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ORIGINAL PAPER

Clinical outcomes of advanced non-small cell lung cancer patients screened for epidermal growth factor receptor gene mutations

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Abstract
Purpose To evaluate the relationship between the epidermal growth factor receptor (EGFR) mutation status and the effectiveness of gefitinib monotherapy or chemotherapy in patients with advanced non-small cell lung cancer (NSCLC).
Methods We retrospectively analyzed a cohort of 100 patients with stage IIIB/IV NSCLC screened for two major EGFR mutations (exon 19 deletions and L858R mutation).
Results Forty-six out of 48 EGFR mutation-positive patients (96%) received gefitinib, whereas only 3 out of 52 EGFR mutation-negative patients (6%) received gefitinib. Favorable objective response rates to gefitinib as first- and second-line treatment (87 and 80%, respectively) were observed in EGFR mutation-positive patients. Overall response rate to chemotherapy as first-line treatment did not differ significantly between patients with EGFR mutations and those without mutation (32 vs. 28%, respectively; $P = 0.7198$). As to first-line treatment, EGFR mutation-positive patients treated with gefitinib experienced significantly longer progression-free survival (PFS) than did patients who received chemotherapy (median survival, 7.8 months vs. 5.1 months, respectively; $P = 0.0323$). Similarly, as to second-line treatment, EGFR mutation-positive patients treated with gefitinib had significantly longer PFS than did patients who received chemotherapy (median survival, 6.5 months vs. 4.0 months, respectively; $P = 0.0048$). Patients with EGFR mutations survived longer than those without EGFR mutations after first-line treatment (median, 24.3 vs. 12.6 months, respectively; $P = 0.0029$).
Conclusion EGFR mutation-positive patients benefit from either first- or second-line gefitinib monotherapy. Further large-scale prospective studies to confirm this finding are needed.

Keywords Epidermal growth factor receptor · Mutation screening · Gefitinib · Non-small cell lung cancer · Cytotoxic chemotherapy · Clinical outcomes

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Introduction

Gefitinib, an orally bioavailable, selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), was the first targeted drug for non-small cell lung cancer (NSCLC). Phase II trials of gefitinib monotherapy in unselected NSCLC patients showed antitumor activity, but demonstrated objective response rates of only 8–18% (Fukuoka et al. 2003; Kris et al. 2003). However, subset analyses of these trials and a retrospective study showed that favorable response to gefitinib was observed in certain

patient subgroups, such as females, patients with adenocarcinoma, Asian patients, and nonsmokers (Fukuoka et al. 2003; Kris et al. 2003; Miller et al. 2004). These results suggest that identifying predictive molecular or genetic biomarkers for gefitinib sensitivity may help to select patients who are most likely to benefit from treatment.

In 2004, three independent groups of investigators reported that somatic EGFR mutations correlate with sensitivity of NSCLC to the EGFR TKIs, gefitinib or erlotinib (Lynch et al. 2004; Paez et al. 2004; Pao et al. 2004). Subsequently, multiple groups of researchers confirmed and extended this striking correlation between EGFR mutations and gefitinib sensitivity, reporting response rates ranging from approximately 60 to 94% in retrospective analyses (Cortes-Funes et al. 2005; Han et al. 2005; Huang et al. 2004; Kim et al. 2005; Mitsudomi et al. 2005; Takano et al. 2005; Taron et al. 2005; Tokumo et al. 2005). Recently, several prospective phase II studies also confirmed the correlation (Asahina et al. 2006; Inoue et al. 2006; Sequist et al. 2008; Sugio et al. 2009; Sunaga et al. 2007; Sutani et al. 2006; Tamura et al. 2008; Yang et al. 2008; Yoshida et al. 2007), and a combined analysis from seven phase II trials in Japan (I-CAMP; Iressa Combined Analysis of Mutation Positives) demonstrated a total response rate of 76.4% (Morita et al. 2009).

To date, many types of mutations in NSCLC patients have been reported, but only four types of TKI-sensitive mutations, including exon 18 and 21 point mutation (G719A/C, L858R and L861Q) and exon 19 in-frame deletion, have been elucidated (Greulich et al. 2005). Of these mutations, the two most common, representing approximately 90% of all EGFR mutations, are the exon 19 deletions and L858R point mutation (Uramoto and Mitsudomi 2007). In a previous study on prospective validation for prediction of gefitinib sensitivity by these two common hot spots for EGFR mutations, we reported a promising overall response rate of 90.5% (Yoshida et al. 2007). Therefore, in order to select patients who might benefit from gefitinib treatment, we continued to screen patients for the two hot spot mutations.

To clarify the relationship between EGFR mutation status and the effectiveness of gefitinib monotherapy or cytotoxic chemotherapy in patients with NSCLC, we performed a retrospective analysis of clinical outcomes of consecutive patients who were screened for two major EGFR mutations.

Patients and methods

Patients

A cohort of 100 patients with inoperable stage IIIB/IV NSCLC were screened for EGFR mutations prior to selection for gefitinib treatment or cytotoxic chemotherapy at Aichi Cancer Center Hospital in Nagoya, Japan, between November 2004 and December 2006. Eligibility criteria were adults (defined as ≥20 years of age) with cytological or histological confirmation of locally advanced (stage IIIB for which thoracic irradiation was not indicated) or metastatic (stage IV) NSCLC who underwent prospective screening of EGFR mutations; ≥1 measurable or assessable lesion, according to the Response Evaluation Criteria in Solid Tumors (Therasse et al. 2000); and written informed consent, in accordance with institutional regulations. Eligible patients were admitted to the study regardless of prior chemotherapy, performance status (PS), or functions of main organs. Exclusion criteria were pulmonary fibrosis, interstitial pneumonia, or prior treatment with an EGFR TKI or antibody. This study was approved by the institutional review board of Aichi Cancer Center Hospital.

EGFR mutation analysis

Mutational analysis of the exon 19 deletion and the L858R mutation in the EGFR gene was performed as described previously (Yatabe et al. 2006). Briefly genomic DNA was extracted from tumors embedded in paraffin blocks or from aspirated tumors obtained from pleural effusions, superficial lymph nodes, or subcutaneous metastases. One reference pathologist (Y.Y.) reviewed all specimens and marked grossly near the tumor-rich lesion on an unstained slide in order to enrich the tumor cell population as much as possible. The exon 19 deletion mutation was determined by common fragment analysis using polymerase chain reaction (PCR) with an FAM-labeled primer set; the PCR products were subjected to electrophoresis on an ABI PRISM 310 instrument (Applied Biosystems, Foster City, CA, USA). The shorter segment of DNA amplified by PCR showed a deletion mutation in a new peak in the electropherogram. The L858R mutation was detected by the Cycleave real-time quantitative PCR technique, using the Cycleave PCR core kit (Takara Co. Ltd., Ohtsu, Japan) with an L858R-specific cycling probe and a probe specific for the wild-type gene. Fluorescence intensity was measured with a Smart Cycler system (SC-100, Cepheid, Sunnyvale, CA, USA).

Statistical analysis

Data were analysed using the chi-square test; $P < 0.05$ was regarded as statistically significant. Confidence intervals (CIs) were calculated using binomial values. Progression-free survival (PFS) and overall survival (OS) were calculated using the Kaplan–Meier method; survival differences were analysed by log-rank test. All analyses were performed with Stat View version 5 software (SAS institute Inc, Cary, NC, USA) on a Macintosh computer.

Results

Patients characteristics

From November 2004 through December 2006, 100 consecutive patients with NSCLC at Aichi Cancer Center hospital were examined to detect EGFR mutations. Patient characteristics are shown in Table 1. All patients were Japanese. EGFR mutations were detected in 48% (48/100) of the patients. Of the patients with EGFR mutations, 23 had the exon 19 deletions, and 25 had the L858R mutation. EGFR mutations were detected more frequently in women and patients who never smoked, whereas fewer EGFR mutations were detected in stage IIIB patients.

Figure 1 depicts the treatment of EGFR mutation-positive patients. Of the 48 EGFR mutation-positive patients, 96% (46/48) received gefitinib monotherapy; 47.9% (23/48), 31.3% (15/48), and 25% (12/48) of the EGFR mutation-positive patients received gefitinib as first-, second- and third-line treatment, respectively. Of the 12 patients with EGFR mutation who were treated with gefitinib as third-line treatment, two patients received gefitinib monotherapy as first-line, two patients received gefitinib monotherapy as second-line, and eight patients received cytotoxic chemotherapy as both first- and second-line.

Only 6% (3/52) of the 52 patients without EGFR mutations received gefitinib monotherapy as first- (two patients) or second-line (one patient) treatment, whereas 96.2% (50/52) of the patients without EGFR mutations received cytotoxic chemotherapy as first-line treatment.

In this study, all patients received first-line treatment, 65% (65/100) of the patients received second-line treatment and median follow-up time for the survivors was 20.2 months (ranging from 9.5 months to 74.6 months).

Table 1 Patient characteristics according to EGFR mutation status

	EGFR mutation status		*P*
	Mutation	Wild-type	
All cases	48	52	
Sex			<0.0001
Male	15	38	
Female	33	14	
Age, years			0.4942
≤60	18	23	
>60	30	29	
Histology			0.1985
Adenocarcinoma	47	48	
Non-adenocarcinoma	1	4	
Smoking status			<0.0001
Never smoker	32	11	
Smoker	16	41	
Stage at initial diagnosis			0.0341
IIIB	7	17	
IV	41	35	
ECOG PS at initial diagnosis			0.169
0/1	42	40	*P* (0/1 vs. ≥2)
2	2	7	
3	3	3	
4	1	2	
Timing of mutation screening			0.4803
Pre-treatment	31	30	
After first-line treatment	11	16	
After second-line treatment	6	5	
After third-line treatment	0	1	
Mutation genotype			
Exon 19 deletion	23	–	
L858R	25	–	

EGFR epidermal growth factor receptor, *PS* performance status

EGFR mutations and response to gefitinib

Objective response rate (complete response rate + partial response rate) to first-line gefitinib therapy was 87% in patients with EGFR mutations. Disease control rate (complete response rate + partial response rate + stable disease rate) in response to first-line gefitinib therapy was 87% in patients with EGFR mutations. Objective response rate for second-line gefitinib therapy was 80% in patients with EGFR mutations. Disease control rate in response to second-line gefitinib therapy was 86.7% in patients with EGFR mutations (Table 2). No objective responses were observed in patients with wild-type EGFR treated with first- or second-line gefitinib.

No statistically significant differences in rates of objective response and disease control between first- and second-line gefitinib treatments were observed. Furthermore, response rate to gefitinib monotherapy in patients with exon 19 deletions, compared with that in patients with the L858R mutation, did not differ significantly in either first- or second-line treatment (data not shown).

EGFR mutations and response to cytotoxic chemotherapy

Objective response to first- and second-line cytotoxic chemotherapy was not influenced by EGFR mutation status (Table 3). Objective response rate to first-line cytotoxic chemotherapy was 32% in patients with EGFR mutations and 28% in patients with wild-type EGFR ($P = 0.7198$).

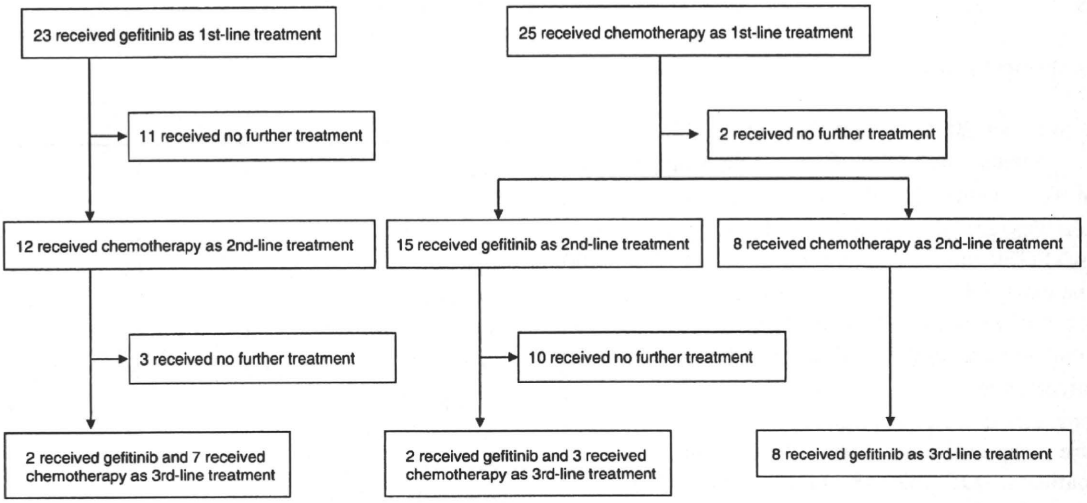

Fig. 1 Treatment flow chart for 48 EGFR mutation-positive patients

Table 2 Response to gefitinib monotherapy in EGFR mutation-positive patients (%)

	First-line ($n = 23$)	Second-line ($n = 15$)
CR	1 (4.3)	1 (6.7)
PR	19 (82.6)	11 (73.3)
SD	0 (0)	1 (6.7)
PD	3 (13.0)	2 (13.3)
OR	20 (87.0)	12 (80.0)
DC	20 (87.0)	13 (86.7)

CR complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *OR* objective response (CR + PR), *DC* disease control (CR + PR + SD)

Objective response rate to second-line cytotoxic chemotherapy was 20% in patients with EGFR mutations and 6.9% in patients with EGFR wild-type ($P = 0.1690$).

EGFR mutation status significantly affected the disease control rate to first-line cytotoxic chemotherapy, but not to second-line cytotoxic chemotherapy. The disease control rate to first-line cytotoxic chemotherapy was 88% in patients with EGFR mutations and 60% in patients with wild-type EGFR ($P = 0.0132$). The disease control rate to second-line cytotoxic chemotherapy was 60% in patients with EGFR mutations and 48.3% in patients with EGFR wild-type ($P = 0.4190$).

PFS in EGFR mutation-positive patients

As illustrated by the Kaplan–Meier curves in Fig. 2a and b, EGFR mutation-positive patients treated with gefitinib monotherapy as first-line treatment experienced significantly longer PFS than did patients who received first-line cytotoxic chemotherapy (median survival, 7.8 months vs. 5.1 months, respectively; $P = 0.0323$). Similarly, EGFR mutation-positive patients treated with gefitinib monotherapy as second-line treatment had significantly longer PFS than did patients who received cytotoxic chemotherapy as second-line treatment (median survival, 6.5 months vs. 4.0 months, respectively; $P = 0.0048$). All 15 patients who received gefitinib monotherapy as second-line treatment had previously received cytotoxic chemotherapy as first-line treatment.

Of the 20 patients who received cytotoxic chemotherapy as second-line treatment, 12 of the patients had received gefitinib as first-line treatment and 8 of the patients had received cytotoxic chemotherapy as first-line treatment previously; no statistically significant difference in PFS was observed between these two groups that had been treated with gefitinib monotherapy as first-line vs. cytotoxic chemotherapy as first-line (data not shown).

In patients treated with gefitinib as first- or second-line treatment, no statistically significant difference in PFS was observed in patients with exon 19 deletions, as compared with patients with the L858R mutation (data not shown).

PFS after cytotoxic chemotherapy, according to EGFR mutation status

In patients treated with cytotoxic chemotherapy as first-line treatment, no significant difference in PFS was observed in patients with EGFR mutations vs. patients who were EGFR wild-type (median survival, 5.1 months vs. 4.4 months, respectively; $P = 0.7184$) (Fig. 3a). Similarly, in patients treated with cytotoxic chemotherapy as second-line treatment, no significant difference in PFS was observed in

Table 3 Response to chemotherapy according to EGFR mutation status (%)

	Chemotherapy as first-line treatment			Chemotherapy as second-line treatment		
	Mutation ($n = 25$)	Wild-type ($n = 50$)	P	Mutation ($n = 20$)	Wild-type ($n = 29$)	P
Type of chemotherapy regimen						
Platinum plus newer agents[a]	22 (88.0)	41 (82.0)		17 (85.0)	18 (62.1)	
Single newer agent	3 (12.0)	9 (8.0)		3 (15.0)	11 (37.9)	
Response						
CR	0 (0)	0 (0)		0 (0)	0 (0)	
PR	8 (32.0)	14 (28.0)		4 (20.0)	2 (6.9)	
SD	14 (56.0)	16 (32.0)		8 (40.0)	12 (41.4)	
PD	3 (12.0)	17 (34.0)		6 (30.0)	14 (48.3)	
NE	0 (0)	3 (6)		2 (10.0)	1 (3.4)	
OR	8 (32.0)	14 (28.0)	0.7198	4 (20.0)	2 (6.9)	0.1690
DC	22 (88.0)	30 (60.0)	0.0132	12 (60.0)	14 (48.3)	0.4190

CR complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *OR* objective response (CR + PR), *DC* disease control (CR + PR + SD

[a] Newer agents were consisted of paclitaxel, docetaxel, vinorelbine, gemcitabine, irrinotecan, amurubicin, and TS-1

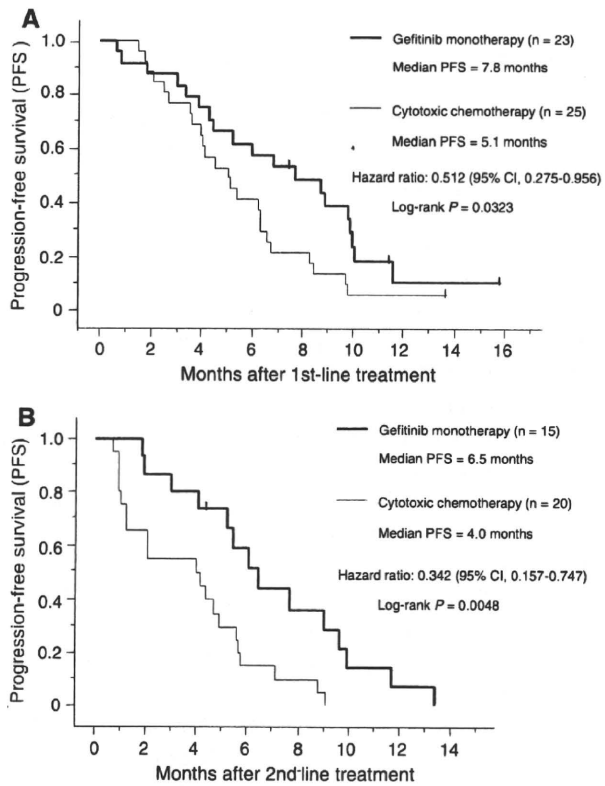

Fig. 2 **a** Kaplan–Meier estimates of progression-free survival of patients with EGFR mutations treated with first-line gefitinib or cytotoxic chemotherapy. **b** Kaplan–Meier estimates of progression-free survival of patients with EGFR mutations treated with second-line gefitinib or cytotoxic chemotherapy

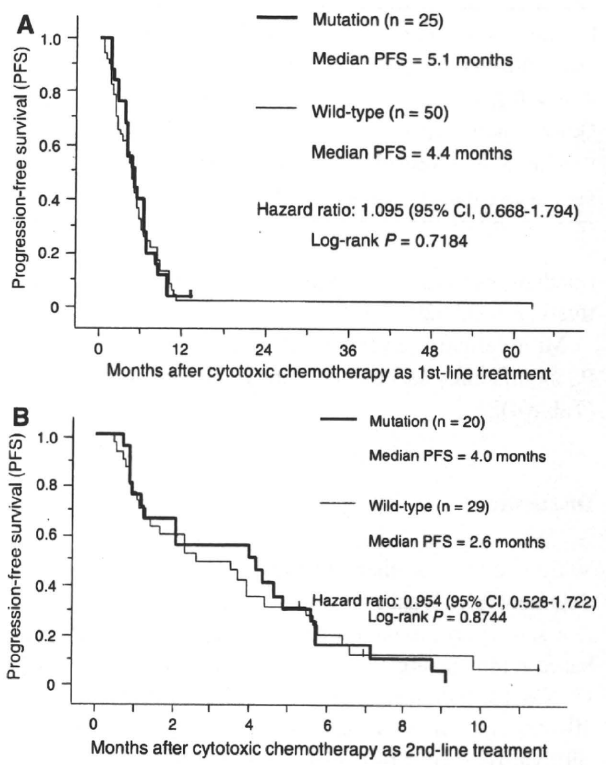

Fig. 3 Kaplan–Meier estimates of progression-free survival of patients grouped by EGFR mutation status who were treated with either first-line (**a**) or second-line (**b**) cytotoxic chemotherapy

patients with EGFR mutations vs. patients with EGFR wild-type (median survival, 4.0 months vs. 2.6 months, respectively; $P = 0.8744$) (Fig. 3b).

Overall survival and multivariate analysis

Patients with EGFR mutations survived for a significantly longer time, as calculated from the initial day of first-line treatment, than did patients who were EGFR wild-type

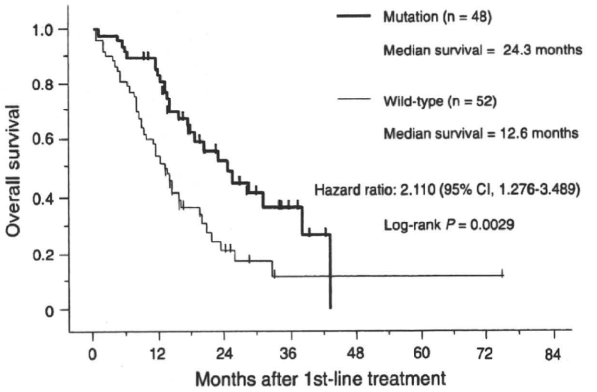

Fig. 4 Kaplan–Meier estimates of overall survival for patients, according to EGFR mutation status

Table 4 Multivariate analysis for overall survival after first-line treatment

Variables	Hazard ratio	95% CI	*P*
EGFR mutation (yes/no)	1.928	1.048–3.545	0.0347
Stage (IIIB/IV)	0.663	0.337–1.306	0.2348
Age (>60/≤60)	1.250	0.741–2.107	0.4028
Gender (male/female)	1.093	0.482–2.481	0.8312
Smoking history (yes/no)	1.268	0.551–2.916	0.5769
Performance status (0–1/2–4)	0.148	0.078–0.282	<0.0001

(median survival, 24.3 months vs. 12.6 months, respectively; $P = 0.0029$; Fig. 4).

Multivariate analysis revealed that EGFR mutations and PS significantly and independently affected overall survival (Table 4).

Discussion

Various cytotoxic chemotherapy agents are utilized in the treatment of advanced or metastatic NSCLC. In the first-line setting, combination chemotherapy such as platinum-based regimens are given empirically to most stage IIIB or IV NSCLC patients, resulting in objective response rates of 30–40%, median survival times of 8–10 months, and 1-year survival rates of 30–40% (Kelly et al. 2001; Schiller et al. 2002). Recently, novel, small molecule therapeutic agents that specifically target certain molecular pathways, including the EGFR TKIs, gefitinib and erlotinib, have been developed. A new approach for selecting patients by the presence of molecular or genetic biomarkers, such as EGFR mutations and gene copy number, is evolving (Cappuzzo et al. 2005, 2007; Han et al. 2006).

Cappuzzo et al. demonstrated that, in NSCLC patients treated with gefitinib, a high gene copy number, rather than EGFR mutations, was a better predictor of survival (Cappuzzo et al. 2005). Furthermore, molecular analyses from large placebo-controlled phase III trials of TKIs also showed that EGFR gene copy number was superior to mutations as a predictor of clinical benefit (Hirsch et al. 2006; Tsao et al. 2005). These studies included mostly Caucasian patients with NSCLC. On the other hand, studies in Japan and Korea demonstrated that EGFR mutation was the most important biomarker to identify NSCLC patients for treatment with gefitinib (Han et al. 2006; Ichihara et al. 2007; Sone et al. 2007; Takano et al. 2005).

In the INTACT and TRIBUTE studies, which were conducted to compare TKIs (gefitinib in the INTACT trial, and erlotinib in the TRIBUTE trial) with placebo in combination with cytotoxic chemotherapy, patients with EGFR mutations exhibited better PFS after cytotoxic chemotherapy than did patients without mutations (Bell et al. 2005; Eberhard et al. 2005). Similarly, Hotta et al. reported that EGFR mutation-positive patients treated with first-line cytotoxic chemotherapy yielded better PFS than did EGFR mutation-negative patients, and furthermore, no significant difference in PFS in patients (with and without mutations) who were treated with cytotoxic chemotherapy following gefitinib monotherapy. Therefore, they suggested that early use of cytotoxic chemotherapy prior to gefitinib treatment was advantageous for EGFR mutation-positive patients (Hotta et al. 2007).

This study assessed whether EGFR mutation-positive status of NSCLC patients influenced clinical outcome of first- and second-line treatment with cytotoxic chemotherapy or gefitinib monotherapy. In contrast to the findings of Hotta et al. (2007), we observed that PFS following first- and second-line cytotoxic chemotherapy was not associated with EGFR mutation status (Fig. 3a, b). Moreover, in our study, EGFR mutation-positive patients treated with first- or second-line gefitinib exhibited better PFS than did patients treated with first- or second-line cytotoxic chemotherapy (Fig. 2a, b). Thus, our findings suggest that patients with EGFR mutations might benefit from either first- or second-line gefitinib monotherapy. The reason for different clinical outcomes in our study and previous studies by other investigators (Bell et al. 2005; Eberhard et al. 2005; Hotta et al. 2007) is unclear. However, possible explanations include differences in ethnicity of study participants and eligibility criteria (e.g., stage of disease and prior treatment) in the various studies. Most of the study participants in the INTACT and TRIBUTE trials were non-Asian patients. In our study, which was conducted in Japan, the EGFR mutation-positive patients had stage IIIB and IV disease. In the study conducted by Hotta et al. (2007), 82% of the EGFR mutation-positive patients had recurrent disease after surgery. Previous research by other investigators has not elucidated how EGFR mutations affect clinical outcomes in

Asian vs. non-Asian NSCLC patients, or in early-stage operable NSCLC patients vs. NSCLC patients with advanced/metastatic disease (Jackman et al. 2006). Recent reports from Asia demonstrated that there was no significant difference in OS between gefitinib-first group and chemotherapy-first group (Morita et al. 2009; Wu et al. 2008).

Because our study population consisted of NSCLC patients screened for EGFR mutation in order to select patients for gefitinib treatment, only three patients who were EGFR wild-type received gefitinib treatment. Therefore, it is difficult to assess whether gefitinib treatment affects clinical outcomes according to EGFR mutation status of NSCLC patients. Nevertheless, we observed similar PFS in patients treated with first- or second-line cytotoxic chemotherapy, regardless of the EGFR mutation status of the patients (Fig. 3a, b). Thus, the longer PFS seen in EGFR mutation-positive patients treated with gefitinib than in patients treated with cytotoxic chemotherapy (Fig. 2a, b) might be attributable to a superior OS than that exhibited by patients who were EGFR wild-type. Our finding is consistent with a subset analysis of a recently completed phase III study (Iressa Pan-Asia Study) showing that gefitinib monotherapy significantly improved the PFS of EGFR mutation-positive patients compared with carboplatin and paclitaxel in the first-line setting (Mok et al. 2009).

Our multivariate analysis indicated that PS and EGFR mutations were significant prognostic factors (Table 4 and Fig. 4), which is consistent with the first report of prospective EGFR mutation screening for NSCLC patients by Sutani et al. (2006). Many investigators believe that patients with EGFR mutations who are treated with EGFR TKIs have significantly longer survival than do patients with EGFR wild-type who are treated with EGFR TKIs (Han et al. 2005; Mitsudomi et al. 2005; Takano et al. 2005). However, this point is still controversial, because some researchers demonstrated that chemotherapy patients with EGFR mutations survived for a longer period than did chemotherapy patients who were EGFR wild-type (Bell et al. 2005; Eberhard et al. 2005). Takano et al. (2008) reported that EGFR mutations are both prognostic and predictive factors. Furthermore, after approval of gefitinib in Japan, median survival of EGFR mutation-positive patients with advanced lung adenocarcinoma was 27.2 months. The median survival time, which was similar to that observed in our study, was never observed in advanced/metastatic NSCLC patients treated with conventional chemotherapy. According to Takano et al. (2008), the favorable median survival time was caused mainly by gefitinib treatment.

Several recent studies have reported that patients with exon 19 deletions had superior response rates, PFS, and OS, as compared with patients with the L858R mutations (Jackman et al. 2006; Mitsudomi et al. 2005; Riely et al. 2006). In this study, clinical outcomes in patients with exon 19 deletions, compared with outcomes in patients with the L858R mutation, did not differ significantly (data not shown). This finding is consistent with previous reports from East Asia showing almost the same survival benefit of gefitinib in patients with either type of mutation (Morita et al. 2009; Takano et al. 2008; Wu et al. 2008).

Recently, a Japanese phase II trial of first-line gefitinib for patients with advanced NSCLC harboring EGFR mutations without indication for chemotherapy demonstrated the benefit of first-line gefitinib for EGFR mutation-positive patients with extremely poor PS and/or with high age, yielding a favorable response rate of 66%, median survival time of 17.8 months and 1-year survival rate of 63% (Inoue et al. 2009). However, we cannot make a conclusion with respect to the timing of gefitinib therapy in the gefitinib-first group and chemotherapy-first group for EGFR mutation-positive patients with good PS and an age <75 years. Our results suggest that EGFR mutation-positive patients benefit from either first- or second-line gefitinib monotherapy. Currently in Japan, two ongoing, prospective, randomized trials are exploring treatment with gefitinib or standard chemotherapy (cisplatin + docetaxel in the trial conducted by the West Japan Oncology Group; carboplatin + paclitaxel in the trial conducted by the North-East Japan Gefitinib Study Group), with the primary endpoint of PFS in patients with EGFR mutations. Results from these trials will provide conclusive results with respect to gefitinib timing for NSCLC patients with EGFR mutations in terms of both PFS and OS.

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Conflict of interest statement All authors have no financial or personal relationships with other people or organizations that could inappropriately influence our work.

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ORIGINAL ARTICLE

Osteopontin-mediated enhanced hyaluronan binding induces multidrug resistance in mesothelioma cells

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Malignant pleural mesothelioma (MPM) is resistant to chemotherapy and thus shows a dismal prognosis. Osteopontin (OPN), a secreted noncollagenous and phosphoprotein, is suggested to be involved in the pathogenesis of MPM. However, the precise role of OPN, especially in the multidrug resistance of MPM, remains to be elucidated. We therefore established stable transfectants (ACC-MESO-1/OPN), which constitutively express OPN, to determine its role in the chemoresistance observed in MPM. The introduction of the OPN gene provides MPM cells with upregulated multidrug resistance through the mechanism of enhanced hyaluronate (HA) binding. The expression of CD44 variant isoforms, which inhibit HA binding, significantly decreased in ACC–MESO–1/OPN cells in comparison to control transfectants. Interestingly, the inhibition of the HA-CD44 interaction abrogated multidrug resistance in the ACC–MESO–1/OPN, thus suggesting the involvement of the surviving signal emanating from the HA-CD44 interaction. An enhanced level of the p-Akt in ACC–MESO–1/OPN cells was observed, and was diminished by CD44 siRNA. Inhibition of the Akt phosphorylation increased in number of the cells underwent apoptosis induced by NVB, VP-16 and GEM. Collectively, these results indicate that OPN is strongly involved in multidrug resistance by enhancing the CD44 binding to HA.
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Keywords: osteopontin; mesothelioma; multidrug resistance; hyaluronan; CD44

Introduction

Malignant pleural mesothelioma (MPM) is an extremely aggressive tumor, which has been shown to be resistant to all conventional therapeutic regimens. A surgical resection is possible in only a minority of patients, and fewer than 15% of these patients live beyond 5 years (Sugarbaker *et al.*, 1996; Boutin *et al.*, 1998; Rusch and Venkatraman, 1999). For those who are not treated with a curative resection, the median survival has been reported to be 6 months (Ruffie, 1991; De Pangher Manzini *et al.*, 1993). As a result, chemotherapy is still the mainstay of disease therapy. Various drugs including doxorubicin, cyclophosphamide, cisplatin (CDDP), carboplatin, gemcitabine (GEM), and pemetrexed have been tested in different combinations (Samson *et al.*, 1987; Chahinian *et al.*, 1993; White *et al.*, 2000; Kindler *et al.*, 2001; Hughes *et al.*, 2002). However, the limited combinations of these agents have marginally provided some clinical benefit because of multidrug resistance. Moreover, none of the molecular targeting agents have shown any of their clinical benefit in the patients with MPM (Moore *et al.*, 2007; Jackman *et al.*, 2008). Therefore, it is hoped that a better understanding of the mechanism of the multidrug resistance in MPM may provide the rationale for the development of new therapeutic strategies.

Drug resistance arises in numerous ways, such as through a decreased access to or uptake of drugs, the activation of repair and detoxification mechanisms, and an increased drug efflux. Among these numerous mechanisms, resistance against anti-cancer agents has been recently reported upon cell adhesion to the extracellular matrix (ECM) (Damiano *et al.*, 1999; Elliott and Sethi, 2002; Hazlehurst *et al.*, 2003), thus suggesting that tumor–microenvironment interaction regulates the sensitivity of anti-cancer agents. For instance, it has been reported that small cell lung cancer cells, myeloma cells, glioma cells and colon cancer cells were protected from apoptosis induced by various anticancer agents when the cells were plated on ECM (Damiano *et al.*, 1999; Sethi *et al.*, 1999; Uhm *et al.*, 1999; Kouniavsky *et al.*, 2002). MPM cells are surrounded by pleural effusion, which contains a variety of ECM including hyaluronate (HA) and osteopontin (OPN) (Thylen *et al.*, 1997; Pass *et al.*, 2005; Grigoriu *et al.*, 2007). The ability to grow in pleural fluid suggests

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that the MPM cells are capable of surviving and proliferating against apoptosis under the influence of this particular microenvironment. These findings indicate that the biological function of MPM appears to be strictly regulated by the interaction with ECM.

OPN, a secreted noncollagenous, phosphoprotein, functions as both an ECM component and cytokine through the binding to its receptors; integrin and CD44 (Denhardt *et al.*, 2001). OPN has been associated with cancer progression, metastasis and apoptosis (Rangaswami *et al.*, 2006). Several studies have revealed elevated levels of OPN in serum and pleural effusion to be observed in patients with MPM (Pass *et al.*, 2005; Grigoriu *et al.*, 2007), thus suggesting the involvement of OPN in the pathogenesis of MPM. In fact, OPN was recently demonstrated to mediate MPM cell proliferation and migration (Ohashi *et al.*, 2009). OPN has also been reported to be involved in resistance to chemotherapy of mouse breast cancer cells by inhibiting apoptosis (Graessmann *et al.*, 2007). However, the role of OPN especially in multidrug resistance in MPM has not yet been clarified.

HA is a linear glycosaminoglycan, which is ubiquitously distributed in the ECM, and interacts with cell surface receptors including CD44 (Toole, 2004). HA facilitates cell adhesion, cell motility, cellular proliferation and tumor progression (Lokeshwar *et al.*, 1997). An increased HA production was found to upregulate drug resistance in cancer cells (Misra *et al.*, 2003). It is therefore possible that the effect of CD44 binding to HA on cell-survival signaling might alter drug resistance. In fact, HA–CD44 interaction was recently revealed to have a pivotal role in the chemoresistance in non-small cell lung cancer cells (Ohashi *et al.*, 2007). Very interestingly, recent studies have strongly supported the notion that the OPN could modulate the CD44 isoform expression, which closely regulates HA binding. For instance, Khan *et al.* (2005) reported that OPN modulates the specific CD44 isoform expression to facilitate breast cancer cell migration. Moreover, an overexpression of endogenous OPN results in increased hyaruronan synthase 2 activities, thus leading to an increased HA production and an enhanced anchorage-independent growth (Cook *et al.*, 2006).

Based on these findings, we hypothesized that OPN could regulate chemosensitivity through the alteration of CD44 binding to HA. We also discuss the potential mechanisms of the multidrug resistance in MPM.

Results

Generation of stable transfectant that secretes OPN

BMGNeo–OPN and BMGNeo were transfected into the ACC-MESO-1 cells and we thus obtained two stable OPN-overexpressing clones (ACC-MESO-1/OPN#7 and ACC-MESO-1/OPN#8) and two control clones (ACC-MESO-1/Neo#1 and ACC-MESO-1/Neo#2). To verify the secretion of OPN protein from the transfectant, we conducted the ELISA for OPN. As shown in Figure 1a, ELISA demonstrated the ACC-MESO-1/OPN cells to secrete significant amounts of OPN.

Transfection with OPN gene result in increased multidrug resistance

The IC_{50} of NVB against OPN7 cells and OPN8 cells were 3.87 ± 0.47 ng/ml and 4.56 ± 0.89 ng/ml, respectively, whereas those against Neo1 cells, Neo2 cells and parental cells were 1.70 ± 0.04 ng/ml, 1.97 ± 0.71 ng/ml and 1.91 ± 0.11 ng/ml, respectively (Figure 1b). The IC_{50} of VP16 against OPN7 cells and OPN8 cells were 8.71 ± 0.34 μM and 15.76 ± 1.49 μM, respectively, whereas those against Neo1 cells, Neo2 cells and parental cells were 4.47 ± 1.51 μM, 3.69 ± 1.00 μM and 2.17 ± 0.19 μM, respectively (Figure 1c). As IC_{50} never reached at any concentration of GEM in OPN transfectants, we could not show the IC_{50} regarding OPN transfectants. The IC_{50} of GEM against Neo1 cells, Neo2 cells and parental cells were 0.05 ± 0.002 μM, 0.03 ± 0.02 μM and 0.04 ± 0.018 μM, respectively (Figure 1d). The IC_{50} of CDDP against OPN7 cells and OPN8 cells were 6.44 ± 0.18 μg/ml and 5.85 ± 0.40 μg/ml, respectively, whereas those against Neo1 cells, Neo2 cells and parental cells were 12.00 ± 1.52 μg/ml, 11.56 ± 2.68 μg/ml and 3.39 ± 1.68 μg/ml, respectively (Figure 1e). These results indicate that the ACC-MESO-1/OPN cells were more resistant to NVB, VP-16 and GEM than the ACC-MESO-1/Neo or parental cells, whereas ACC-MESO-1/OPN cells did not demonstrate resistance to CDDP. We next evaluated the amount of apoptotic cells (Figure 1f). We observed that ACC-MESO-1/OPN cells were more significantly resistant to apoptosis mediated by anticancer agents than ACC-MESO-1/Neo cells. These data indicate that the transfection with OPN gene thus resulted in an increased multidrug resistance. Moreover, transfection with OPN gene also increased resistance to apoptosis induced by NVB, VP-16 and GEM.

OPN regulates cell adhesion to HA

To evaluate the effect of OPN transfected to ACC-MESO-1 cells on OPN or HA binding, an *in vitro* cell adhesion assay was performed using ACC-MESO-1/OPN, ACC-MESO-1/Neo and parental cells. The cells were investigated for adhesion to OPN, HA or BSA (Figure 2a). Interestingly, the ratio of adherence to HA (percent-specific adhesion to HA/percent-specific adhesion to BSA) of ACC-MESO-1/OPN cells was significantly greater than that of the ACC-MESO-1/Neo and parental cells, whereas neither ACC-MESO-1/OPN cells nor ACC-MESO-1/Neo cells demonstrated adherence to OPN (Table 1). To investigate whether the silencing of the OPN expression abrogates enhanced adhesion to HA, we downregulated the OPN expression in ACC-MESO-1/OPN#8 cells by siRNA. siRNA transfection downregulated the OPN expression by >80% (Figure 2b) and then we next performed an adhesion assay. As expected, the silencing of OPN expression in ACC-MESO-1/OPN#8 cells decreased the adhesion to HA (Figure 2c). These results therefore suggest that OPN regulates the cell adhesion to HA.

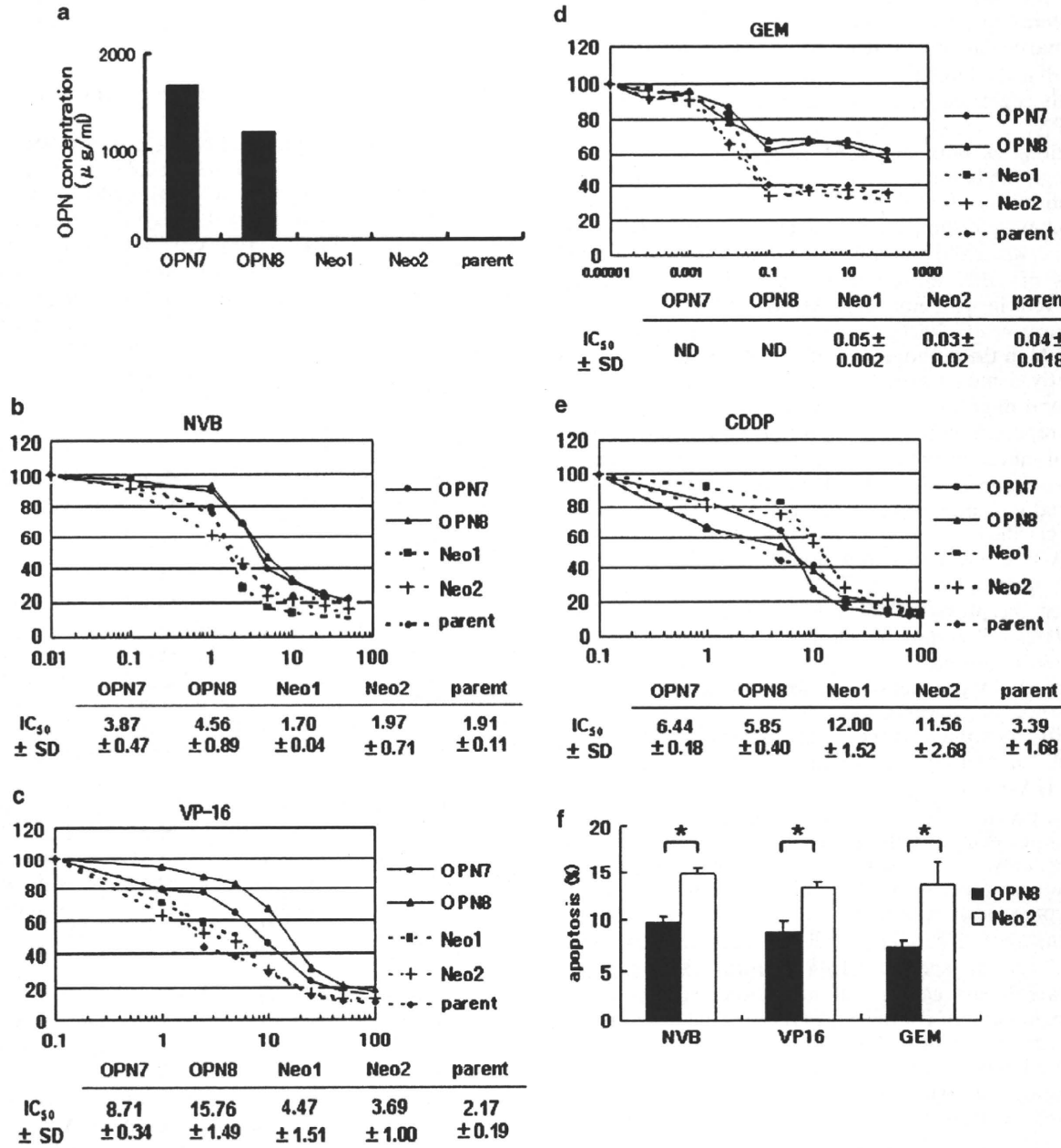

Figure 1 Establishment of ACC-MESO-1 cells transfected with an empty vector (ACC-MESO-1/Neo) or OPN gene (ACC-MESO-1/OPN). (**a**) OPN protein secretion was determined with ELISA. *In vitro* chemosensitivity assay. Both ACC-MESO-1/OPN and ACC-MESO-1/Neo clones were cultured in the absence or the presence of various concentration of vinorelbin (VNB; **b**), etoposide (VP-16; **c**), gemcitabine (GEM; **d**), and cisplatin (CDDP; **e**). Data are representative of the findings of one of three independent experiments with similar results. IC_{50}s are presented as the mean ± s.d. in triplicates. ND: not determined. (**f**) Apoptosis induction was evaluated by Annexin V staining method. ACC-MESO-1/OPN#8 cells were cultured in the presence of VNB (2.5 ng/ml), VP-16 (10 μM) and GEM (0.1 μM). Closed and open squares indicate ACC-MESO-1/OPN#8 and ACC-MESO-1/Neo#2, respectively. Data are presented as the mean ± s.d. of apoptotic cells of three independent experiments. $*P<0.05$ vs OPN8.

Expression of CD44 and CD44 variant isoforms

As ACC-MESO-1/OPN cells showed an enhanced adhesion to HA, we therefore compared the expression levels of CD44, a principle receptor for HA, on the surface of ACC-MESO-1/OPN, ACC-MESO-1/Neo and parental cells with FACScan. As shown in Figure 3a, there was no difference in the total CD44 surface expression among ACC-MESO-1/OPN, ACC-MESO-1/Neo and parental cells. As certain CD44 variant isoforms have been reported to display significantly less HA binding than CD44s (Iida and Bourguignon, 1997), we next examined the expression pattern

of the CD44 variant isoforms using RT–PCR (Figure 3b) and western blotting (Figure 3c). It was noteworthy that the fragment of CD44s, which was expected to exhibit a PCR amplification product of 375 bp and several other larger fragments were expressed in each clone, thus suggesting the presence of alternatively spliced transcripts (Figure 3b). To identify these larger fragments, a direct DNA sequence analysis was performed. The 550 bp product corresponds to exon14 (v10), whereas the 750 bp product corresponds to exons12, 13 and 14 (v8, v9 and v10, respectively) (data not shown). CD44 protein expression was analyzed by western blotting using BU52. The expression of high molecular weight CD44 variant isoforms was significantly decreased in the ACC-MESO-1/OPN cells in comparison compared to that of the ACC-MESO-1/Neo and parental cells (Figure 3c). To determine whether exogenous OPN regulates the expression of CD44 variant isoforms on ACC-MESO-1 cells, a western blot analysis was performed to evaluate the CD44 isoform expression on the ACC-MESO-1 cells cultured on OPN and BSA. The expression of high molecular weight CD44 variant isoforms significantly decreased in the ACC-MESO-1 cells cultured on OPN compared with that of the ACC-MESO-1 cells cultured on BSA (Figure 3d).

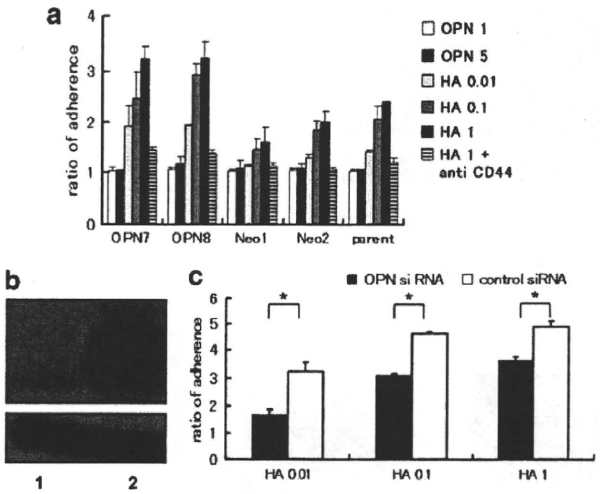

Figure 2 Adhesion assay. *In vitro cell* adhesion assay of ACC-MESO-1/OPN cells and ACC-MESO-1/Neo cells. (**a**) ACC-MESO-1/OPN, ACC-MESO-1/Neo or parental cells, were allowed to adhere to wells coated with OPN (1, 5 μg/ml), HA (0.01, 0.1, 1 mg/ml) or BSA (10 mg/ml) in the presence or absence of anti-CD44 antibody (BU75, 1 μg/ml). Data are presented as the mean ± s.d. in triplicates. (**b**) OPN expression by ACC-MESO-1/OPN cells transfected with OPN siRNA (lane 1) or negative control siRNA (lane 2). OPN protein expression was detected by western blotting. Bottom panel shows β-actin expression as loading control. (**c**) *In vitro* cell adhesion assay was performed using ACC-MESO-1/OPN OPN#8 siRNA and ACC-MESO-1/OPN#8 control siRNA. Closed and open squares indicate OPN siRNA and control siRNA, respectively. Data are presented as the mean ± s.d. in triplicates. $^{*}P<0.05$ vs control siRNA.

Downregulation of CD44v10 expression increases the multidrug resistance

To investigate whether the downregulation of the CD44v10 expression is involved in the mechanism of the multidrug resistance, we downregulated the CD44v10 expression in ACC-MESO-1 cells by siRNA. siRNA transfection downregulated the CD44v10 expression by >80% and then a chemosensitivity assay was performed (Figure 4a). The IC_{50} of NVB against the ACC-MESO-1 cells transfected with CD44v10 siRNA was 17.47 ± 7.00 ng/ml, whereas those against the cells transfected with control siRNA was 2.54 ± 0.31 ng/ml (Figure 4b). The IC_{50} of NVB against the ACC-MESO-1 cells transfected with CD44v10 siRNA was significantly higher than that against the cells transfected with control siRNA ($P<0.05$). The IC_{50} of VP16 against the ACC-MESO-1 cells transfected with CD44v10 siRNA was 10.53 ± 0.11 μM, whereas those against the cells transfected with control siRNA was 2.36 ± 0.20 μM (Figure 4c). The IC_{50} of VP-16 against the ACC-MESO-1 cells transfected with CD44v10 siRNA was significantly higher than that against the cells transfected with control siRNA ($P<0.05$). As IC_{50} was never

Table 1 The ratio of adherence to HA and OPN (percent-specific adhesion to HA or OPN/percent-specific adhesion to BSA)[a]

	OPN 1	*OPN 5*	*HA 0.01*	*HA 0.1*	*HA 1*
OPN7	1.02 ± 0.09	1.00 ± 0.02	1.92 ± 0.40*	2.47 ± 0.48	3.21 ± 0.24#,##,###
OPN8	1.05 ± 0.03	1.15 ± 0.15	1.93 ± 0.01*,**,***	2.93 ± 0.22+,++,+++	3.25 ± 0.30#,##,###
Neo1	1.02 ± 0.03	1.08 ± 0.15	1.11 ± 0.01	1.45 ± 0.22	1.60 ± 0.30
Neo2	1.03 ± 0.05	1.08 ± 0.09	1.29 ± 0.06	1.85 ± 0.18	2.00 ± 0.21
Parent	1.01 ± 0.05	1.03 ± 0.03	1.40 ± 0.02	2.07 ± 0.24	2.39 ± 0.01

[a]Data are presented as the mean ± s.d. in triplicates.
$^{*}P<0.05$ vs Neo1 cells treated with HA 0.01.
$^{**}P<0.05$ vs Neo2 cells treated with HA 0.01.
$^{***}P<0.05$ vs parent cells treated with HA 0.01.
$^{\#}P<0.05$ vs Neo1 cells treated with HA 1.
$^{\#\#}P<0.05$ vs Neo2 cells treated with HA 1.
$^{\#\#\#}P<0.05$ vs parent cells treated with HA 1.
$^{+}P<0.05$ vs Neo1 cells treated with HA 0.1.
$^{++}P<0.05$ vs Neo2 cells treated with HA 0.1.
$^{+++}P<0.05$ vs parent cells treated with HA 0.1.

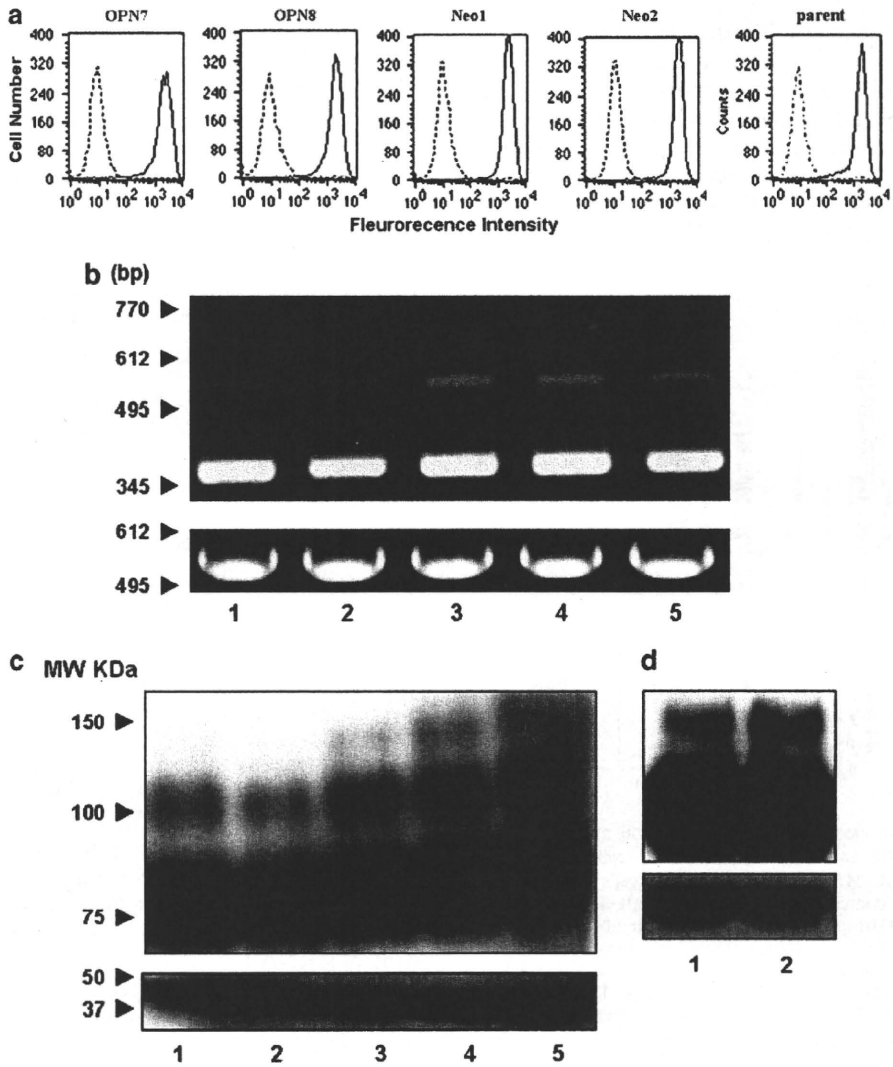

Figure 3 The expression of CD44 and CD44variant forms on ACC-MESO-1/OPN, ACC-MESO-1/Neo and parental cells. **(a)** To determine the CD44 expression, ACC-MESO-1/OPN, ACC-MESO-1/Neo and parental cells were incubated with monoclonal anti-CD44 (BU52, 1 μg/ml) antibody and analyzed with FACScan. **(b)** CD44 mRNA expression by ACC-MESO-1/OPN#7 (lane 1), ACC-MESO-1/OPN#8 (lane 2), ACC-MESO-1/Neo#1 (lane 3), ACC-MESO-1/Neo#2 (lane 4) and ACC-MESO-1 cells (lane 5) were shown. A bottom panel of each set shows β-actin expression as a control. DNA size markers are shown on the left side. **(c)** The expression of CD44s and variants on ACC-MESO-1/OPN#7 (lane 1), ACC-MESO-1/OPN#8 (lane 2), ACC-MESO-1/Neo#1 (lane 3), ACC-MESO-1/Neo#2 (lane 4) and ACC-MESO-1 (lane 5) were assessed by western blotting with anti-CD44 antibody (BU52). A bottom panel shows β-actin expression as loading control. The molecular weight size markers are indicated on the left side. **(d)** The expression of CD44s and variants on ACC-MESO-1 cells cultured on OPN (lane 1) and BSA (lane 2). CD44 protein expression was detected by western blotting. Bottom panel shows β-actin expression as loading control.

reached at any concentration of GEM in the ACC-MESO-1 cells transfected with CD44v10 siRNA, we could not show the IC_{50} against these cells. The IC_{50} of GEM against the cells transfected with control siRNA was $0.08 \pm 0.009\,\mu M$ (Figure 4d). As expected, the silencing of the CD44v10 expression in ACC-MESO-1 cells increases the multidrug resistance. The silencing of CD44v10 expression in ACC-MESO-1 cells also increases adhesion to HA (data not shown). These results suggest that the downregulation of CD44v10, accompanied by the enhanced adhesion to HA, is involved in the multidrug resistance.

Inhibition of the HA–CD44 interaction abrogated multidrug resistance and resistance to apoptosis

To determine whether HA–CD44 interaction is involved in the mechanism of the multidrug resistance, the expression of CD44 in ACC-MESO-1/OPN and ACC-MESO-1/Neo cells were downregulated by siRNA. siRNA transfection downregulated the CD44 expression by >70% and then a chemosensitivity assay was performed (Figure 5a). The IC_{50} of NVB against the OPN8 cells transfected with CD44 siRNA, OPN8 cells transfected with control siRNA, Neo2 cells transfected with CD44 siRNA and Neo2 cells transfected with

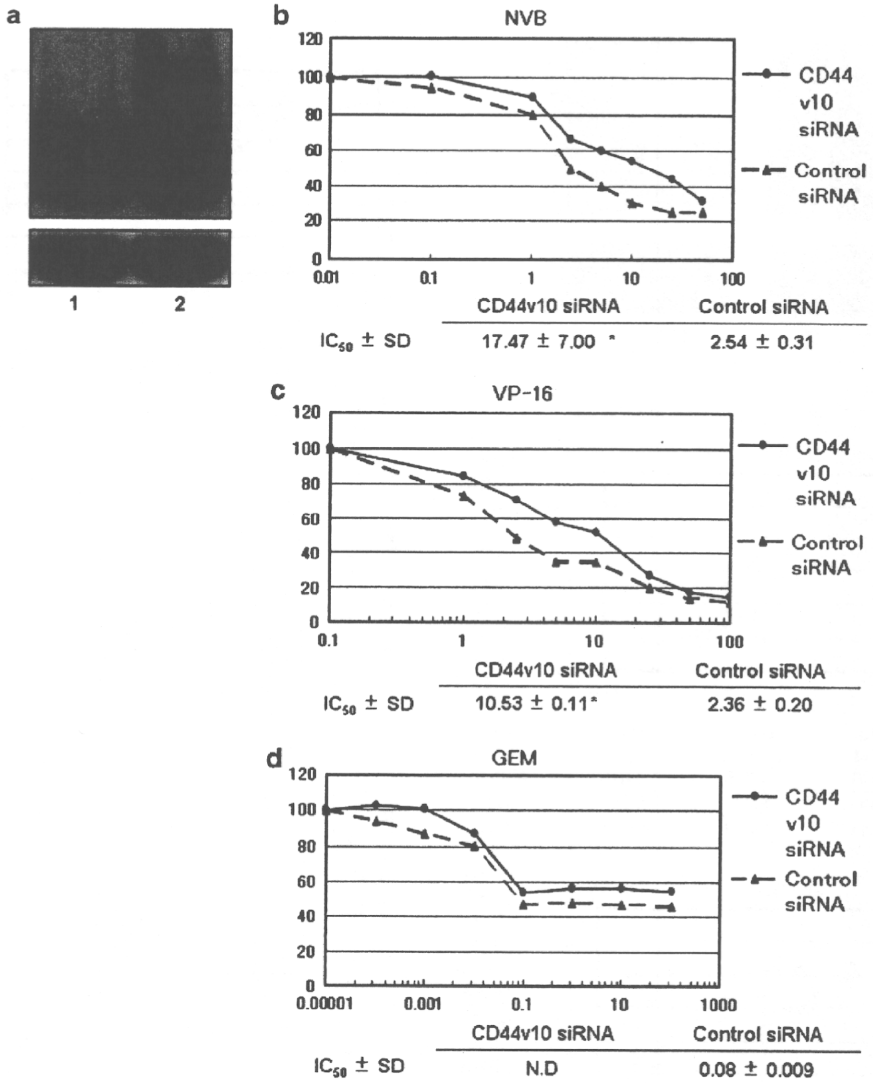

Figure 4 Effect of CD44v10 downregulation by siRNA on chemosensitivity of ACC-MESO-1 cells. (**a**) CD44v10 expression of ACC-MESO-1 cells transfected with CD44v10 siRNA (lane 1) or negative control siRNA (lane 2). A bottom panel shows β-actin expression as loading control. ACC-MESO-1 CD44 siRNA and negative control siRNA cells were cultured in the absence or the presence of various concentrations of vinorelbin (VNB; **b**), etoposide (VP-16; **c**) and gemcitabine (GEM; **d**). IC_{50}s are presented as the mean ± s.d. in triplicates. $^*P<0.05$ vs control siRNA. ND, not determined.

control siRNA were 1.10 ± 0.11 ng/ml, 5.33 ± 0.49 ng/ml, 1.65 ± 0.13 ng/ml and 1.61 ± 0.20 ng/ml, respectively (Figure 5b). The IC_{50} of VP-16 against the OPN8 cells transfected with CD44 siRNA, OPN8 cells transfected with control siRNA, Neo2 cells transfected with CD44 siRNA and Neo2 cells transfected with control siRNA were 17.54 ± 0.74 μM, 19.95 ± 1.05 μM, 11.52 ± 0.26 μM and 12.95 ± 0.75 μM, respectively (Figure 5c). As IC_{50} was never reached at any concentration of GEM in the OPN8 cells transfected with CD44 siRNA and control siRNA, we could not show the IC_{50} regarding the OPN8 cells. The IC_{50} of GEM against the Neo2 cells transfected with CD44 siRNA and Neo2 cells transfected with control siRNA were 0.07 ± 0.009 μM and 0.06 ± 0.008 μM, respectively (Figure 5d). As expected, the silencing of the CD44 expression in ACC-MESO-1/OPN#8 cells abrogated the multidrug resistance and increased apoptotic cells in number (Figure 5e). In contrast, silencing of CD44 expression did not influence the chemosensitivity in ACC-MESO-1/Neo#2 cells. ACC-MESO-1/OPN cells in the absence or the presence of BU75 were also cultured with VNB, VP-16 and GEM. As shown in Figure 5f, inhibition of the HA–CD44 interaction increased apoptosis and abrogated resistance to apoptosis. To confirm that the resistance to apoptosis was mediated by overexpression of OPN, we downregulated OPN expression by siRNA and performed the same experiment (Figure 5g). We again observed the downregulation of OPN expression to increase apoptosis. These results together with Figure 3c suggest that OPN-mediated alteration in HA–CD44 binding is involved in the mechanism of multidrug

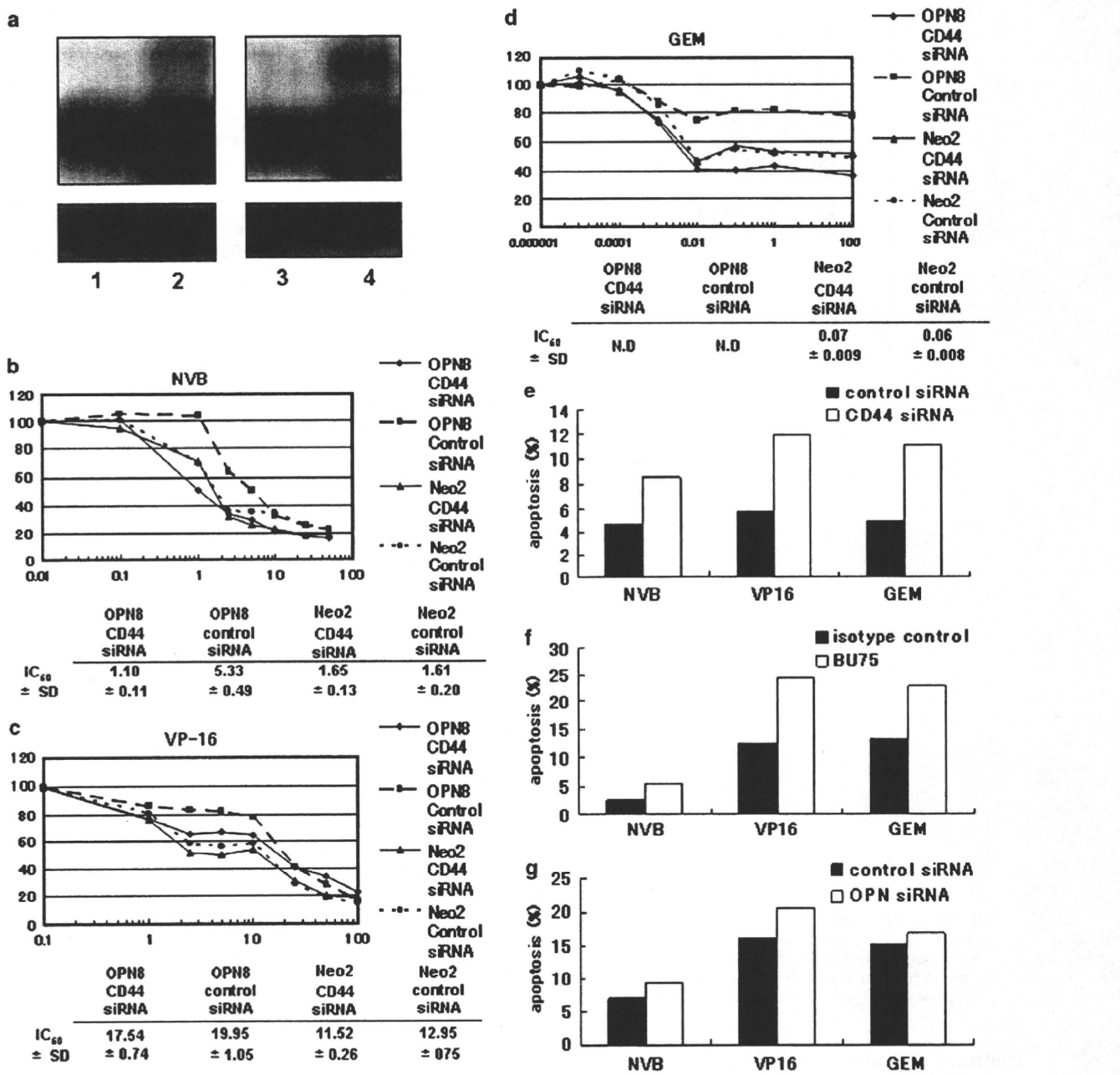

Figure 5 The effect of CD44 downregulation by siRNA on chemosensitivity of ACC-MESO-1/OPN and ACC-MESO-1/Neo cells. (**a**) The CD44 expression of ACC-MESO-1/OPN#8 or ACC-MESO-1/Neo#2 cells transfected with CD44 siRNA (lane 1, 3) or negative control siRNA (lane 2, 4). A bottom panel shows β-actin expression as loading control. ACC-MESO-1/OPN#8 or ACC-MESO-1/Neo#2 CD44 siRNA and negative control siRNA cells were cultured in the absence or the presence of various concentrations of vinorelbin (VNB; **b**), etoposide (VP-16; **c**) and gemcitabine (GEM; **d**). IC_{50}s are presented as the mean ± s.d. in triplicates. ND: not determined. (**e**) Effect of CD44 downregulation by siRNA on apoptosis of ACC-MESO-1/OPN cells exposed to indicate chemotherapeutic agents. Apoptosis was evaluated by the Annexin V staining method. ACC-MESO-1/OPN#8 cells treated with CD44 siRNA or negative control siRNA were cultured in the presence of VNB (2.5 ng/ml), VP-16 (10 μM) and GEM (0.1 μM). Closed and open squares indicate the percentage of apoptotic cells treated with negative control siRNA or CD44 siRNA, respectively. (**f**) The effect of anti-CD44 antibody (BU75) on apoptosis of ACC-MESO-1/OPN cells exposed to indicated chemotherapeutic agents. Apoptosis was evaluated by Annexin V staining method. ACC-MESO-1/OPN#8 cells were incubated with BU75 (1 μg/ml) or isotype control mouse IgG2a (1 μg/ml) in the presence of VNB (2.5 ng/ml), VP-16 (10 μM) and GEM (0.1 μM). Closed and open squares indicate the percentage of apoptotic cells treated with isotype control mouse IgG2a or BU75, respectively. (**g**) ACC-MESO-1/OPN#8 treated with OPN siRNA or negative control siRNA cells were cultured in the presence of VNB (2.5 ng/ml), VP-16 (10 μM) and GEM (0.1 μM). Closed and open squares indicate the percentage of apoptotic cells treated with negative control siRNA or OPN siRNA, respectively. All results (**e**–**g**) are representative ones of three independent experiments with similar results.

resistance and resistance to apoptosis induced by NVB, VP-16 and GEM.

CD44-mediated resistance to apoptosis involves the Akt survival pathway

As the Akt pathway is a well-characterized kinase that promotes cellular survival and several researchers have already shown that HA activates the PI3k–Akt signaling pathway (Sohara *et al.*, 2001; Ghatak *et al.*, 2002; Zoltan-Jones *et al.*, 2003), we therefore investigated whether the survival signal emanating from the HA–CD44 interaction is mediated by the activation of the Akt pathway. To assess Akt phosphorylation, immunoblotting with anti-Akt and phospho-Akt-specific antibodies was carried out in ACC-MESO-1/OPN and ACC-MESO-1/Neo cells (Figure 6a). As expected, the enhanced level of phosphorylation of Akt (p-Akt) was observed in the ACC-MESO-1/OPN cells. To investigate whether the HA–CD44 interaction mediates p-Akt, the CD44 expression was thus downregulated by siRNA and then the p-Akt expression was assessed. As shown in Figure 6b, the elevation of p-Akt in the ACC-MESO-1/OPN cells decreased by the downregulation of CD44. To substantiate the functional role of Akt activation in the resistance to apoptosis, Akt inhibitor (LY294002) was used to block Akt activation.

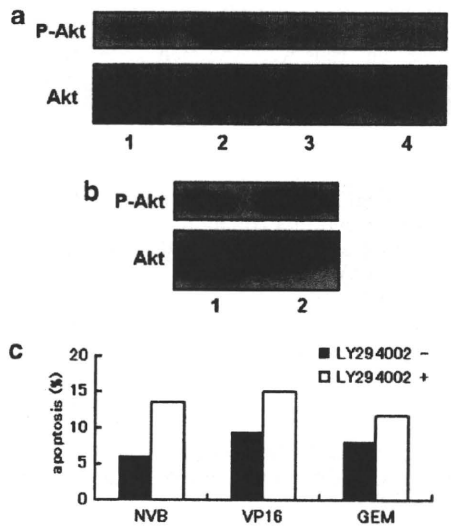

Figure 6 Effect of OPN upregulation or CD44 downregulation on phosphorylated Akt expression. (**a**) Expression of phosphorylated Akt and total Akt in ACC-MESO-1/OPN#7 (lane 1), ACC-MESO-1/OPN#8 (lane 2), ACC-MESO-1/Neo#1 (lane 3) and ACC-MESO-1/Neo#2 cells (lane 4) were determined by a western blot analysis. (**b**) ACC-MESO-1/OPN#8 cells were transfected with CD44 siRNA (lane 1) or negative control siRNA (lane 2). The expression of phosphorylated Akt and total Akt were determined by western blot analysis. (**c**) ACC-MESO-1/OPN#eight cells were incubated with or without LY294002 (10 μM) in the presence of VNB (2.5 ng/ml), VP-16 (10 μM) and GEM (0.1 μM). The percentage of apoptotic cells was determined by Annexin V staining method. Closed and open squares indicate the percentage of apoptotic cells treated without and with LY294002, respectively. The results are representative of three independent experiments with similar results.

ACC-MESO-1/OPN cells with or without LY294002 were cultured in the presence of VNB, VP-16 and GEM and apoptosis was detected by Annexin V (Figure 6c). As expected, the inhibition of the Akt phosphorylation increased the number of cells that underwent apoptosis. These results suggest that the activation of the Akt survival pathway, induced through the CD44, is therefore involved in the resistance to apoptosis induced by NVB, VP-16 and GEM.

Discussion

We here demonstrated that OPN provides MPM cells with an increased multidrug resistance and resistance to apoptosis to anti-cancer agents through HA–CD44 interaction. OPN-mediated alteration in CD44 binding to HA appears to have an important role in obtaining multidrug resistance by ACC-MESO-1/OPN cells.

In this study, the transfection of the OPN gene mediated the upregulation of HA binding in MPM cells. In contrast, the silencing of the OPN gene in ACC-MESO-1/OPN cells abrogated an enhanced adhesion to HA. These results indicate that OPN either directly or indirectly regulates HA binding. How does OPN gene transfer confer enhanced HA binding? First of all, we quantified and compared the amount of HA secreted into medium by both OPN transfectants. As opposed to our hypothesis, there was no difference in the amount of secreted HA (data not shown). In contrast, the expression of high molecular weight CD44 variant isoforms containing v8-10 and v10 was significantly reduced in ACC-MESO-1/OPN cells in comparison with control transfectants, whereas total CD44 expression levels on both transfectants are equivalent. As Iida and Bourguignon, (1997) demonstrated that cells coexpressing both transfected CD44v10 and endogeneous CD44s display a significant reduction in HA-mediated cell adhesion in comparison with parental cells expressing only CD44s, OPN-mediated enhanced HA binding in this study appears to be attributable to the downregulation of the CD44 variant isoforms, but not to the upregulation of CD44 expression.

Recent studies have revealed that HA strongly promotes anchorage-independent growth and that the resistance of cancer cells to growth arrest and apoptosis under anchorage-independent conditions is dependent on the constitutive interactions between HA and CD44 (Li and Heldin, 2001; Zoltan-Jones *et al.*, 2003). The treatment of tumor cells with hyaluronidase was observed to increase the activities of various chemotherapeutic agents. In contrast, an increased HA production has been reported to induce resistance in drug-sensitive tumors (Misra *et al.*, 2003). In our study, ACC-MESO-1/OPN cells which showed an enhanced adhesion to HA obtained multidrug resistance, and a disruption of HA–CD44 interaction by siRNA or neutralizing anti-CD44 antibody abrogated the resistance to apoptosis. Several groups have revealed that HA activates the PI3K–Akt signaling pathway through CD44, thereby

promoting cell survival (Sohara *et al.*, 2001; Ghatak *et al.*, 2002; Zoltan-Jones *et al.*, 2003). In fact, hyaluronan oligomers (oHA), which compete for endogenous polymeric HA, suppresses the PI3K/Akt cell survival pathway and retains chemosensitivity to anti-cancer agents (Cordo Russo *et al.*, 2008). In our study, the downregulation of the CD44 expression by siRNA suppressed Akt phosphorylation and increased apoptotic cells in number by the treatment with anti-cancer agents. These results suggested that OPN-induced resistance to apoptosis in the mesothelioma is mediated by the PI3K/Akt signaling pathway.

Khan *et al.* (2005) demonstrated that OPN upregulates the CD44v6, nine expressions, which have a key role in cancer metastasis, and the upregulation of variant CD44 isoforms has been reported to facilitate breast cancer cell migration . In contrast, OPN gene transfer in MPM cells reduced the CD44v8-10, ten expressions, and the downregulation of these variant CD44 isoforms confers chemoresistance of MPM in this study. The difference in OPN-induced alteration of CD44 isoforms may reflect the difference in the cell type between breast cancer and MPM. In MPM, local invasiveness is characteristic, whereas distant metastasis is infrequently observed. Such invasive growth requires ECM for MPM cells as previously reported (Li and Heldin, 2001). Moreover, the acquisition of chemoresistance by HA–CD44 interaction could be advantage for MPM cells to protect themselves by anti-cancer agents. These ideas are supported by previous reports which demonstrated MPM to be associated with elevated levels of OPN and HA in the pleural effusion (Thylen *et al.*, 1997; Pass *et al.*, 2005; Grigoriu *et al.*, 2007).

In this study, ACC-MESO-1/OPN cells, which showed an enhanced HA binding were shown to be more resistant to anti-cancer agents, NVB, VP-16 and GEM. These agents confer antitumor ability by a different mechanism. NVB binds to tubulin and inhibits its polymerization to form microtubules. VP-16 acts through inhibition of DNA topoisomerase II. GEM requires intracellular activation to its triphosphate derivative dFdCTP, which incorporates into DNA and then inhibits DNA synthesis. However, whether resistance to these three agents is mediated by the same mechanism still remains to be elucidated. We found the inhibition of the Akt phosphorylation by LY294002 to increase the number of cells that underwent apoptosis by the treatment with all three agents. These results suggested that the activation of the Akt survival pathway, induced through the CD44, may therefore be involved in the resistance to these three agents. Although, there still remains the question of what downstream events of the Akt pathway are involved in multidrug resistance, the inhibition of HA–CD44 or Akt pathway in combination with conventional agents may be more useful than conventional chemotherapy in the treatment of MPM.

To confirm that these findings can be generalized, we also examined other mesothelioma cell lines, such as H28 and ACC-MESO-4. Unfortunately, all mesothelioma cell lines tested, except ACC-MESO-1, expressed significant mRNA amounts of OPN by RT–PCR (data not shown). We therefore tried to downregulate the OPN expression in H28, which secreted significant amounts of OPN, by siRNA or miRNA to evaluate the role of OPN in chemoresistance. However, we were unable to establish stable transfectant by miRNA. siRNA transfection also could not downregulate the OPN expression. Although our data, in which OPN is involved in the chemoresistance, is promising, we still need to confirm that these results can be generalized by using animal models in the future.

In summary, we herein demonstrated that OPN mediated the alteration in HA–CD44 binding and that HA–CD44 interaction therefore has an important role in the acquisition of multidrug resistance by MPM. These results highlight the potential importance of OPN, which modulates HA–CD44 interaction, as a therapeutic target in multidrug resistance in patients with MPM.

Materials and methods

Cell lines

The human mesothelioma cell lines, ACC-MESO-1 cells were established at the Aich Cancer Center Research Institute (Nagoya, Japan) (Usami *et al.*, 2006). The cells were maintained in RPMI-1640 (Kohjin Bio, Sakado-city, Saitama Japan) containing 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C in 5% CO_2 atmosphere. The cells were routinely tested for Mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Cambrex, Rockland, ME, USA).

Reagents

The monoclonal anti-CD44 antibody (BU52), which is directed against epitopes common to all CD44 isoforms and the monoclonal anti-CD44 antibody (BU75), which blocks hyaluronate (HA) binding to CD44, were purchased from Ancell Corp (Bayport, MN, USA). The rabbit anti-Osteopontin (OPN) polyclonal antibody was purchased from Immuno-Biological and Laboratories (Gunma, Japan). The rabbit anti-Akt polyclonal antibody and the rabbit anti-phospho-Akt antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). LY294002 was purchased from Sigma Chemicals (St Louis, MO, USA). To evaluate cell viability, the Cell Counting Kit-8 was purchased from Wako (Osaka, Japan).

Transfection

We have previously described the eukaryotic cDNA expression vector BMG Neo, conferring neomycin resistance, and BMGNeo containing the murine OPN cDNA was designated as BMGNeo–OPN (Takahashi *et al.*, 2002). BMGNeo and BMGNeo–OPN were transfected into ACC-MESO-1 cells using Lipofectamine 2000 Reagent (Invitrogen Corporation, Camarillo, CA, USA) according to the manufacture's instructions. The cells were selected with medium containing 0.5 mg/ml of 418 sulfate (Geneticin; Invitrogen Corporation). Several clones were isolated with limiting dilution. The resulting selected and isolated cells transfected with BMGNeo–OPN and BMGNeo were designated as ACC-MESO-1/OPN and ACC-MESO-1/Neo, respectively.

Detection of CD44 transcription by reverse transcriptase–polymerase chain reaction

The expression of CD44 mRNA was assessed by RT–PCR. TaKaRa FastPure RNA kit (Takara, Japan) was used to extract RNA according to the manufacturer. cDNA was synthesized using SuperscriptIII reverse transcriptase (Invitrogen Corporation). To detect CD44 variant isoform expression, the sense primer; 5′-GACAAGTTTTGGTGGCACGCA-3′, and antisense primer; 5′-TCAGATCCATGAGTGGTATGGG AC-3′ were used. This amplifies the intervening region of the transcripts including any inserted exons of the variant (CD44v) region. Amplifications for β-actin (sense primer, 5′-AGAAA ATCTGGCACCACACC-3′; antisense primer, 5′-AGGAGG GAAGGCTGGAAGAG-3′) were performed in TaKaRa-ExTaq polymerase (Takara, Japan). The PCR conditions were 2 min at 95 °C; 25 cycles of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C, followed by a 7-min incubation at 72 °C.

Sequence

RT-PCR was performed under the same conditions as described above. The PCR products were purified by using MinElute (Qiagen, Maryland, MD, USA). Sequencing was performed using commercial reagents and an automated sequencer (ABI Prism BigDye Terminator v1.1 Cycle Sequencing Kit and ABI 3130 Genetic Analyzer; both Applied Biosystems, Foster city, CA, USA).

Detection of OPN protein secretion by ELISA

To determine OPN secretion in culture supernatant, a commercial ELISA kit (Immuno-Biological and Laboratories) was used according to the manufacture's instructions. Briefly, ACC-MESO-1/OPN and ACC-MESO-1/Neo were plated at 5×10^5 cells/well in 6-well 35 mm culture plates in 3 ml medium with 10% FCS. After 24 h, the culture medium was replaced with a medium containing 1% FCS for an additional 48 h. The culture supernatants were collected and subjected to an ELISA analysis.

In vitro *chemosensitivity assay*

The ells (2.0×10^3) were seeded onto 96-well microtiter plates in the absence or the presence of various concentration of chemotherapeutic agents including vinorelbine (VNB, Kyowa Hakko, Tokyo, Japan), etoposide (VP-16, Sigma), gemcitabine (GEM, Eli Lilly, Kobe, Japan) and cisplatin (CDDP, LKT Laboratories, St Paul, MN, USA). After 72 h of incubation, 10 μl of Cell Counting Kit-8 was added to each well. Four hours later, the optical density was measured at 450 nm with a microplate reader (Bio-Rad, Richmond, CA, USA). The results are expressed as the percentage of cell viability.

Adhesion assay

Ninety-six-well flat bottom plates (Corning Incorporated, Corning, New York, USA) were coated with recombinant OPN (1 and 5 μg/ml) or HA (0.01, 0.1 and 1 mg/ml) or 10 mg/ml BSA in PBS overnight at 4 °C. The following procedures were previously described (Takahashi *et al*., 2003).

RNA interference assay

ACC-MESO-1/OPN cells were transfected with 5 nM OPN siRNA using Hiperfect Transfection Reagent (Qiagen) or 10 nM CD44 and CD44v10 siRNA using Lipofectamine RNAiMAX (Invitrogen Corporation) according to the manufacturer's instructions. A knockdown efficacy was evaluated by Western bloting. Small interfering RNAs directed against OPN (spp1) (Mm_Spp1_1_HP siRNA), CD44 (5′-AAAUGG UCGCUACAGCAUCTT-3′), CD44v10 (5′-CACACGAAGG AAAGCAGGACCUUCA-3′) and a negative control (Allstars Negative Control siRNA) were purchased from either Qiagen or Invitrogen. The ACC-MESO-1/OPN cells transfected with siRNA for OPN, CD44 and negative control siRNA were designated as ACC-MESO-1/OPN OPN siRNA, ACC-MESO-1/OPN CD44 siRNA and ACC-MESO-1/OPN control siRNA, respectively.

Western bloting

For the Western blot analyses, the cells were homogenized in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% NaN3, 1 mM phenylmethylsulfonylfluoride, 1 μg/ml aprotinin, 1% Triton-X-100). Samples containing equal amounts of protein were separated on acrylamide gels and transferred to a nitrocellulose filter with electroblotting. The filters were blocked for 1 h in PBS containing 0.1% Tween-20 (PBS-T) and 5% dry milk, washed in PBS-T, and then incubated with BU52 (1:500), rabbit anti-Osteopontin polyclonal antibody (1:500), rabbit anti-Akt polyclonal antibody (1:1000), rabbit anti-phospho-Akt antibody (1:1000) and monoclonal anti-β-actin antibody (1:4000) at 4 °C overnight. The filters were again washed and then incubated with horseradish-peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 1 h. Filters were then washed in PBS-T, and specific proteins were detected using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Flowcytometric analysis

The adherent cells were detached from plates with 0.05% EDTA in PBS and were washed in PBS containing 0.1% BSA. Then, the cells (5×10^5) were incubated with BU52 (1 μg/ml) in PBS containing 0.1% BSA at 4 °C for 30 min. After washing the cells, the cells were incubated with fluorescent-labeled anti-mouse IgG (Chemicon, Temecula, CA, USA). The Propidium Iodide (Sigma) was added to final concentration of 10 μg/ml to exclude dead cells. Fluorescence was analyzed with a FACScan (Becton-Dickson Co., Mountain view, CA, USA).

Evaluation of apoptosis by Annexin V

ACC-MESO-1/OPN cells transfected with CD44 siRNA, OPN siRNA and negative control cells were treated with VNB, VP-16, or GEM for 48 h in the presence or absence of LY294002. Cells were harvested and the Annexin V-FITC –PI Kit (Sigma Inc.) was used according to the manufacturer's instructions. Early-stage apoptotic cells were Annexin positive and PI negative (right lower quadrant) and late-stage apoptotic cells were labeled by positivity with Annexin V and PI (right upper quadrant). The percentage of apoptotic cells was assessed by adding the percentage of cells in the two right quadrants.

Statistics

A statistical analysis was performed with an analysis of variance (ANOVA). The differences between the means were considered to be statistically significant at $P<0.05$.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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