response with chemoradiotherapy might also contribute to avoiding a pneumonectomy. Regarding the identification of pathological downstaging as a prognostic factor in our study, many studies have reported similar results [17, 18].

Our study showed that while no significant difference in the rate of distant metastasis was observed between the chemoradiotherapy group (34.3%) and the chemotherapy group (46.7%), the rate of distant relapse seems to be lower in the chemoradiotherapy group than in the chemotherapy group. Whether radiotherapy is necessary for patients receiving induction chemotherapy if the local disease lesion is controlled by surgical resection has been a point of discussion, since thoracic radiation does not seem to directly affect pre-existing micrometastases at distant sites. When considering distant metastasis, two possibilities should be taken into account: (i) the presence of micrometastasis before induction therapy, and (ii) the occurrence of micrometastasis during induction therapy and prior to surgical resection. The fact that the incidence of distant metastasis seemed to be lower in the chemoradiotherapy group implied that intensive local therapy may reduce the chance of micrometastasis during induction therapy. From this perspective, the addition of radiotherapy to chemotherapy may lower the rate of distant metastasis, compared with that for chemotherapy alone. The other possibility explaining the difference in the rates of distant metastasis is the difference in the chemotherapeutic regimens used for the induction therapies in our study.

We did not analyze the difference in surgical outcomes such as operating time and length of hospital stay between the chemotherapy and chemoradiotherapy groups. Our impression is that the surgery after chemoradiotherapy is technically more demanding compared with that after chemotherapy. For example, mediastinal lymph node dissection after a mediastinoscopy is often hard in cases after chemoradiotherapy. In addition, we basically use the greater omentum to cover anastomosis for sleeve bronchial resection after chemoradiotherapy. For these cases, operating time was longer in chemoradiotherapy than in chemotherapy. Regarding the usage of the omental flap to cover anastomosis, there may be the criticism that a laparotomy for omental harvest is too invasive. However, we intended to make a maximum effort to prevent anastomotic complication because the surgical outcome could be unfavorable when the bronchopleural fistula occurs in patients after induction therapy. Of note, the omental flap could not always prevent the bronchopleural fistula, suggesting the importance of appropriate surgery [19]. Recently, we have been using laparoscopic surgery to harvest the greater omentum in order to reduce the invasiveness of laparotomy.

To enable our study to be interpreted appropriately, its strengths and limitations need to be considered. A strength of the current report is that its subjects comprised consecutive patients who were prospectively cared for by the same treatment team according to preset institutional treatment policies. On the other hand, some of the inherent limitations of our study include its lack of a randomized design and chronological differences in the induction chemotherapy (1995–1999) and chemoradiotherapy (1999–2010) regimens and the discrepancy in sample size between the chemotherapy and chemoradiotherapy groups, especially the small number of the chemotherapy group. However, a randomized study comparing chemoradiotherapy followed by surgery with chemotherapy followed by surgery was closed due to the lack of interested participants (Radiation Therapy Oncology Group–0412). While the chronological difference may have affected the longer OS in the chemoradiotherapy group, compared with that in the chemotherapy group, the fact that a lower rate of incomplete resection, a favorable DFS time, and a more advanced disease stage were observed in the chemoradiotherapy group, compared with the chemotherapy group, seems to support the advantage of induction chemoradiotherapy in this patient population.

As one of the future directions of the induction therapy, identifying clinicopathological or biological factors that can be indicators of the induction regimen is critical for further improvement of therapeutic outcomes. Our sample size presented here was not large enough for this analysis, but data accumulation will lead to the establishment of a personalized induction therapy for NSCLC.

In conclusion, our results indicate that induction chemoradiotherapy is superior to induction chemotherapy with regard to the outcome of NSCLC patients with mediastinal lymph node metastasis.

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ORIGINAL ARTICLE

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Original Study

Knockdown of the *Epidermal Growth Factor Receptor* Gene to Investigate Its Therapeutic Potential for the Treatment of Non–Small-Cell Lung Cancers

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Epidermal growth factor receptor (EGFR) can be a therapeutic target in non–small-cell lung cancer with EGFR activation, even if *EGFR* mutation is not present. By contrast, EGFR cannot be a target when EGFR is not activated, even if EGFR protein is expressed. For acquired resistant cells to EGFR–tyrosine kinase inhibitors, EGFR can be a target if those cells depend on EGFR and are not driven by other oncogenes.

Background: Epidermal growth factor receptor (EGFR) is often overexpressed in non–small-cell lung cancer (NSCLC). Anti-EGFR agents, including EGFR-tyrosine kinase inhibitors are considered to be effective when a drug-sensitive *EGFR* mutation is present. However, inherent and acquired resistances are major problems of EGFR-targeting therapies. In this study, we performed *EGFR* knockdown by using small interfering RNAs in NSCLC cell lines to examine the significance of targeting EGFR for NSCLC therapy. **Methods:** We treated 13 NSCLC cell lines, including 8 *EGFR* mutant and 5 *EGFR* wild type by using gefitinib or small interfering RNAs against *EGFR* (siEGFR). Three cell lines (PC-9-GR1, RPC-9, and HCC827-ER) were experimentally established with acquired resistance to EGFR-tyrosine kinase inhibitors. The antitumor effect was determined by using an 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt (MTS) or colony formation assay. The protein expression was evaluated by using Western blotting. **Results:** All 13 cell lines expressed EGFR protein, and siEGFR downregulated EGFR protein expression in all. The cell viability was suppressed by siEGFR in 6 of 8 *EGFR*-mutant cell lines (suppressed 57%-92% of control cells), including PC-9-GR1 and RPC-9. The NCI-H1650 and HCC827-ER harbored *EGFR* mutations but were not suppressed. Of note, PTEN (phosphatase and tensin homolog) was deleted in NCI-H1650, and *c-MET* was amplified in HCC827-ER. It was not suppressed in any of the *EGFR* wild-type cells except in the NCI-H411, in which EGFR is phosphorylated, which indicates its activation. **Conclusions:** Analysis of the results indicated that EGFR can be a therapeutic target in NSCLCs with EGFR activation. In contrast, targeting EGFR is not appropriate for tumors in which EGFR is not activated, even if EGFR is expressed.

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Keywords: Epidermal growth factor receptor, Epidermal growth factor receptor–tyrosine kinase inhibitors, Non–small-cell lung cancer, Oncogene addiction, Small interfering RNA

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Introduction

Epidermal growth factor receptor (EGFR) is a transmembrane protein that consists of an extracellular ligand-binding domain, and its phosphorylation of tyrosine kinase domain causes its activation and leads to a multitude of effects, including cell proliferation, cell differentiation, angiogenesis, metastasis, and anti-apoptosis.[1] EGFR is overexpressed in various human cancers and is considered to be a therapeutic target for the treatment of cancer. Various therapeutic agents, such as anti-EGFR antibody and EGFR-tyrosine kinase inhibitors (EGFR-TKI), have been developed for clinical use. Previous reports have shown that activating *EGFR* mutations are present in non–small-cell lung cancer (NSCLC).[2,3] Activating *EGFR* mutations are known to cause oncogene addiction, and first-generation EGFR-TKIs such as gefitinib and erlotinib have shown significant antiproliferative effects against NSCLC with *EGFR* mutations.[3,4] Thus, mutant *EGFR* is an appropriate target of EGFR-TKIs.

However, in many clinical trials, targeting EGFR that uses first-generation EGFR-TKIs has not been remarkably effective against NSCLCs with wild-type *EGFR*. Cetuximab, an anti-EGFR antibody, is not effective as a monotherapy against NSCLC.[5] Furthermore, NSCLCs that initially exhibit a remarkable response to EGFR-TKIs can unexpectedly become resistant to EGFR-TKIs after the long-term use of EGFR-TKIs. In view of these observations, the inhibition of EGFR may not be a therapeutic target for NSCLCs with wild-type *EGFR* or acquired resistance.

However, whether EGFR can serve as a therapeutic target for NSCLCs with wild-type *EGFR* remains uncertain because EGFR-TKIs only target the tyrosine kinase domain and anti-EGFR antibodies only inhibit ligand binding to EGFR. In addition to its tyrosine kinase activity, EGFR can serve as a partner in heterodimer parings with other EGFR family members, such as human epidermal growth factor receptor (HER) 2 and HER3.[6] Thus, EGFR may have other functional roles in cancer cell proliferation and survival, and the disruption of EGFR by using other means may be a therapeutic strategy for the treatment of NSCLC.

RNA interference is a powerful technique for studying gene function. Small interfering RNAs (siRNA) have provided unprecedented opportunities to examine the roles of genes in many kinds of diseases. Gene-specific siRNA provides a powerful tool for demonstrating the specific knockdown of a targeted gene. Although there are many studies of the impact of EGFR-TKIs or anti-EGFR antibodies on NSCLC, the role of EGFR has rarely been studied by inhibiting EGFR protein production in cells. In the current study, we investigated the therapeutic possibility of targeting EGFR by depleting EGFR protein by using siRNA-mediated knockdown in NSCLC. In particular, we focused on NSCLC cell lines with inherent or acquired resistance to EGFR-TKIs.

Materials and Methods

Estimation of Cell Viability by Using an MTS Assay

Cell viability after gefitinib treatment and *EGFR* knockdown was estimated by using an 3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt (MTS) assay. The detailed experiment for gefitinib treatment has previously been described.[7] The cell viability after gefitinib treatment was determined on the basis of gefitinib IC_{50} (half maximal inhibitory concentration) as follows: sensitive ($IC_{50} \leq 1$ μmol/l), and resistant ($IC_{50} > 1$ μmol/l).[8] For *EGFR* knockdown, siRNAs that target *EGFR* (siEGFR) were designed through the RNAi Co Ltd (Tokyo, Japan) Web site by using the siDirect online software system and were synthesized by RNAi Co Ltd as previously described.[9] Two siEGFR that target different regions of *EGFR* were used as follows: siEGFR-1 (5′-GCU ACG AAU AUU AAA CAC UUC-3′ and 5′-AGU GUU UAA UAU UCG UAG CAU-3′) and siEGFR-2 (5′-CCG CAA AUU CCG AGA CGA AGC-3′ and 5′-UUC GUC UCG GAA UUU GCG GCA-3′) (sense and antisense, respectively). The cells were transfected with 10 nmol/l of siRNA by using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) in a 60-mm dish and were incubated for 48 hours. Control cells were treated with a scrambled negative-control siRNA sequence (siScramble). Subsequently, the cells were collected and plated in 96-well plates at a density of 3×10^3 cells per well. Cell viability was evaluated 72 hours later by using an MTS assay with CellTiter 96 Aqueous one solution Reagent (Promega, Madison, WI).[7] Cell viability was calculated as the average of values obtained by using both siEGFR-1 and siEGFR-2. The cell viability after siEGFR knockdown was determined on the basis of percentage of control cell viability treated with siScramble: strongly suppressed (≥50% of control was suppressed), and poorly suppressed (<50% of control was suppressed).

Cell Lines

Ten lung cancer cell lines (PC-9, HCC827, NCI-H441, NCI-H460, NCI-H661, NCI-H820, NCI-H1650, NCI-H1975, NCI-H1993, and A549) were used in this study. These cell lines were kind gifts from Adi F. Gazdar, MD (Hamon Center for Therapeutic Oncology Research and Department of Pathology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX). These cell lines were proven to have individual genetic origins by using the Powerplex 1.2 system (Promega) at the University of Texas Southwestern Medical Center at Dallas. The cells were maintained in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO), supplemented with 10% fetal bovine serum and incubated in 5% CO_2.

We also used experimentally established TKI-resistant cell lines (PC-9-GR1, RPC-9, and HCC827-ER). PC-9-GR1 was established by cultivation in the presence of gradually increasing concentration of gefitinib. In addition, we obtained RPC-9, which acquired a T790M mutation after a long period of exposure of gefitinib,[10] and HCC827-ER that acquired *c-MET* amplification after a long period of exposure of erlotinib.[11] The *EGFR* mutation status and other gene alterations in all the cell lines tested are shown in Table 1.

Colony Formation Assay

In vitro cell proliferation was tested by using a liquid colony formation assay. Fifty viable cells were plated onto 6-well plates in triplicate. The cells were cultured and counted 14 days later after staining with 0.1% crystal violet in 20% ethanol for 5 minutes at room temperature. The number of visible colonies (>50 cells) was counted.

Western Blotting Analysis

Cells were treated with siRNAs in 60-mm dishes and were collected 72 hours later. The detailed protocol for Western blotting has previously been described.[12] The primary antibodies were as follows: polyclonal

Table 1 Summary of NSCLC Cell Lines and Antitumor Effect of Gefitinib and siRNA for *EGFR* Knockdown

Cell Lines	*EGFR* Mutational Status	Other Gene Alterations	IC_{50} of Gefitinib (μmol/l)	Antitumor Effect of siEGFR			
				MTS Assay (% of Control Suppressed)			Colony Formation Assay (% of Visible Colonies, Average)
				siEGFR-1	siEGFR-2	Average	
PC-9 (AD)	Exon 19 del (E746-A750)	–	0.20[a]	98	50	74[b]	23[b]
RPC-9 (AD)	Exon 19 del (E746-A750) + T790M	–	7.5	54	62	58[b]	17[b]
PC-9-GR1 (AD)	Exon 19 del (E746-A750)	–	14.0	86	32	59[b]	73[b]
HCC827 (AD)	Exon 19 del (E746-A750)	–	0.001[a]	61	53	57[b]	60[b]
HCC827-ER (AD)	Exon 19 del (E746-A750)	*MET* amp	28.6	44	2.0	23	ND
NCI-H820 (AD)	Exon 19 del (E746-A749) + T790M	–	14.1	51	63	57[b]	ND
NCI-H1975 (AD)	Exon 21 (L858R) + T790M	–	11.7	92	92	92[b]	ND
NCI-H1650 (AD)	Exon 19 del (E746-A750)	*PTEN* del	19.4	2.0	0	1.0	92
NCI-H441 (AD)	WT	*KRAS* (G12V)	10.3	51	62	57[b]	ND
NCI-H460 (LC)	WT	*KRAS* (Q61H), *PIK3CA* (E545K)	53.4	5	0	2.5	102
NCI-H661 (LC)	WT	–	31.3	16	5	11	104
A549 (AD)	WT	*KRAS* (G12S)	22.0	4	1	2.5	95
NCI-H1993 (AD)	WT	*MET* amp	7.7	0	0	0	102

Abbreviations: AD = adenocarcinoma; amp = amplification; del = deletion; EGFR = epidermal growth factor receptor; IC_{50} = half maximal inhibitory concentration; LC = large-cell carcinoma; MTS = 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; ND = not done; NSCLC = non–small-cell lung cancer; siEGFR = small interfering RNA against *EGFR*; siRNA = small interfering RNA; WT = wild type.
[a] Gefitinib sensitive cell lines.
[b] The cell viability was strongly suppressed by siEGFR.

anti-EGFR, phospho-EGFR (Tyr1068), phospho-HER-2, phospho-HER-3, Akt, phospho-Akt (Ser473), p44/p42 MAPK (mitogen-activated protein kinase), and phospho-p44/p42 MAPK. All the antibodies were purchased from Cell Signaling Technology (Beverly, MA). Monoclonal anti-actin antibody, used as an equal loading control, was purchased from Sigma-Aldrich. The following secondary antibodies were used: goat antirabbit or antimouse immunoglobulin G conjugated horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). To detect specific signals, the membranes were examined by using ECL plus Western blotting Detection Reagents (Amersham Biosciences, UK Limited, Buckinghamshire, UK).

Statistical Analysis

An unpaired Student *t* test was used to compare data between the 2 groups. $P < .05$ was considered statistically significant.

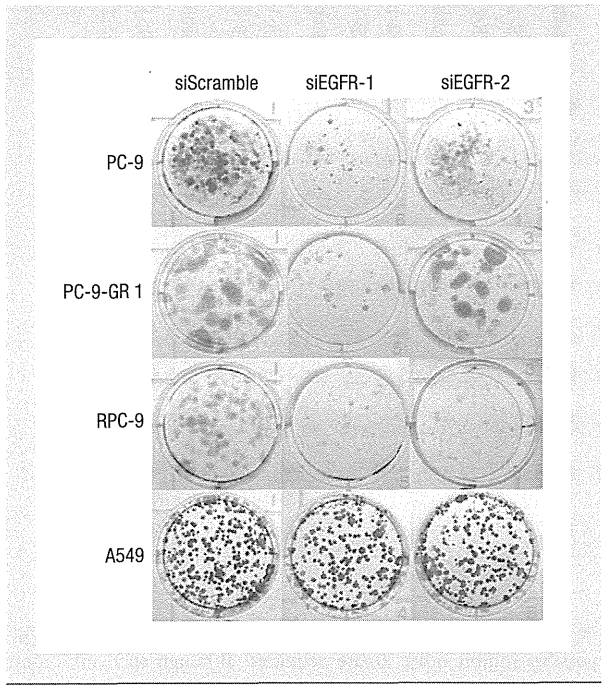
Results

Antitumor Effect of siRNA Against EGFR

The antitumor effect of siEGFR was evaluated as cell viability by using an MTS assay for all 13 NSCLCs: 8 *EGFR*-mutant and 5 *EGFR* wild-type cell lines (Table 1). The cell viabilities of 4 *EGFR*-mutant cell lines (PC-9, HCC827, NCI-H820, and NCI-1975) were strongly suppressed by siEGFR (range of control, 57%-92%). Cells with the T790M mutation in *EGFR* (NCI-H820 and NCI-H1975), which is tolerant of EGFR-TKIs, were significantly suppressed as EGFR-TKI-sensitive cells (57% and 92% of control, respectively). However, 4 cell lines with wild-type *EGFR* (NCI-H460, NCI-H661, A549, and NCI-H1993) were poorly suppressed by siEGFR except for NCI-H441, in which EGFR is phosphorylated. Of interest, gefitinib was not effective against NCI-H441 (IC_{50} = 10.3 μM).

Regarding the experimentally established TKI-resistant cell lines, RPC-9 carries the T790M mutation and HCC827-ER exhibits *c-MET* amplification, while the resistance mechanism of PC-9-GR1 remains unknown (neither *c-MET* amplification nor secondary *EGFR* mutation). The cell viabilities of RPC-9 and PC-9-GR1 were suppressed by siEGFR (58% and 59% of control, respectively), although HCC827-ER was resistant to siEGFR when compared with the sensitivity of the parental cells

Figure 1 Representative Data for a Colony Formation Assay Are Shown. PC-9, RPC-9, and PC-9-GR1 Were Inhibited by Small Interfering RNAs for EGFR (Epidermal Growth Factor Receptor) Knockdown, and A549 was Resistant. The Antitumor Effect of EGFR Knockdown as Assessed by Using the Colony Formation Assay was Consistent With the Results of the 3-[4,5-Dimethylthiazol-2-yl]-5-[3-Carboxymethoxyphenyl]-2-[4-Sulfophenyl]-2H-Tetrazolium, Inner Salt (MTS) Assay



(23% of control). A colony formation assay was performed to examine cell proliferation in the 9 cell lines. Representative data for the colony formation assay are shown in Figure 1. The cell proliferation of examined *EGFR*-mutant cell lines except for NCI-H1650 (PC-9, RPC-9, PC-9-GR1, and HCC827) were inhibited by siEGFR, and the cell proliferation of examined *EGFR* wild-type cell lines (NCI-H460, NCI-H661, A549, and NCI-H1993) were not inhibited. The antitumor effect of *EGFR* knockdown as assessed by using the colony formation assay was consistent with the results of the MTS assay (Table 1).

We previously determined the IC_{50} values of gefitinib by using an MTS assay in the 7 cell lines (PC-9, RPC-9, HCC827, NCI-H820, NCI-H1650, NCI-H1975, and A549).[7] We newly examined the IC_{50} values for gefitinib of the other 6 cell lines (PC-9-GR1, HCC827-ER, NCI-H441, NCI-H460, NCI-H661, and NCI-H1993). The results are shown in Table 1. Among the 8 *EGFR*-mutant cell lines, PC-9 and HCC827 were gefitinib sensitive; NCI-H1650, which exhibited a PTEN (phosphatase and tensin homolog) protein deficiency, and NCI-H820 and NCI-H1975, which had the T790M mutation in *EGFR*, were gefitinib resistant. The remaining 3 experimentally established cell lines were resistant.

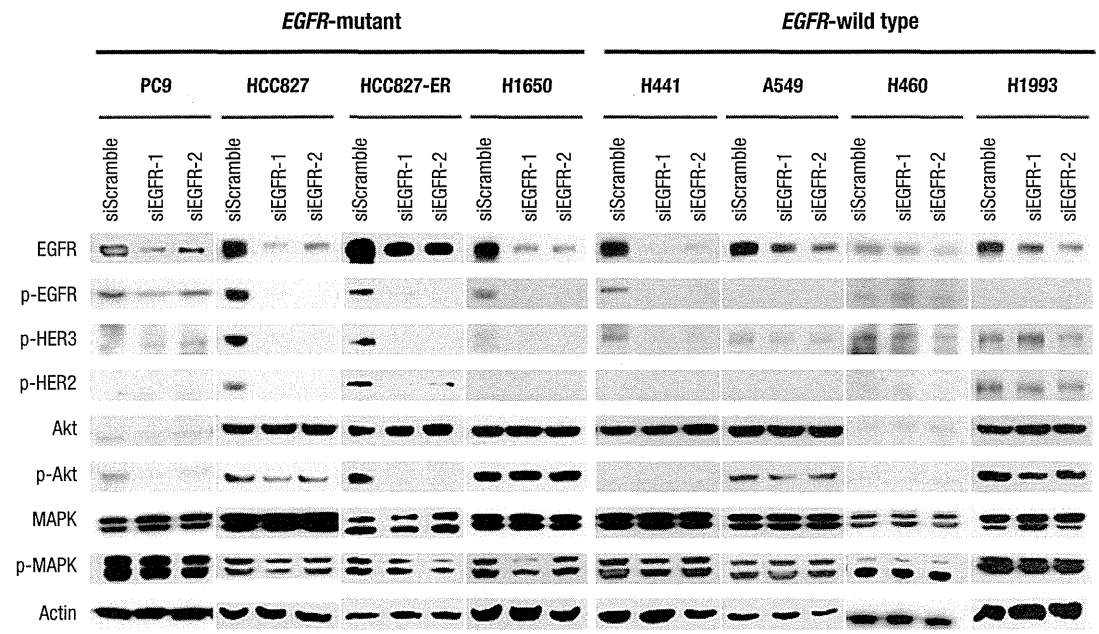
Effect of EGFR Knockdown on Protein Profile of EGFR Signaling

The expression of EGFR, its effector proteins, and other EGFR family proteins and representative data are shown in Figure 2. Although EGFR protein was expressed in all the cell lines that were tested, the expression of phosphorylated EGFR (p-EGFR) varied, even among the *EGFR*-mutant cell lines. p-EGFR was expressed in all of the *EGFR*-mutant cell lines and was completely suppressed by siEGFR, except in the NCI-H1650 cell line. However, a low level of activated p-EGFR expression was observed in most of the *EGFR* wild-type cells, except for NCI-H441. Western blotting revealed that the expression of p-EGFR under untreated conditions was correlated with the effect of siEGFR. The suppressions of p-EGFR and its effectors in HCC827-ER were similar to those in the parental cells.

Discussion

Whereas, EGFR was expressed in all of the cell lines that were examined, siEGFR was only effective when EGFR was phosphorylated, and the depletion of EGFR protein did not affect cell proliferation when EGFR was not activated. *EGFR* mutations are a major and strong activator of EGFR because mutations cause constitutive phosphorylation of tyrosine in the EGFR tyrosine kinase domain, which results in oncogene addiction.[13,14] EGFR was phosphorylated in only 1 cell line without an *EGFR* mutation. EGFR kinase with an exon 19 deletion or the L858R mutation increases the affinity of the kinase to EGFR-TKIs and decreases the affinity to adenosine triphosphate (ATP), compared with wild type kinase.[15,16] However, the *EGFR* T790M mutation, which causes acquired resistance, is thought to increase ATP affinity to a level comparable with that of wild-type *EGFR*.[17] In view of these points, cells that have acquired resistance to reversible EGFR-TKIs through the *EGFR* T790M mutation still addict on the mutant *EGFR* gene. Thus, the targeting of EGFR is thought to be a useful strategy if the ATP binding site is not the therapeutic target. Indeed, results of our study demonstrated that the downregulation of the EGFR protein overcame the reversible EGFR-TKI resistance. In addition, PC-9-GR1, the resistance mechanism of which is not known, was inhibited by *EGFR* knockdown, which indicates that targeting EGFR itself is effective in some resistant cases of which mechanism is unclear. The T790M mutation, *c-MET* amplification, and other mechanisms account for approximately 50%, 20%, and 30% of all resistance mechanisms, respectively.[18-21] Therefore, targeting EGFR may be an appropriate strategy for more than 50% of NSCLCs with reversible EGFR-TKI resistance. As strategies for targeting EGFR, there seems to be several promising agents, including anti-EGFR antibodies, non-ATP competitive EGFR-TKIs, and irreversible EGFR-TKIs. Some of these have shown clinical benefit in treatment of NSCLC. The addition of cetuximab, an anti-EGFR monoclonal antibody, to chemotherapy would improve overall survival and overall response rate.[22] In addition, afatinib, an irreversible EGFR-HER2 inhibitor, suppresses wild-type and activated EGFR and HER2 mutants, and has a higher affinity for binding to EGFR with the T790M mutation than first-generation EGFR-TKIs.[23] It shows clinical benefit as a second- or third-line

Figure 2 Protein Expression Profiles of Non–Small-Cell Lung Cancer Cell Lines After Treatment With Small Interfering RNAs Against *EGFR*



treatment in patients who have acquired resistance to EGFR-TKIs.[24]

As mentioned, *c-MET* amplification is one mechanism of EGFR-TKI resistance.[20] HCC827-ER, which carries an *EGFR* mutation and exhibits *c-MET* amplification, became resistant to siEGFR, whereas the parental HCC827 cell line was sensitive. This cell line appears to escape the addiction to EGFR. To overcome resistance in NSCLC through a similar oncogene switch, another strategy than targeting EGFR may be necessary.

Among the *EGFR*-mutant cells, NCI-H1650 was unique in that the *PTEN* gene was homozygously deleted with a consequent loss of expression.[25] PTEN is known to regulate PI3K activity.[26] The loss of PTEN results in the constitutive activation of PI3K and the subsequent activation of Akt independent of the EGFR status. Thus, the inhibition of EGFR by EGFR-TKI and siEGFR may not have influenced cell proliferation. However, PTEN deletion is a rare event in NSCLC.[27]

Our study also indicated that targeting EGFR is not appropriate for the treatment of NSCLCs without EGFR activation. However, an EGFR-targeting strategy is useful in NCI-H441, in which EGFR is phosphorylated. NCI-H441 is insensitive to gefitinib because EGFR is wild type. For this category of NSCLC, although first-generation EGFR-TKIs are not effective, other inhibitors that are not ATP competitors in the tyrosine kinase domain may show antitumor activity, and targeting EGFR may be useful.

In this study, we used siEGFR to elucidate the therapeutic role of EGFR in NSCLC. For this purpose, irreversible EGFR-TKI might be an appropriate agent. However, irreversible EGFR-TKI could possibly inhibit other kinase activities, although such inhibitions would probably not be as strong as the inhibition of EGFR. Although an off-target effect of siEGFR introduction cannot be completely excluded, such effects are likely to be minimal in the examined cells.

Conclusion

Our results indicated that EGFR can be used as a therapeutic target in NSCLCs with EGFR activation, even after resistance has been acquired. In contrast, targeting EGFR is not an appropriate strategy in tumors in which EGFR is not activated, even if EGFR is expressed.

Clinical Practice Points

- Anti-EGFR agents, including EGFR-TKIs, are considered to be effective for NSCLC when drug-sensitive *EGFR* mutation is present. However, inherent and acquired resistances are major problems of EGFR targeting therapies.
- Our results indicate that EGFR can be a therapeutic target of NSCLCs with p-EGFR expression with or without EGFR mutation, which strongly suggests that tumors depend on an activated EGFR pathway. Furthermore, EGFR still can be a target in acquired resistant tumors to EGFR-TKIs if they express p-EGFR.
- Various strategies that target EGFR will be developed and available. In clinical relevance, p-EGFR–positive NSCLCs estimated by immunohistochemistry by using biopsied or surgically resected

specimens may be a candidate of EGFR targeting therapy, including those who have acquired resistance to currently available EGFR-TKIs.

Disclosure

The authors have stated that they have no conflicts of interest.

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ORIGINAL ARTICLE – THORACIC ONCOLOGY

Induction Chemoradiotherapy Followed by Surgical Resection for Clinical T3 or T4 Locally Advanced Non–Small Cell Lung Cancer

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ABSTRACT

Purpose. To examine the usefulness of trimodality therapy in patients with clinical T3 or T4 (cT3–4) locally advanced non–small cell lung cancer (LA-NSCLC).

Methods. Between 1997 and 2009, a total of 76 LA-NSC LC patients with cT3–4 underwent surgery. Among them, 36 patients underwent induction chemoradiotherapy with docetaxel and cisplatin plus concurrent radiation followed by surgery (IC group). The other 40 patients initially underwent surgery (IS group). The outcomes of the IC and IS groups were then investigated. To minimize possible biases caused by confounding treatment indications, we performed a retrospective cohort analysis by applying a propensity score (PS). Patients were divided into three groups according to PS tertiles, and comparisons between the IC and IS groups were made by PS tertile-stratified Cox proportional hazard models.

Results. For the entire cohort, which had a median follow-up duration of 48 months, the 3- and 5-year overall survival rates were 83.8 and 78.9%, respectively, in the IC group, versus 66.8 and 56.5%, respectively, in the IS group ($P = 0.0092$). After adjustments for potentially confounding variables, the IC group continued to have a significantly longer overall survival than the IS group ($P = 0.0045$). In addition, when the analysis was limited to 52 patients with cT3–4N0 or N1 disease, the IC group had a significantly longer overall survival than the IS group after adjustments for confounding variables ($P = 0.019$).

Conclusions. Our study indicates that trimodality therapy is highly effective in patients with cT3–4 LA-NSCLC.

Lung cancer continues to be a major cause of cancer mortality worldwide. Almost half of all patients develop inoperable stage IV disease with metastasis to distant sites, and a quarter of all patients are diagnosed after the cancer has already spread to regional lymph nodes or directly beyond the primary site, resulting in a stage II or III disease.[1] For stage II or III non–small cell lung cancer (NSCLC), surgical resection remains an important part of treatment.[2]

The N and T statuses determine the degree of local disease extension. N2 or N3 (N2–3) disease and T3 or T4 (T3–4) disease are considered locally advanced (LA) diseases. An N2–3 status generally indicates a need for definitive chemoradiotherapy (CRT) or induction CRT followed by surgery, rather than initial surgery.[3] On the other hand, initial surgery is generally the treatment of choice for N0 or N1 (N0–1) disease. Regarding the T factor, whereas T3 tumors are basically candidates for initial surgery, such treatment is not recommended for T4 tumors, in principle, because of the large risk of an incomplete resection, especially when the tumors have

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highly invaded important structures. However, complete resections may be possible in patients with limited invasion of some structures including the carina, left atrium, superior vena cava, or pulmonary artery.[2] In addition, T4 staging tends to be clinically overstaged, rather than understaged.[4] Considering these factors, clinical T4 (cT4) disease is not a contraindication for surgery.

In the literature, the 5-year survival rate of patients with T3 disease who were treated with initial surgery has been reported to be 18–37%.[5–7] Thus, the clinical outcome remains unsatisfactory, especially in patients with incomplete resection. We have used induction chemotherapy or CRT followed by surgery for the treatment of N2–3 LA-NSCLC disease since 1995.[8, 9] Trimodality therapy for stage III NSCLC has yielded a 3-year survival rate of more than 60%, which seems to be superior to the outcome of reported T3 disease even though N2–3 diseases were included in our study cohort. These results strongly suggested the usefulness of trimodality therapy for LA-NSCLC including T3 disease. Considering these, whereas the use of induction CRT for LA-NSCLC remains controversial except in patients with superior sulcus tumor, we have applied trimodality therapy for less advanced diseases including T3N0M0 and T3N1M0 based on the physician's discretion in individual cases.[10]

As evidence regarding trimodality therapy for clinical T3 or T4 (cT3–4) without N2–3 disease in randomized controlled trials remains scarce, second-best evidence from retrospective studies comparing two approaches should be considered.[2] In the present retrospective study, we examined the usefulness of trimodality therapy for patients with cT3–4 LA-NSCLC, compared with patients who underwent initial surgery. One serious concern with this approach is that the results may be biased by confounding indications. To avoid this possibility, we conducted a retrospective cohort analysis by applying a propensity score (PS) analysis in addition to the usual cohort analysis.

PATIENTS AND METHODS

Patient Selection and Evaluation

The records of 76 consecutive patients with cT3–4 invasive NSCLC who underwent pulmonary resection with nodal dissection between March 1997 and October 2009 at Okayama University Hospital, Okayama, Japan, were reviewed. The patient characteristics are shown in Table 1. Patients whose cT status was determined by tumor size more than 7 cm or presence of satellite nodules were excluded from this study. Among cN2 patients, pathologic N2 was confirmed in 8 patients who underwent a mediastinoscopy. Among 76 patients, 36 patients received induction CRT before resection (IC group), and 40 patients underwent initial surgery without receiving induction CRT (IS group). All 8 patients who had confirmed pathologic mediastinal nodal metastasis were treated with induction CRT followed by surgery. The International Association of the Study of Lung Cancer tumor, node, metastasis system for NSCLC, 7th edition, was used for disease staging.[11]

Clinical stage was determined by chest radiography, bronchoscopy, computed tomography (CT) of the chest and upper abdomen, magnetic resonance imaging (MRI) of the brain, and a radionuclide bone scan or ^{18}F-fluorodeoxyglucose positron emission tomography (PET)/CT scan. The institutional review board approved the study, and informed consent was obtained from all the patients.

Induction CRT

The application of trimodality therapy was left to the physician's discretion. Basically, induction CRT was administered to patients who had mediastinal nodal metastases confirmed by a mediastinoscopy or large and invasive tumors that would have made the achievement of a complete resection with a pathologic safety margin difficult. The criteria and treatment regimen for induction CRT have been previously described.[9] In brief, docetaxel (40 mg/m2) was administered intravenously followed by cisplatin (40 mg/m^2) before radiotherapy on days 1 and 8. The chemotherapy was repeated at a 3- or 4-week interval. Radiotherapy was started on the first day of chemotherapy and total radiation dose of 46 gray (Gy) was planned, in principle, using a conventional fractionation (2 Gy/day). Dose escalation of the radiotherapy was allowed for poorly responding tumors up to 60 Gy.

After induction CRT, the patients were restaged and those without progressive disease were scheduled to receive surgery within 6 weeks of the completion of the induction therapy. The radiologic response of lesions was classified into four categories as described in previous study: complete response, partial response, stable disease, and progressive disease.[9,12] The pathologic response to induction CRT was classified into three groups: pathologic complete response, pathologic major response, and pathologic minor response.[13]

Surgical Resection, Adjuvant Treatment, and Follow-up

The surgical procedure was determined on the basis of the extent of disease. Although a lobectomy was preferred, a bilobectomy, sleeve resection, or pneumonectomy was performed in cases requiring such procedures because of the location of the primary tumor or metastatic nodal invasion. All the patients underwent complete ipsilateral mediastinal and subcarinal nodal dissection. For cN3 disease ($n = 4$), the contralateral hilar ($n = 1$) or ipsilateral

TABLE 1 Patient characteristics (unadjusted)

Variable		IC ($n = 36$)	IS ($n = 40$)	P
Age, y, median (range)		58 (33–74)	68 (48–81)	0.0005
Sex, M/F		30/6	35/5	0.75
Histology, Sq/Ad/AdSq/LC		17/17/1/1	22/15/1/2	0.80
Performance status, 0/1		26/10	31/9	0.61
Period of treatment, 1997–2003/2004–2009		14/22	23/17	0.10
c stage	T3 N0	5	18	<0.001
IIB	T3 N1/N2	3/7	11/4	
IIIA	T4 N0/N1	3/5	5/2	
IIIB	T3 N3	4	0	
	T4 N2	9	0	
Involved structures				
cT3	Chest wall	12	28[a]	
	Parietal pleura	5	12	
	Rib or muscle	7	16	
	Diaphragm	0	3[a]	
	Pericardium	1	4[a]	
	Mediastinal pleura	3	1	
	<2 cm carina	3	1	
cT4	Atrium	1	0	
	Great vessel	13[a]	7	
	Esophagus	2[a]	0	
	Vertebral body	1[a]	0	
	Carina	2	0	
Superior sulcus		4	1	

IC induction chemoradiotherapy, *IS* initial surgery, *Sq* squamous cell carcinoma, *Ad* adenocarcinoma, *AdSq* adenosquamous carcinoma, *LC* large cell carcinoma

[a] Multiple structures were involved: chest wall with diaphragm ($n = 1$), with pericardium ($n = 2$), diaphragm with pericardium ($n = 1$), great vessel with esophagus ($n = 1$), with vertebral body ($n = 1$)

supraclavicular ($n = 3$) lymph nodes were dissected according to the initial disease extent. Resection with reconstruction of the chest wall or major vessels was performed, if necessary. In the IC group, the bronchial stump was covered with pericardial fat tissue or an intercostal muscle pedicle. When a sleeve resection was performed, the greater omentum was used to wrap the anastomosis. Postoperative treatment was left to the physician's discretion. Follow-up protocol after surgery is as follows: chest and abdominal CT or PET/CT scan and enhanced brain MRI were repeated every 3 months for 2 years. From 3 to 5 years, these image analyses were repeated every 6 months. After 5 years, chest X-ray was repeated every year.

Statistical Methods

We calculated the PS by logistic regression based on available factors that were considered to be potentially associated with patient selection and the pscore command in Stata, version 11 (StataCorp, College Station, TX). Seven such factors that were included in the PS calculation were age, sex, histology, performance status, clinical stage, operation type, year of treatment (Supplementary Material 1). After the PS calculation, the subjects were divided into 3 groups according to the PS tertile to sustain comparability between the IC and the IS group within each stratum.

In this study, overall survival (OS) was defined as the primary end point and disease-free survival (DFS) as the secondary end point. OS and DFS were calculated from the date of initial treatment until the date of death or the last follow-up for OS and until confirmed disease recurrence by cross-sectional imaging studies or death for DFS. We used the PS to control potential confounding by treatment indication for the induction CRT on survival. We compared end points for the IC and IS groups in three patterns: (1) comparison using all the patients in the cohort ($n = 76$), (2) comparison among cN0–1 patients ($n = 52$), and (3) comparison of a selected cohort in which IC patients were individually matched with IS patients by PS

in all the patients ($n = 32$), and cN0–1 patients ($n = 24$). For pattern 1 and 2, the IC and IS groups were compared by a stratified logrank test and a stratified multivariate Cox proportional hazard model with PS tertile as a stratification factor. For pattern 3, the IC and IS groups were compared by usual logrank test and multivariate Cox proportional hazard model.

The baseline characteristics of the IC and IS groups were compared by the Wilcoxon rank sum test for continuous variables and the Fisher's exact test for categorical variables, as appropriate. Data were analyzed by Stata, version 11. P values of less than 0.05 were considered significant for comparison of characteristics. We applied P value less than 0.01 as significant for survival analysis to avoid inflation of alpha error by multiple comparisons.

RESULTS

Patient Characteristics

Between March 1997 and October 2009, a total of 76 LA-NSCLC patients with cT3–4 disease underwent surgical resection. The patient characteristics stratified according to therapeutic modality are shown in Table 1. A difference in the patient backgrounds was noted for age and clinical stage. The median age was significantly younger and the clinical stage was significantly higher in the IC group than in the IS group (age, $P = 0.0005$; stage, $P < 0.001$).

Toxicities and Response to Induction CRT

Out of the 36 patients in the IC group, 23 completed the planned CRT. The toxicities were similar to those described in our previous report.[9] The chemotherapy dose was modified because of toxicity in 12 patients. The total radiation dose was 20 Gy in 1 patient, 40 Gy in 8 patients, 46 Gy in 19 patients, and more than 50 Gy in 8 patients. The clinical response to induction therapy was partial response in 25 patients, stable disease in 11 patients, and complete response or progressive disease in none. The pathologic response in the resected specimens was estimated. Nineteen patients (53%) exhibited a pathologic complete response, 17 patients (47%) exhibited a pathologic major response, and none of the patients exhibited a pathologic minor response.

Surgical Resection, Postoperative Complications, and Adjuvant Treatment

The surgical procedures and postoperative complications are shown in Table 2. All 76 patients underwent surgical resection. In the IC group, all the patients underwent complete tumor resection. In the IS group, 37 (93%) of the 40 patients underwent complete tumor resection; two patients had pleural dissemination that was diagnosed pathologically after surgery, and one patient had a microscopically positive surgical margin.

One patient in the IS group died after a left pneumonectomy because of acute respiratory failure. Pulmonary complications were the most common type of postoperative complication in

TABLE 2 Surgical procedure and postoperative complications

Variable	IC ($n = 36$)	IS ($n = 40$)	P
Operation type			0.015
Lobectomy	21	34	
Sleeve lobectomy	7	1	
Bilobectomy	5	1	
Pneumonectomy	3	4	
Combined resection, yes/no	27/9	35/5	0.24
Chest wall	12[a]	25[a]	
Parietal pleura	8	10	
Rib or muscle	4	15	
Diaphragm	0	6[a]	
Pericardium	0	5[a]	
Mediastinal pleura	6	1	
Atrium	1	0	
Great vessel	7[a]	5[a]	
Recurrent nerve	1[a]	0	
Esophagus	1	0	
Carina	1	0	
Complete resection, yes/no	36/0	37/3	0.24
Postoperative complication,[b] yes/no	18/18	15/25	0.36
Pneumonia	2	6	
Empyema	2	0	
Hemorrhage, reoperation	0	1	
Chylothorax	1	0	
Bronchopleural fistula	1	0	
Atrial arrhythmia	2	2	
Hoarseness	2	4	
Effusion	0	2	
Brachial plexus palsy	1	0	
Radiation pneumonitis	4	0	
Postoperative morality	0	1	

IC induction chemoradiotherapy, *IS* initial surgery

[a] Multiple structures were resected; (Induction CRT group) chest wall with great vessel ($n = 1$), with recurrent nerve ($n = 1$), (Initial surgery group) chest wall with diaphragm ($n = 1$), with pericardium ($n = 2$), diaphragm with pericardium ($n = 2$), chest wall with diaphragm and great vessel ($n = 1$)

[b] The number of each event was described

both groups. No significant difference in the rate of postoperative complications was observed between the groups.

In the IC group, 14 patients underwent adjuvant chemotherapy. In the IS group, 14 patients underwent adjuvant chemotherapy, seven patients underwent adjuvant radiotherapy, three patients underwent adjuvant CRT.

Survival and Relapse Pattern

At a median follow-up period of 48 months, five patients in the IC group and 13 patients in IS group had died of NSCLC. One patient in the IC group and three patients in the IS group had died of other causes. Seven patients in the IC group and 17 patients in the IS group had experienced a disease relapse. The disease recurrence patterns were classified as local sites (surgical margin, intrapulmonary, regional lymph node, and pleural cavity) and distant sites. The initial recurrent patterns were as follows: in the IC group, 1 patient (3%) developed a recurrence at local site, 3 patients (8%) developed recurrences at distant sites, and 3 patients (8%) developed recurrences at both sites; in the IS group, 8 patients (20%) developed recurrences at local sites, 5 patients (13%) developed recurrences at distant sites, and 4 patients (10%) developed recurrences at both sites.

In analysis pattern 1 with the unadjusted model, the 3- and 5-year OS rates were 83.8 (95% confidential interval [CI] 65.3–92.9) and 78.9% (95% CI 58.2–90.1) in the IC group, versus 66.8 (95% CI 46.6–80.8) and 56.5% (95% CI 35.1–73.2) in the IS group (Fig. 1). The patients in the IC group showed significantly longer OS and DFS times than those in the IS group (OS, $P = 0.0092$; DFS, $P = 0.0037$). Subset analyses identified several factors that were related to a favorable outcome: the initial disease stage, sex, histology, and pathologic response. Only the use of induction therapy was significantly associated with the outcome. There was no significant difference in OS and DFS between those with or without adjuvant therapy in both the IC and IS groups.

The comparison of the characteristics between the IC and IS groups according to the PS tertile indicated an equivalent distribution of the background characteristics (Supplementary Material 2). When the PS was taken into consideration, the patients in the IC group continued to exhibit a significantly longer OS than those in the IS group according to a stratified logrank test ($P = 0.0045$). A multivariate analysis showed a significantly better outcome in the IC group (hazard ratio [HR], 0.068; 95% CI 0.013–0.35; $P = 0.001$) (Supplementary Material 3).

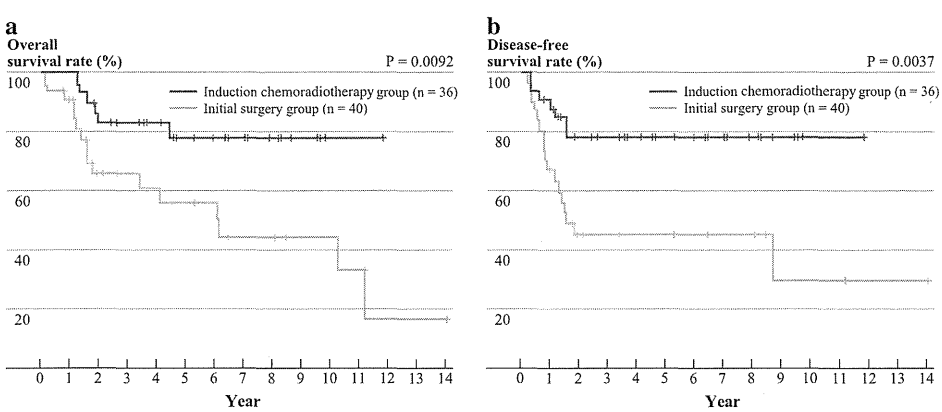

FIG. 1 Kaplan-Meier curves showing the unadjusted **a** OS and **b** DFS rates for patients in the IC and IS groups

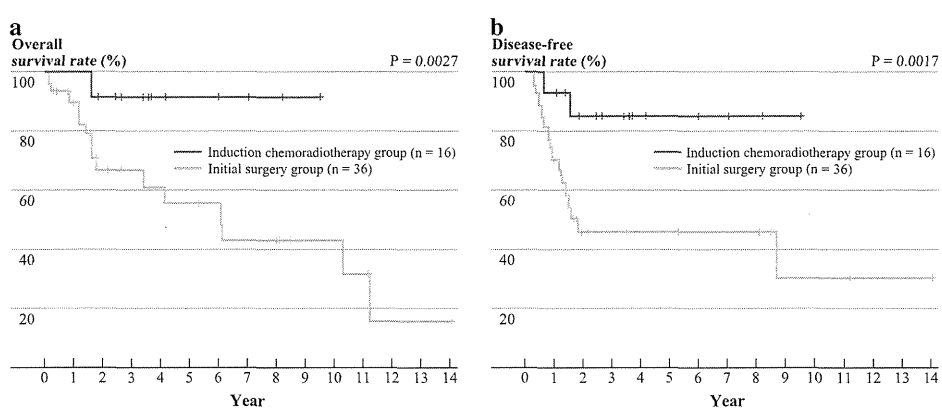

FIG. 2 Kaplan-Meier curves showing the unadjusted **a** OS and **b** DFS rates for patients with cT3–4 and cN0–1 disease

TABLE 3 Patient characteristics of PS matched cohort

Variable		IC ($n = 16$)	IS ($n = 16$)	P
Age, y, median (range)		64 (51–74)	64 (48–75)	0.82
Sex, M/F		13/3	13/3	1.00
Histology, Sq/Ad/AdSq		7/8/1	9/6/1	0.85
Performance status, 0/1		13/3	13/3	1.00
c stage, IIB/IIIA/IIIB		3/9/4	3/13/0	0.14
Involved structures				
cT3	Chest wall	7	9	
	Parietal pleura	3	5	
	Rib or muscle	4	4	
	Diaphragm	0	1[a]	
	Pericardium	0	2[a]	
	Mediastinal pleura	2	0	
	<2 cm carina	1	0	
cT4	Great vessel	6[a]	5	
	Esophagus	1[a]	0	
Superior sulcus		2	0	
Operation type				0.79
Lobectomy		13	12	
Sleeve lobectomy		1	0	
Bilobectomy		1	1	
pneumonectomy		1	3	
Period of treatment, y, 1997–2003/2004–2009		7/9	9/7	0.72
Combined resection, yes/no		14/2	15/1	1.00
Chest wall		6	9[b]	
Parietal pleura		2	6	
Rib or muscle		4	3	
Diaphragm		0	4[b]	
Pericardium		0	1[b]	
Mediastinal pleura		4	1[b]	
Great vessel		4	3	

PS propensity score, *IC* induction chemoradiotherapy, *IS* initial surgery, *Sq* squamous cell carcinoma, *Ad* adenocarcinoma, *AdSq* adenosquamous carcinoma

[a] Multiple structures were involved: diaphragm with pericardium ($n = 1$), great vessel with esophagus ($n = 1$)

[b] Multiple structures were resected; chest wall with diaphragm ($n = 1$), with diaphragm and great vessel ($n = 1$), diaphragm with pericardium ($n = 1$)

The OS and DFS curves for 52 patients with clinical N0 or N1 (cN0–1) (analysis pattern 2) are shown in Fig. 2. The 3- and 5-year OS rates were both 92.3% (95% CI 56.6–98.9) in the IC group ($n = 16$) and were 67.7 (95% CI 46.6–81.9) and 56.4% (95% CI 33.9–73.8) in the IS group ($n = 36$), respectively. In this cohort, the OS and DFS times in the IC group were significantly longer than those in the IS group (OS, $P = 0.027$; DFS, $P = 0.017$). In addition, the PS values were calculated in this cohort, and survivals were analyzed after stratification according to the PS tertile (Supplementary Material 1). A comparison of the characteristics between the IC and IS groups according to the PS tertile is shown in Supplementary Material 4. When the PS was taken into the consideration, the patients in the IC group continued to exhibit a significantly longer OS than those in the IS group (a stratified logrank test, $P = 0.019$). A multivariate analysis continued to exhibit a significantly better outcome in the IC group in this cohort (HR, 0.068; 95% CI 0.00043–0.28; $P = 0.006$, Supplementary Material 5).

PS Matching Analysis (Analysis Pattern 3)

Among all 76 patients, 32 patients were extracted from each group using PS matching. The patient characteristics were matched as shown in Table 3. After adjustments for

potentially confounding variables, the patients in the IC group continued to exhibit a better outcome than those in the IS group ($P = 0.00067$). In addition, among 52 patients with cN0–1 disease, 24 patients were extracted using PS matching (Supplementary Material 6). After adjustments, the patients in the IC group also exhibited a better outcome those than the IS group ($P = 0.026$).

DISCUSSION

We previously reported that induction CRT followed by surgery is a promising treatment for patients with stage III NSCLC.[9] The present study focused on cT3–4 LA disease involving adjacent structures and compared the outcome of trimodality therapy with initial surgery in this population. Our study showed that trimodality therapy significantly prolonged the OS and DFS times. Although the present study was retrospective in design, a PS matching analysis reduced the biases inherent in this series.

A significantly high incidence of postoperative complications after induction treatments has been reported, particularly after a pneumonectomy.[14] In the present study, no treatment-related mortalities were observed in the IC group. Although no significant difference in the rate of postoperative complication was observed between the groups, severe complications including bronchopleural fistula and empyema occurred in the IC group. This outcome suggests the importance of a meticulous surgical procedure and perioperative management.

The rationale for the use of induction treatments is to prevent cancer cell microresidues at local sites and to eradicate micrometastatic disease at distant sites.[15] Indeed, the outcomes of the present study support this rationale. First, local recurrences occurred in 11% of the patients in the IC group and 30% in the IS group. The control of local recurrences using induction CRT is thought to contribute to a better likelihood of achieving a complete resection. Furthermore, nodal metastasis is known to be significantly more common among patients with visceral pleura invasion, possibly through the subpleural lymphatics to the hilar and mediastinal lymph nodes.[16] The therapeutic effect of induction CRT on this afferent pathway may have contributed to the better outcome of the IC group. Second, distant metastases occurred in 17% of the patients in the IC group and 23% of the patients in the IS group. These frequencies are similar even though the IC group included more patients with an advanced disease stage, compared with the IS group. This outcome suggests the effectiveness of induction therapy for eradicating distant micrometastasis.

In this study, all 13 patients with c-IIIB disease (4 of T3N3M0 and 9 of T4N2M0) were treated with trimodal therapy, but mediastinal nodal metastasis was only confirmed in three patients. Recent guidelines do not recommend surgery for T4N2 or more advanced disease, nor do they recommend induction chemotherapy or CRT followed by surgery for N3 disease.[1, 3, 17] Although the c-IIIB stage may have been overestimated in our series, all the c-IIIB patients were treated with trimodality therapy, and their clinical outcomes seemed to be acceptable (3-year survival rate of 75%).

Finally, our induction CRT regimen was thought to contribute to the favorable outcome of the trimodality therapy. Our regimen originated from the same combination of docetaxel and cisplatin with concurrent radiation that is used for unresectable LA-NSCLC.[18] A recent study showed that concurrent CRT with docetaxel and cisplatin is associated with a favorable prognosis compared with mitomycin, vindesine, and cisplatin (MVP) in patients with unresectable LA-NSCLC.[19] Of note, Stupp et al. reported the excellent prognosis (40% of 5-year OS rate) of stage IIIB patients treated with trimodal therapy with docetaxel and cisplatin.[20]

In conclusion, our results strongly suggest that this combined modality treatment is highly effective in patients with cT3–4 LA-NSCLC, compared with initial surgery.

CONFLICT OF INTEREST None of the authors have any conflict of interest to report.

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Prognostic impact of cancer stem cell-related markers in non-small cell lung cancer patients treated with induction chemoradiotherapy

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ABSTRACT

The expression of several cancer stem cell (CSC)-related markers has been confirmed in non-small cell lung cancer (NSCLC). The aim of this study was to clarify the clinical role of CSC-related markers in patients with NSCLC undergoing induction chemoradiotherapy (CRT). Fifty patients with clinically diagnosed N2 or N3 NSCLC who underwent induction CRT with docetaxel and cisplatin concurrently with thoracic radiation followed by surgery were examined in this study. The expressions of CSC related markers (CD133, ALDH1, ABCG2, and Bmi-1) were examined using immunohistochemical staining in surgically resected specimens. Among the 50 patients, 20 patients had no residual tumor cells in the resected specimen when examined pathologically; CSC-related marker expressions and their correlation to survival were evaluated in the other 30 patients. After a median follow-up period of 72 months, the 5-year overall survival rate of the patients with CD133-positive or ALDH1-positive specimens was significantly worse than that of the patients with both CD133-negative and ALDH1-negative expressions (44.9% vs. 90.0%, respectively; $P = 0.042$). In a multivariate analysis, CD133 and ALDH1 negativity ($P = 0.047$) and cN2-3 single station metastasis ($P = 0.03$) were significant independent prognostic factors for prolonged survival. The expressions of CSC-related markers after CRT were significantly correlated with a poor prognosis in patients with NSCLC. The development of therapeutic strategies including adjuvant therapy that take CSC-related marker positivity into consideration is likely to be a key factor in further improvements of the prognosis of patients undergoing trimodality therapy.

1. Introduction

Lung cancer is the leading cause of death among patients with malignant tumors worldwide. For locally advanced non-small cell lung cancer (NSCLC), multimodal therapy including chemotherapy, radiotherapy and surgery can improve the survival of patients, compared with single-treatment modalities. Definitive chemoradiotherapy is one of the treatments of choice for locally advanced NSCLC, especially when N2 or N3 disease is apparent. While surgical resection after induction therapy is not currently considered an established standard approach, surgery after induction therapy is often performed by experienced institutions worldwide [1]. Stupp et al. recently reported an excellent outcome (5-year survival rate of 40%) for stage IIIB patients who were treated with docetaxel and cisplatin followed by accelerated radiotherapy and surgery [2]. We also reported a promising clinical outcome of trimodality therapy for NSCLC patients with stage III disease [3].

To further improve the outcome of induction therapy, prognostic factors for induction therapy needed to be identified. The histological response in resected specimens is usually examined, since a pathological complete response (pCR) or mediastinal downstaging are prognostic factors of induction therapy followed by surgical treatment. However, the rate of pCR or downstaging is approximately 30%, and the exploration of novel prognostic factors may be useful [1–3].

Recently, cancer stem cells (CSCs), which are characterized by the capacity for self-renewal and pluripotency, have been attracting interest as a source of cancer cells [4]. Various molecules are being investigated as putative markers of CSCs in malignancies including lung cancer [5]. CD133, which was initially described

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as a surface antigen specific for human hematopoietic stem cells [6,7], is now being used to identify and isolate putative CSC populations from malignant tumors including cancers of the brain, prostate, liver, pancreas, and colon as well as melanomas [8–14]. CD133 has also been used to isolate cancer-initiating (stem) cells from lung cancer [15]; however, its clinical role in lung cancer remains unclear. As another candidate marker for CSCs, aldehyde dehydrogenase (ALDH), which is known to occur in brain tumors and breast cancer, is widely regarded as a surface marker of CSCs in lung cancer [16–18]. Serrano et al. reported that ALDH-positive lung cancer stem-like cells have longer telomeres but similar telomerase activity levels, compared with the non-CSC fraction [19].

A previous study reported that the expression of some CSC-related markers was related to a poor prognosis among patients with initially resected NSCLC, suggesting their potential use as prognostic markers such as CD133, ATP-binding cassette superfamily G member 2 (ABCG2), Bmi-1, and Octamer-4 [20–22]. Indeed, CSCs are known to have drug or radiation-resistant features [23,24]. These reports suggest that the presence of residual CSC-like cells in specimens treated with induction CRT may indicate the resistance of the cancer cells and may be related to a poor prognosis.

In this study, we examined the expression of four CSC-related markers (CD133, ALDH1, ABCG2, and Bmi-1) using immunohistochemical (IHC) staining in surgically resected specimens that had been subjected to induction CRT and evaluated the prognostic impact of these CSC-related markers in viable cells after induction therapy.

2. Materials and methods

2.1. Patients, treatment plan and study design

A total of 50 patients with locally advanced N2 or N3 NSCLC who underwent induction chemotherapy concurrently with thoracic radiation followed by surgery between January 2000 and June 2009 at Okayama University Hospital were enrolled in this study. Twenty eight patients had pathological N2 disease, as confirmed by the mediastinoscopy. All the patients underwent a previously reported induction therapy regimen [3]. Briefly, the regimen consisted of docetaxel (40 mg/m^2) and cisplatin (40 mg/m^2) administered on days 1, 8, 29 and 36 plus concurrent thoracic irradiation at a dose of 40–60 Gray. Following induction CRT, the response of the patients was evaluated using the results of chest radiography and computed tomography (CT) or ^{18}F-fluorodeoxyglucose positron emission tomography/CT. Patients without progressive disease were scheduled to receive surgery within 6 weeks of the completion of the induction therapy. The surgical procedure was determined based on the extent of the disease. A lobectomy was preferred; however, a bilobectomy, sleeve resection, or pneumonectomy was performed in patients requiring these procedures because of primary tumor or metastatic lymph node invasion. Postoperative adjuvant treatment was left to the physician's discretion, and 21 of the 50 patients received adjuvant chemotherapy. Study approval was obtained from the institutional review board, and informed consent was obtained from each of the enrolled patients.

2.2. IHC staining

Surgical specimens of the primary lesion were evaluated using IHC staining in the cases with residual tumor cells. Evaluations of pre-treatment biopsy samples of the primary lesion could not be performed because sufficient amounts of the samples necessary for the evaluation of CSC-related markers using IHC staining could not be obtained in many cases.

Surgical specimens were fixed in 10% formaldehyde, embedded in paraffin and cut into 4 μm-thick sections. The sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. For antigen retrieval, the sections were heated in a microwave in 10 mM of sodium citrate (pH 6.0) for 30 min. The sections were incubated in 3.0% H_2O_2 solution for 10 min to block endogenous peroxidase activity. IHC staining was performed using the ImmPRESS Reagent Kit (Vector Laboratories, CA) according to the manufacturer's instructions. Following a blocking step with normal horse serum, the sections were incubated for 60 min with the primary antibodies at room temperature. The primary antibodies consisted of a mouse monoclonal anti-CD133 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany; diluted 1:50 in PBS), a rabbit polyclonal anti-ALDH1 antibody (Abcam, Cambridge, UK; diluted 1:400 in PBS), a mouse monoclonal anti-ABCG2 antibody (Abcam; diluted 1:400 in PBS), and a rabbit polyclonal anti-Bmi-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:50 in PBS). After a brief wash, the appropriate ImmPRESS Reagent (anti-mouse or anti-rabbit immunoglobulin) was added to the sections and incubated for 30 min. Antibody binding was detected using a ImmPACT DAB Peroxidase Substrate Kit (Vector Laboratories), and Mayers hematoxylin was used for counterstaining.

2.3. Assessment of IHC staining

Two investigators (KS and KI) who were unaware of the clinical data independently evaluated the marker staining under a light microscope at a ×400 magnification. According to the immunostaining intensity, tumor cells with moderate or strong staining were considered positive, while tumor cells with no or weak staining were defined as negative. The IHC staining for CD133, ALDH1, ABCG2, and Bmi-1 was semi-quantitatively assessed based on the approximate percentage of positive cells over the total number of tumor cells and was determined according to previously described criteria as follows: CD133 negative $\leq$ 1%, positive > 1% [21,25]; ALDH1 negative $\leq$ 10%, positive > 10% [17]; ABCG2 negative $\leq$ 10%, positive > 10% [21,26]; and Bmi-1 negative $\leq$ 5%, positive > 5% [27]. Omission of primary antibody served as a negative control in each marker. The bronchial epithelial cells (in ALDH1, ABCG2, and Bmi-1) or macrophages (in ALDH1) were used as internal control (Supplementary Fig. 1). In CD133, strongly stained sample was used as positive control.

2.4. Statistical analysis

In this study, the overall survival (OS) period was defined as the primary endpoint and the disease-free survival (DFS) period was defined as the secondary endpoint. The OS and DFS were calculated from the date of initial treatment until the date of death or the last follow-up for OS and until confirmed disease recurrence or death for DFS.

The baseline characteristics of the patients were compared using the Wilcoxon rank sum test for continuous variables and the Fisher exact test for categorical variables, as appropriate. A univariate analysis of OS and DFS was performed using the Kaplan–Meier method with logrank testing, and a multivariate analysis was performed using the Cox proportional hazard model. All the data were analyzed using JMP, version 9.0.0 (SAS Institute Inc, Cary, NC). For each analysis, probability values of less than 0.05 were considered significant.

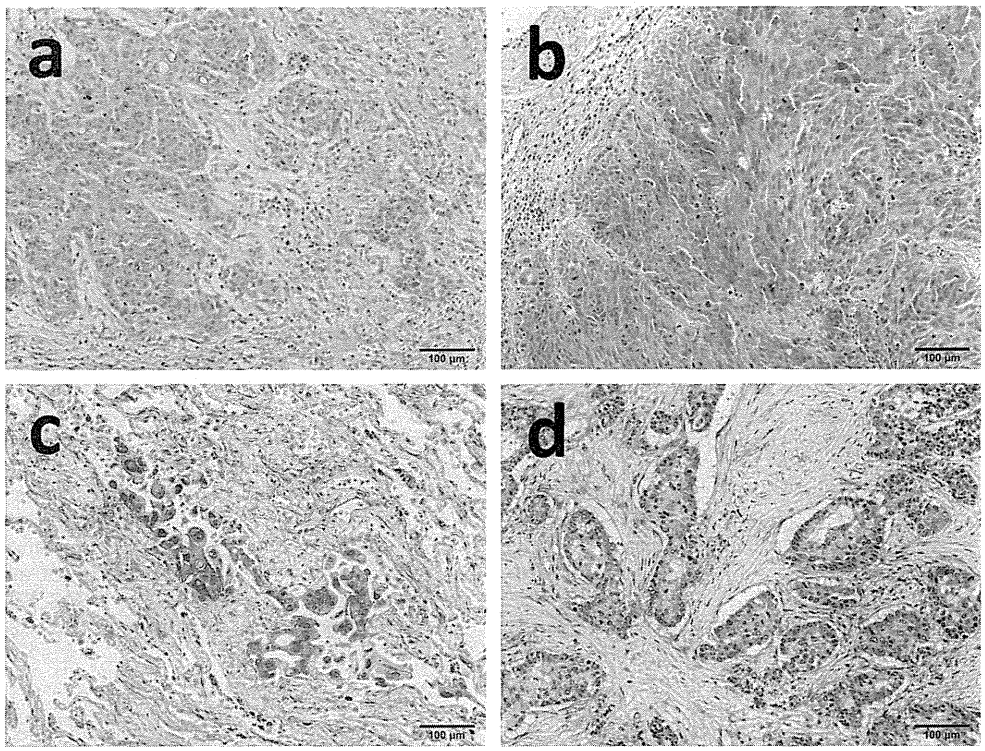

Fig. 1. Representative examples of immunohistochemical staining for each of the markers in a surgical resected primary tumor. (a) CD133 staining; strong, 15% positive, (b) ALDH1 staining; strong, 72% positive (c) ABCG2 staining; strong, 60% positive, (d) Bmi-1 staining; strong, 80% positive.

3. Results

3.1. Expression of CSC related markers in NSCLC

Among the 50 surgically resected specimens, viable tumor cells remained in the specimens from 30 patients. Among these 30 patients, 9 patients (30%) were positive for CD133 expression, 18 patients (60%) were positive for ALDH1 expression, 14 patients (47%) were positive for ABCG2 expression, and 25 patients (83%) were positive for Bmi-1 expression. Details of each marker expression in each patient are shown in Supplementary Table 1. Examples of the IHC staining patterns are shown in Fig. 1, Supplementary Figs. 1 and 2. The relations between the IHC staining patterns and the clinicopathological factors were examined (Table 1). With the exception of Bmi-1 expression, which was significantly higher in smokers than in non-smokers ($P=0.011$), no significant associations were observed between the marker expression statuses and clinicopathological factors (age, sex, smoking history, histology, serum carcinoembryonic antigen [CEA] level, clinical or pathological stage, or cN2-3 station). Regarding each marker expression, ALDH1 expression correlated with Bmi-1 expression (Supplementary Table 2).

3.2. Clinical outcomes and impact of CSC-related marker expressions

The relationships between the CSC-related marker statuses and the clinical outcomes were examined. After a median follow-up duration of 72 months, nine patients had died. Seventeen patients had experienced a disease relapse. The 3-year and 5-year OS rates were 71.4% (95% confidential interval [CI]: 85.1–52.2) and 61.5% (95% CI: 78.3–41.4), respectively. The 3-year and 5-year DFS rates were 52.1% (95% CI: 34.3–69.3) and 39.0% (95% CI: 22.6–58.4), respectively. The 5-year OS rate in the CD133-positive or ALDH1-positive patients was significantly poorer than that of the patients who were negative for both CD133 and ALDH1 (44.9% [95% CI: 22.3–69.9] vs. 90.0% [95% CI: 53.3–98.6], respectively; $P=0.042$) (Fig. 2). The relationships between other clinicopathological factors and survival were also examined. The selected clinicopathological factors were as follows: age, sex, smoking history, histology, CEA level, clinical disease stage (cStage), clinical N status (cN), clinical N2 or N3 metastasis station (cN2-3 station), and adjuvant chemotherapy (administered/not administered) (Table 2). Among them, the cN and cN2-3 station were significantly associated with the OS period. In a multivariate analysis, which included significant prognostic factors by the univariate analysis, CD133 and ALDH1 negativity (Hazard ratio [HR]: 0.16, 95% CI: 0.0086–0.98, $P=0.047$) and single mediastinal nodal metastasis (not multinodal metastasis) (HR: 0.19, 95% CI: 0.028–0.91, $P=0.03$) were significant independent prognostic factors of a prolonged survival (Table 3).

Postoperative adjuvant chemotherapy was performed in 15 of the 30 patients. Although the number was small, the CSC related marker status of the 15 patients who received adjuvant chemotherapy was not significantly related to the OS or DFS periods.

4. Discussion

We showed that the positive expression of CD133 or ALDH1 is independent predictor of disease relapse and a poor prognosis among patients who have received induction CRT using docetaxel and cisplatin. The positive expression of CD133 or ALDH1 in resected specimens may be an indicator of tumor resistance, indicating the failure to control preexisting micrometastasis or to prevent metastasis during or after CRT.

The four markers that were examined in this study have been previously reported as candidate CSC-related proteins in NSCLC. Tirino et al. reported that CD133-positive cells isolated from surgically resected specimens of previously untreated NSCLC were able

Table 1
Clinicopathological factors and CSC related markers expression.

Characteristics (*n*)	CD133		ALDH1		ABCG2		Bmi-1	
	+/−	*P*	+/−	*P*	+/−	*P*	+/−	*P*
Age		0.43		0.71		0.27		1
<60 (15)	6/9		10/5		9/6		13/2	
≥60 (15)	3/12		8/7		5/10		12/3	
Sex		0.68		1		0.44		0.14
Male (21)	7/14		13/8		11/10		19/2	
Female (9)	2/7		5/4		3/6		6/3	
Smoking history		0.37		0.21		0.85		0.011*
Non-smoker (8)	1/7		3/5		3/5		4/4	
Smoker (22)	8/14		15/7		11/11		21/1	
Histology		1		0.46		0.26		0.3
Ad (20)	6/14		13/7		11/9		18/2	
Non-Ad (10)	3/7		5/5		3/7		7/3	
CEA		1		0.71		0.27		0.33
<4.1 (15)	5/10		10/5		9/6		11/4	
≥4.1 (15)	4/11		8/7		5/10		14/1	
Clinical stage		0.2		0.14		1		1
IIIA (20)	8/12		14/6		9/11		17/3	
IIIB (10)	1/9		4/6		5/5		8/2	
cN2/3 station		1		0.71		0.72		1
Single (15)	4/11		8/7		6/9		12/3	
Multi (15)	5/10		10/5		8/7		13/2	
Recurrence		0.69		0.061		0.48		1
−(13)	3/10		5/8		5/8		11/2	
+(17)	6/11		13/4		9/8		14/3	

Ad: adenocarcinoma.
* $p < 0.05$.

Table 2
5-year overall survival and disease-free survival rate.

Subsets (*n*)	5-year OS (%)	*P*	5-year DFS (%)	*P*
Age, <60 (15)/≥60 (15)	47.9/75.4	0.098	23.3/56.1	0.077
Sex, Male (21)/Female (9)	57.6/71.1	0.42	36.7/44.4	0.82
Smoking, Non-smoker (8)/Smoker (22)	72.9/57.6	0.43	50.0/35.4	0.76
Histology, Ad (20)/Non-Ad (10)	62.2/55.6	0.55	33.9/48.0	0.97
CEA, <4.1 (15)/≥4.1 (15)	71.5/46.6	0.28	66.0/9.5	0.020*
Clinical stage, IIIA (20)/IIIB (10)	65.0/50.0	0.18	34.6/48.0	0.15
cN status, cN2 (26)/cN3 (4)	66.9/25.0	0.0098*	43.2/0.0	0.25
cN2-3 station, Single (15)/Multi (15)	85.7/37.7	0.014*	51.2/24.0	0.087
Adjuvant therapy, adm (15)/not adm (15)	55.0/70.7	0.69	26.7/55.6	0.24
CD133 expression, −(21)/+(9)	69.0/38.9	0.38	43.7/27.8	0.58
ALDH1 expression, −(12)/+(18)	75.0/50.9	0.33	65.6/24.4	0.073
ABCG2 expression, −(16)/+(14)	63.5/58.0	0.55	50.0/21.8	0.46
Bmi-1 expression, −(5)/+(25)	80.0/57.7	0.46	40.0/39.9	0.82
CD133/ALDH1 expression, Both negative (10)/Either positive (20)	90.0/44.9	0.042*	70.0/24.6	0.042
CD133/ABCG2 expression, Both negative (10)/Either positive (20)	78.6/51.7	0.26	60.0/27.1	0.29
CD133/Bmi-1 expression, Both negative (5)/Either positive (25)	80.0/57.7	0.46	40.0/39.9	0.82
ALDH1/ABCG2 expression, Both negative (7)/Either positive (23)	71.4/58.4	0.63	57.1/33.5	0.55
ALDH1/Bmi-1 expression, Both negative (5)/Either positive (25)	80.0/57.7	0.46	40.0/39.9	0.82
ABCG2/Bmi-1 expression, Both negative (4)/Either positive (26)	75.0/59.8	0.68	50.0/38.1	0.99

Ad: adenocarcinoma; adm: administered.
* $p < 0.05$.

Table 3
Multivariate Cox proportional hazard model.

Factors	Hazard ratio	*P*	95% CI
cN status, cN2 vs. cN3	0.68	0.61	0.16–3.43
cN2-3 station, single vs. multi	0.19	0.037*	0.028–0.91
CD133/ALDH1 expression, Both negative vs. Either positive	0.16	0.047*	0.0086–0.98

CI: confidential interval.
* $p < 0.05$.