for Aurora-A was done as reported previously (14). All the animal experiments were done in accordance with institutional guidelines of the Kyoto University.

Treatment with docetaxel in nude mice. 510 m and 510 A tumors were generated as above. Briefly, suspensions of 1.0×10^6 cells in PBS (50 μL) were injected s.c. into the left flanks of 5-week-old male BALB/c *nu/nu* mice at day 0. The inoculation was conducted in five mice, and mice were treated with docetaxel (7.0 mg/kg body weight; i.p., thrice weekly) or with 0.1 mL PBS (pH 7.4; i.p., thrice weekly) as described previously with modifications (27). Tumor growth was estimated from the average volume of tumors, and tumor volume was calculated as already described. At 39 days after inoculation, all mice were sacrificed, and s.c. tumors were resected, and tumor size was compared. All the animal experiments were done in accordance with institutional guidelines of the Kyoto University.

Statistical analysis. All experiments were done in duplicate or triplicate. The Bonferroni multiple comparison test and t test were used for the statistical analysis of comparative data using StatView version 5 (SAS Institute, Cary, NC). Values of $P < 0.05$ were considered significant and are indicated by asterisks in the figures.

Results

Expression of Aurora-A and sensitivity to docetaxel in ESCC cell lines. Levels of Aurora-A protein expression in three cancer cell lines were determined by Western blotting (Fig. 1A). KYSE 510 and KYSE 1440 had higher levels of expression than KYSE 110. Next, the sensitivity of the cell lines to docetaxel was checked by WST assay. As shown in Fig. 1B, IC_{50} of KYSE 510 and KYSE 1440 ($4.9 \times 10^{-7} \pm 0.20 \times 10^{-7}$ mol/L and $4.6 \times 10^{-7} \pm 0.46 \times 10^{-7}$ mol/L, respectively) was higher than that of KYSE 110 ($2.4 \times 10^{-8} \pm 0.21 \times 10^{-8}$ mol/L; Fig. 1B). Thus, the cell lines with the higher levels of Aurora-A protein (KYSE 510 and KYSE 1440) were more resistant to docetaxel than the cell line with the lower level (KYSE 110).

Vector-based Aurora-A shRNA decreased proliferation in ESCC cell lines. To assess the role of the overexpression of Aurora-A in ESCC cells, we first established subclones via the transfection of vector-based shRNA for Aurora-A in KYSE 510 and KYSE

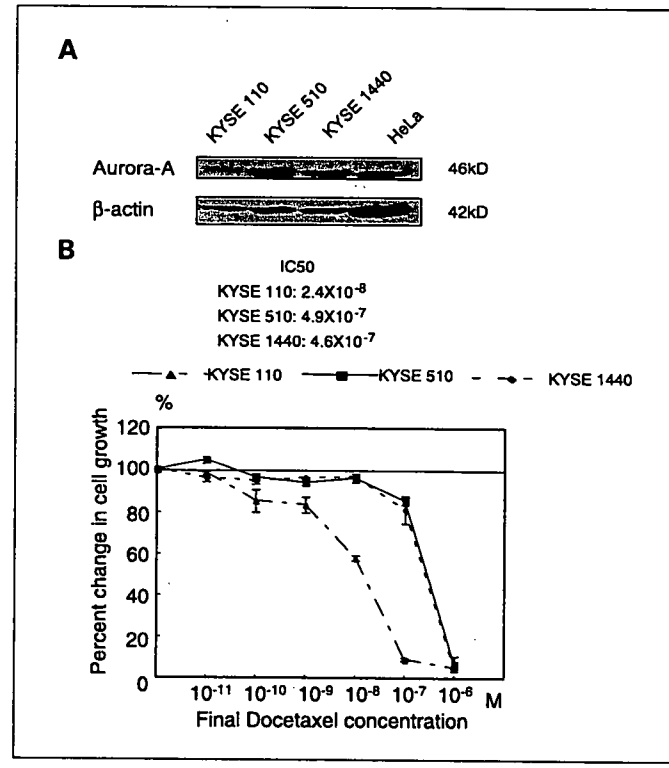

Fig. 1. Aurora-A expression and chemosensitivity to docetaxel in ESCC cell lines. *A*, expression of Aurora-A protein in ESCC cell lines. Immunoblots were probed with the anti-Aurora-A antibody and the anti-β-actin antibody. The HeLa cell line was included as a positive control of Aurora-A expression. *B*, cytotoxic activities of docetaxel in ESCC cell lines were measured by WST-8 assay. Graded concentrations of docetaxel (10^{-5} to 10^{-10} mol/L) were added to the wells, and the cells were cultured for 48 h. Control cultures received normal growth medium only. Cell viability was measured as absorbance at 450 nm, and values were compared with the control. The IC_{50} value was defined as the concentration that reduced the absorbance in each test by 50%. X axis, final docetaxel concentration (10^{-6} to 10^{-11} mol/L). The experiments were repeated thrice.

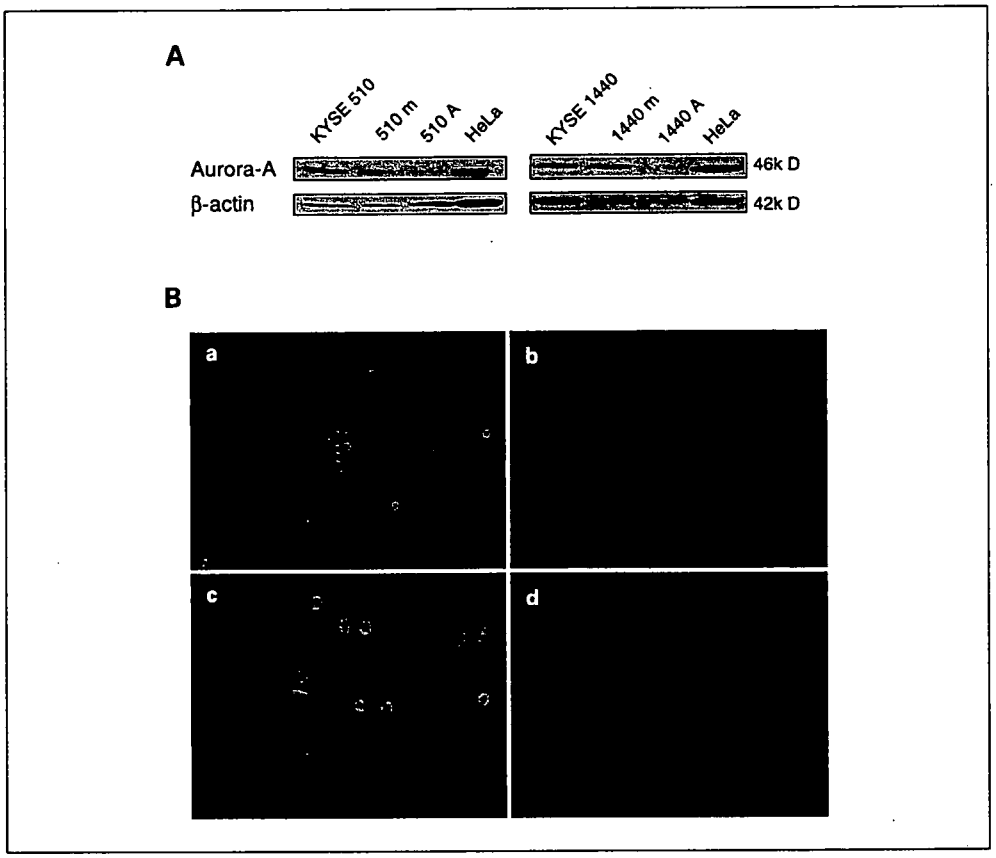

Fig. 2. Vector-based Aurora-A shRNA decreased proliferation of ESCC cell lines. *A*, suppression of Aurora-A protein expression with the Aurora-A shRNA vector. The KYSE 510 and KYSE 1440 cells were stably transfected with an empty vector (510 m or 1440 m) or an Aurora-A shRNA vector (510 A or 1440 A). The expression of Aurora-A protein in these clones was examined by Western blotting. Bottom, protein expression of β-actin. *B*, representative immunofluorescent staining of Aurora-A in an Aurora-A shRNA-transfected cells (510 A or 1440 A) or an empty vector – transfected cells (510 m or 1440 m). a, immunofluorescent staining of Aurora-A in empty vector – transfected cells (510 m). Magnification, ×200. Strong nuclear staining was observed. b, immunofluorescent staining of Aurora-A shRNA-transfected cells (510 A). Magnification, ×200. Weak nuclear staining was observed. c, immunofluorescent staining of Aurora-A in empty vector – transfected cells (1440 m). Magnification, ×200. Strong nuclear staining was observed. d, immunofluorescent staining of Aurora-A in Aurora-A shRNA-transfected cells (1440 A). Magnification, ×200. Weak nuclear staining was observed.

1440 cells. The levels of Aurora-A expression were efficiently reduced by 87% in the stable subclone 510 A and by 90% in 1440 A but were not reduced in empty vector–transfected clones (510 m and 1440 m; Fig. 2A). We also confirmed suppression of Aurora-A expression by immunofluorescent staining and found that Aurora-A immunoreactivity in established stable subclone 510 A and 1440 A was homogeneously depleted (Fig. 2B). We investigated the effect of suppressing the expression of Aurora-A on proliferation in ESCC cell lines. With suppression of Aurora-A expression, cell growth was inhibited by 38.7 ± 10.5% in 510 A and by 24.3 ± 10.0% in 1440 A compared with empty vector–transfected cells (510 m and 1440 m; $P < 0.01$ and 0.01, respectively; Fig. 2C). To investigate the growth suppression caused by inhibition of Aurora-A expression, cell cycle changes in 510 A and 1440 A as well as in empty vector–transfected cells were examined by flow cytometry. The population of cells in G_2-M phase was significantly larger in 510 A (41.13 ± 6.94%) and 1440 A (38.35 ± 1.06%) than that in the empty vector–transfected cells (510 m: 24.12 ± 2.33%, $P < 0.01$; 1440 m: 22.55 ± 2.25%, $P < 0.01$; Fig. 2D).

***The effect of suppressing Aurora-A on chemosensitivity to docetaxel* in vitro.** Chemosensitivity to docetaxel was investigated by WST assay and cell number determinations. In the WST assay, we found that IC_{50} of stable transfectants of shRNA for Aurora-A was lower than that of the empty vector transfectant (510 A, $2.7 \times 10^{-7} \pm 0.4 \times 10^{-7}$ mol/L; 510 m, $4.8 \times 10^{-7} \pm 0.7 \times 10^{-7}$ mol/L; 1440 A, $2.6 \times 10^{-7} \pm 0.8 \times 10^{-7}$ mol/L; 1440 m, $4.9 \times 10^{-7} \pm 0.4 \times 10^{-7}$ mol/L; Fig. 3A and B). We also confirmed the effect of suppressing Aurora-A expression on chemosensitivity to docetaxel by cell number determinations. In the cell number determinations, the suppression of the expression of Aurora-A allowed even 0.3 nmol/L docetaxel to be effective (Fig. 3C and D). Discrepancy in effective concentration of docetaxel between WST assay and cell number determinations could be explained by the difference in the initial numbers of cells, cell concentration at the beginning of each experiment, as well as the difference in size of culture plates and observation period. We repeated the cell number determinations with the same cell concentration and observation period as WST assay and confirmed that growth-inhibitory effect of docetaxel in these two different experiments was similar (Supplementary Data 2). Importantly, in respective experiments, we found that the suppression of Aurora-A expression enhanced chemosensitivity to docetaxel. To investigate the effect of chemosensitivity to docetaxel by the suppression of Aurora-A expression, apoptotic cells in the 510 A and 1440 A clones as well as empty vector–transfected clones were examined by flow cytometry and the type of cell death induced by docetaxel was assessed. In the Aurora-A shRNA-transfected cells, 0.3 nmol/L docetaxel induced a notable level of apoptosis compared with the empty vector–transfected cells (510 A, 71.3%; 510 m, 36.12%; 1440 A, 78.1%; 1440 m, 26.28%; Fig. 3E and F). These findings suggested that the suppression of Aurora-A expression augmented the apoptosis induced by docetaxel. To confirm the validity of the experiment, cells treated with 3% formalin for 30 min and stained with both Annexin V and propidium iodide served as a positive control for double staining (Supplementary Data 3).

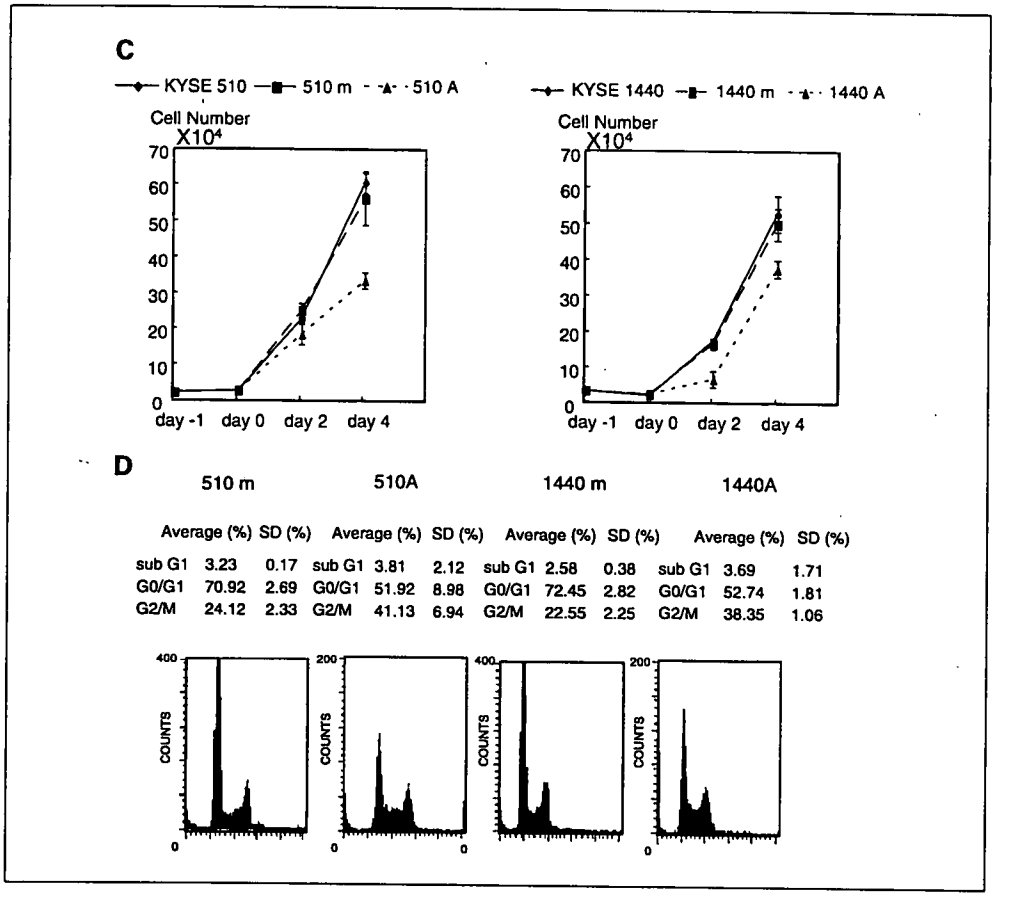

Fig. 2 *Continued. C, in vitro* growth assay in cells transfected with the Aurora-A shRNA vector (510 A or 1440 A) or an empty vector (510 m or 1440 m). The number of cells was counted every 2 d. The experiments were repeated thrice. *D*, cell cycle profiles obtained by flow cytometry. Ratios of cell populations in G_1 and G_2-M were the average and SD. These experiments were done thrice, and representative results are shown. The experiments were repeated thrice, and the representative results are shown.

***The effect of Aurora-A suppression on tumor growth* in vivo.** To examine the possible effect of Aurora-A shRNA on tumor growth *in vivo*, s.c. tumor formation assay in nude mice was done. As shown in Fig. 4A, the tumors formed from Aurora-A shRNA-transfected cells (510 A) were clearly smaller than that formed from empty vector–transfected cells (510 m). Then, we examined tumor volume and weight at 46 days after inoculation. The average tumor volume of the mice at day 46 was reduced by 36.1% in 510 A compared with 510 m (P = 0.03; Fig. 4C), and the average tumor weight was also decreased by 31.5% in 510 A compared with 510 m (P = 0.03; Fig. 4C). All of the tumors were stained with H&E and for Aurora-A (Fig. 4D), and Aurora-A immunoreactivity was confirmed to be reduced in tumors grown from Aurora-A shRNA-transfected cells but not in tumors grown from empty vector–transfected cells.

***The effect of Aurora-A suppression on chemosensitivity to docetaxel* in vivo.** To examine the possible effect of Aurora-A shRNA on chemosensitivity to docetaxel *in vivo*, s.c. tumors were generated in nude mice followed by treatment with docetaxel or PBS. The tumors formed from Aurora-A shRNA-transfected cells (510 A) were apparently smaller than those formed from empty vector–transfected cells (510 m) after the treatment with docetaxel at day 39 (Fig. 5A, *b* and *d*). To confirm this, we measured tumor volume at 39 days after inoculation. As shown in Fig. 5C, following the treatment with docetaxel, the average tumor volume of 510 A and 510 m was 175.3 ± 70.4 mm^3 and 312.8 ± 28.0 mm^3, respectively, and thus, the suppression of Aurora-A expression resulted in a 44.0% inhibition of tumor growth (P = 0.03).

Discussion

We previously reported that the expression of Aurora-A was frequently up-regulated in ESCC tissues and cell lines and contributed to a poor prognosis (14). In an attempt to determine the potential of Aurora-A as a therapeutic target, we used a vector-based shRNA technique to knock out its expression and analyzed its phenotypes.

In the current study, we were able to suppress the expression of Aurora-A using vector-based shRNA for two different target sequences in two different ESCC cell lines and obtained very similar results at each examination *in vitro*. That is, a reduction of Aurora-A protein expression was clearly related to cell growth inhibition and increased sensitivity to docetaxel.

Recently, a relationship between Aurora-A activity and G_2-M transition was reported in cancer cells (28). In the current study, we showed that the suppression of Aurora-A expression caused an accumulation of the cells in the G_2-M phase *in vitro*, resulting in the inhibition of proliferation of ESCC cell lines. Moreover, in recent report, Hata et al. (17) showed that the suppression of Aurora-A expression had an antitumorigenic effect *in vivo* in pancreatic cancer, and we similarly showed that the suppression of Aurora-A expression in ESCC cell lines inhibited tumor growth *in vivo* using our vector-mediated shRNA strategy. Our results were consistent with Hata et al. in

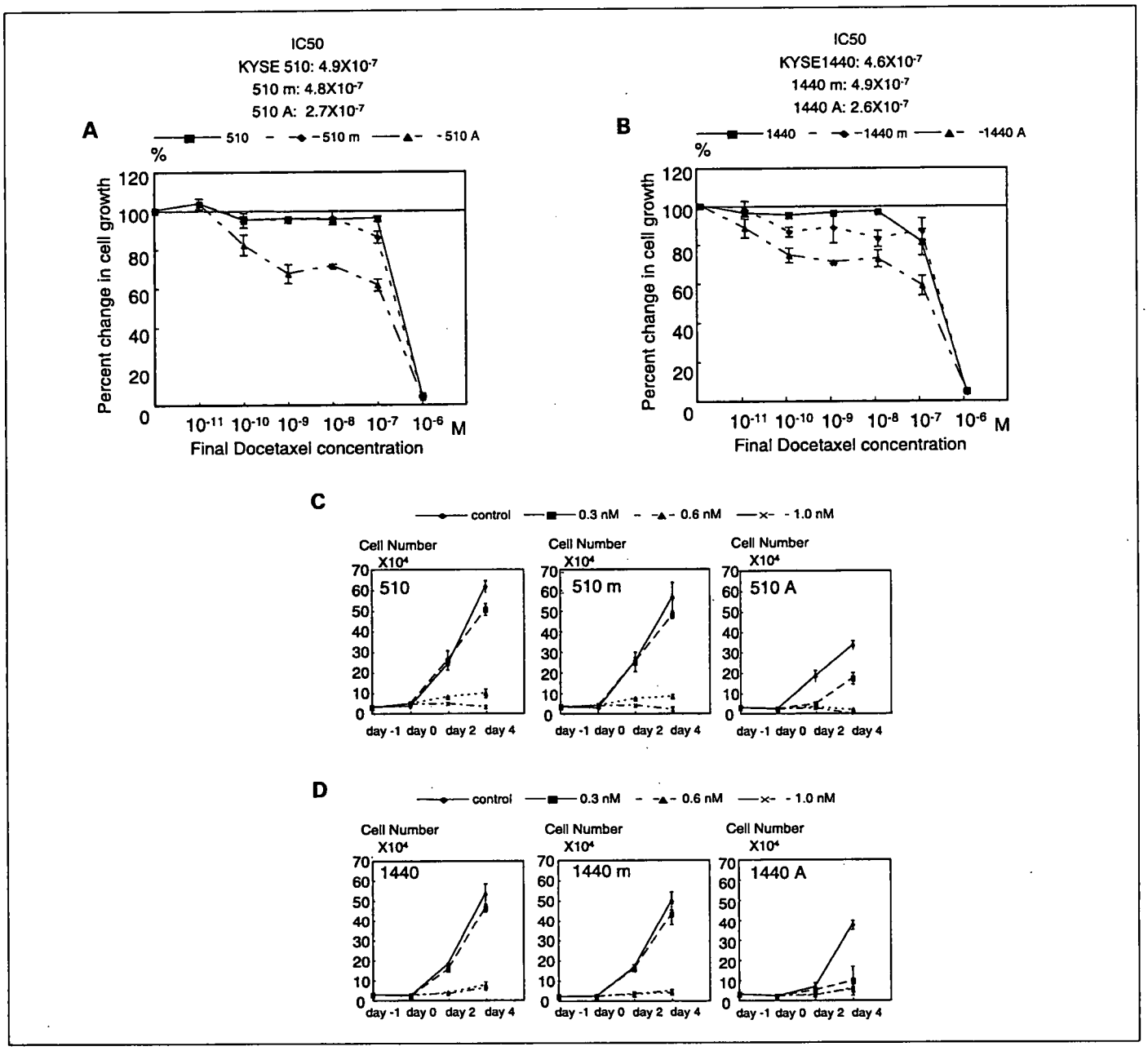

Fig. 3. Effect of the suppression of Aurora-A expression on sensitivity to docetaxel. *A* and *B*, cytotoxic activities of docetaxel were measured by WST-8 assay. Graded concentrations of docetaxel (10^{-5} to 10^{-10} mol/L) were added to the wells, and the cells were cultured for 48 h. Control cultures received normal growth medium only. Cell viability was measured as absorbance at 450 nm, and values obtained were compared with that of the control. The IC_{50} value was defined as the concentration that reduced the absorbance in each test by 50%. X axis, final docetaxel concentration (10^{-6} to 10^{-11} mol/L). The experiments were repeated thrice. *C* and *D*, cytotoxic activities of docetaxel were confirmed by cell counting. Graded concentrations of docetaxel (0, 0.3, 0.6, and 1.0 nmol/L) were added for the first 2 d, and normal growth medium was added for the next 2 d. The number of cells was counted every 2 d. These experiments were repeated thrice.

terms of antitumor activity that the suppression of Aurora-A expression caused in ESCC. Consequently, Aurora-A seemed to be a critical factor for the proliferation of cancer cells, and therefore, it should be a good therapeutic target for halting proliferation of ESCC.

Furthermore, we found that the suppression of Aurora-A expression enhanced the sensitivity to docetaxel-induced apoptosis both *in vitro* and *in vivo*. Taxanes bind to free tubulin and promote the assembly of tubulin into stable microtubules. Hence, they stop cell cycle progression, causing cells in the M phase to accumulate at the metaphase-anaphase transition and subsequently leading them to apoptosis, which is consistent with our findings that the suppression of Aurora-A expression resulted in the accumulation of cells in the G_2-M phase. As previously discussed, Aurora-A is essential for the proper arrangement of centrosomes and microtubules, and Hata et al. showed that a combination of the suppression of Aurora-A expression and use of taxanes resulted in an augmented induction of apoptosis in pancreas cancer *in vitro* (17). Additionally, Anand et al. (15) showed that the overexpression of Aurora-A induced increased resistance to taxanes via a decrease in spindle checkpoint activity *in vitro*. Our results

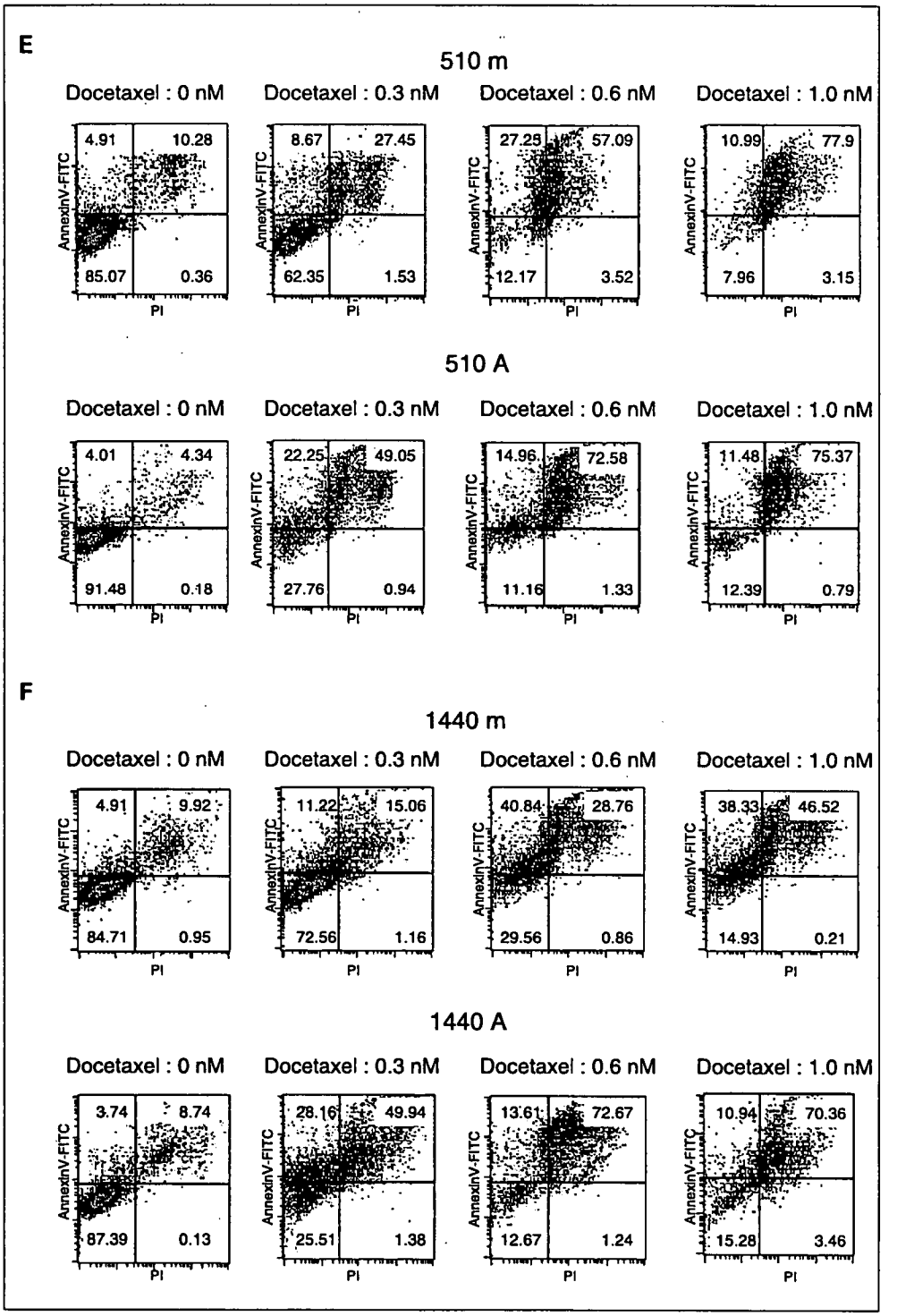

Fig. 3 *Continued. E* and *F*, to assess the type of cell death induced by docetaxel, flow cytometry was done. Cells in bottom left quadrant (unstained), top left quadrant (stained only with Annexin V), and top right quadrant [stained with both Annexin V and propidium iodide (*PI*)] represent viable cells, cells in early apoptosis, and cells in late apoptosis, respectively. The experiments were repeated thrice, and the representative results are shown.

are consistent with this study and were able to show that the suppression of Aurora-A expression enhanced chemosensitivity to docetaxel not only *in vitro* but also *in vivo*. These results suggest that, by suppressing Aurora-A expression, spindle checkpoint activity might have recovered and, thus, increased the sensitivity to taxanes. In the meantime, the mechanism that triggers apoptosis after inhibition of Aurora-A expression, as well as the complicated biological activity of Aurora-A, remains to be clarified.

RNA interference has become conventional applications for *in vivo* cancer therapy (29, 30), and an efficient way of delivering small interfering RNA into solid tumors has been developed (31). In the current study, we explored the possibility that the RNA interference–mediated suppression of Aurora-A could be used as a specific gene-targeting therapy to suppress the progression of ESCC. Moreover, the function of Aurora kinase inhibitors (including the patent literature) has been studied recently, revealing potentially promising anticancer

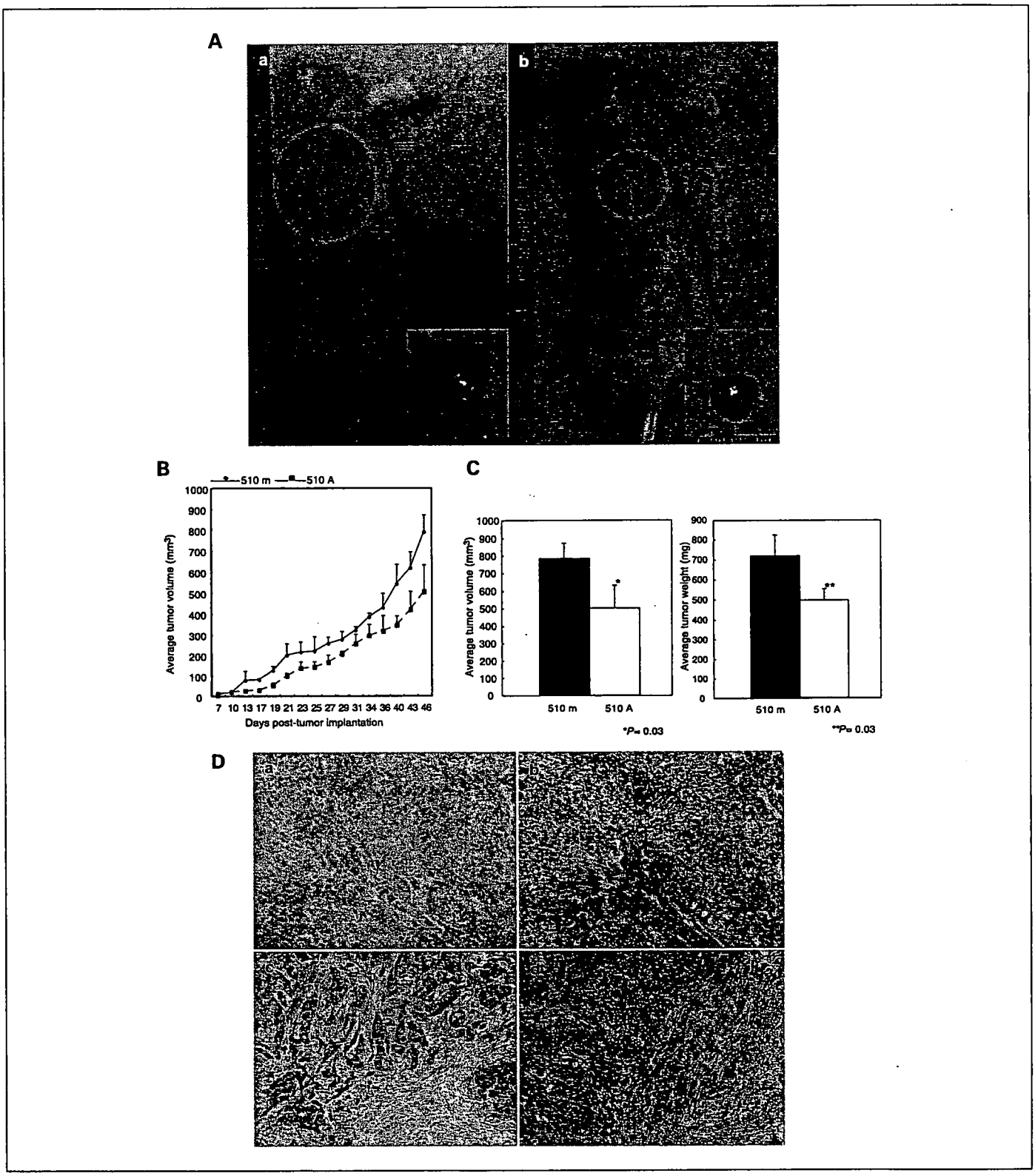

Fig. 4. Effect of the down-regulation of Aurora-A expression on the formation of tumors *in vivo*. *A*, representative features of tumors in a mouse 46 d after the inoculation. a, tumors formed from empty vector – transfected cells (510 m) in the left flank; b, tumors formed from Aurora-A shRNA-transfected cells (510 A) in the left flank. *B*, growth of tumors in the mice injected with Aurora-A shRNA-transfected cells (510 A) or empty vector – transfected cells (510 m). The inoculation was done in five mice. *C*, tumor volume and weight at day 46 after inoculation. Left, black column, average tumor volume at day 46 after the inoculation of empty vector – transfected cells (510 m); white column, average tumor volume at day 46 after the inoculation of Aurora-A shRNA-transfected cells (510 A; $n = 5$). *, $P = 0.03$. Right, black column, average tumor weight at day 46 after the inoculation of empty vector – transfected cells (510 m); white column, average tumor weight at day 46 after the inoculation of Aurora-A shRNA-transfected cells (510 A; $n = 5$). **, $P = 0.03$. *D*, a, immunohistochemical staining of Aurora-A in s.c. tumors at day 46 after the inoculation of Aurora-A shRNA-transfected cells (510 A). Magnification, ×200. b, H&E staining of s.c. tumors at day 46 after the inoculation of Aurora-A shRNA-transfected cells (510 A). Magnification, ×200. c, immunohistochemical staining of Aurora-A in s.c. tumors at day 46 after the inoculation of empty vector – transfected cells (510 m). Magnification, ×200. d, H&E staining of s.c. tumors at day 46 after the inoculation of empty vector – transfected cells (510 m). Magnification, ×200.

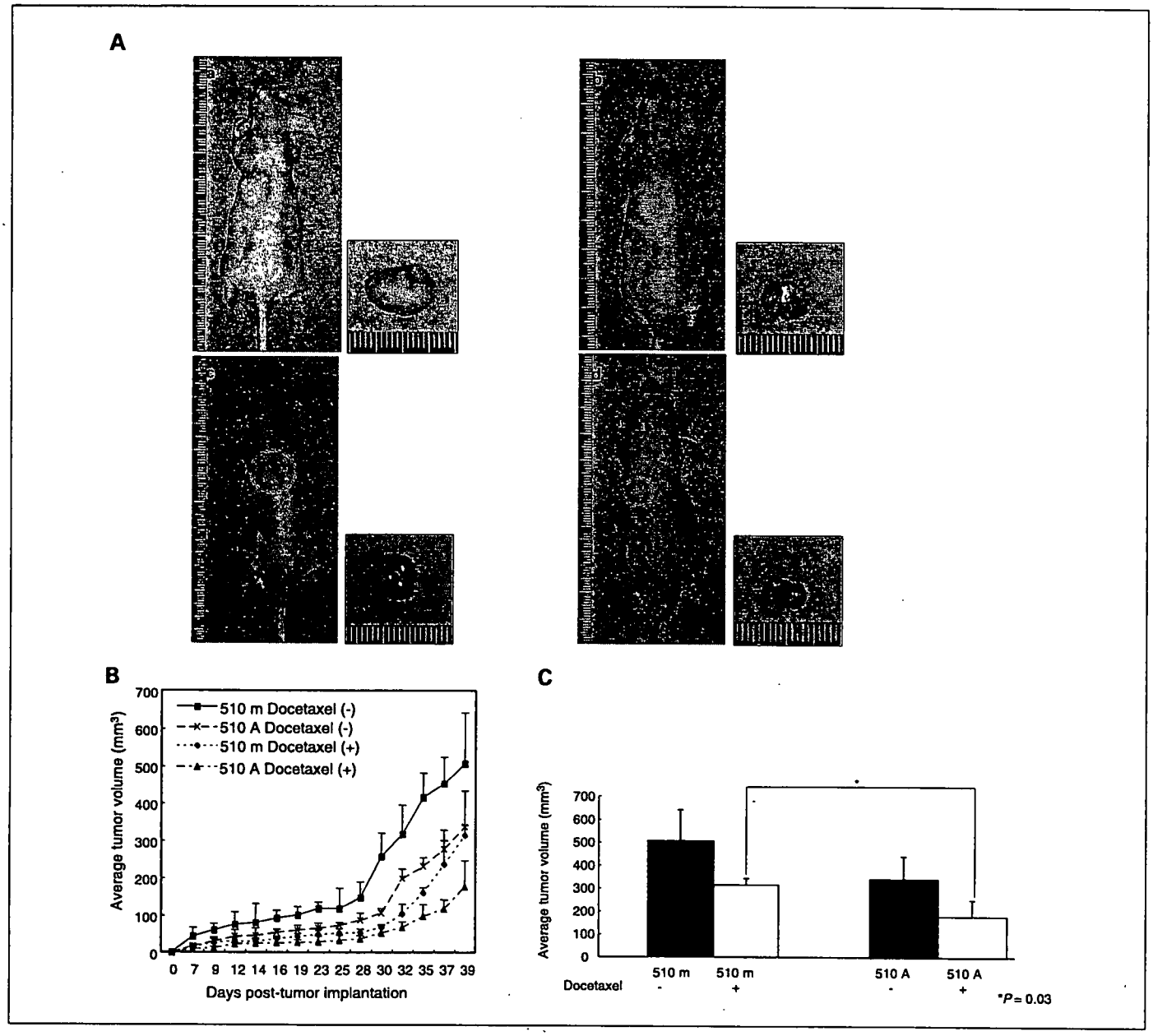

Fig. 5. Effect of the suppression of Aurora-A expression on sensitivity to docetaxel *in vivo*. Mice were treated with docetaxel (7.0 mg/kg body weight; i.p., thrice weekly) or with 0.1 mL PBS (pH 7.4; i.p., thrice weekly). *A*, representative features of tumors 39 d after inoculation. a, empty vector – transfected cells (510 m) were injected s.c. into the left flank and the mice were treated with PBS (i.p., thrice weekly). b, empty vector – transfected cells (510 m) were injected s.c. into the left flank and the mice were treated with docetaxel (7.0 mg/kg body weight; i.p., thrice weekly). c, Aurora-A shRNA-transfected cells (510 A) were injected s.c. into the left flank and the mice were treated with PBS (i.p., thrice weekly). d, Aurora-A shRNA-transfected cells (510 A) were injected s.c. into the left flank and the mice were treated with docetaxel (7.0 mg/kg body weight; i.p., thrice weekly). *B*, growth of tumors in the mice injected with Aurora-A shRNA-transfected cells (510 A) or empty vector – transfected cells (510 m) with or without docetaxel. The inoculation was done in five mice. *C*, tumor volume at day 39 after the inoculation. Left, black column, average tumor volume at day 39 after the inoculation of empty vector – transfected cells (510 m) in mice treated with PBS; white column, average tumor volume at day 39 after the inoculation of empty vector – transfected cells (510 m) in mice treated with docetaxel ($n = 5$). Right, black column, average tumor volume at day 39 after the inoculation of Aurora-A shRNA-transfected cells (510 A) in mice treated with PBS; white column, average tumor volume at day 39 after the inoculation of Aurora-A shRNA-transfected cells (510 A) in mice treated with docetaxel ($n = 5$). *, $P = 0.03$.

effects (32, 33). Therefore, our results in combination with these findings suggest that taxane-mediated chemotherapy could be more effective in combination with these anti-Aurora agents in ESCC.

In summary, the suppression of Aurora-A expression is shown to inhibit tumor growth of ESCC and enhanced chemosensitivity to docetaxel both *in vitro* and *in vivo*. Consequently, the therapeutic regimen to suppress the Aurora-A expression is a feasible candidate to become a novel therapeutic strategy for the treatment of ESCC.

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