bipartite sequence in human tumors are extremely rare. In this context, Goldman *et al* showed that in a neuroblastoma cell line expressing cytoplasmically sequestered wild-type p53, p53 target genes (*p21WAF1* and *MDM2*) were up-regulated following cell irradiation (18). These results also suggest that wild-type p53 retains some functional activity when it is sequestered in the cytoplasm, although p53 homologues, such as p63 and p73, may have been involved in the result. By contrast, our experimental system was a p53-specific inducible system; therefore, involvement of p53 homologue activation is unlikely.

Our previous knowledge of p53-dependent apoptosis was that after genotoxic stress, activated p53 transactivated its downstream genes in a sequence-specific manner in the cell nucleus and induced apoptosis in cells through the direct or indirect induction of the downstream protein(s); however, a transactivation-independent mechanism for p53-dependent apoptosis has been reported by several laboratories (19,20). In addition, we previously indicated a lack of correlation between p53-dependent transactivation activity and the ability to induce apoptosis, and speculated that a transactivation-independent mechanism may exist (17). We excluded the nuclear function of p53, including the sequence-specific transactivation function, by introducing R306G, a mutation in the bipartite sequence at residues 305 and 306. A conditional expression system of cytoplasmically sequestered p53 was constructed and we found that cytoplasmically sequestered p53 retains its ability to arrest cell proliferation (wild-type p53) and induce apoptosis (S121F). These results strongly support a cytoplasmic apoptotic function of p53. Notably, however, cytoplasmically sequestered p53 transactivated downstream genes. Therefore, we did not clarify whether cytoplasmic p53-dependent apoptosis depends on either a direct or an indirect transactivation mechanism or is independent of transactivation.

Additional experiments are required to evaluate which mechanism is crucial for p53-dependent apoptosis and to clarify the mechanism underlying super p53 (S121F)-dependent apoptosis.

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●原　著●

食道癌化学療法におけるエレンタールの口内炎予防・軽減効果についての検討

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Elental Prevented and Reduced Oral Mucositis during Chemotherapy in Patients Esophageal Cancer: Tadahisa Fukui[*1], Yuriko Itoh[*1], Mika Orihara[*2], Kazuya Yoshizawa[*2], Hiroaki Takeda[*2], Sumio Kawada[*2] and Takashi Yoshioka[*1]
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Summary
Stomatitis is a side effect caused frequently by chemotherapy in patients with esophageal cancer, but a standard treatment for it has not been established. Reactive oxygen species are known to be among the causes of stomatitis induced by chemotherapy or radiotherapy, and some reports suggest that their influences might be reduced by the oral supplementation of glutamine. Elental® is one of the widely-used nutritional supplements, and its pack contains 1, 932 mg of L-glutamine (an especially high amount.). Therefore, we examined the preventive or reducing effects Elental® mey have on oral mucositis. Fifteen patients with esophageal cancer received chemotherapy, six of whom had grade one oral mucositis. All of those six patients entered the investigation, and seven courses of Elental® were administered. After seven courses, all six patients oral mucositis declined from grade 1 to grade 0. This result suggests that Elental® has preventive or reducing effects on oral mucositis. **Key words**: Oral mucositis, Chemotherapy, Esophageal cancer, Glutamine, Elental® (*Received Jan. 18, 2011/Accepted Apr. 14, 2011*)

要旨　食道癌における化学療法では口内炎は頻度の高い有害事象の一つである。口内炎に対する有効な治療法は確立されておらず，標準的な治療法も存在しない。グルタミンは活性酸素による組織障害を軽減させる作用があり，抗癌剤や放射線による口内炎を軽減させる報告がある。今回われわれは，グルタミンを含有する栄養剤であるエレンタール®（L-グルタミン1,932 mg 含有/1袋）を用いて，口内炎予防・軽減作用について検討した。15人の化学療法施行食道癌患者のうち，6人に登録前の治療で grade 1の口内炎が認められた。これらの患者を対象に，口内炎がでた次コースの化学療法時開始前7日から開始後第7日まで計14日間，エレンタールを1日1袋内服させた。エレンタールは6人に計7サイクル投与され，全例全サイクルで grade 0となった。エレンタール内服にて口内炎の予防・軽減効果を期待できる可能性があると考えられた。

はじめに

近年は食道癌に対する標準治療が確立されつつあり，進行病期Ⅱ～Ⅳでは放射線化学療法あるいは抗癌剤治療が行われる。抗癌剤治療は5-FU持続静注とプラチナ製剤との併用療法が第一選択となる。日本では5-FU＋シスプラチン療法[1]あるいは，5-FU＋ネダプラチン療法[2,3]が使用されるが，ネダプラチンは腎機能が低下している症例に選択される。5-FUでよくみられる副作用には，口内炎や下痢などの消化器症状（粘膜炎），吐気や食欲低下などがあり，シスプラチンでよくみられる副作用としては，腎毒性と吐き気・嘔吐がある。このうち5-FUによる口内炎はしばしば経験される有害事象の一つであり[4,5]，特に放射線を併用した場合は発症頻度が高いとされる。軽症の口内炎は患者も申告せず見過ごされがちで，好中球減少を来し感染を併発し，重症化して初めて見つかることもある。重症化した場合は，治癒するまでに2週間ほどかかるために栄養状態が悪化し，入院の長期化

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表 1 グルタミンの有効性を示した原著論文，総説

著者，文献		グルタミン投与	抗癌剤	調査項目	結論
Anderson PM, *et al*: *Cancer* 83: 1433-1439, 1998.	原著 13)	4 g po×14 days	CAD, VAdrC, IA, CDDPAdr, MTX	ECOG grading system	口内炎軽減，治癒日数短縮
Decker-Baumann C, *et al*: *Eur J Cancer* 35: 202-207, 1999.	原著 14)	0.4 g/kg iv×5 days 治療1日前から	5-FU 550 mg/m^2, CF 170 mg/m^2 d1-5	血中濃度 粘膜の病理所見	胃・十二指腸の粘膜炎軽減
Daniele B, *et al*: *Gut* 48: 28-33, 2001.	原著 15)	18 g po×15 days 治療5日前から	5-FU 450 mg/m^2, FA 100 mg/m^2 d1-5	消化吸収試験	吸収・透過性の変化を軽減
Cerchietti LC, *et al*: *Int Radiat Oncol Biol Phys* 65: 1330-1337, 2006.	原著 16)	0.4 g/kg iv×5 days	5-FU 1,000 mg/m^2 d1-5, CDDP 100 mg/m^2 d1	WHO Scale	口内炎軽減
Choi K, *et al*: *Clin Nutr* 26: 57-62, 2007.	原著 17)	30 g po×15 days 治療3日前から	5-FU 500 mg/m^2, LV 100 mg/m^2 d1-5	^{51}Cr-EDTA	腸管の透過性抑制
Peterson DE, *et al*: *Cancer* 109: 322-331, 2007.	原著 18)	Saforis (glutamine in Up Tec) 7.5 g po×14 days	CAF, FAC, AC	WHO Scale	全体的な Scale を低下
Sharma R, *et al*: *Lancet Oncol* 6: 93-102, 2005.	総説 10)				Level Ⅰb

CAD: cyclophosphamide, doxorubicin, dacarbazine, VAdrC: vincristine, doxorubicin, cyclophosphamide, IA: ifosfamide, doxorubicin, CDDPAdr: cisplatin, doxorubicin, MTX: high dose methotrexate, CF: Calcium-Folinate, FA: folinic acid, CDDP: cisplatin, LV: Leucovorin, CAF: cyclophosphamide, doxorubicin, 5-FU, FAC: 5-FU, doxorubicin, cyclophosphamide, AC: doxorubicin, cyclophosphamide

や quality of life（QOL）低下の原因となる。このような場合，化学療法の延期や抗癌剤の投与量の減量が必要となり，dose intensity（DI）が保てず生存期間にも影響を及ぼすことになる[6]。

口内炎の有効な対処法は少なく，標準治療も存在しない。事前の歯科受診やブラッシング，フロスなどによる口腔ケアが重要視されており，他にはクライオセラピー，生理食塩水やアロプリノール，キシロカイン含嗽液などによる治療が，いくつかの報告や総説・ガイドラインでのほぼ共通した対処法である[7-12]。

グルタミンは抗酸化作用と抗炎症作用があることで知られ，口内炎や粘膜炎を軽減させる報告や総説がある（表1）[7,10,13-18]。5-FU や口内炎に限定すると報告はやや少なくなるが，ハムスターにおいて 5-FU 投与時における口内炎軽減作用を病理学的に検討した報告[19]，実地臨床において口内炎や胃・十二指腸炎，腸管粘膜の透過性を軽減させた報告[13-18]，乳癌患者におけるアンスラサイクリン併用レジメンでの無作為試験では有意に口内炎が減少したとする報告[18]，放射線治療時における口内炎や粘膜炎軽減作用を検討する報告などがある[20,21]。

エレンタール®（味の素製薬: 東京）はもともと低残渣食として宇宙滞在を視野に入れて開発されたが，現在は栄養吸収のよさから炎症性腸疾患の食事療法として処方されることが多く，L-グルタミンは 1,932 mg/包含まれる。成人1日の食餌にはおおよそ 4～5%（約 2,700 mg）のグルタミンが含まれており[22]，エレンタール内服によりほぼ1日分に相当する量のグルタミンを摂取できることから，口内炎予防ないし軽減効果が期待できると考えられる。そこで，食道癌における化学療法ないし化学放射線療法でみられる口内炎に対するエレンタールの予防効果・軽減効果に関して検討を行った。

Ⅰ．対象・方法

1．対象患者

2010年4月1日～9月30日まで山形大学医学部附属病院腫瘍内科にて2サイクル以上の抗癌剤治療された食道癌患者15人を対象とした。適格基準は，病理学的に食道癌の確定診断がなされている，performance status（PS）（ECOG）が 0～2，主要臓器機能が保たれている，本試験に書面にて同意が得られた者とした。臨床病期や放射線併用の有無は問わないこととし，必要な場合は初回治療前に歯科受診させ口腔ケアを行った。ただし，胃ろうを造設し経腸栄養を行っている患者は除外した。なお，本試験は当院の倫理審査委員会承認の下に行われた。

2．方　法

抗癌剤治療時の口内炎の程度を有害事象共通用語規準（Common Terminology Criteria for Adverse Events: CTCAE v4.0）で評価し，抗癌剤治療で grade 1 以上の口内炎が起きた症例に対して，エレンタール内服投与を行った。エレンタールは，口内炎のでた次のコースの抗癌剤投与開始前7日から14日間1日1包投与された。抗癌剤治療レジメンは 5-FU＋シスプラチン療法（5-FU

表 2 対象患者一覧

患者	性別年齢	病期	抗癌剤治療		放射線治療		血清アルブミン (mg/dL)		エレンタール内服状況
			前回治療時	登録時	前回治療時	登録時	登録前	第8病日	
No. 1	男性 73歳	Ⅳa	5-FU 1,250 mg×5 days シスプラチン 100 mg×1 day	5-FU 1,250 mg×5 days ネダプラチン 120 mg×1 day	なし	なし	2.8	2.0 (−28.6%)	14日間
No. 2	男性 70歳	CRT後再発	5-FU 1,100 mg×5 days シスプラチン 80 mg×1 day	5-FU 1,000 mg×5 days ネダプラチン 100 mg×1 day	なし	なし	3.8	3.6 (−5.3%)	7日間
No. 3	男性 71歳	Ⅳb	5-FU 1,300 mg×5 days シスプラチン 130 mg×1day	5-FU 1,300 mg×5 days シスプラチン 130 mg×1 day	なし	なし	3.3	3.6 (+9.1%)	14日間
					なし	なし	3.3	3.2 (−3.0%)	14日間
No. 4	女性 61歳	Ⅳb	5-FU 1,000 mg×5 days シスプラチン 100 mg×1 day	5-FU 1,000 mg×5 days シスプラチン 100 mg×1 day	同時照射	同時照射	3.8	3.8 (±0%)	14日間
No. 5	男性 59歳	Ⅳa	5-FU 850 mg×5 days シスプラチン 85 mg×1 day	5-FU 850 mg×5 days シスプラチン 85 mg×1 day	同時照射	同時照射	2.5	2.1 (−16.0%)	9日間
No. 6	男性 73歳	CRT後再発	5-FU 1,200 mg×5 days ネダプラチン 120 mg×1 day	5-FU 1,200 mg×5 days ネダプラチン 120 mg×1 day	なし	なし	3.6	4.0 (+11.1%)	14日間

800 mg/m^2 civ d1〜5, シスプラチン 80 mg/m^2 d1 q4week）で，腎機能の悪い場合5-FU＋ネダプラチン療法（5-FU 800 mg/m^2 civ d1〜5, ネダプラチン 80 mg/m^2 d1 q4week）を使用した。エレンタール内服後の口内炎の評価は，化学療法開始8病日から聴取しCTCAE v4.0に準拠して記載した。栄養状態の評価も併せて行い，指標は血清アルブミン値とし，治療開始前と第8病日に測定した。

Ⅱ. 結 果

対象15人中で口内炎を発症した患者は，grade 1が6人，grade 3が1人，全体では計7人46.7%（7/15）であった。grade 3の1人はエレンタール内服前に拒否で脱落となり，6人/計7サイクルの患者で検討を行った（表2）。平均年齢は67.8歳で，男性5人，女性1人。放射線化学療法後の再発2人，臨床病期Ⅳa 2人，Ⅳb 2人。5-FU＋シスプラチン療法での治療3人（4サイクル），腎障害により5-FU＋ネダプラチン療法が行われた患者3人（3サイクル）であった。前回・登録時ともに放射線照射中であった患者は2人（2サイクル），前回・登録時ともに放射線照射をしていない患者は4人（5サイクル）であった。前回治療での口内炎は全例grade 1で，全例がエレンタールの内服によりgrade 0となった（7サイクル/7サイクル: 100%）。4人（5サイクル）はエレンタールを14日間内服できたが，1人（1サイクル: No. 2）は7日間，1人（1サイクル: No. 5）は9日間の内服であった。いずれも吐気が原因ではなく，食欲低下と腹部膨満感によるものであった。

治療前後の血清アルブミン値の測定結果（表2）は，10%以上低下した症例は2人（2サイクル）あり，症例No. 1（−28.6%）と症例No. 5（−16.0%）であった。10%未満の低下に留まった症例は2人（2サイクル）で，症例No. 2（−5.3%）と症例No. 3（−3.0%）。変化なしまたは増加が3人（3サイクル）で，症例No. 3（+9.1%）と症例No. 4（±0%）と症例No. 6（+11.1%）であった。

Ⅲ. 考 察

薬剤別にみた口内炎発症頻度（grade 3〜4）は5-FU単剤持続注14%，5-FU併用レジメンでは5-FU＋シスプラチン18%，5-FU＋シスプラチン（放射線併用）38%で，臓器別の口内炎発症頻度（grade 3〜4）は頭頸部癌42%，食道癌46%との海外の報告がある[7]。日本における報告では，食道癌治療での口内炎発症頻度（grade 1〜2）は5-FU＋シスプラチン（放射線併用なし）18.3〜35%となっている[4,5]。今回の試験中の口内炎発症頻度は46.7%と他の報告より少し高めであった。5-FUと放射線を併用することで口内炎の頻度が高まることが示唆されており，併用した放射線の影響が今回の口内炎の頻度

表 3 口内炎発症の Five-Stage Process[7]

1．Initiation	放射線や抗癌剤の直接作用により粘膜上皮細胞，粘膜下層の脈管や線維芽細胞の DNA が損傷し細胞増殖や組織修復が抑制される。活性酸素による組織障害も同時に起こり，粘膜炎を起こす主要経路と考えられ前述の細胞や脈管へ直接的な損傷を与える。この段階の組織炎症は可逆性の状態である。
2．Message generation	NF-κB を介して，TNF-α，IL-1，IL-6 などの炎症性サイトカインが放出されることで組織炎症が拡大し細胞障害が促進される。
3．Signaling and amplification	アポトーシスや組織障害によって，さらに炎症性サイトカインが放出され悪化していく。
4．Ulceration	粘膜構造は破壊され細菌感染などにより炎症細胞が誘導され重症化していく。
5．Healing	治癒過程

NF-κB: Nuclear factor-kappaB, TNF-α: tumor necrosis factor-alfa, IL-1: interleukin-1, IL-6: interleukin-6

を高めた原因と推定される。

口内炎発症のメカニズムはしだいに解明されてきており，様々な炎症性サイトカインや活性酸素が段階的に関与していると考えられ，Five-Stage Process が提唱されている（表 3）。grade 1 の口内炎は「1．Initiation」の病態と思われ，口内炎の主因である活性酸素の影響は可逆性である[6,7,23]。今回の検討は結果的に grade 1 の口内炎のみが対象となったが，5～7 日ごろに発症しはじめ，7～14 日ごろに最も悪化する症例が多かった。エレンタール内服を行った 6 人，計 7 サイクル全例で grade 0 に回復した。前回と同じ抗癌剤投与量での治療がなされているため，口内炎も同様に起こってくることが多いために，エレンタールによる口内炎改善効果はあった可能性が高い。また，予防効果について，5-FU 開始前からの投与が有効との報告がある[14,15,17]。今回，化学療法開始 7 日前からエレンタールの内服を開始して，口内炎の発症がなかったことから予防効果を示唆するものと考えられる。しかし，少数例での検討でもあり，比較対象がなく無作為割付もしていないために確定的な結論を述べるのは難しい。今後さらなる検討が必要である。

治療前後の血清アルブミン測定も行ったが，血清アルブミン値の変化なし，または増加例も計 3 人（症例 No. 3, 4, 6）おり，減少幅が 5.3～3.0％に留まった者も 2 人（症例 No. 2, 3）おり，エレンタール内服が栄養状態維持につながり，口内炎改善の一つの助けとなった可能性が示唆される。しかし，治療前から血清アルブミン値が 3.0 未満で栄養状態不良症例（症例 No. 1, 5）では，エレンタール内服後もアルブミン値は 16.0～28.6％低下しているのにもかかわらず（表 2），口内炎は改善した。エレンタールによる口内炎改善には，グルタミン補給が主役であることを示しているのかもしれない。

今回われわれが行ったエレンタール内服による検討で，エレンタールには口内炎予防効果の可能性があること，軽症の口内炎の改善効果が期待し得る可能性があることが示唆された。また，これにはある程度エレンタールによる栄養補助が貢献している可能性もある。口内炎は軽症では見落とされがちな有害事象であるが，患者の精神状態や闘病意欲とも結び付き QOL に重要な影響を及ぼし得る。エレンタール内服が口内炎の軽減や予防に対する積極的アプローチとなれば，QOL の向上や生存期間延長につながると考えられる。今後，食道癌に限らず，多くの癌腫と症例で検討を行っていく必要があると思われる。

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Targeting colon cancer stem cells using a new curcumin analogue, GO-Y030

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BACKGROUND: Persistent activation of signal transducers and activators of transcription 3 (STAT3) is commonly detected in many types of cancer, including colon cancer. To date, whether STAT3 is activated and the effects of STAT3 inhibition by a newly developed curcumin analogue, GO-Y030, in colon cancer stem cells are still unknown.
METHODS: Flow cytometry was used to isolate colon cancer stem cells, which are characterised by both aldehyde dehydrogenase (ALDH)-positive and CD133-positive subpopulations ($ALDH^+/CD133^+$). The levels of STAT3 phosphorylation and the effects of STAT3 inhibition by a newly developed curcumin analogue, GO-Y030, that targets STAT3 in colon cancer stem cells were examined.
RESULTS: Our results observed that $ALDH^+/CD133^+$ colon cancer cells expressed higher levels of phosphorylated STAT3 than ALDH-negative/CD133-negative colon cancer cells, suggesting that STAT3 is activated in colon cancer stem cells. GO-Y030 and curcumin inhibited STAT3 phosphorylation, cell viability, tumoursphere formation in colon cancer stem cells. GO-Y030 also reduced STAT3 downstream target gene expression and induced apoptosis in colon cancer stem cells. Furthermore, GO-Y030 suppressed tumour growth of cancer stem cells from both SW480 and HCT-116 colon cancer cell lines in the mouse model.
CONCLUSION: Our results indicate that STAT3 is a novel therapeutic target in colon cancer stem cells, and inhibition of activated STAT3 in cancer stem cells by GO-Y030 may offer an effective treatment for colorectal cancer.
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Colorectal cancer is the third leading cause of cancer-related deaths in the United States. For patients with advanced colon cancer, the 5-year survival rate is less than 10%. Recent evidence suggests the existence of a small population of tumourigenic stem cells responsible for tumour initiation, metastasis and resistance to chemotherapy and radiation. Increasing evidence suggests that cancer stem cells are also relevant to colorectal cancer, and that they have an important role in cancer spread and recurrence (Barker *et al*, 2007; O'Brien *et al*, 2007; Ricci-Vitiani *et al*, 2007; Boman and Huang, 2008). It is important to identify the regulatory mechanisms and signalling pathways involved in colon cancer stem cells and develop novel reagents to target this refractory colon cancer stem cell population.

The signal transducers and activators of transcription (STAT) protein family represents a group of transcription factors that have a role in relaying extracellular signals initiated by cytokines and growth factors from the cytoplasm to the nucleus (Calo *et al*, 2003; Frank, 2007; Germain and Frank, 2007). Following activation, phosphorylated STATs dimerise and translocate to the nucleus, where they regulate the expression of numerous critical genes involved in cell cycle progression, proliferation, invasion, and survival. However, the constitutive activation of STAT3 is frequently detected in primary human cancer cells, including colorectal carcinoma cells (Corvinus *et al*, 2005; Kusaba *et al*, 2005). Persistent STAT3 activation is associated with enhanced proliferation and invasion of colorectal cancer cells *in vitro* and tumour growth in a colorectal tumour model *in vivo*, and inhibition of STAT3 induces apoptosis and reduces tumour cell invasion in colorectal cancer cells (Corvinus *et al*, 2005; Lin *et al*, 2005; Tsareva *et al*, 2007; Xiong *et al*, 2008). These reports indicate that constitutively activation of STAT3 is one of the important pathways that contribute to the oncogenesis in colorectal cancer and can serve as an attractive therapeutic target for colorectal carcinoma.

During the past decade, a number of developmental pathways that regulate cancer stem cells, especially in breast cancer stem cells, have been elucidated. These pathways include Notch, Hedgehog, Wnt, human epidermal growth factor receptor 2, AKT etc (Liu and Wicha, 2010). However, the role of STAT3 in colon cancer stem cells and the effect of STAT3 inhibition in colon cancer stem cells are still unknown.

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Many markers and features of cancer stem cells have been defined. The transmembrane protein CD133 (Prominin-1 or AC133) is one of the markers that was first used to identify and isolate stem cells in brain cancers (Singh *et al*, 2004). Subsequently, CD133 was used to isolate stem cells from a host of other normal and cancerous tissues, including colon cancer (O'Brien *et al*, 2007; Ricci-Vitiani *et al*, 2007). Another potential colon cancer stem cell marker is aldehyde dehydrogenase 1 (ALDH1), a detoxifying enzyme that oxidises intracellular aldehydes and thereby confers resistance to alkylating agents (Magni *et al*, 1996; Yoshida *et al*, 1998). Implantation of as few as 100 $ALDH^+$ cells was capable of tumour initiation (Huang *et al*, 2009). When using ALDH and CD133 together to form tumour xenografts, $ALDH^+/CD133^+$ cells showed an increased ability to generate tumour xenografts compared with $ALDH^+/CD133^-$ or $ALDH^+$ alone (Huang *et al*, 2009). The present study uses both ALDH and CD133 together as markers for colorectal stem cells and examines the role of the STAT3 pathway in these cancer stem cells. Our results indicated that $ALDH^+/CD133^+$ subpopulation of colorectal cancer stem cells expressed higher levels of STAT3 phosphorylation compared with $ALDH^-/CD133^-$ subpopulations.

Curcumin is the primary bioactive compound isolated from turmeric, the popular Indian curry spice. Curcumin has anti-inflammatory, antioxidant, chemopreventive and chemotherapeutic properties by regulating multiple cell signalling pathways, including the STAT3 pathway (Aggarwal and Shishodia, 2006). It has been used against various types of cancers, including colon cancer, with little to no toxicity (Hatcher *et al*, 2008). Our results indicated that curcumin inhibited STAT3 phosphorylation, cell viability, and tumoursphere formation in $ALDH^+/CD133^+$ colon cancer stem cells. A novel curcumin analogue, GO-Y030 (Shibata *et al*, 2009), also inhibited STAT3 phosphorylation, the expression of STAT3 downstream target genes, cell viability, tumoursphere-forming capacity, and induced apoptosis in $ALDH^+/CD133^+$ cells. The effects of GO-Y030 were more potent than curcumin. Furthermore, GO-Y030 inhibited tumour growth of $ALDH^+/CD133^+$ cells in the mouse model *in vivo*. Our results suggest that STAT3 is a novel therapeutic target in colorectal cancer stem cells, and the novel curcumin analogue, GO-Y030, might be used as a new therapeutic reagent to target colon cancer stem cells in future.

MATERIALS AND METHODS

Colon cancer cell lines

Human colorectal cancer cell lines (DLD-1, HCT-116, SW480, and HT29) were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS), $4.5\,g\,l^{-1}$ L-glutamine, sodium pyruvate, and 1% penicillin/streptomycin. All cell lines were stored in a humidified 37°C incubator with 5% CO_2.

GO-Y030 and curcumin

Curcumin was purchased from Sigma-Aldrich (St Louis, MO, USA). GO-Y030 (Supplementary Figure 1), a new curcumin analogue (Shibata *et al*, 2009), was provided by Dr Shibata's laboratory.

Computational binding studies of GO-Y030

Molecular docking program MLSD based on AutoDock 4 was used to dock GO-Y030 to the binding sites of the STAT3 SH2 domain (PDB code 1BG1). ADT tool was used to prepare parameter and input files as previously reported (Huey *et al*, 2007; Li and Li, 2010). The small molecule GO-Y030 was docked to STAT3 SH2 using Lamarckian Genetic Algorithms (LGA) and Particle Swarm Optimisations (PSO) as searching methods. Docking simulations were repeated for 100 runs, with 1.0 and 6.5 million energy evaluations being used for PSO and LGA, respectively, in each run. The Docking found a few distinct conformational clusters. The binding modes of GO-Y030 were clustered with an RSMD of 2.0 Å. The major clusters with top binding energies were visually examined for binding modes.

Cell viability assay

Colon cancer stem cells (3000 per well in 96-well plates) were incubated with desired concentrations of compounds in triplicate at 37°C for 72 h. 3-(4,5-Dimethylthiazolyl)-2,5-diphenyltetrazolium bromide viability assays were performed and the absorbance was read at 595 nm. Half-maximal inhibitory concentrations (IC_{50}) were determined using Sigma Plot 9.0 Software (Systat Software Inc., San Jose, CA, USA).

Isolation of cancer stem cells

The ALDEFLUOR kit (StemCell Technologies, Durham, NC, USA) was used to isolate subpopulations with high ALDH enzymatic activity as previously described (Ginestier *et al*, 2007). Briefly, cells were trypsinised to single cells using 0.05% trypsin and subsequently suspended in ALDEFLUOR assay buffer containing ALDH substrate (BAAA, $1\,\mu mol\,l^{-1}$ per 1×10^6 cells) and then incubated for 40 min at 37°C. For each sample, an aliquot of cells was stained under identical conditions with $15\,mmol\,l^{-1}$ diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor, as a negative control. In all experiments, the ALDEFLUOR-stained cells treated with DEAB served as ALDH-negative controls. Anti-human PE-CD133 antibody (Miltenyi Biotec, Auburn, CA, USA) were used to identify CD133-positive cells. $ALDH^+/CD133^+$ and $ALDH^-/CD133^-$ subpopulations were separated from SW480, HCT116, DLD-1, and HT29 colon cancer cells by a FACS Wantage SE (Becton Dickinson, Palo Alto, CA, USA) Flow Cytometer. After sorting, $ALDH^+/CD133^+$ cells were cultured in serum-free stem cell medium (mammary epithelial basal medium) to maintain cancer stem cell characteristics. Cancer stem cells were grown in a serum-free mammary epithelial basal medium (Clonetics division of Cambrex BioScience, Walkerville, MD, USA) supplemented with B27 (Invitrogen, Carlsbad, CA, USA), $20\,ng\,ml^{-1}$ EGF (BD Biosciences, San Jose, CA, USA), antibiotic-antimycotic ($100\,U\,ml^{-1}$ penicillin-G sodium, $100\,\mu g\,ml^{-1}$ streptomycin sulphate), $4\,\mu g\,ml^{-1}$ gentamycin, $1\,ng\,ml^{-1}$ hydrocortisone, $5\,\mu g\,ml^{-1}$ insulin, and $100\,\mu M$ β-mercaptoethanol (Sigma-Aldrich) in a humidified incubator (5% CO_2) at 37°C. $ALDH^-/CD133^-$ cells and un-separated cells were cultured in regular medium and replaced with the stem cell medium above for 3 days before harvesting.

Western blot analysis

After treatment with GO-Y030 ($5\,\mu M$ or $10\,\mu M$) or DMSO for 24 h, $ALDH^+/CD133^+$, $ALDH^-/CD133^-$ and un-separated DLD-1, HCT-116, SW480, and HT29 colorectal cancer cells were lysed in cold RIPA lysis buffer containing protease inhibitors and subjected to SDS-PAGE. Proteins were transferred to a PVDF membrane and probed with antibodies (Cell Signaling Tech., Danvers, MA, USA). Membranes were probed with a 1:1000 dilution of antibodies (Cell Signaling Tech.) against phospho-specific STAT3 (Tyrosine 705), phospho-independent STAT3, phospho-specific ERK1/2 (Threonine 202/Tyrosine 204, T202/Y204), cleaved caspase-3, cleaved PARP, Phospho-Rb (Ser780), and GAPDH. Membranes were analysed using enhanced chemiluminescence Plus reagents and scanned with the Storm Scanner (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). The intensity of bands was quantified and normalised to GAPDH. For interferon-γ (IFN-γ), IL-4, and IL-6 stimulation experiments, HT29 colon cancer cells were serum-starved for 24 h and left untreated or

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pre-treated with GO-Y030 (2.5–10 μM) or DMSO for 2 h. Then, 50 ng ml^{-1} IFN-γ, IL-4, or IL-6 were added and the cells were harvested for western blot analysis 30 min later.

Reverse transcriptase–polymerase chain reaction

ALDH$^+$/CD133$^+$ subpopulations of DLD-1, HCT-116, and SW480 colon cancer cells were treated with GO-Y030 (5 μM) or DMSO for 24 h. RNA was then collected using RNeasy Kits (Qiagen, Valencia, CA, USA). Primer sequences and source information of STAT3 downstream target genes can be found in Supplementary Table 1.

Annexin-V apoptosis assay

Apoptotic cell death induced by GO-Y030 was quantified by flow cytometry with Annexin-V/propidium iodide (PI) double staining (BD Pharmingen, San Jose, CA, USA). After treatment with GO-Y030 or DMSO for 48 h, ALDH$^+$/CD133$^+$ SW480 colon cancer stem cells were collected and washed with cold PBS. The cell pellet was then re-suspended in 1 × binding buffer. Annexin V-FITC and PI (5 μl per 100 μl buffer) were added for 15 min at room temperature (RT) in darkness, and then analysed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) within 1 h.

Tumoursphere culture

The ALDH$^+$/CD133$^+$ and ALDH$^-$/CD133$^-$ subpopulations of DLD-1, HCT-116, SW480, and HT29 colorectal cancer cells were plated as single cells in ultra-low attachment six-well plates (Corning, Lowell, MA, USA) at a density of 250 to 50 000 viable cells per well in duplicate. Cells were grown in a serum-free stem cell medium described as above in a humidified incubator (5% CO_2) at 37°C. On the second day after seeding, the ALDH$^+$/CD133$^+$ cells were treated with 2.5–5 μM of GO-Y030. Tumourspheres were observed under microscope 10 to 15 days later. For counting tumourspheres, the content of all wells was collected, pooled, and transferred onto a collagen-coated six-well dish in differentiating medium (DMEM supplemented with 10% FBS). Tumourspheres adhered in these conditions in approximately 24 h, after which they were stained with crystal violet and counted under low magnification.

Mouse xenograft tumour model

Animal studies were conducted in accordance with the principles and standard procedures approved by IACUC at the Research Institute at Nationwide Children's Hospital. SW480 and HCT-116 ALDH$^+$/CD133$^+$ cells (1×10^5) were injected subcutaneously into the right flank area of 4- to 5-week-old female, non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, which were purchased from Jackson Laboratory (Bar Harbor, ME, USA). After 10 days, mice were divided into two treatment groups consisting of six mice per group: Control vehicle (100% DMSO) and 50 mg kg^{-1} of GO-Y030. Tumour growth was determined by measuring the length (L) and width (W) of the tumour every other day with a caliper and tumour volume was calculated on the basis of the following formula: volume = (π/6) LW^2.

RESULTS

The phosphorylation of STAT3 in ALDH$^+$/CD133$^+$ subpopulation of colorectal cancer cells compared with the ALDH$^-$/CD133$^-$ subpopulation

To determine whether STAT3 is activated in colorectal cancer stem cells, we separated ALDH$^+$/CD133$^+$ and ALDH$^-$/CD133$^-$ subpopulations from DLD-1, HCT-116, SW480, and HT29 colorectal cancer cell lines by flow cytometry, as previously described (Ginestier *et al*, 2007). The percentage of ALDH$^+$/CD133$^+$

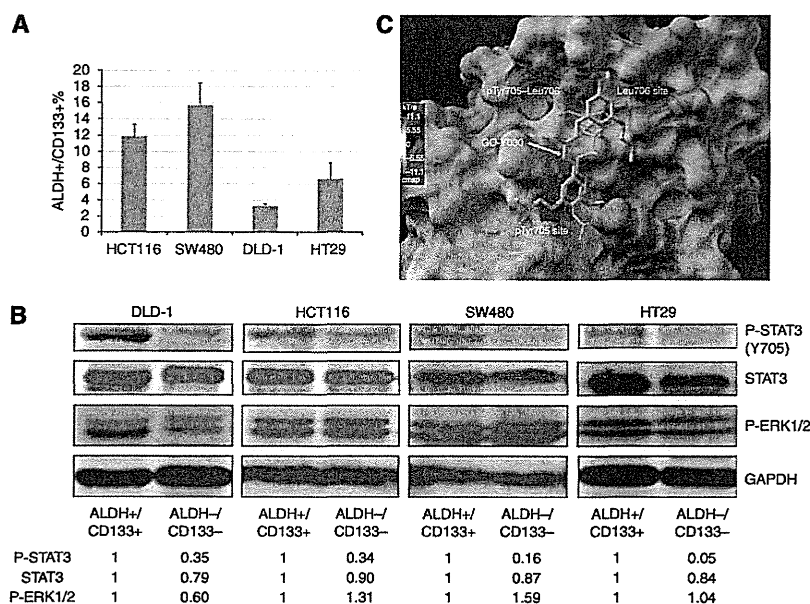

Figure 1 STAT3 phosphorylation of ALDH$^+$/CD133$^+$ subpopulation of colon cancer cells is higher than un-separated and the ALDH$^-$/CD133$^-$ subpopulations. (**A**) ALDH$^+$/CD133$^+$ and ALDH$^-$/CD133$^-$ subpopulations were separated from DLD-1, HCT-116, and SW480 colon cancer cells by flow cytometer. The percentage of ALDH$^+$/CD133$^+$ subpopulations was shown. (**B**) Phosphorylation of STAT3 (Y705), ERK 1/2 (T202/Y204), phospho-independent STAT3 of ALDH$^+$/CD133$^+$, and ALDH$^-$/CD133$^-$ subpopulations were detected by western blot. (**C**) Computer modelling of GO-Y030 binding to STAT3 SH2 domain. GO-Y030 is in Thick Stick-Ball (S-B) model and in grey colour. The native pTyr–Leu706 phospho-peptide binding of the partnering SH2 in homo-dimerisation is in green colour. GO-Y030 occupied both pTyr705- and Leu706-binding sites, which very effectively displaced the native pTyr705–Leu706 peptide with a stronger binding affinity than native peptide in the binding site of STAT3 SH2 domain. The colour reproduction of this figure is available at the *British Journal of Cancer* online.

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Table 1 $ALDH^+/CD133^+$ cells generated more tumourspheres than $ALDH^-/CD133^-$ cells

	SW480	HCT-116	DLD-1	HT29
250 cells per well				
$ALDH^+/CD133^+$	2±1*	2±0*	19±3*	12±2*
$ALDH^-/CD133^-$	0	0	0	1±1
500 cells per well				
$ALDH^+/CD133^+$	7+1*	8±2*	29±10*	21±3*
$ALDH^-/CD133^-$	0	2±0	1±1	3±2
1000 cells per well				
$ALDH^+/CD133^+$	8±2*	12±2*	42±9 *	21±2*
$ALDH^-/CD133^-$	3±1	2±1	1±1	5±1

$ALDH^+/CD133^+$ and $ALDH^-/CD133^-$ subpopulations of colorectal cancer cells were separated by flow cytometry and cultured in stem cell medium as described in Materials and Methods. The numbers of tumoursphere generated per 250, 500, or 1000 cells were counted 2 weeks later. *$P<0.01$.

subpopulations from HCT-116, DLD-1, SW480, and HT-29 colon cancer cells were shown in Figure 1A. ALDH+/CD133+ subpopulations of colorectal cancer cells have been reported as having an increased ability to generate tumour xenografts compared with $ALDH^+/CD133^-$ or $ALDH^+$ alone, and exhibits properties of colorectal cancer stem cells *in vitro* and *in vivo* (Huang *et al*, 2009). To confirm the cancer stem cell properties of $ALDH^+/CD133^+$ subpopulations, we first compared the tumourspere-forming ability between $ALDH^+/CD133^+$ and $ALDH^-/CD133^-$ subpopulations. As shown in Table 1 and Supplementary Figure 2, $ALDH^+/CD133^+$ cells of SW480, HCT-116, DLD-1, and HT29 all generated more tumoursperes than $ALDH^-/CD133^-$ cells. We also tested the expression of other stem cell markers, such as CD44, Oct-4, and Nestin in $ALDH^+/CD133^+$ and $ALDH^-/CD133^-$ cells (Supplementary Figure 3). CD44 expression of $ALDH^+/CD133^+$ was higher than $ALDH^-/CD133^-$cells. However, Oct-4 expression was lower and there was no difference in Nestin expression between them. There are a few papers that reported CD44 as well as ALDH and CD133 are markers of colon cancer stem cells (Dalerba *et al*, 2007; Du *et al*, 2008; Todaro *et al*, 2010). To date, the experimental data to support Oct-4 and Nestin as colon cancer stem cell markers are still lacking.

The level of STAT3 phosphorylation at Tyrosine residue 705 (Y705) was then examined in $ALDH^+/CD133^+$ and $ALDH^-/CD133^-$ cells. Interestingly, our results showed that the $ALDH^+/CD133^+$ subpopulations of SW480, HCT-116, DLD-1, and HT29 (Figure 1B) colorectal cancer cells expressed higher levels of STAT3 phosphorylation (Y705) compared with the $ALDH^-/CD133^-$ subpopulation cells. The phosphorylation of ERK (Threonine 202/Tyrosine 204) in the $ALDH^+/CD133^+$ subpopulations was not higher than that of $ALDH^-/CD133^-$ subpopulations in the all four cell lines. Here we demonstrated that colorectal cancer stem cells ($ALDH^+/CD133^+$cells) expressed higher phosphorylated or activated STAT3 compared with $ALDH^-/CD133^-$ cells. These results suggested that the STAT3 pathway has a more important role in colorectal cancer stem cells.

Computational binding modelling of GO-Y030

GO-Y030 is a newly development curcumin analogue (Supplementary Figure 1; Shibata *et al*, 2009). It has been demonstrated to inhibit colorectal carcinoma cells growth *in vitro* and in a mouse model *in vivo* (Shibata *et al*, 2009). However, the mechanism of GO-Y030 inhibition of colorectal carcinogenesis is still not very clear. We previously reported that Curcumin analogue GO-Y030 inhibits STAT3 activity and cell growth in breast and pancreatic carcinomas (Cen *et al*, 2009). Here, we used molecular docking program MLSD based on the AutoDock 4 to investigate that if GO-Y030 binds to the STAT3 SH2 domain. In a major conformational cluster, GO-Y030 occupied both the pTyr705 and Leu706 binding sites in the STAT3 SH2 domian, which contributed a binding energy of $-8.2\,kcal\,mol^{-1}$ (Figure 1C). GO-Y030 binding to both pTyr705 and Leu706 binding sites could displace the native pTyr705-Leu706 peptide more effectively than the binding of Curcumin to pTyr705 and the side pocket (Figure 1C).

GO-Y030 inhibited the STAT3 phosphorylation in colorectal cancer cells

To confirm the inhibition of phosphorylated or activated STAT3 by GO-Y030 in colon cancer cells, we examined STAT3 phosphorylation (Y705) in three independent colon cancer cell lines (cells were cultured in 10% FBS) using phospho-STAT3 (Tyrosine 705) antibodies (Supplementary Figure 4). Phosphorylation at Y705 is important in the activation of STAT3 (Kaptein *et al*, 1996; Schaefer *et al*, 1997; Faruqi *et al*, 2001). Our results indicated that GO-Y030 significantly inhibited STAT3 phosphorylation (Y705) in DLD-1, HCT-116, and SW480 human colon cancer cell lines (Supplementary Figure 4). The inhibition of STAT3 phosphorylation by GO-Y030 was consistent with the induction of apoptosis, as evidenced by the cleavages of PARP and caspase-3 (Supplementary Figure 4).

There are seven known mammalian STAT proteins (1–4, 5a, 5b, and 6), which can be activated by certain cytokines or growth factors (Turkson and Jove, 2002; Calo *et al*, 2003; Frank, 2007; Germain and Frank, 2007). After activation, STAT1 regulates the expression of genes that promote growth arrest and apoptosis, and is considered as a putative tumour suppressor (Calo *et al*, 2003; Yu *et al*, 2009). STAT3 and STAT6 are involved in inhibiting antitumour immunity (Yu *et al*, 2009). To investigate the specific inhibition of GO-Y030, we detected the phosphorylation of STAT3, STAT1, or STAT6 induced by IL-6, IFN-γ, or IL-4 in HT29 colon cancer cell lines. GO-Y030 inhibited un-induced (Supplementary Figure 4) and IL-6 ($50\,ng\,ml^{-1}$)-induced phosphorylation of STAT3 (Y705) (Supplementary Figure 5A). However, GO-Y030 did not inhibit phosphorylation of STAT1 or STAT6 induced by $50\,ng\,ml^{-1}$ of IFN-γ or IL-4 (Supplementary Figures 5B, 5C). This indicated the selectivity of GO-Y030 on STAT3, but not STAT1 and STAT6. The inhibition of STAT3 phosphorylation by GO-Y030 is unlikely through JAK2, as JAK2 phosphorylation is not reduced (Supplementary Figure 5A).

GO-Y030 inhibited STAT3 phosphorylation and induced apoptosis in $ALDH^+/CD133^+$ subpopulations of colorectal cancer cells

To confirm the important role of STAT3 in colon cancer stem cells, we next examined the effect of GO-Y030 in colorectal cancer stem cells. We observed that GO-Y030 inhibited STAT3 phosphorylation (Y705), but not ERK1/2 phosphorylation (T202/Y204) in the $ALDH^+/CD133^+$ subpopulation of SW480, HCT-116, DLD-1, and HT29 (Figure 2A) colorectal cancer cell lines. Curcumin also inhibited STAT3 phosphorylation (Y705) in the $ALDH^+/CD133^+$ subpopulations of SW480 and HCT-116 colorectal cancer cell lines (Figure 2B) at higher concentration (50 μM). These results indicated that GO-Y030 was a potent inhibitor of STAT3 phosphorylation in these colorectal cancer stem cells. GO-Y030 also reduced the percentage of $ALDH^+/CD133^+$ cells in HCT-116 and SW480 colorectal cancer cell lines (Supplementary Figure 6).

The inhibition of STAT3 by GO-Y030 also downregulated the expression of many known STAT3-regulated genes in colorectal cancer stem cells related to cancer cell proliferation, survival, and angiogenesis, such as Cyclin D1 (Bromberg *et al*, 1999), survivin (Gritsko *et al*, 2006), Bcl-2, and Bcl-XL (Bromberg *et al*, 1999; Figure 2C). Furthermore, GO-Y030 inhibited Notch-1 and Notch-3

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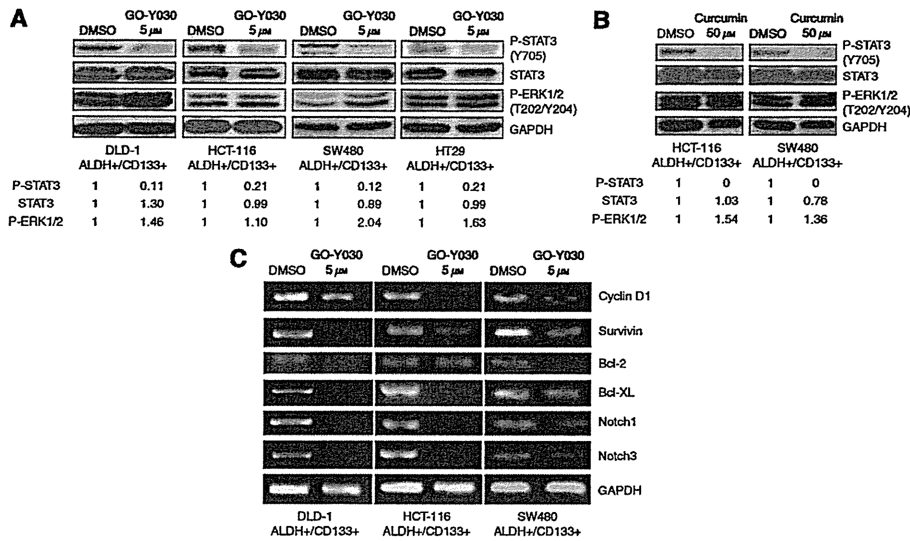

Figure 2 GO-Y030 inhibited STAT3 phosphorylation and downregulated STAT3-regulated genes expression in ALDH$^+$/CD133$^+$ cells. (**A**) ALDH$^+$/CD133$^+$ cells were treated with DMSO or 5 μM of GO-Y030 for 24 h. Phosphorylation of STAT3 in DLD-1, HCT-116, and SW480 colon cancer stem cells were detected by western blot. (**B**) ALDH$^+$/CD133$^+$ cells were treated with DMSO or 50 μM of curcumin for 24 h. (**C**) ALDH$^+$/CD133$^+$ cells were treated with GO-Y030 (5 μM) or DMSO for 24 h. Reverse transcriptase–polymerase chain reaction reveals decreased expression of STAT3 downstream target genes in GO-Y030-treated cells as compared with DMSO control.

expression (Figure 2C) in ALDH$^+$/CD133$^+$ cells, which have recently been reported as a putative STAT3 downstream target gene (Grivennikov and Karin, 2008). The Notch signalling pathway is known to be essential for normal stem cell self-renewal and differentiation in a variety of tissues, and is involved in human cancer stem cells' self-renewal capacity and tumourigenicity (Dontu *et al*, 2004; Grivennikov and Karin, 2008).

We further detected the effect of GO-Y030 on colon cancer stem cell apoptosis and cell cycle. The results showed that GO-Y030 increased the expression of cleaved PARP and cleaved caspase-3, which indicated cell apoptosis (Figure 3A). GO-Y030 also inhibited RB phosphorylation (Ser780), which should arrest cell cycle progression in G1 in HCT116 and SW480 colon cancer stem cells (Figure 3A). The effects of GO-Y030 on colon cancer stem cell apoptosis was also detected by flow cytometry after staining with Annexin-V/PI. The results showed that GO-Y030 led to a dose-dependent increase in apoptosis. The percentage of apoptosis cells increased from 5.3 ± 1.3 to 39.1 ± 4.6% (5 μM GO-Y030, $P<0.05$) and 52.4 ± 0.8% (10 μM GO-Y030, $P<0.05$) in SW480 colon cancer stem cells (Figures 3B and C). These results indicated that GO-Y030 induces apoptosis and cell cycle arrest in colon cancer stem cells.

GO-Y030 inhibited cell viability and tumoursphere-forming capacity of ALDH$^+$/CD133$^+$ cells

We next examined the inhibitory effects of GO-Y030 and curcumin on cell viability in colorectal cancer stem cells. Our results observed that GO-Y030 and curcumin could inhibit cell viability of the ALDH+/CD133+ subpopulation from SW480, HCT-116, DLD-1, and HT29 (Figure 4A) colorectal cancer cells, further supporting the idea that this subpopulation of colorectal cancer stem cells is sensitive to GO-Y030. GO-Y030 was more potent than curcumin in inhibiting cell viability of the ALDH+/CD133+ subpopulations from SW480, HCT-116, DLD-1, and HT29 (Figure 4A). We compared the IC_{50} of colon cancer cells with tumour stem cells after GO-Y030 treatment in Supplementary Table 2. There is no significant difference between the IC_{50} values, they are both sensitive to GO-Y030. Furthermore, we examined the efficacy of GO-Y030 in inhibiting colorectal cancer stem cells to survive and proliferate in anchorage-independent conditions and their ability to form tumourspheres. Our results indicated that GO-Y030 and curcumin can inhibit tumoursphere-forming capacity in the ALDH+/CD133+ subpopulation of SW480, HCT-116, DLD-1, and HT29 (Figure 4B) colorectal cancer cells. Again, we also found that GO-Y030 was more potent than curcumin (Figure 4B). The GO-Y030-treated cells remaining in the plates were not viable as verified by Trypan blue exclusion assay (data not shown). Therefore, we demonstrated that colorectal cancer stem cells in the ALDH$^+$/CD133$^+$ cells expressed an activated form of STAT3, and this is the first report that demonstrates that these cancer stem cells are sensitive to GO-Y030 inhibition. These results indicated that GO-Y030 was a good drug candidate for targeting colorectal cancer stem cells for inhibition of phosphorylated or activated STAT3.

GO-Y030 suppresses tumour growth of colon cancer stem cells in the mouse tumour model

We have demonstrated that GO-Y030 inhibits STAT3 phosphorylation, cell viability, and tumoursphere growth in colorectal cancer stem cells expressing elevated levels of STAT3 phosphorylation *in vitro*. To determine whether GO-Y030 may have therapeutic potential for clinical colorectal carcinoma treatment, we further tested GO-Y030 against ALDH$^+$/CD133$^+$ cells isolated from the SW480 and HCT-116 colon cancer cells in NOD/SCID mice xenograft models *in vivo*. SW480 and HCT-116 cancer stem cells (1×10^5 cells per mouse) were injected subcutaneously into nude mice in two groups, DMSO vehicle group with six mice and GO-Y030 group with six mice. GO-Y030 (50 mg kg^{-1}) was administrated via intraperitoneal injection beginning on day 15 or day 19. Caliper measurements of the longest perpendicular tumour diameters were performed every other day to estimate the tumour volume, using the following formula: $4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)$, which represents the three-dimensional volume of an ellipse. The results from the administration of GO-Y030 showed that GO-Y030 significantly suppresses ($P<0.01$) the tumour growth in SW480 (Figure 5A) and HCT-116 (Figure 6A), tumour weight in SW480 (Figure 5B) and HCT-116 (Figure 6B), and

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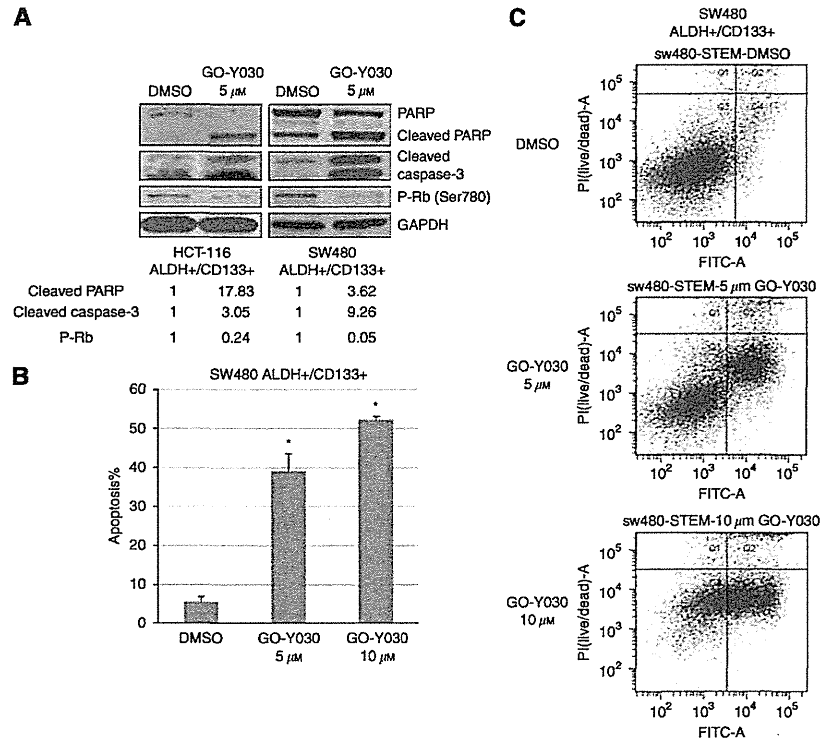

Figure 3 GO-Y030 induced apoptosis in ALDH$^+$/CD133$^+$ cells. (**A**) GO-Y030 increased the expression of cleaved PARP and cleaved caspase-3 and inhibited RB phosphorylation (Ser780) in HCT116 and SW480 colon cancer stem cells. (**B, C**) After treatment with GO-Y030 or DMSO for 48 h, ALDH$^+$/CD133$^+$ SW480 colon cancer stem cells were collected and analysed by flow cytometry. GO-Y030 led to a dose-dependent increase in apoptosis (*$P<0.05$).

tumour mass in SW480 (Figure 5C) and HCT-116 (Figure 6C) colon cancer stem cells. The average reduction in SW480 tumour weight was 57.96% in GO-Y030-treated mice compared with the DMSO vehicle in xenograft mouse model (Figure 5B). The average reduction in HCT-116 tumour weight was 58.10% in GO-Y030-treated mice compared with the DMSO vehicle in xenograft mouse model (Figure 6B). However, the body weight of the mice treated with GO-Y030 was not reduced at the end of the treatment compared with mice treated with the DMSO vehicle (Figure 6D). These results from two independent tumour models demonstrate that GO-Y030 is potent in suppressing tumour growth from colon cancer stem cells *in vivo*.

DISCUSSION

Currently, the main effort to target constitutive STAT3 signalling is only focused on the bulk of cancer cells. No report has been published to target STAT3 in colon cancer-initiating cells or colon stem cells. Both CD133 and ALDH have been used to isolate colorectal cancer stem cells (O'Brien *et al*, 2007; Ricci-Vitiani *et al*, 2007; Boman and Huang, 2008). When using ALDH and CD133 together to form tumour xenografts, ALDH$^+$/CD133$^+$ cells showed an increased ability to generate tumour xenografts compared with ALDH$^+$/CD133$^-$ or ALDH$^+$ alone (Huang *et al*, 2009). ALDH$^+$/CD133$^+$ cells tended to elicit larger tumours and elicited them more rapidly than ALDH$^+$/CD133$^-$ cells. Taken together, the data suggest that using both ALDH and CD133 appears to be better at enriching colorectal cancer stem cells than using ALDH or CD133. This study extends previous research by using both ALDH and CD133 together as markers for colorectal stem cells from colon cancer cell lines and examines STAT3 phosphorylation in these cancer stem cells. Our data showed that ALDH$^+$/CD133$^+$ cells generated more tumourspheres than ALDH$^-$/CD133$^-$ cells, suggesting that ALDH$^+$/CD133$^+$ cells possess cancer stem cell properties. Our results also indicated that colorectal cancer-initiating cells or colon stem cells, characterised by the ALDH$^+$/CD133$^+$ subpopulations of colorectal cancer cells, expressed higher levels of STAT3 phosphorylation than the un-separated and ALDH$^-$/CD133$^-$ subpopulations. These results suggest that STAT3 is a novel therapeutic target in colorectal cancer stem cells.

To explore the inhibition of STAT3 in colon cancer stem cells, we examined the inhibitory effects of a newly developed curcumin analogue, GO-Y030. Curcumin is one of the most widely characterised phytochemicals and is the active ingredient of the rhizome of the plant turmeric, which has both antioxidant and anti-inflammatory properties (Aggarwal and Shishodia, 2006). From published literature, curcumin has showed inhibitory effects in colon cancer cells (Hanif *et al*, 1997; Chauhan, 2002). Curcumin also has a chemopreventive potential in the context of colon cancer as seen in a mouse model and in human clinical trials (Kawamori *et al*, 1999; Johnson and Mukhtar, 2007). Curcumin has also been shown to inhibit STAT3 but with higher doses (Bharti *et al*, 2003; Aggarwal and Shishodia, 2006; Ohori *et al*, 2006). These results suggest that curcumin might be an ideal agent to target STAT3 in colon cancer. However, the growth suppressive activity and bioavailability of curcumin in human may still not be sufficient as an effective preventive or therapeutic agent for cancer. Therefore, more potent analogues of curcumin that can inhibit the STAT3 pathway with lower doses are needed as a more efficient form of treatments for colorectal cancer. We examined the

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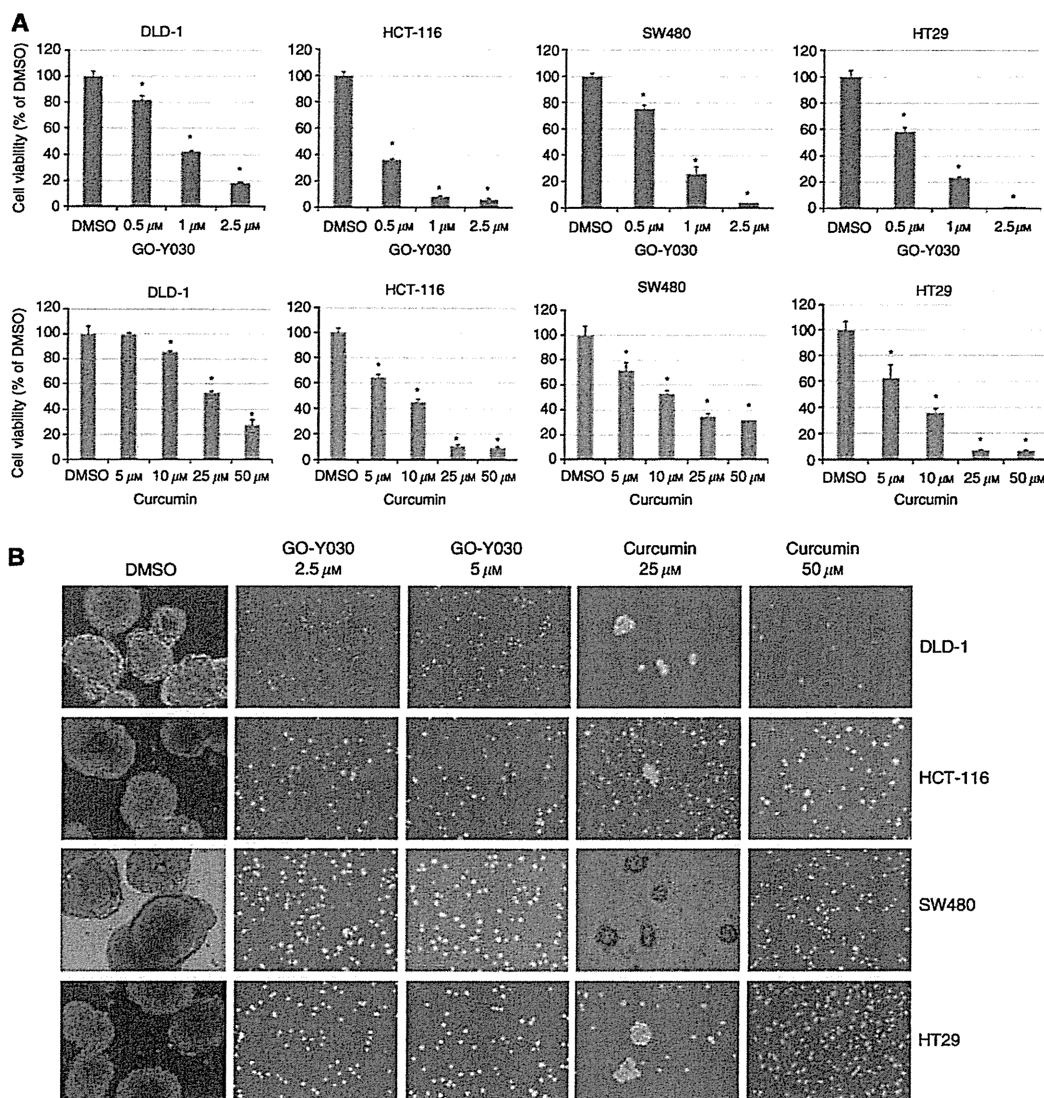

Figure 4 GO-Y030 and curcumin inhibited cell viability and tumoursphere formation of colon cancer stem cells. (**A**) The ALDH$^+$/CD133$^+$ cells were seeded in 96-well plates (3000 cells per well) in triplicates in a serum-free mammary epithelial basal medium (MEBM). The following day, cancer stem cells were treated with 0.5 to 2.5 μM of GO-Y030 or 5–50 μM of curcumin for 72 h. At the end of each time point, 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide assay was used to determine cell viability (*$P<0.05$). (**B**) The ALDH$^+$/CD133$^+$ cells were plated as single cells in ultra-low attachment six-well plates (Corning) at a density of 50 000 viable cells per well. Cells were grown in a serum-free MEBM as described in Materials and Methods. Twenty-four hours after seeding, the ALDH$^+$/CD133$^+$ cells were treated with 2.5 or 5 μM of GO-Y030 or 25 or 50 μM of curcumin.

inhibitory effects of GO-Y030 in the inhibition of STAT3 in colon cancer stem cells. GO-Y030 is one of the most potent curcumin analogues in the growth suppression of cancer cells (Ohori et al, 2006). Our results presented here show for the first time that GO-Y030 could efficiently inhibit STAT3 phosphorylation and cell viability, tumoursphere-forming capacity, and induce apoptosis in colorectal cancer stem cells. GO-Y030 can also downregulate putative IL-6/STAT3 downstream target genes that are involved in stem cell growth and survival such as Notch 1 (Grivennikov and Karin, 2008) as well as known STAT3 downstream target genes, such as Cyclin D1 (Bromberg et al, 1999), survivin (Diaz et al, 2006; Gritsko et al, 2006), Bcl-2 (Catlett-Falcone et al, 1999; Real et al, 2002), and Bcl-XL (Bromberg et al, 1999), that are involved in proliferation and survival. This provides possible molecular mechanisms of GO-Y030-mediated inhibition of STAT3 in colorectal cancer stem cells. Furthermore, our results show that GO-Y030 exhibits growth suppressive activity on the tumour growth of SW480 colon cancer stem cells.

These results suggested that constitutive active STAT3 in these cancer stem cells enhances proliferation and survival, as well as tumour growth in mice, whereas STAT3 blockade by GO-Y030 suppressed tumour stem cell growth *in vitro* and *in vivo*. The *in vivo* results are consistent with the *in vitro* cancer stem cell data, indicating that GO-Y030 is a potent inhibitor for the STAT3 pathway to suppress tumour growth of colon cancer stem cells in mouse models *in vivo*. In summary, this study is the first report to demonstrate that STAT3 is activated in colorectal cancer stem cells. Targeting STAT3 may be able to deplete the colorectal cancer stem cells and provide a promising approach to treat advanced colorectal cancer. Our study also demonstrated that GO-Y030 is a

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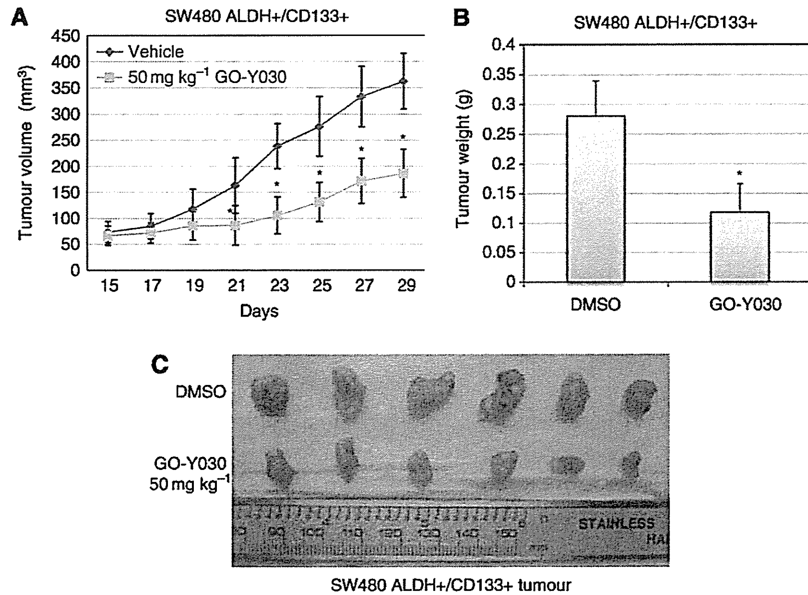

Figure 5 (**A**) GO-Y030 suppressed tumour growth in mouse xenografts with SW480 colon cancer stem cells. The mice were given daily intraperitoneal dosages of 50 mg kg^{-1} GO-Y030 or DMSO. Tumour volume (**A**), tumour weight (**B**), and tumour mass (**C**) were reduced in GO-Y030-treated mice compared with DMSO vehicle group (*P<0.05).

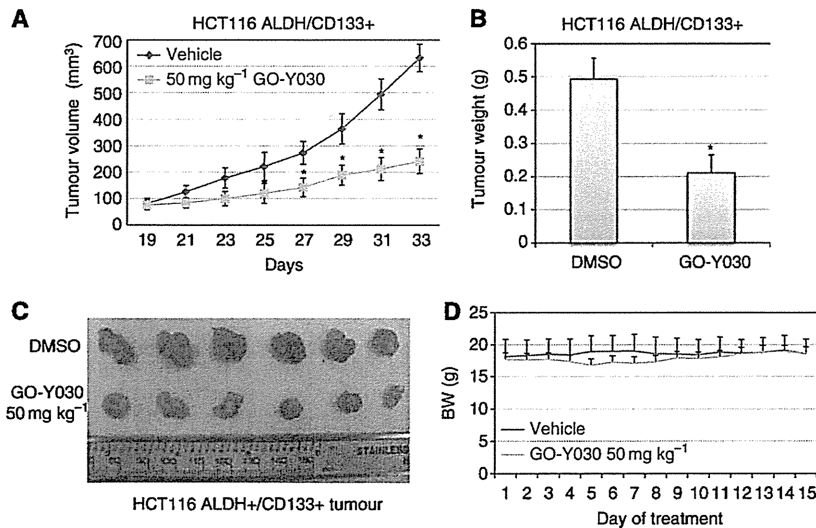

Figure 6 (**A**) GO-Y030 suppressed tumour growth in mouse xenografts with HCT-116 colon cancer stem cells. The mice were given daily intraperitoneal dosages of 50 mg kg^{-1} GO-Y030 or DMSO. Tumour volume (**A**), tumour weight (**B**), and tumour mass (**C**) were reduced in GO-Y030-treated mice compared with DMSO vehicle group (*P<0.05). (**D**) The reduction of bodyweights of GO-Y030-treated mice was similar to that of the vehicle-treated mice over 15 days of treatments.

potent inhibiting STAT3 for cancer stem cells and is a good drug candidate to target constitutive STAT3 signalling in colorectal cancer stem cells or cancer-initiating cells.

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Acquired/intratumoral mutation of *KRAS* during metastatic progression of colorectal carcinogenesis

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Abstract. Mutations at codons 12 and 13 of the *KRAS* gene have been identified as level I predictive biomarkers against the treatment of advanced colorectal cancer with anti-epidermal growth factor receptor (EGFR) monoclonal antibodies. It is thought that the genetic analysis of *KRAS* mutations associated with metastatic colorectal cancer can be routinely conducted using DNA obtained on one occasion from one organ, from the primary or a metastatic site, whichever is preferentially available. However, the issue of tumor heterogeneity resulting from acquired/intratumoral mutations remains. Recently, the possibility of acquired/intratumoral mutations in the *KRAS* gene has been reported by two research groups and has ranged from 7.4 to 15.4%. Specimens were collected from advanced colorectal cancer patients with resected primary, and at least one metastatic, site. Direct sequence analysis was performed for *KRAS*, *BRAF* and *PIK3CA*, and immunohistochemistry for glutathione S-transferase II (GSTP) and EGFR. In the current study, we identified an acquired mutation rate of approximately 11.1% in the *KRAS* gene (1/9). This figure is not negligible. Our observation indicates, particularly in the case of metastatic recurrence after a long interval, that there may be considerable tumor heterogeneity resulting from acquired or intratumoral mutations of the *KRAS* gene.

Introduction

In the last decade, two anti-epidermal growth factor receptor monoclonal antibodies (EGFR mAbs), cetuximab and panitumumab, were approved for the treatment of EGFR-positive colorectal cancer (CRC) (1,2). EGFR signals are transduced by *KRAS* and follow two signaling pathways, the RAS-RAF-MEK-ERK and RAS-PI3 kinase-AKT/PKB pathways. Mutations at codons 12 and 13 of the *KRAS* gene have been identified as a level I predictive biomarker against the treatment of advanced CRC with anti-EGFR mAbs according to the College of American Pathologists (CAP) level of evidence classification; that is, these mutations have been definitively proven as biomarkers based on evidence from multiple, statistically robust, published trials, and they are generally used in patient management (3). BRAF is a serine-threonine kinase located downstream of KRAS, which is a component of the RAS-RAF-MEK-ERK signaling pathway (4). A valine to glutamate substitution mutation at codon 600 (V600E) of the *BRAF* gene is a hot spot and is observed in 5-22% of CRCs (4). *BRAF* has a level IIA CAP predictive value, which means that extensive biological and clinical studies have repeatedly shown it to have predictive value for therapy; however, this remains to be validated in statistically robust studies (3). Phosphatidylinositol 3 kinase (PI3K) is composed of a regulatory and a catalytic subunit (5). The latter is encoded by the *PIK3CA* gene. Mutations in *PIK3CA* are observed in 15% of CRCs (6); approximately 70% of *PIK3CA* mutations are located at exon 9 [a glutamic acid to lysine substitution at codons 542 (E542K) and 545 (E545K)] and 20% at exon 20 [a histidine to arginine substitution at codon 1047 (H1047R)] (7). *PIK3CA* has a level IIB CAP predictive value, indicating that it has shown promise in multiple studies; however, sufficient data for its inclusion in categories I or IIA are lacking (3). Although EGFR is a direct target of EGFR mAbs, the EGFR expression level does not have any predictive value in a clinical setting (3). Glutathione S-transferase II (GSTP) is involved in detoxification and may be used as a cancer marker (8). Overexpression of GSTP has been reported to be closely correlated with KRAS mutations; the GSTP expression level is higher in CRCs with KRAS mutations compared to wild-type KRAS (9). Expression of mutant KRAS activates GSTP at a transcriptional level. If this observation is reproducible in a clinical setting, the presence of a KRAS mutation may be distinguishable by GSTP immunohistochemistry (IHC).

One report, analyzing 233 genes, indicated that there may be differences in as few as 3% of genes between primary and metastatic sites (10). Moreover, mutations in the *KRAS*, *BRAF* and *PIK3CA* genes occur around the adenoma stage (10). In

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these situations, it is thought that the routine performance of one genetic test for *KRAS* mutations associated with metastatic CRC using DNA obtained from one organ, either from the primary or a metastatic site, whichever is preferentially available, is sufficient. However, the possibility of considerable tumor heterogeneity remains an issue. Recently, the possibility of acquired or intratumoral mutations of the *KRAS* gene was reported (11,12). Although the number of cases surveyed was small, the frequency of acquired mutations identified was not negligible. In our study, we identified 9 cases in which synchronous or metachronous metastasis was resectable, together with the primary CRC, and determined the status of target genes, including *KRAS*, *BRAF*, *PIK3CA*, EGFR and GSTP at each of these sites to determine the incidence of acquired mutations that may affect treatment with EGFR mAbs.

Materials and methods

Patient selection. Samples were collected from the primary site, and from at least one site of distant synchronous or metachronous metastasis, from 9 patients with colorectal adenocarcinoma whose tumors were resected at Akita University Hospital (Japan). This study was approved by the institutional ethics committee for clinical studies at Akita University, Graduate School of Medicine, on July 20th, 2010, and each of the patients gave their informed consent to the procedure.

Direct sequencing. Direct sequencing of codons 12 and 13 of *KRAS*, codon 600 of *BRAF*, and exons 9 and 20 of *PIK3CA* was outsourced to SRL Inc. (Tokyo, Japan) or Falco Biosystems Ltd. (Kyoto, Japan). Briefly, the tumor cell-rich area of a hematoxylin and eosin-stained section was identified by microscopy. Tissue was then removed from the same area of a deparaffinized, unstained section. DNA from sections of that tissue sample was then isolated using the QIAamp FFPE Tissue kit (QIAGEN K.K.; Tokyo, Japan) and exon 1 of the *KRAS* gene, exon 15 of the *BRAF* gene, and exons 9 and 20 of the *PIK3CA* gene were amplified by polymerase chain reaction (PCR). The PCR products were visualized using agarose gel electrophoresis with ethidium bromide staining. PCR DNA fragments were directly sequenced using an ABI 3130 Genetic Analyzer (Applied Biosystems; Foster City, CA, USA) according to the manufacturer's instructions.

Immunohistochemistry. Almost all of the procedures were performed using a BenchMark XT IHC/ISH Staining Module (Roche Diagnostics K.K; Tokyo, Japan). Deparaffinized 4-μm specimens were used for IHC along with anti-human EGFR mouse monoclonal antibody (clone 2-18C9, Dako Japan; Tokyo, Japan), anti-human KRAS mouse monoclonal antibody (clone ab55391, Abcam Japan; Tokyo, Japan), and polyclonal rabbit anti-human GSTP (311-H, Medical and Biological Laboratories Co., Ltd.; Nagoya, Japan). Immunopositivity for EGFR was judged as positive if there were >0.1% positive cells. Immunoreactivities for KRAS and GSTP were graded as negative (0 to <10% positive cells), + (≥10 to <30% positive cells), ++ (>30 to <70% positive cells) and +++ (>70% positive cells). The percentage of immunopositive cells was calculated by counting at least 400 cancer cells in contiguous fields with the greatest immunopositivity.

Results

Patient characteristics. A total of 9 patients (3 females and 6 males) were included in this observational study. The median age was 67 years (range, 56-75). The patients were diagnosed as having CRC adenocarcinomas (2 rectal and 7 colon cancers). Three synchronous and 5 metachronous liver metastases, 2 synchronous and 5 metachronous lung metastases, and 1 synchronous ovarian metastasis were included. Resection of the primary region and at least one metastasis site was conducted either simultaneously or independently (Table I).

Sequence analyses of the KRAS, BRAF and PIK3CA genes. Regarding *KRAS* mutations, a glycine to aspartic acid mutation at codon 12 (G12D) was observed in the primary region of case 5, and a glycine to aspartic acid mutation at codon 13 (G13D) was observed in the primary region of case 1. In the remaining cases, no mutations were observed in the primary regions (Table I, Fig. 1A). The *KRAS* mutation frequency in the primary region was thus estimated to be 22.2% (2/9). At the metastatic sites, a G12D mutation was observed in both the lung and liver metastatic sites of case 5, and a G13D mutation was observed in the liver metastatic site of case 1. In case 8, a *KRAS* mutation involving a glycine to valine substitution at codon 12 (G12V) was observed in the liver metastatic site (Fig. 1B). In the remaining cases, no mutations were observed in the metastatic regions. The mutation frequency of *KRAS* at each metastatic site was thus estimated to be 27.3% (3/11).

No BRAF mutations were observed at exon 15 in the primary regions of all cases other than for case 7 (Fig. 1C). In case 7, a leucine to arginine mutation was observed at codon 597 (L597R) (Fig. 1D). This L597R mutation was also observed at the site of lung metastasis in case 7. No other mutations were observed at any of the remaining metastatic sites. According to the genomic information found in the Catalogue of Somatic Mutations of Cancer (COSMIC), released by the Sanger Institute, L597R was confirmed as a somatic variant (http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=sample&id=749760).

As for *PIK3CA*, no mutations were observed at exons 9 and 20 in the primary region of all cases with the exception of case 4 (Fig. 1E). In case 4, a glutamine to glutamic acid mutation was observed at codon 546 (Q546E) at exon 9 (Fig. 1F). This Q546E mutation was also observed at the site of liver metastasis in case 4. No mutations were observed at the remaining metastatic sites. Q546E was confirmed as a somatic variant by COSMIC (http://www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=mut_summary&id=6147).

Immunohistochemical analyses of EGFR, KRAS and GSTP. Immunopositivity for EGFR was observed at the primary site in 4 out of the 9 cases (cases 2, 3, 4 and 9) (Table I). A corresponding immunopositivity was observed at the metastatic sites in these 4 cases. However, a different immunopositivity was observed for cases 7 and 8, where the immunoreacitivity for EGFR was negative at the primary site but positive at the metastatic site (Fig. 2A and B). No immunopositivity was observed at the primary or metastatic sites in the remaining cases.

Table I. Clinical profiling of 9 mCRC patients and their biomarker status.

Characteristic	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9
Age	72	66	65	56	67	75	71	72	57
Gender	M	M	M	F	M	F	M	M	F
Primary	R	A	R	A	A	A	A	T	S
Hist	Wel-mod	Mod	Mod>wel	Mod	Mod	Wel	Mod	Wel	Wel>mod
Meta	Liver	Liver	Liver Lung	Liver	Liver Lung	Liver	Lung	Liver LN	Ovary
Occurence	S	S	M M	S	S S	M	M	M M	S
Interval (D)	-	-	483 1435	-	- -	382	1652	2321 2321	-
KRAS	G13D G13D	Wild Wild	Wild Wild Wild	Wild Wild	G12D G12D G12D	Wild Wild	Wild Wild	Wild G12V G12V	Wild Wild
BRAF	Wild Wild	Wild Wild	Wild Wild Wild	Wild Wild	Wild Wild Wild	Wild Wild	L597R L597R	Wild Wild ND	Wild Wild
PIK3CA	Wild Wild	Wild Wild	Wild Wild Wild	Q546E Q546E	Wild Wild Wild	Wild Wild	Wild Wild	Wild Wild ND	Wild Wild
EGFR	(-) (-)	(+) (+)	(+) (+) (+)	(+) (+)	(-) (-) (-)	(-) (-)	(-) (+)	(-) (+) ND	(+) (+)
GSTP	(+++) (++)	(+++) (++)	(+++) (++) ND	(+++) (+++)	(++) (+++) ND	(-) (-)	(+) ND	(-) (-) ND	(-) (-)

S, synchronous metastasis; M, metachronous metastasis; Wel, well-differentiated; Mod, moderately differentiated; ND, non-defined. Interval indicates days between primary and metastatic lesion resection. CRC, colorectal cancer; LN, lymph node; GSTP, glutathione S-transferase II; EGFR, epidermal growth factor receptor; R, rectal; A, ascending; T, transverse; S, sigmoid.

Immunoreactivity for KRAS is apparently not dependent on the mutational status of KRAS (Table I, Fig. 2C and D). Moreover, a correlation between the immunoreactivities or mutational status was not observed between *KRAS* and GSTP in this study (Fig. 2E-G). Therefore, we were unable to diagnose the mutational status of *KRAS* by GSTP IHC in a clinical setting.

Case presentation. In this observational study, a difference in the *KRAS* gene status between the primary and metastatic sites was observed in 1 (case 8) out of 9 cases (11.1%). This was independently confirmed by a separate analysis. Furthermore, the same *KRAS* mutation was detected in the resected mediastinal lymph node in case 8. Differences in immunopositivity for EGFR were observed in 2 (cases 7 and 8) out of 9 cases (22.2%). Case 7 was a 71-year-old male with ascending colon cancer. Following the primary resection, a 5-fluorouracil (5-FU) regimen (RPMI regimen) was administered for 6 months as adjuvant chemotherapy but 1,652 days following resection of the primary site, metastasis was evident in one lung and the site was resected (13). Modified leucovorin, fluorouracil and oxaliplatin (mFOLFOX6) was then administered as adjuvant chemotherapy for 180 days following resection until completion (April, 2011). Case 8 was a 72-year-old male with transverse colon cancer. Following primary resection, adjuvant chemotherapy was similarly administered for 6 months. However, 2,321 days after the primary resection, metastasis was detected at one site in the liver and in one mediastinal lymph node. The two sites were resected and mFOLFOX6 initiated as adjuvant chemotherapy (14). However, on day 159, mFOLFOX6 was terminated due to lung and abdominal lymph node metastases. A folinic acid-fluorouracil-irinotecan (FOLFIRI) regimen was then initiated and continued for 232 days up to the time of writing (15).

Discussion

In this study, we identified a possibility that acquired or intratumoral mutations may occur in the EGFR signaling pathway during CRC progression. Regarding *KRAS*, mutations in codons 12 and 13 were observed in 2 out of 9 cases at the primary site, and an acquired mutation was found in 1 case at a distal

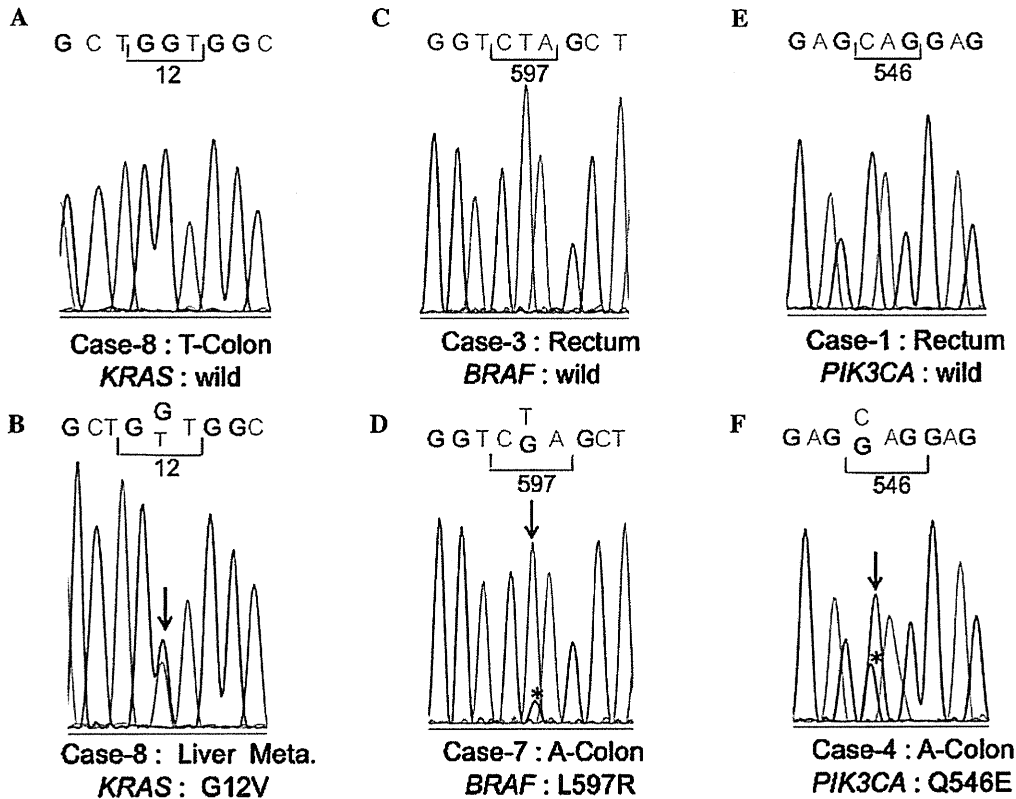

Figure 1. Sequence analyses of *KRAS*, *BRAF* and *PIK3CA*. The representative sequence analysis is shown for each case. Heterozygous mutations are shown by perpendicular lines.

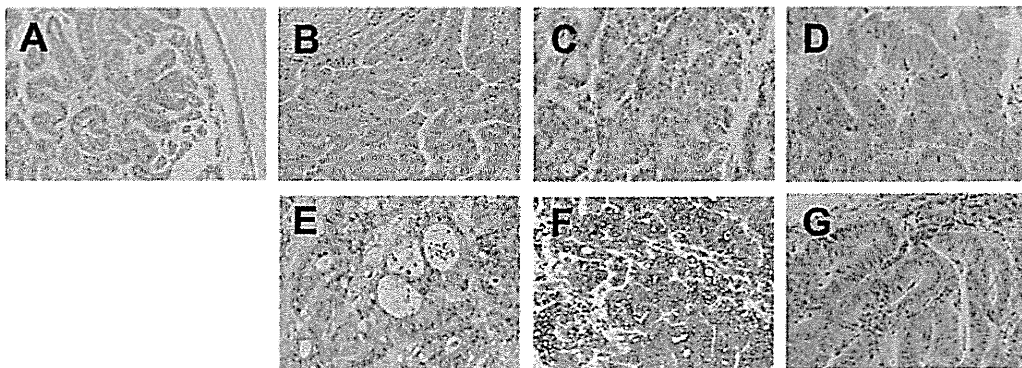

Figure 2. Immunohistochemical analyses of EGFR, KRAS and GSTP. (A) Negative immunoreactivity for EGFR at the primary lesion and (B) positive immunoreactivity at the site of lung metastasis in case 7, (C) negative immunoreactivity for KRAS (G12D) at the primary lesion and (D) positive immunoreactivity for KRAS (G12D) at the site of lung metastasis in case 5, (E) immunoreactivity for GSTP is denoted by (++) at the primary lesion, (F) (+++) at the site of liver metastasis, and (G) (-) at the site of lung metastasis in case 5; all sites had the same KRAS G12D mutation. EGFR, epidermal growth factor receptor; GSTP, glutathione S-transferase II; G12D, glycine to aspartic acid mutation at codon 12.

metastatic site. In previous reports, the mutation frequency of *KRAS* at codons 12 and 13 has ranged from 27 to 53% in CRC, which is similar to our finding (30%). The mutation frequencies of *BRAF* (V600E) and *PIK3CA* (exons 9 and 12) have been reported as 5-22% and 15%, respectively, in CRC. In our study, no oncogenic mutations of *BRAF* or *PIK3CA* were observed at either the primary or the metastatic sites. Differences in EGFR immunoreactivity were observed between the primary and metastatic sites in two instances, cases 7 and 8. In these two cases, the duration between the date of resection of the primary site and the date of metastatic recurrence was much longer (1,652 and 2,321 days, respectively) than that for the other cases (7 synchronous and 7 metachronous metastatic sites). In the remaining cases, the duration between the date of resection of the primary site and the date of onset of metastatic recurrence ranged from 217 to 952 days (median, 395). Since protein is easily degraded, the IHC analysis of EGFR may be affected by long-term storage. Therefore, the failure to detect immunoreactivity at the primary sites in cases 7 and 8 may be due to protein degradation during long-term storage. However, the direct

sequencing of *KRAS* was successfully performed using DNA obtained from the archived specimens of the primary sites for these cases (Fig. 1). DNA is more stable than protein over longer periods; therefore, the quality of DNA in this study was sufficient for direct sequencing. In case 8, the possibility of an acquired or intratumoral mutation was suspected. The overall incidence of acquired or intratumoral mutations of *KRAS* was approximately 10% in this study, which is nearly identical to that of previous reports. Bouchahda *et al* reported acquired *KRAS* mutations (G12D and G13D) in 2 out of 13 cases (15.4%) (11). Richman *et al* reported intratumoral *KRAS* mutations at codons 12 and 13 in 5 out of 68 cases (7.4%) and an intratumoral *BRAF* mutation (V600E) in 2 cases (2.9%). Thus, in total, mutations in the EGFR pathway were identified in 7 out of 68 cases (10.3%) in their study (12). Although only 9 cases were analyzed in our study, each case had at least one resectable metastatic site and the total number of sites (combining primary and metastatic sites) was 18. Thus, it may be better to report an acquired mutation rate of approximately 11.1% (2/18). In conclusion, when metastatic recurrence occurs after a long interval, it is likely that acquired *KRAS* mutations may be identified.

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