与していることが強く示唆された[11].

3．CGRP，ADM の作用

両ペプチドの生理学的作用の共通点は多く，強い末梢血管拡張作用もその一つである．さらに CGRP は腸管運動亢進作用，分泌作用があり，ADM には抗炎症性サイトカイン作用がある．したがって，大建中湯の多彩な作用を理解するうえできわめて重要な鍵となる内因性ペプチドである．

両ペプチドの大きな違いは産生部位にある．CGRP はおもに神経終末など神経組織，ADM はおもに上皮細胞，平滑筋細胞など非神経組織である．大建中湯が腸管粘膜上皮細胞と感覚神経終末を刺激することで ADM と CGRP および受容体関連因子が動員され血流増加が起こることが推察された．

4．薬効成分と ADM

次に腸管粘膜上皮培養細胞を用いた成分レベルでの解析を進めた．解析前に行った大建中湯の成分解析の結果，多くの薬効成分が含まれていることが判明した(**図 1**)．最初に，腸管粘膜上皮細胞が ADM を産生，放出することを確認し，大建中湯によって濃度依存性，時間依存性に ADM 産生，放出が起こり，生薬レベルでは山椒と乾姜が ADM 産生，放出を起こすことを確認した．さらに山椒と乾姜の主成分のランダム刺激試験を行い，hydroxy-α-sanshool と 6-shogaol が有効成分であることが判明した[12),13)].

5．腸管粘膜上皮細胞の刺激機序

次に腸管粘膜上皮細胞に対する刺激機序を明らかにした．そのヒントとなったのは生体センサーである TRP (transient receptor potential) チャネルである．自然物の多くが特有の TRP チャネルを刺激するアゴニストとなっている．たとえば冷覚に関する TRPM8 チャネルのアゴニストはハッカの成分メントールである．冷湿布で冷たいと感じるのは血流が低下するのではなくて冷湿布に含まれるメントールによって TRPM8 チャネルが刺激され冷たいと感じるのである．

そこで漢方の原料となる自然物にも多くの TRP チャネルに対する刺激物が含まれているという仮説をたて，ADM 産生，放出に関与する hydroxy-α-sanshool と 6-shogaol の文献的検索を行った結果，TRPA1 と TRPV1 という二つのチャネルのアゴニストであることが判明．次に腸管粘膜上皮細胞にこの二つのチャネルが発現しているか否かを検討した結果，TRPA1 のみ強く発現し，TRPA1 のアゴニストで刺激すると ADM が放出されることが判明．一方，TRPV1 やほかの TRP チャネルのアゴニストではまったく反応しないことから，hydroxy-α-sanshool と 6-shogaol が TRPA1 チャネルを介して ADM を刺激していることが確認された．この研究過程で TRPA1 チャネルを高発現している腸管粘膜上皮細胞としてエンテロクロマフィン細胞を同定確認した．

この細胞はセロトニンを分泌して腸管運動を亢進させることが知られている．そこで，大建中湯の腸管運動亢進作用がこのエンテロクロマフィン細胞の TRPA1 チャネルを刺激し，セロトニン分泌を促すことで腸管運動亢進作用を発現していると考え，研究を進めた結果，hydroxy-α-sanshool と 6-shogaol が TRPA1 チャネルを介してエンテロクロマフィン細胞からセロトニン分泌を促していることが明らかとなった．つまり，大建中湯は腸管粘膜上皮細胞から ADM とセロトニンを放出させることで腸管血流増加と腸管運動亢進作用を発現させている可能性が強く示唆された(**図 2**)．

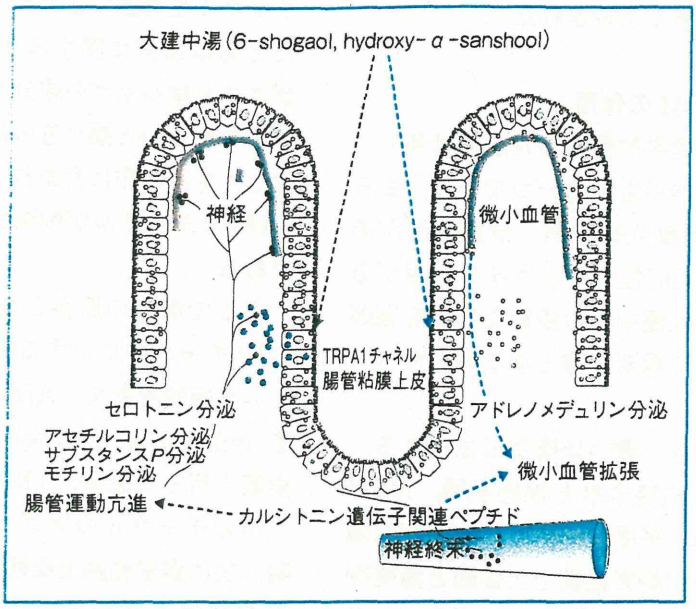

図2　大建中湯の腸管血流増加と腸管運動亢進作用の発現機序

6．PGE_2抑制作用

さらに，最近のわれわれの研究で乾姜の成分が強いPGE_2抑制作用があることが示唆されている．PGE_2を抑制する機序としてPGE_2の原料となるアラキドン酸の産生を抑制することなどが明らかとなりつつある．PGE_2は腸管運動の重要な担い手である平滑筋の動きを抑制することが知られていることから，大建中湯の術後早期の麻痺性イレウスに効果をもたらしている機序としてきわめて魅力的である．しかしながら，これら有効成分がどのようなルートで標的細胞に到達するのかは吸収試験を行うまで不明のままであった．

Ⅳ．吸収試験(薬理動態)

この項のポイント

- 血中に吸収されることで効果を発現している部分が相当数ある．

これまで，漢方を含めたハーバルメディシンにおいて薬物動態，吸収試験はまったく行われてこなかった．理由は技術的問題であった．最近のプロテオーム解析テクノロジーの発展によって可能となってきた．

最初に行ったのは健康人における大建中湯の吸収試験である[14),15)]．その結果，大建中湯の有効生薬である山椒のhydroxy-α-sanshoolを含めて多くの成分が大量に瞬時に吸収され，血中濃度が高まることが判明．乾姜も6-shogaolなど多くの成分が吸収されるが比較的ゆっくり吸収され，その吸収率は山椒の成分に比べて遙かに小さいものであった．一方，人参成分はほとんどが吸収されないことが判明．また，肝臓や腸管にて乾姜の成分の多くが抱合されることも判明した．

次に動物レベルでも吸収試験を行い，同様の結果を得ただけでなく，回腸末端部まで到達した抱合体が腸内細菌によって脱抱合され，再度活性物質となり大腸に到達することが判明した．これまで大建中湯は直接的な働きが主体であると考えられてきたが，吸収されることで効

果を発現している部分が相当数あることが推察され，これまで説明できなかった臨床試験結果を説明できるようになった．

V．臨床試験

この項のポイント
- エビデンスレベルが高い臨床試験で証明されている．

大建中湯が開腹手術後の腸閉塞の発生や再発を予防し，手術の必要性を減らす効果があることがいくつかのエビデンスレベルが高い臨床試験で証明されている．

1．クロスオーバー試験

単一施設において，胃がんで噴門部切除後の空腸パウチ間置術を行った17例を対象に，大建中湯投与15 g/dayあり，なしのランダム化クロスオーバー試験が行われ，大建中湯が液状物，固形物ともに胃からの排出を有意に促進し，パウチによる胃内容物停滞に伴う不快な症状を有意に改善する結果が得られた[16]．

2．プラセボ対照試験

1）腸閉塞例

単一施設において，154例の消化管開腹手術後早期に腸閉塞をきたした24例(16%)を，大建中湯を2週間15 g/day投与した群と，プラセボ投与群に分けたランダム化比較試験が行われ，大建中湯投与群はプラセボ群に比べ，腸閉塞手術の頻度が有意に50%以上減少したという結果が得られている．また，有意差はないものの腸閉塞の再燃頻度は大建中湯投与群で減少傾向が認められた[17]．

2）肝腫瘍例

全国26施設で行われたプラセボ対照二重盲検試験であるDKTフォーラムの一つであるJFMC40-1001試験は肝腫瘍で肝切した231例を，大建中湯を術後3日目から1週間15 g/day投与した群と，プラセボ投与群に分けたランダム化二重盲検試験が行われ，排ガス出現時期を指標に腸管運動再開までの期間を比較した結果，大建中湯群が有意に改善する結果が得られた(2012年米国肝臓学会で発表)．

3）健康人60人の例

手術後ではないが，大建中湯にとって大変重要な臨床試験が米国メイヨークリニックで行われた．健康白人60例を対象にプラセボ対照二重盲検試験がラジオアイソトープを使用して行われ，大建中湯を投与した群が有意に結腸排出，小腸運動促進効果を認められた．これまで，12種類の新薬について，腸管運動改善薬としてプラセボ投与群に分けたランダム化二重盲検試験が行われたがすべてネガティブな結果で，大建中湯が13番目の新薬として初めてポジティブな結果を出すことに成功した[18]．

VI．副作用

この項のポイント
- 発生率が0.1%以下で重篤なものは報告されていない．

米国食品医薬局(FDA)からの要請で大規模な大建中湯の副作用調査が最近行われたが，過去においても大規模調査が行われている．いずれも発生率が0.1%以下で重篤なものは報告されていない．重要なポイントとして併用時の注意としてα-グルコシダーゼ阻害薬(糖尿病治療薬)との併用は同薬のおもな副作用である腹部膨満感を増悪させるので糖尿病患者の腸管運動改善を目的に大建中湯を投与するときは注意を要する[19]．

おわりに

大建中湯は成分レベルの研究が着々と進んで

おり，薬物動態も明らかとなり，さらにはプラセボ対照二重盲検試験で薬効が証明されつつある[20]．とくに，術後の麻痺性イレウス改善や癒着性イレウス発生抑制効果が期待される．漢方が代替補完医療から脱出し，西洋薬と同じように使用される時代はもうそこまできている[9]．しかしながら，漢方薬は合剤であり，各成分の組み合わせで効果を発揮していることは明らかで，残念ながらその点をエビデンスレベルで明快に説明できていない．将来の漢方薬の国際化に向けて必ず明らかにしなくてはならない大きな課題である．

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Summary

Daikenchuto and Postoperative Ileus

Toru Kono*

Kampo is gaining a measure of recognition in the modern medical community through validation by scientific evidence. This acceptance is driven in part by molecular studies elucidating the pharmacologic mechanisms of daikenchuto. These studies have identified neurotransmitters involved in intestinal motility. They have also identified the active constituents of Japanese pepper and dried ginger which mobilize the endogenous calcitonin family of peptides and bring about serotonin release from intestinal epithelial cells, which improves blood flow and motility. They thereby intervene in the postoperative inflammatory cascade. Moreover, the results of these studies have lead to a number of double-blind, placebo-controlled clinical trials in the United States and Japan to determine the effects of daikenchuto on accelerating intestinal motility. In addition, pharmacokinetic studies have revealed that the active constituents of Japanese pepper and dried ginger are absorbed from the gastrointestinal tract and enter the bloodstream. The administration of daikenchuto during early postoperative periods represents a potential advance in the management of postoperative ileus.

Key words : Kampo, daikenchuto, ileus, Japanese pepper, dried ginger

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Clinical Efficacy of Paclitaxel/Cisplatin as an Adjuvant Chemotherapy for Patients With Cervical Cancer Who Underwent Radical Hysterectomy and Systematic Lymphadenectomy

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Background and Objectives: The aim of this study was to compare the clinical efficacy of paclitaxel/cisplatin (TP) as an adjuvant chemotherapy to adjuvant radiotherapy (RT) after radical hysterectomy and systematic lymphadenectomy for patients with cervical cancer.
Methods: A total of 125 patients with early-stage cervical cancer, who underwent radical hysterectomy, and received adjuvant therapy due to recurrent risk factors were retrospectively analyzed. Forty-nine patients were treated with RT, and 32 received paclitaxel/cisplatin (TP) for three to six cycles at 4-week interval. Survival and postoperative complications were compared between two modalities.
Results: There was no significant difference of 3-year disease-free survival between two groups ($P = 0.23$), while significantly better 3-year overall survival in TP group than RT group ($P = 0.02$). Seven of 32 patients (21.9%) treated with adjuvant TP, 16 of 49 patients (32.7%) treated with RT showed disease recurrence. Median of survival time after recurrence in RT group and TP group was 8.5 months, 12.0 months, respectively. Postoperative bowel obstruction was significantly more frequent in the RT group compared to the TP group ($P = 0.01$).
Conclusions: Postoperative chemotherapy using TP might be more beneficial for survival than adjuvant RT and can reduce postoperative complications for cervical cancer patients treated with radical hysterectomy.
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KEY WORDS: **cervical cancer; radical hysterectomy; adjuvant chemotherapy; paclitaxel/cisplatin; survival**

INTRODUCTION

Early-stage cervical cancer is generally treated by surgery, radiotherapy (RT), or a combination of both. At many institutions in Japan, cervical cancer [International Federation of Gynecology and Obstetrics (FIGO) stage Ib1-IIb] is treated with radical surgery and adjuvant RT when postoperative pathological examinations reveal risk factors for recurrence, including deep stromal invasion (DSI), lymph-vascular space invasion (LVSI), parametrial invasion (PI), lymph node metastasis (LNM), and bulky tumor [tumor diameter >4 cm (BT)]. We traditionally performed radical surgery first, and then used RT as an adjuvant therapy for patients with stage Ib1-IIb disease in many Japanese institutions. However, there is no definitive evidence that RT is beneficial for survival after radical surgery for cervical cancer [1,2]. Adjuvant chemotherapy combined with radical hysterectomy and systematic lymphadenectomy may also provide a survival benefit, while there are no randomized controlled studies comparing the clinical efficacy of chemotherapy and RT in the literature.

In our institution, adjuvant RT was used after radical hysterectomy. However, we have sometimes encountered severe postoperative complications (lower-limb lymphedema, bowel obstruction, and urinary disturbance) among patients receiving adjuvant RT, which significantly reduced their quality of life. Based on these observations and some data suggesting that the therapeutic effects of adjuvant chemotherapy and adjuvant RT were similar [3], we began to use more frequent adjuvant chemotherapy after surgery after the year 2000. We previously demonstrated that adjuvant chemotherapy using another regimen (BOMP; bleomycin, vincristine, mitomycin C, and cisplatin) has similar survival effect to adjuvant RT in patients with cervical squamous cell carcinoma (SCC) without multiple lymph-node metastatic sites, and significantly reduced the postoperatve complications compared with adjuvant RT [4]. However, BOMP may not be the best regimen for cervical cancer, because the clinical efficacy of BOMP was not compared with other regimens, and paclitaxel/cisplatin (TP) combination is currently recognized as a standard chemotherapy regimen by Gynecologic Oncology Group (GOG) for patients with metastatic, recurrent or persistent cervical cancer [5]. Moreover, standard chemotherapy regimen has not been established for cervical cancer in adjuvant setting yet. We have, therefore, employed TP regimen as an adjuvant chemotherapy for early-stage cervical cancer after radical surgery instead of BOMP since 2003.

In the present retrospective study, we investigated the clinical efficacy of adjuvant TP after radical hysterectomy and systematic lymphadenectomy in women with cervical cancer by comparing patients' survival and postoperative complications.

METHODS

Two hundred seventy-one patients with FIGO stage Ib1-IIb cervical cancer have been treated with radical surgery at Hokkaido University Hospital between 1991 and 2007. All patients underwent radical hysterectomy with removal of a vaginal cuff of at least 2 cm, total resection of parametrial tissue and systematic retroperitoneal lymphadenectomy. This operation is a nerve-sparing modification of

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the Okabayashi operation [6]. The nerve-sparing procedure was further refined by introducing the preservation of vesical branches of pelvic plexus after 1997. Patients with risk factors for recurrence, including DSI (>2/3 thickness), LVSI, PI, LNM, and BT, received adjuvant RT or chemotherapy. RT consisted of whole pelvic external irradiation by four-field technique with 50 Gy for 25 fractions beginning 4 weeks after surgery. CT consisted of paclitaxel 135 mg/m^2 intravenously (IV) for 24 hr on day 1, followed by cisplatin 50 mg/m^2 IV for 2 hr on day 2. Patients received at least three courses at 4-week intervals beginning 2–3 weeks after surgery. The patients' 3-year overall survival and disease-free survival were evaluated. We also assessed postoperative complications, including lower-limb lymphedema, bowel obstruction and urinary disturbance. Patients with stage II lower-limb lymphedema were considered positive. We utilized the grading of lower-limb lymphedema severity proposed by the International Society of Lymphology [7]. At stage II, the swelling becomes irreversible and develops into pitting edema. Patients treated for bowel obstruction with intravenous infusion and/or surgery were considered positive. Patients with long-term self-catheterization or incontinence were considered positive for postoperative urinary disturbance. Patient survival was calculated using the Kaplan–Meier method. The significance of the survival difference was evaluated using the log-rank test. The chi-square test was used to analyze correlations between variables. Significance was set at $P < 0.05$. Statistical analyses were performed with the Statview software package (SAS Institute, Cary, NC).

RESULTS

Patients' Characteristics

Patient's characteristics are listed in Table I. Of the 271 patients who underwent radical hysterectomy from 1991 to 2007, 146 patients did not receive adjuvant therapy because 141 patients did not show risk factors for recurrence, and five had post operative complications. One hundred twenty-five patients had risk factors for recurrence. Forty-nine patients with risk factors have been treated with adjuvant RT between 1991 and 2000. In this group, the median age was 52 years (range 28–74 years). Nine of the patients treated with adjuvant RT presented with clinical stage Ib1 cancer, 12 with stage Ib2 cancer, three with stage IIa cancer, and 25 with stage IIb cancer. Postoperative pathological examination confirmed that 27 patients had DSI, 40 had LVSI, 16 had PI, 25 had BT and 22 had multiple LNM sites.

Between 2000 and 2007, 60 patients with risk factors for recurrence were treated with adjuvant chemotherapy. Twenty-eight patients with SCC received adjuvant chemotherapy with BOMP as previously described [4] from 2000 to 2003. Sixteen patients with adenocarcinoma were treated with adjuvant chemotherapy other than BOMP, including 10 patients with IEP (ifosphamide, etoposide, and cisplatin), and six patients with MEP (mitomycin C, etoposide, and cisplatin). Thirty-two patients received TP irrespesctive of histologic type from 2003 to present. Median age was 48 years (range 20–71 years). Nine of these patients presented with clinical stage Ib1 cancer, eight with stage Ib2 cancer, and 15 stage IIb cancer. Postoperative pathological examination confirmed that 19 had DSI, 24 had LVSI, seven had PI, 16 had BT, and 11 had multiple LNM sites. There has been found to be no statistically significant difference of distribution of risk factors described above among each group. However, there was statistically significant difference of distribution of histologic types between two groups ($P = 0.01$), that is, two of 49 had non-SCC in RT group, while eight of 32 had non-SCC in TP group.

Treatment Outcome

Treatment outcomes are shown in Table II. In the RT group, 33 showed disease-free, 16 of 49 patients (32.7%) had recurrent disease and 11 of 41 patients (22.4%) died of disease within 36 months. Seven patients had pelvic tumor recurrence affecting the vaginal stump, pelvic wall, or pelvic lymph node. Nine patients had extrapelvic recurrence affecting the lung, liver, brain, or supraclavicular lymph node. In the TP group, 25 patients showed disease-free, seven of 32 patients (21.9%) experienced recurrence and one of 32 patients (3.1%) died of disease within 36 months. Two patients had pelvic recurrence affecting pelvic lymph node and pelvic wall. Four patients had extrapelvic recurrence, affecting lung, para-aortic lymph node, or supraclavicular lymph node. One had both of pelvic and extrapelvic failure. There was no statistically significant difference between two groups with regard to clinical stage, DSI, LVSI, BT, VI, PI, and multiple LNM sites. Adenosquamous/adenocarcinoma histology was significantly more frequent in TP group than in RT

TABLE I. Patients' Characteristics

	Adjuvant therapy		
	Radiotherapy (RT)	Chemotherapy (CT)	*P*-value
Number of patient	49	32	
State			
Ib1	9	9	
Ib2	12	8	
IIa	3	0	Stage I vs. II; N.S.
IIb	25	15	
Pathological subtype			
Squamous	47	24	
Adeno/adenosquamous	2	8	Squamous vs. adeno/adenosquamous: 0.1
Bulky+	25	16	N.S.
DSI+	27	19	N.S.
LVSI+	40	24	N.S.
PI+	16	7	N.S.
VI+	13	4	N.S.
The number of LNM part			
Under 2	27	21	Under 2 vs. equal or over 2
Equal or over 2	22	11	N.S.

DSI, deep stromal invasion; LVSI, lymph-vascular space involvement; PI, parametrial invasion; VI, vaginal invasion; LNM, lymph node metastasis.

TABLE II. Treatment Outcome According to the Type of Adjuvant Therapy

Adjuvant therapy	Number of patient	No recurrence	Recurrent part: Intra pelvic	Recurrent part: Extra pelvic	Dead of disease
Radiotherapy	49	33	7	9	11
Chemotherapy	32	25	3	5[a]	1
Total	81	58	10	14	12

[a]Including one case who had recurrent tumor intra and extra pelvic.

group ($P = 0.01$) (Table I). The 3-year DFS rate was 78.1% for the TP group, and 67.3% for the RT group; the difference was not statistically significant ($P = 0.23$) (Fig. 1A). The 3-year OS rate was 93.8% for the TP group, and 69.4% for the RT group. There was significantly better OS rate in TP group than in RT group ($P = 0.02$) (Fig. 1B).

Median of survival time after recurrence in TP group and RT group was 12.0 months (range; 7–35 months), 8.5 months (range; 3–162 months), respectively. There is no statistically significant difference of survival after recurrence between two groups. Details of recurrent cases in TP and RT groups were described in Tables IIIA and IIIB, respectively. Five of seven patients (71.4%) in TP group received RT, while eight of 16 patients (50.0%) received RT. All

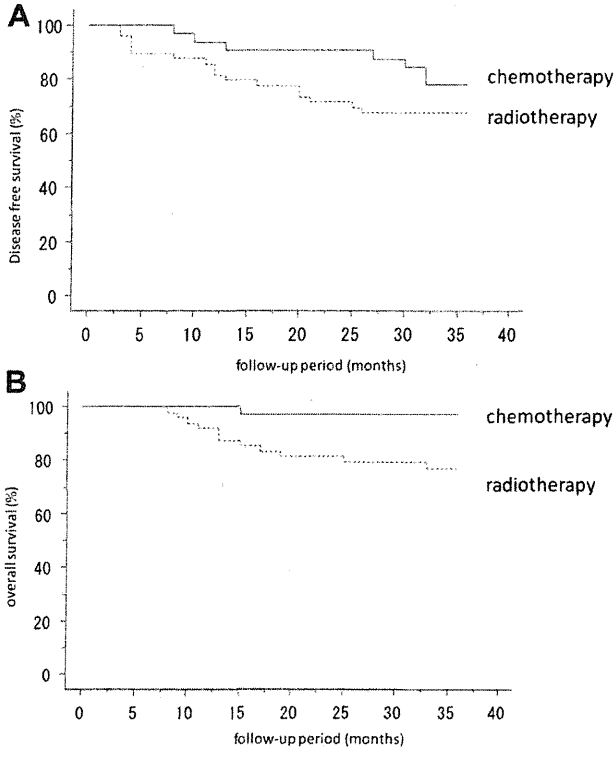

Fig. 1. Kaplan–Meyer survival curve according to the type of adjuvant therapy. **A**: There was no statistically significant difference of 3-year disease-free survival rate between TP group (78.1%) and RT group (67.3%) ($P = 0.23$). **B**: There was significantly better overall survival rate in TP group (93.8%) than in RT group (69.4%) ($P = 0.02$).

three cases with pelvic recurrence in TP group received combination of RT and chemotherapy, but all six cases with pelvic recurrence in RT group were treated with chemotherapy alone.

The incidences of postoperative complications are shown in Table IV. Among the 49 patients who received adjuvant RT, 11 (22.4%) had lower-limb lymphedema, 17 (34.7%) had urinary disturbance, and 12 (24.5%) had bowel obstruction. Ten cases of bowel obstruction were cured by conservative treatment, two necessitated intestinal surgery. Among the 32 patients in the TP group, four (11.4%) had lower-limb lymphedema, five (15.6%) had urinary disturbance, and one (3.1%) had bowel obstruction. The incidence of lower-limb lymphedema tended to be higher in the RT group compared to the TP groups, while the difference was not statistically significant ($P = 0.2$). Urinary disturbance was more frequent in the RT group than the TP group with marginal significance ($P = 0.07$). The incidence of bowel obstruction was significantly higher in the RT group than in the TP group ($P = 0.01$).

DISCUSSION

In this retrospective analysis, we have shown that TP regimen might have survival effect on patients with cervical cancer who underwent radical hysterectomy and systematic LND. There was no significant difference of 3-year disease-free survival according to the type of postoperative adjuvant therapy, while 3-year overall survival in TP group was significantly better than that in RT group (Fig. 1B).

Adjuvant RT has been generally introduced for stages I–II cervical cancer patients who are at high risk for recurrence. To date, several guidelines for cervical cancer treatment, including those launched by the American College of Obstetricians and Gynecologists and the Japan Society of Gynecologic Oncology, recommend RT as a first-line postoperative adjuvant therapy for patients with a high risk for recurrence. In 1998, a prospective randomized study conducted by the Gynecologic Oncology Group to investigate the efficacy of adjuvant RT for stage I cervical cancer cases at risk for recurrence showed that adjuvant RT significantly reduces cancer recurrence by 44% [8]. Similar results have been demonstrated by several retrospective studies [4,9,10]; however, it has not yet been demonstrated by randomized controlled trials that adjuvant RT prolongs overall survival. In fact, recent meta-analysis by cochrane review failed to demonstrate survival effect with adjuvant RT compared to no further treatment [11]. To establish a survival benefit of adjuvant chemotherapy, we need to establish a standard chemotherapeutic regimen because no standard chemotherapeutic regimen has been established in cervical cancer in an adjuvant setting yet. TP is recognized as a current standard chemotherapeutic regimen for metastatic, recurrent, or persistent cervical cancer, because phase III trial comparing four cisplatin-containing doublet combinations, including TP, gemcitabine/cisplatin, topotecan/cisplatin, and vinorelbine/cisplatin, revealed that patients treated with TP showed a more favorable prognosis than others [5]. Paclitaxel/carboplatin (TC) combination might be a promising regimen as a standard adjuvant chemotherapy in cervical cancer, because Japanese Clinical Oncology Group (JCOG) has conducted phase III trial to compare TP with TC for recurrent and metastatic cervical cancer, and will get the result soon [12,13]. Another promising regimen for cervical cancer is irinotecan/platinum (cisplatin or nedaplatin) combination, which has been tested in phase II trial with objective response rate of 80.4% [14], and phase II trial is currently on going to evaluate clnical efficacy of irinotecan/nedaplatin as an adjuvant chemotherapy in node-positive patients with cervical cancer who underwent radical hysterectomy in Japan.

In our previous report, 3-year disease-free and overall survival in chemotherapy (BOMP) group was similar to those in RT group in

TABLE IIIA. Outcome After Recurrence of Seven Patients Who Received Adjuvant TP

Cases	Age	Pathological subtype	PTNM classification	Recurrent period after operation (months)	Recurrent part	Therapeutic regimen after recurrence	Survival time after recurrence (months)	Outcome
1	53	M	pT2b.N1M0	8	Lung	CT	7	DOD
2	56	E	pT1b2.N1.M0	30	Lnguinal node, perlvic lymph node, PAN	RT,CT	26	DOD
3	57	SCC	pT1b2.N1.M0	10	Lung	CT	35	AWD
4	43	SCC	pT1b2.N1.M0	13	PAN	RT	23	NED
5	38	ad-sq	pT1b2.N1.M0	32	Pelvic lymph node	CCRT,CT	9	AWD
6	41	M	pT2b.N1.M0	27	PAN, virchow	RT,CT	13	AWD
7	53	M	pT2a2.N1.M0	31	Pelvic wall	CCRT,CT	11	SOD

M, mucinous adenocarcinoma; E, endometrioid adenocarcinoma; SCC, squamous cell carcinoma; ad-sq, adenosquamous cell carcinoma; PAN, para aortic lymph node; RT, radioyherapy; CT, chemotherapy; CCRT, concurrent chemoradiotherapy; DOD, dead of disease; AWD, alive with disease; NED, no evidenceof disease.

patients with cervical SCC with intermediate risks treated with radical hysterectomy and lymphadenectomy [4]. However, BOMP is not the best chemotherapy regimen for cervical cancer, because Japanese Clinical Oncology Group (JCOG) study to compare the efficacy of neoadjuvant chemotherapy using BOMP followed by radical hysterectomy to primary radical hysterectomy in cervical cancer patients with stage Ib2-IIb disease failed to show the clinical benefit of neoadjuvant chemotherapy using BOMP, and objective response rate of BOMP was 61.0%, which seems lower than generally expected [15].

Survival time after recurrence in TP group (median of 12.0 months) was longer than that in RT group (median of 8.5 months). The exact reason why adjuvant TP has survival effect in our patient cohort remains unclear. Since incidence rate of extrapelvic failure was similar between two groups, the difference of survival after recurrence might be due to disease-control for pelvic recurrence. RT or concurrent chemoradiotherapy (CCRT) could be applied to pelvic failure for three cases of TP group in our patient cohort. (Table IIIA) They can subsequently receive systemic chemotherapy again. In patients who received adjuvant RT, RT, or CCRT can not be generally applied for pelvic failure. Indeed, RT was applied to recurrent disease in paraaortic nodes, subclavicular nodes in three cases, but not to pelvic failure in our patient cohort. (Table IIIB) The patients who received adjuvant RT can receive systemic chemotherapy alone for pelvic control. Mean survival time after recurrence in patients with pelvic failure was 16.4 months, 8.8 months in TP group and RT group, respectively. We, therefore, speculate that multimodal treatment using RT followed by systemic chemotherapy could contribute to longer survival after recurrence.

In the present study, we have shown that incidence of postoperative complications is less frequent in adjuvant TP group than in adjuvant RT group. Bowel obstruction was significantly less frequent in TP group as we previously described with BOMP regimen [4]. Incidence rate of lower-limb lympedema seemed higher in RT group than TP group, but was not statistically different in our patient cohort. However, our recent result of the retrospective analysis on lower-limb lymphedema after radical hysterectomy and systematic lymphadenectomy demonstrated that postsurgical RT was an independent risk factor for stage II/III disease evaluated with the same diagnostic criteria in different patients' cohort [16]. Thus, adjuvant RT might be an important risk factor for severe lower-limb lymphedema. Incidence of urinary disturbance was higher in RT group than that in TP group with marginal significance ($P = 0.07$) as we previously described [4]. Moreover, our recent result of the retrospective analysis revealed that adjuvant RT was one of the risk factors for persistent low bladder compliance after radical hysterectomy and systematic lymphadenectomy [17].

CONCLUSIONS

We found 3-year survival effect of adjuvant TP in patients with cervical cancer treated with radical hysterectomy and systematic lymphadenectomy. We believe that it is worth considering a

TABLE IIIB. Outcome After Recurrence of 16 Patients Who Received Adjuvant RT

Cases	Age	Pathological subtype	PTNM classification	Recurrent period after operation (months)	Recurrent part	Therapeutic regimen after recurrence	Survival time after recurrence (months)	Outcome
1	31	SCC	pT1b2.N1.M0	11	PAN	RT.CT	6	DOD
2	60	SCC	pT2b.N1.M0	16	Vaginal stump	RT (RALS)	162	NED
3	59	SCC	pT1b1.N1.M0	3	Pelvic wall	CT	7	DOD
4	49	SCC	pT1b2.N1.M0	12	Lung, virchow	CT	3	DOD
5	46	SCC	pT1b2.N1.M0	3	PAN, virchow	CT	8	DOD
6	52	SCC	pT2b.N1.M0	4	Lung	CT	5	DOD
7	51	SCC	pT2b.N1.M0	25	Plelvic lymph node	CT	15	DOD
8	53	SCC	pT2b.N1.M0	13	Lung	CT	6	DOD
9	58	SCC	pT1b1.N0.M0	20	Brain	CT	27	DOD
10	38	SCC	pT2b.N1.M0	26	Liver	CT	13	DOD
11	50	SCC	pT2a.N0.M0	8	Pelvic wall	CT	5	DOD
12	41	SCC	pT1b1.N1.M0	4	Pelvic wall	CT	4	DOD
13	49	SCC	pT2b.N1.M0	21	Lung	CT	22	DOD
14	48	SCC	pT2a.N1.M0	4	Pelvic wall	CT	9	DOD
15	55	SCC	pT1b1.N1.M0	20	Virchow	RT.CT	13	DOD
16	43	SCC	pT2b.N1.M0	2	Pelvic wall	CT	13	DOD

SCC, squamous cell carcinoma; PAN, para aortic lymph node; RT, radiotherapy; RALS, remote after loading system; CT, chemotherapy; DOD, dead of disease; NED, no evidence of disease.

TABLE IV. The Incidence of Postoperative Complications According to the Type of Adjuvant Therapy

Adjuvant therapy	Number of patient	Lower-limb lymphedema	Bowel obstruction	Urinary disturbance	Not available
Radiotherapy	49	11 (22.4%)	12* (24.5%)	17 (34.7%)	4
Chemotherapy	32	4 (11.4%)	1 (3.1%)	5 (15.6%)	2
Radiotherapy vs. chemotherapy		$P = 0.2$	$P = 0.01$	$P = 0.07$	

*Two cases of bowel obstruction necessitated intestinal surgery.

prospective randomized trial of chemotherapy using TP versus RT as an optional adjuvant therapy to patients with risk factors for recurrence. We need to further establish a standard regimen of adjuvant chemotherapy for future trials.

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A Prospective Study on the Efficacy of Octreotide in the Management of Malignant Bowel Obstruction in Gynecologic Cancer

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Objective: Malignant bowel obstruction (MBO), of which symptoms lead to a poor quality of life, is a common and distressing clinical complication in advanced gynecologic cancer. The aim of this study was to prospectively assess the clinical efficacy of octreotide to control vomiting in patients with advanced gynecologic cancer with inoperable gastrointestinal obstruction.

Methods: Patients with advanced gynecologic cancer, who presented at least one episode of vomiting per day due to MBO, were enrolled in this prospective study from 2006 to 2009. Octreotide was administered when necessary at doses starting with 300 μg up to 600 μg a day by continuous infusion for 2 weeks. Primary end point was vomiting control, which was evaluated by common terminology criteria for adverse events version 3 (CTCAE v3.0). Adverse events were also evaluated by CTCAE v3.0.

Results: Twenty-two cases were enrolled in this study. Octreotide controlled vomiting in 15 cases (68.2%) to grade 0 and 3 cases (13.6%) to grade 1 on CTCAE v3.0. Overall response rate to octreotide treatment was 81.8% in our patients' cohort. Among 14 cases without nasogastric tube, the overall response rate was 93.1% (13/14). Among 8 cases with nasogastric tube, 4 cases were free of tube with decrease of drainage, and overall response rate was 62.5% (5/8). No major adverse events related to octreotide were reported.

Conclusions: We conclude that 300-μg/d dose of octreotide was effective and safe for Japanese patients with MBO by advanced gynecologic cancer. Octreotide could contribute to better quality of life by avoiding placement of nasogastric tube.

Key Words: Octreotide, Malignant bowel obstruction, Gynecologic cancer, Quality of life

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Malignant bowel obstruction (MBO) is a common and distressing clinical complication in advanced gynecologic cancer and is reported in 5.5% to 42% of terminal patients with ovarian cancer.[1–3] Vomiting secondary to MBO is a great problem in terminal patients.

Current treatments for MBO for patients with advanced cancer include the following: (1) surgery to bypass/remove the obstruction, (2) gastrointestinal drainage via a nasogastric tube, and (3) medication (antimimetics or others). Surgical treatment is often contraindicated owing to poor performance status, and many patients with gynecologic malignancies (especially ovarian cancer cases) would not be eligible for surgery because of the presence of diffuse intraperitoneal carcinomatosis, multiple partial obstruction points, ascites, and/or previous radiotherapy. Placement of nasogastric tube may be the only treatment available for inoperable cases. A nasogastric tube can achieve symptomatic relief but sometimes causes mucosal erosion, esophagitis, and aspiration pneumonia, which lead to poor quality of life. Thus, from the standpoint of the quality of life of terminal patients, the best way is to control symptoms relating to MBO without placement of nasogastric tube.

Octreotide, an analog of somatostatin, is a drug to control symptoms due to MBO. It inhibits the release and activity of gastrointestinal hormones,[4] and modulates gastrointestinal function by reducing gastric acid secretion, slowing intestinal motility, decreasing bile flow, increasing mucus production, and reducing splanchnic blood flow.[4,5]

Although several retrospective studies on the clinical efficacy of octreotide have been reported in gynecologic cancer,[6,7] there have been no prospective studies on the efficacy and safety of octreotide for MBO by patients with gynecologic malignancy in the literature. Additionally, clinical study on octreotide conducted in Japan[8] included few patients with gynecologic malignancies, which raises questions whether octreotide is effective for MBO of Japanese patients with gynecologic malignancies. Thus, the aim of this study was to prospectively evaluate the efficacy of octreotide in controlling vomiting of patients with terminal gynecologic cancer with MBO.

PATIENTS AND METHODS

From March 2006 to December 2009, 22 patients with abdominal recurrence of advanced gynecologic cancer were enrolled in this prospective study.

The patients in this study were required to be hospitalized, to be between 20 and 80 years of age, to have MBO that was refractory to conventional medical treatment, and to have a life expectancy of at least 3 weeks. Before being enrolled in this study, the patients also had at least one episode of vomiting per day on one designated day or had marked drainage of bowel contents (≥300 ml/day) from a nasogastric tube. Patients who retained normal hepatic function, as indicated by a total bilirubin of 2.0 mg/dL or less, were eligible for the study. The study excluded patients with serious complications (eg, active infection, pleural effusion, and gastrointestinal hemorrhage) and those with symptomatic brain metastasis. After enrollment, the patients received octreotide (300 μg/d) subcutaneously or intravenously as a continuous injection for 7 days. Patients who responded to this 7-day course of treatment continued to receive the drug with the same dose up to 14 days. The dose of octreotide could be increased to 600 μg/d for another 7 days if no improvement of symptoms was observed with 300 μg/d at day 7. The patients were assessed daily to determine the number of vomiting episodes, the severity of their nausea, and (if relevant) the volume of fluid draining from the nasogastric tube.

Response criteria were based on the change from baseline (24 hours before the start of treatment) to days 4, 8, and 15 in the severity of vomiting, which was graded using CTCAE v3.0. The response to treatment was graded using 3 categories (complete control [CC], partial control [PC], and no control [NC]). Patients with grade 0 vomiting on day 8 were assigned a rating of CC. The rating was PC if the grade for vomiting was decreased by one grade or more from baseline on day 8. No change or an increase of grade was regarded as NC. In patients with a nasogastric tube at baseline, extubation was allowed if drainage was reduced to less than baseline. After extubation, the response to the treatment was graded according to the following 3 categories defined by grade of nausea/vomiting: CC (grade 0), PC (only one episode of vomiting per day or nausea only), and NC (no change or increase of grade). Change of other symptoms including nausea, anorexia, abdominal distension, and fatigue were also evaluated by CTCAE v3.0. The occurrence of adverse events and abnormal laboratory findings were considered for the evaluation of safety, and the severity of adverse drug reaction was graded in accordance with CTCAE v3.0. With regard to the clinical laboratory testing, hematology, biochemistry, and urine tests were performed just before the start of the treatment with study medication and after 8 and 15 days of treatment. This study was approved by the institutional review board of Hokkaido University Hospital and was conducted in compliance with Ethical Guidelines for Clinical Studies. In accordance with the declaration of Helsinki, written informed consent was obtained from all patients before enrollment.

The results reported by Shima et al[8] were taken into consideration to calculate the sample size. Because the clinical response rate (CC/PC) for octreotide treatment was expected to be 54% based on previous Japanese study,[8] we calculated that 22 patients would be needed to detect in response to octreotide of 55%, with 80% power and a 2-sided 5% significance level.

Steel test was used to analyze the statistical difference of the number of vomiting episodes, drainage from nasogastric tube, and other symptoms including nausea, appetite loss, abdominal distension, general fatigue between baseline and each point for evaluation (days 4, 8, and 15). Significance was set at $P < 0.05$. Statistical analyses were performed with the excel 2008 (Social Survey Research Information Co, Ltd).

RESULTS

Patients' Characteristics

Demographic and baseline characteristics of 22 patients are listed in Table 1. Median age of the patients was 62 years (range, 43–79 years). Ovarian cancer was the most frequent type of malignancy (n = 12 [54.5%]), followed by cervical or endometrial cancer (n = 6 [27.3%]), primary peritoneal cancer (n = 3 [13.6%]), and double cancer of endometrium and ovary

TABLE 1. Patients' characteristics (n = 22)

Characteristics	No. Patients
Age, median (range)	62 (43–79)
Cancer	
Endometrial or cervical	6
Ovarian	12
Peritoneal	3
Endometrial-ovarian	1
Major site of obstruction	
Small intestine	14
Large intestine	3
Rectum	1
Undetermined (include carcinomatous peritonitis	4
Nasogastric tube	
Yes	8
No	14
ECOG PS	
1	2
2	7
3	9
4	4

ECOG, Eastern Cooperative Oncology Group; PS, performance status.

(n = 1 [4.6%]). The most prevalent major site of obstruction was the small intestine in 14 cases (63.6%). At baseline, a nasogastric tube was already placed in 8 patients (36.4%), whereas not in 14 patients (63.6%). The baseline performance status (PS) was 3 to 4 in 13 patients (59.1%).

Response

The response to treatment with octreotide, which was evaluated by the change of vomiting episodes, is summarized in Table 2. Among all patients treated, 15 patients (68.2%) had a response of CC and 3 patients (13.6%) had a response of PC, with an overall response rate (CC/PC) of 81.8%. Among the 14 patients without a nasogastric tube at baseline, 11 patients (78.6%) achieved CC and 2 patients (14.3%) achieved PC with an overall response rate of 92.9% (13/14). Among the 8 patients with a nasogastric tube at baseline, 4 patients (50.0%) achieved CC and one (12.5%) achieved PC with an overall response rate of 62.5%. In the entire study population, the median number of vomiting episodes per day was significantly reduced from 3.0 (range, 0–12) at baseline to 0 (range, 0–11) on day 4 ($P = 0.0029$), 1.0 (range, 0–6) on day 8 ($P = 0.0003$), and 0 (range: 0–4) on day 15 ($P < 0.0001$; Fig. 1) Among the 7 patients with a nasogastric tube at baseline, all patients showed a significant decrease in drainage from 793 mL at baseline to 219 mL on day 4 ($P = 0.0201$) and 301 mL on day 8 ($P = 0.0492$; Fig. 2). Change of other symptoms including nausea, anorexia, abdominal distension, and fatigue were also evaluated by the severity of grades using CTCAE version 3. Among them, grade of nausea was significantly reduced from 2.7 at baseline to 1.7 on day 4 ($P = 0.0311$), 1.5 on day 8 ($P = 0.0085$), and 1.5 on day 15 ($P = 0.0544$). Anorexia, abdominal distension, and fatigue were also reduced from the baseline without significant difference on days 4, 8, and 15 (Fig. 3).

TABLE 2. Efficacy of control vomiting episodes

Efficacy	n (%)
CC	15 (68.2)
PC	3 (13.6)
NC	4 (18.2)
Without gastric tube	14
CC	11 (78.6)
PC	2 (14.3)
NC	1 (7.1)
With gastric tube	8
CC	4 (50.0)
PC	1 (12.5)
NC	3 (37.5)

Adverse Events

Adverse events were graded irrespective of the relation to octreotide treatment (Supplemental Digital Content, Table 1, http://links.lww.com/IGC/A80). All of them were not related to octreotide treatment itself but to the worse general condition of the patients. Overall, treatment with octreotide was well tolerated and did not cause any serious or clinically significant adverse reactions.

DISCUSSION

Baines et al[9] published the first study on a pharmacological approach with octreotide in treating symptoms

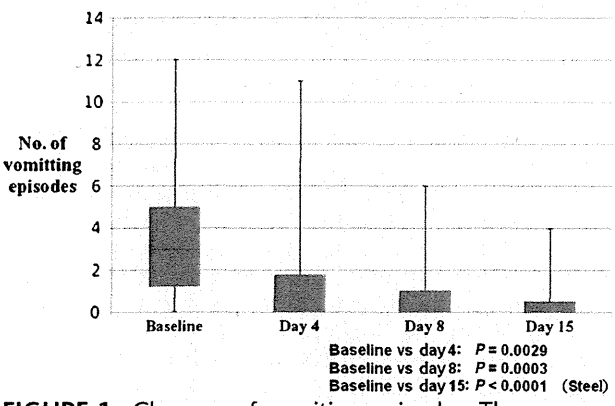

FIGURE 1. Changes of vomiting episodes. The mean number of vomiting episodes per day was significantly reduced from 4.5 at baseline to 1.5 on day 4 ($P = 0.0029$), 1.0 on day 8 ($P = 0.0003$), and 0.5 on day 15 ($P < 0.0001$). P value was calculated by Steel test.

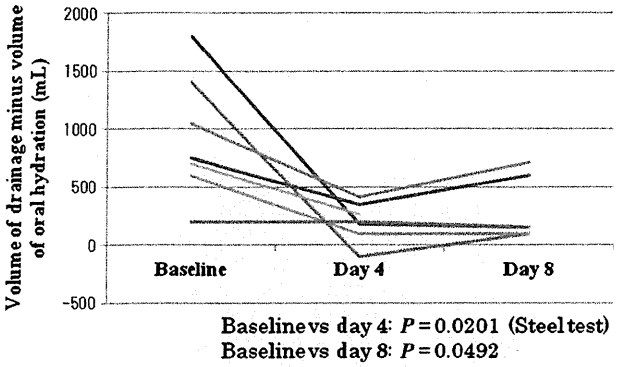

FIGURE 2. Changes in the drainage volume from nasogastric tube. The mean actual daily drainage volume was calculated: the total drainage volume − oral water intake. Of 7 patients with a nasogastric tube at baseline, all patients showed a decrease in drainage from 793 to 219 mL on day 4 ($P = 0.0201$) and 301 mL on day 8 ($P = 0.0492$). P value was calculated by Steel test.

including nausea, vomiting, pain, and others due to MBO in advanced cancer, thereby avoiding the nasogastric tube and intravenous hydration.

Many recent studies have shown that octreotide is useful for controlling gastrointestinal symptoms due to MBO in patients with advanced cancer, whereas there has been no prospective study in the patients with gynecologic cancer in the literature. In this study, we prospectively evaluated the efficacy and safety of 300-μg/d initial dose of octreotide in Japanese patients with gynecologic cancer with MBO. The primary efficacy end point was the change in vomiting episodes after treatment. To ensure objectivity of assessment, we used CTCAE v3.0 to grade the severity of emesis. In contrast, previous retrospective clinical studies have often used the World Health Organization (WHO) toxicity criteria[10] (grade 1, nausea; grade 2, transient vomiting; grade 3, vomiting requiring therapy; and grade 4, intractable vomiting). Compared with the WHO criteria, CTCAE v3.0 (grade 1, only nausea; grade 2, 1–5 vomiting episodes per 24 hours; grade 3, ≥6 vomiting episodes per 24 hours) seems to provide a more quantitative assessment of the severity of emesis.

Of the patients in this study, 81.8% responded to the treatment with octreotide; and the overall response rate was higher than that previously reported in Japanese patients.[8] Possible explanations for the favorable response to octreotide in the present study include the underlying malignancies. Regarding the type of underlying malignancies, more than half of the patients enrolled in the previous study had gastric cancer (n = 14 [56.0%]). Additionally, only 2 cases of gynecologic cancer (ovarian cancer) were included. Analysis of response data obtained in previous study revealed that 5 (35.7%) of 14 patients with gastric cancer and 6 (54.5%) of 11 patients with other cancers had a response of PC or better. The patients with gastric cancer tended to have a lower response rate. The lower response rate of the patients with gastric cancer was partly responsible for the lower overall response rate in the previous study. Current study clearly indicated that octreotide is more effective for MBO by gynecologic malignancy than by gastric cancer, probably because of the difference of location of obstruction, and/or milder obstruction than gastric and/or colon cancer, in which direct obstruction by tumors causes severe symptoms. In the previous prospective study for Japanese patients, they described that the timing of assessment (day 6) might have affected the difference in the response rate compared to the previous reports. In overseas clinical studies,[11,12] the response to octreotide was assessed after only 3 days of treatment so the longer treatment period before examination in previous Japanese study might also have contributed to the lower response rate. We, therefore, assessed the change of symptoms at several points after starting octreotide treatment—at days, 4, 8, and 15—and found that octreotide was highly effective for Japanese patients with MBO by gynecologic malignancy at any points.

Among cases with nasogatric tube, 4 of 8 cases became free of the tube in our study. Long-term placement of nasogastric tube can interfere with coughing to clear pulmonary secretions and may be associated with nasal cartilage erosion, otitis media, aspiration pneumonia, esophagitis, and bleeding. The nasogastric tube is sometimes even more unpleasant for the patient than the basic condition itself.

Mangili et al[6,13] described that octreotide controlled the vomiting in all cases of ovarian cancer in 2 reports. However, both are retrospective studies; and WHO criteria, which are less objective than CTCAE v3.0 used in this study, was used to grade severity of vomiting in the first report,[6] and they did not describe how they evaluated the severity of symptoms in the second report.[13] Matulonis et al[7] reported overall response rate of 46.2% (6/13) for patients with advanced ovarian cancer in a retrospective study, which is worse than our

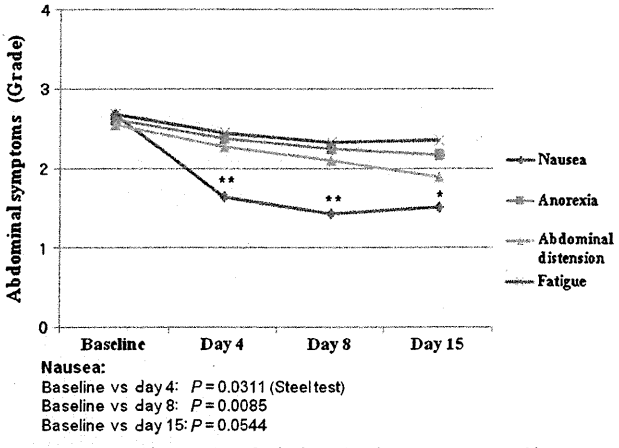

FIGURE 3. Changes of abdominal symptoms. Change of the abdominal symptoms including nausea, anorexia, abdominal distension, and fatigue. Among of them, the mean grade of nausea was reduced from 2.68 at baseline to 1.64 on day 4 ($P = 0.0311$), 1.43 on day 8 ($P = 0.0085$), and 1.5 on day 15 ($P = 0.0544$). P value was calculated by Steel test.

result. Our current prospective study clearly demonstrated that octreotide is effective for MBO by gynecologic malignancies using the more objective criteria for grading the severity of symptoms. For patients without nasogastric tube, octreotide was highly effective (92.9%) in this study. Thus, from the standpoint of quality of life, the first choice of treatment for MBO by gynecologic malignancy might be the administration of octreotide, not placement of nasogastric tube.

In summary, initial treatment with 300 μg/d of octreotide for 14 days was confirmed to be effective and safe for controlling vomiting in Japanese patients with MBO by gynecologic malignancies. We, therefore, need to introduce octreotide, if applicable, at the early phase of best supportive care for the terminal patients with MBO by gynecologic malignancies, resulting in avoidance of placement of nasogastric tube, which can impair their quality of life.

ACKNOWLEDGMENT

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ORIGINAL ARTICLE

Mutant p53 gain-of-function induces epithelial–mesenchymal transition through modulation of the miR-130b–ZEB1 axis

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The tumor suppressor gene p53 has been implicated in the regulation of epithelial–mesenchymal transition (EMT) and tumor metastasis by regulating microRNA (miRNA) expression. Here, we report that mutant p53 exerts oncogenic functions and promotes EMT in endometrial cancer (EC) by directly binding to the promoter of miR-130b (a negative regulator of ZEB1) and inhibiting its transcription. We transduced p53 mutants into p53-null EC cells, profiled the miRNA expression by miRNA microarray and identified miR-130b as a potential target of mutant p53. Ectopic expression of p53 mutants repressed the expression of miR-130b and triggered ZEB1-dependent EMT and cancer cell invasion. Loss of an endogenous p53 mutation increased the expression of miR-130b, which resulted in reduced ZEB1 expression and attenuation of the EMT phenotype. Furthermore, re-expression of miR-130b suppressed mutant p53-induced EMT and ZEB1 expression. Importantly, the expression of miR-130 was significantly reduced in EC tissues, and patients with higher expression levels of miR-130b survived longer. These data provide a novel understanding of the roles of p53 gain-of-function mutations in accelerating tumor progression and metastasis through modulation of the miR-130b–ZEB1 axis.

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Keywords: EMT; cancer; gain-of-function; miRNA; p53 mutation

INTRODUCTION

Epithelial–mesenchymal transition (EMT) is a transcriptional process that has a key role in regulating embryonic morphogenesis and cancer metastasis. During EMT, epithelial cells lose their polarization and homotypic cell adhesion, resulting in a more motile, spindle-like morphology with increased invasiveness.[1] At the molecular level, EMT occurs as a result of the activity of several transcriptional factors, such as ZEB1/2, Twist, BMI-1, Snail, and Slug, which suppress expression of the epithelial marker E-cadherin and induce the mesenchymal genes *N-cadherin* and *Vimentin*.[1] However, the mechanisms and pathways that drive EMT programs are not fully understood.

Non-coding microRNAs (miRNAs), including miR-200 and miR-194/192 family members, have been identified as negative regulators of EMT and metastasis by repressing the expression of ZEB1/2.[2] The overexpression of miR-200 and let-7b in gemcitabine-resistant pancreatic cancer cells induces the mesenchymal–epithelial transition, which is the reverse process of EMT.[3] Moreover, miR-194 is critical for maintaining the hepatic epithelial cell phenotype and inhibits metastasis by targeting several EMT activator genes.[4]

Recently, a regulatory connection between p53 signaling and miRNA-mediated EMT has been demonstrated. Wild-type (WT) p53 directly activates the transcription of miR-200c and miR-192 family members, which leads to ZEB1/2 downregulation and repression of EMT.[5] Furthermore, mutation of p53 can promote EMT and the aggressive potential of tumor cells by inhibiting WT p53-miR-200c pathways through dominant-negative effects on WT p53.[6] However, besides the dominant-negative effects upon WT p53, increasing evidence suggests that p53 mutations acquire additional oncogenic functions, such as a gain-of-function (GOF), which actively drive cells toward invasion and metastasis[7] through transactivation or transrepression of a large set of genes involved in regulation of cell adhesion, migration and proliferation.[8] In agreement with these findings, previous studies have found that overexpression of miRNAs (miR-181b and miR-200c) is associated with either p53 mutations or shorter patient survival in human colon cancer,[9] indicating that mutant p53 may exert GOF activities and promote EMT by modulating miRNAs.

Here, we identified a novel mechanism by which mutant p53 demonstrates GOF effects to facilitate EMT and cancer cell invasion by repressing miR-130b, an inhibitor of ZEB1. We further demonstrated that the expression of miR-130 was significantly reduced in endometrial cancer (EC) tissues, and patients with higher expression levels of miR-130b survived longer. Thus, these data suggest that restoration of miR-130b may have therapeutic value in tumors expressing mutant p53.

RESULTS

Mutant p53 GOF contributes to EMT in EC cells

Although mutant p53 GOF has been shown to promote EMT by upregulating Twist in prostate cancer cells,[10] to date, the role of mutant p53 GOF in initiating EMT during EC progression remains unknown. To explore this issue, we used p53-null HEC-50 cells[11] stably transduced with vectors encoding p53 mutations R273H,

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R175H or C135Y, as well as an empty vector. Polyclonal cell lines were generated to omit clonal variation. The p53 protein level was verified using the anti-p53 (DO-7) antibody, which recognizes both WT and mutant p53.

Enforced expression of these p53 mutants induce a shift in cell morphology from a paved stone epithelial appearance to more mesenchymal phenotypes, with loss of cell-to-cell contact and increased cell spreading (Figure 1a). These morphological changes were accompanied by the upregulation of mesenchymal genes, including *Twist*, *ZEB1*, *BMI-1*, *Snail*, *N-cadherin* and *Vimentin*, and decreased expression of the epithelial marker E-cadherin (Figure 1b). To examine whether overexpression of mutant p53 can promote cell invasion, we next performed a cell invasion assay and observed a significant increase in the invasive capacity of mutant p53-expressing cells compared with empty vector-transfected control cells (Figure 1c). These findings were supported by concomitantly enhanced expression of metastatic-associated genes *osteopontin*, *MMP-2* and *MMP-9* in HEC-50 cells containing the p53 mutants (Supplementary Figure S1A).

Recently, EMT has been shown to have critical roles in modulating the cancer stem-like cell phenotype and conferring increased drug resistance of cancer cells.[12] To test the roles of mutant p53 GOF in acquiring stemness and drug-resistant properties in EC cells, we investigated the effects of stable expression of mutant p53 R175H on the self-renewal potential of cells using a sphere formation assay. We also assessed the chemosensitizing properties of this cell line after treatment with paclitaxel using the Cell Counting Kit-8. We found that transfection of this mutant, but not empty vector, enabled HEC-50 cells to form floating spheres in a serum-free medium (Figure 1d) and became more resistant to paclitaxel treatment (Figure 1e). To further explore the mechanisms of mutant p53 GOF-mediated cancer stemness and drug resistance, Quantitative reverse transcription (qRT–PCR) was performed to show that the mRNA levels of well-characterized stem cell markers (*CD133*, *KLF4* and *NANOG*) and chemoresistance-related genes (*MDR-1* and *MRP-1*) was highly enhanced in R175H-expressing cells (Figure 1f).

Similar to the findings obtained from stable transfection experiments, transient transfection of a vector encoding mutant p53 R248Q, but not empty vector, promoted cell invasion (Supplementary Figure S2C). In addition, this mutant also promoted EMT-like changes, including enhanced expression of ZEB1, BMI-1, N-cadherin and Vimentin, as well as repression of E-cadherin in HEC-50 cells (Supplementary Figure S2A). Taken together, these observations suggested a crucial role of mutant p53 GOF in driving EMT and invasive phenotypes of EC cells.

Knockdown of mutant p53 in EC cells causes a reversal of EMT and inhibition of cell invasion ability

To further examine whether loss of endogenous mutant p53 can inhibit EMT features, we performed shRNA-mediated knockdown of mutant p53 in HEC-1 cells, which express endogenous mutant

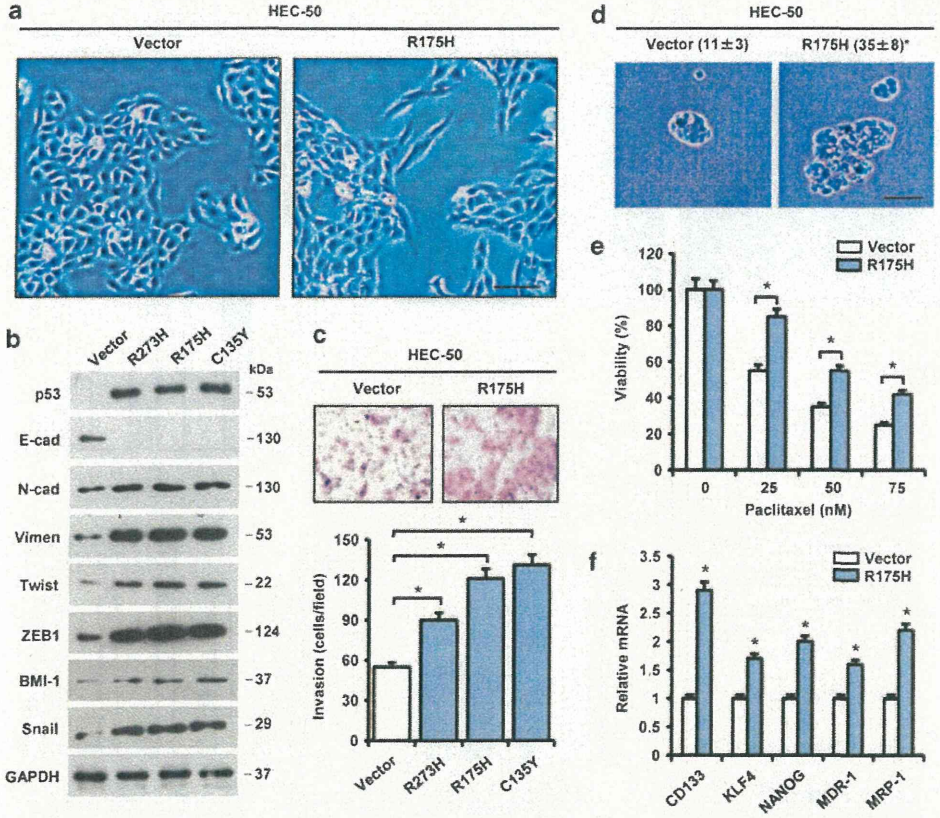

Figure 1. Mutant p53 GOF contributes to EMT in EC cells. (**a**) Morphology of endometrial cancer HEC-50 cells containing a control vector or mutant p53 R175H. Scale bars represent 100 μm. (**b**) Protein expression of p53 and EMT markers as analyzed by immunoblot. (**c**) Invasion of HEC-50 cells following overexpression of mutant p53s (mean ± s.d.; $n = 3$; $^*P < 0.01$). Representative images of invaded cells are shown. (**d**) Images indicate mammosphere formation in HEC-50 cells expressing the indicated constructs. The number of spheres obtained from 1000 cells at 12 days after plating (scale bar = 50 μm; mean ± s.d.; $n = 3$; $^*P < 0.01$). (**e**) Mutant R175H- or empty vector-transfected HEC-50 cells were treated with paclitaxel (0, 25, 50 and 75 nmol/l) for 48 h. Cell viability were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (mean ± s.d.; $n = 3$; $^*P < 0.01$). (**f**) Relative mRNA expression of stemness markers (normalized to GAPDH) in HEC-50 cells transfected with control or R175H vector, determined by qRT–PCR (mean ± s.d.; $n = 4$; $^*P < 0.01$).

p53 R248Q. Silencing of mutant p53 resulted in significant changes in cell morphology, and the scattered, mesenchymal-like HEC-1 cells began to exhibit a more epithelial-like cobblestone appearance (Figure 2a). Downregulation of this p53 mutant increased the expression of epithelial marker E-cadherin and repressed the expression of mesenchymal markers Twist, ZEB1, BMI-1, Snail, N-cadherin and Vimentin (Figure 2b). In agreement with these findings, knockdown of mutant p53 markedly reduced cell invasion (Figure 2c) and reduced the expression of osteopontin, MMP-2 and MMP-9 (Supplementary Figure S1B). To investigate if reduction of mutant p53 expression can suppress cancer stem-like and drug resistance properties, a sphere formation assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay were used. We found that HEC-1 cells transfected with *p53* shRNA displayed decreased sphere formation (Figure 2d) and were more sensitive to paclitaxel (Figure 2e). These results were supported by qRT–PCR experiments in which the mRNA expression of *CD133*, *KLF4*, *NANOG*, *MDR-1* and *MRP-1* were significantly attenuated following knockdown of endogenous p53 using shRNA (Figure 2f). Collectively, these results demonstrate that knockdown of mutant p53 can reverse the EMT phenotype and rescue cell invasion of EC cells.

ZEB1 is a key downstream mediator in p53 GOF mutant-induced EMT

Previous studies have shown that ZEB1 has a crucial role in the promotion of EMT and cancer stem cell properties in human cancer cells.[13] Overexpression of ZEB1 has been detected in aggressive EC.[14] Therefore, induction of ZEB1 expression by a p53 GOF mutant and reduction of its expression after mutant p53 silencing allowed us to postulate that ZEB1 may be essential for p53 GOF mutant-induced EMT in EC cells. Transfection of HEC-50 cells with *ZEB1* siRNA inhibited mutant p53 R175H-induced BMI-1 and Snail expression, restored E-cadherin expression (Supplementary Figure S3A) and greatly impaired p53 R175H-mediated cell invasion (Supplementary Figure S3B). However, this treatment did not significantly affect the protein level of Twist. Following transfection with *ZEB1* siRNA in HEC-1 cells, the mRNA expression of *BMI-1* and *Snail* was suppressed and *E-cadherin* was elevated (Supplementary Figure S3C). These data indicate that ZEB1 acts as an important downstream effector of these p53 mutants to mediate the EMT process in EC cells.

The p53 GOF mutants contribute to global repression of miRNA expression

To identify miRNAs mediated by the p53 GOF mutants, we performed array-based miRNA profiling of HEC-50 cells transduced with either p53 mutants or empty vector. Of 188 human miRNAs assayed, 23 miRNAs were expressed above background levels. Ectopic overexpression of mutant p53 R273H, R175H and C135Y in HEC-50 cells led to a global downregulation of all these miRNAs (Supplementary Figure S4A). We further validated the microarray results using qRT–PCR (Figure 3b). Notably, the expression of several miRNAs with known tumor suppressor activity, including let-7b,[3] miR-143,[15] miR-194,[16] miR-424,[17] miR-451,[18] and miR-

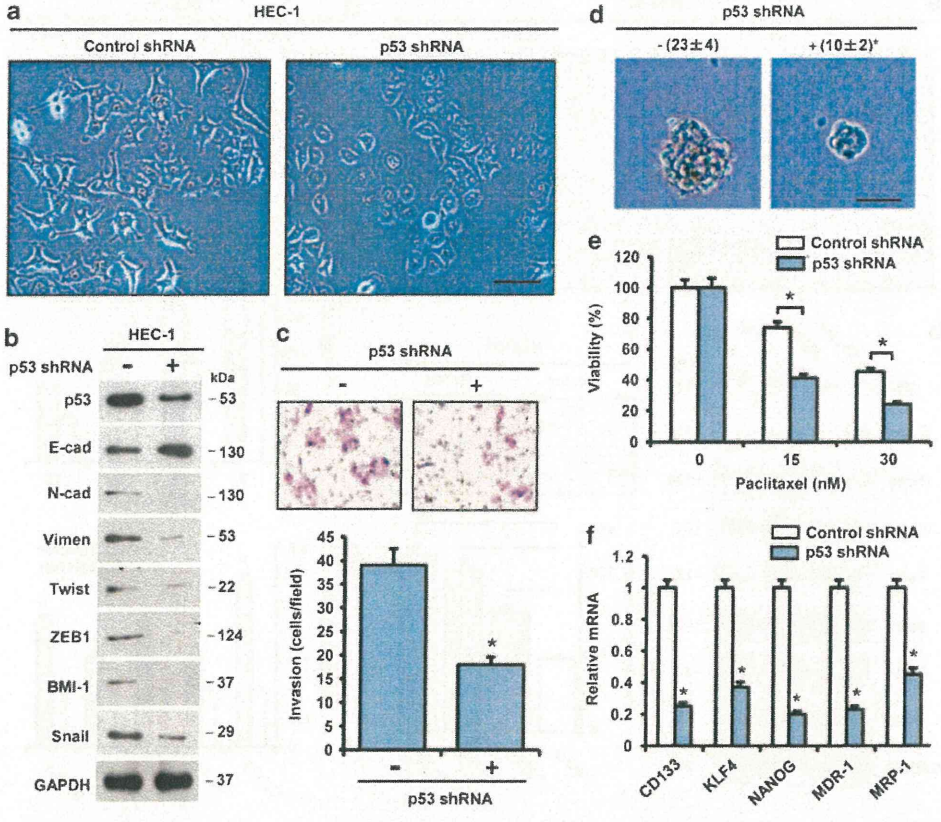

Figure 2. Knockdown of mutant p53 in EC cells causes a reversal of EMT and inhibition of cell invasion ability. (**a**) Morphology of endometrial cancer HEC-1 cells transfected with control shRNA vector or p53 shRNA vector (scale bar = 100 μm). (**b**) Protein levels of p53 and EMT markers as analyzed by western blot. (**c**) Invasion of HEC-1 cells after p53 shRNA transfection (mean ± s.d.; $n = 3$; $^*P < 0.01$). Representative images of invaded cells are shown. (**d**) Images show mammosphere formation in HEC-1 cells after p53 silencing by shRNA. Number of spheres obtained from 1000 cells at 12 days after plating (scale bar = 50 μm; mean ± s.d.; $n = 3$; $^*P < 0.01$). (**e**) Control- or p53 shRNA-transfected HEC-1 cells were treated with paclitaxel (0, 15, and 30 nmol/l) for 48 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (mean ± s.d.; $n = 3$; $^*P < 0.01$). (**f**) Relative mRNA expression of stemness markers (normalized to GAPDH) in HEC-1 cells after p53 silencing, determined by qRT–PCR (mean ± s.d.; $n = 4$; $^*P < 0.01$).

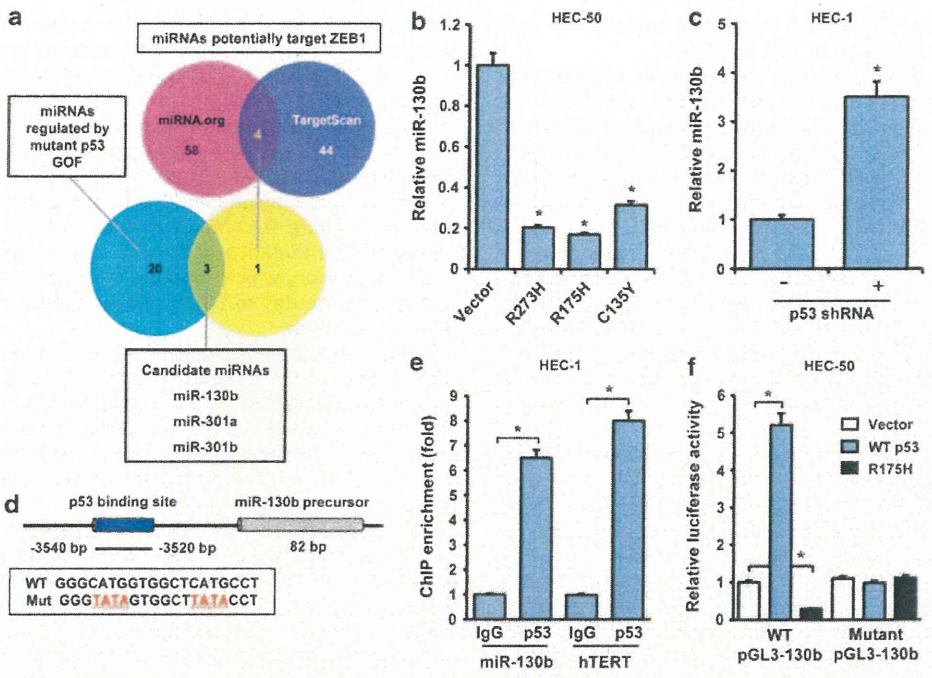

Figure 3. Mutant p53 binds to and transrepresses the promoter of miR-130b. (**a**) Schematic of algorithm used to select candidate microRNAs that potentially target ZEB1, and are negatively regulated by mutant p53s. (**b**, **c**) Relative miR-130b expression levels in HEC-50 cells transfected with mutant p53 vector (**b**), or in HEC-1 cells after p53 silencing by shRNA (**c**), were determined by qRT–PCR (mean ± s.d.; $n=4$; $^*P<0.01$). (**d**) Location and sequence of predicted p53-binding sites in the promoter of miR-130b gene. Mutated residues (red) are indicated at the bottom. (**e**) ChIP–qPCR analysis of mutant p53 (DO-7 antibody) binding to the miR-130b promoter region in HEC-50 cells. Human telomerase (hTERT) was used as a positive control. The fold enrichment over the IgG control is represented (mean ± s.d.; $n=3$; $^*P<0.01$). (**f**) HEC-50 cells were transfected with luciferase reporter plasmid pGL3-130b or empty pGL3-basic vector, along with control vector, wild-type p53 or mutant p53 R175H vector, and relative luciferase activity were assayed (mean ± s.d.; $n=3$; $^*P<0.01$). All qRT–PCR or luciferase values were normalized to GAPDH or Renilla activity, respectively.

146[19] were significantly reduced in p53-mutant-expressing cells (Supplementary Figure S4B). Thus, these results suggest that global repression of miRNA expression is likely to be a critical mechanism for p53 GOF mutant-enhanced EC tumorigenesis.

Mutant p53 binds to and transrepresses the promoter of miR-130b

Considering the important roles of ZEB1 in regulating EMT, we next sought to determine whether any of the 23 miRNAs may target ZEB1, and if repression of these miRNAs by mutant p53 may contribute to increased ZEB1 expression in EC cells. We first searched for all predicted miRNA-*ZEB1* interactions by using two target-prediction algorithms: TargetScan (http://www.targetscan.org) and microRNA.org (http://www.microrna.org), and detected four miRNAs that potentially bind to the 3′ untranslated regions (3′-UTR) of *ZEB1* mRNA. We then cross-referenced these four miRNAs with the 23 miRNAs identified by miRNA microarray. We found three miRNAs (miR-130b, miR-301a and miR-301b) that were downregulated in p53-mutant-expressing cells and predicted to bind to *ZEB1* 3′-UTR (Figure 3a). We selected *miR-130b* to investigate its effects on EMT and EC cell invasion because transfection of miR-301a and miR-301b did not substantially alter the protein expression of ZEB1 in EC cells (data not shown).

To evaluate whether the p53 mutants (R273H, R175H and C135Y) control the expression of miR-130b, we examined the effects of overexpression of mutant p53 on the expression of miR-130b in HEC-50 cells. The qRT–PCR analysis confirmed a significant decrease in mature miR-130b levels following transfection with the p53 mutants (Figure 3b). Similarly, the p53 mutation R248Q, but not the empty vector, inhibited the expression of *miR-130b* when expressed transiently (Supplementary Figure S2B). On the other hand, HEC-1 cells transfected with *p53* shRNA exhibited a marked elevation in the level of miR-130b (Figure 3c). These results suggest that the endogenous expression of miR-130b is negatively regulated by p53 mutants.

Recent evidence has established an association between p53 and several miRNAs, such as miR-34,[20] miR-192[21] and miR-200c.[5] Transcription of these miRNAs is directly regulated by p53. In particular, several studies have suggested that miR-200c is downregulated in EC tissues,[22] and restoration of miR-200c expression in HEC-50 cells decreases cell invasion.[23] Using qRT–PCRs to compare miRNA levels in HEC-50 cells, we found that overexpression of mutant p53 R175H and C135Y decrease the expression of miR-200c by 30–20%, whereas mutant R273H has no effects on its expression (Supplementary Figure S5), indicating that downregulation of miR-200c is involved in mutant p53 GOF-induced EC cell invasion.

However, it remains unknown whether p53 mutants function as a transcription regulator of miR-130b. Therefore, we searched for p53-binding sites in the miR-130b promoter using a bioinformatics approach.[24] Importantly, we found a conserved p53-binding site (5′-GGGCATGGTGGCTCATGCCT-3′) with a ranking score of 83 (Figure 3d). To determine whether an endogenous p53 mutant can bind this site, chromatin immunoprecipitation (ChIP)–qPCR analysis was performed on HEC-1 cells. The human telomerase (*hTERT*) promoter served as a positive control, as it has been previously shown that p53 mutants can bind this promoter.[25] Both miR-130b (sixfold) and *hTERT* (eightfold) promoter sequences were specifically enriched by anti-p53 antibodies, but not by

non-specific antibodies (Figure 3e). These data suggest that miR-130b is a direct target of mutant p53 in EC cells.

To assess if the downregulation of *miR-130b* expression is mediated by transrepression of the p53 mutants, we cloned the p53-binding sequence of the miR-130b promoter upstream of firefly luciferase to yield a WT plasmid pGL3-130b, and further generated mutant pGL3-130b luciferase vectors containing mutations in the candidate p53-binding site. The WT pGL3-130b or mutant pGL3-130b vector was transfected into HEC-50 cells with either a control vector, mutant p53 R175H or WT p53. Interestingly, the luciferase activity of WT pGL3-130b was significantly repressed by R175H, but was transactivated by WT p53. However, expression of mutant p53 or WT p53 did not affect the luciferase activity of mutant pGL3-130b (Figure 3f). Therefore, our observations by qRT–PCR, ChIP–qPCR and the luciferase assay collectively demonstrate that a GOF p53 mutant binds to and transrepresses the miR-130b promoter.

Our results showing a fivefold increase in the ability of WT p53 to transactivate the promoter of miR-130b (Figure 3f, lane 2) raised an interesting possibility that WT p53 controls metastasis through modulation of miR-130b. Therefore, we transiently transfected the WT p53 expression vector into HEC-50 cells (Figure 4a). A qRT–PCR analysis revealed that expression of WT p53 protein significantly induced the levels of miR-130b and also slightly increased the expression of miR-200c (Figure 4b), which is a known target of WT p53.[6] In WT p53-expressing HHUA cells, activation of p53 in response to the Mdm2 antagonist Nutlin-3 (Figure 4c) enhanced the level of miR-130b, but this was abolished by the shRNA-mediated knockdown of p53 (Figure 4d). Consistent with an earlier report,[6] the knockdown of WT p53 in HHUA cells induced changes associated with EMT, such as a mesenchymal morphology (Figure 4e), low expression of *E-cadherin*, upregulation of *ZEB1* and *BMI-1* (Figure 4f), and increased cell invasion (Figure 4g). We also observed an enrichment of WT p53 binding to both the miR-130b and p21 promoters using a ChIP–PCR analysis (Figure 4h). In addition, WT p53 was able to transactivate a pGL-3-miR-130b luciferase reporter gene (Figure 4i). Silencing of p53 by shRNA abrogated Nutlin-3-stimulated luciferase activities of the miR-130b promoter (Figure 4i, compare lane 5 to lanes 7 and 8). These data indicated that mutant and WT p53 exert opposite effects on miR-130b expression, which supports the hypothesis that a p53 GOF mutant contributes to EC carcinogenesis by altering the expression of miR-130b.

Despite direct transcriptional regulation by mutant p53, some GOF effects of the p53 mutants may depend on their ability to inactivate p53 family members p63 or p73.[26] Furthermore, p63 has been shown to inhibit metastasis through transactivation of miR-130b.[27] Therefore, we determined whether p63 inhibition by the p53 mutants is involved in the p53 GOF mutant-induced suppression of *miR-130b* in HEC-50 cells. We found that downregulation of p63 protein expression by *p63* siRNA (Supplementary Figure S6A) resulted in a dose-dependent decrease in *p21* luciferase activity (Supplementary Figure S6C). As expected, transient transfection of the WT p53 expression vector markedly transactivated the *p21* promoter (Supplementary Figure S6C). However, the mRNA expression of *miR-130b* did not substantially change after *p63* knockdown (Supplementary Figure S6B). Thus, p63 inhibition is not likely to be responsible for p53 GOF mutant-induced suppression of miR-130b in EC cells.

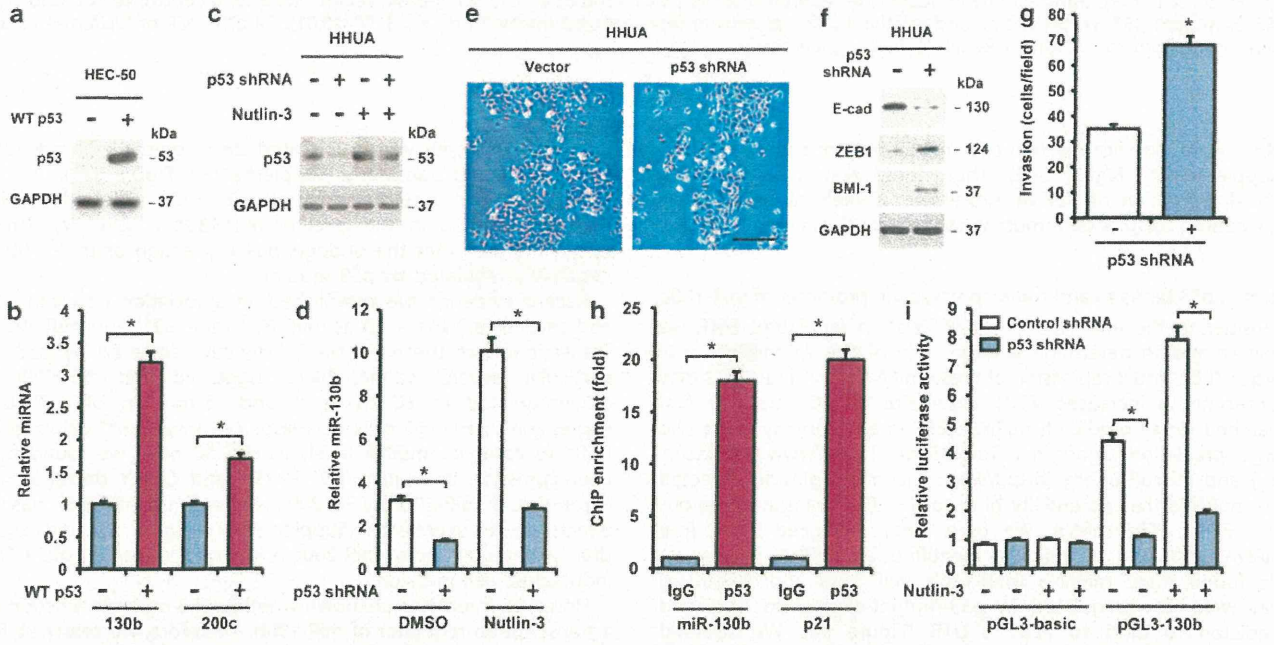

Figure 4. WT p53 transactivates the promoter of miR-130b. (**a**) WT p53 protein level in HEC-50 cells transfected with WT p53 expression vector or control vector. (**b**) qRT–PCR for miR-130b and miR-200c in HEC-50 cells transfected with WT p53 expression vector or control vector (mean ± s.d.; $n=4$; $^*P<0.01$). (**c**, **d**) HHUA cells transfected with p53 shRNA vector or control vector were treated with 5 μmol/l of Nutlin-3 or dimethyl sulfoxide (DMSO) for 12 h. WT p53 protein (**c**) and miR-130b expression (**d**) were detected by western blot analysis and qRT–PCR (mean ± s.d.; $n=4$; $^*P<0.01$), respectively. (**e**) Morphology of HHUA cells after p53 silencing. Scale bars represent 200 μm. (**f**) Western blot analysis for EMT markers in HHUA cells after p53 silencing. (**g**) Invasion assay of HHUA cells after transfection with p53 shRNA (mean ± s.d.; $n=3$; $^*P<0.01$). (**h**) ChIP–qPCR analysis of WT p53 (DO-7 antibody) binding to the miR-130b promoter region in HHUA cells. *p21* was used as a positive control. The fold enrichment over the IgG control is represented (mean ± s.d.; $n=3$; $^*P<0.01$). (**i**) Indicated HHUA cells were transfected with luciferase reporter plasmid pGL3-130b or empty pGL3-basic vector, and treated with 5 μmol/l of Nutlin-3 or DMSO for 12 h. Relative luciferase activity was determined (mean ± s.d.; $n=3$; $^*P<0.01$). All qPCR or luciferase values were normalized to GAPDH or Renilla activity, respectively.