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再発小細胞肺癌に対する 標準的治療法の確立に関する研究

平成24年度　総括研究報告書

研究代表者　後 藤　功 一

平成 25（2013）年　4 月

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Ⅰ．総括研究報告

厚生労働科学研究費補助金（がん臨床研究事業）
総括研究報告書

再発小細胞肺癌に対する標準的治療法の確立に関する研究

研究代表者　後藤　功一
独立行政法人国立がん研究センター東病院
呼吸器内科外来医長

研究要旨

再発小細胞肺がんに対する標準治療の確立を目的に、標準治療と見なされているノギテカン(NGT)療法に対して、我が国で新しく開発されたシスプラチン＋エトポシド＋イリノテカン(PEI)療法の優越性を多施設共同第III相比較試験において検証する。本臨床試験は、平成19年8月 Japan Clinical Oncology Group (JCOG)プロトコール審査委員会の承認を経て、平成19年9月20日より開始となり、参加各施設における倫理審査委員会の承認を経て、平成20年1月より本格的に症例登録が始まった。症例集積ペースが予定よりもやや遅かったが、当初の集積期間 4 年間より約 1 年間遅れて、平成24年11月19日に180例の予定症例集積が完了した。今後は、2年間の追跡期間となる。

研究分担者

氏名	所属	職名
田村友秀	国立がん研究センター中央病院	科長
森　清志	栃木県立がんセンター	部長
岡本浩明	横浜市立市民病院	部長
山本信之	静岡県立静岡がんセンター	副院長
横山　晶	新潟県立がんセンター新潟病院	院長
樋田豊明	愛知県がんセンター中央病院	部長
今村文生	大阪府立成人病センター	部長
中川和彦	近畿大学医学部	教授
武田晃司	大阪市立総合医療センター	部長
木浦勝行	岡山大学大学院 医歯薬学総合研究科	教授
細見幸生	東京都立駒込病院	医長
里内美弥子	兵庫県立がんセンター	部長
近森研一	山口宇部医療センター	医長
瀬戸貴司	九州がんセンター	医師
工藤新三	大阪市立大学大学院医学研究科	准教授
湊　浩一	群馬県立がんセンター	医療局長
澤　祥幸	岐阜市民病院	部長
西尾誠人	がん研究会有明病院	副部長
野上尚之	四国がんセンター	医長

A. 研究目的

再発小細胞肺がん（初回治療が奏効して、治療終了から 90 日以上経過して再発を認めた sensitive relapse）を対象にして、現在の標準的治療法と見なされるノギテカン(NGT)療法に対するシスプラチン＋エトポシド＋イリノテカン療法(PEI 療法)の優越性を検証することを目的とする。

B. 研究方法

全国40施設の研究グループによる多施設共同第 III 相比較試験で、エンドポイントは生存期間である。生存期間中央値（MST）を8ヶ月から12ヶ月に向上させることを見込んでいる。

対象患者は、再発小細胞肺がん（初回治療が奏効して、治療終了から90日以上経過して再発を認めた sensitive relapse）であり、小細胞肺がんに対する外科的切除術の既往がなく、初回治療としてプラチナ製剤を含む併用化学療法または放射線化学療法を受けており、75才以下、ECOG Performance Status (PS) 0-2、主要臓器機能が保持されており、患者本人の自由意思による文書同意が得られた患者である。

JCOG データーセンターでの中央登録、無作為化割り付けを行う。なお、割付調整因子は、PS、再発時病期、施設である。

治療内容は、NGT 療法、あるいは PEI 療法を行う。NGT 療法は、ノギテカン 1.0 mg/m^2(day1-5)、3週間隔、4コースとする。PEI 療法は、第1週目：シスプラチン(25 mg/m^2, day 1)＋エトポシド(60 mg/m^2, day 1-3)、第2週目：シスプラチン(25 mg/m^2, day 1)＋イリノテカン(90 mg/m^2, day 1)の2週間を1コースとして5コース(計10週）の治療法である。PEI 療法は、1コース目の第8日目より G-CSF を抗癌剤投与日以外に連日投与する。

中間解析は1回、安全性モニタリングは原則年2回。予定症例数は180例で、症例集積期間は当初は4年間の予定であったが、症例集積ペースが遅いため、6年間に延長した。最新のモニタリングレポートにおいて、全患者の MST が16ヶ月であり、研究計画時に想定された10ヶ月よりもかなり良好なため、最終解析は、症例集積終了後1年から2年へ延長した。

(倫理面への配慮)

参加患者の安全性確保については、適格条件やプロトコール治療の中止変更規準を厳しく設けており、試験参加による不利益は最小化される。また、「臨床研究に関する倫理指針」およびヘルシンキ宣言などの国際的倫理原則に従い以下を遵守する。

1) 研究実施計画書の IRB 承認が得られた施設のみから患者登録を行う。

2) すべての患者に登録前に充分な説明と理解に基づく自発的同意を本人より文書で得る。

3) データの取り扱い上、直接個人が識別できる情報を用いず、かつデータベースのセキュリティを確保し、個人情報の保護を厳守する。

4) JCOG のプロトコール審査委員会、効果・安全性評価委員会、監査委員会、放射線治療委員会などによる第三者的監視を受けることを通じて、科学性と倫理性の確保に努める。

C. 研究結果

全国の肺がん臨床研究の主要 40 施設で研究グループを組織し、平成19年9月20日より本試験を開始した。症例集積ペースが予定よりもやや遅かったため、登録期間を2年間延長した結果、平成24年11月19日に目標症例数である180例の登録が完了した。今後は2年間の追跡期間となる。

平成 24 年度前期定期モニタリングレポートによる138例の解析では、NGT 療法と PEI 療法それぞれにおけるグレード3以上の好中球減少 84.3% vs 80.9%、ヘモグロビン減少 27.1% vs 79.4%、血小板減少 30.0% vs 45.6%、下痢 0% vs 10.3%、発熱性好中球減少 7.1% vs 32.4%であり、治療関連死亡は両群1例ずつ認められ、毒性は明らかに PEI 療法が強いと考えられる。一方、平成24年5月21日現在の両群合わせた155例の MST は1.39年であり、現時点で両群の差を知ることは出来ないが、高い治療効果が期待される。

なお、平成22年9月11日 JCOG 効果・安全性評価委員会の中間解析審査において本試験の継続が承認されている。

D. 考察

小細胞肺がんは全肺がんの 10-15%を占め、非小細胞肺がんに比べると化学療法や放射線療法の感受性が高く、初回治療に対する奏効率は限局型で 80-100%, 進展型で 60-80%である。しかし、80-90%の小細胞肺がんは再発を来し、5年生存率は限局型で約 25%、進展型で 0-5%であり、小細胞がん全体の5年生存率は10%未満と不良である。再発後の化学療法に対する反応は悪く、再発から死亡までの MST は 3-4 ヶ月と言われて来た。

近年、再発小細胞肺がんは、初回化学療法が奏効し、治療終了から 60-90 日以上経過して再発を認める sensitive relapse と、初回治療が奏効しない、あるいは奏効しても 60-90 日以内に再発を認める refractory relapse の2つに分類されて、臨床研究が行われてきた。これは、この2群で化学療法の効果や生存期間に差を認めるためである。例えば、NGT 療法でみると、奏効率、MST は、sensitive relapse では 14-37%、25-37週、refractory relapse では 6-11%、16-20週である。

現在までに再発小細胞肺がん(sensitive relapse)を対象とした 4 つの大規模な第 III 相試験が報告されている。NGT 療法とシクロホスファミド+アドリアマイシン+ビンクリスチン(CAV)療法を比較した第 III 相試験では、MST:25.0 週対 24.7 週と有意差を認めなかったが、再発に伴う症状の改善では NGT 療法が優れていた。NGT 療法の経口投与法と静脈投与法の比較試験では、奏効率、生存に有意差を認めず、毒性も同程度であった。また、NGT 療法の経口投与と無治療の第 III 相試験では、NGT 療法の有意な MST の延長(26 週対 14 週)を認めた。2011 米国臨床腫瘍学会(ASCO)では、NGT 療法とアムルビシン療法の第 III 相試験の結果が報告されたが、生存に有意差を認めなかった。再発小細胞肺がんに対する標準的化学療法は確立していないが、上記 4 つの第 III 相試験の結果に基づいて、世界的に NGT 療法が再発小細胞肺がんに対する標準治療とみなされている。そこで、再発小細胞肺がん(sensitive relapse)に対する標準治療の確立を目指して、NGT 療法と我々が開発した PEI 療法の第 III 相比較試験を実施し、今年度で症例集積が完了したところである。

E. 結論

「再発小細胞肺癌に対する標準的治療法の確立に関する研究」では、「再発小細胞肺癌に対する NGT 療法と PEI 療法を比較する第 III 相試験(JCOG0605)」を平成 19 年 9 月 20 日より多施設共同試験として開始し、平成 24 年 11 月 19 日に 180 例の予定症例集積が完了した。

F. 健康危険情報

厚生労働省に報告した健康危険情報なし。

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得

   なし

2. 実用新案登録

   なし

3. その他

   なし

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kubo T, Takigawa N, Osawa M, Harada D, Ninomiya T, Ochi N, Ichihara E, Yamane H, Tanimoto M, Kiura K.	Subpopulation of small-cell lung cancer cells expressing CD133 and CD87 show resistance to chemotherapy.	Cancer Sci	104(1)	78-84	2013
Rossi A, Di Maio M, Chiodini P, Rudd RM, Okamoto H, Skarlos DV, Früh M, Qian W, Tamura T, Samantas E, Shibata T, Perrone F, Gallo C, Gridelli C, Martelli O, Lee SM.	Carboplatin- or cisplatin-based chemotherapy in first-line treatment of small-cell lung cancer: the COCIS meta-analysis of individual patient data.	J Clin Oncol	30(14)	1692-98	2012
Ono A, Naito T, Ito I, Watanabe W, Shukuya T, Kenmotsu H, Tsuya A, Nakamura Y, Murakami H, Kaira K, Takahashi T, Kameya T, Nakajima T, Endo M, Yamamoto N.	Correlations between serial pro-gastrin-releasing peptide and neuron-specific enolase levels, and the radiological response to treatment and survival of patients with small-cell lung cancer.	Lung Cancer	76(3)	439-444	2012
NaitoT, Tanaka F, Ono F, Yoneda K, Takahashi T, Murakami H, Nakamura Y, Tsuya A, Kenmotsu H, Shukuya T, Kaira K, Koh Y, Endo M, Hasegawa S, Yamamoto N.	Prognostic impact of circulating tumor cells in patients with small-cell lung cancer.	J Thorac Oncol	7(3)	512-519	2012

Ogawa S, Horio Y, Yatabe Y, Fukui T, Ito S, Hasegawa Y, Mitsudomi T, Hida T.	Patterns of recurrence and outcome in patients with surgically resected small cell lung cancer.	Int J Clin Oncol	17(3)	218-224	2012
Ochi N, Hotta K, Takigawa N, Oze I, Fujiwara Y, Ichihara E, Hisamoto A, Tabata M, Tanimoto M, Kiura K.	Treatment-related death in patients with small-cell lung cancer in phase III trials over the last two decades.	PLoS One	7(8)	e42798	2012
Fujii M, Hotta K, Takigawa N, Hisamoto A, Ichihara E, Tabata M, Tanimoto M, Kiura K.	Influence of the timing of tumor regression after the initiation of chemoradiotherapy on prognosis in patients with limited-disease small-cell lung cancer achieving objective response.	Lung Cancer	78(1)	107-111	2012
Tsuchida T, Yamane H, Ochi N, Tabayashi T, Hiraki A, Nogami N, Takigawa N, Kiura K, Tanimoto M.	Cytotoxicity of activated natural killer cells and expression of adhesion molecules in small-cell lung cancer.	Anticancer Res	32	887-892	2012

Ⅲ. 研究成果の刊行物・別刷

Cancer Science
The official journal of the Japanese Cancer Association JCA

Subpopulation of small-cell lung cancer cells expressing CD133 and CD87 show resistance to chemotherapy

Toshio Kubo,[1] Nagio Takigawa,[2,4] Masahiro Osawa,[1] Daijiro Harada,[1] Takashi Ninomiya,[1] Nobuaki Ochi,[1] Eiki Ichihara,[1] Hiromichi Yamane,[2] Mitsune Tanimoto[1] and Katsuyuki Kiura[3]

[1]Department of Hematology, Oncology, Respiratory Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama; [2]Department of General Internal Medicine 4, Kawasaki Medical School, Okayama; [3]Department of Respiratory Medicine, Okayama University Hospital, Okayama, Japan

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Tumors are presumed to contain a small population of cancer stem cells (CSCs) that initiate tumor growth and promote tumor spreading. Multidrug resistance in CSCs is thought to allow the tumor to evade conventional therapy. This study focused on expression of CD133 and CD87 because CD133 is a putative marker of CSCs in some cancers including lung, and CD87 is associated with a stem-cell-like property in small-cell lung cancer (SCLC). Six SCLC cell lines were used. The expression levels of CD133 and CD87 were analyzed by real-time quantitative reverse transcription-polymerase chain reaction and flow cytometry. CD133+/− and CD87+/− cells were isolated by flow cytometry. The drug sensitivities were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Non-obese diabetic/severe combined immunodeficiency mice were used for the tumor formation assay. SBC-7 cells showed the highest expression levels of both CD133 and CD87 among the cell lines. CD133−/CD87−, CD133+/CD87−, and CD133−/CD87+ cells were isolated from SBC-7 cells; however, CD133+/CD87+ cells could not be obtained. Both CD133+/CD87− and CD133−/CD87+ subpopulations showed a higher resistance to etoposide and paclitaxel and greater re-populating ability than the CD133−/CD87− subpopulation. CD133+/CD87− cells contained more G0 quiescent cells than CD133−/CD87− cells. By contrast, CD133−/CD87− cells showed the highest tumorigenic potential. In conclusion, both CD133 and CD87 proved to be inadequate markers for CSCs; however, they might be beneficial for predicting resistance to chemotherapy. (*Cancer Sci* 2013; 104: 78–84)

Small-cell lung cancer (SCLC) is highly sensitive to chemotherapy. More than 80% of patients achieve an objective response; however, most responders eventually relapse because of drug resistance. Less than 30% of patients with limited disease and 1–2% of patients with extensive disease survive to 5 years.[1]

Cancer stem cells (CSCs) have been proposed as one of the causes of treatment resistibility. Cancer stem cells are a rare population of undifferentiated cells that are responsible for tumor initiation, maintenance, and spreading. They are resistant to anticancer agents and can self-renew and generate progeny in the form of differentiated cells that constitute most of the cells in tumors.[2,3] Because a surviving population of CSCs after conventional treatment might be responsible for tumor regrowth, identifying and eradicating the CSC population are very important.

Cancer stem cells were isolated initially from leukemia and subsequently from solid tumors, including brain, breast, prostate, colon, and liver cancer.[2–6] The methods used to isolate CSCs include cell surface marker analysis,[2–6] side-population analysis,[7] and the sphere-formation assay.[5,8] Putative CSC markers were reported to be CD34-positive/CD38-negative for acute myeloid leukemia, CD44-positive/CD24-negative/α2β1-low/Lin-negative for breast cancer, CD44-positive/α2β1-high/CD133-positive for prostate cancer, and CD133-positive/nestin-positive for brain cancer.[9] The present study focused on expression of CD133 and CD87 as putative cell-surface markers. CD133 is reported to be a marker of CSCs in some cancers, such as brain, prostate, and colorectal cancer.[3–5] Freshly dissociated human SCLC and non-small-cell lung cancer contain CD133-positive cells, which could generate long-term lung tumor spheres *in vitro* that could both differentiate and preferentially form tumors *in vivo*.[8] However, CD133 was reported to be both a positive and a negative marker of CSCs in lung cancer.[10,11] Meanwhile, in human SCLC cell lines, a small population of urokinase plasminogen activator receptor (uPAR/CD87)-positive cells was identified, of which a subset demonstrated enhanced clonogenic activity *in vitro*.[12] CD87 has been implicated in the growth, metastasis, and angiogenesis of several solid and hematologic malignancies, and its increase was associated with a poor clinical outcome.[13] Targeting CD87 can have broad-spectrum antitumor effects.[14]

We hypothesized that both CD133 and CD87 might be useful as CSCs markers in SCLC. To test this hypothesis, we investigated the expression levels of CD133 and CD87 using six SCLC cell lines. Additionally, we examined whether amrubicin might be effective for such cancer stem-like cells because it was demonstrated to be effective for refractory SCLC patients.[15]

Material and Methods

Drugs. Drugs were obtained from the following sources: cisplatin and amrubicinol from Nippon Kayaku (Tokyo, Japan); etoposide and paclitaxel from Bristol-Myers Squibb (Tokyo, Japan); 7-ethyl-10-hydroxy-campthothecin (SN-38), an active metabolite of irinotecan, from Yakult Honsha Co. Ltd. (Tokyo, Japan); and 3-[4,5-dimethyl-thizol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) from Sigma Chemical Co. (St. Louis, MO, USA).

Cell culture. The SBC-3, 4, 5, 6, 7, and 9 cell lines were established in our laboratory from SCLC patients.[16] The SBC-3 cell line was derived from bone marrow aspirates of an untreated patient.[17] The other cell lines were established from pleural effusion or pericardial effusion of patients who had

[4]To whom correspondence should be addressed.
E-mail: ntakigaw@med.kawasaki-m.ac.jp

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received chemotherapy. All cell lines were characterized by Tsuchida *et al.*,[18] and some were stored at the Japanese Collection of Research Bioresources (http://cellbank.nibio.go.jp/cellbank.html). These cell lines were cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin in a tissue culture incubator at 37°C under 5% CO_2.

Reverse transcription-polymerase chain reaction. RNA samples were prepared for reverse transcription-polymerase chain reaction (RT-PCR) using an RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol, and cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Duplex TaqMan real-time PCR was used to analyze the CD133 and CD87 expression levels in each cell line using an ABI PRISM 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Sequences of the Taqman probe and primers for CD133, CD87, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: CD133: Taqman probe (5′-FAM-TGGCATCGTGCAAACCTGTGGCC-TAMRA-3′), forward primer (5′-AGTGGATCGAGTTCTCTATCA GTG-3′), reverse primer (5′-CAGTAGCTTTTCCTATGCCAA ACC-3′); CD87: Taqman probe (5′-FAM-ACAGCCCGGCC AGAGTTGCCCT-TAMRA-3′), forward primer (5′-CCACTCA GAGAAGACCAACAGG-3′), reverse primer (5′-GGTAACGG CTTCGGGAATAGG-3′). GAPDH was co-amplified in the same reaction mixture as an endogenous reference gene. Sequences of the probe and primers for GAPDH were as follows: Taqman probe: 5′-FAM-CGTCGCCAGCCGAGCCA-CATCG-TAMRA-3′; forward primer: 5′-CGACAGTCAGCC GCATCTTC-3′; and reverse primer: 5′-CGACCTTCACCTT CCCCATG-3′. The average levels of CD133 and CD87 expression were determined from differences in the threshold amplification cycles between CD133 and CD87 and GAPDH.

Flow cytometry. Cells were harvested and re-suspended at 1×10^6 cells/mL of staining buffer. Fluorescent-labeled monoclonal antibodies were added in concentrations recommended by the manufacturer. After washing, the labeled cells were analyzed and sorted using a FACS Aria flow cytometer (Becton Dickinson, Mountain View, CA, USA). The antibodies used were allophycocyanin (APC)-conjugated mouse anti-human CD133 (Clone AC 133; Miltenyi Biotec, Auburn, CA, USA) and FITC-conjugated mouse anti-human uPAR (CD87; American Diagnostica, Stamford, CT, USA) and phycoerythrin (PE)-conjugated mouse anti-human MDR1 (eBioscience, San Diego, CA, USA). Gating was implemented on the basis of negative-control staining profiles. The sort was performed in four-way purity mode (the purity was >98%). The cell-cycle analysis was performed after staining with Hoechst 33342 and Pyronin Y (Sigma-Aldrich, St. Louis, MO, USA). Cells were stained according to the manufacturer's instructions.

Limiting dilution assay. To determine the clonogenicity and regenerative ability of single cells, a limiting dilution assay was carried out. The cells were resuspended in fresh medium, diluted to 3 cells/mL, and seeded at approximately 0.3 cells/well with 100 μL of medium into 96-well plates. Wells containing no cells or more than one cell were excluded after careful microscopic examinations, and those containing a single cell were marked and monitored daily under a microscope. After colony formation, the colonies were counted, dissociated, harvested, and cultured again.

Cell proliferation assay. Cell proliferation was examined on days 1, 2, 3, and 4. Isolated cells (1×10^5) were seeded in a cell culture flask at a final volume of 5 mL. After incubation, proliferation was evaluated by enumerating cells. Growth inhibition was determined using a modified MTT dye reduction assay with Cell Counting Kit-8 (Dojindo, Tokyo, Japan). Briefly, cells were plated on 96-well plates at a density of 3000 cells per well with RPMI 1640 with 10% FBS. Several concentrations of each drug were added to wells, and incubation was continued for 72 h. MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was then added to all wells, and incubation was continued for a further 2 h. After the dark blue crystals had dissolved, the absorbance was measured with a microplate reader. The percentage of growth is shown relative to that of untreated controls. Each assay was performed in triplicate or quadruplicate. The mean ± standard error of the 50% inhibitory concentration (IC_{50}) of the drugs in cells was determined.

Immunoblotting. Proteins were extracted from each cell line and incubated in lysis buffer [1% Triton X-100, 0.1% SDS, 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerol phosphate, 10 mM NaF, and 1 mM Na-orthovanadate] containing protease inhibitors (Roche Diagnostics, Basel, Switzerland) and centrifuged at 20 630*g* for 20 min at 4°C. Proteins were separated by SDS-PAGE using 5–15% precast gels (Bio-Rad, Hercules, CA, USA) and transferred onto nitrocellulose membranes. Specific proteins were detected by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) using the antibodies to aldehyde dehydrogenase 1A1 (1:100 dilution; Abcam, Cambridge, MA) and β-actin (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA). The secondary antibody; anti rabbit IgG (HRP-linked, species-specific whole antibody) (GE Healthcare), was used at a 1:5000 dilution.

Xenograft model. Sorted cells were injected subcutaneously into the backs of 7-week-old female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (Charles River, Yokohama, Japan). Groups of mice were inoculated with CD133+/CD87−, CD133−/CD87+, or CD133−/CD87− cells at 5×10^3 and 2×10^3 cells. Tumor growth was monitored twice per week, and tumor volume ($\text{width}^2 \times \text{length}/2$) was determined periodically. A lack of tumor formation at 8 weeks after sorted-cell injection was described as "no tumor formation".

Statistical analysis. The differences between the groups were compared using Student's *t*-test and χ^2 test. $P < 0.05$ was considered statistically significant. All data were analyzed using Microsoft Office Excel 2007 (Microsoft Japan Corporation, Tokyo, Japan).

Results

SBC-7 cells showed high expression levels of both CD133 and CD87. Expression levels of CD133 and CD87 mRNA by real-time quantitative RT-PCR were determined. SBC-7 cells showed the highest expression of both CD133 and CD87 among the six cell lines. SBC-9 cells also showed both CD133 and CD87 expression, and SBC-4 and SBC-5 cells showed expression of only CD133 and CD87, respectively. SBC-3 cells demonstrated neither CD133 nor CD87 expression (Fig. 1a). We confirmed expression of CD133 and CD87 in each cell line by flow cytometry (Fig. 1b,c). SBC-7 cells displayed some subpopulations: CD133+/CD87− (41.1%), CD133−/CD87+ (10.1%), and CD133−/CD87− (48.3%); however, CD133+/87+ double-positive cells were very rare (0.6%). The cell-surface expression of CD133 was confirmed in SBC-7 and SBC-9, and that of CD87 was in SBC-5 and SBC-7, respectively. Although there seemed to be a correlation between the mRNA levels and cell surface expressions, cell surface expression was not detected at moderate mRNA levels, such as CD133 in SBC-4 and CD87 in SBC-9. Because only SBC-7 cells showed both CD133 and CD87 expressions in flow cytometry analysis, we selected SBC-7 cells and investigated their characteristics as CSCs.

CD133+/CD87− and CD133−/CD87+ subpopulations showed repopulating ability. We used SBC-7 cell lines and examined the properties of each subpopulation. To compare the re-populating ability of each subpopulation, we sorted the CD133+/

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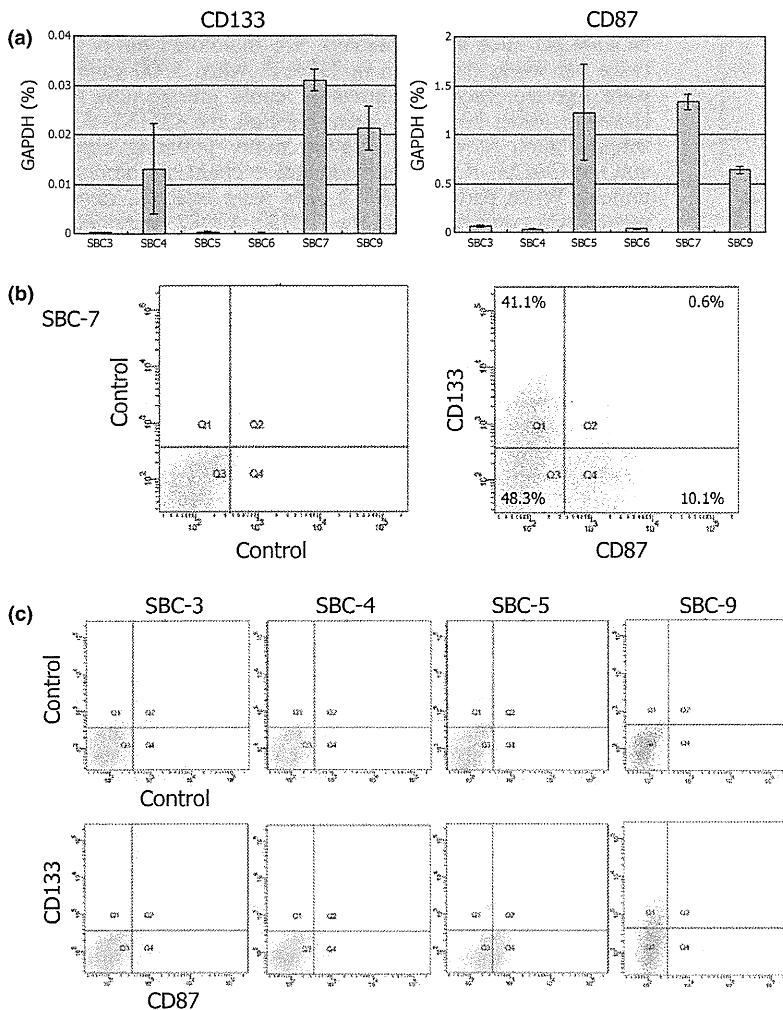

	SBC-3	SBC-4	SBC-5	SBC-9
CD133+ / 87+	0 %	0.1 %	0.1 %	0.1 %
CD133+ / 87–	0 %	0 %	0.1 %	14.4 %
CD133–/ 87+	0.1 %	0.3 %	26.5 %	0.3 %
CD133–/ 87-	99.9 %	99.6 %	73.4 %	85.2 %

Fig. 1. (a) The mRNA expression levels of CD133 and CD87 in each cell line using real-time quantitative reverse transcription-polymerase chain reaction. SBC-7 cells showed the highest expression levels of both CD133 and CD87 among the six cell lines. SBC-4 cells expressed only CD133, and SBC-5 cells expressed only CD87. SBC-3 cells expressed neither CD133 nor CD87. Bars indicate the standard deviation. (b) Flow cytometry analysis of SBC-7 cells stained with CD133 and CD87 antibodies. SBC-7 cells showed CD133+/CD87–, CD133–/CD87+, and CD133–/CD87– subpopulations; however, a CD133+/CD87+ subpopulation was not obtained. (c) Flow cytometry analysis of SBC-3, 4, 5, and 9 cells stained with CD133 and CD87 antibodies. SBC-5 showed a CD133–/CD87+ subpopulation. SBC-9 cells showed a CD133+/CD87– but not a CD133–/CD87+ subpopulation.

CD87–, CD133–/CD87+, CD133–/CD87–, and CD133+/CD87+ cells by flow cytometry (Suppl. Fig. S1), cloned the sorted cells with limiting dilutions, and cultured them separately under the same conditions for 6 weeks. Although we attempted to select CD133+/CD87+ cells several times, no double-positive cells could be obtained for further examination, including *in vivo* study. Therefore, we investigated the characteristics of three subpopulations: CD133+/CD87–, CD133–/CD87+, and CD133–/CD87–. We then re-stained the cultured cells with CD133 and CD87 antibodies and analyzed them by flow cytometry. The CD133+/CD87– population generated both CD133+/CD87– and CD133–/CD87– subpopulations, and the CD133–/CD87+ population generated both CD133–/CD87+ and CD133–/CD87– subpopulations. However, the CD133–/CD87– population produced only CD133–/CD87– cells. CD133+/CD87+ were not obtained from any cultured subpopulation (Fig. 2).

Drug sensitivity, cell cycle and aldehyde dehydrogenase 1A1 expression in the subpopulations. Next, we examined the sensitivity of each subpopulation to the chemotherapeutic drugs cisplatin, etoposide, paclitaxel, and 7-ethyl-10-hydroxy-camptothecin (SN-38: active metabolite of irinotecan). Cells expressing either CD133 or CD87 were more resistant to etoposide and paclitaxel than were double-negative cells (Table 1). In addition, CD133+/CD87– cells showed the highest resistance to etoposide among the three groups ($P < 0.05$). The IC_{50}s (μM) to cisplatin were 5.19 ± 0.19 in CD133–/CD87–, 3.49 ± 0.68 in CD133+/CD87–, 4.72 ± 0.64 in CD133–/CD87+, and 2.14 ± 0.22 in parent SBC-7 (Table 1). Although CD133- and CD87-positive cells tended to be more sensitive to cisplatin than double-negative cells, there was no significant difference among the cell lines tested. When compared with SBC-7 parental cells, CD133+/CD87– cells showed more resistance to etoposide ($P = 0.01$) and paclitaxel ($P = 0.02$), and CD133–/CD87+ cells were more resistance to paclitaxel ($P = 0.03$).

Additionally, we analyzed the cell cycle of each subpopulation by flow cytometry. The CD133+/CD87– subpopulation contained more G0 quiescent cells than did CD133–/CD87+ and CD133–/CD87– subpopulations (Fig. 3). Aldehyde dehydrogenase 1A1 levels seemed similar among the three subpopulations (Suppl. Fig. S2).

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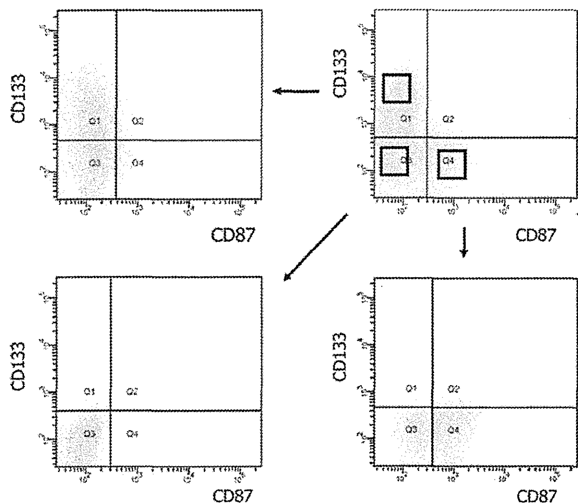

Fig. 2. Re-analysis of each subpopulation after limiting dilatation by flow cytometry. CD133+/CD87− and CD133−/CD87+ subpopulations in SBC-7 cells showed re-populating ability. However, the CD133−/CD87− subpopulation could produce only CD133−/CD87− cells.

Growth rate and MDR1 expression in the subpopulations. We also investigated the cell proliferation rates of each subpopulation (Suppl. Fig. S3). The growth rate of CD133−/CD87+ cells was greater than that of CD133−/CD87− and CD133+/CD87− cells. The growth rates of CD133−/CD87− and CD133+/CD87− cells were similar. Although rapid proliferation makes a cell line appear more drug-sensitive compared with a more-slowly growing cell line, the drug sensitivity of the SBC-7 subclones could not be explained by the growth rate alone. Next, we examined the expression levels of MDR1 on each subpopulation by flow cytometry. The expression of MDR1 was higher in CD133−/CD87+ cells than that in CD133−/CD87− cells (8.1% *vs* 3.1%) (Suppl. Fig. S4).

Drug exposure did not induce CD133 or CD87 expression. We investigated whether the expression levels of CD133 and CD87 were upregulated in cells resistant to chemotherapeutic drugs. We used the SBC-3 cell line as a parent cell, which expressed neither CD133 nor CD87, and its resistant cell lines to cisplatin, SN-38, or etoposide (SBC-3/CDDP, SBC-3/SN-38, or SBC-3/ETP, respectively).[19–21] The CD133 mRNA levels in SBC-3/CDDP and CD87 in SBC-3/ETP were slightly upregulated compared with those in SBC-3 (Fig. 4a). However, in flow cytometry analysis, there was no significant upregulation of CD133 or CD87 expression in the resistant cells (Fig. 4b). Thus, the surface expression of CD133 or CD87 at least was unlikely to be induced by the chronic exposure of chemotherapeutic drugs *in vitro*.

CD133−/CD87− subpopulations showed high tumor formation ability *in vivo*. The tumorigenic potential of each subpopulation through subcutaneous injection of each sorted cell line in NOD/SCID mice was evaluated. We monitored tumor growth twice per week. As shown in Table 2, when 5000 sorted cells were injected, each subpopulation could initiate new tumors. However, when 2000 cells were injected, the CD133−/CD87− subpopulation showed the highest tumor initiating capability, and the CD133−/CD87+ subpopulation could not produce new tumors. When parental SBC-7 cells were injected, tumor formation was confirmed as in the CD133–/CD87– subpopulation. The pathological feature of the tumors with hematoxylin-eosin staining was similar to parental SBC-7 xenograft tumors (Suppl. Fig. S5). Re-analysis of each derived tumor using CD133 and CD87 antibodies in flow cytometry showed that the surface markers of the tumor cells were similar to those of each subpopulation cultured *in vitro* (data not shown).

CD133-positive cells were also resistant to amrubicinol. Although CD133- and CD87-positive cells could not satisfy the requirements for CSCs, these cells showed chemoresistant characteristics. Additionally, CD133+/CD87− cells had higher tumorigenicity and higher resistance to chemotherapeutic drugs than CD133−/CD87+ cells. The IC_{50}s of amrubicinol in CD133-positive and -negative cells were 0.732 ± 0.119 μM and 0.172 ± 0.038 μM, respectively (P = 0.009).

Discussion

The need to target therapies at the self-renewal capacity of the stem-cell compartment, effectively interrupting the source of recurrence in tumors sensitive to conventional therapeutic approaches, has also evolved under the CSC hypothesis in the lung cancer field.[9] However, identifying a phenotypic marker in lung CSCs has been unsuccessful. In this study, we investigated whether CD133 or CD87 might be a putative marker of CSCs. At first, we examined the expression levels of CD133 and CD87 mRNA by real-time quantitative RT–PCR. Then, we confirmed the expression of CD133 and CD87 on cell surface by flow cytometry. Although there were discrepancies between the expression levels of mRNA and protein in some cell lines, such as SBC-4 and SBC-9, only SBC-7 cells displayed both CD133 and CD87 cell-surface markers. The ambivalence might be explained by following reasons. (i) Although mRNA was induced, the protein might not be detected because of small quantity; (ii) The protein might be subject to degradation easily, and (iii) It might stay in the cytoplasm and could not appear on the cell surface.

Both CD133- and CD87-positive cells showed higher resistance to chemotherapeutic drugs and a higher re-populating ability and contained more G0 quiescent cells than did the double-negative subpopulation *in vitro*. However, the double-negative subpopulation showed the highest tumor-initiating capability *in vivo*. Thus, CD133 and CD87 did not satisfy the requirements for CSCs in SCLC cells. The reason that double-negative cells showed the highest tumor-initiating capability remains unclear. We used SCLC cell lines to examine the characteristics of CD133- and CD87-positive cells. In cell

Table 1. Sensitivity of each subpopulation to chemotherapeutic drugs

	Cisplatin (μM)	SN-38 (nM)	Etoposide (μM)	Paclitaxel (nM)
CD133−/CD87−	5.19 ± 0.19	37.31 ± 5.88	5.17 ± 0.20	2.87 ± 0.08
CD133+/CD87−	3.49 ± 0.68	107.96 ± 40.24	>100 (P < 0.01)*	7.02 ± 0.36 (P = 0.01)*
CD133−/CD87+	4.72 ± 0.64	12.66 ± 2.74	23.46 ± 4.00 (P = 0.04)*	6.94 ± 0.31 (P = 0.01)*
SBC-7	2.14 ± 0.22	22.37 ± 3.59	37.54 ± 7.12	3.66 ± 0.26

SN-38, 7-ethyl-10-hydroxy-campthothecin, an active metabolite of irinotecan. The asterisk indicates that the IC_{50}s (mean ± standard error) of the CD133+/CD87− and CD133−/CD87+ subpopulations were significantly higher than that of the CD133−/CD87− subpopulation (P < 0.05). The P-value was calculated with Student's t-test compared with CD133−/CD87− subpopulation.

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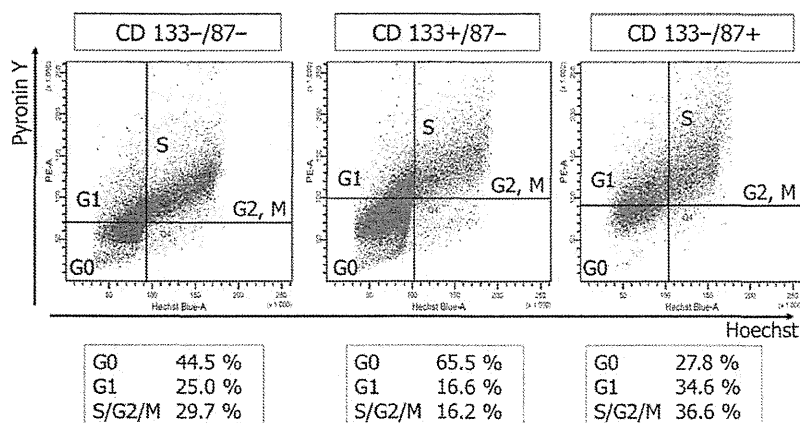

Fig. 3. Cell-cycle analysis of each subpopulation with Hoechst 33342 and Pyronin Y. The CD133+/CD87− subpopulation contained more G0 quiescent cells than did CD133−/CD87+ and CD133−/CD87− subpopulations.

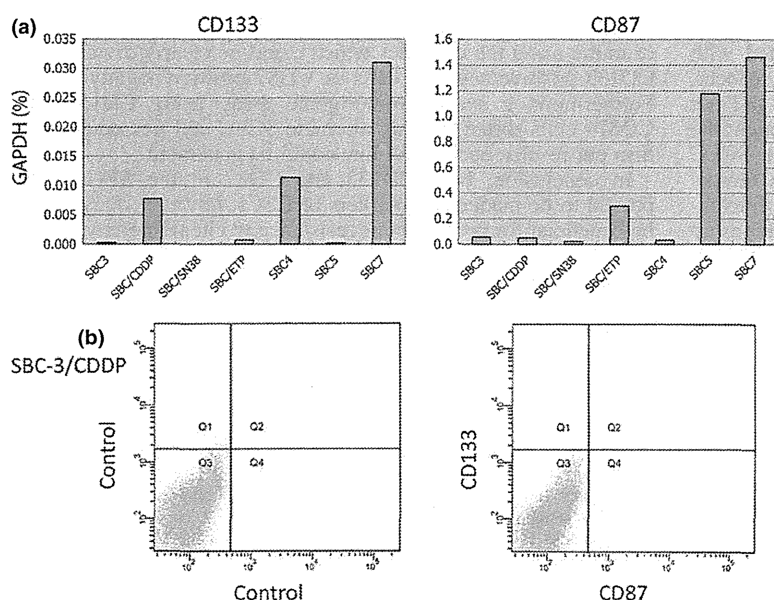

Fig. 4. (a) CD133 and CD87 mRNA levels in parental (SBC-3) and resistant (SBC-3/CDDP, SBC-3/SN38, and SBC-3/ETP) cell lines using real-time quantitative reverse transcription–polymerase chain reaction. CD133 in SBC-3/CDDP and CD87 in SBC-3/ETP were more highly expressed than those in SBC-3. (b) Flow cytometry analysis of SBC-3/CDDP cells stained with CD133 and CD87 antibodies. The expression of CD133 or CD87 was not increased in resistant cells.

Table 2. Tumorigenicity of sorted subpopulations

Injected cell numbers	2×10^3	5×10^3
CD133−/CD87−	3/3 (100%) $P = 0.014$	6/6 (100%) $P = 0.121$
CD133+/CD87−	1/2 (50%) $P = 0.171$	7/7 (100%) $P = 0.089$
CD133−/CD87+	0/3 (0%)	4/6 (67%)

Data are presented as number of tumors/injections of sorted cells. The *P*-value was calculated with χ-test comparing each sub-population to CD133-/CD87+ population.

lines, the characteristics of tumor cells can be changed from primary cultured cells or fresh cells; thus, the double-negative subpopulations might acquire some specific ability to initiate new tumors. In addition, Meng *et al.* previously reported that lung cancer cell lines regardless of CD133 expression could initiate new tumors in nude mice.[11] Thus, CD133 alone might not be useful as a stem cell marker for lung cancer.

Particularly, because CD133-positive cells showed a higher tumor-initiating capability than CD87-positive cells, we investigated the strategy to overcome the resistance to conventional chemotherapy in CD133-positive cells. Amrubicin, a synthetic 9-aminoanthracycline, is converted to the active metabolite amrubicinol via reduction of its C-13 ketone group to a hydroxyl group by carbonyl reductase.[22] Adriamycin-resistant cells show partial resistance to amrubicin *in vitro.*[23] Phase II studies of previously treated SCLC patients showed that amrubicin was effective in both sensitive and refractory relapse.[15] Unfortunately, CD133-positive cells were 4.3 times more resistant to amrubicinol than were CD133-negative cells.

In the present study, both CD133 and CD87 proved to be inadequate markers for CSCs; however, they seemed to predict resistance to chemotherapy. We could not clarify the mechanism why CD133- or CD87-positive cells showed higher resistance to etoposide and paclitaxel. Etoposide targets the cells in S/G2/M phase. CD133+/CD87− fraction, which harbored 16.2% of S/G2/M fraction, showed a higher level of IC_{50} in etoposide than CD133−/CD87− containing 29.7% of that fraction. However, CD133−/CD87+ fraction, which harbored higher levels S/G2/M phase was also more resistant against etoposide compared with CD133−/CD87−. Therefore, the resistant mechanism of CD133 or CD87 was not clarified only by cell cycle analysis. Gutova *et al.* reported that CD87-positive cells

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showed higher expression of MDR1.[12] In our study, the expression level of MDR1 was higher in CD133−/CD87+ subpopulation. However, the expression rate of MDR1 (8.1%) was lower than that (10–40%) in their report.[12] Chen *et al.* indicated that CD133-positive cells were highly co-expressed with ABCG2 transporter and were significantly resistant to conventional treatment methods compared with CD133-negative non-small-cell lung cancer cells.[24] Thus, the CD133- or CD87-positive subpopulation in SBC-7 might be related to drug resistance. Meanwhile, cisplatin seemed effective irrespective of the CD133 or CD87 status because cisplatin resistance was not associated with MDR1 or ABCG2 overexpression.[25,26] The surface expressions of both CD133 and CD87 were not increased after chronic exposure of SBC-3 cells to chemotherapeutic drugs, resulting in acquisition of resistance. The upregulation of CD133 or CD87 expression might be a part of a complicated chemoresistance mechanism.

Increased levels of urokinase plasminogen activator and its receptor CD87 were strongly correlated with poor prognosis and unfavorable clinical outcome in patients with acute myeloid leukemia and breast cancer.[13] In many solid tumors, such as glioblastoma, the presence of CD133 was correlated with poor survival.[3] In patients with non-small cell lung cancer, CD133 was indicative of a resistance phenotype, but did not represent a prognostic marker for survival.[27] Although the clinical outcome of CD133 or CD87 expression in SCLC patients remains unclear, our data suggested that the tumors expressing CD133 and/or CD87 might be resistant to conventional chemotherapy. To prove the hypothesis, the relationship between CD133 and/or CD87 expression levels on human SCLC materials and corresponding chemosensitivity should be investigated. The drugs should be screened for their ability to overcome the resistant SCLC cells.

The limitation of our study was that we were unable to generate CD133+/CD87+ double-positive cells, which might have true CSC characteristics. Thus efficient sorting of a small population of double-positive cells for *in vivo* experimentation is necessary. Characterization of the CD133+/CD87+ might be relevant for this study and could reveal some remarkable properties of this subset (e.g., an enhanced tumorigenic ability) compared with single-positive CD133 or CD87 fractions. In addition, we extensively examined the SBC-7 line, which was the only cell line that exhibited surface expression of both CD133 and CD87 among the cells we used. We tried to confirm that CD133 or CD87 positive cells showed higher chemoresistance than negative cells using the SBC-9 cells. SBC-9 cells were divided into CD133+/CD87− and CD133−/CD87− subpopulations. Unfortunately, CD87 positive cells in the SBC-9 cells were not obtained because it might be due to the small amount of the cells (0.4%). We investigated cell viability of both subpopulations after 96 h exposure to cisplatin, etoposide and paclitaxel at the IC_{50} of each drug for the SBC-9 cells. CD133+/CD87− cells were resistant to only etoposide than CD133−/CD87− cells (Suppl. Fig. S6). We should further examine using the cell lines which could be clearly divided into CD133-positive/negative cells or CD87-positive/negative cells. Furthermore, a second tumorigenic assay using CD133+and CD87+ cells sorted from an alternate SCLC cell line could confirm our results; such a cell line could be generated.

In conclusion, both CD133 and CD87 in the SBC-7 line proved to be inadequate markers of CSCs; however, they might be beneficial for prediction of resistance to chemotherapy.

Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. CD133 and CD87 expression and sort position in SBC-7 cell line.

Fig. S2. The expression levels of aldehyde dehydrogenase 1A1 (ALDH1A1) in each subpopulation by western blotting.

Fig. S3. Growth curves of each subpopulation.

Fig. S4. The cell surface expression levels of MDR1 on each subpopulation by flow cytometry.

Fig. S5. Hematoxylin-eosin staining of xenograft tumors.

Fig. S6. The cell viability of CD133+/CD87− cells and CD133−/CD87− cells in the SBC-9 after treatment with cisplatin, etoposide or paclitaxel.

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ORIGINAL REPORT

Carboplatin- or Cisplatin-Based Chemotherapy in First-Line Treatment of Small-Cell Lung Cancer: The COCIS Meta-Analysis of Individual Patient Data

Antonio Rossi, Massimo Di Maio, Paolo Chiodini, Robin Michael Rudd, Hiroaki Okamoto, Dimosthenis Vasilios Skarlos, Martin Früh, Wendi Qian, Tomohide Tamura, Epaminondas Samantas, Taro Shibata, Francesco Perrone, Ciro Gallo, Cesare Gridelli, Olga Martelli, and Siow-Ming Lee

ABSTRACT

Purpose
Since treatment efficacy of cisplatin- or carboplatin-based chemotherapy in the first-line treatment of small-cell lung cancer (SCLC) remains contentious, a meta-analysis of individual patient data was performed to compare the two treatments.

Patients and Methods
A systematic review identified randomized trials comparing cisplatin with carboplatin in the first-line treatment of SCLC. Individual patient data were obtained from coordinating centers of all eligible trials. The primary end point was overall survival (OS). All statistical analyses were stratified by trial. Secondary end points were progression-free survival (PFS), objective response rate (ORR), and treatment toxicity. OS and PFS curves were compared by using the log-rank test. ORR was compared by using the Mantel-Haenszel test.

Results
Four eligible trials with 663 patients (328 assigned to cisplatin and 335 to carboplatin) were included in the analysis. Median OS was 9.6 months for cisplatin and 9.4 months for carboplatin (hazard ratio [HR], 1.08; 95% CI, 0.92 to 1.27; P = .37). There was no evidence of treatment difference between the cisplatin and carboplatin arms according to sex, stage, performance status, or age. Median PFS was 5.5 and 5.3 months for cisplatin and carboplatin, respectively (HR, 1.10; 95% CI, 0.94 to 1.29; P = .25). ORR was 67.1% and 66.0%, respectively (relative risk, 0.98; 95% CI, 0.84 to 1.16; P = .83). Toxicity profile was significantly different for each of the arms: hematologic toxicity was higher with carboplatin, and nonhematologic toxicity was higher with cisplatin.

Conclusion
Our meta-analysis of individual patient data suggests no differences in efficacy between cisplatin and carboplatin in the first-line treatment of SCLC, but there are differences in the toxicity profile.

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Antonio Rossi and Cesare Gridelli, S.G. Moscati Hospital, Avellino; Massimo Di Maio and Francesco Perrone, National Cancer Institute, "G. Pascale" Foundation, Naples; Paolo Chiodini and Ciro Gallo, Medical Statistics, Second University, Naples; Olga Martelli, San Giovanni-Addolorata Hospital, Rome, Italy; Robin Michael Rudd, London Lung Cancer Group; Wendi Qian, Medical Research Council Clinical Trials Unit; Siow-Ming Lee, University College London (UCL) Cancer Institute and UCL Hospitals, London, UK; Hiroaki Okamoto, Yokohama Municipal Citizen's Hospital, Yokohama; Tomohide Tamura, National Cancer Center Hospital; Taro Shibata, Japan Clinical Oncology Group Data Center, National Cancer Center, Tokyo, Japan; Dimosthenis Vasilios Skarlos, Metropolitan Hospital; Epaminondas Samantas, Agioi Anargiroi Hospital, Athens, Greece; and Martin Früh, Kantonsspital, St. Gallen, Switzerland.

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A.R. and M.D.M. contributed equally to this work.

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Corresponding author: Antonio Rossi, MD, Division of Medical Oncology, S.G. Moscati Hospital, Avellino, Città Ospedaliera, Contrada Amoretta, 8, Avellino, Italy 83100; e-mail: arossi_it@yahoo.it.

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INTRODUCTION

Small-cell lung cancer (SCLC) accounts for approximately 15% of all lung cancers. At presentation, approximately 70% of patients are diagnosed as having extensive disease and the remaining patients are diagnosed as having limited disease.[1]

The main international guidelines recommend platinum-based chemotherapy as the standard of care for first-line therapy of SCLC.[2-4] However, whether cisplatin or carboplatin are equally effective in the treatment of SCLC is still contentious. These two platinum compounds have different toxicity profiles. Cisplatin is associated with more GI adverse effects, neurotoxicity, and renal function impairment, and its administration requires a prolonged hydration,[5,6] but carboplatin is associated with more myelosuppression.

Although the mechanisms of action are similar, it is unclear whether carboplatin and cisplatin have the same clinical efficacy. For some tumors such as ovarian cancer, randomized studies[7,8] supported the use of carboplatin instead of cisplatin; for other tumors, such as germ cell and head and neck tumors, cisplatin is superior to carboplatin.[9] Several meta-analyses have addressed the issue of cisplatin-based versus carboplatin-based chemotherapy in the first-line treatment of advanced non–small-cell lung cancer. Cisplatin-based regimens resulted in slightly superior outcomes compared

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