かし、実際の生体内における PTX 代謝への CYP3A5 の影響は不明であり、CYP3A5 遺伝子多型がヒト CYP3A 活性の個人差の一因となることも示唆されていることから、本研究では日本人における主な CYP3A5 遺伝子多型である CYP3A5*3 の遺伝子型と PTX 体内動態との関連性を検討した。CYP3A5*3 の遺伝子型と PTX の AUC との相関、遺伝子型と PTX に対する *p*-3'-OH-PTX の AUC 比との相関は認められなかったことから、CYP3A5*3 の PTX の *p*-3'-水酸化代謝や体内動態への影響は乏しいことが示唆された。この結果から、CYP3A5 は生体内においても PTX の代謝には関与しないか、あるいは関与していても CYP3A5*3 が CYP3A5 の薬物代謝能に及ぼす影響は小さいことが考えられた。

MDR1 の発現量や基質輸送能の変化により PTX の胆汁への排泄が影響を受ける可能性があるため、本研究では発現量や基質薬物の動態に影響を及ぼすことが示唆されている MDR1 遺伝子多型に関して、PTX 体内動態との関連性を検討した。その結果、C3435T を除くすべての SNPs において、変異型アレル保有者での PTX AUC の低下傾向が認められた。更に、MDR1 の全 SNPs を含めた変異型アレル数と PTX の AUC 及び CLtot との間には有意な相関性が認められた。これらの結果より、MDR1 の T-129C、T1236C 及び G2677(A,T)は MDR1 による PTX 排泄能を増大させ、血中濃度を低下させる可能性が示された。また、変異型アレル数の増加に従い PTX 血中濃度が低下するという、いわゆる遺伝子量効果（gene dose effect）が認められたことから、これらの SNPs の組み合わせが、PTX 体内動態の個人差の一因となる可能性が示された。これまで、PTX 体内動態に関連する遺伝子多型と体内動態パラメータとの相関解析に関する報告はなく、MDR1 の遺伝子多型の PTX 体内動態に及ぼす影響は本研究が初めての報告となる。

E. 結論

PTX 体内動態の個人差には MDR1 の遺伝子多型が密接に関与し、総合的な MDR1 遺伝子型診断が PTX 投与患者の副作用発現回避に有益である可能性が示された。

F. 健康危険情報

特記すべきことなし

G. 研究発表

1. 論文発表

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2. 学会発表

なし

H. 知的財産権の出願・登録状況（予定含）

1．特許取得：なし
2．実用新案登録：なし
3．その他：なし

厚生労働科学研究費補助金（臨床研究基盤整備推進研究事業）
分担研究報告書

卵巣癌患者に対するCD-DST法による感受性試験の妥当性の検討

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研究要旨

初発の卵巣がん(原発性の上皮性卵巣悪性腫瘍)69例を対象にCD-DST法による薬剤感受性試験を施行した。使用した抗がん剤は、パクリタキセル、カルボプラチン、イリノテカン、マイトマイシン、ドセタキセル、エトポシド、アドリアマイシン、シスプラチンの８薬剤であった。組織型の内訳は漿液性腺癌26例、明細胞腺癌15例、粘液性腺癌13例、類内膜腺10例、癌肉腫3例、悪性ブレンナー腫瘍2例であった。進行期の内訳はIa期9例、Ic期15例、IIC期4例、IIIb期3例、IIIc期31例、IV期7例であった。CD-DST法による薬剤感受性試験は卵巣がんにも十分応用可能と考えられる。

A. 研究目的

卵巣がんの標準的治療としてTJ療法が施行されている。しかし薬剤感受性には個体差があることはよく知られており、TJ療法の奏効率は70%前後と報告されている。また全ての組織型の薬剤感受性は同一とは言えず、例えば粘液性腺癌や明細胞腺癌においてはTJ療法が不応である場合も多い。さらに感受性を示していた化学療法であっても再発した場合には耐性獲得していることもあり、これが患者を死に至らしめる要因ともなっている。このようなTJ療法に対して耐性を有する腫瘍では他の薬剤に対する奏効率も20%以下との報告が多く、有効な薬剤の選択は卵巣癌治療の重要な課題の一つである。

CD-DST法(collagen gel droplet embedded culture drug sensitivity test)は比較的近年に開発されたin vitroの薬剤感受性試験である。本邦は単離細胞をコラーゲンゲル小滴内に包埋した三次元培養と画像定量法を組み合わせた全く新しい原理に基づく。すなわち培養環境がコラーゲンを細胞外マトリック

スとする、いわゆる生体内を再現した立体的環境にあることから、細胞は生体内に近い増殖形態を維持することが可能である。結果として培養系に接触させる薬剤の濃度は他方に比べてはるかに低くなり、臨床上使用される治療量に近似した生理学的条件下での薬剤感受性の評価が可能である。

本研究では、卵巣がんに対する化学療法の感受性をCD-DST法を用いて検討することにより、本法の臨床的有効性を評価することを目的とした。

B. 研究方法

術前に初発卵巣がんを疑った症例を仮登録した。仮登録した症例は、術中に組織の一部を感受性試験に提出し、かつ術後の病理診断で卵巣原発の卵巣がん（卵巣上皮性悪性腫瘍）と診断された症例を本登録した。

薬剤感受性試験はCD-DST法で行った。感受性試験に供する癌細胞の培養条件を良くするために検体を24時間以内にコラゲナーゼ処理して培養を開始した。感受性試験の対象薬剤数は原則として8薬剤とした。しかし検体量が十分でない場合、あるいは培養により細胞が十分に発育せずに結果的に細胞数が不足した場合には、検査する薬剤の優先順位をパクリタキセル、カルボプラチン、イリノテカン、マイトマイシン、ドセタキセル、エトポシド、アドリアマイシン、シスプラチンの順とした。

研究開始に際し東北大学の倫理委員会の承認を得た。患者の不利益を防止する為の措置として、すべてのデータを連結可能匿名化した。

C. 研究結果

術前に卵巣がん（原発性かつ初発）を疑った103例を仮登録した。手術中、腫瘍摘出直後に腫瘍に割を入れ、腫瘍の一部（約1ｇ）を感受性試験の組織培養検体として供した。103例中に術後に原発性卵巣がん（上皮性卵巣悪性腫瘍）と病理診断された症例は71例であった。71例中2例は病巣が小さすぎたため、もし感受性試験に検体を採取すると病理診断に影響が出ると判断し、感受性試験への組織提出を中止した。以上、卵巣がんと病理診断確定しかつ感受性試験に組織検体を提出した69症例を本登録し本研究の対象とした。対象者の年齢は18歳から75歳で、平均年齢は55.2歳であった。

対象の組織型の内訳は漿液性腺癌26例、明細胞腺癌15例、粘液性腺癌13例、類内膜腺10例、癌肉腫3例、悪性ブレンナー腫瘍2例であった。

対象の進行期の内訳はIa期9例、Ic期15例、IIC期4例、IIIb期3例、IIIc期31例、IV期7例であった。

69例中、感受性試験に成功した（薬剤感受性の結果が出た）ものが59例、不成功（培養不成功）が10例であった。すなわち感受性試験成功率は86%(59/69)であった。59例全例でTJ療法を施行した。59例のTJ化学療法に対する反応、再発、生存については現在経過観察中である。

D. 考察

今年度は臨床検体69例と比較的大規模でCD-DST法による薬剤感受性試験の成功率を検討した。その結果、86%の成功率は他の抗がん剤感受性試験に比較しても十

分高い成功率であることが示された。

感受性試験の成功例中、術後化学療法を施行した59症例については経過観察により化学療法の臨床効果と感受性試験の成績を合わせて検討する予定である。

E. 結論

CD-DST法による抗がん剤感受性試験の成功率は約90%である。感受性試験成績と臨床効果とのすりあわせを来年度以降に行う予定である。

F. 健康危険情報

特記すべきことなし

G. 研究発表

なし

H. 知的財産権の出願・登録状況（予定含）

なし

厚生労働科学研究費補助金（臨床研究基盤整備推進研究事業）
分担研究報告書

進行・再発卵巣明細胞腺癌に対するパクリタキセル、プラチナ製剤併用療法の有用性に関する検討

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研究協力者　高野忠夫、吉永浩介　（東北大学病院）

研究要旨

卵巣明細胞癌(CC)は卵巣癌の中でも予後不良で、化学療法抵抗性の癌とされている。今回PTX, PLT併用療法を施行した評価病変のあるCC28症例(初回治療例12、再発例16)と対照として漿液性腺癌初発22例に関して奏効率を後方視的に比較検討した。CCにおける奏効率は初回治療例58%、再発例19%と有意に初回治療例の方が高かった。漿液性腺癌での奏効率は77%でありCCより高かったが、両者の間に有意差はなかった。初回治療例の奏効率はCAP療法などの従来の治療法に比較すれば高く、将来CCに対する第1選択の治療レジメの一つとして考えられる。

A. 研究目的

卵巣明細胞癌(CC)は卵巣癌の中でも予後不良で、従来のCAP療法に対する奏効率が10ー20%にすぎない化学療法抵抗性の癌とされる。またCCは早期癌が多く評価可能病変を持つ症例が少ないために、化学療法に対する奏効率を検討することが困難である。本研究では評価病変のある進行、再発CCに対してPTX、PLT製剤併用療法の奏効率を求めることを目的とした。

B. 研究方法

症例は10施設で1998年から2003年にTJ療法(T;175mg/m2, J;AUC;5)またはTP療法(T;175mg/m2, CDDP;50mg/m2)を施行した評価病変を有する進行、または再発のCCで、28症例(初回治療12、再発例16)を対象とした。また同時期に治療した卵巣漿液性腺癌初回治療例22例を対照とした。CCはcentral pathological reviewによりCC成分が全体の50%以上を占めるものとした。治療前と上記治療3コース後にCTにてRECISTの基準に従って治療効果を判定し、CR, PRを治療効果有効と判断した。カイ2乗検定、Fisher's exact testで解析し、$P<0.05$を有意差ありと判断した。

C. 研究結果

全体の奏効率はCRが3例、PRが7例で36%(10/28)であった。初回治療例のみでは58%(7/12)、再発症例では19%(3/16)であり、両者間で$p<0.05$と有意差を認めた。一方、漿液性腺癌での初回治療例の奏効率は77%(17/12)であり、CCより高かったが有意差はなかった。

D. 考察

これまでに、漿液性腺癌と比べて、CCはプラチナ製剤でPDの率が有意に高いという報告や進行CCでCAPまたはCP療法で生存率が有意に低いという報告があるがいずれもPTXを含んだ化学療法ではなかった。2000年のCancerのデータでは測定可能病変のある進行再発CCに対するCAP療法の奏効率は11%と低かった。また2003年のASCOの報告をみてみるとTJ療法の奏効率の検討において漿液性腺癌81%に比べてCCは18%で有意に低いという結果であったが11例という少数例の解析であった。今回のPTXとプラチナ製剤の併用療法の結果では再発症例は従来の報告と同様の奏効率であったが、初回治療例は従来の奏効率より明らかに高かった。またin vitroの実験でPTXがCCの細胞株に対して有効であるが、漿液性腺癌で効果が低いとの報告もある。今回の検討と併せて考えるとPTXを加えることにより高い奏効率を得ることができると考えられ、CCの初回治療としてPTXとプラチナ製剤の併用療法は有効な治療と考えられた。

E. 結論

CCに対するPTXとプラチナ製剤の併用療法の奏効率は漿液性腺癌に対する奏効率に比べて若干低い。しかしPTXとプラチナ製剤の併用療法は従来の治療法に比較すれば十分に高く、CCの初回治療の化学療法として有効な治療である可能性が示唆された。

F. 健康危険情報

特記すべきことなし

G. 研究発表

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H. 知的財産権の出願・登録状況（予定含）

ない

Ⅳ. 研究成果の刊行に関する一覧表

IV　　研究成果の刊行に関する一覧表

A　書籍

なし

B　雑誌

Promoter methylation status of the Cyclin D2 gene is associated with poor prognosis in human epithelial ovarian cancer.　Sakuma M, Akahira J, Ito K, Niikura H, Moriya T, Okamura K, Sasano H, Yaegashi N. Cancer Sci. 2007;98:380-6.

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V. 研究成果の刊行物・別冊

Promoter methylation status of the Cyclin D2 gene is associated with poor prognosis in human epithelial ovarian cancer

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Gene silencing associated with aberrant DNA methylation of promoter CpG islands is one mechanism through which several genes may be inactivated in human cancers. Cyclin D2, a member of the D-type cyclins, implicated in cell cycle regulation, differentiation and malignant transformation, is inactivated due to aberrant DNA methylation in several human cancers. In the present study, we examined the promoter methylation status and expression of Cyclin D2 in human epithelial ovarian cancer, and then determined the relationship between methylation status and various clinicopathological variables. Twelve ovarian cancer cell lines and 71 surgical specimens were examined by methylation-specific polymerase chain reaction and quantitative reverse transcription–polymerase chain reaction to evaluate the methylation status and expression of the Cyclin D2 gene. The relationship between methylation status and various clinicopathological variables was evaluated using statistical analysis. Aberrant methylation of Cyclin D2 was present in five of 12 ovarian cancer cell lines and 16 of 71 primary ovarian cancer tissues. In five cell lines with methylation, expression of the Cyclin D2 gene tended to be lower than in cell lines without methylation. In ovarian cancer tissues, methylation bands were detected in 16 of 71 cases. The methylation status of Cyclin D2 was associated with advanced stage and a residual tumor size (>2 cm) ($P = 0.027$ and $P = 0.031$, respectively). Based on univariate analysis, patients with aberrant methylation of the Cyclin D2 promoter had a significantly worse chance of disease-free survival than those without methylation ($P = 0.021$). Our results suggest that aberrant promoter methylation of the Cyclin D2 gene is significantly associated with patient prognosis in epithelial ovarian cancer. (*Cancer Sci* 2007; 98: 380–386)

Epithelial ovarian cancer is the most common and deadliest gynecological malignancy in developed countries. Early stages of ovarian cancer are generally asymptomatic and difficult to detect. By the time clinical diagnosis is made, most patients have widespread tumor dissemination.[1] Despite a high response rate to first-line chemotherapy, the prognosis of these women is poor, with an overall 5-year survival rate of only 10–20%.[1,2]

Epigenetic alterations, changes that affect gene expression but not the gene sequence itself, are believed to be one mechanism by which tumor suppressor genes are inactivated in human cancers.[3,4] In particular, hypermethylation of cytosine residues in CpG islands leads to heritable gene silencing via the formation of a repressive chromatin structure.[5,6] Studies of DNA hypermethylation in human ovarian cancer have identified some key genes as targets for epigenetic downregulation, including some hormone receptors,[7] cytokines, cell signaling intermediates, adhesion molecules,[8] DNA damage checkpoint genes,[9] and regulators of the cell cycle.[10] The cell cycle regulators, notably the cyclins, have the potential to function as oncogenes when regulated inappropriately.

The cyclins are a family of proteins that dictate transitions between phases of the cell cycle by regulating the activity of their downstream effectors, the cyclin-dependant kinases (cdk). The D-type cyclins, D1, D2 and D3, play a critical role in early checkpoint regulation of the G_1 phase of the cell cycle. They activate cdk4 and cdk6, leading to the phosphorylation of the retinoblastoma tumor suppressor protein (Rb). This, in turn, dissociates Rb from the transcription factor E2F, thereby permitting DNA transcription. Given the critical role of the D-type cyclins in cell cycle regulation, their abnormal or untimely expression could disrupt the normal cell cycle, resulting in cell proliferation.[11] In fact, Cyclin D1 is considered by some to be a putative protooncogene, as it is overexpressed in a number of tumor types, including breast cancer, thyroid carcinoma, stomach cancer and lymphomas.[12] Aberrant expression of Cyclin D2 has also been demonstrated in human ovarian granulose cell tumors and testicular germ cell tumor cell lines.[13]

Although well known for their proliferation-promoting activity, the D-type cyclins (notably D2) also have growth-inhibitory effects. Cyclin D2 has been shown to be dramatically upregulated under conditions of growth arrest in human and murine fibroblasts. Furthermore, transient overexpression of Cyclin D2 efficiently inhibits cell cycle progression and DNA synthesis. This suggests that an alternative role for Cyclin D2 may be to promote exiting from the cell cycle and maintenance of a nonproliferative state.[14] The expression of Cyclin D2 is frequently lost in human breast cancers, gastric cancers, lung cancers and ovarian granulose cell tumors. This loss of expression is the result of promoter hypermethylation.[10,15–18]

In the present study, we examined the promoter methylation status and gene expression of Cyclin D2 in human epithelial ovarian cancer cell lines. We also evaluated the correlation between methylation status of the Cyclin D2 promoter and various clinicopathological parameters in patients with epithelial ovarian cancer.

Materials and Methods

Cell lines. Twelve ovarian carcinoma cell lines were used. OVCAR3, SKOV3 (both adenoarcinomas), Caov3, OV90 (both serous adenocarcinoma), TOV21G, ES2 (both clear cell adenocarcinoma) and TOV112D (endometrioid adenocarcinoma) were purchased from American Type Culture Collection. JHOS2, JHOS3, HTOA (all serous adenocarcinoma), OMC3 (mucinous adenocarcinoma) and JHOC5 (clear cell adenocarcinoma) were purchased from Riken Cell Bank (Tsukuba). Cell lines were maintained in DMEM/F12 medium (Invitrogen), supplemented

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with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen), and incubated in a 5% CO_2 atmosphere at 37°C.

Surgical specimens and clinical data. The research protocol was approved by the Ethics Committee of Tohoku University Graduate School of Medicine, Sendai, Japan. We examined 71 ovarian cancer specimens obtained from patients treated between 1988 and 2002 at Tohoku University Hospital, Sendai, Japan. All specimens were retrieved from the surgical pathology files at Tohoku University Hospital. Informed consent was obtained from each patient. Specimens were fixed in 10% formalin and embedded in paraffin. Patient age, performance status on admission, histology, stage, grade, residual tumor after primary surgery, and overall survival were obtained from a chart review. The median follow-up time for patients was 59 months (range, 4–120 months). Performance status was defined according to the WHO criteria.[19] Histology, stage and grading followed the FigO criteria.[20] Residual tumor was defined as the amount of unresectable tumor left following primary volume reductive surgery. Optimal volume reduction was achieved when the residual tumor was less than 2 cm. Patients with a residual tumor greater than 2 cm were considered to have suboptimal volume reduction. Overall survival was calculated from the time of initial surgery to death or the date of the last contact. Survival times of patients still alive or lost to follow-up were censored as of December 2002.

An ovarian tissue obtained from a 50-year-old woman who had received surgical treatment for benign uterine tumor was used as a normal ovarian tissue for methylation-specific polymerase chain reaction (MSP) and reverse transcription–polymerase chain reaction (RT-PCR).

Methylation-specific polymerase chain reaction. The methylation status of the samples was assessed using MSP as described previously.[21] Genomic DNA from ovarian cancer cell lines was extracted using the AquaPure Genomic DNA kit (Bio-Rad). Genomic DNA from ovarian tumor specimens was extracted from paraffin blocks. For each tissue, the presence of carcinoma was confirmed on a H&E stained section. For DNA extraction, three 5-μm tissue sections from the same block were scraped from the slide and treated with Dexpat (Takara). The quality and integrity of the DNA were evaluated in terms of the $A_{260/280}$ ratio. Genomic DNA (1 μg) was treated with sodium bisulfite using a CpGenome DNA modification kit (Intergen) according to the manufacturer's protocol. Amplification was conducted in a 20-μL reaction volume containing 2 μL of 10× ExTaq buffer, 1.5 μL of 2.5 mM $MgCl_2$, 1 mM of each primer, 1.5 mL of 2.5 mM dNTPs, and 1 unit of Takara ExTaq polymerase (Takara). The reaction was cycled for 40 cycles, each of which consisted of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s, followed by a 7-min extension at 72°C. The primers used were 5′-AGAGTATGTGTTAGGGTTGATT-3′ and 5′-ACATCCTCACCAACCCTCCA-3′ (−1431 to −1326, 106-bp) for the unmethylated reaction (U), and 5′-GGCGGATTTTATCGTAGTCG-3′ and 5′-CTCCACGCTCGATCCTTCG-3′ (−1404 to −1304, 101-bp) for the methylated reaction (M).[18] Universal unmethylated human genomic DNA (Intergen) was used as a positive control for the unmethylated reaction. Universal methylated human male genomic DNA (Intergen) was used as a positive control for the methylated reaction. Reaction products were separated by electrophoresis on 3% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

Quantitative RT-PCR. Total RNA was isolated from cells by phenol–chloroform extraction using Isogen reagent (Nippon Gene). RNA was treated with RNase-free DNase (Roche Diagnostics; 1 μg/μL) for 2 h at 37°C, followed by heat inactivation at 65°C for 10 min. Total RNA (5 μg) was reversed transcribed using the Superscript II first-strand synthesis system (Invitrogen) with random hexamers according to the manufacturer's protocol. Quantitative polymerase chain reaction (PCR) was carried out using an iCycler system (Bio-Rad). For the determination of Cyclin D2 cDNA content, a 25-μL reaction mixture consisting of 23 μL iQSYBR Green MasterMix, 1 μL of each primer and 1 μL of cDNA template was cycled as follows: 2-min denaturation at 90°C, 30-s annealing at either 60°C (for Cyclin D2) or 62°C (for β-actin), and 1.5-min extension at 72°C. Primers for PCR reactions were as follows: Cyclin D2-F, 5′-TACTTCAAGTGCGTGCAGAAGGAC-3′ and Cyclin D2-R, 5′-TCCCACACTTCCAGTTGCGATCAT-3′;[22] and β-actin-F, 5′-CCAACCGCGAGAAGATGAC-3′ and β-actin-R, 5′-GGAAGGAAGGCTGGAAGAGT-3′.[23] β-Actin primers were utilized as an internal positive control and Cyclin D2 expression level was calculated by dividing the quantity obtained for Cyclin D2 by the quantity obtained for β-actin. Two independent RT-PCR reactions were carried out for each sample.

5-Aza-2′-deoxycitidine and trichostatin A treatment. To confirm that epigenetic change contributed to loss of Cyclin D2 gene expression, we assessed the effect of 5-aza-2′-deoxycitidine (5azaC) (Sigma), a demethylating agent, and trichostatin A (TSA) (Sigma), a histone deacetylase inhibitor, on Cyclin D2 mRNA expression and cell growth of ovarian cancer cell lines by quantitative RT-PCR and cell count, respectively.

Ovarian cancer cell lines (OMC3, OVCAR3, JHOS2, JHOC5 and SKOV3) were cultured at a point of 70% confluence in 10-cm cell dishes. They were treated with 1.0 μM 5azaC for 3 or 5 days. They were also treated with 0.5 μM TSA.[24,25] We set up TSA treatment times of 4, 8, 16 and 32 h, and the treatments for 8 and 16 h appeared the most effective for gene expression compared to control culture (data not shown). Total RNA was prepared at each time point and the expression of Cyclin D2 mRNA was analyzed by quantitative RT-PCR. Furthermore, we investigated the effects of these chemical agents on cell growth of ovarian cancer cell lines by cell count at each time point.

Immunohistochemistry. For the purpose of investigating cell proliferation we examined the immunohistochemical expression of Ki-67 in ovarian cancer tissue. Immunohistchemical analysis was carried out with the streptavidin–biotin amplification method using the NX/ES IHC system (Ventana Medical Systems). Monoclonal antibody for Ki-67 (MIB-1) was purchased from DAKO. For antigen retrieval, the slides ware heated in an autoclave at 120°C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dyhydrate [pH 6.0]). The dilution of primary antibody was 1:50. Scoring of Ki-67 in carcinoma cells was counted independently by two of the authors (M. S. and J. A.), and the percentage of immnunoreactivity in at least 500 carcinoma cells (i.e. the labeling index) was determined.

Statistical analysis. Statistical analysis was carried out using Stat View 5.0 software (SAS Institute). The correlation between the Cyclin D2 mRNA expression level and methylation status was assessed using the Mann–Whitney *U*-test. The statistical significance between methylation status and various clinicopathological parameters was evaluated using Friedman's χ^2 *r*-test and the Mann–Whitney *U*-test. A univariate analysis of prognostic significance for prognostic factors was carried out using the log-rank test after each survival curve was obtained by the Kaplan–Meier method. Multivariate analysis was carried out using the Cox regression model to evaluate the predictive power of each variable independently. All patients who could be assessed were included in the intention-to-treat analysis. A result was considered significant when the *P*-value was less than 0.05.

Results

Methylation status of the Cyclin D2 gene in ovarian cancer cell lines and tissues. Bands corresponding to methylated Cyclin D2 were

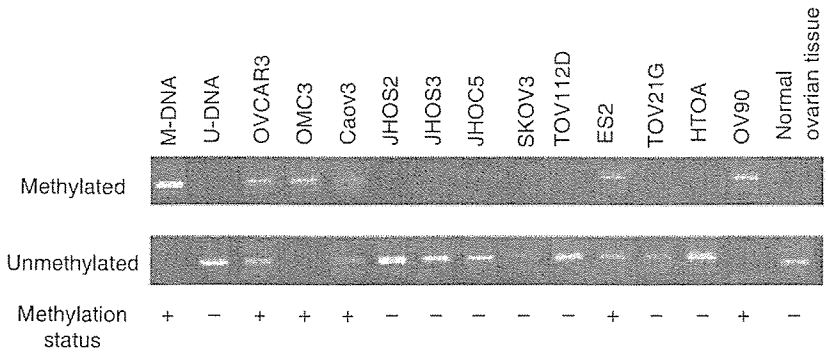

Fig. 1. Methylation status of the Cyclin D2 gene in ovarian cancer cell lines and a normal ovarian tissue. The 101-bp bands in the 'Methylated' lanes indicate the presence of methylated alleles of the Cyclin D2 gene. The 106-bp bands in the 'Unmethylated' lanes correspond to the unmethylated alleles. Methylation status is denoted as follows: +, methylated alleles with or without unmethylated alleles; −, purely unmethylated alleles. M-DNA, universal methylated human male genomic DNA, was used for positive control of methylated reaction. U-DNA, universal unmethylated fetal genomic DNA, was used for positive control of unmethylated reaction.

Table 1. Patient characteristics and cyclin D2 methylation status

Variable	*n*	Cyclin D2 methylation			*P*-value
		+	−	%	
Age (years)					
<50	29	8	21	27.6	
≥50	42	8	34	19	NS
Performance status†					
0–1	51	9	42	17.6	
2–4	19	7	12	36.8	NS
FigO stage					
I, II	35	4	31	2.9	
III, IV	36	12	24	33.3	0.027
Histological type of adenocarcinoma					
Serous	26	6	20	23.1	
Endometrioid	15	3	12	20	
Mucinous	7	3	4	75	
Clear cell	23	4	19	17.4	NS
Grade					
1	24	5	19	20.8	
2	22	7	15	31.8	
3	17	3	14	17.6	NS
Residual tumor size (cm)					
<2	47	7	40	14.9	
≥2	24	9	15	37.5	0.031
Ki-67 labeling index (median)		21.6	23.6	20.4	NS

†0, asymptomatic and fully active; 1, symptomatic, fully ambulatory, restricted in physically strenuous activity; 2, symptomatic, ambulatory, capable of self-care, more than 50% of walking hours are spent out of bed; 3, symptomatic, limited self-care, more than 50% of time is spent in bed, but not bedridden; 4, completely disabled, no self-care, bedridden.

detected in five of 12 cell lines, three of which also contained the unmethylated band, as shown in Fig. 1. The methylated band was detected in two of five cell lines derived from serous adenocarcinoma (Caov3, OV90), in one of three cell lines from clear cell carcinoma (ES2), in the one mucinous adenocarcinoma (OMC3), but not in the endometrioid adenocarcinoma. The normal ovarian tissue was negative for the methylated band. The methylated band was detected in 16 of the 71 surgical specimens (6/26 serous, 4/23 clear cell, 3/15 endometrioid and 3/7 mucinous adenocarcinoma), as shown in Table 1.

Expression of the Cyclin D2 gene in ovarian cancer cell lines and normal ovarian tissue. The expression of the Cyclin D2 gene in the cell lines is presented in Fig. 2. Quantitative RT-PCR was carried out and the ratio of Cyclin D2 to β-actin was calculated to allow for comparison among the cell lines. The median value of relative Cyclin D2 gene expression in cell lines with methylation (0.015) tended to be lower than that in cell lines without methylation (0.03), although the difference was not significant ($P = 0.19$, Mann–Whitney U-test). The expression level of the Cyclin D2 gene in normal ovarian tissue was relatively high compared with ovarian cancer cell lines.

Effects of 5azaC and TSA treatment on methylated cell lines. To confirm that promoter methylation contributed to the loss of Cyclin D2 gene expression, we assessed the effect of 5azaC, a demethylating agent, on Cyclin D2 mRNA expression by quantitative RT-PCR. OMC3 and OVCAR3 cells, which were positive for the methylated band in MSP, were treated. From MSP analysis OMC3 had only methylated alleles, but OVCAR3 had both methylated and unmethylated alleles. We also assessed the effect of TSA, a histone deacetylase inhibitor, to investigate whether another epigenetic change, histone deacetylation, contributed to the silencing of Cyclin D2 gene expression. Treatment of OMC3 cells with 5azaC for 5 days led to a 2.64-fold increase in expression (Fig. 3a). Treatment of OVCAR3 cells with 5azaC for 5 days resulted in a 222-fold increase in expression (Fig. 3b). Treatment with TSA also contributed to re-expression of the Cyclin D2 gene in OMC3 and OVCAR3 cells (2.3-fold and 119-fold, respectively) (Fig. 3). These results suggested that the decreased expression of Cyclin D2 in these cell lines was related to epigenetic change, including DNA methylation or histone deacetylation.

The effects of 5azaC and TSA on cell growth are summarized in Fig. 4. Compared with cell growth in control culture, cell growth with 5azaC or TSA treatment was suppressed in each culture. These chemical agents resulted in inhibition of cell growth in these ovarian cancer cell lines simultaneous with re-expression of the Cyclin D2 gene.

Effects of 5azaC and TSA treatment on unmethylated cell lines. In the MSP and quantitative RT-PCR analyses, expression of the Cyclin D2 gene was decreased in some cell lines without promoter methylation. We assessed the effect of 5azaC or TSA treatment in these cell lines (JHOS2, JHOC5 and SKOV3) to investigate the participation of epigenetic change in the silencing of this gene. Treatment of JHOS2 cells with TSA resulted in higher re-expression than treatment with 5azaC (Fig. 5a). Treatment of JHOC5 cells with TSA for 16 h resulted in an 84.4-fold increase in expression, and treatment with 5azaC also led to a 137-fold increase in expression (Fig. 5b). As for SKOV3 cells, treatment with TSA did not increase the expression of this gene. These results suggest that histone deacetylation may contribute to silencing of the Cyclin D2 gene in JHOS2 and JHOC5 cells, but not in SKOV3.

Correlation between clinicopathological parameters and methylation status of Cyclin D2 in epithelial ovarian cancer. The clinicopathological parameters relative to the methylation status of Cyclin D2 are presented in Table 1. Methylation status was significantly associated with advanced stage and residual tumor size >2 cm.

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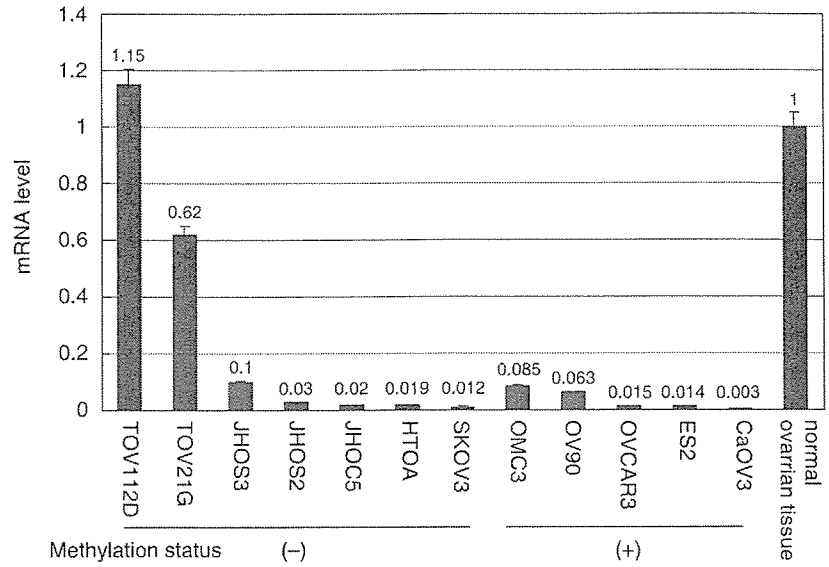

Fig. 2. Expression of the Cyclin D2 gene in ovarian cancer cell lines and normal ovarian tissue. Two independent reverse transcription–polymerase chain reactions were carried out for each sample, and the ratio of Cyclin D2: β-actin was calculated and normalized with the level of normal ovarian tissue. Methylation status is indicated in the same way as in Fig. 1.

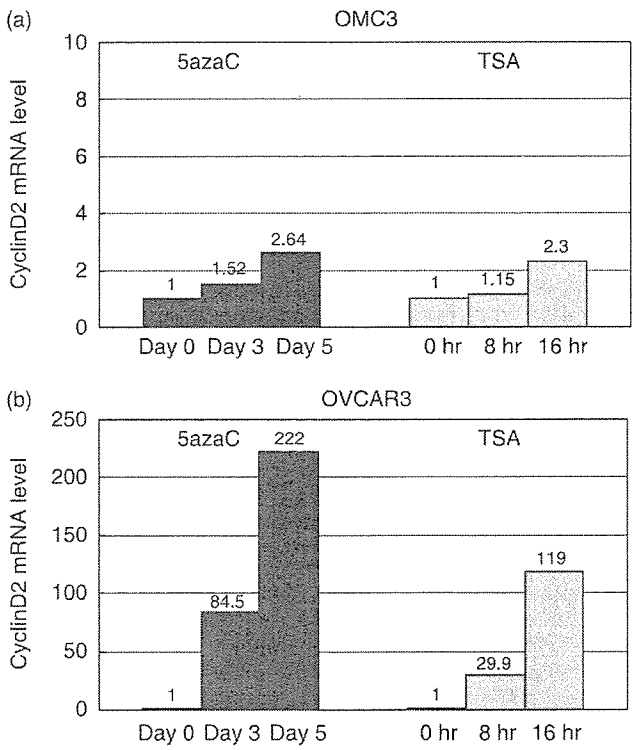

Fig. 3. Expression level of the Cyclin D2 gene as determined by quantitative reverse transcription–polymerase chain reaction in OMC3 and OVCAR3 cells following treatment with (a) 5-aza-2′-deoxycitidine (5azaC) or (b) trichostatin A (TSA). The ratio of Cyclin D2 : β-actin was calculated and normalized with the level before treatment.

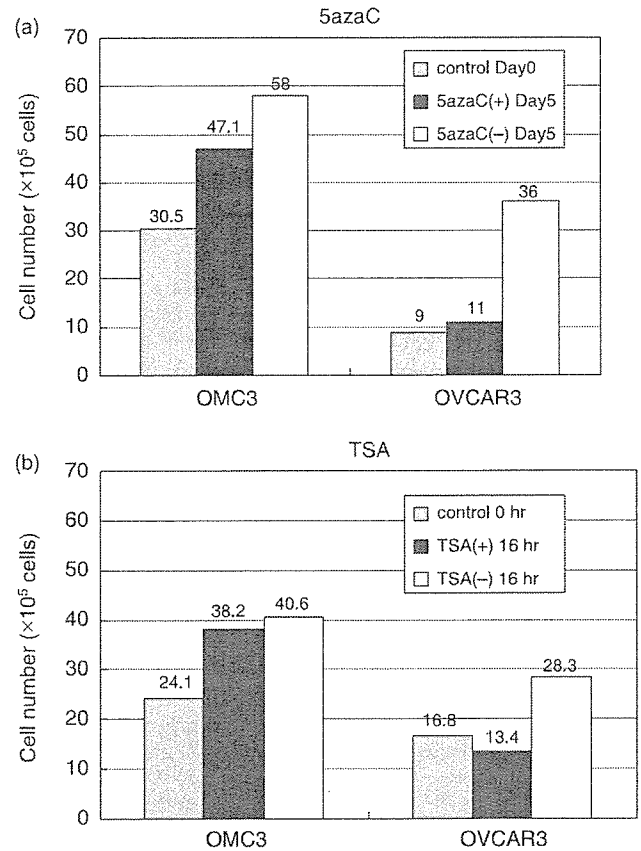

Fig. 4. Cell number of OMC and OVCAR3 cells following treatment with (a) 5-aza-2′-deoxycitidine (5azaC) or (b) trichostatin A (TSA). *Control treatment with medium alone.

There was no association between methylation status and age, performance status, histological type, histological grade or Ki-67 labeling index

The results of the univariate analysis of prognostic significance for each variable with respect to survival are summarized in Tables 2 and 3. Of the clinicopathological parameters evaluated, performance status, stage, histological grade and residual tumor size were significantly associated with disease-free and overall survival. The methylation status of Cyclin D2 was significantly associated with disease-free survival; the cases with methylation had significantly worse rates of disease-free survival than those without methylation (Fig. 6; $P = 0.021$). With

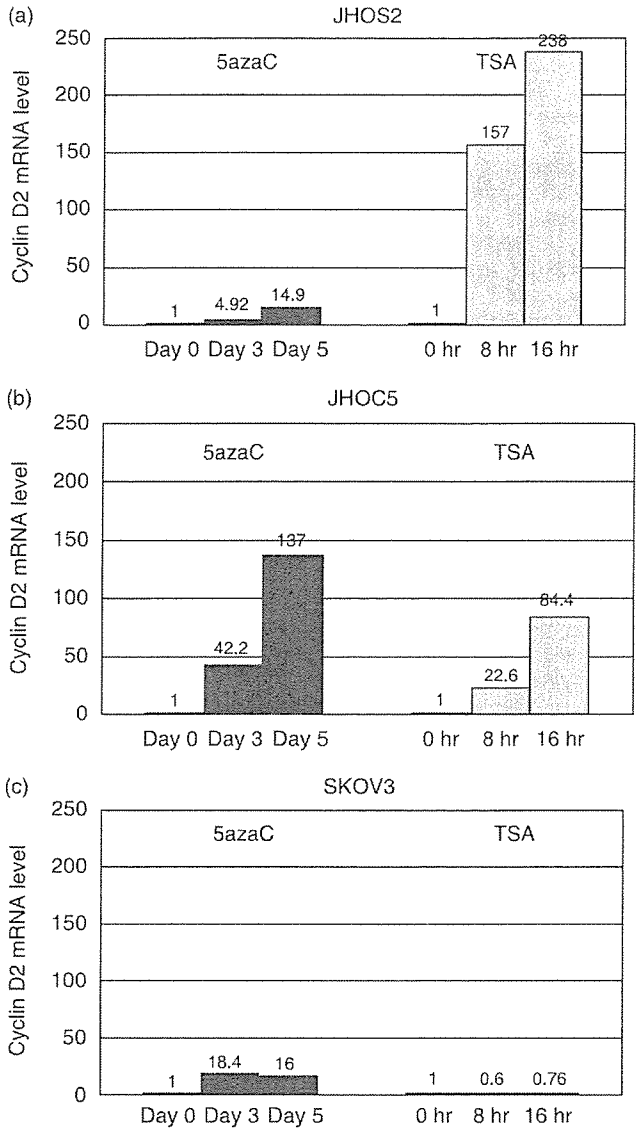

Fig. 5. Expression level of the Cyclin D2 gene as determined by quantitative reverse transcription–polymerase chain reaction in (a) JHOS2, (b) JHOC5 and (c) SKOV3 cells following treatment with 5-aza-2′-deoxycitidine (5azaC) or trichostatin A (TSA). The ratio of Cyclin D2 : β-actin was calculated and normalized with the level before treatment.

regard to overall survival, methylated cases had a worse prognosis than unmethylated cases, but the difference was not significant (Fig. 7; $P = 0.063$). In multivariate analysis, methylation status of cyclin D2 turned out not to be an independent prognostic factor (data not shown).

Discussion

Aberrant promoter methylation is found in many types of human cancer and is a common mechanism for transcriptional inactivation of various genes, including tumor suppressor genes, DNA repair genes, cell cycle regulatory genes and apoptosis-related genes. In the present study, we determined the Cyclin D2 promoter methylation status of several ovarian cancer cell lines and ovarian cancer surgical specimens, measured the levels of Cyclin D2 gene expression in ovarian cancer cell lines and

Table 2. Univariate analysis of disease-free survival

Variable	*P*-value
Cyclin D2 methylation status	0.0212
Age	0.6657
Performance status	<0.0001
FigO stage	0.0001
Histological type	0.4709
Grade	0.1332
Residual tumor	0.0008

Table 3. Univariate analysis of overall survival

Variable	*P*-value
Cyclin D2 methylation status	0.0625
Age	0.4195
Performance status	0.0003
FigO stage	0.0003
Histological type	0.0637
Grade	0.1983
Residual tumor	0.0016

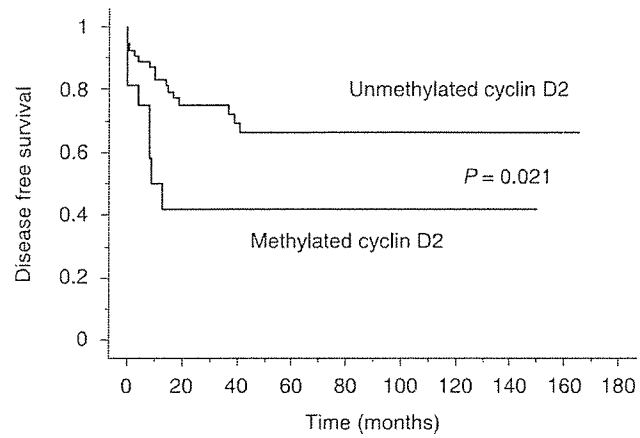

Fig. 6. Association between Cyclin D2 promoter methylation status and disease-free survival in patients with epithelial ovarian cancer.

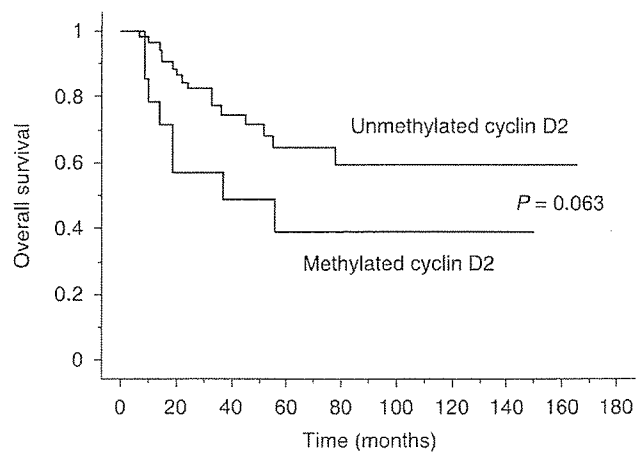

Fig. 7. Association between Cyclin D2 promoter methylation status and overall survival in patients with epithelial ovarian cancer.

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linked the methylation status of the Cyclin D2 promoter to various clinical and pathological variables in ovarian cancer patients.

From MSP and quantitative RT-PCR analysis, there was a trend towards a reduction in gene expression in the presence of hypermethylation; however, this association was not significant, and it was suggested that expression of the Cyclin D2 gene in ovarian cancer cell lines, as a whole, was considerably low in comparison with that in normal ovarian tissue. There was an increase in Cyclin D2 gene expression following the 5azaC treatment of cell lines with promoter methylation of the Cyclin D2 gene in MSP. However, TSA or 5azaC treatment of the cell lines without methylation in MSP resulted in re-expression of the Cyclin D2 gene. Together with these findings, it is suggested that some epigenetic changes, including promoter methylation or histone deacetylation, might contribute to silencing of the Cyclin D2 gene in epithelial ovarian cancer cell lines. The re-expression by treatment with 5azaC in the unmethylated cell lines JHOS2 and JHOC5 suggests that the Cyclin D2 gene may be secondary re-expressed owing to activating other suppressed gene by promoter methylation with treatment of 5azaC, or there is a possibility that aberrant methylation did exist but in a different region of the Cyclin D2 promoter to that which we analyzed. Further investigation and data regarding the acetylation status of histones, a different DNA methylation analysis to decipher the MSP results, and DNA methylation of the transcription factor of Cyclin D2 are needed to supplement our hypothesis.

Epithelial ovarian cancer cell growth following treatment with 5azaC or TSA was suppressed in OMC3 and OVCAR3 cell lines. Treatment with these chemical agents resulted in inhibition of cell growth as well as re-expression of the Cyclin D2 gene. However, another tumor suppressor gene was also re-expressed by these treatments, and these chemicals could have cell toxicity in itself[26–28]. The present data suggests that 5azaC and TSA could be therapeutic agents targeting epigenetic changes in epithelial ovarian cancer, and epigenetic gene silencing of the Cyclin D2 gene could used as a marker of tumor growth.

The D-type cyclins are early checkpoint regulators at the G_1 phase of the cell cycle. Although well known for their proliferation-promoting activity, the D-type cyclins also have growth-inhibitory effects.[14] Thus, decreased expression of Cyclin D2 could result in abnormal cell proliferation and contribute to malignant transformation. Indeed, Cyclin D2 gene silencing secondary to DNA promoter methylation has been demonstrated in several human cancers.[15–17,29] Cyclin D2 promoter hypermethylation has also been detected in nearly half of breast cancers and is associated with gene silencing. Cyclin D2 hypermethylation has also been demonstrated in small cell and non-small cell lung cancer tumor tissues and cell lines,[17] and in approximately half of gastric cancer specimens.[16] In the present study, 22.5% of the surgical specimens and 41.7% of the cell lines had aberrant Cyclin D2 promoter hypermethylation. Our results, though somewhat higher than what has been reported for ovarian granulosa cell tumors,[10] are similar to the percentages seen in several other cancers. Howver, some reports say that aberrant methylation of the Cyclin D2 promoter is an early event in tumorigenesis, as is suggested by its presence in ductal carcinoma *in situ* in breast cancer and its absence in normal ducts;[15,18,29] however, this epigenetic change was associated with advanced ovarian cancer in the present study. Our results suggest that aberrant methylation of this gene could be related to tumor progression rather than tumorigenesis of epithelial ovarian cancer.

A number of biological tumor variables, such as DNA ploidy, steroid hormone receptor status and the expression of certain oncogenes, are associated with prognosis in epithelial ovarian cancer.[30–32] The promoter methylation status of several genes, such as 14-3-3 sigma, BRCA1, hMLH1 and TMS1, has been used to predict poor survival in epithelial ovarian cancer patients.[9,24,33–35] In the present study, Cyclin D2 promoter methylation was significantly associated with advanced stage, a larger residual tumor size and poor prognosis. Because there was a trend toward the repression of gene expression in the presence of promoter hypermethylation in ovarian cancer cell lines, we presume that Cyclin D2 gene silencing might occur in primary tissues with methylation, though the levels of the Cyclin D2 gene have not been analyzed in this study. These results suggest that the aberrant promoter methylation of Cyclin D2, or decreased expression of this gene caused by methylation, may be associated with aggressive biological characteristics, and may play a significant role in disease progression in epithelial ovarian cancer.

The contribution of Cyclin D2 to the pathophysiology of epithelial ovarian cancer is not known at a rudimentary level. Though numerous studies have classified it as an oncogene, our data and that of others strongly supports the hypothesis that it functions as a tumor suppressor gene. Further studies are needed to better clarify the relationship between Cyclin D2 gene expression level and its function as either an oncogene or a tumor suppressor. A deeper understanding of the role of D-type cyclins in ovarian cancer tumor biology could provide a foundation on which to base new diagnostic tests or molecular therapies.

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ORIGINAL ARTICLE

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Dose escalation study of docetaxel and nedaplatin in patients with relapsed or refractory squamous cell carcinoma of the esophagus pretreated using cisplatin, 5-fluorouracil, and radiation

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Abstract
Background. Definitive chemoradiation with cisplatin (CDDP) and 5-fluorouracil (5FU) has been playing an important role in the treatment of esophageal cancer, but some patients are not curable or have recurrent lesions. However, few chemotherapeutic regimens are available for such patients. Docetaxel and nedaplatin are active for esophageal cancer. We conducted a dose-escalation study of docetaxel and nedaplatin as second line-chemotherapy after definitive chemoradiation in patients with relapsed or refractory squamous cell carcinoma of the esophagus after chemoradiation.
Methods. Nedaplatin was administered on day 1 and docetaxel was administered on days 1 and 15, every 4 weeks. Dose escalation was based on the dose-limiting toxicity (DLT) observed during the first cycle.
Results. Twelve patients were enrolled. At a docetaxel dose of 30 mg/m^2 and a nedaplatin dose of 80 mg/m^2, one grade 4 neutropenia occurred and caused one treatment break longer than 2 weeks, but there were few DLTs. At doses of 35 and 80 mg/m^2, respectively, two grade 4 neutropenias and one grade 2 thrombopenia occurred and caused three treatment breaks longer than 2 weeks. Therefore, the maximum tolerated dose was established at this dose level. Two grade 3 anorexias and one grade 3 nausea occurred, but other non-hematological toxicities were generally mild. Responses were seen in one-fourth of the 12 patients, including one complete remission.
Conclusion. The recommended doses of docetaxel and nedaplatin were 30 and 80 mg/m^2, respectively. This combination could be a potential second-line treatment for this target population.

Key words Docetaxel · Nedaplatin · Esophageal cancer · Definitive chemoradiation · Dose escalation study

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Introduction

Carcinoma of the esophagus is a highly aggressive neoplasm. Surgical resection has improved the survival of patients with esophageal cancer during the past two decades, but the survival remains relatively poor, with 5-year survival rates of 20%–40%.[1,2] Since the results of an intergroup randomized controlled trial (Radiation Therapy Oncology Group 85-01), which compared chemoradiation (CRT) with radiation alone, were reported, CRT for esophageal cancer has been revealing promising results.[3,4] Recently, several reports have shown curability by using definitive CRT[5,6] and almost equal survival compared to surgical resection.[7] Preoperative CRT and surgery has also been compared with surgery alone, but the survival benefit is not yet clear.[8–10]

The standard chemotherapy regimen in CRT for esophageal cancer has been a combination of cisplatin (CDDP) and 5-fluorouracil (5FU). It has shown the best clinical outcome, not only because of the synergism of the two agents[11] but also because of the radiosensitizing effects.[12] However, it was reported that the complete remission rates were 33%–75% after definitive CRT[5,6,13] and the incidences of recurrences were also high after preoperative CRT and surgery.[8–10] It is clear that there is an urgent need for active and tolerable chemotherapeutic regimens that can be available to patients with relapsed or refractory lesions after CRT involving CDDP and 5FU. However, there have been few trials of second-line treatment[14–16] and their results have been poor.

Docetaxel (TXT; Taxotere; Sanofi-Aventis, Paris, France) is a novel semisynthetic taxoid obtained from 10-deacetylbaccatin III, a precursor extracted from the needles of the European yew, *Taxus baccata*. It works as an antimitotic agent, enhancing microtubule assembly and inhibiting the depolymerization of tubulin, resulting in the inability of cells to divide.[17] A phase II study of TXT in advanced or recurrent esophageal cancer was conducted in Japan.[16] The overall response rate for the 49 eligible patients was 20.4%. Of the 36 patients previously treated with chemotherapy or CRT, 16.6% responded, compared with 36.4% of the 11 untreated patients. The incidences of grade 3/4 toxicities were: neutropenia, 87.8%; leucopenia, 73.5%; febrile neutropenia, 18.4%; anorexia, 18.4%; infection, 16.3%; anemia, 12.2%; and general fatigue, 12.2%, but there was no treatment-related death.[16]

Nedaplatin (cis-diammine-glycolatoplatinum; CDGP; Shionogi Pharma, Osaka, Japan) is a second-generation platinum derivative that was developed with the aim of decreasing the renal and gastrointestinal toxicities but maintaining the effectiveness of CDDP.[18] A phase II study of CDGP in 29 patients with esophageal cancer showed a response rate of 51.7%, involving 2 responders of 4 patients who were resistant to CDDP-based regimens.[19] The incidences of grade 3/4 toxicities were: thrombocytopenia, 27.9%; leucopenia, 19.7%; anemia, 19.7%; nausea and vomiting, 11.5%; and anorexia, 4.9%, but there was no severe renal toxicity and no treatment-related death.[19]

We considered a combination chemotherapy with TXT and CDGP in patients with relapsed or refractory squamous cell carcinoma of the esophagus after definitive CRT with CDDP and 5FU. TXT was administered on days 1 and 15, and CDGP was administered on day 1, every 4 weeks. The rationale for this combination is that the drugs have different action mechanisms and safety profiles.[16,19] The biweekly administration of TXT might make it possible to reduce neutropenia, which is the dose-limiting toxicity (DLT) of TXT.[20] CDGP may be used safely in patients who were treated with CDDP, because it has lower renal toxicity than CDDP.[18] Moreover, in the phase II study noted above,[19] CDGP was effective in patients with esophageal cancer who were resistant to a CDDP-based regimen, although the number of patients was small.

We performed a dose-escalation study of TXT and CDGP in patients with relapsed or refractory squamous cell carcinoma of the esophagus who had received definitive CRT using CDDP and 5FU. The objectives of this study were to assess the safety and toxicity profiles of this regimen and to determine the maximum tolerated dose (MTD), DLT, and recommended dose (RD) for a phase II study.

Patients and methods

Patient selection

Patients with histologically confirmed squamous cell carcinomas of the esophagus were enrolled at our institutes. Eligible patients with metastatic, or locally recurrent, or residual disease not curable with surgery had been previously treated with CDDP, 5FU, and radiation, with total dosages of more than 160 mg/m^2 of CDDP, more than 8 g/m^2 of 5FU, and more than 50 gray (Gy) of radiation. Other eligibility criteria included an Eastern Clinical Oncology Group (ECOG) scale performance status of 2 or less; age between 20 and 79 years; life expectancy of at least 3 months; provision of written informed consent in accordance with government and institutional guidelines; and adequate organ functions, with a WBC count of more than 3000/mm^3; absolute neutrophil count of more than 1500/mm^3; platelet count of more than 10×10^4/mm^3; aspartate aminotransferase (AST) and alanine aminotransferase (ALT levels) within three times the upper limit of normal (ULN) or five times the ULN in the presence of liver metastasis; total bilirubin less than 1.5 mg/dl; serum creatinine less than 1.5 mg/dl and/or creatine clearance more than 50 ml/min. Exclusion criteria included the following: concomitant uncontrolled, nonmalignant disease (malignant hypertension; cardiac, pulmonary, renal, or hepatic disease; active infection), neuropathy of more than grade 2, active double cancer, pregnant women, brain metastases with any symptoms, or a prior history of treatment for psychiatric diseases. Patients with active interstitial pneumonitis or severe pulmonary fibrosis on chest X-rays or computed tomography (CT) were also excluded. The protocols were approved by the ethics committee of our institution.

Study treatment

CDGP was dissolved in 500 ml of saline and administered as a 2-h IV infusion, followed by the administration of TXT, on day 1. Antiemetic therapy with dexamethasone and 5-hydroxy-tryptamine-3 receptor antagonists was administered as a 30-min IV infusion before the administration of CDGP. TXT was diluted in 250 ml of 5% glucose and administered as a 90-min IV infusion on days 1 and 15. The protocol of this study included the criteria for starting and continuing this treatment. To start the first course of this treatment, the eligibility criteria had to be fulfilled. To receive TXT on day 15, patients were required to maintain a WBC count of more than 2000/mm^3; absolute neutrophil count of more than 1000/mm^3; platelet count of more than 7.5×10^4/mm^3; and serum creatinine of less than 1.6 mg/dl. To start the next course, patients were required to maintain a WBC count of more than 3000/mm^3; absolute neutrophil count of more than 1500/mm^3; platelet count of more than 10×10^4/mm^3; and serum creatinine of less than 1.5 mg/dl. If these conditions were not fulfilled, TXT on day 15 and the next course was administered after recovery from these toxicities. If patients did not recover within 2 weeks, they were withdrawn from the study.

Five escalating dose levels of TXT/CDGP were prepared (Table 1). Level 1 was the starting dosage level, but level 0 was also prepared, because level 1 could have been the MTD. The starting doses of TXT and CDGP were 30 mg/m^2 and 80 mg/m^2, respectively. The initial dose of TXT was half

Table 1. Dose escalation scheme

Dosage level	TXT (mg/m^2)	CDGP (mg/m^2)	No. of enrolled patients
0	30	70	none
1	30	80	6
2	35	80	6
3	35	90	none
4	40	90	none

Five dose levels of docetaxel (TXT) / nedaplatin (CDGP) were prepared. Level 1 was the starting dosage level, but level 0 was also prepared, because level 1 may have been the MTD

the dose approved in Japan. The initial dose of CDGP was based on the lower limit of the dose recommended by the Japanese Government Health Care insurance. Individual drug escalations were alternated, and at least three new patients were entered at each level. Dose escalation was not allowed in individual patients.

Dose-limiting toxicity (DLT)

All toxicities were graded according to the Japanese version of the National Cancer Institute common toxicity criteria (NCI-CTC).[21] DLT was defined as follows: (1) grade 4 neutropenia for 3 days or more; (2) grade 3 febrile neutropenia; (3) grade 4 thrombocytopenia or anemia; (4) more than grade 2 renal toxicity; (5) grade 3 or 4 non-hematologic toxicity, except for alopecia; and (6) delay of more than 14 days in carrying out any treatment or in initiating the second cycle of therapy and discontinuation of this protocol treatment due to hematologic adverse effects. For purposes of determining the MTD, only DLTs occurring during the first cycle of therapy were considered. If one patient at a dose level experienced DLT, then three additional patients were treated at the same dose level. The MTD was defined as the dose level that resulted in two of six patients developing the DLTs. The recommended dose was to be the dose immediately below the MTD.

Pretreatment and follow-up studies

Pretreatment evaluation included complete patient histories, physical examinations, complete blood cell counts, biochemistry involving liver and renal functions, urinalysis, tumor markers (e.g., squamous cell carcinoma; [SCC]), electrocardiogram, esophagogastro-endoscopy, and radiologic studies (roentgenograms, CT scans and magnetic resonance imaging [MRI]). Bone scintigraphy was performed if serum alkaline phosphatase was elevated, and audiography was performed if clinically indicated.

While the patients were receiving the treatment course, complete blood cell counts, biochemistry involving liver and renal function, and urinalysis were performed weekly. If necessary, other appropriate examinations were added. CT scans were performed after every course to assess tumor response, although that was not the purpose of this study. Esophagogastro-endoscopy and the measurement of tumor markers were also performed if considered necessary. Tumor responses of measurable or assessable lesions were evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines.[22] Recurrences or residues in the esophagus were also evaluated by the criteria of the Japanese Society for Esophageal Diseases.[23]

Table 2. Patient characteristics

Characteristic	No. of patients
Patients enrolled	12
Male	11
Female	1
Age (years)	
Median	70
Range	54–75
Clinical stage prior to CRT	
II	4
III	7
IVa	1
ECOG performance score	
0	5
1	5
2	2
Prior treatment except for CRT	2
Salvage surgery	1
Bypass surgery	1
Sites of lesions	
Esophagus	5
Lymph node	8
Cervix	1
Mediastinum	5
Abdomen	2
Lung	3
Liver	1
Pl C	1

Baseline characteristics of all 12 patients are listed. All patients had received definitive chemoradiation
ECOG, Eastern Clinical Oncology Group; CRT, chemoradiotherapy

Results

Twelve patients were enrolled in this study and patient characteristics are listed in Table 2. The patients were 11 men and one woman, and the median age was 70 years (range, 54 to 75 years). All patients had histologically confirmed squamous cell carcinomas of the esophagus before the definitive CRT, and 4, 7, and 1 patients, respectively, had stages II, III, and IVa prior to CRT. Five, 5, and 2 patients showed performance status 0, 1 and 2, respectively. All patients had previously received treatment with CDDP, 5FU, and radiation, for which the total dosages were more than 160 mg/m^2 of CDDP, more than 8 g/m^2 of 5FU, and more than 50 Gy of radiation. One patient had received a salvage operation because of a locally relapsed lesion in the esophagus after the definitive CRT, but metastases occurred in the cervical lymph nodes 6 months after the operation. One patient received a bypass operation upon demand, because the primary site was refractory to the definitive CRT and did not alleviate dysphagia. All patients