11. Nakamura T, Hayashi K, Ota M, Eguchi R, Ide H, Takasaki K, et al. Salvage esophagectomy after definitive chemotherapy and radiotherapy for advanced esophageal cancer. Am J Surg 2004;188:261–6.
12. Sobin LH, Wittekid C (eds) TNM classification of malignant tumors. International Union Against Cancer. 5th ed. New York: Wiley; 1997.
13. Japanese Society for Esophageal Diseases. Guidelines for clinical and pathologic studies on carcinoma of the esophagus. Ninth English edition. Tokyo: Kanehara; 2001.
14. Ishikura S, Nihei K, Ohtsu A, Boku N, Hironaka S, Mera K, et al. Long-term toxicity after definitive chemoradiotherapy for squamous cell carcinoma of the thoracic esophagus. J Clin Oncol 2003;21:2697–702.
15. Brücher BLDM, Weber W, Bauer M, Fink U, Avril N, Stein HJ, et al. Neoadjuvant therapy of esophageal squamous cell carcinoma: response evaluation by positron emission tomography. Ann Surg 2001;233:300–9.

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# 進行・再発食道癌に対するsecond-lineとしてのDocetaxel + Nedaplatin併用療法の検討

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要旨　食道扁平上皮癌に対する化学療法を含む前治療後の無効，再発例に対するsecond-line治療としてのドセタキセル（TXT）／ネダプラチン（CDGP）併用化学療法を2002年から2004年に13例施行，評価可能な10例（男9例，女1例）を対象とした。年齢の中央値は65歳（56～70歳），前治療は切除手術＋術後補助化学放射線療法が4例，切除手術＋術後補助化学療法1例，切除術後再発に対する化学療法3例で，切除不能例は2例であった。治療回数は1回が8例，2回が1例，3回が1例であった。投与はTXT 60 mg/m$^2$，CDGP 80 mg/m$^2$を静脈内投与とし，3週間以上の休薬期間をおいた。効果はPR 2例，SD 6例，PD 2例でありCRはなかった。PDを除いた8例の本治療開始日からの50%無増悪生存期間は135日（88～370日）で，50%生存期間は170日（88～570日）であった。有害事象は白血球減少が主でGrade 2が1例，3が2例，4が5例であった。本併用療法はsecond-lineの化学療法として有用である。

キーワード：ドセタキセル，ネダプラチン，進行・再発食道癌，セカンドライン，
化学療法

## I．はじめに

食道扁平上皮癌に対する化学療法としては，シスプラチン（CDDP）・5FU併用療法（FP療法）がゴールドスタンダードとして広く行われており，根治または導入治療のみならず術前後の補助化学療法として広く普及している[1,2]。当科でもこの2剤，またはこれにアドリアマイシン（ADR）を加えたFAP療法を第一選択として行っている[3]。一方，これを上回る有効性を有する化学療法は開発されておらず，またこの一次治療で十分な効果が得られなかった症例に対するsecond-lineの化学療法も確立されていない。そうした中でNedaplatin（CDGP）とDocetaxel（TXT）の2剤はそれぞれ単剤での食道扁平上皮癌を対象とした第II相臨床試験で優れた奏効率を示し[4～6]，また他の薬剤で化学療法が先行した症例にも奏効することが報告され注目されている[4～6]。そこで，当科では2002年より食道原発扁平上皮癌でFP療法またはFAP療法の無効例あるいはその後の再発例に対して，second-lineの薬剤としてCDGPとTXTを併用した治療を行ってきた。今回，その有効性や有害事象につき臨床的検討を行った。

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## II．対象と方法

当科ではsecond-line化学療法の適応を食道原発の扁平上皮癌で，① FP療法またはFAP療法が無効あるいはその後再発したことが画像上確認されて

**Table 1** Characteristics of Patients

| Patient no. | Sex | Age (yr) | TNM classification | Prior operation | Prior chemotherapy regimens | Prior radiation | Target lesion (overlapped) |
|---|---|---|---|---|---|---|---|
| 1 | M | 65 | T4N2M0 | + | FAP 1 course | − | Lung mediastinal lymph node |
| 2 | M | 69 | T3N2M0 | + | FAP 1 course | − | Mediastinal lymph node |
| 3 | F | 63 | T1N1M0 | + | 5 FU・Nedaplatin | + | Lung mediastinal lymph node |
| 4 | M | 66 | T3N2M0 | + | FAP 1 course | + | Lung mediastinal lymph node |
| 5 | M | 70 | T4N2M0 | − | FAP 2 courses | − | Liver bone mediastinal lymph node |
| 6 | M | 56 | T4N2M0 | − | FAP 2 courses | + | Lung abdominal lymph node |
| 7 | M | 67 | T4N2M0 | + | FP 1 course | + | Lung |
| 8 | M | 66 | T3N1M0 | + | FP 1 course | + | Lung pleura mediastinal lymph node |
| 9 | M | 59 | T4N4M0 | + | FP 1 course | + | Mediastinal and abdominal lymph nodes |
| 10 | M | 61 | T3N2M0 | + | FP 1 course | + | Mediastinal lymph node |

いる，② 治療開始時の年齢が20歳以上70歳以下，③ 評価可能病変を有する，④ performance status（PS）が0～2である，⑤ 重篤な合併症を有しない，⑥ 本人から文書による Informed Consent が得られている，⑦ 前治療終了から1カ月以上の間隔がある，としている。また，前治療として放射線治療の有無を問わなかった。

2002年から2004年までの期間で，当科で化学療法を行った食道原発扁平上皮癌患者のうち，上記の適格基準すべてを満たしかつ TXT・CDGP 併用療法を施行しえた13例の中で，十分かつ詳細な効果判定が行えた10例について臨床的検討を行った。残りの3例は現在化学療法中で，今後評価を行う予定である。

症例10例の内訳は，男性9名，女性1名，本治療開始時の年齢は中央値65歳（56～70歳）であった。初治療前の病期は Stage II が1例，III が3例，IV a が6例であった。前治療は切除手術＋術後補助化学放射線療法4例，切除手術＋術後補助化学療法1例，切除術後再発に対する化学療法が3例に施行されていた。切除不能は2例で1例は化学放射線療法，1例に化学療法が行われていた。前化学療法の効果は PR が1名，SD が1名，8名が PD であった。また，本治療開始時の評価対象病変は肺転移5例，肝転移1例，骨転移1例，縦隔リンパ節転移8例，腹部リンパ節転移2例，胸膜転移1例であった（重複あり）（Table 1）。

用量設定に関して，1999年犬山らの多施設共同研究の進行・再発頭頸部癌に対する第II相臨床試験の結果に基づき[7]，TXT は60 mg/m$^2$とした。また，1992年田口らの CDGP 100 mg/m$^2$，4週ごと点滴静注を用いた第II相臨床試験の結果[4]と1998年室らの CDGP 90 mg/m$^2$，5 FU 800 mg/m$^2$ 5日間の併用療法，第I－II相臨床試験の用量設定，投与方法[8,9]を参考とし，2剤を併用した場合の安全性，実行可能性を試みる意味もあり CDGP は80 mg/m$^2$を使用することとした。その他に，second-line ということで患者の予備力が少ないことなども考慮した。

投与は，TXT 60 mg/m$^2$を1時間，CDGP 80 mg

**Table 2** Responses to Treatment

| Patient no. | Response | Survival time with TXT/CDGP therapy (days) | Survival time with prior chemotherapy (days) | Survival time with primary therapy (days) | Prognosis |
|---|---|---|---|---|---|
| 1 | PR | 218 | 411 | 512 | Death |
| 2 | SD | 255 | 315 | 516 | Death |
| 3 | SD | 516 | 640 | 2572 | Death |
| 4 | SD | 88 | 392 | 493 | Death |
| 5 | PD | 117 | 218 | 218 | Death |
| 6 | SD | 89 | 310 | 310 | Death |
| 7 | PD | 232 | 472 | 597 | Death |
| 8 | SD | 158 | 365 | 451 | Alive |
| 9 | PR | 170 | 380 | 459 | Death |
| 10 | SD | 111 | 463 | 511 | Death |

$/m^2$ を1時間でこの順に点滴静注した。これを1コースとし，その後3週間以上の休薬期間をおいた。原病の明らかな増悪（PD）あるいは再発または有害事象を含め全身状態の悪化がなければ可能な限り反復することとした。

効果判定は，Response Evaluation Criteria in Solid Tumors（RECIST）基準[10]に従い，10 mm以上の標的病変に対する長径和を用いて評価を行った。有害事象は米国National Cancer Institute の Common Toxicity Criteria for Adverse Events Version 2.0, Jan. 30, 1998/JCOG[11]に従い評価した。生存期間の検定にはKaplan-Meier法を用いた。また，その他の記載は食道癌取扱い規約第9版[12]に従った。

## III．結　果

### 1．抗腫瘍効果

本治療施行回数は3コースが1例，2コースが1例で，残りは1コースのみ施行しえた。化学療法が1コースのみで終了した原因は2名がPDであったため，残りの6名はGrade 3以上の骨髄抑制を認めたためで，1例（症例8）は用量を減量して2コース目を施行した。また，治療効果に関して，10例中CRとなった症例はなかった。PRは2例（20%），SDは6例（60%），PDは2例（20%）であり，奏効率は20%であった。PR 2例の奏効期間はそれぞれ135日と170日であった。PDを除いた8例の50%無増悪生存期間は135日（88～370日）であった。2004年11月31日現在9例は死亡し，うち2例は感染性の他病死であった（それぞれ，感染性心内膜炎，細菌性髄膜炎）。また，初治療日からの50%生存期間は511日（218～2573日），前化学療法開始日からの50%生存期間は392日（218～640日），TXT・CDGP開始日からの50%生存期間は170日（88～570日）であった（Table 2, Fig. 1）。

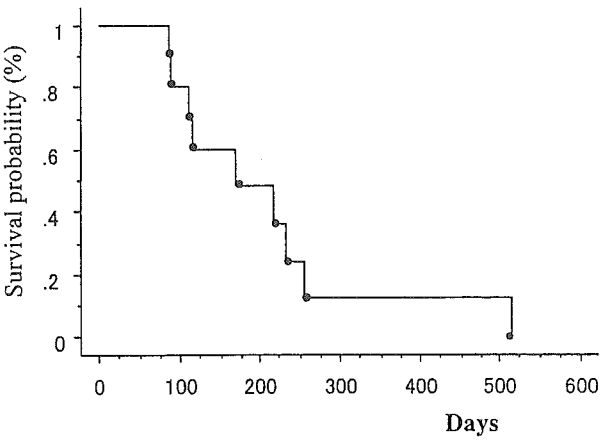


**Fig. 1** Overall survival with TXT/CDGP therapy.

### 2．有害事象

本症例による有害事象では白血球減少，好中球減少が著明に認められ，Grade 2が1例，Grade 3が2例，Grade 4が5例であった（Table 3）。Grade 3以上の症例に対しては全例GCSFを投与したが，Grade 4症例の2例が敗血症を発症した。Grade 3以上の7例の治療開始後のnadirの時期は中央値で8日目（7～10日），白血球数は1000 /μl（300～2000 /μl），白血球数2000以下の期間は中央値で3日間（1～4日間）であった。症例8では，1

**Table 3** Toxicity Associated with This Chemotherapy

| Toxicity | Grade | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| Hematological toxicity | | | | | |
| Leucopenia | 2 | 0 | 1 | 2 | 5 |
| Neutropenia | 2 | 0 | 1 | 2 | 5 |
| Anemia | 10 | 0 | 0 | 0 | 0 |
| Non-hematological toxicity | | | | | |
| Nausea/Vomitting | 7 | 1 | 1 | 1 | 0 |
| Alopecia | 6 | 1 | 3 | | |
| Stomatitis | 9 | 1 | 0 | 0 | 0 |
| Rash | 9 | 1 | 0 | 0 | 0 |

コース終了後，Grade 4の白血球減少を生じたため，doseを3割減量し，白血球数の回復を待ち，2コース目を施行した。しかし，1コース終了時にはSDであった転移巣が2コース終了後にPDとなった。その他のGrade 3以上の有害事象は悪心，嘔吐が1名のみで，Grade 2以下では，悪心，嘔吐（2例），脱毛（4例），口内炎（1例），皮疹（1例）をそれぞれ認めた。

## IV．考　察

本検討例では，全例FP療法，FAP療法あるいは5FU・CDGPといった化学療法を前治療に行った中での無効もしくは再発症例であったにもかかわらず，PR 2例，SD 6例であった。また，術後の補助化学療法も含めた前化学療法開始日からの50%生存期間は392日，TXT・CDGP開始日から170日であった。本対象例におけるsecond-line化学療法の多くは1コースのみであり，HayashiらのJCOG 9407でのFP療法における50%生存期間は201.5日[2]であり，今後TXT，CDGPの薬剤自体が生存期間に影響する可能性についても検討する必要がある。

TXTはヨーロッパいちいの針葉樹抽出物より半合成し得られた抗がん剤で，その作用機序は微小管の重合促進および脱重合抑制であり，既存の微小管に作用する抗がん剤とは異なっておりtaxane系の薬剤に分類される[13]。また，taxane系の薬剤の抗腫瘍作用発現はp 53非依存性でp 53に関連するといわれているCDDPや5FUとの交差耐性がないとの報告もある[14,15]。

本邦における報告は進行・再発頭頸部癌に対する第Ⅱ相試験が最初で，今回の用量設定の基本となったが，TXT 60 mg/m$^2$の用量で奏効率22.2%を示した[7]。食道癌に対するTXT 70 mg/m$^2$を用いた第Ⅱ相試験では，20.4%の奏効率を示し，原発巣以上にリンパ節転移，肺，肝といった臓器転移に縮小効果が得られた[5,6]。また，CDDP，CDGP，先行投与例に対しても15.8%の奏効率を示した[5,6]。併用療法では，本邦では田中らの術後＋FP療法後の再発食道癌におけるsecond-lineでの10例の検討があり[16]，5FU 500 mg/body 5日間，CDDP 10 mg/body 5日間，TXT 60 mg/m$^2$ 1日投与の3剤併用療法で，PR 4例，SD 2例，PD 4例と報告されている。

一方，CDGPは，CDDPの有効性を落とすことなく腎毒性を軽減する目的で開発された第2世代の白金化合物で，薬効はCDDPと同様であるが，血漿中でほとんどが蛋白非結合型白金化合物として存在し，DNAの複製を阻害し抗腫瘍効果を示す。本邦では田口らのCDGP 100 mg/m$^2$を単剤で用いた第Ⅱ相臨床試験で，奏効率51.7%と単剤投与としてはきわめて優れた成績がえられ[4]，CDDPによる先行治療後の無効例に対しても50%の奏効率が得られた[4]。また，併用療法では術後再発または切除不能食道癌に対する5FUとCDGPを併用した第Ⅱ相臨床試験でYoshiokaらはCDGP 80または100 mg/m$^2$ 1日投与，5FU 350または500 mg/m$^2$ 5日間投与で53.8%の奏効率を認め[17]，室らはCDGP 90 mg/m$^2$ 1日，5FU 800 mg/m$^2$ 5日間投与で39.5%の奏効率を示し[18]，FP療法より高い奏効率が得られている[2]。以上に加えて腎毒性などCDDPより軽いことも考慮するsecond-lineの薬剤として有望と考えられる。

有害事象は，TXTにおける第Ⅱ相試験で，Grade 3以上の有害事象として血液毒性が顕著で，白血球減少（73.5%），好中球減少（87.8%），貧血（12.2%），発熱性好中球減少（18.4%）と報告されている[5,6]。今回われわれはTXT 60 mg/m$^2$の用量設定で本検討を開始したが，同様に白血球減少，好中球減少がGrade 3以上で70%と高率に認められ，その中の2名に敗血症の発症をみた。いずれの症例も救命しえたが，前治療に手術，化学療法，放射線治療を行っており，患者自身の予備力が少なかったことが影響したと考えられた。また，症例8は

血液毒性により2コース目で減量がなされ，効果もSDからPDとなっており，投与量が関係した可能性がある。

欧米での第Ⅱ相試験では75～100 mg/m$^2$の用量設定で[9,19,20]，日本でも室らは70 mg/m$^2$と設定している[5,6]。second-lineの化学療法ということを考慮すると患者の予備力もなく，Grade 4の有害事象は避けたいが，最大限の効果を得ることも必須である。症例8から，現在のdose設定を検討するよりは，分割投与など，投与方法の検討をした方が良いと考えられた。しかし，TXTに関して，一括あるいは低用量分割投与の比較試験は行われておらず，今後の検討課題といえる。

本検討のように過去の報告例でもTXTの有害事象に骨髄抑制，特に白血球減少，それに伴う感染症を併発した症例は報告されている[5,6,9,19,20]。しかし，進行胃食道癌に対するSchullらの報告ではTXT 50 mg/m$^2$，CDDP 50 mg/m$^2$を1，15日分割投与で奏効率も46%を示しているにもかかわらず，Grade 3以上の白血球減少は24%と低値を示している[9]。彼らは総白血球数1000～2000 /$\mu$lにてGCSFを5 $\mu$g/kg/dayを5日間投与する方法をとっており，GCSF投与も化学療法の1つと位置付けている。人種や投与方法の違いもあり，有害事象の出現の差異に関しての比較は難しいところである。しかし，われわれの検討でもGCSFはGrade 3以上で全例使用しており，結果としてnadirは投与後8日前後で迎え，適切なGCSFの投与により3日前後で改善することなども考慮すると，本化学療法においては，血液毒性である白血球減少は不可避な有害事象のひとつと位置付け，積極的な血液検査やGCSFの投与，予防的抗生剤の投与など，管理上での改善を検討する必要があると思われる。

## V．まとめ

進行・再発食道癌のsecond-lineの化学療法としてTXT・CDGP併用療法を行い，PR 2例，SD 6例，PD 2例と比較的良好な成績を示した。しかし，いくつかの問題点も提起された。特に，有害事象において，血液毒性である白血球減少を起こす頻度が高率であり，抗生剤の予防投与など検討が必要であることが示唆された。

## 文　献


1）Iizuka, T., Kakegawa, T., Ide, H., et al.: Phase II evaluation of cisplatin and 5-fluorouracil in advanced squamous cell carcinoma of the esophagus: a Japanese Esophageal Oncology Group Trial. Jpn. J. Clin. Oncol., 22: 172–176, 1992.

2）Hayashi, K., Ando, N., Watanabe, H., et al.: Phase II evaluation of protracted infusion of cisplatin and 5-fluorouracil in advanced squamous cell carcinoma of the esophagus: a Japanese Esophageal Oncology Group（JEOG）Trial (JCOG 9407). Jpn. J. Clin. Oncol., 31: 419–423, 2001.

3）安田卓司，甲　利幸，石黒信吾・他：局所進行食道癌に対するFAP療法の試み．癌と化療，22：1538–1541，1995.

4）田口鐵男，涌井　昭，鍋谷欣市・他：254-S（Cis-diammine glycolato platinum）の消化器癌に対する第Ⅱ相臨床試験．癌と化療，19：483–488，1992.

5）陳　勁松，室　　圭，兵頭一之介・他：進行・再発食道癌に対するRP 56976（Taxotere）の臨床第Ⅱ相試験．日癌治，37：300，2002.

6）Muro, K., Hamaguchi, T., Ohtsu, N., et al.: A phase II study of single-agent docetaxel in patients with metastatic esophageal cancer. Ann. Oncol., 15: 955–959, 2004.

7）犬山征夫，形浦昭克，戸川　清・他：進行・再発頭頸部癌に対するRP 56976（Docetaxel）の後期第Ⅱ相臨床試験　多施設共同研究．癌と化療，26：107–116，1999.

8）室　　圭，白尾國昭，島田安博・他：進行または切除後再発食道癌に対するネダプラチン（254-S）＋5FU併用療法の第Ⅰ–Ⅱ相臨床試験．日癌治，33：213，1998.

9）Schull, B., Kornek, G.V., Schmid, K., et al.: Effective combination chemotherapy with bimonthly docetaxel and cisplatin with or without hematopoietic growth factor support in patients with advanced gastroesophageal cancer. Oncology, 65: 211–217, 2003.

10）Therasse, P., Arbuck, S.G., Eisenhauer, E.A., et al.: New guidelines to evaluate the response to treatment in solid tumors. J. Natl. Cancer Inst., 92: 205–216, 2000.

11）JCOG運営委員会：National Cancer Institute-Common Toxicity Criteria（NCI-CTC Version 2.0, Jan. 30, 1998）～日本語訳JCOG版～．癌と

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# 進行食道癌治療への挑戦

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日常診療の指針

# 進行食道癌治療への挑戦

*Tactics for advanced esophageal cancer*

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## はじめに

食道癌は予後不良の癌の1つであったが，頸部リンパ節郭清を含めた3領域リンパ節郭清を伴う胸部食道切除術が安全に行われるようになり，予後の向上を認めた．手術施行例における詳細なリンパ節転移の検討から頸部リンパ節転移の頻度は少なくなく，主占居部位がLtで深達度sm症例を除き，頸部リンパ節郭清がR2手術をさけるためには必要であることが判明してきた．一方，内視鏡的粘膜切除術(EMR)により早期食道癌の治療は侵襲も少なく，予後も良好であり満足すべき治療結果が得られている．このため術前診断はきわめて重要となり，壁深達度LPMまではEMRの絶対適応としてコンセンサスが得られている．

近年，切除範囲が広くても一括切除可能なESDも行われるようになってきており，分割切除のEMRに比し局所再発が減少するとの報告が多い．壁深達度に関してはさらに，MM-SM1まで適応拡大している施設も増加してきている．SM2以深の癌は前述のLt，SMを除き3領域リンパ節郭清の対象となる．MM-SM1については今後の解析を待つ必要があるが，少なくともSM2以深の癌は治療法がきわめて異なり，進行食道癌の範疇に含まれると判断する．取扱い規約(第9版)による早期食道癌の定義は原発巣が粘膜層にとどまりリンパ節転移を認めない症例と規定されている．

## 1．進行食道癌の治療

UICCのTNM分類に従うと，頸部リンパ節転移はすべてM1(LYM)となる．当科の解析では主占居部位がUtで47.1%，Mt 31.1%，Lt 19.8%とそれぞれの縦隔内リンパ節転移が43.5，50.6，41.3%であることを考慮すると，頸部リンパ節転移をすべてM1と規定するにはその転移頻度があまりにも高率である．さらに頸部リンパ節郭清を行うことにより，R2の手術とならず良好な予後が期待できる．今後，術前診断の進歩により頸部郭清を省略できる症例は期待できるが，現状ではリンパ節転移診断には限界があり，積極的に行うべきだと考えている．当科の検討や，梶山らの検討においても多くのリンパ節転移は1 cm以下のリンパ節に認められ，その診断は今後の重要な検討課題である．

化学放射線治療はその進歩により，多くの症例に行われるようになった．治療が長期間となり，有害事象も少なくなく，放射線肺臓炎，胸水，心嚢炎など晩期合併症を考慮する必要もあり，侵襲については決して少なくないが，臓器温存の面から治療後のQOLに関しては有利な治療法である．SM癌に対して多施設による手術との第III相臨床試験が計画されているが，あまりにもmodalityが違う治療のため参加する被験者が少ないとの予測が懸念されている．

98年から当科では統合化した低侵襲化手術を行っている．皮膚を縦切し広背筋温存開胸，後縦隔経路再建，メチルプレドニゾロンの使用(Day 0：250 mg，Day 1，2：125 mg i.v.)により手術死亡は認めておらず，9割以上の症例で当日抜管，術後在院日数は中央値18日である．後述の高度進行食道

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***Key words*** : 進行食道癌／集学的治療／術前化学放射線治療／遺伝子治療／重粒子線治療

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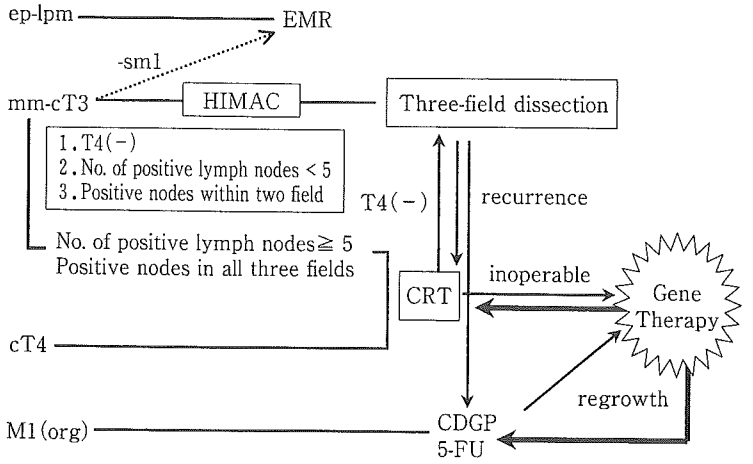


**図1 Strategy for Esophageal Cancer**

癌を除いた術前未治療症例の5年生存率は68.1%であり，組織学的進行度ではStage II以上が68.8%を占めていることを考慮すると良好な成績である．放射線化学療法が6週間要することと比較しても，術前未治療手術治療は治療を受ける側にとっても有用な治療法であると考えられる．

短期死亡症例の解析ではR2の1例を除きリンパ節転移が5個以上の症例であり，そのほとんどは腹部リンパ節転移の診断が不十分な症例であった．術前診断の進歩が今後さらなる予後の改善に必要である．

### 2．高度進行食道癌の治療

当科では1983年から3領域郭清を施行してきたが，術式は安定し安全に手術可能となった．予後不良症例について解析したところ，頸部・胸部・腹部の3領域すべてにリンパ節転移を認めた症例，リンパ節転移を5個以上認めた症例，T4症例の3群は3領域リンパ節郭清を施行しても有意に予後不良であった．当科では98年からこれらの3群を高度進行食道癌として術前化学放射線治療を施行した後，3領域リンパ節郭清を伴う食道切除を行っている．術前治療の侵襲を考慮し，手術は放射線照射終了後3週間の間隔をあけている．手術死亡は認めておらず，術後合併症については呼吸器合併症がやや多かったが有意差を認めず，安全に施行可能であった．

従来15%未満であった予後不良群の5生率が42.5%まで改善された．T4症例についてはR2手術の予後が非切除症例の予後と差がないことから実際の臨床上他臓器浸潤を否定できない症例に対しては積極的に化学放射線治療を施行し，T4が解除された症例については積極的に切除を行うことが望ましいと考えている．原発巣のみならずとくにリンパ節転移の残存の有無に関する術前診断は困難であり，癌が残存していないことの証明がきわめて難しい現時点の医療レベルでは，残存した癌に対する保存的な治療でのコントロールは難しいことを考慮し，根治照射後のサルベージ手術ではなく術前化学放射線治療後予定手術として積極的に手術をすることが望ましいと考えている．

再発症例に対し，切除，抗癌剤，放射線と施行可能な治療法をすべて考慮し，再発巣に応じ適切に選択して治療を積極的に行っている．また，当科では切除不能，他治療抵抗性進行食道癌に対する癌抑制遺伝子p53をアデノウイルスベクターを用いて局注する臨床試験を実施した．局所効果は著効例を認めなかったが多くの症例がSDであり，安全に施行可能であった．現在，最長3年5ヵ月生存中の症例も認めている．手技はきわめて容易であり，有害事象は従来の化学療法より少ないので，今後の発展が期待される結果であった．

また，放射線医学総合研究所の協力を得て，術前短期(2週間)重粒子線照射の第I/II相臨床試験を計画し，現在実施中である．今後の解析によりその有用性を検討したい．

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# Cisplatin-dependent upregulation of death receptors 4 and 5 augments induction of apoptosis by TNF-related apoptosis-inducing ligand against esophageal squamous cell carcinoma

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**TNF-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily known to induce apoptosis in a variety of cancers. The purpose of our study was to examine the effects of TRAIL in combination with cisplatin against esophageal squamous cell carcinoma (ESCC) cell lines *in vitro* and *in vivo*, and to elucidate underlying molecular mechanisms. Expression profiles of TRAIL receptors were investigated in 19 ESCC (KYSE) cell lines using RT-PCR. Crystal violet staining assays were performed to reveal the sensitivity against TRAIL. Flow cytometric analyses of apoptosis induction and TRAIL receptor expression were performed. Furthermore, Western blot was used to clarify the apoptosis pathway involved, and a nude-mouse xenograft model was used to show effects *in vivo*. Results show that death receptors (DR) 4 and 5 were expressed in 100% of the cell lines, and 79% (15/19) expressed 4 TRAIL receptors. There was only 1 cell line without decoy receptor expression. Eighteen cell lines were resistant to TRAIL, but in some, the combination treatment with cisplatin could overcome this resistance. They underwent apoptosis *via* activation of caspase-8 and -3, and cisplatin-dependent upregulation of DR4 and 5 was detected. Furthermore, pretreatment with cisplatin followed by TRAIL resulted in significant tumoricidal effects. Finally, systemic administration of TRAIL with cisplatin synergistically suppressed tumor growth of ESCC xenografts in nude mice. These results provide a significance of cisplatin-induced upregulation of death receptors as apoptosis-inducing machinery, and it was suggested that sequential administration of cisplatin and TRAIL might be a feasible chemotherapeutic regimen against ESCC.**


**Key words:** TNF-related apoptosis-inducing ligand (TRAIL); apoptosis; cisplatin; esophageal squamous cell carcinoma; chemotherapy

Esophageal SCC has extremely poor prognosis among gastrointestinal cancers because the diagnosis is not made until at an advanced stage and there are few effective therapies. Esophagectomy with extensive lymphadenectomy is far from being satisfactory with a dismal 5-year survival rate, and available chemotherapeutic regimens are yet to show promising outcomes. Thus, we are still in the midst of searching for a more effective modality to conquer this highly mortal malignancy.

TRAIL (also known as Apo2L) is a relatively new member of the TNF family that has been discovered from the expression sequence tag.[1,2] TRAIL is highly homologous to FasL and other members of the TNF ligand family, and its constitutive expression has been detected in a variety of human tissues including spleen, thymus, prostate, peripheral blood lymphocytes, ovary, small intestine, colon, placenta and lung, but not in the brain, liver or testis.[1] Four cognate receptors have been identified: death receptor (DR) 4 (TRAIL receptor (TR) 1)[3] and DR5 (KILLER/TR2[4–8] are receptors with fully functional cytoplasmic death domains. On the other hand, decoy receptor (DcR) 1 (TRID / TR 3)[4–6] and DcR 2 (TRUNDD / TR 4)[9–11] have either an obliterated (DcR1) or a truncated (DcR2) death domain. These decoy receptors have been proposed to competitively inhibit TRAIL-induced apoptosis by acting as nonfunctional receptors. All 4 TRAIL receptors are highly expressed in a wide variety of normal cells and the expression of decoy receptors is substantially limited in tumor cells.[3,5,10] These findings in addition to normal cells' resistance to TRAIL have lead to the presumptive belief that normal cells are protected from TRAIL-mediated apoptosis by decoy receptors[1,2,4,6,9,10] However, later studies revealed that DcR1 and DcR2 are also expressed in some cancers sensitive to TRAIL, thereby defying their putative function[12–15] Currently, exact functions of decoy receptors remain unknown and the mechanism for normal cells' resistance to TRAIL is still obscure. In the meantime, some studies raised questions regarding toxicity of TRAIL against normal tissues,[12,16] but this controversy seemed to be resolved by further findings that clinical-grade recombinant human TRAIL has minimal toxicity against normal human cells.[17,18]

Although TRAIL has been shown to be capable of inducing apoptosis in tumor cells of diverse origin,[19–23] studies on esophageal cancer have been very rare. In our study, ESCC cell lines (KYSE series) that have been established at our institution[24,25] are subjected to a series of experiments to demonstrate the potential usefulness of TRAIL against ESCC and underlying mechanisms behind this therapeutic model is discussed.

## Material and methods

### *Cell lines*

Nineteen genetically distinct KYSE cell lines were used. HeLa cells were used as positive controls for surface expression of DR4 and DR5 in flow cytometry. Peripheral blood mononuclear cells (PBMCs) isolated from whole blood of 2 of the authors (KK and NT) using Ficoll-Paque solution (Amersham Biosciences, London, UK) served as normal controls. All cells were cultured and maintained in RPMI 1640 (Nikken Biomedical Laboratory, Kyoto, Japan) plus 2% Fetal Bovine Serum (Biofluids, MD) at 37°C, in humidified 5% $CO_2$ atmosphere.

### *Patients*

Original ESCC from which 19 KYSE series were established had been obtained during esophagectomy performed at the Department of Surgery and Surgical Basic Science of Kyoto University. All patients had a pathological diagnosis of squamous cell carcinoma, and their histological grading and staging were classified according to the pathological tumor/node/metastasis (pTNM) system (5th edition). The subjects consisted of 16 men and 3 women of age, 39–79 years (average age of 57.7 ± 9.8 years). There were no patients with distant organ metastasis, so all of the stage IV patients had distant lymph node metastasis. Details of patient characteristics are displayed in Table I. Written consent was obtained from the patients for the performance of surgery and for the use of

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TABLE I – CLINICOPATHOLOGICAL CHARACTERISTICS OF 19 ORIGINAL TUMORS[1]

| Parameter | n | Sensitivity to combination Tx | | p Value |
|---|---|---|---|---|
| | | + | – | |
| Age (years) | | | | |
| <50 | 3 | 1 | 2 | >0.999 |
| >50 | 16 | 6 | 10 | |
| Sex | | | | |
| Female | 4 | 2 | 2 | = 0.603 |
| Male | 15 | 5 | 10 | |
| N status | | | | |
| N(+) | 16 | 5 | 11 | = 0.523 |
| N(–) | 3 | 2 | 1 | |
| M status | | | | |
| M(+) | 5 | 2 | 3 | >0.999 |
| M(–) | 14 | 5 | 9 | |
| Tumor location | | | | |
| Ce | 4 | 1 | 3 | = 0.930 |
| Ut | 3 | 1 | 2 | |
| Mt | 10 | 4 | 6 | |
| Lt | 2 | 1 | 1 | |
| TNM stage | | | | |
| 2a | 3 | 2 | 1 | = 0.640 |
| 2b | 2 | 0 | 2 | |
| 3 | 9 | 3 | 6 | |
| 4a | 2 | 1 | 1 | |
| 4b | 3 | 1 | 2 | |
| Histological differentiation | | | | |
| Well | 6 | 1 | 5 | = 0.365 |
| Moderate | 8 | 4 | 4 | |
| Poor | 4 | 2 | 2 | |

[1]Values represent numbers of patients except otherwise indicated.

resected samples for research in accordance with the Kyoto University Institutional Review Board.

*Cytokines and antibodies*

Soluble recombinant human TRAIL was purchased from DAKO (Denmark); cisplatin was obtained from Nippon Kayaku Co. Ltd. (Tokyo, Japan). Monoclonal mouse anti-caspase-8, anti-human BAX, anti-FADD and anti-XIAP antibodies were purchased from Medical & Biological Laboratories Co., Ltd (Nagoya, Japan). A monoclonal mouse anti-Bcl-X antibody, and polyclonal rabbit anti-caspase-8 and anti-caspase-3 antibodies were purchased from BD Pharmingen (San Diego, CA). A polyclonal rabbit anti-cFLIP antibody, a monoclonal mouse anti-β-actin antibody, and a mouse IgG1 isotype control were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). A monoclonal mouse anti-FLIP antibody was purchased from Apotec (Geneva, Switzerland). Rabbit polyclonal anti-NFκB antibodies (anti-p50 and anti-p65) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Peroxidase-conjugated sheep anti-mouse IgG and donkey anti-rabbit IgG were purchased from Amersham Pharmacia Biotech (London, UK). Monoclonal mouse antibodies to TRAIL-R1, -R2, -R3, and -R4, and a biotinylated polyclonal antibody to mouse IgG1, were purchased from Alexis Biochemicals (UK). Streptavidin/FITC was purchased from Ancell Corporation (Bayport, MN).

*Total RNA extraction and RT-PCR*

Total RNA from KYSE cell lines was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's recommendations. PBMCs were incubated overnight in a 6-well tissue culture plate (Iwaki glass, Tokyo, Japan) before total RNA extraction. RNA quantification was performed spectrophotometrically. First strand cDNA was synthesized using 1 μg of total RNA, 1 μl of oligo $dT_{(12-18)}$ (Invitrogen), 4 μl of 5× first strand buffer (Invitrogen), 4 μl of dNTP mix (2.5 mM each) (Invitrogen) and 2 μl of 0.1 M DTT (Invitrogen), in a total volume of 20 μl. Previously published sequences of PCR primers were adopted: DR4 sense, 5′-CTG-AGC-AAC-GCA-GAC-TCG-CTG-TCC-AC-3′; DR4 antisense, 5′-AAG-GAC-ACG-GCA-GAG-CCT-GTG-CCA-T-3′;[26] DR5 sense, 5′-CTG-AAA-GGC-ATC-TGC-TCA-GGT-G-3′; DR5 antisense, 5′-CAG-AGT-CTG-CAT-TAC-CTT-CTA-G-3′[19] DcR1 sense, 5′-GTT-TGT-TTG-AAA-GAC-TTC-ACT-GTG-3′; DcR1 antisense, 5′-GCA-GGC-GTT-TCT-GTC-TGT-GGG-AAC-3′;[13] DcR2 sense, 5′-CTT-TTC-CGG-CGG-CGT-TCA-TGT-CCT-TC-3′; DcR2 antisense, 5′-GTT-TCT-TCC-AGG-CTG-CTT-CCC-TTT-GTA-G-3′;[26] glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5′-TGG-TAT-CGT-GGA-AGG-ACT-CAT-GAC-3′ and GAPDH antisense, 5′-ATG-CCA-GTG-AGC-TTC-CCG-TTC-AGC-3′. PCR was performed using 5 μl of 10× PCR buffer (Sawady Technology, Tokyo, Japan), all 4 dNTPs at 2.5 mM, 2.5 U Taq DNA polymerase (Sawady Technology), 1/20 of cDNA and each primer at 0.5 μM, in a total volume of 50 mgr:l using PTC-200 Peltier thermal cycler (MJ Research, Inc., Watertown, MA). Thirty-five cycles of amplification were performed (melting step at 94°C for 1 min (DR4), 45 sec (DR5), 30s (DcR1), and 45 sec (DcR2); annealing step at 62°C for 1 min (DR4), 57°C for 45 sec (DR5), 56°C for 30 sec (DcR1), and 63°C for 45 sec (DcR2); elongation step at 72°C for 1 min (DR4), 45 sec (DR5), 30 sec (DcR1) and 45 sec (DcR2); followed by a final step at 72°C for 10 min). PCR product lengths were 505 bp (DR4), 583 bp (DR5), 140 bp (DcR1), 464 bp (DcR2) and 189 bp (GAPDH). Samples without reverse transcriptase served as negative controls to confirm that there was no genomic DNA contamination.

*Crystal violet staining assay*

Each cell line was cultured until subconfluent in 12-well tissue culture plates (Iwaki glass), and TRAIL (0 ng/ml, 50 ng/ml or 100 ng/ml) and cisplatin (0 μg/ml, 2.5 μg/ml or 5 μg/ml) were added either alone or in combinations for 24 hr. Then medium was discarded and adherent cells were washed with PBS, stained and fixed with 0.2% crystal violet in 10% phosphate-buffered formaldehyde (Wako Pure Chemical, Japan) for 30 sec. Excess crystal violet solution was discarded, and adherent cells, considered viable, were observed. After completely air-dried, stained cells were lysed with 2% SDS solution by shaking plates in room temperature for 1 hr. OD absorbance was measured at 560 nm using SJeia II microplate reader (Sanko Jun-yaku Co., Ltd., Tokyo, Japan), and the percent absorbance of every well was determined.

*Flow cytometry for detection of apoptotic cells and surface TRAIL receptor expression*

KYSE cell lines, HeLa cell line ($5 \times 10^5$ cells) and PBMCs ($3 \times 10^6$ cells) were seeded in 24-well tissue culture plates (Iwaki glass). For detection of apoptosis, TRAIL (50 ng/ml) and cisplatin (5 μg/ml) were added either alone or in combination for 24 hr. Subsequently, floating cells in the medium and adherent cells were collected. Using Annexin V-FITC Apoptosis Detection Kit (Medical & Biological Laboratories Co., Ltd.), cells were stained with Annexin-V FITC and propidium iodide (PI) according to manufacturer's instructions. Untreated cells and cells treated with 3% formaldehyde for 30 min served, respectively, as negative and positive controls for double staining. For detection of surface TRAIL receptor expression, cells were either untreated (control) or treated with 5 μg/ml cisplatin for 6 hr, harvested, and stained with monoclonal antibodies against TR1–TR4, followed sequentially by incubation with biotinylated goat anti-mouse IgG, and then with streptavidin-FITC. HeLa cells served as positive controls for DR4/5 surface expression, and PBMCs served as positive controls for DcR1/2 surface expression. Cells were analyzed immediately after staining using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and the Cell Quest software (Becton Dickinson). For each measurement, >10,000 cells were counted.

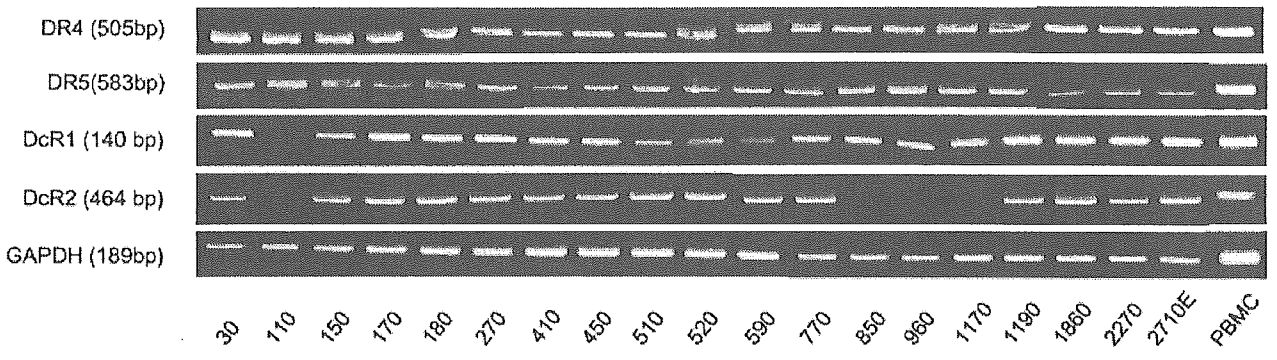


FIGURE 1 – RT-PCR detection of TRAIL receptors in 19 KYSE cell lines and peripheral blood mononuclear cells (PBMCs): RT-PCR was run for 35 cycles. DR4 and DR 5 were expressed in all cell lines. Four cell lines lacked DcR2 expression, and KYSE 110 was the only decoy receptor (−) cell line. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression is shown as a loading control. Experiments were repeated at least 3 times and the most representative results are shown.

*Morphological analysis using Hoechst33342 and propidium iodide staining*

In some cell lines, nuclear morphology was assessed using Hoechst 33342 and PI staining. After 24 hr treatment with TRAIL and cisplatin, cells were harvested, labeled with Hoechst 33342 (5 μg/ml) and PI (1 μg/ml) at 37°C for 10 min and examined under fluorescent microscopy according to the method reported previously.[27] Intact blue nuclei, condensed/fragmented blue nuclei, condensed/fragmented pink nuclei and intact pink nuclei were considered viable, early apoptotic, late apoptotic and necrotic cells, respectively.

*Immunoblotting analyses*

Lysis buffer containing 2% SDS solution supplemented with Tris-HCl, pH 6.5 and 25% glycerol, and a mixture of protease inhibitors in DMSO solution (Wako Pure Chemicals) was used to destruct cellular structures. Lysates were then sonicated on ice, centrifuged and protein concentration determined using BCA protein assay kit (Pierce, Rockford, IL). Next, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12.5% (w/v) gel, followed by transblotting to Immobilon-P transfer membranes (Millipore, Bedford, MA). Nonspecific bindings were blocked using Blockace solution (Dainippon Pharmaceutical Co., Osaka, Japan), and incubation with primary antibodies was carried out. After washing 3 times with tris-buffered saline with Tween 20, pH 8.0 (0.05 M Tris, 0.138 M NaCl, 0.0027 M KCl and 0.05% Tween 20; Sigma-Aldrich), the membrane was successively incubated with secondary antibodies and peroxidase activity was revealed using an ECL plus chemiluminescence kit (Amersham Pharmacia Biotech).

*Combination treatment with sequential addition of TRAIL and cisplatin*

KYSE cell lines sensitive to the combination treatments were cultured to subconfluence in 12-well tissue culture plates and 50 ng/ml TRAIL and 5 μg/ml cisplatin were added sequentially either TRAIL first, washed 3 times with PBS and then cisplatin, or *vice versa*, for 12 hr each. Crystal violet staining assays were then performed.

In vivo *treatments*

KYSE 170 cells ($5 \times 10^6$) were suspended in 100 μl PBS and inoculated subcutaneously into the right flank of the female nude mice, 5–6 weeks of age, of BALB/c background (Charles River Japan, Inc., Kanagawa, Japan). For the treatment of the established xenografts, the tumors were permitted to establish to the diameter of 6–7 mm for 10–14 days. Either cisplatin (1 mg/kg, 2 mg/kg, or 3 mg/kg), sterile normal saline (150 μl/body), or TRAIL (1 μg/body) was administered intraperitoneally (i.p.) daily for 4 consecutive days followed by 3 off-days per 1 course of a treatment. For the combination treatment, 2 mg/kg cisplatin and 1 μg/body-TRAIL were administered i.p. in the same dose schedule. Mice in all groups were treated for 2 consecutive weeks and then observed. Tumor growth was followed by measurements of tumor diameters with a sliding caliper 3 times a week, and mice were monitored daily. The tumor volume was calculated according to the following formula: $TV = L \times W^2/2$, where L and W are the major and minor dimensions, respectively. Systemic toxicity of the treatments was assessed by change in body weights. All treatment protocols were approved by the animal care and use committee of Kyoto University.

*Statistical analyses*

Commercially available software, Stat View version 4.5 (Berkeley, CA), was utilized for all statistical analyses. The correlations between various clinicopathological factors and sensitivity to the combination therapy were evaluated using Fisher's exact test and Kruskal-Wallis test. ANOVA Bonferroni test was used to evaluate the significance of differences in rates of apoptosis induction between combination therapies and single drug therapies. Mann-Whitney U-test was used to analyze the significance of difference of tumoricidal effects of the sequential treatment. In all tests, $p$ values less than 0.05 were considered significant.

## Results

*TRAIL receptor expression profiling in KYSE cell lines and PBMCs*

A summary of RT-PCR results on 19 KYSE cell lines and PBMCs is shown in Figure 1. DR4 and DR5 expression was detected in all of the cell lines; thus, they were ubiquitously expressed among KYSE cell lines. DcR1 was expressed in 18 of 19 cell lines (95%) and DcR2, in 15 of 19 cell lines (79%). There was only 1 cell line (KYSE 110) that was completely devoid of the decoy receptor transcripts. All 4 TRAIL receptor transcripts were detected in PBMCs.

*Tumoricidal effects of TRAIL and cisplatin against KYSE cell lines*

KYSE 110, 170 and 520 were subjected to the preliminary crystal violet staining assays. They had been selected because KYSE 110 is our only decoy-receptor negative cell line, and KYSE 170 and 520, which express all 4 TRAIL receptors, are 2 of the most frequently studied cell lines in our laboratory.[25,28] KYSE 110 and 170 were sensitive to the combination treatments, while KYSE 520 appeared to be resistant (Fig. 2*a*). Reduction of viable KYSE 110 and 170 cells by the treatment using 50 ng/ml TRAIL and 5 μg/ml cisplatin was statistically significant compared to either

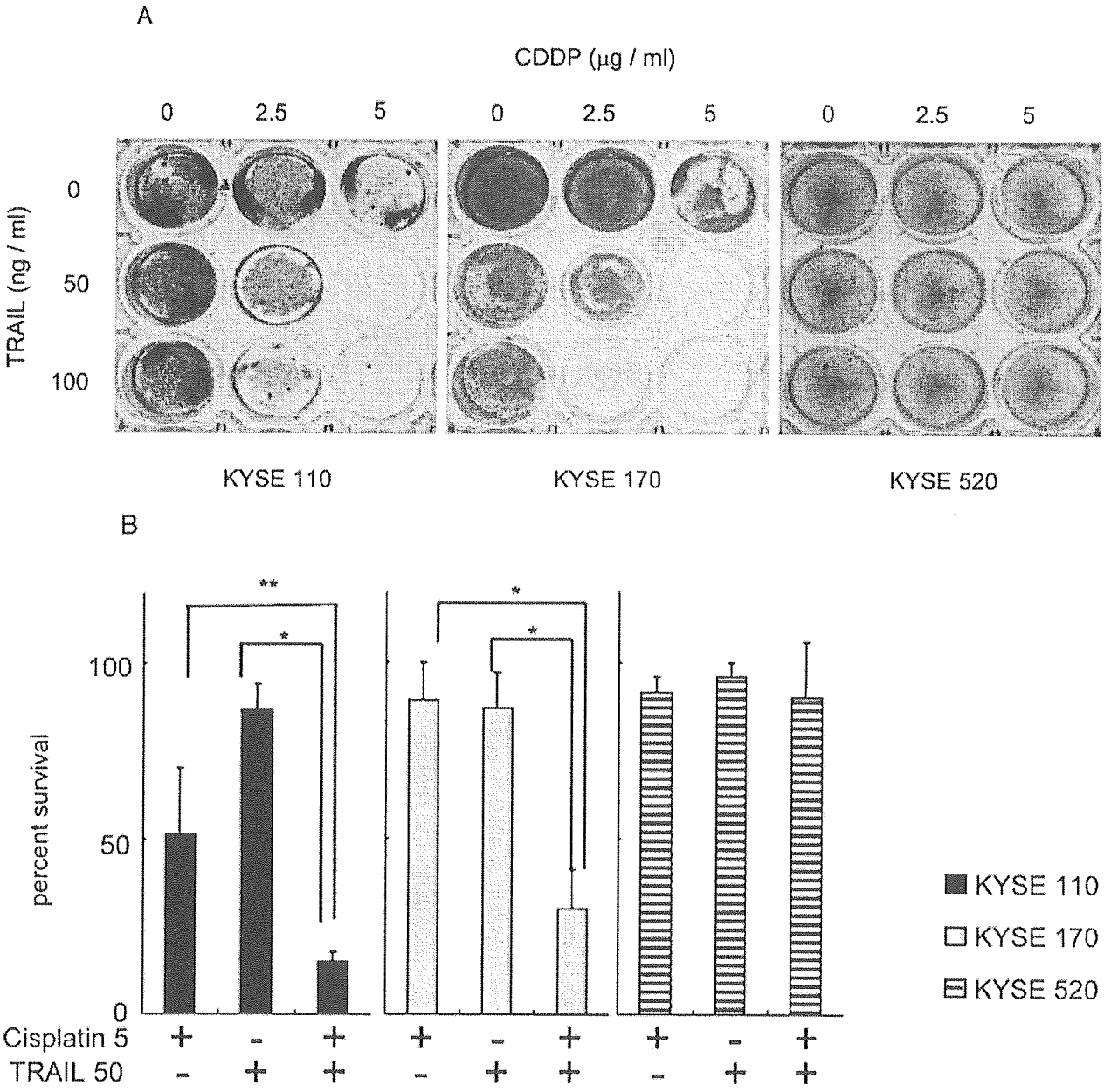


FIGURE 2 – (*a*) Crystal violet staining results after treatments with various concentrations of TRAIL and cisplatin for 24 hr are shown. KYSE 110 and KYSE 170 were sensitive to combination treatments, whereas KYSE 520 was resistant. Experiments were repeated 6 times per cell line, and the most representative results are shown. (*b*) Crystal violet staining results were quantified with OD absorbance. For each cell line, OD absorbance value for untreated control was arbitrarily set for 100%. All data are the mean of 4 independent experiments; bars represent SE. Cytotoxic effects in both KYSE 110 and KYSE 170 by the combination treatment was statistically significant compared to either single agent alone ($*p < 0.001$, $**p < 0.05$).

single agent alone, displaying synergistic effects (Fig. 2*b*). Hence, 50 ng/ml TRAIL and 5 μg/ml cisplatin were adapted to pursue further studies. Among 19 KYSE cell lines, KYSE 110, 170, 510, 850, 1190, 1860 and 2270 showed variable levels of sensitivity against the combination treatment and KYSE 2270 was the only cell line sensitive to TRAIL alone (Fig. 3). In the subsequent studies, KYSE 110 170, 1860 and 2270 had been selected because in these cell lines, viable cells were reduced significantly by the combination treatment, compared to either single agent alone. KYSE 960 and 1170 were used as resistant cell lines that lack DcR2 expression.

*Apoptosis induced by the combination treatment in KYSE cell lines*

To assess the type of cell death induced by TRAIL and cisplatin, flow cytometry was performed: Annexin-V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained) from early apoptotic cells (stained only with annexin-V) and late apoptotic or necrotic cells (stained with both annexin-V and PI). Cisplatin alone could induce a notable level of apoptosis only in KYSE 1860 (4.7% control *vs.* 30.8%) (Fig. 4*a*).

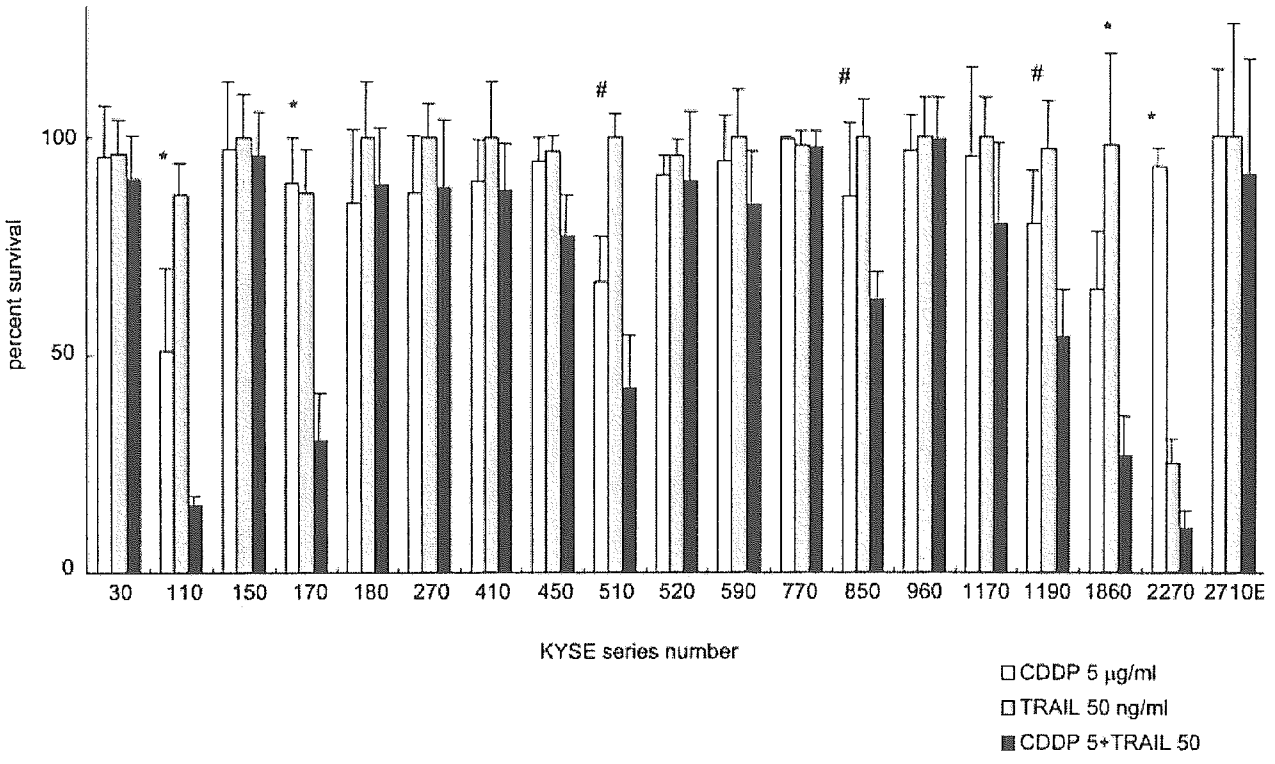


FIGURE 3 — After in contact with given doses of TRAIL and cisplatin for 24 hr, sensitivities of 19 KYSE cell lines to these agents either alone or in combination were evaluated with the crystal violet staining assays. Three cell lines (KYSE 110, 510 and 1860) and 1 cell line (KYSE 2270) showed some sensitivities to cisplatin and TRAIL, respectively. With the combination treatment, variable levels of cytotoxic effects were seen in 7 cell lines (KYSE 110, 170, 510, 850, 1190, 1860 and 2270). Asterisk indicates the cell lines in which cytotoxicity by the combination treatment was statistically significant ($p < 0.05$) compared to either single agent alone. Number sign denotes the cell lines in which cytotoxicity induced by the combination treatment was statistically significant compared to 1 of the 2 agents. Results indicate the mean of 4 independent experiments for each cell line.

TRAIL alone induced extensive apoptosis in KYSE 2270 (7.7% control *vs.* 88.5%) and some apoptosis in KYSE 170 (14.6% control *vs.* 35.3%) and 1860 (4.7% control vs. 21.9%). KYSE 110, despite absence of decoy receptors, did not undergo significant apoptosis when treated with TRAIL alone. However, the combination treatment resulted in synergistic cytotoxic effects. A majority of apoptotic cells in KYSE 110 was in early apoptosis while those in KYSE 170, 1860 and 2270 were in late apoptosis. By extending treatment periods to 48 hr, however, most of the apoptotic cells in KYSE 110 proceeded to a late apoptotic stage, revealing relatively slow progression of apoptotic process in KYSE 110 (data not shown). On the contrary, there was hardly any increase of apoptotic cells in KYSE 410, 520, 960 and 1170 (Fig. 4*b*), and these results were comparable with those in crystal violet assays. To further confirm that KYSE 170, 1860 and 2270 underwent apoptosis by the combination treatment, nuclear morphology was observed under fluorescent microscope, which revealed cells either in early or late apoptosis, but not in necrosis (Fig. 5). Meanwhile, PBMCs were resistant to both TRAIL and cisplatin, and the combination treatment could not induce apoptosis either (Fig. 4*b*).

*Demographic data analyses*

Clinicopathological data of 19 original ESCC are shown in Table I. Parameters compared are age and gender of the patients, TNM status, location of the primary tumor, TNM stage and histological grading of the resected tumor, against sensitivity to the combination treatment of TRAIL and cisplatin. In 1 of the patients, histological grading was not available. Although significant statistical differences could not be reached between cell lines sensitive to, and resistant to, the combination treatment in any of the criteria examined, well-differentiated SCC tended to be more resistant to the combination treatment, as 5 of 6 (83.3%) well-differentiated tumors were resistant, as opposed to 6 of 12 (50%) moderately and poorly differentiated tumors ($p = 0.316$).

*Activation of caspase cascade by the combination treatment*

TRAIL is known to induce apoptosis in target cells *via* activation of the caspase cascade (extrinsic pathway).[15,29] It has also been disclosed that caspase-8 can activate Bid of the Bcl-2 family to initiate the mitochondrial (intrinsic) pathway of apoptosis.[20,30,31] To unveil the TRAIL-mediated apoptosis pathway in KYSE cell lines, the expression of pro-apoptotic proteins caspase-8, -3, Bax, FADD and anti-apoptotic proteins Bcl-XL, FLIPL and XIAP has been investigated. First, in order to determine the optimal timing of protein extraction, KYSE 170 was treated with TRAIL and cisplatin, proteins extracted every 2 hr and activation of caspase-8 examined. An active form of caspase-8 was detectable within 4 hr and appeared to peak at 8 hr; hence, 8 hr contact time was deemed sufficient for protein extraction (Fig. 6). The baseline expression levels of these proteins in untreated controls are shown in Figure 7*a*. The expression levels of FADD, FLIPL and XIAP were variable among cell lines but there was no correlation between their expression levels and cell lines' sensitivity to the combination treatment. After the combination treatment, activation of caspase-8 and -3 was observed in KYSE 110, 170, 1860 and 2270 but not in KYSE 410, 520, 960 and 1170 (Fig. 7*b*). In order to elucidate intracellular changes involved in cisplatin-induced sensitization of esophageal SCC, Western blotting detection of FLIPS, FLIPL, XIAP and NFκB was further performed on both untreated cells and cells treated with 5 μg/ml cisplatin for

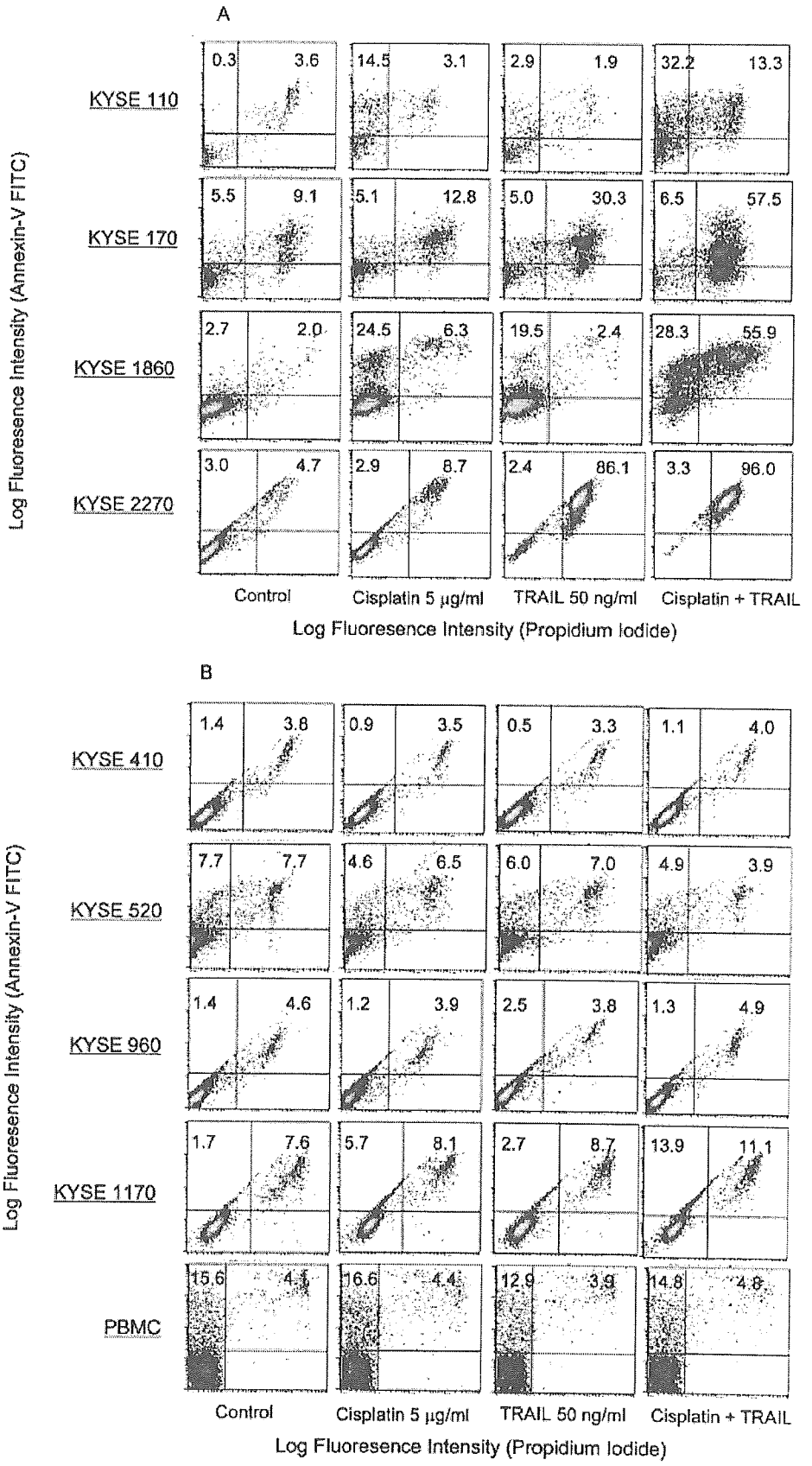


FIGURE 4 – Flow cytometric detection of apoptotic cells: Concentration of each agent used for "cisplatin + TRAIL" is 5 µg/ml and 50 ng/ml, respectively. Y-axis: Annexin-V conjugate. X-axis: Propidium Iodide uptake. Lower left quadrant, upper left quadrant, and upper right quadrant represent viable cells, cells in early apoptosis, and cells in late apoptosis respectively. Numbers in the diagrams show the percentage of cells represented in respective quadrants. (*a*) Cell lines sensitive to the combination treatment. Enhanced induction of apoptosis by the combination treatment was clearly observed in KYSE 110, 170, 1860 and 2270. B, KYSE 410, 520, 960, 1170 and PBMCs were resistant to the combination treatment and did not undergo apoptosis. Experiments were repeated at least 3 times for each cell line, and the most representative results are shown.

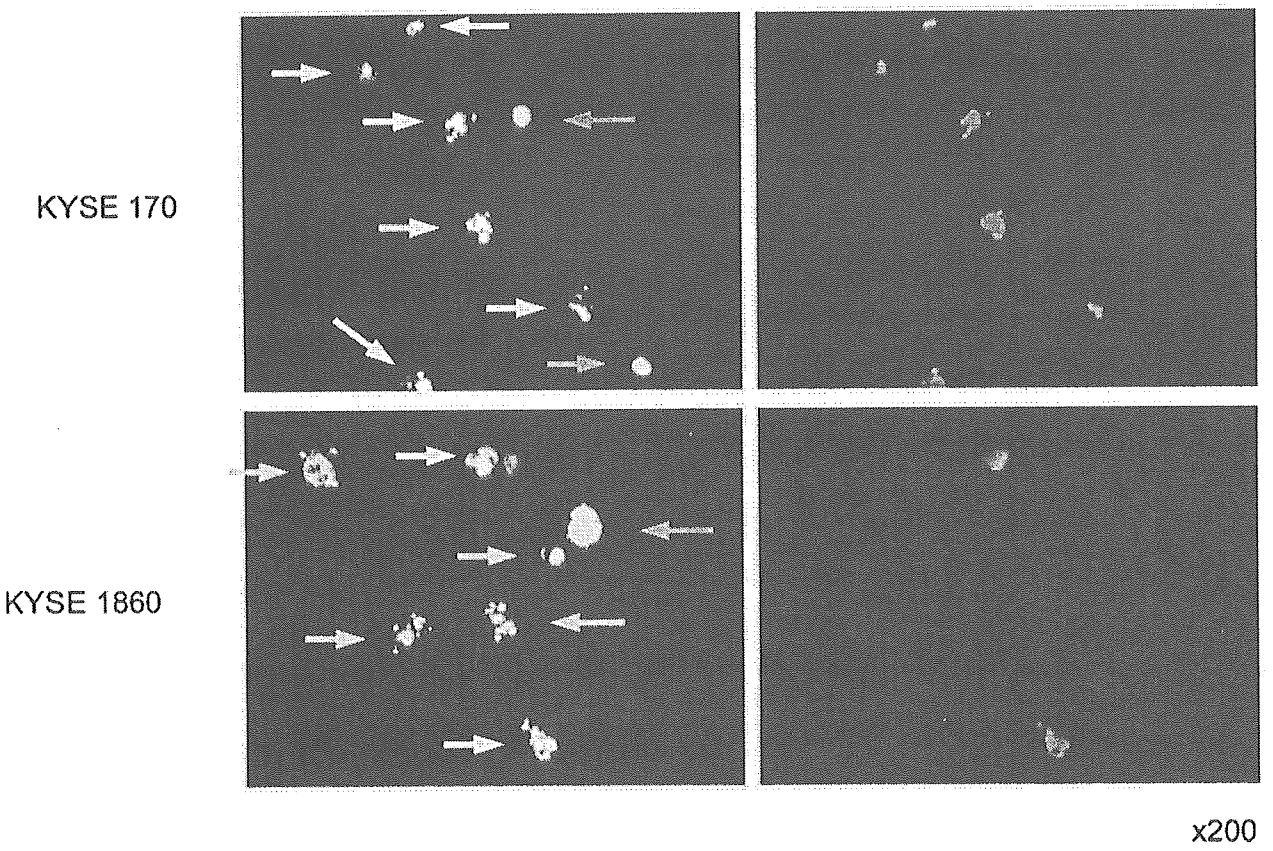


FIGURE 5 – Results of the morphological analyses of the nucleus using Hoechest 33342 and propidium iodide (PI) performed on KYSE 170 and 1860 after they had been treated with 50 ng/ml TRAIL and 5 μg/ml cisplatin for 24 hr. Left panels show Hoechest 33342 and PI staining. Right panels show the same view of the PI staining only. Most cells are either in early or late apoptosis, and necrotic cells were rather scarce. Intact blue nuclei (red arrow), condensed/fragmented blue nuclei (green arrow), condensed/fragmented pink nuclei (yellow arrow) and intact pink nuclei (not seen in the figure) represent viable, early apoptotic, late apoptotic and necrotic cells, respectively.

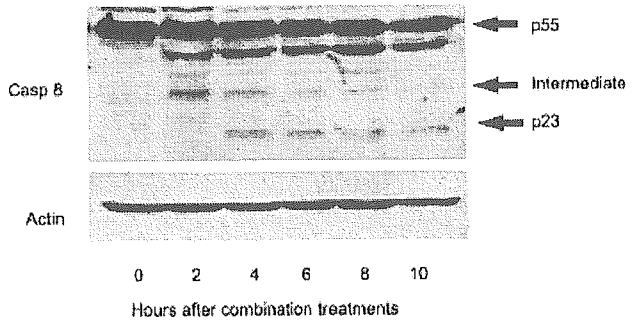


FIGURE 6 – Activation of procaspase-8 in KYSE 170. Cells were treated with TRAIL and cisplatin for indicated hours, and cell lysates were obtained for immunoblotting. An intermediate product of caspase-8 started to appear after 2 hr, and an active form was observable after 4 hr. Detection of the active form appeared to peak after 8 hr. Beta-actin expression is shown as a loading control. Experiments were repeated 3 times, and the most representative results are shown.

8 hr. Results are shown in Figure 7*c*. All 8 cell lines expressed FLIPL, FLIPS , XIAP and NFκB in various levels, and direct correlation between expression levels of these proteins and cell lines' sensitivity to the combination treatment was not apparent. However, slight decreases in expression levels of XIAP and FLIPS were noted in sensitive cell lines after the cisplatin treatment while their expression levels remained unchanged in the resistant cell lines. Meanwhile, expression levels of NFκB (p50, p65 and p115) and FLIPL remained unaffected by the cisplatin treatment (Fig. 7*c*).

*Cisplatin-induced upregulation of DR4 and DR5 in KYSE cell lines*

Influences of cisplatin on the surface expression of TRAIL receptors in KYSE cell lines were next investigated by flow cytometry, which clearly showed cisplatin-induced upregulation of both DR4/5 expression in KYSE 110, 170,1860 and 2270 after 6 hr exposure (Fig. 8*a*). On the other hand, their expression levels in KYSE 410, 520, 960 and 1170 remained nearly unchanged (Fig. 8*b*). Expression of the surface DcR1/2 paralleled that of mRNA transcripts but neither cisplatin-dependent upregulation nor downregulation was observed in any of the cell lines (Fig. 8*b*). Similar experiments were performed with PBMCs; DR4 was not detected on the surface of PBMCs even though DR4 mRNA was present by RT-PCR. DR5, DcR1 and DcR2 were detectable, but their expression levels were not influenced by the exposure to cisplatin.

*Sensitization of KYSE cell lines to TRAIL by pretreatment with cisplatin*

From the results thus far, upregulation of DR4/5 by cisplatin may be a critical factor rendering cells sensitive to the combination treatment. To confirm this, crystal violet staining studies were carried out in sensitive cell lines by sequentially adding cisplatin and TRAIL. Since KYSE 2270 was sensitive to TRAIL, signifi-

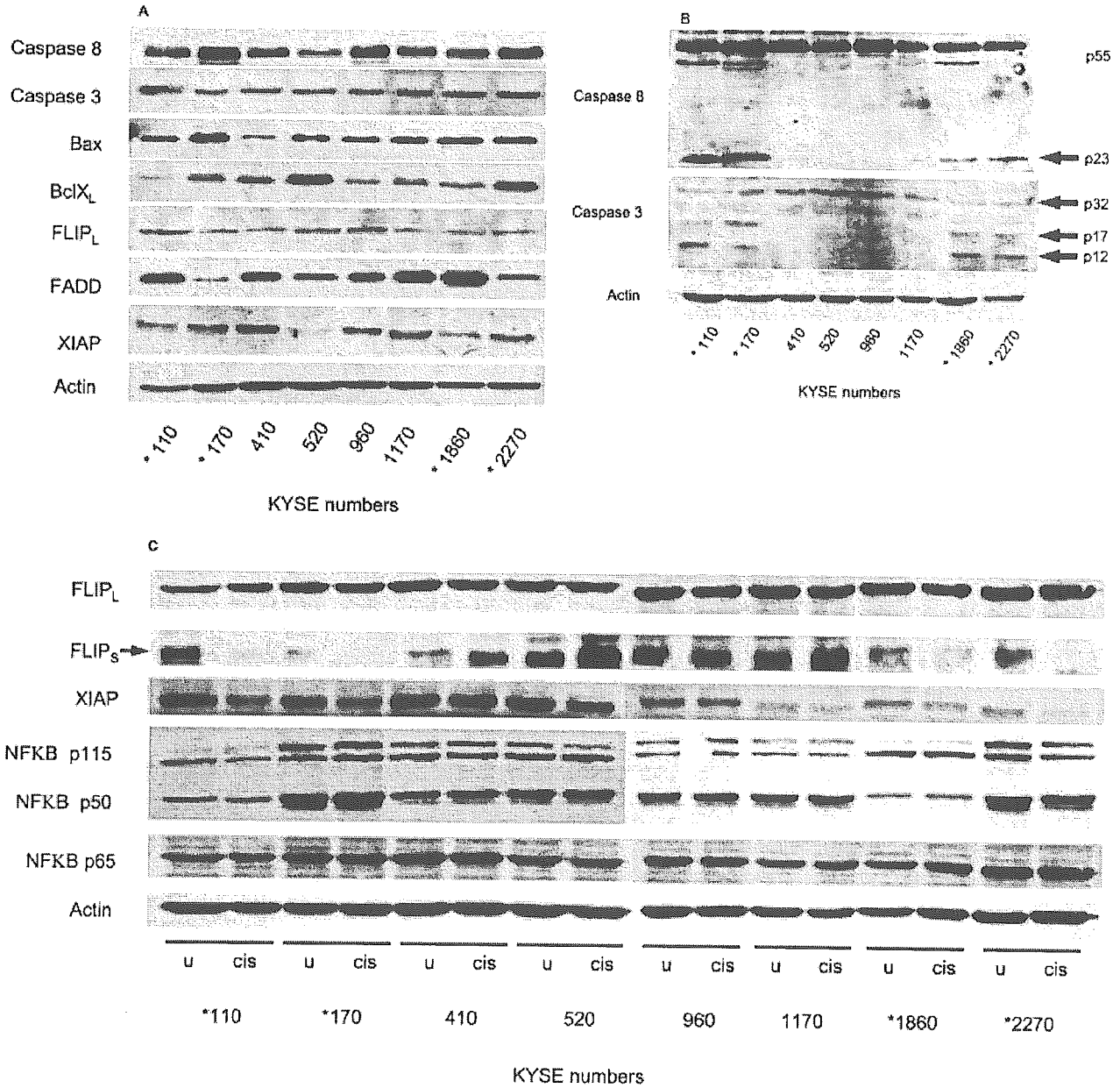


FIGURE 7 – Detection of key apoptotic proteins by immunoblotting analyses: (*a*) in untreated cells, procaspase-8, procaspase-3, Bax, Bcl-XL, cFLIP, FADD and XIAP were expressed in all 8 cell lines. The numbers below the figure represent the cell line numbers. (*b*) Eight hours after the combination treatment with TRAIL and cisplatin, proteolytic activation of procaspase-8 and procaspase-3 were observed in sensitive cell lines KYSE 110, 170, 1860 and 2270 but not in resistant cell lines KYSE 410, 520, 960 and 1170. (*c*) Eight hours after the treatment with 5 μg/ml cisplatin, slight decreases in expression level of anti-apoptotic proteins XIAP and FLIPS were observed in sensitive cell lines. Expression levels of NFκB (p115, p65 and ad p50) remained unchanged. u: untreated samples, cis: samples treated with 5 μg/ml cisplatin, p55: Procaspase-8, p23: cleaved caspase-8, p32: Procaspase-3, p17/p12: cleaved caspase-3. Beta-actin expression is shown as a loading control. Experiments were repeated at least 3 times, and the most representative results are shown. Asterisk denotes cell lines sensitive to the combination treatment.

cant cytotoxicity was observed regardless of the treatment sequence, but in other 3 cell lines, pretreatment with cisplatin followed by treatment with TRAIL resulted in significantly more cytotoxic effects than when the sequence was reversed (Fig. 9).

### In vivo *suppression of tumor growth by the treatment of nude mice with TRAIL and cisplatin*

Because cisplatin enhanced the apoptosis-inducing potential of TRAIL by upregulating DR4/5 *in vitro*, we sought to examine whether this combination is effective in an established ESCC tumor model *in vivo*. KYSE 170 was implanted into the right thigh of the nude mice. Similar to *in vitro* experiments, TRAIL and cisplatin were simultaneously injected i.p. to observe their effects. KYSE 170 tumors carried by mice treated with the combination of TRAIL and cisplatin grew slower after the first course of treatment and remained smaller than any other tumors except those treated with 3 mg/kg cisplatin (Fig. 10*a*). In contrast, tumors in mice treated with normal saline or with TRAIL singly grew significantly faster. The suppression of tumor growth by the combination

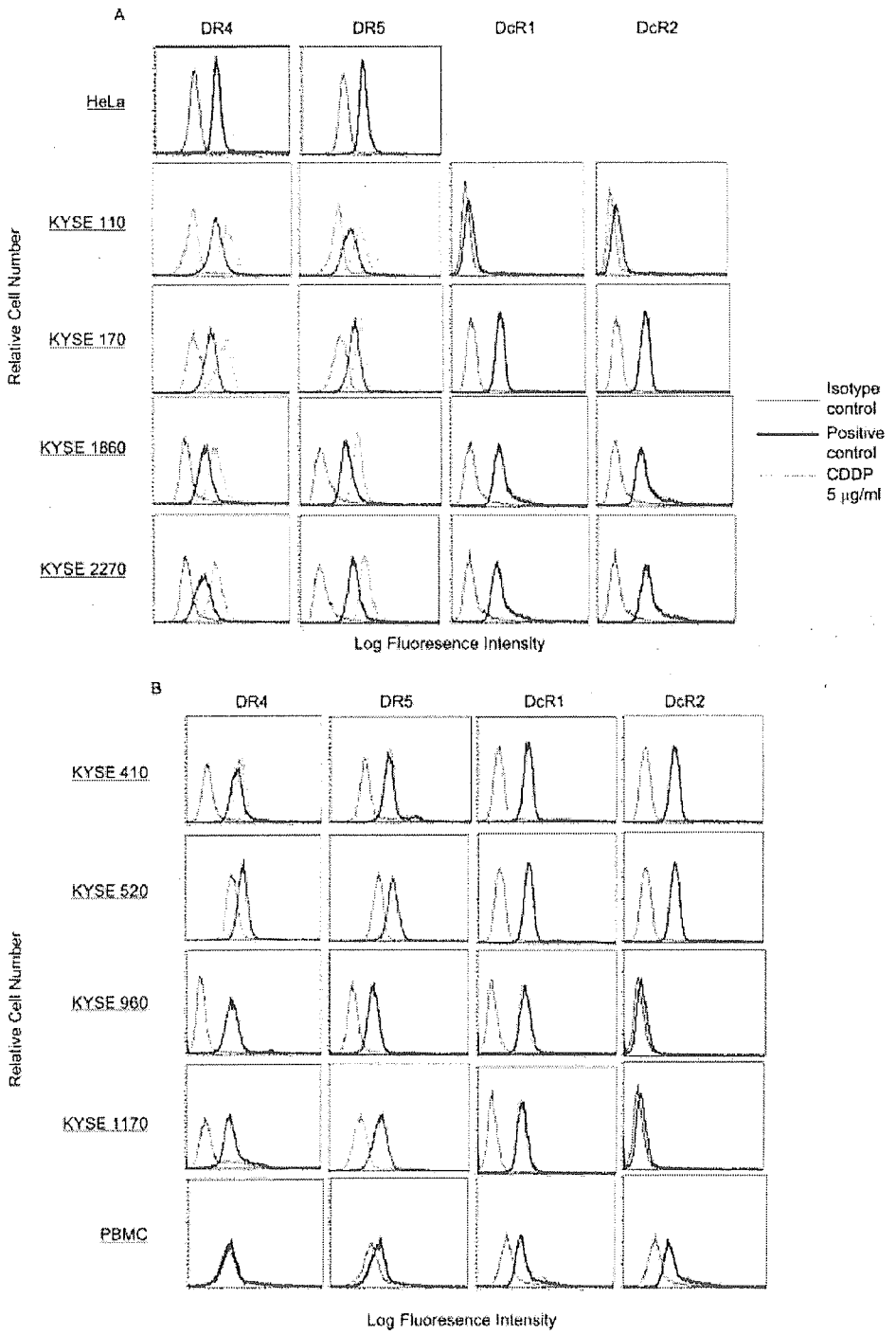


FIGURE 8 – Flow cytometric analysis of surface DR4 and DR5 expression. (*a*) Cell lines sensitive to the combination treatment. (*b*) Cell lines resistant to the combination treatment. HeLa cells served as the positive control for the surface expression of DR4 and DR5. PBMCs served as the positive control for DcR1 and DcR2. The baseline expression patterns of TRAIL receptors in KYSE cell lines paralleled those of the mRNA transcripts. In PBMCs, however, the surface expression of DR4 could not be detected. The cell lines sensitive to the combination treatment revealed cisplatin-induced upregulation of both DR4 and DR5 but in the resistant cell lines including PBMCs, expression levels of DRs was unaffected. Expression of decoy receptors was not influenced by the addition of cisplatin in any of the cell lines. Experiments were repeated at least 3 times for each cell line, and the most representative results are shown.

treatment was statistically significant compared to TRAIL alone ($p = 0.0006$) or normal saline ($p < 0.0005$), but the statistical significance was not reached when compared to the effect of 2 mg/kg cisplatin ($p = 0.253$). On the contrary, weight loss of the mice treated with 3 mg/kg cisplatin was much more than mice in any other groups, though statistical significance was not reached (Fig. 10*b*). Both the suppression of tumor growth and weight loss of mice were dose dependent for cisplatin, but the addition of TRAIL did not appear to influence the weight of mice, consistent with other reports that TRAIL causes minimal or no side effects in mice.[19,32]

## Discussion

The present results indicate that ESCC cell lines are generally resistant to TRAIL, but in some cell lines that resistance can be overcome by adding cisplatin. Death signals originate from the

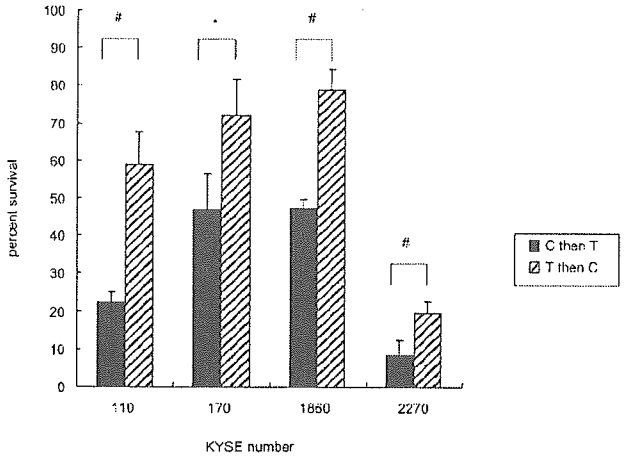


FIGURE 9 – KYSE 110, 170, 1860 and 2270 were subjected to the sequential administration of cisplatin and TRAIL. They were either pretreated with 5 μg/ml cisplatin for 12 hr, followed by treatment with 50 ng/ml TRAIL for 12 hr (C then T), or *vice versa* (T then C), stained with the crystal violet solution and OD absorbance was measured. OD absorbance of untreated cells was set for 100%. For all 4 cell lines, pretreatment with cisplatin followed by TRAIL resulted in statistically significant cytotoxic effects compared to the reverse sequence. KYSE 2270, which is sensitive to TRAIL, revealed significant cytotoxicity regardless of the treatment sequence. Graphs represent the mean of 8 independent experiments for each cell line; bars, SE. #$p < 0.001$. *$p < 0.005$.

TRAIL receptors and we have demonstrated that cisplatin can upregulate DR4/5 to enhance ligand-induced cytotoxicity. All KYSE cell lines expressed both DR4 and DR5, showing a quintessential pattern of death receptor expression in malignant cell lines. The decoy receptors had been thought to play a cytoprotective role against TRAIL in normal cells[4–6,9,10] until TRAIL-sensitive tumors expressing decoy receptors were reported[13,14,20,29,33] to keep their roles remain ambiguous. When compared to other tumor cell lines, incidences of DcR1/2 expression in KYSE cell lines are higher than expected, with only 1 decoy-receptor negative cell line. High incidences of decoy receptor expression may be one of the unique molecular characteristics reflecting TRAIL-resistant nature of ESCC.

Statistical analysis of clinicopathological data of the original cancers revealed that sensitivity to the combination treatment was unrelated to age, gender, location of the tumor, lymph node metastasis, distant lymph node metastasis or TNM staging of the tumor. However, although statistical significant was not reached, well-differentiated ESCC tended to be more resistant to the combination treatment compared to moderately and poorly differentiated ESCC. One of the possible explanations for this is that well differentiated ESCC retains more characteristics of normal esophageal epithelial cells. Since normal cells are generally resistant to TRAIL, well-differentiated ESCC might remain more resistant to the treatment with TRAIL. Furthermore, although statistical significance was not reached, 100 % of the well-differentiated tumors expressed both DcR1 and DcR2 whereas some of the moderately and poorly differentiated tumors lacked decoy receptor expression. By increasing the number of samples, resistant characteristics and differences in expression levels of decoy receptors may become more evident.

Several chemotherapeutic agents including cisplatin, one of the most common anticancer drugs against ESCC, have been reported to upregulate DR4/5 to augment TRAIL-mediated apoptosis in cancer cells.[19,21,22,32] Consequently, we selected cisplatin to evaluate whether or not it can break TRAIL resistance in ESCC. Although cisplatin alone was largely ineffective at the given dose, when combined with TRAIL, synergistic cytotoxicity was exerted in 7 out of 19 (37%) cell lines. Further examination revealed that the addition of cisplatin did not influence decoy receptor expression, but it was rather capable of upregulating DR4/5 to augment TRAIL-mediated apoptosis. Following the time course of death receptor upregulation in these cell lines with flow cytometry, they became clearly bright after 6 hr, but even after the 4 hr contact, the upregulation seemed to have begun (data not shown). These results demonstrated that upregulation of DR4/5 by cisplatin begins relatively early in the sensitive cell lines. However, DR expression of the resistant cell lines was not affected. Several different mechanisms have been suggested for upregulation of DRs. Sheikh *et al.*[34] has shown the involvement of p53 at the transcriptional level in upregulation of DR5, and Gibson *et al.*[22] has suggested differential activation of NFκB could upregulate both DR4 and DR5. Still others suggested the involvement of post-translational mechanisms such as stabilization of cellular microtubules,[35] differential translocation of intracellularly stored DRs upon stimulation by the chemotherapeutic agents[33,36] and chemotherapeutic agent-mediated changes in the rate of receptor turnover at the cell surface.[37] Since none of the mechanisms could sufficiently explain DR upregulation in all of the cancers, this mechanism might differ in different types of cancer. In our study, semiquantitative RT-PCR performed on KYSE 110 and 170 also revealed upregulation of DR4/5 transcripts after the treatment with 5 μg/ml cisplatin (data not shown); therefore, an increase in mRNA transcription may be responsible for DR upregulation in ESCC. Interestingly, in PBMCs, DR4 was not expressed on the surface even though DR4 mRNA was detected in RT-PCR, while surface DR5, DcR1 and DcR2 were expressed, as were their respective mRNA transcripts. The expression of these receptors was not influenced by cisplatin. These results indicate that deficient DR4 expression and lack of DR upregulation in addition to the decoy receptors might contribute to PBMCs' resistance to the combination treatment. It appears that the control of DR4 expression is at the post-transcriptional level in PBMCs, and differs from ESCC. Overall, it may be deduced that the induction of death receptor upregulation by cisplatin in esophageal SCC is indicative of their sensitivity to the combination treatment with TRAIL.

In ESCC, DcR1 and DcR2 do not seem to be essential with regard to their sensitivity against TRAIL for following 2 reasons. First, KYSE 110, the decoy receptor negative cell line, is resistant to TRAIL itself. Second, some of the decoy receptor positive cell lines became susceptible to TRAIL in the presence of cisplatin even though their expression of DcR1 and DcR2 are not influenced by cisplatin. It appears that physiological expression levels of DcR1 and DcR2 are not sufficient to interfere with TRAIL-induced apoptosis as was demonstrated in overexpression experiments of the initial reports investigating TRAIL receptors.[3,6,9,13,14,20,29,33] Furthermore, although a possible role of DcR2 as a NFκB activator has been disclosed,[11] functional roles of DcR1 is yet to be elucidated. Taken together, it seems unlikely that signals originating from DcR1/2 upon binding of TRAIL overcome those from DR4/5 in ESCC to inhibit TRAIL-mediated apoptosis, and the mechanisms of TRAIL resistance seemingly resides on the intracellular factors rather than DcR1 and DcR2.

Apoptosis-inducing mechanisms by TRAIL is thought to be similar to its kin, FasL and TRAIL-mediated apoptosis *via* the extrinsic pathway has been delineated by Griffith *et al.* and others.[15,20,23,29] While TRAIL receptor-specific cytoplasmic adapter protein is yet to be discovered, some studies have reported that FADD may act as the adapter protein for TRAIL receptors.[38] Our study clearly indicated the involvement of caspases in TRAIL-mediated apoptosis of ESCC. However, expression of FADD had been variable among ESCC cell lines and its role as an adapter protein for TRAIL receptors remained unclear. Recently, activation of the intrinsic pathway in TRAIL-mediated apoptosis has been described; Bax seems to take a critical pro-apoptotic role while Bcl-XL is an important inhibitory factor in this pathway of

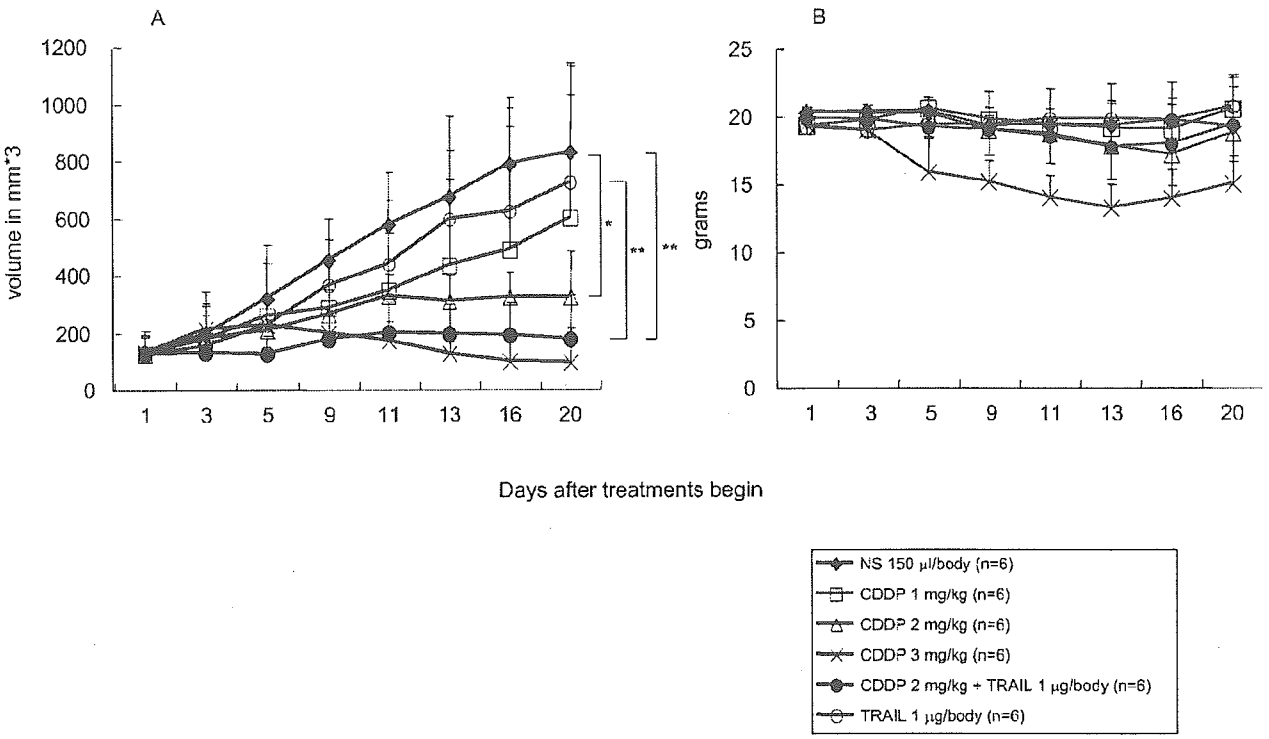


FIGURE 10 – Synergistic antitumor effect of the combination treatment with TRAIL and cisplatin *in vivo*. (*a*) KYSE 170 cells ($5 \times 10^6$) were inoculated into the right thigh of Balb/c nude mice. After tumor formation, mice received i.p. injection of either TRAIL, cisplatin, normal saline or the combination of TRAIL (1 µg/body) and cisplatin (2 mg/kg). Cisplatin alone induced dose-dependent suppression of xenografted tumor growth, although low-dose cisplatin (1 mg/kg) was largely ineffective. The combination treatment resulted in statistically significant tumor growth suppression compared to both the control and TRAIL-alone groups. (*b*) injection of TRAIL alone resulted in no weight loss of nude mice, while cisplatin caused weight loss of nude mice in a dose-dependent manner. Weight loss was particularly prominent when 3 mg/kg cisplatin was administered. Closed circle, Control group with normal saline; open circle, TRAIL (1 µg/body); open square, cisplatin (1 mg/kg); open triangle, cisplatin (2 mg/kg); ×, cisplatin (3 mg/kg); closed circle, TRAIL (1 µg/kg) + cisplatin (2 mg/kg). Data are shown as the means; bars, SE. $*p < 0.05$. $**p < 0.001$.

apoptosis.[38–42] We detected varying levels of Bax and Bcl-XL in 8 KYSE cell lines but the evidence for the intrinsic pathway activation in the sensitive cell lines could not be obtained. Meanwhile, detection of anti-apoptotic proteins by Western blotting disclosed slight decreases in FLIPS and XIAP expression in the combination-treatment sensitive cell lines after the treatment with 5 µg/ml cisplatin. Such changes were not observed in the resistant KYSE cell lines. FLIP blocks the binding of caspase-8 to the death domains of DR to inhibit apoptosis induction and has been shown to be critical in TRAIL resistance of other cancers,[13,34,43,44] while XIAP inhibits the intrinsic pathway of apoptosis.[45] Cisplatin-mediated decreases of these 2 proteins might partially explain the cell lines' sensitivity to the combination treatment. However, more definitive intracellular mechanisms that determine ESCC's sensitivity to TRAIL and cisplatin are likely to exist, and this shall be the topic of further study. On the contrary, Western blotting did not prove any significant changes in the NFκB expression levels after the cisplatin treatment in any of the cell lines. While NFκB is generally considered anti-apoptotic,[11,46] a moderate amount of activated NFκB has been suggested to upregulate DRs.[22] Subsequently, it will be necessary to detect the active form of NFκB to evaluate its roles in DR upregulation of ESCC.

Importantly, pretreatment of KYSE cell lines with cisplatin followed by TRAIL resulted in significant cytotoxic effects compared to the reverse sequence in the treatment-sensitive KYSE cell lines, thus supporting the significance of cisplatin-induced upregulation of DRs. Furthermore, *in vivo* experiments with nude mice revealed synergistic effects of the combination treatment similar to *in vitro* results when compared to the TRAIL-alone (1 µg/body) group. Although statistical significance was not reached when compared to cisplatin-alone group (2 mg/kg), tendency toward synergy was observed; an encouraging outcome that may lead to further investigation. Notably, we were able to obtain these results with much lower dose of TRAIL than previously reported.[19,32] Growth of the xenografted KYSE 170 was suppressed by administration of cisplatin alone even though this cell line was resistant to cisplatin *in vitro*. We believe this seemingly discrepant result was obtained probably because with the administration of the given concentration of cisplatin, the serum concentration became sufficiently high to cause some apoptosis. TRAIL was shown to have negligible influences on weight loss at 1 µg/body, implying minimal side effects.

These results support the cisplatin-dependent DR upregulation as a critical factor in augmenting TRAIL-mediated apoptosis in ESCC and in addition may provide the following clinical implication: first, there is a possibility that the combination therapy can overcome not only TRAIL resistant but also cisplatin resistant ESCC. Second, synergistic effects of the combination treatment against ESCC obtained *in vitro* can be translated into *in vivo* experiments. Third, in the clinical settings, sequential administration of cisplatin and TRAIL, which may be safer, might be the better way of treating the patients. Since the critical issue regarding toxicity of recombinant human TRAIL to normal human cells appeared to be resolved by alternating its