とから，治療方針の検討の一助になるものと思われる．

骨転移を伴う原発不明がんの全身治療と局所治療

骨転移を伴う原発不明がんに対する治療方法は，前述したように予後良好なサブグループとそれ以外に分けて考える必要がある．予後良好なサブグループの一つである，造骨性転移のみ有し，かつPSA値が上昇している男性症例では，進行性前立腺がんに準じた内分泌療法が試みられる[5]．

一方，予後良好なサブグループに分類されていない原発不明がんに対しては，一般的にはプラチナ製剤とタキサン系薬剤の併用療法がおこなわれており，前記サブグループからはずれた骨転移を有する原発不明がんに対する方針も，多くの施設でこれに準じていると思われる．

また，一般臓器からの骨転移に対する処置同様に，疼痛緩和や病的骨折を回避する目的での放射線療法やビスホスホネート（BP）治療，放射性ストロンチウムの投与，セメント注入による椎体形成術は積極的に施行される．一方，生体に侵襲的な外科的治療法については，原発不明がんで期待される予後が通常6～9ヵ月とされるため，リスクとベネフィットを十分勘案する必要があると思われる．

BP製剤の適応

Rosenら[11]は骨転移を有する固形がん733例を対象にゾレドロン酸投与が骨関連事象（skeletal related events：SRE）を減少させるかについての大規模な無作為化比較試験をおこない，ゾレドロン酸が病的骨折や脊椎圧迫骨折などのSREを減少させることを報告している．この試験のなかには原発不明がんも51例も含まれており，ゾレドロン酸による骨転移の症状マネジメントは原発不明がんでも有用であると思われる．

ただ，この結果を含め，ゾレドロン酸投与による予後改善効果についての解析報告はないことから，『原発不明がん診療ガイドライン2010年版』[5]でも，同効果を意図したゾレドロン酸の積極的な使用根拠にはならないものとしている．

今後の課題と展望

以上，骨転移を有する原発不明がんの病態と特徴について概説してきた．原発不明がんはその時点における診断レベルによる除外診断であるため，今後，分子生物学的診断技術の向上により，ほとんどの原発不明がんは各臓器の遠隔転移という診断に帰着していく可能性は高い．また，現時点においては骨転移に対する局所的治療方法やBP製剤などを用いた薬物療法の治療方針については，原発不明がんでも各種臓器がんの骨転移であっても大きな違いはない．しかし，原発不明がんと各種臓器がんとの骨転移発生頻度パターンの違いから，骨転移を起こした細胞間には分子生物学的特徴の違いが存在している可能性もあるかもしれず，その差異の解明とともに原発不明がんの病態にあわせた特有の治療法も必要になってくる可能性もあるかもしれない．

（加藤俊介）

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―症例報告―

低用量ネダプラチン・5FU 併用放射線治療が著効した，重度肝障害をともない全身状態不良の肝転移を有する食道癌の1例

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要旨：重度肝障害をともない全身状態不良の肝転移を有する食道癌に対して，緩和的放射線療法に低用量ネダプラチンと5FUによる化学療法を併用し著効を示した症例を経験したので報告する．治療開始後，肝転移巣は著明に縮小し肝機能も軽快した．外来での化学療法に移行可能となり，その後も2次，3次治療を行い，結果として600日以上の延命効果を得ることができた．

索引用語：食道癌，肝転移，放射線化学療法，低用量ネダプラチン

はじめに

今日の食道癌治療は手術・放射線・抗癌剤を組み合わせた集学的治療が行われる．Stage III までは治癒を目指して積極的な手術や放射線化学療法を行うが，転移性病変（M1b）を有する場合は根治が不可能であるために，一般的には化学療法などによる治療が推奨されている．食道癌診療ガイドライン（2007年）[1)]においては，化学療法・放射線治療，あるいはバイパス術，食道ステント挿入術，腸瘻・胃瘻造設などの緩和医療が提示されている．NCCN ガイドライン（2010年）[2)]でも Performance status（PS）2以下の全身状態が良好な患者であれば化学療法または緩和医療，PS 3以上の全身状態不良な患者であれば緩和医療と記載されている．また，化学療法に関しては，NCCN のガイドラインでは DCF 療法（ドセタキセル 75 mg/m^2 d1，シスプラチン 75mg/m^2 d1，5FU 750 mg/m^2 civ d1～5，これを3週ごとに繰り返す）（category 1）[3)]，FP 療法（シスプラチン 75mg/m^2 d1，5FU 1000mg/m^2 civ d1～4，これを3～4週ごとに繰り返す）（category 1）[4)5)]などが推奨されており，本邦においても遠隔転移症例に対する化学療法として FP 療法（シスプラチン 70mg/m^2 d1，5FU 700mg/m^2 civ d1～5，これを4週ごとに繰り返す）の有用性[6)]が示されている．

ネダプラチンはシスプラチンによる腎毒性を軽減する目的で本邦にて開発された第3世代白金製剤で，用量規定毒性は骨髄抑制である．水分負荷がほとんど必要ないのが特徴であるが，十分な臨床試験の蓄積がないために標準治療の確立までには至っていない．今回われわれは，多発肝転移を有し重篤な肝機能障害をともなう全身状態不良食道癌症例に対して，低用量ネダプラチンと5FU を使った化学放射線療法を行い奏効した症例を経験したので報告する．本稿での低用量ネダプラチンとは，連日少量（10mg/body/day）での投与法とした．

I 症 例

症例：64歳男性．

主訴：心窩部痛，嚥下困難．

既往歴：54歳：尿管結石，59歳：冠動脈狭窄にて経皮的冠血管拡張術．

現病歴：心窩部の重苦感があり，約3カ月様子を見ていたが症状が軽快しないため近医を受診

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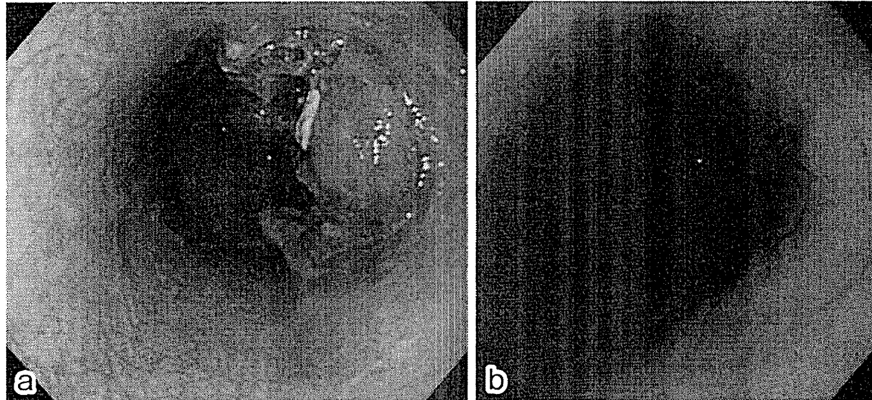

Figure 1. 上部消化管内視鏡検査　a）治療前：下部食道に2型腫瘍を認める．b）治療後第405病日：原発巣は瘢痕化し再発を認めない．

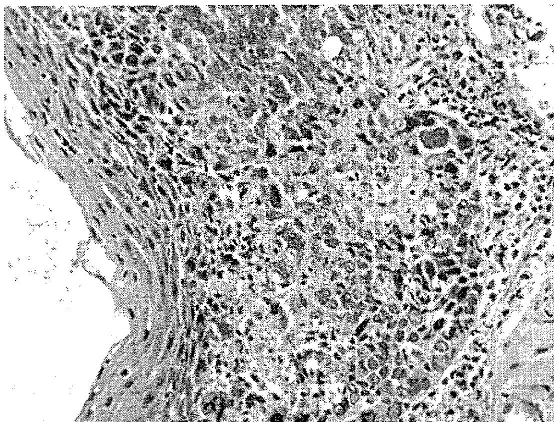

Figure 2. HE染色×400：角化はほとんど認められず，低～中分化型扁平上皮癌の像を呈する．

し，同日の上部消化管内視鏡検査にて食道癌と診断された．また，腹部超音波検査にて多発肝腫瘍を認め，精査加療目的に当院紹介となった．

入院時現症：身長：167.5cm，体重：73.0kg，血圧：124/80mmHg，体温：36.2℃，脈拍：80回/分，胸部：異常なし，腹部：上腹部正中にて腫大した肝臓を4横指触知し，圧痛をともなった．

各種検査：上部消化管内視鏡検査（Figure 1a）：胸部下部食道に2型腫瘍を認めた．前医での生検診断は低～中分化型扁平上皮癌（Figure 2）であった．

食道超音波内視鏡検査：大動脈への浸潤所見はなかった．

腹部造影CT検査（Figure 3a）：所属リンパ節転移の所見なし．肝両葉に大小の結節影を認めた．S1の腫瘤は下大静脈を圧排していた．

入院時血液検査所見：T.Bilの上昇はなかったがLDH 2370IU/L，ALP 1035IU/Lと異常高値を示した．血清クレアチニンも1.11mg/dLと軽度高値であった（Table 1）．

II　臨床経過

十分なインフォームド・コンセントを得た後，低用量ネダプラチン・5FU療法（ネダプラチン10 mg/body/day div d1～5，8～12，15～19，5FU 500 mg/body/day civ d1～5，8～12，15～19，これを4週間ごとに繰り返す）[7～11]と，病巣局所に絞った緩和的放射線照射20回分割総線量40Gy同時併用を開始した．治療開始後，第15病日の血液生化学検査はFigure 4のように改善傾向となり，第27病日にCT（Figure 3b）を行いPRと判断した．第29病日に化学療法第2コース目を開始する予定であったが，グレード2の血小板数減少（56000/μL）をきたしたため化学療法は休止した．治療再開時に入院期間の短縮希望と抗癌剤連日投与は拒否されたために，第49病日からはネダプラチン・5FU療法（ネダプラチン50mg/m^2 div d1，5FU 500mg/m^2 civ d1～5，これを2～3週間ごとに繰り返す）[8]にレジメンを変更し第56病日に退院した．外来ではインヒューザーポンプ

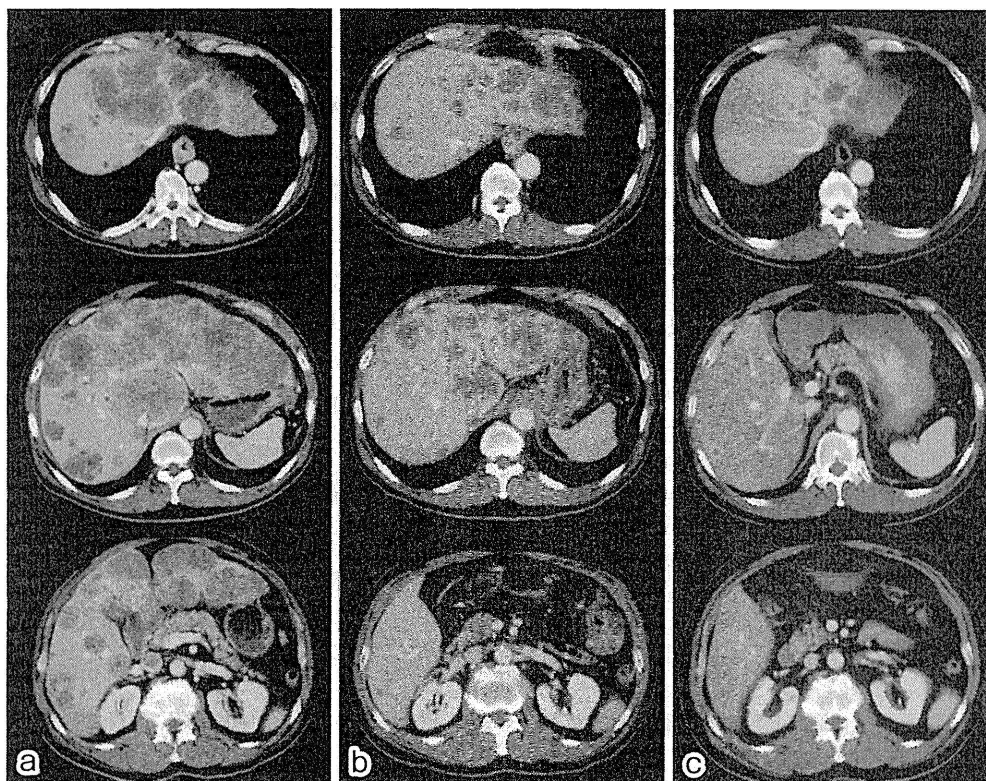

Figure 3. 腹部造影CT検査　a）治療開始前：肝両側に転移性病巣が多発し肝は著明に腫大しており，原発巣の食道壁は肥厚している．b）第27病日：腫瘍は縮小し肝腫大も軽減している．c）第179病日：さらに腫瘍は縮小している．

Table 1. 入院時血液検査所見

項目	値	項目	値
総蛋白	7.4 IU/dL	Na	141 mEq/L
ALB	4.0 g/dL	K	4.7 mEq/L
T.Bil	0.8 g/dL	Cl	106 mEq/L
AST	171 IU/L	WBC	6990 /μL
ALT	54 IU/L	RBC	417×10^4 /μL
LDH	2370 IU/L	Hb	12.9 g/dL
ALP	1035 IU/L	Ht	38.2 %
Ch-E	283 IU/L	PLT	24.2×10^4 /μL
γ-GTP	631 IU/L	AFP	7.1 ng/mL
BUN	23 mg/dL	CEA	1.05 ng/mL
Cr	1.11 mg/dL	CA19-9	4.8 U/mL
UA	8.3 mg/dL	CYFRA	2.3 ng/mL
		SCC	1.9 U/mL

などを使用して，第245病日まで計7コース施行した．第179病日のCT（Figure 3c）では治療最良効果となり肝腫大はほぼなくなり，生化学検査所見も正常化した（Figure 4）．その後PRを維持したが，第255病日のCTにて転移性肝腫瘍の再増大を認めたためPDと判定，3次治療としてドセタキセル・シスプラチン療法（ドセタキセル60mg/m^2 d1，シスプラチン60mg/m^2 d1，これを3～4週間ごとに繰り返す）[12]に変更して2コース施行した．第308病日のCTで腫瘍の縮小を認めPRと判定したが，倦怠感や食欲低下，入院治療の拒否のためドセタキセル単独療法（ドセタキセル70mg/m^2 d1，これを3週ごとに繰り返す）[13]に変更し4コース施行した．しかし，第394病日のCTにてPD判定となり，5次治療として第399病日よりビンデシン・ネダプラチン療法（ビンデシン3mg/m^2 d1，8，ネダプラチン90mg/m^2 d1，これを4週間ごとに繰り返す）[8][14]に変更し外来にて5コース行ったが，肝腫瘍の急速な増大と癌性腹膜炎により第611病日に永眠された．原発巣に関しては第76病日の上部消化管内視鏡検査でCRを確認し，第405病日（Figure 1b）の内視鏡

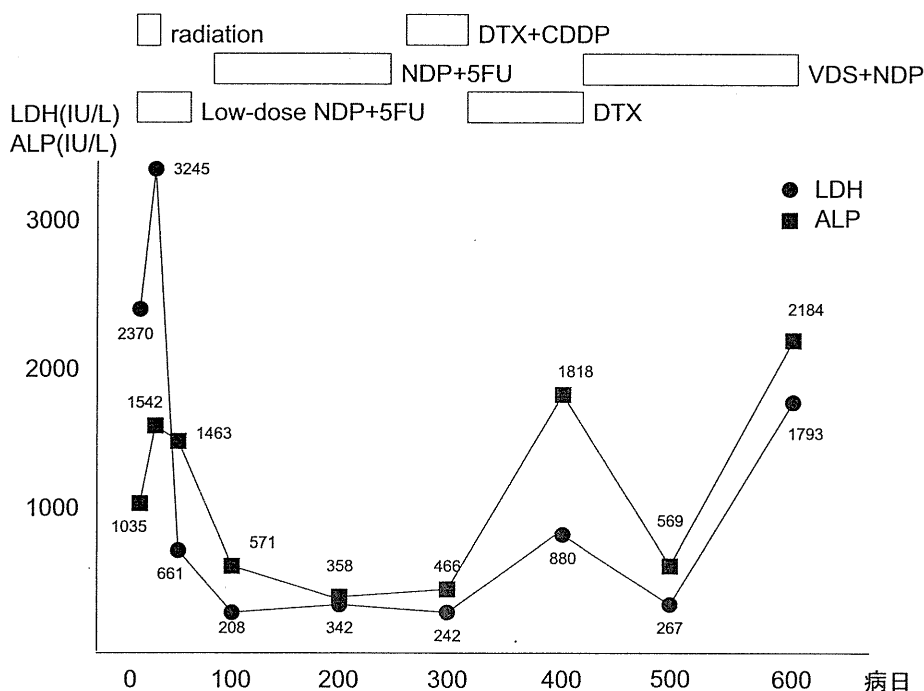

Figure 4. 臨床経過：低用量ネダプラチン＋5FUで治療を開始し，肝機能は正常化した．

検査でもCRを維持しており，亡くなる直前まで経口摂取が可能であった．

III 考 察

本症例は入院時PS 1であったが，その後PS 3に悪化し腹部触診上も上腹部正中で肝腫大は10横指となり，血液生化学検査（Figure 4）や血清クレアチニンは1.29mg/dLと悪化傾向であった．また，転移性肝腫瘍のS1部分が下大静脈を圧排しており急激な進行で血流障害をきたす可能性もあったため，通常のFP療法[6]は危険性が高いと判断した．初回治療であること，2剤併用での効果増強，患者は比較的若く治療に対して積極的であり，PS 3であるが肝腫大による疼痛の影響が強いと思われたことからネダプラチン併用レジメンで治療を行うこととした．また，通過障害が最も患者の苦痛となっていたことから，放射線照射を同時併用で行うことにした．

進行食道癌は，一般的に予後不良であり，標準療法も存在しない．しかし，全身状態が良好（PS 0～2）で根治的化学放射線療法が可能なStage IVa（T4/M1 Lym）例に関しては，シスプラチンと5FUに放射線療法を併用した化学放射線療法で，87％と高い奏効率と約20％の根治が得られることが報告され参照治療と考えられる[15]．しかし，本症例のような遠隔転移やPS 3～4の全身状態不良例に関しては，参照治療も存在しない．

食道癌診療ガイドライン（2007年）[1]，NCCNガイドライン（2010年）[2]，ESMOガイドライン（2010年）[16]では遠隔転移症例にはbest supportive care（BSC）あるいは化学療法単独，悪性狭窄には放射線照射や食道ステントなどが推奨されている．しかし，近年では症例に応じた治療が選択されるようになってきており，いくつかの総説や報告では化学放射線療法による有効性も示されている[17)～20]．特に緩和的放射線治療により長期間経口摂取可能になりQOLが維持された長期生存例も報告されている．緩和的放射線治療においては，照射線量は根治線量より少ない40～50Gy前後で行われ照射による副作用出現を配慮しQOLを損なわないことが重要と考えられる[20]．一方，遠隔転移症例に対する化学療法は単剤治療での検討や，2～3剤の併用療法での検討などが行われており，実臨床においては個々の患者の全身状態に応じて治療選択がなされている．一般的

には，高齢者などは単剤治療，全身状態が良好な患者は併用療法で治療される傾向にある[21]．ネダプラチンの腎毒性に関しては，動物実験や臨床試験フェーズ1においては非血液毒性を含めて軽度であったとする報告がある[22)23]．各臓器のフェーズ2（100mg/m² d1，これを4週ごとに繰り返す）[24)~26]におけるグレード2～4の血清クレアチニンの上昇は，肺癌1.5％[24]・頭頸部癌0.0％[25]・消化器癌3.3％[26]，食道癌のフェーズ2（ネダプラチン90mg/m² d1，5FU 800mg/m² civ d1～5，これを4週ごとに繰り返す）でのグレード2～4の血清クレアチニンの上昇は2.4％[27]と腎毒性の発現は低頻度であった．シスプラチンとネダプラチンを直接比較した報告はないが，腎機能が低下した症例や高齢者，循環器系疾患の合併などにより大量の水負荷ができない場合にはネダプラチンは使用しやすい薬剤であると思われる．

まとめ

今回，低用量ネダプラチン併用化学療法で腫瘍縮小導入が安全に可能で，さらなるレジメンへ継続可能となり，長期間の病勢進行がコントロール可能であった症例を経験した．低用量ネダプラチン併用化学療法は，状態の悪い患者に対しても適応可能で安全かつ有効なレジメンとして，今後さらに検討されるべき治療法と考えられた．

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A case of advanced esophageal cancer with multiple liver metastases accompanying poorly general conditions and serious liver dysfunctions, successfully treated using concurrent radiotherapy and chemotherapy with low-dose nedaplatin and 5-fluorouracil

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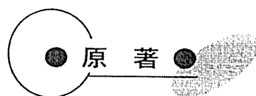
A 64-year-old male visited our hospital complaining of epigastric pain, and was diagnosed as esophageal cancer with multiple liver metastases. Histological examinations revealed squamous cell carcinoma. He had serious liver dysfunctions and his general conditions were severe, expressed as 3 of the performance status. He was treated using radiotherapy and chemotherapy with low-dose nedaplatin and 5-fluorouracil after the fully sufficient informed consent was taken. As for adverse events, Grade 2 of thrombocytopenia was only observed. Liver tumors responded to anticancer drug treatment immediately, and liver functions were almost normalized. The chemotherapy using low-dose nedaplatin and 5-FU combined with the radiotherapy was feasible and effective to patients with poor conditions.



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:
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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 前回治療時	抗癌剤治療 登録時	放射線治療 前回治療時	放射線治療 登録時	血清アルブミン(mg/dL) 登録前	血清アルブミン(mg/dL) 第8病日	エレンタール内服状況
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 activatic phosphorylated STATs dimerise and translocate to the nuclei where they regulate the expression of numerous critical gen involved in cell cycle progression, proliferation, invasion, a survival. However, the constitutive activation of STAT3 is frequen detected in primary human cancer cells, including colorec carcinoma cells (Corvinus *et al*, 2005; Kusaba *et al*, 2005). Persiste STAT3 activation is associated with enhanced proliferation a invasion of colorectal cancer cells *in vitro* and tumour growth in colorectal tumour model *in vivo*, and inhibition of STAT3 induc apoptosis and reduces tumour cell invasion in colorectal cancer ce (Corvinus *et al*, 2005; Lin *et al*, 2005; Tsareva *et al*, 2007; Xiong *et* 2008). These reports indicate that constitutively activation of STA' is one of the important pathways that contribute to the oncogenesis colorectal cancer and can serve as an attractive therapeutic target f colorectal carcinoma.

During the past decade, a number of developmental pathwa that regulate cancer stem cells, especially in breast cancer ste cells, have been elucidated. These pathways include Notc Hedgehog, Wnt, human epidermal growth factor receptor 2, AK etc (Liu and Wicha, 2010). However, the role of STAT3 in colo cancer stem cells and the effect of STAT3 inhibition in colo 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, t content of all wells was collected, pooled, and transferred onto collagen-coated six-well dish in differentiating medium (DME supplemented with 10% FBS). Tumourspheres adhered in the conditions in approximately 24 h, after which they were stained wi crystal violet and counted under low magnification.

Mouse xenograft tumour model

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

RESULTS

The phosphorylation of STAT3 in ALDH^{+}/CD133^{+} subpopulation of colorectal cancer cells compared with t ALDH^{-}/CD133^{-} subpopulation

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

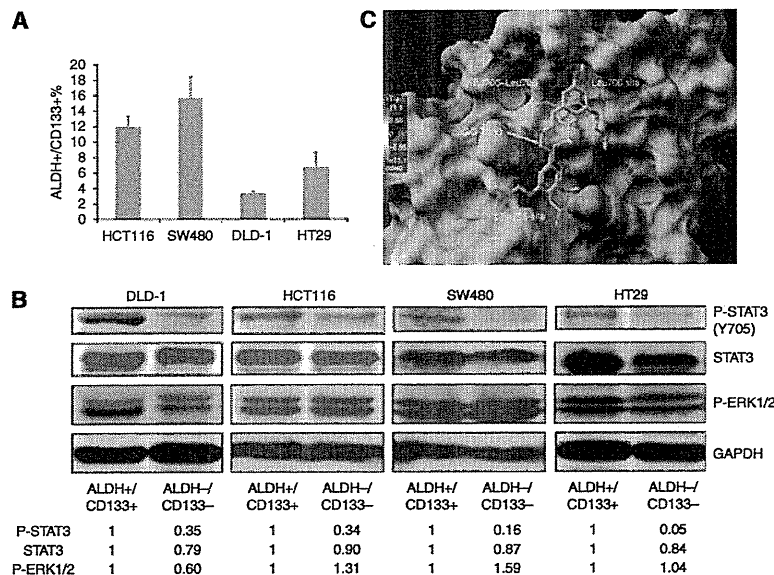

Figure 1 STAT3 phosphorylation of ALDH^{+}/CD133^{+} subpopulation of colon cancer cells is higher than un-separated and the ALDH^{-}/CD13 subpopulations. (**A**) ALDH^{+}/CD133^{+} and ALDH^{-}/CD133^{-} subpopulations were separated from DLD-1, HCT-116, and SW480 colon cancer cells flow cytometer. The percentage of ALDH^{+}/CD133^{+} subpopulations was shown. (**B**) Phosphorylation of STAT3 (Y705), ERK 1/2 (T202/Y204), phosph independent STAT3 of ALDH^{+}/CD133^{+}, and ALDH^{-}/CD133^{-} subpopulations were detected by western blot. (**C**) Computer modelling of GO-Y0 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 t partnering SH2 in homo-dimerisation is in green colour. GO-Y030 occupied both pTyr705- and Leu706-binding sites, which very effectively displaced t native pTyr705–Leu706 peptide with a stronger binding affinity than native peptide in the binding site of STAT3 SH2 domain. The colour reproduction 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 $-8.2\,\mathrm{kcal\,mol^{-1}}$ (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 anti-tumour 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 ($50\,\mathrm{ng\,ml^{-1}}$)-induced phosphorylation of STAT3 (Y705) (Supplementary Figure 5A). However, GO-Y030 did not inhibit phosphorylation of STAT1 or STAT6 induced by $50\,\mathrm{ng\,ml^{-1}}$ 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 ($50\,\mu\mathrm{M}$). 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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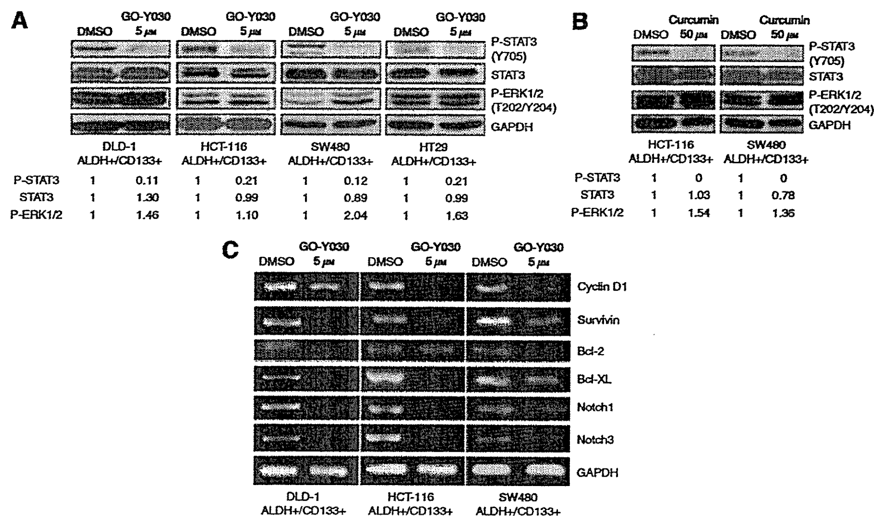

Figure 2 GO-Y030 inhibited STAT3 phosphorylation and downregulated STAT3-regulated genes expression in ALDH$^+$/CD133$^+$ cells. (**A**) ALDH CD133$^+$ cells were treated with DMSO or 5 μM of GO-Y030 for 24 h. Phosphorylation of STAT3 in DLD-1, HCT-116, and SW480 colon cancer stem ce were detected by western blot. (**B**) ALDH$^+$/CD133$^+$ cells were treated with DMSO or 50 μM of curcumin for 24 h. (**C**) ALDH$^+$/CD133$^+$ cells we treated with GO-Y030 (5 μM) or DMSO for 24 h. Reverse transcriptase–polymerase chain reaction reveals decreased expression of STAT3 downstrea 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 $39.1 \pm 4.6\%$ (5 μM GO-Y030, $P<0.05$) and $52.4 \pm 0.8\%$ (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 ai their ability to form tumourspheres. Our results indicated th GO-Y030 and curcumin can inhibit tumoursphere-formii capacity in the ALDH+/CD133+ subpopulation of SW48 HCT-116, DLD-1, and HT29 (Figure 4B) colorectal cancer cel Again, we also found that GO-Y030 was more potent th curcumin (Figure 4B). The GO-Y030-treated cells remaining the plates were not viable as verified by Trypan blue exclusi assay (data not shown). Therefore, we demonstrated that colorec cancer stem cells in the ALDH$^+$/CD133$^+$ cells expressed activated form of STAT3, and this is the first report th demonstrates that these cancer stem cells are sensitive to G Y030 inhibition. These results indicated that GO-Y030 was a goo drug candidate for targeting colorectal cancer stem cells f 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 phosph rylation, cell viability, and tumoursphere growth in colorec cancer stem cells expressing elevated levels of STAT3 phosph rylation *in vitro*. To determine whether GO-Y030 may ha therapeutic potential for clinical colorectal carcinoma treatmer we further tested GO-Y030 against ALDH$^+$/CD133$^+$ cells isolat from the SW480 and HCT-116 colon cancer cells in NOD/SCI mice xenograft models *in vivo*. SW480 and HCT-116 cancer ste cells (1×10^5 cells per mouse) were injected subcutaneously in nude mice in two groups, DMSO vehicle group with six mice ar GO-Y030 group with six mice. GO-Y030 (50 mg kg^{-1}) w administrated via intraperitoneal injection beginning on day or day 19. Caliper measurements of the longest perpendicul tumour diameters were performed every other day to estimate tl tumour volume, using the following formula: $4\pi/3 \times$ (width/2 $\times$ (length/2), which represents the three-dimensional volume an ellipse. The results from the administration of GO-Y030 showe that GO-Y030 significantly suppresses ($P<0.01$) the tumo growth in SW480 (Figure 5A) and HCT-116 (Figure 6A), tumo weight in SW480 (Figure 5B) and HCT-116 (Figure 6B), ar

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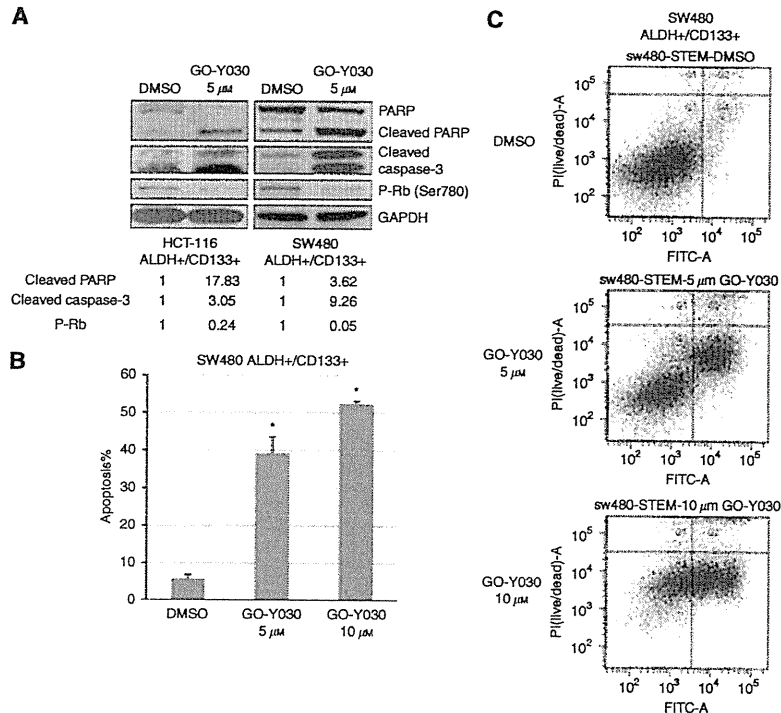

Figure 3 GO-Y030 induced apoptosis in $ALDH^+/CD133^+$ cells. (**A**) GO-Y030 increased the expression of cleaved PARP and cleaved caspase-3 and inhibited RB phosphorylation (Ser780) in HCT116 and SW480 colon cancer stem cells. (**B, C**) After treatment with GO-Y030 or DMSO for 48 h, $ALDH^+/CD133^+$ 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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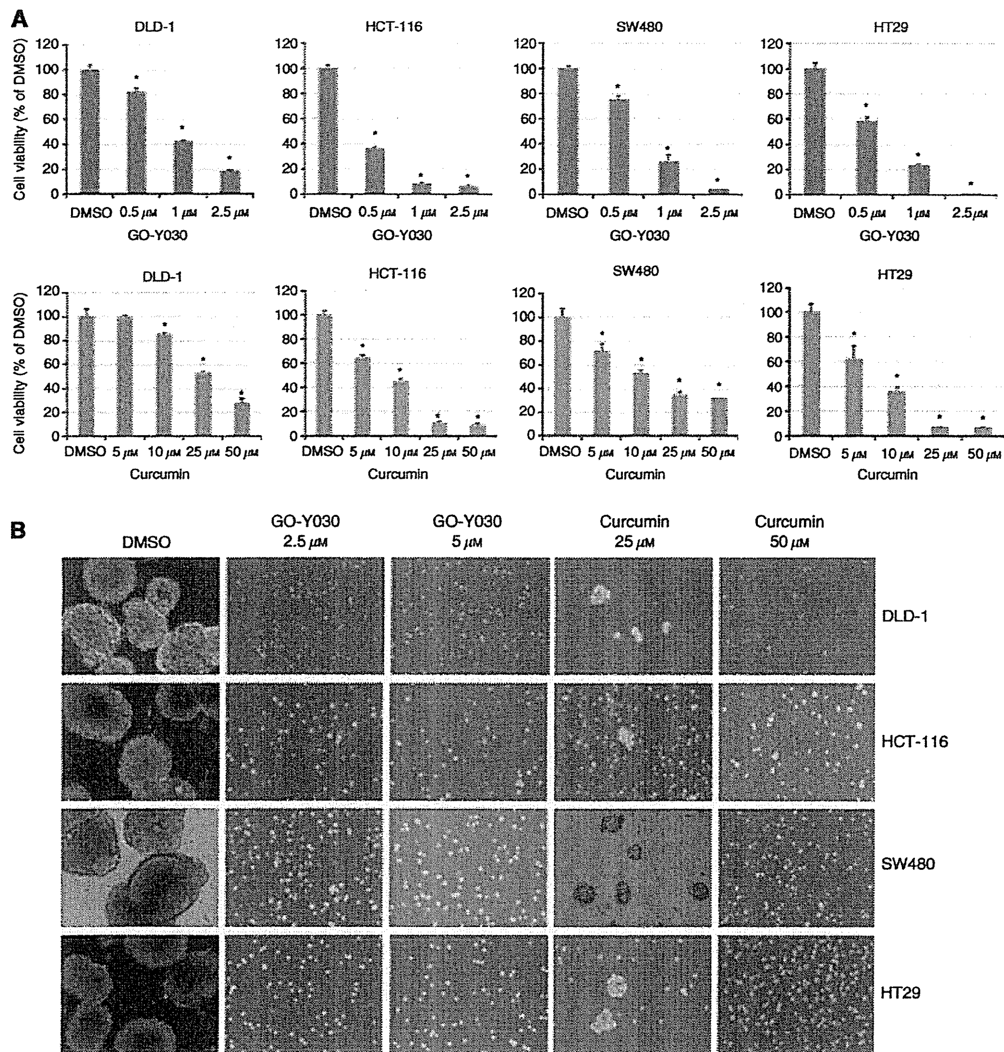

Figure 4 GO-Y030 and curcumin inhibited cell viability and tumoursphere formation of colon cancer stem cells. (**A**) The ALDH$^+$/CD133$^+$ cells we seeded in 96-well plates (3000 cells per well) in triplicates in a serum-free mammary epithelial basal medium (MEBM). The following day, cancer stem ce 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 diphenyltetrazolium bromide assay was used to determine cell viability (*$P<0.05$). (**B**) The ALDH$^+$/CD133$^+$ cells were plated as single cells in ultra-lc 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 a 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 th GO-Y030 exhibits growth suppressive activity on the tumo growth of SW480 colon cancer stem cells.

These results suggested that constitutive active STAT3 in the cancer stem cells enhances proliferation and survival, as well tumour growth in mice, whereas STAT3 blockade by GO-Y0: suppressed tumour stem cell growth *in vitro* and *in vivo*. Tl *in vivo* results are consistent with the *in vitro* cancer stem cell dat indicating that GO-Y030 is a potent inhibitor for the STA' pathway to suppress tumour growth of colon cancer stem cells mouse models *in vivo*. In summary, this study is the first report demonstrate that STAT3 is activated in colorectal cancer ste cells. Targeting STAT3 may be able to deplete the colorectal canc stem cells and provide a promising approach to treat advanc colorectal cancer. Our study also demonstrated that GO-Y030 is

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