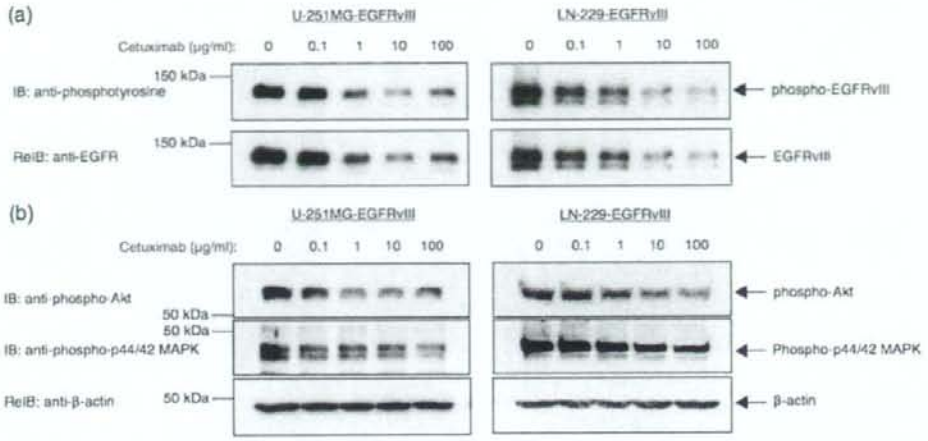

Fig. 3. Effect of cetuximab on epidermal growth factor receptor variant III (EGFRvIII) and its downstream signaling molecules. EGFRvIII-expressing glioma cells were exposed to 0, 0.1, 1, 10, or 100 μg/mL cetuximab for 24 h. (a) 250 μg of total cell lysates were immunoprecipitated with an anti-EGFR antibody (Ab). The immunoprecipitates were probed by immunoblotting with an antiphosphotyrosine Ab (upper lane) and the membranes were reblotted with an anti-EGFR antibody (lower lane). (b) Equal amounts of cell lysates were immunoblotted with a specific antihuman antibody as the first antibody, and then with a horseradish peroxidase–conjugated secondary antibody.

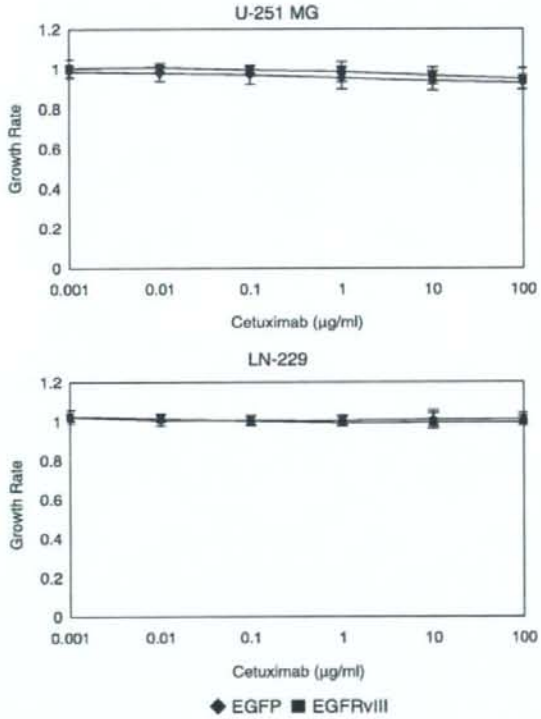

Fig. 4. MTS assays showing the growth-inhibitory effects of cetuximab on malignant glioma cells with and without epidermal growth factor receptor variant III (EGFRvIII) overexpression. 3000 glioma cells per well were placed onto a 96-well tissue culture plate and incubated overnight. Cetuximab was added at concentrations of 0–100 μg/mL and incubated for 48 h before an MTS assay was performed. This figure is representative of three independent experiments. Bars, SD.

Cetuximab induces ADCC in the presence of human PBMCs against malignant glioma cells expressing EGFRvIII. In molecular targeting using mAb, ADCC or CDC activity should be considered as one of the potent antitumor mechanisms.[16] First, we investigated the ADCC activity by morphological observation, PI stain, and an MTS assay. As our results in Fig. 5(a) show, cetuximab in the presence of PBMCs induced extensive lysis of the cells in culture. In contrast, treatment with cetuximab alone, human PBMCs alone, or PBMCs plus human IgG control (data not shown), did not apparently induce lysis of the cells. Correspondingly, PI nucleic acid stain revealed remarkable cell death in the cell population treated with cetuximab and PBMCs (Fig. 5b). To further evaluate cetuximab-induced ADCC against EGFRvIII expressed in the cells, we performed the MTS assay in the presence of human PBMCs at an E/T ratio of 10. In the mock control with low expression levels of wt EGFR, mild ADCC activity was detected (Fig. 5c). On the other hand, cetuximab treatment significantly inhibited the proliferation of the EGFRvIII-overexpressing glioma cells in a dose-dependent manner. There was no significant percentage ADCC at the zero μg/mL of cetuximab in the EGFRvIII-expressing cells as well as in mock controls (EGFP), suggesting that both cells could not be susceptible to PBMCs alone. Next, we examined CDC activity against these cells using the MTS assay in the presence of 25% human serum containing human complement. No CDC-mediated cytolytic effect was observed even at the highest concentration of cetuximab tested (data not shown). These results suggest that this mAb also recognizes the EGFRvIII expressed on the cell surfaces and exerts potent ADCC activity against the glioma cells overexpressing this mutant receptor.

Discussion

Overexpression of EGFR can be a promising characteristic as a molecular target for malignant glioma therapy.[12] Malignant gliomas that are wt EGFR–positive may simultaneously overexpress EGFRvIII, which is reported to be associated with aggressive phenotypes and resistance to chemo- and radiotherapy.[2,10,11] Although anti-EGFR mAb cetuximab may play an important role and be hopefully evaluated in preclinical studies, limited

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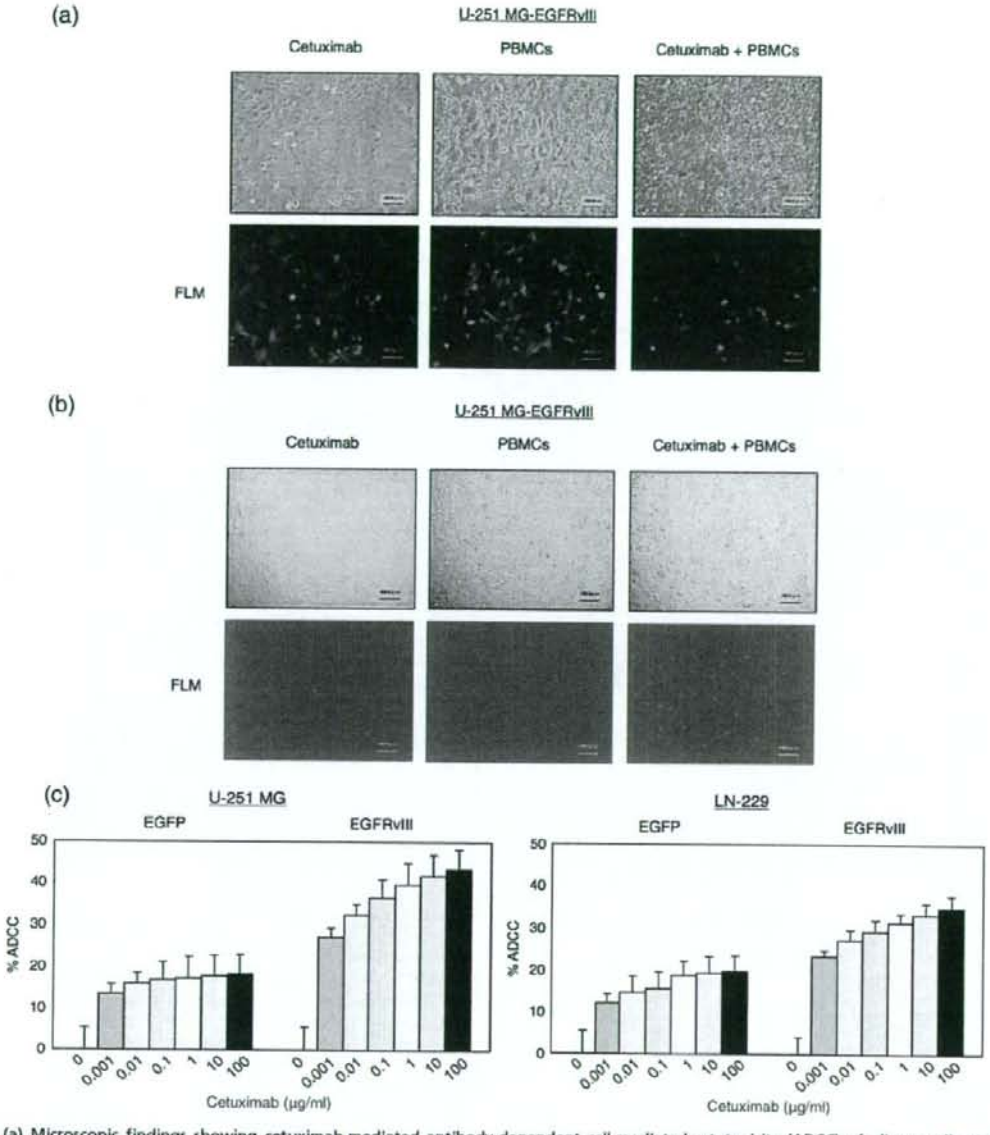

Fig. 5. (a) Microscopic findings showing cetuximab-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) of glioma cells expressing epidermal growth factor receptor variant III (EGFRvIII). Monolayers of the glioma cells were treated with cetuximab (10 μg/mL) and human peripheral blood mononuclear cells (PBMCs) at an effector target ratio of 10:1 at 37°C for 24 h. Controls included treatment with cetuximab alone (left panels), PBMCs alone (middle panels), or PBMCs plus human IgG (data not shown). Note the extensive lysis of the cells in the presence of both cetuximab and PBMC (right panels) but not in any of the control cultures. (b) Propidium iodide (PI) stain showing cetuximab-mediated ADCC of the EGFRvIII-expressing glioma cells. Under the same condition as (a), PI solution was added and incubated for 15 min. Note the remarkable increase of the dead cells in the presence of both cetuximab and PBMC (right panels). (c) MTS assays showing the ADCC activity of cetuximab against malignant glioma cells with and without EGFRvIII expression. Under the same condition as in Fig. 4, human PBMCs were added at an effector target ratio of 10:1 and incubated for 48 h before an MTS assay was performed. This figure is representative of three independent experiments. Bars, SD.

data have been reported regarding the potential of cetuximab to target the EGFRvIII in malignant glioma cells.[3] The present data provide evidence that cetuximab has a capability to recognize EGFRvIII as well as wt EGFR. Although cetuximab produces modest activities to block the EGFRvIII signaling or inhibit the growth of glioma cells with this receptor directly, this mAb has great potential to induce ADCC activity against the EGFRvIII-expressing glioma cells. These results suggest that cetuximab therapy might be effective against malignant gliomas expressing EGFRvIII.

In any anti-EGFR mAb strategy against EGFRvIII, it is of great importance to determine whether or not the mAb can recognize this deletion mutant receptor as the target. EGFR is composed of three major domains: extracellular domains including a ligand-binding site, a hydrophobic transmembrane segment, and a tyrosine-kinase containing cytoplasmic region.[13] EGFRvIII is a mutant form of EGFR encoded by a mutated gene characterized by in-frame deletion of 801 bp coding 6–273 amino acids from the extracellular domain.[11] As the extracellular part is composed of domain I to IV, all of domain I and the amino-terminal 2/3 of domain II are absent in this mutant receptor, while domain III and IV remain intact. Cetuximab has been produced as one of mAbs directed against the extracellular ligand-binding domain of the EGFR, and recent structural studies have demonstrated that the interaction between cetuximab and the EGFR is with domain III of the receptor, not with other domains.[18] Our experiments using immunoprecipitation and immunofluorescence revealed that cetuximab has the ability to bind to EGFRvIII. These findings suggest that EGFRvIII preserves the cetuximab-binding structure regardless of its possible conformational change due to 268 amino acids deletion.[18,23] There have been some recent reports on the possibility of cetuximab for detecting EGFRvIII.[24,25] Aerts *et al.* developed cetuximab-based imaging probe to target EGFR and demonstrated its potential as an imaging agent for not only wild type but also EGFRvIII.[24] Yang's group used cetuximab as a delivery tool of radio-isotope and evaluated boronated mAb for boron neutron capture therapy of a rat glioma expressing either wild-type EGFR or EGFRvIII.[25] This evidence reveals that cetuximab has a potential role in the advancement of anti-EGFR strategy.

Cetuximab competes with ligands for binding to the EGFR.[13] Binding of cetuximab to the EGFR prevents phosphorylation and activation of the receptor tyrosine kinase, resulting in the inhibition of its downstream signal transduction which controls cellular biology.[13,14] This mode of action is considered as the primary mechanism for the antitumor activity of cetuximab. Therefore, EGFRvIII, constitutively activated regardless of ligand-binding, may be insusceptible to such direct inhibition by cetuximab. Indeed, our experiments demonstrated that cetuximab, despite binding to the EGFRvIII, did not have clear inhibitory effects on the phosphorylation of EGFRvIII, Akt, and MAPK.

In mAb therapies, indirect growth-inhibition by activating host immune effector cells is a hopeful mechanism for antitumor activity.[16] Previously, we found that ADCC activity is a major mode of action of anti-HER2/neu mAb trastuzumab for breast cancer cell lines.[20] Therefore, even though cetuximab alone cannot inhibit the growth of the EGFRvIII-expressing cells in culture, it may have cytolitic potential in the treatment of EGFRvIII-expressing tumors *in vivo* by immunological mechanisms such as ADCC or CDC.[26] ADCC has rarely been discussed as one of the antitumor mechanisms of cetuximab. Recently, it has been reported that this mAb causes EGFR-expressing tumor cells to die through this mode of action and elicits effective ADCC activity against lung, head, and neck cancer.[17,27,28] In the literature, the authors describe the potential of cetuximab to exhibit ADCC activity mediated by targeting EGFR expressed in tumor cells, but do not discuss in detail whether cetuximab-mediated ADCC can be evoked against mutant EGFR, especially a deletion mutant form of extracellular domain that is vital for cetuximab binding, which is often discussed in malignant glioma. Our *in vitro* study showed that in the presence of human PBMCs, cetuximab induced strong ADCC, presumably due to additional activation of the immune effector functions by this antibody. A new insight drawn from our study is that even though the target is EGFRvIII, which has a partial deletion of EGF-binding site, once cetuximab binds to the mutant receptor, ADCC could be substantially produced against malignant glioma cells. These findings suggest that *in vivo* treatment of cetuximab could generate antitumor activity through ADCC even against malignant gliomas expressing EGFRvIII, although it should be considered that the growth advantage conferred by EGFRvIII is mostly found *in vivo*, not *in vitro*.

Previous studies have shown that cetuximab binding results in internalization of the antibody-receptor complex, which leads to down-regulation of EGFR expression on the cell surface and the blockading of its downstream signaling.[13] Patel and colleagues examined the ability of cetuximab as an effective drug for EGFRvIII-expressing tumor cells and concluded that down-regulation of EGFRvIII resulted in inhibition of cell proliferation.[29] In our experimental conditions, cetuximab treatment attenuated EGFRvIII expression in a dose-dependent manner. However, despite the decreased levels of this mutant receptor, cetuximab did not apparently inhibit the phosphorylation of Akt and MAPK and the growth of glioma cells with this receptor. Akt functions in one of the major signaling cascades, the phosphatidylinositol-3-kinase-Akt pathway, and controls the balance between glioma cell survival and apoptosis.[13,30,31] p44/42 MAPK functions in another major cascade, the ras–raf–MAPK pathway, which plays a critical role in cell growth and proliferation.[13] There are several possible mechanisms to explain why cetuximab-mediated attenuation of EGFRvIII did not produce antitumor activity in the EGFRvIII-overexpressing glioma cells. The simplest explanation rests in the possibility that, because the EGFRvIII is not completely depleted even at the highest dose of cetuximab tested, EGFRvIII signaling still exists to maintain its downstream activation. Another potentially related explanation is that once constitutive activation of EGFRvIII is established in a cell, it may be difficult to disturb the constant downstream signaling. In any case, we expected cetuximab-induced ADCC against EGFRvIII-expressing cells and clearly demonstrated that cetuximab binding to EGFRvIII could exhibit ADCC activity, inducing glioma cell death. This evidence might hopefully have an impact in anti-EGFR mAb therapy for malignant glioma.

When discussing the clinical relevance of cetuximab therapy against malignant glioma, there are two major points to be elucidated: (i) delivery of cetuximab to the central nervous system; and (ii) recruitment of immune-effector cells into brain tumors. In chemotherapy for brain tumors including malignant glioma, it is necessary to consider whether the drug can effectively reach the tumor through the blood–brain barrier (BBB). Some encouraging findings regarding this problem have been reported. Eller *et al.* demonstrated that intraperitoneal injection of cetuximab significantly increased median survival in nude mice bearing intracranial xenografts of glioblastoma.[3] Arwert and colleagues showed that intravenous injection of cetuximab resulted in a considerable reduction of intracranial glioma burden.[32] This evidence suggests that systemic administration could achieve effective concentration in the brain. On the other hand, there is some information that intact antibodies do not reach significant levels in malignant gliomas after systemic administration. Therefore, in the clinical practice of mAb therapy for brain tumors, several methods to deliver the agent to tumors have been tried out, such as convection-enhanced delivery with stereotactic infusion-catheter placement and osmotic BBB disruption with selective intra-arterial mannitol infusion, producing promising results. Recently, non-invasive localized delivery of mAb to the mouse brain was reported by magnetic resonance imaging–guided focused ultrasound-BBB disruption.[33] These modalities would enable effective delivery of cetuximab to malignant gliomas in the brain. Next, recruitment of immune-effector cells into the brain tumor is also vital for cetuximab to induce ADCC reaction against malignant glioma. In previous literature on mAb treatment for brain tumors, peritumoral infiltrates of macrophages were shown in mice treated with the mAb which was found to induce ADCC *in vitro*, whereas a paucity of T cells and natural killer cells was also described.[34] Although it should be elucidated

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whether macrophages or microglia would be able to mediate the ADCC in the brain, the promising result in the paper suggested that the mAb-mediated ADCC reaction could be evoked in *in vivo* models, which might be encouraging for cetuximab to produce an effective ADCC activity *in vivo*. In conclusion, the above information provides us with some hope that cetuximab may be used to treat patients with malignant glioma. As the next step, further investigation is needed in a clinical setting.

In summary, we have reported that cetuximab can target EGFRvIII and although this mAb appears to be less effective in direct inhibition of EGFRvIII activity, intervention of effector cells such as human PBMCs can produce antitumor efficacy of cetuximab even against EGFRvIII-expressing glioma cells. In view of the concept that cetuximab has been previously shown to develop chemosensitizing and radiosensitizing effects, the use of this mAb may have great therapeutic potential against malignant gliomas.[3,4] Moreover, conjugation of cytotoxic agents such as drugs or radioisotopes might produce enhanced antitumor activity.[12] Thus, we emphasize that targeted therapy using the anti-EGFR mAb cetuximab could play a significant role in the development of multidisciplinary treatment strategies for these tumors.

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nature medicine

RPN2 gene confers docetaxel resistance in breast cancer

Kimi Honma[1,2], Kyoko Iwao-Koizumi[3], Fumitaka Takeshita[1], Yusuke Yamamoto[1], Teruhiko Yoshida[4], Kazuto Nishio[5], Shunji Nagahara[6], Kikuya Kato[3] & Takahiro Ochiya[1]

Drug resistance acquired by cancer cells has led to treatment failure. To understand the regulatory network underlying docetaxel resistance in breast cancer cells and to identify molecular targets for therapy, we tested small interfering RNAs (siRNAs) against 36 genes whose expression was elevated in human nonresponders to docetaxel for the ability to promote apoptosis of docetaxel-resistant human breast cancer cells (MCF7-ADR cells). The results indicate that the downregulation of the gene encoding ribophorin II (RPN2), which is part of an *N*-oligosaccharyl transferase complex, most efficiently induces apoptosis of MCF7-ADR cells in the presence of docetaxel. RPN2 silencing induced reduced glycosylation of the P-glycoprotein, as well as decreased membrane localization, thereby sensitizing MCF7-ADR cells to docetaxel. Moreover, *in vivo* delivery of siRNA specific for *RPN2* markedly reduced tumor growth in two types of models for drug resistance. Thus, RPN2 silencing makes cancer cells hypersensitive response to docetaxel, and RPN2 might be a new target for RNA interference–based therapeutics against drug resistance.

Breast cancer is the most common malignancy in women. Either neoadjuvant or adjuvant chemotherapy administered to subjects with stage 1–3 breast cancers can improve their survival rates[1–3]. Among chemotherapeutic agents, docetaxel, which belongs to the group of taxanes (mitotic inhibitors and antimicrotubule agents), has been shown to have well-established benefits in breast cancer[4]. The response rate to docetaxel, however, is 50% even in first-line chemotherapy, and it decreases to 20–30% in second- or third-line chemotherapy[5–7]; nearly half of the treated subjects do not respond to it and suffer side effects. There is currently no method to reliably predict tumor responses to docetaxel before therapy or to detect when resistance or hypersensitivity develops. Therefore, the identification of molecular biomarkers in docetaxel-resistant breast cancer that could help in a more accurate assessment of individual treatment and the development of molecular-target therapies that could lead to better tumor reduction are of considerable interest.

It has been reported that the expression of the multidrug transporter P-glycoprotein, encoded by the *MDR1* gene (official gene symbol *ABCB1*), is one of the causes of clinical drug resistance to taxanes[8,9]. Other molecules, such as the multidrug resistance–associated protein MRP1[10,11], breast cancer resistance protein (*ABCG2*) and other transporters[12], which act as energy-dependent efflux pumps capable of expelling a large range of xenobiotics, and GSTpi, which is one of the isoenzymes of the glutathione-*S*-transferase (GST)[13–15], have been extensively reported to be overexpressed in tumor cells showing the multidrug-resistant phenotype. It was recently shown that high thioredoxin expression is associated with resistance to docetaxel in breast cancer[16,17]. These molecules might be clinically useful in the prediction of a response to anticancer drugs. Currently, however, none have proven to be specific target molecules for increasing the efficacy of chemotherapy in breast cancer.

To better understand the regulatory network underlying docetaxel resistance in breast cancer cells and to identify molecular targets for therapy, we initiated gene expression profiling of 44 subjects with breast tumors (22 responders and 22 nonresponders) by adaptor-tagged competitive PCR[18] to identify the genes capable of predicting a docetaxel response in human breast cancer and reported the preliminary results of 85 genes whose expression potentially correlated with docetaxel resistance[16]. In the current study, we used an atelocollagen-based siRNA cell transfection array[19,20] to identify the genes responsible for conferring drug resistance. Among the siRNAs targeting genes that were elevated in nonresponders to docetaxel, siRNA designed for *RPN2* (RNP2 siRNA) significantly promoted docetaxel-dependent apoptosis and cell growth inhibition of MCF7-ADR human breast cancer cells that are resistant to docetaxel. Furthermore, atelocollagen-mediated *in vivo* delivery of RPN2 siRNA significantly reduced drug-resistant tumor growth in mice given docetaxel. RPN2 confers drug resistance via the glycosylation of P-glycoproteins and regulates antiapoptotic genes. Thus, RPN2 siRNA introduction hypersensitizes cancer cell response to chemotherapeutic agents, making RPN2 a potential key target for future RNA interference (RNAi)-based therapeutics against a drug-resistant tumor.

[1]Section for Studies on Metastasis, Japanese National Cancer Center Research Institute, 1-1, Tsukiji, 5-chome, Chuo-ku, Tokyo 104-0045, Japan. [2]Koken Bioscience Institute, KOKEN, 2-13-10 Ukima, Kita-ku, Tokyo 115-0051, Japan. [3]Research Institute, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-2 Nakamichi, Higashinari-ku, Osaka 537-8511, Japan. [4]Genetics Division and [5]Pharmaceutical Division, Japanese National Cancer Center Research Institute, 1-1, Tsukiji, 5-chome, Chuo-ku, Tokyo 104-0045, Japan. [6]Dainippon Sumitomo Pharma, 3-45, Kurakakiuchi, 1-chome, Ibaraki, Osaka 567-0878, Japan. Correspondence should be addressed to T.O. (tochiya@ncc.go.jp).

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RESULTS

RNAi-based screening for identification of molecular target

As an extension of our previous strategy of analyzing docetaxel resistance in breast cancer cells and of identifying molecular targets for therapy[16], we conducted a study of RNAi-induced gene knockdown in docetaxel-resistant MCF7-ADR human breast cancer cells. Among the 85 genes listed[16], 61 genes that are potentially targets for siRNA strategy were upregulated in human nonresponders. We selected 36 genes with more than a 0.365 signal-to-noise ratio and successfully designed and synthesized siRNAs specific to these genes (**Table 1**). The siRNAs were conjugated to atelocollagen and arrayed on a 96-well microplate. Then, MCF7-ADR cells expressing the luciferase gene (MCF7-ADR-Luc) were seeded into the microplate (the target validation process by cell transfection array is schematically shown in **Supplementary Fig. 1** online.). To evaluate the efficiency of the atelocollagen-mediated cell transfection array, we used GL3 siRNA against the gene encoding luciferase. Atelocollagen-mediated GL3 siRNA delivery caused an approximate 75% reduction of the luciferase activity in MCF7-ADR-Luc cells relative to the control nontargeting siRNA (data not shown). To identify the genes responsible for docetaxel resistance, we assessed siRNAs for their ability to inhibit cell growth and induce apoptosis in the presence of docetaxel compared with the control nontargeting siRNA. We measured cell growth by luciferase activity and examined apoptosis by caspase-7 activation. The results indicated that the downregulation of eight genes (*PTPLB, GSTP1, TUBB, RPN2, SQRDL, NDUFS3, PDCD5* and *MRPL17*) resulted in marked inhibition of cell growth ($P < 0.05$, **Fig. 1a**). Induction of apoptosis was evidenced in cells by downregulation of six genes (*PTPLB, APRT, CFL1, RPN2, SQRDL* and *MRPL17*; $P < 0.05$, **Fig. 1b**). In particular, RPN2 siRNA strongly enhanced caspase-7 activity in the presence of docetaxel ($P < 0.001$, **Supplementary Fig. 2a** online). We validated these results by counting Hoechst-stained cells showing apoptotic nuclear condensation and fragmentation (**Fig. 2a**) and found that there was a significantly higher apoptotic cell death rate in cells given RPN2 siRNA and docetaxel relative to that in cells given RPN2 siRNA alone ($P < 0.02$, **Fig. 2b**). No significant difference was observed in cells with nontargeting control siRNA (**Fig. 2b**). At 72 h after treatment with siRNA and docetaxel, there was substantial cell death induced by RPN2 siRNA compared with the control nontargeting siRNA (**Fig. 2c**). At 96 h after the transfection, almost all RPN2 siRNA–treated cells were detached and disappeared from the culture dishes.

Table 1 The list of 36 genes whose expression is elevated in nonresponders to docetaxel in subjects with breast cancer

No	Gene	Description	Accession number
1	*UFM1*	Ubiquitin-fold modifier 1	BC005193
2	*PTPLB*	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member b	AF052159
3	*S100A10*	S100 calcium binding protein A10	M38591
4	*APRT*	Adenine phosphoribosyltransferase	Y00486
5	*CFL1*	Cofilin-1 (non-muscle)	X95404
6	*GSTP1*	Glutathione *S*-transferase pi 1	M24485
7	*HSPA5*	Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	M19645
8	*GNB2L1*	Guanine nucleotide binding protein (G protein), β polypeptide 2 like 1	M24194
9	*TUBB*	Tubulin, β	BC001002
10	*MX1*	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	M33882
11	*COX7C*	Cytochrome *c* oxidase subunit VIIc	BC001005
12	*RPN2*	Ribophorin II	Y00282
13	*DYNLL1*	Dynein, light chain, LC8-type 1	U32944
14	*FXR1*	Fragile X mental retardation, autosomal homolog 1	U25165
15	*SQRDL*	Sulfide quinone reductase-like (yeast)	AF151802
16	*NDUFS3*	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30 kDa (NADH-coenzyme Q reductase)	AL135819
17	*EST*	ESTs	AL358933
18	*C19orf10*	Chromosome 19 open reading frame 10	BC003639
19	*ATP5E*	ATP synthase, H^+ transporting, mitochondrial F1 complex, ε subunit	AF052955
20	*PDCD5*	Programmed cell death 5	AF014955
21	*CLPTM1L*	CLPTM1-like	AL137440
22	*PPP1R14B*	Protein phosphatase 1, regulatory (inhibitor) subunit 14B	X91195
23	*MRPL17*	Mitochondrial ribosomal protein L17	AK026857
24	*TUBA1B*	Tubulin, α1b	BC006481
25	*IFI6*	Interferon, α-inducible protein 6	X02492
26	*GAPDH*	Glyceraldehyde-3-phosphate dehydrogenase	AF261085
27	*SLC25A3*	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	BC006455
28	*MAD2L2*	MAD2 mitotic arrest deficient-like 2 (yeast)	AF157482
29	*CTNNB1*	Catenin (cadherin-associated protein), β1, 88 kDa	X87838
30	*CALR*	Calreticulin	M84739
31	*MRPS6*	Mitochondrial ribosomal protein S6	BC000547
32	*ANGPTL2*	Angiopoietin-like 2	AF007150
33	*RPL38*	Ribosomal protein L38	Z26876
34	*ANAPC7*	Anaphase promoting complex subunit 7	AY007104
35	*ENO1*	Enolase 1, (α)	BC004325
36	*ALDH2*	Aldehyde dehydrogenase 2 family (mitochondrial)	M20456

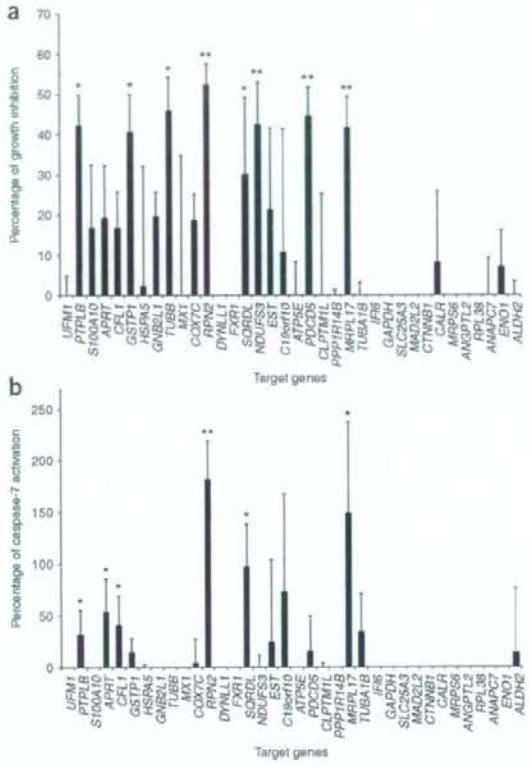

Figure 1 RNAi cell transfection array analysis in cultured breast cancer cells. **(a)** Inhibition of cell growth by 36 siRNAs on atelocollagen-based cell transfection arrays in the presence of docetaxel (1 nM) 72 h after transfection. The cell growth was estimated by luciferase activity in MCF7-ADR-Luc cells, which stably express luciferase ($n = 4$ per group; $^*P < 0.05$, $^{**}P < 0.01$). **(b)** Activation of caspase-7 by 36 siRNAs on atelocollagen-based cell transfection arrays in the presence of docetaxel (1 nM) 72 h after transfection. After the detection of luciferase activity, the same cell transfection arrays were assigned to the measurement of caspase-7 activity, which is elevated in apoptotic MCF7-ADR-Luc cells ($n = 4$ per group; $^*P < 0.05$, $^{**}P < 0.01$). Values are means ± s.d.

However, the expression of other genes was also found to correlate with docetaxel resistance by RNAi-based screening, and they could have some possible additive or synergistic effects with RPN2. Thus, we examined the induction of apoptosis after cotransfection of RPN2 siRNA and siRNAs against other genes that caused cell growth inhibition, apoptosis induction or both in docetaxel-resistant MC7-ADR cells. Knockdown of *PTPLB*, *APRT*, *CFL1*, *GSTP1*, *TUBB*, *SQRDL*, *NDUFS3*, *PDCD5* or *MRPL17* genes with simultaneous knockdown of *RPN2* did not significantly enhance caspase-7 activity relative to the knockdown of *RPN2* alone (**Supplementary Fig. 2b**). This result shows that knockdown of the other genes does not have an additive effect when used together with knockdown of RPN2.

We confirmed the efficacy of RPN2 siRNA for the knockdown of RPN2 messenger RNA by cell-direct real-time RT-PCR analysis. This analysis revealed that RPN2 siRNA inhibited 80% of the mRNA level relative to the control nontargeting siRNA (**Fig. 2d**). Immunofluorescence staining of the RPN2 protein revealed that the RPN2 protein localized in the cytoplasm and its expression was decreased by RPN2 siRNA (**Fig. 2e**). In further experiments, a liposome-mediated RPN2 siRNA transfection was performed. The western blot analysis showed a 45% reduction in RPN2 protein abundance (90% reduction of mRNA by real-time RT-PCR analysis) by RPN2 siRNA transfection in comparison with the control nontargeting siRNA (**Fig. 2f**). These results suggest that downregulation of RPN2 expression by siRNA inhibits cell growth and induces apoptosis in the presence of docetaxel.

In addition, we established stable clones expressing short hairpin RNA (shRNA) against RPN2 (shRNP2) and examined the effect on apoptosis induction in MCF7-ADR cells. The clone that expressed the lowest RPN2 mRNA level showed a 70% reduction of RPN2 expression relative to the control clone ($P < 0.001$, **Supplementary Fig. 2c**), and this shRPN2 clone showed a high caspase-7 activity as compared to the control clone in the presence of docetaxel ($P < 0.001$, **Supplementary Fig. 2d**). We examined seven independent clones and found that they all showed a similar phenotype of increasing drug sensitivity (data not shown). Therefore, consistent with our results with synthetic RPN2 siRNA, the data from the shRPN2 experiments provide evidence for the involvement of RPN2 in drug resistance.

To evaluate the effect of RPN2 siRNA on the drug response of MCF7-ADR cells, we measured the half-maximal inhibitory concentration (IC_{50}) for taxanes. The IC_{50} values for docetaxel in MCF7 and MCF7-ADR cells were 9.48 ± 1.48 nM and 40.22 ± 5.14 nM, respectively ($P < 0.001$). RPN2 siRNA–transfected MCF7-ADR cells were 3.5-fold more sensitive to docetaxel compared with nontargeting siRNA–transfected cells (IC_{50} of 11.47 ± 1.97 nM versus 39.48 ± 2.98 nM, $P < 0.001$). Thus, RPN2 silencing makes MCF7-ADR cells sensitive to docetaxel to a degree similar to that in drug-sensitive MCF7 cells. For paclitaxel, another taxane, the IC_{50} values in MCF7 and MCF7-ADR cells were 13.00 ± 2.02 nM and 89.74 ± 3.43 nM, respectively ($P < 0.001$). RPN2 siRNA–transfected MCF7-ADR cells were 2.6-fold more sensitive to paclitaxel compared with nontargeting siRNA–transfected cells (IC_{50} of 32.92 ± 3.89 nM versus 84.39 ± 5.48 nM, $P < 0.001$). These results indicate that RPN2 silencing bestows a hypersensitive response to taxanes to drug-resistant breast cancer cells.

In addition, we examined docetaxel-resistant EMT6/AR10.0 cells with high expression of the mouse *Rpn2* gene and the *Mdr1* (*Abcb1b*) and *Mdr3* (*Abcb1a*) genes, which reportedly have a similar role in drug resistance to that of the *MDR1* gene in humans, to see whether they have a similar phenotype to MCF7-ADR cells in terms of RPN2 expression status and drug resistance. The siRNA-mediated knockdown of mouse Rpn2 (70% reduction of mRNA by real-time RT-PCR analysis) significantly induced apoptosis of cells in the presence of docetaxel (**Supplementary Fig. 2e–g**). In contrast, nontargeting control siRNA showed no effect (**Supplementary Fig. 2e–g**). Therefore, these results suggest that RPN2 confers docetaxel resistance to both human and mouse cell lines.

Induction of RPN2 expression by docetaxel treatment

Real-time RT-PCR analysis showed that docetaxel-resistant MCF7-ADR cells expressed a slightly increased level of RPN2 mRNA (approximately 20%, $P < 0.01$) relative to parental MCF7 cells (**Fig. 3a**). However, RPN2 mRNA expression in parental MCF7 cells was markedly induced by docetaxel in a dose-dependent manner at

48 h after treatment (**Fig. 3b**). These data indicate that RPN2 mRNA induction may correlate with the observed antiapoptotic phenotype of MCF7-ADR cells.

Furthermore, MCF7-ADR cells expressed abundant MDR1 mRNA, which is a major cause of docetaxel resistance, whereas docetaxel-sensitive MCF7 cells did not (**Fig. 3c**). Additionally, MDR1 mRNA expression in MCF7 cells was strongly induced by docetaxel at 48 h after treatment (**Fig. 3d**). Together, these data provide a new insight into the development of docetaxel resistance in MCF7 cells: when breast cancer cells coordinately express a high amount the of *MDR1* and *RPN2* gene products, the cells become drug-resistant.

RPN2 expression associates with response to docetaxel

In this study, subjects with breast cancer with complete response and partial response were defined as responders, whereas subjects with no change and progressive disease were defined as nonresponders, in accordance with World Health Organization criteria[16] (**Supplementary Note** online).

Of the 44 subjects, 22 showed a pathologic response to docetaxel, and the other 22 showed no response[16]. To understand the clinical importance of the status of RPN2 expression in the subjects, we compared the expression level (signal log ratio) for RPN2 transcript between nonresponder and responder subjects by the Mann-Whitney *U*-test. The subjects with higher RPN2 expression showed a significantly lower response rate to docetaxel than did those with relatively low expression of RPN2 (signal log ratio expressed as mean ± s.e.m. in nonresponders was 0.347 ± 0.062 versus 0.111 ± 0.052 in responders; $P = 0.0052$). Thus, there is a significant association of RPN2 expression with the pathologic response to docetaxel. Although the data are not shown, RPN2 mRNA expression was significantly increased in cancerous tissues compared to that in normal tissues.

Furthermore, we also assessed validated sets of new samples from 26 subjects with breast tumors (12 responders and 14 nonresponders). The expression of RPN2 was higher in nonresponders than in responders (nonresponders, 0.240 ± 0.066, versus responders, 0.025 ± 0.194). Because of the small sample size in the validation set, we have not obtained conclusive results at this time. We are currently seeking larger samples that will be tested in the near future. However, when we combined studies with subjects in the learning and validation sets, RPN2 expression was significantly higher in nonresponders (34 subjects) than in responders (36 subjects) (nonresponders, 0.306 ± 0.046, versus responders, 0.080 ± 0.075; $P = 0.0219$).

Downregulation of RPN2 in orthotopic breast tumors

To extend our *in vitro* findings and to determine whether RPN2 could be an effective therapeutic target for docetaxel-resistant breast cancer, we examined the effect of RPN2 siRNA on an animal model of breast tumors by orthotopically implanting MCF7-ADR cells into mice and using an atelocollagen-mediated *in vivo* siRNA delivery[21,22]. We injected the RPN2 siRNA or nontargeting control siRNA (1 nmol per tumor) with 0.5% atelocollagen in a 200 µl volume into tumors that had reached 4–5 mm in diameter 7 d after inoculation of MCF7-ADR cells. At the time of siRNA administration, docetaxel was intraperitoneally (i.p.) injected into the mice. Subsequent tumor development was monitored by measuring the tumor size for a week. Mice that had been administered the RPN2 siRNA–atelocollagen complex and docetaxel (20 mg kg^{-1} i.p.) showed a significant decrease in tumor size (mean ± s.d.; day 0, 52 ± 8 mm^3; day 7, 21 ± 8 mm^3) relative to mice that had been administered the control nontargeting siRNA–atelocollagen (day 0, 37 ± 7 mm^3; day 7, 35 ± 12 mm^3; $P < 0.01$) (**Fig. 4a**). The tumor size was markedly reduced by administration of RPN2 siRNA with docetaxel at 7 d after treatment (**Fig. 4b**). In the absence of docetaxel, RPN2 siRNA treatment slightly reduced MCF7-ADR tumor size relative to controls; however, there were no statistically significant differences (**Supplementary Fig. 3a** online). We also observed that docetaxel alone had no significant effect on tumor growth (**Supplementary Fig. 3a**). Furthermore, no significant differences were observed in tumor growth between mice treated with control nontargeting siRNA and untreated mice in the presence or in the absence of docetaxel (data not shown). Thus, RPN2 siRNA is useful for reducing the size of orthotopic MCF7-ADR breast tumors in the presence of docetaxel. Additionally, to evaluate the effect of sustained treatment with siRNA, we treated mice with tumors twice by injection of siRNA-atelocollagen complex. RPN2 siRNA or nontargeting control siRNA (1 nmol per tumor) were injected into the tumors (diameter, 4 mm) at days 0

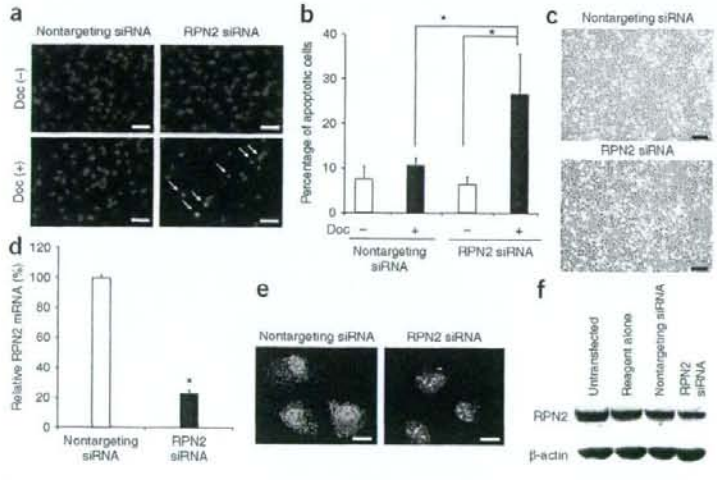

Figure 2 Apoptosis of MCF7-ADR cells transduced with RPN2 siRNA. (**a**) Hoechst staining of cells in the presence or absence of docetaxel (Doc, 1 nM) 72 h after the transfection of RPN2 siRNA. Scale bar, 50 µm. The arrows indicate cells with nuclear condensation and fragmentation. (**b**) Numbers of apoptotic cells from **a**. The data show the percentage of apoptotic cells in the presence or absence of docetaxel (1 nM) 72 h after the transfection of RPN2 siRNA. As a control, nontargeting control siRNA was used ($n = 4$ per group, $^*P < 0.02$). (**c**) Phase-contrast micrograph of MCF7-ADR cells 72 h after treatment with RPN2 siRNAs or control nontargeting siRNAs in the presence of docetaxel. Scale bar, 200 µm. (**d**) Knockdown of RPN2 mRNA by RPN2 siRNA in a cell transfection array, as monitored by cell-direct real-time RT-PCR analysis. As a control, nontargeting siRNA was used ($n = 4$ per group, $^*P < 0.001$). (**e**) Immunofluorescence staining of the RPN2 protein in MCF7-ADR cells 72 h after treatment with RPN2 siRNAs or control nontargeting siRNAs. Scale bar, 5 µm. (**f**) Western blot analysis of RPN2 protein in MCF7-ADR cells treated with RPN2 siRNAs or control nontargeting siRNAs 72 h after the liposome-mediated transfection. Values are means ± s.d.

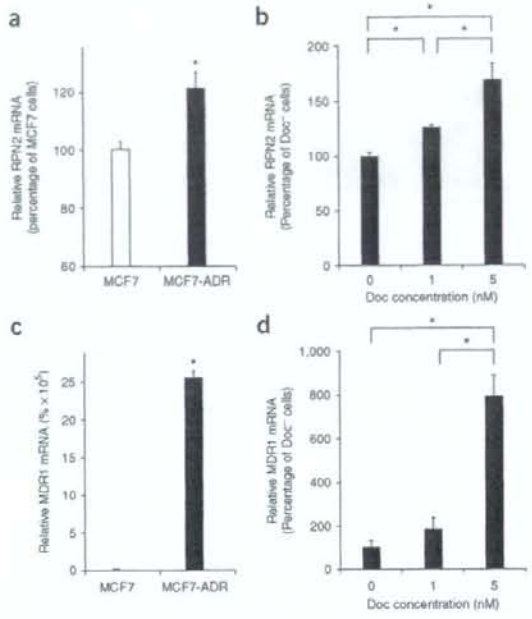

Figure 3 Induction of RPN2 and MDR1 expression by docetaxel treatment. RPN2 mRNA and MDR1 mRNA expression were analyzed by real-time RT-PCR. (**a**) RPN2 expression in drug-resistant MCF7-ADR cells and parental drug-sensitive MCF7 cells ($n = 3$ per group, $^*P < 0.01$). (**b**) Expression of RPN2 induced by docetaxel treatment in parental MCF7 cells. The data shown are from 48 h after the treatment ($n = 3$ per group, $^*P < 0.01$). (**c**) MDR1 expression in drug-resistant MCF7-ADR cells and parental drug-sensitive MCF7 cells ($n = 3$ per group, $^*P < 0.001$). The numbers on the *y* axis represent percentage ($\times 10^5$) of MCF7 cells. (**d**) Expression of MDR1 induced by docetaxel treatment in parental MCF7 cells. The data shown are from 48 h after the treatment ($n = 3$ per group, $^*P < 0.01$). Values are means ± s.d.

and 10. Simultaneously, docetaxel (20 mg kg^{-1} i.p.) was injected into the mice. We observed the mice for 20 d. Mice that had been given RPN2 siRNA and docetaxel showed significantly suppressed tumor growth relative to the mice that were administered control nontargeting siRNA at day 20 after the treatment ($P < 0.05$, **Supplementary Fig. 3b,c**). Mice showed no toxic effect during the observation period.

Furthermore, we examined the effect of RPN2 siRNA on a second animal model of breast tumors by orthotopically implanting MDA-MB-231/MDR1 cells. First, we established an MDA-MB-231/MDR1 cell line, which expresses the *MDR1* gene inducing docetaxel resistance. In this study, MDR1 expression is a key factor, because we are proposing that the coordinate expressions of RPN2 and P-glycoprotein may participate in the mechanism of docetaxel resistance. We injected the RPN2 siRNA or nontargeting control siRNA (2 nmol per tumor) with 0.5% atelocollagen in a 200 µl volume into tumors that were 5–6 mm in diameter 8 d after inoculation of MDA-MB-231/MDR1 cells. At the same time of siRNA administration, we injected docetaxel i.p. into the mice. Because docetaxel at a dose of 20 mg kg^{-1} in mice slightly suppressed MDA-MB-231/MDR1 tumor growth, we reduced the dose of docetaxel to 7 mg kg^{-1}, corresponding to the IC_{50} value of docetaxel in MDA-MB-231/MDR1 cells, which was 35% of that of MCF7-ADR cells. At a dose of 7 mg kg^{-1} docetaxel, mice treated with docetaxel alone showed no significant change in tumor growth. Subsequent tumor development was monitored by measuring the tumor size for a week. Mice that had been administered the RPN2 siRNA–atelocollagen complex and docetaxel (7 mg kg^{-1} i.p.) showed a significant inhibition of tumor growth (day 0, 61 ± 21 mm^3; day 7, 97 ± 24 mm^3) relative to mice that had been administered the control nontargeting siRNA–atelocollagen complex (day 0, 68 ± 9 mm^3; day 7, 154 ± 23 mm^3) (**Fig. 4c,d**). The value was statistically significant, with $P < 0.002$. Tumors treated with RPN2 siRNA in the absence of docetaxel showed no significant inhibition relative to control tumors that had been given nontargeting siRNA or docetaxel alone (data not shown). These results show that the growth of docetaxel-resistant MDA-MB-231/MDR1 tumors was suppressed by administration of RPN2 siRNA and docetaxel. Thus, RPN2 silencing is effective for the suppression of tumor growth in two models for docetaxel-resistant breast cancer in the presence of docetaxel.

RPN2 siRNA delivery augments docetaxel-induced apoptosis

MCF7-ADR tumors treated with RPN2 siRNA were investigated for apoptotic activity after docetaxel treatment for 3 d. TUNEL staining of tumor tissue treated with RPN2 siRNA revealed a significant number of apoptotic cells relative to the number in nontargeting control siRNA–treated tumors ($P < 0.01$, **Fig. 4e,f**). In contrast, RPN2 siRNA–transduced tumors in the absence of docetaxel showed no marked apoptotic cell death (**Fig. 4e,f**). We have also previously shown that atelocollagen alone does not induce any cytotoxic or inflammatory effect when it is injected into mice[23,24]. In a subsequent experiment, the mRNA levels of RPN2 in treated tumors were measured. RPN2 expression was significantly reduced in mouse tumors after combined treatment with RPN2 siRNA and docetaxel ($P < 0.05$, **Fig. 4g**). Furthermore, the RPN2 protein abundance in treated tumors was markedly downregulated by RPN2 siRNA (**Fig. 4h**). Thus, these results altogether indicate that RPN2 siRNA induces tumor inhibition via augmentation of docetaxel-induced apoptotic cell death *in vivo*.

To examine docetaxel retention in the tumors in the *in vivo* experiment, we performed drug disposition analysis. Eleven hours after docetaxel administration, we dissected the tumors and determined the amount of docetaxel incorporated into the tumors by HPLC with ultraviolet detection at 225 nm after solid-liquid extraction. We detected docetaxel in tumors that had received RPN2 siRNA ($n = 4$) at a range of 667 to 1400 ng per wet gram of tissue (**Fig. 4i**). In contrast, the tumors that received control siRNA ($n = 4$) showed a very low amount of docetaxel (~10 ng per wet gram of tissue). Thus, the results clearly indicate that abrogation of RPN2 expression in drug-resistant tumors results in docetaxel accumulation in those tumors.

RPN2 siRNA reduces *N*-linked glycosylation of MDR1

The mammalian *RPN2* gene encodes a type I integral membrane protein found only in the rough endoplasmic reticulum[25,26]. The RPN2 protein is part of an *N*-oligosaccharyl transferase complex that links high mannose oligosaccharides to asparagine residues found in the N-X-S/T consensus motif of nascent polypeptide chains[27]. The expression of the multidrug transporter P-glycoprotein, encoded by *MDR1*, is one of the causes of clinical drug resistance to taxanes. Real-time RT-PCR analysis showed that MCF7-ADR cells expressed abundant MDR1 mRNA, whereas parental cells did not (**Fig. 3c**). In addition, the MDR1 mRNA amount was not significantly decreased

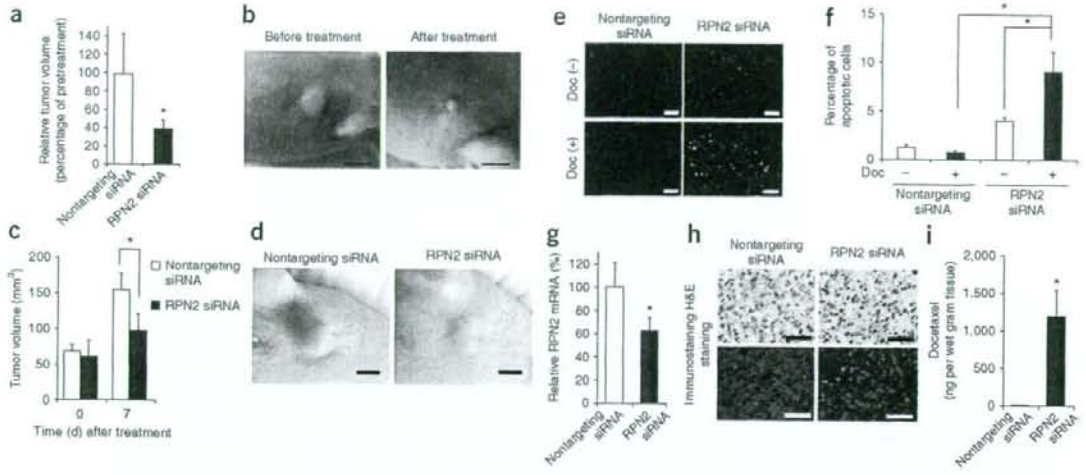

Figure 4 Delivery of RPN2 siRNA to docetaxel-resistant breast tumors. The effect of RPN2 siRNA was examined in orthotopic breast tumor models. (**a**) Reduction of MCF7-ADR breast tumor volume in mice given RPN2 siRNA or control nontargeting siRNA along with docetaxel ($n = 6$ per group, $^*P < 0.01$). (**b**) siRNA-treated MCF7-ADR tumors in mice before and 7 d after docetaxel treatment. Scale bar, 5 mm. (**c**) Growth of MDA-MB-231/MDR1 breast tumor in mice administered RPN2 siRNA or nontargeting siRNA along with docetaxel ($n = 6$ per group, $^*P < 0.002$). (**d**) MDA-MB-231/MDR1 tumors in mice 7 d after treatment with siRNA and docetaxel. Scale bar, 5 mm. (**e**) TUNEL staining of MCF7-ADR tumor tissues treated with RPN2 siRNAs or nontargeting siRNAs in the presence or absence of docetaxel. Scale bar, 50 μm. (**f**) TUNEL-positive cells were counted and are represented in the graph ($n = 3$ per group, $^*P < 0.01$). (**g**) Expression of RPN2 mRNA in MCF7-ADR tumors treated with RPN2 siRNAs or nontargeting siRNAs ($n = 3$ per group, $^*P < 0.01$). (**h**) Expression of RPN2 protein in MCF7-ADR tumors. H&E staining and RPN2 immunofluorescence staining (green, RPN2; blue, nuclei) of tissues treated with RPN2 siRNA or nontargeting siRNA. Scale bar, 50 μm. (**i**) Docetaxel retention in MCF7-ADR tumors in mice treated with RPN2 siRNAs or nontargeting siRNAs ($n = 4$ per group, $^*P < 0.001$). Values are means ± s.d.

in MCF7-ADR cells transduced by RPN2 siRNA (**Supplementary Fig. 4** online). For this reason, and to assess the potential involvement of *RPN2* gene overexpression in MDR1 functions, we tested the glycosylation status of MDR1 protein in MCF7-ADR cells transfected with RPN2 siRNA. We analyzed the glycosylation patterns by western blotting of P-glycoprotein, which appears on blots as mature 170-kDa, immature (partially glycosylated) 150-kDa and unglycosylated 140-kDa bands[28]. The 150-kDa immature and 140-kDa unglycosylated P-glycoproteins were clearly found in MCF7-ADR cells with RPN2 knockdown (90% inhibition of mRNA by real-time RT-PCR analysis; **Fig. 5a**). More than 80% of P-glycoproteins were unglycosylated or partially glycosylated in RPN2-silenced cells (composition of P-glycoproteins, 170 kDa:150 kDa:140 kDa = 18:40:42). In contrast, MCF7-ADR cells transduced with nontargeting control siRNA expressed more than half of their P-glycoproteins as170-kDa mature P-glycoprotein (170 kDa:150 kDa:140 kDa = 52:17:31). This result showed that RPN2 knockdown inhibits glycosylation of P-glycoproteins in MCF7-ADR cells. The western blot of P-glycoprotein, particularly in cells transduced with RPN2 siRNA, showed 'smear' patterns (**Fig. 5a**). We speculated that the smear pattern was caused by the presence of intermediately glycosylated forms in various sizes. We treated the cell lysate samples with peptide:*N*-glycosidase F (PNGase F) to remove *N*-glycan chains, which shifted the P-glycoprotein in the blot from a smear pattern to a 140-kDa unglycosylated protein band in MCF7-ADR cell lysates. After PNGase F treatment, both nontargeting control siRNA– and RPN2 siRNA–transduced cells showed a 140-kDa unglycosylated P-glycoprotein band (**Fig. 5a**). This indicates that the smear pattern resulted from the presence of intermediately glycosylated P-glycoprotein and that there were a number of intermediately glycosylated P-glycoproteins in the RPN2-silenced cells because of inhibition of glycosylation on P-glycoprotein.

We further evaluated the RPN2 siRNA effects on cell surface P-glycoprotein expression in MCF7-ADR cells by immunofluorescence staining. As expected, immunofluorescence staining indicated that P-glycoprotein was predominantly localized to the cell membrane in MCF7-ADR cells transduced with control nontargeting siRNAs, whereas the intensity of membrane P-glycoprotein in RPN2-downregulated cells was considerably reduced (**Fig. 5b**). Moreover, retention of rhodamine-123, which is a substrate of P-glycoprotein, was strongly enhanced in MCF7-ADR cells transfected with RPN2 siRNA compared to those transfected with nontargeting siRNA (**Fig. 5c**). This indicates that downregulation of RPN2 restores drug retention and inhibits P-glycoprotein function by suppressing the glycosylation of P-glycoproteins in MCF7-ADR cells.

To further bolster these findings, we performed immunostaining analysis of RPN2 and P-glycoprotein in MCF7-ADR tumors in mice. The RPN2 shutdown resulted in a marked disappearance of the membrane-bound P-glycoprotein (**Fig. 5d**), an observation that supports our *in vitro* findings that RPN2 downregulation by siRNA in drug-resistant MCF7-ADR cells results in the loss of membrane-bound P-glycoprotein.

Furthermore, we have examined the status of RPN2 and P-glycoprotein in breast cancer tissues from subjects with RPN2 mRNA high expression ($n = 4$) and RPN2 mRNA low expression ($n = 4$) by immunostaining. P-glycoprotein was predominantly localized to the cell membrane in the primary tumor with a strong signal for RPN2, whereas in the primary tumor with low expression of RPN2, P-glycoprotein was found in the cytoplasm (**Supplementary**

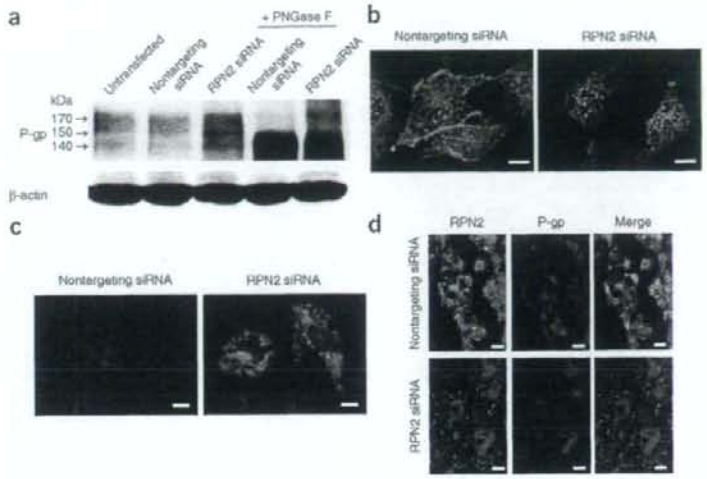

Figure 5 RPN2 siRNA regulates glycosylation of P-glycoprotein (P-gp). (**a**) Western blot analysis shows the glycosylation status of P-gp in MCF7-ADR cells 72 h after transfection of RPN2 siRNA or control nontargeting siRNA. Bands migrating at 170, 150 and 140 kDa represent mature, immature and unglycosylated forms of P-gp, respectively. (**b**) Immunofluorescence staining of P-gp on MCF7-ADR cell membrane surfaces. Cells were treated with RPN2 siRNA or nontargeting siRNA for 72 h. Scale bar, 5 μm. (**c**) Rhodamine-123 retention in MCF7-ADR cells 72 h after transfection with RPN2 siRNA or control nontargeting siRNA. Scale bar, 5 μm. (**d**) Localization of P-gp in tumors of MCF7-ADR in mice. Immunofluorescence staining of RPN2 (green) and P-gp (red) are shown. Nuclei are blue (DAPI). Merged images are also shown. Scale bar, 5 μm.

Fig. 5a,b online). Similar results were observed for other breast cancer tissues (**Supplementary Fig. 5c**).

Thus, these data provide a clear link between the glycosylation status of P-glycoprotein and RPN2 expression in drug-resistant breast cancer cells, and the disappearance of the membrane-bound P-glycoprotein leads to a reversal of the multidrug- resistant phenotype.

DISCUSSION

Cancer researchers today are confronted with how to best identify and select the next generation of molecular targets for oncology. An impressive array of potential new cellular targets, suitable for therapeutic intervention, has been revealed by the recent completion of the human genome sequencing project. Approaches as varied as transcription profiling, proteomics and the use of siRNAs are all being exploited in the race to select the most promising candidate drug targets. We tested the feasibility of using atelocollagen-mediated RNAi delivery *in vitro* and *in vivo* to obtain an unbiased evaluation on the efficacy of a specific siRNA related to drug resistance in human breast cancer. We show here that, among genes whose expression was elevated in nonresponders to docetaxel, the siRNA designed for *RPN2* significantly promoted docetaxel-dependent apoptosis and cell growth inhibition of MCF7-ADR human breast cancer cells that exhibit docetaxel resistance. A clinicopathological study showed that there is a significant association of *RPN2* expression with a pathologic response to docetaxel. Most notably, atelocollagen-mediated *in vivo* delivery of RPN2 siRNA significantly reduced the size of orthotopic MCF7-ADR tumors in mice given docetaxel.

In this study, we demonstrated that the atelocollagen delivery system markedly enhanced the efficiency of siRNA for the inhibition of RPN2 in mouse tumor models of human breast cancer. Because siRNA shows very low efficiency in gene silencing *in vivo*, various delivery methods, such as the use of plasmids and viral vectors encoding siRNA and the use of lipids, have been investigated. We have previously shown that the atelocollagen-mediated systemic delivery of siRNA might be a unique strategy for the inhibition of bone-metastatic prostate tumor growth[22]. The siRNA-atelocollagen complex is a nano-sized particle and is stable *in vitro* and *in vivo*[21,29]. Furthermore, we have previously confirmed that the atelocollagen complex shows low toxicity and low immunogenicity *in vivo*[23,24]. Thus, an atelocollagen-mediated local or systemic delivery system holds great potential for the practical application of gene suppression using siRNAs for cancer therapeutics.

Targeting of P-glycoprotein by small-molecular compounds, antibodies or both is an effective strategy to overcome multiple drug resistance in cancer[30]. Despite promising previous studies showing that the inhibition of P-glycoprotein by pharmacological means can sensitize drug-resistant cells, the ultimate goal of restoring drug sensitivity has met with limited success in clinical trials. Our results indicate that RPN2 is partly responsible for P-glycoprotein–mediated drug resistance in breast cancer and is involved in the regulation of the glycosylation status of P-glycoprotein. In fact, downregulation of *RPN2* restored drug retention, suggesting that P-glycoprotein function is inhibited via suppression of the glycosylation of P-glycoprotein in MCF7-ADR cells. *N*-glycosylation has been shown to contribute to the stability of the P-glycoproteins[31], and it has been reported that reduced glycosylation results in the disappearance of membrane-bound P-glycoprotein, which causes the loss of a multidrug-resistant phenotype[32]. Furthermore, multidrug-resistant cells are hypersensitive to the *N*-linked glycosylation inhibitor tunicamycin, which induces partial inhibition of the glycosylation of GLUT-1, a glucose transporter, and diminishes GLUT-1–mediated transport[33]. Because the amount of MDR1 mRNA was not significantly decreased in MCF7-ADR cells transduced with RPN2 siRNA, it is predicted that RPN2 inhibition may reduce the glycosylation of P-glycoprotein, thereby inducing perturbation of its subcellular localization, inhibition of its protein synthesis and/or acceleration of its degradation, with MCF7-ADR cells inevitably becoming hypersensitive response to docetaxel. In contrast, the RPN2 protein is part of an *N*-oligosaccharyl transferase complex that links to *N*-glycosylation ability; therefore, RPN2 inhibition could affect *N*-oligosaccharyl transferase function, resulting in impaired glycosylation of the P-glycoproteins. We speculate that RPN2 has a key role in drug-resistant tumor cells that overexpress P-glycoprotein and acts as a facilitator, stabilizing factor or both for *N*-glycosylation of P-glycoprotein. The coordinated expression of RPN2 and P-glycoprotein may participate in the mechanism of docetaxel resistance via the glycosylation status of P-glycoprotein.

However, one group has recently reported that the stability of P-glycoprotein is regulated by the ubiquitin-proteasome pathway in multidrug-resistant cancer cells[34]. Furthermore, the P-glycoprotein must be phosphorylated by protein kinase C (PKC) to effectively

function as a drug-efflux pump[35], which suggests that PKC is indirectly involved in the development of the multidrug-resistant phenotype. More recently, it was revealed that wild-type p53, a tumor suppressor, may resensitize soft tissue sarcoma to chemotherapeutic agents by reducing MDR1 phosphorylation via transcriptional repression of PKC expression[36]. There is no direct evidence of RPN2 involvement with the transcriptional repression of PKC. Other transporter proteins mediating drug resistance are the multidrug resistance–associated protein and ABCG2. Whether these different populations of multidrug resistance–associated protein family members and ABCG2 are affected by RPN2 has yet to be determined.

Recently, downregulation of multidrug resistance by the introduction of synthetic siRNAs has been reported[37,38]. However, only partial reversal of the drug-sensitive phenotype of the cells has been obtained. A possible explanation for this low inhibitory effect is that it was the result of a long half-life of P-glycoprotein[39] and the less efficient delivery of synthetic siRNAs into cells. Although the data are not shown, we compared the cell growth inhibition by synthetic RPN2 siRNA versus MDR1 siRNA in the presence of docetaxel *in vitro*. At the mRNA level, the downregulation of RPN2 and MDR1 obtained with the most efficient siRNA was 90% and 80%, respectively. These results indicate that cell growth inhibition was achieved by both siRNAs, although RPN2 siRNA showed a stronger growth inhibitory effect compared to MDR1 siRNA. Thus, though it is impossible at the moment to judge whether MDR1 or RPN2 is a more profitable target for overcoming drug resistance, RPN2 does provide a valuable clue for making multidrug-resistant breast cancer cells sensitive to anticancer drugs.

The continuing interest in apoptosis among cancer biologists has been strengthened by the hope that a molecular understanding of cell death will inform our understanding of cancer drug resistance. In fact, upregulation of antiapoptotic *Bcl2* family genes has been shown to be key in tumor malignancy and drug resistance[40,41]. Overexpression of exogenous Bcl-xL or Bcl-2 suppresses apoptosis[42,43]. In our study, knockdown of RPN2 by siRNA in MCF7-ADR cells selectively downregulated mRNA expression of Bcl-xL and Bcl-w (**Supplementary Fig. 6** online). These results suggest that RPN2 regulates Bcl-xL– and Bcl-w–mediated antiapoptosis and may be partly responsible for the docetaxel resistance of the MCF7-ADR cells. It has already been reported that apoptosis-based therapies[44], such as the downregulation of Bcl-xL expression, Bcl-w expression or both with antisense oligonucleotides, abolish tumorigenicity and enhance chemosensitivity in human malignant glioma cells[45–47]. In addition, Bcl-xL and Bcl-w are upregulated by nuclear factor-κB (NF-κB)[48]. Some chemotherapeutic agents, such as cisplatin and docetaxel, instantly induce the activation of NF-κB in cancer cells, and the cells become drug resistant[49]. In fact, we found that *RPN2* gene expression is also induced by docetaxel treatment of drug-sensitive MCF7 cells. Therefore, it would be useful to know whether RPN2 induces the downregulation of Bcl-xL and Bcl-w in MCF7-ADR cells by direct association with the NF-κB signaling pathway.

It is noteworthy that our findings using docetaxel-resistant human breast cancer cells are commonly found in other multiple cancers. Cisplatin-resistant human non–small cell lung carcinoma cells recover their sensitivity to cisplatin by knockdown of *RPN2* expression and die by apoptosis (Y.Y., K.H. and T.O., unpublished data). In addition, mouse mammary tumor cells resistant to docetaxel express mouse Rpn2, and inhibition of Rpn2 results in apoptotic cell death in the presence of docetaxel (**Supplementary Fig. 2e–g**). Therefore, RPN2 status is responsible for the drug-resistant nature of multiple cancer cell lines both in humans and in mice, and RPN2 expression may confer cross-resistance to a variety of anticancer drugs.

We previously reported that a group of redox genes is useful for the prediction of the clinical response to docetaxel in subjects with breast cancer[16]. Our current results indicate that the RPN2 mRNA level might serve as a predictor of the response to anticancer therapy rather than as a prognostic factor. The determination of the RPN2 mRNA level will be useful in the selection of subjects who are likely to benefit from adjuvant chemotherapy. Furthermore, our animal experiments suggest that treatment of subjects with a pharmacological agent that blocks RPN2 expression or function may induce a complete response to chemotherapeutic drugs. The *RPN2* gene may therefore represent a promising new target for RNAi therapeutics against multidrug-resistant tumors.

METHODS

Cell culture. Human mammary carcinoma cell lines, MCF7 cells and multidrug-resistant MCF7-ADR cells were provided by Shien-Lab, Medical Oncology, National Cancer Center Hospital of Japan. We cultured MCF7, MCF7-ADR and MDA-MB-231 (American Type Culture Collection) cells in RPMI 1640 (Gibco BRL) supplemented with 10% FBS (Gibco BRL) under 5% CO_2 in a humidified incubator at 37 °C. We cultured the mouse mammary tumor cell line EMT6/AR10.0 (European Collection of Cell Cultures), which shows docetaxel resistance, in MEM (EBSS) with 2 mM glutamine, 1% non essential amino acids and 10% FBS. The establishment of bioluminescent MCF7-ADR-Luc cells and docetaxel-resistant MDA-MB-231/MDR1 cells is described in **Supplementary Methods** online.

Design and synthesis of small interfering RNAs. We designed siRNAs and synthesized them with an siRNA duplex for each gene target (Dharmacon) for the preparation of an atelocollagen-based cell transfection array. The siRNA sequences are described in **Supplementary Methods**.

Atelocollagen-based cell transfection array. For RNAi-based functional screening, we prepared an atelocollagen-based cell transfection array, which enables reverse transfection of cells by atelocollagen-mediated gene transfer (**Supplementary Methods**). We performed live-cell luciferase assay for measurement of cell growth, and we performed caspase-7 assays with Apo-ONE Caspase-3/7 Assay Reagent (Promega) and Hoechst staining for apoptosis (**Supplementary Methods**).

Real-time reverse transcription PCR. We purified total RNA from cells and tumor tissues with an RNeasy Mini Kit and RNase-Free DNase Set (QIAGEN) and produced cDNAs with an ExScript RT reagent Kit (Takara). We then subjected cDNA samples to real-time PCR with SYBR Premix Ex Taq (Takara) and specific primers (**Supplementary Methods**). We carried out the reactions in a LightCycler (Roche Diagnostics). We normalized gene expression levels by *HPRT1* or *ACTB*. The cell-direct quantitative RT-PCR method is described in the **Supplementary Methods**.

Atelocollagen-mediated RPN2 small interfering RNA delivery *in vivo*. We performed mouse experiments in compliance with the guidelines of the Institute for Laboratory Animal Research at the National Cancer Center Research Institute of Japan. We used 4-week-old female athymic nude mice (CLEA Japan) to generate an experimental orthotopic breast cancer model. We injected 1.0×10^7 MCF7-ADR cells or MDA-MB-231/MDR1 cells suspended in 100 μl sterile PBS into the fat pad. When the tumor grew to approximately 5 mm in diameter, we injected mice with 200 μl of siRNA-atelocollagen by intratumoral injection. Preparation of the siRNA-atelocollagen complex is described in the **Supplementary Methods**. Simultaneously, we injected docetaxel i.p. into mice. We harvested tumor tissues for analysis of RPN2 mRNA and RPN2 protein at 24 h and 72 h after treatment, respectively.

TUNEL technique. We harvested tumor tissues 72 h after administration of siRNA and prepared frozen sections. We then performed TUNEL staining with an *in situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics), according to the manufacturer's protocol. We stained the nuclei with DAPI. We

determined the number of fluorescein-positive cells in three microscopic fields of each section by fluorescence microscopy.

Docetaxel disposition in tumors. We studied drug disposition of docetaxel in tumors in mice by HPLC with ultraviolet detection at 225 nm after solid-liquid extraction as described elsewhere[30]. Eleven hours after i.p. administration of 20 mg kg^{-1} docetaxel, we harvested the tumors treated with siRNA-atelocollagen complex and then analyzed the docetaxel abundance in the tumor.

Transfection of small interfering RNA. We carried out transfection of MCF7-ADR and EMT6/AR10.0 cells with siRNA using DharmaFECT 1 (Dharmacon) and TransIT-TKO (Mirus), respectively, according to the manufacturers' protocol (**Supplementary Methods**).

Antibodies. We used RPN2-specific antibody (H300, Santa Cruz Biotechnology) and MDR-specific antibody (G-1, Santa Cruz Biotechnology). We visualized staining with Alexa 488 or Alexa 594 (Molecular Probes). We used fluorescence microscopy or confocal fluorescence microscopy (Olympus) for observation of immunofluorescence-stained cells. The procedures of western blotting and immunofluorescence staining are described in the **Supplementary Methods**.

Rhodamine-123 retention assay. We washed cells once with prewarmed Opti-MEM I medium (37 °C, Gibco BRL) and incubated the cells for 30 min at 37 °C in the Opti-MEM I medium containing 10 μM rhodamine-123. We then removed the rhodamine-123 solution from the extracellular medium and washed the cells twice with Opti-MEM I medium. We observed the cells for fluorescence of rhodamine-123 under fluorescence microscopy.

Human samples. The study protocol for clinical samples (results presented in **Table 1**) was approved by the Institutional Review Board of Osaka University Medical School, and written informed consent was obtained from each subject (**Supplementary Note**).

Statistical analyses. We conducted statistical analysis by analysis of variance with the Student's *t*-test. We considered a P value of 0.05 or less as a significant difference.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

K.H. performed the experimental work, data analysis and writing of the first draft of the manuscript. K.K. and T.O. selected the initial set of genes subjected to the screening. K.I.-K., K.K., T.Y. and T.O. participated in the conception, design and coordination of the study. E.T. and Y.Y. performed siRNA delivery *in vivo* and helped with data analysis. K.N. provided drug-resistant cell lines. S.N. provided delivery molecules. The manuscript was finalized by T.O. with the assistance of all authors.

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Association of epidermal growth factor receptor (*EGFR*) gene mutations with *EGFR* amplification in advanced non-small cell lung cancer

Ryotaro Morinaga,[1,2,3] Isamu Okamoto,[1,8] Yoshihiko Fujita,[4] Tokuzo Arao,[4] Masaru Sekijima,[5] Kazuto Nishio,[4] Hiroyuki Ito,[5] Masahiro Fukuoka,[7] Jun-ichi Kadota[2] and Kazuhiko Nakagawa[1]

[1]Department of Medical Oncology, Kinki University School of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-8511; [2]Second Department of Internal Medicine and [3]Department of Medical Oncology, Oita University Faculty of Medicine, 1-1 Idaigaoka, Yuhu, Oita 879-5593; [4]Department of Genome Biology, [5]Department of Pathology, Kinki University School of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-8511; [6]Research Division for Advanced Technology, Kashima Laboratory, Mitsubishi Chemical Safety Institute, 14 Sunayama, Kamisu, Ibaraki 314-0255; [7]Department of Internal Medicine, Kinki University School of Medicine, Sakai Hospital, 2-7-1 Harayamadai, Minami-ku, Sakai, Osaka 590-0132, Japan

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Somatic mutations in the epidermal growth factor receptor (*EGFR*) gene are associated with the response to *EGFR* tyrosine kinase inhibitors in patients with non-small cell lung cancer (NSCLC). Increased *EGFR* copy number has also been associated with sensitivity to these drugs. However, given that it is often difficult to obtain sufficient amounts of tumor tissue for genetic analysis from patients with advanced NSCLC, the relationship between these two types of *EGFR* alterations has remained unclear. We have now evaluated *EGFR* mutation status both by direct sequencing and with a high-sensitivity assay, the Scorpion-amplification-refractory mutation system, and have determined *EGFR* copy number by fluorescence *in situ* hybridization (FISH) analysis in paired tumor specimens obtained from 100 consecutive patients with advanced NSCLC treated with chemotherapy. *EGFR* mutations or FISH positivity (*EGFR* amplification or high polysomy) were apparent in 18% (18/100) and 32% (32/100) of patients, respectively. The Scorpion-amplification-refractory mutation system was more sensitive than direct sequencing for the detection of *EGFR* mutations. Furthermore, *EGFR* mutations were associated with *EGFR* amplification ($P = 0.009$) but not with FISH positivity ($P = 0.266$). Our results therefore suggest the existence of a significant association between *EGFR* mutation and *EGFR* amplification in patients with advanced NSCLC. (*Cancer Sci* 2008; 99: 2455–2460)

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase of the ErbB family and has been implicated in the proliferation and survival of cancer cells. Aberrant expression of *EGFR* has been detected in many human epithelial malignancies, including non-small cell lung cancer (NSCLC).[1,2] This receptor has therefore been identified as a promising target for anticancer therapy, and several agents have been synthesized that inhibit its tyrosine kinase activity. *EGFR* tyrosine kinase inhibitors (TKI) have been evaluated most extensively in individuals with NSCLC, and they have had a substantial impact on the treatment of this disease by offering additional therapeutic options for patients with advanced NSCLC.[3–6]

Somatic mutations in the tyrosine kinase domain of *EGFR* have been detected in a subset of NSCLC patients who respond to EGFR TKI[7–9] and have been shown to be closely associated with sensitivity to these drugs.[10–14] Indeed, we and others have prospectively demonstrated a high response rate to EGFR TKI therapy in NSCLC patients with *EGFR* mutations.[15–21] An increased copy number of the *EGFR* gene, as revealed by fluorescence *in situ* hybridization (FISH), has also emerged as an effective molecular marker of EGFR TKI sensitivity in NSCLC.[22–24] We previously showed that *EGFR* mutation and *EGFR* amplification are associated in human NSCLC cell lines and that endogenous EGFR expressed in such cell lines positive for both of these *EGFR* alterations are activated constitutively.[25] However, the relationship between *EGFR* mutation and FISH positivity for *EGFR*, which reflects gene amplification or high polysomy, has remained unclear.[22–24,26,27] Indeed, only a few studies have evaluated the relationship between mutation and gene copy number for *EGFR* because of the difficulty in obtaining tumor samples suitable for genetic analysis from individuals with advanced NSCLC. We previously showed that the Scorpion-amplification-refractory mutation system (ARMS) is a sensitive technique for the detection of *EGFR* mutations in tumor specimens such as pleural effusion fluid or tissue obtained by transbronchial needle aspiration.[28–30] In the present study, we evaluated *EGFR* mutation status in small tumor specimens from patients with advanced NSCLC both by direct sequencing and by Scorpion-ARMS and compared the sensitivity of these methods for the detection of *EGFR* mutations. Furthermore, we determined *EGFR* copy number by FISH analysis in paired tumor specimens and examined its relationship to *EGFR* mutation.

Materials and Methods

Patients. The present retrospective study recruited consecutive patients with advanced NSCLC who received chemotherapy at Kinki University Hospital between January 2003 and December 2005. Patients eligible for the study had histologically confirmed stage III or IV NSCLC that was not curable by surgical resection or radiotherapy, irrespective of the presence of measurable lesions or good performance status (PS). Patients with recurrence after surgical resection were excluded. Complete clinical information and tissue blocks suitable for genetic analysis were available for 100 patients. We examined the relationship between *EGFR* mutation and *EGFR* copy number as well as the influence of these *EGFR* alterations on clinical outcome. Tumor response was assessed by computed tomography and evaluated according to the Response Evaluation Criteria in Solid Tumors.[31] Survival was calculated from the date of initiation of chemotherapy either to the date of death from any cause or to the date of last contact. Some patients had been receiving EGFR TKI treatment before the demonstration in 2004 that mutations in *EGFR* confer increased sensitivity to these drugs. Moreover, many patients had already died before the initiation of our genetic analysis, preventing us from obtaining informed consent. The institutional review board

[8]To whom correspondence should be addressed.
E-mail: chi-okamoto@dotd.med.kindai.ac.jp

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therefore approved our study protocol with the conditions that samples would be processed anonymously and analyzed only for somatic mutations (not for germline mutations) and that the study would be disclosed publicly, according to the Ethical Guidelines for Human Genome Research published by the Ministry of Education, Culture, Sports, Science, and Technology, the Ministry of Health, Labor, and Welfare, and the Ministry of Economy, Trade, and Industry of Japan. The present study also conforms to the provisions of the Declaration of Helsinki.

Identification of *EGFR* mutations. The tumor specimens were fixed with formalin and embedded in paraffin. DNA was extracted with the use of a QIAamp Micro kit (Qiagen K.K., Tokyo, Japan) from tumor tissue derived either by macrodissection or by laser-capture microdissection carried out to enrich tumor cells. Polymerase chain reaction-based direct sequencing of exons 18–21 and ARMS with designed 'Scorpion' primers were applied for the allele-specific detection of *EGFR* mutations. Only the following previously described mutations[7,8] were classified as mutations in the present study: G719X in exon 18, deletion of E746 to A750 or of neighboring residues in exon 19, as well as L858R and L861Q in exon 21. Patients were regarded as *EGFR* mutation positive if a mutation in *EGFR* was detected either by direct sequencing or by ARMS. All mutations were confirmed by analysis of at least two independent amplification products.

Determination of *EGFR* copy number. *EGFR* copy number was determined by FISH analysis with the use of dual-color DNA probes (LSI *EGFR* SpectrumOrange/CEP 7 SpectrumGreen; Vysis, Downers Grove, IL, USA). The tumor specimens were classified into six categories on the basis of the FISH results, as described previously.[22] Those with high polysomy (≥4 copies of *EGFR* in ≥40% of cells) or gene amplification (presence of a tight *EGFR* gene cluster and a ratio of *EGFR* to chromosome 7 of ≥2 or ≥15 copies of *EGFR* per cell in ≥10% of cells analyzed) were considered FISH positive, with those in the remaining categories being considered FISH negative.

Statistical analysis. The relationships among *EGFR* status, clinical characteristics, and tumor response to EGFR TKI were analyzed with Fisher's exact test as appropriate. Survival curves were constructed by the Kaplan–Meier method, and the differences in survival between patient subgroups were compared by the log-rank test. The impact of various factors on survival was evaluated by univariate and multivariate analysis according to the Cox regression model. A P-value <0.05 was considered statistically significant. All statistical analysis was carried out with StatView software (SAS Institute, Cary, NC, USA).

Results

Patient characteristics. Between January 2003 and December 2005, a total of 125 consecutive patients diagnosed histologically with advanced NSCLC underwent chemotherapy at Kinki University Hospital. Tissue specimens from 100 patients were assessable for both *EGFR* mutation and *EGFR* copy number. Of these specimens, 72 were obtained by bronchoscopic biopsy, 15 by percutaneous needle biopsy (12 from lung, two from bone, and one from lymph node), six by thoracoscopic biopsy, and seven by surgery for diagnosis or palliative therapy. The clinical characteristics of these 100 patients are shown in Table 1. Most of the patients were male (64%) and had a history of smoking (67%), and adenocarcinoma was the most prevalent tumor histology (61%). Most patients (83%) also had a good Eastern Cooperative Oncology Group PS (0 or 1), and 63% received second-line or subsequent rounds of chemotherapy. Fifty-three patients (53%) were treated with EGFR TKI. Seventy patients (70%) had died by the time of genetic analysis, with the median follow-up time for the 30 survivors being 14.6 months.

***EGFR* alterations in non-small cell lung cancer.** Patients were analyzed for *EGFR* mutations by direct sequencing of exons 18 through 21 and by Scorpion-ARMS (Table 2). *EGFR* mutations, consisting of in-frame deletions in exon 19 ($n = 5$) and point mutations in exon 21 ($n = 13$), were detected in 18 patients (18%). Eight *EGFR* mutations were detected by direct sequencing and 16 mutations were detected by Scorpion-ARMS. Ten of the 16 mutations detected by Scorpion-ARMS were not identified by direct sequencing. However, two of the deletions in exon 19 (E746_S752 and E746_T751) that were detected by direct sequencing were not identified by Scorpion-ARMS, given that the Scorpion primers were designed only for detection of the E746_A750 deletion in exon 19. *EGFR* mutations were significantly more frequent in tumors of women than in those of men (33 vs 9%), in adenocarcinomas than in tumors with other histologies (28 vs 3%), and in never-smokers than in smokers (42 vs 6%) (Fig. 1a). One of the 18 *EGFR* mutations was detected in a squamous cell carcinoma. Determination of *EGFR* copy number by FISH analysis revealed gene amplification in six patients and high polysomy in 26 patients, with 32 patients thus being classified as FISH positive (Table 3). In contrast to *EGFR* mutation, FISH

Table 1. Characteristics of patients with advanced non-small cell lung cancer ($n = 100$)

Characteristic	Subset	No. patients
Sex	Male	64
	Female	36
Smoking history	Never-smoker	33
	Smoker	67
Tumor histology	Adenocarcinoma	61
	Other	39
Eastern Cooperative Oncology Group performance status	0	24
	1	59
	≥2	17
No. chemotherapies	1	37
	≥2	63

Table 2. Detection of epidermal growth factor receptor (*EGFR*) mutations by direct sequencing or amplification-refractory mutation system (ARMS) ($n = 100$)

Site	Mutation	Direct sequencing	ARMS	Direct sequencing or ARMS
Exon 19	15-bp deletion	1	3	3
	16-bp deletion	1	0	1
	19-bp deletion	1	0	1
Exon 21	L858R	5	13	13
Total		8 (8%)	16 (16%)	18 (18%)

Table 3. Determination of epidermal growth factor receptor gene copy number by fluorescence *in situ* hybridization (FISH) analysis ($n = 100$)

FISH status	Finding	No. patients
Positive	Gene amplification	6
	High polysomy	26
	Total	32
Negative	Low polysomy	35
	High trisomy	2
	Low trisomy	26
	Disomy	5
	Total	68

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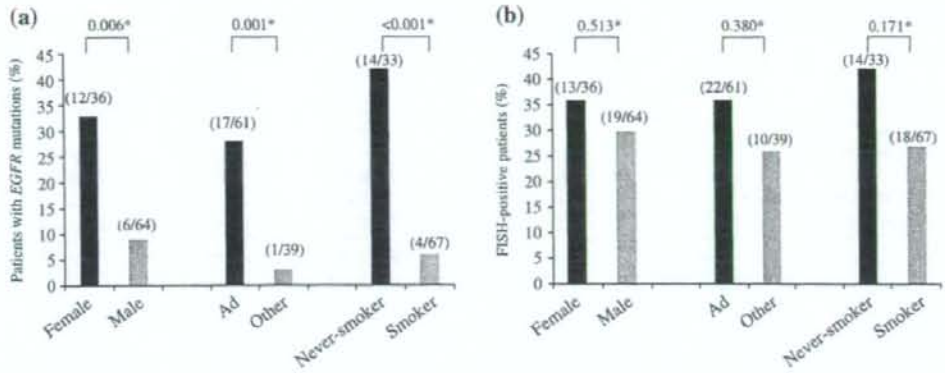

Fig. 1. Sex, tumor histology, and smoking status of patients with advanced non-small cell lung cancer and with either (a) epidermal growth factor receptor (*EGFR*) mutations or (b) a high *EGFR* copy number. Ad, adenocarcinoma. *P-values were determined by Fisher's exact test.

Table 4. Relationship between epidermal growth factor receptor (*EGFR*) mutation and either fluorescence *in situ* hybridization (FISH) status of *EGFR* amplification

Mutation status	FISH status		Gene amplification	
	Positive	Negative	Positive	Negative
Positive ($n = 18$)	8	10	4	14
Negative ($n = 82$)	24	58	2	80
P-value*		0.266		0.009

*Determined by Fisher's exact test.

positivity was not associated with sex, tumor histology, or smoking status (Fig. 1b). Although no relationship was apparent between *EGFR* mutation and FISH positivity (gene amplification or high polysomy), *EGFR* mutation and *EGFR* amplification were significantly associated (Table 4). The clinicopathological and genetic features of patients with *EGFR* mutations are shown in Table 5.

Overall survival. For the total patient population, the median overall survival was 12.3 months, with a 1-year survival rate of 51.7%. Univariate analysis revealed that overall survival was significantly longer in women, never-smokers, patients with a favorable PS, and those with *EGFR* mutations (Table 6; Fig. 2a). In contrast, no difference in overall survival was apparent between FISH-positive and FISH-negative patients (Table 6; Fig. 2b). We also carried out multivariate analysis to identify factors that contribute to overall survival, with covariates including clinicopathological and genetic factors (sex, smoking history, tumor histology, PS, *EGFR* mutation status, FISH status). Female sex and favorable PS were found to be independent prognostic factors (Table 6).

Responsiveness to epidermal growth factor receptor tyrosine kinase inhibitor treatment. Of the 53 patients treated with EGFR TKI, 40 individuals were assessable for objective response. Whereas the rate of response to EGFR TKI treatment for patients with *EGFR* mutations was significantly higher than that for those without such mutations (71.4 vs 11.5%, $P < 0.001$), there was no significant association between FISH status and responsiveness

Table 5. Clinicopathological and genetic features of patients with epidermal growth factor receptor (*EGFR*) mutations

No.	Age (years)	Sex	Smoking status	Histology	Response to EGFR TKI	Type of *EGFR* mutation		*EGFR* copy number
						Sequencing	ARMS	
1	72	F	Never	Ad	PR		L858R	Low trisomy
2	58	F	Never	Ad	PR	L858R	L858R	Gene amplification
3	81	F	Never	Ad	SD	L858R	L858R	High polysomy
4	72	F	Never	Ad	NE		L858R	Gene amplification
5	48	M	Smoker	Ad	SD		L858R	Low trisomy
6	67	F	Never	Ad	SD		L858R	Low trisomy
7	59	F	Never	Ad	PR		L858R	High polysomy
8	78	M	Smoker	Ad			L858R	High trisomy
9	71	F	Never	Ad	PR		L858R	Low polysomy
10	82	F	Never	Ad	PR	L858R	L858R	Low trisomy
11	67	F	Never	Ad		L858R	L858R	High polysomy
12	87	F	Never	Sq	PR	L858R	L858R	Low polysomy
13	78	M	Never	Ad			L858R	Gene amplification
14	56	F	Never	Ad	PR		(E746_A750)del	Low polysomy
15	63	M	Never	Ad	PD	(E746_A750)del	(E746_A750)del	Gene amplification
16	63	M	Smoker	Ad	PR		(E746_A750)del	Low polysomy
17	61	M	Smoker	Ad	PR	(E746_S752)del insV		Low trisomy
18	73	F	Never	Ad	PR	(E746_T751)del insS		High polysomy

Ad, adenocarcinoma; ARMS, amplification-refractory mutation system; NE, not evaluated; PD, progressive disease; PR, partial response; SD, stable disease; Sq, squamous cell carcinoma; TKI, tyrosine kinase inhibitor.

Table 6. Univariate and multivariate analyses of prognostic factors for overall survival

Factor	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Sex (female/male)	0.54	0.32–0.91	**0.021**	0.55	0.32–0.93	**0.025**
Smoking history (never-smoker/smoker)	0.50	0.30–0.85	**0.011**			
Histology (adenocarcinoma/other)	0.64	0.39–1.05	0.077	0.68	0.40–1.14	0.141
ECOG PS (0/≥1)	0.44	0.24–0.79	**0.006**	0.48	0.29–0.86	**0.019**
EGFR mutation status (positive/negative)	0.52	0.28–0.97	**0.039**			
FISH status (positive/negative)	1.36	0.82–2.23	0.231	1.49	0.88–2.50	0.130

CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; EGFR, epidermal growth factor receptor; FISH, fluorescence *in situ* hybridization; HR, hazard ratio; PS, performance status. Multivariate analysis was carried out using the stepwise method (include, <0.05; exclude, >0.2). Significant *P*-values are shown in bold.

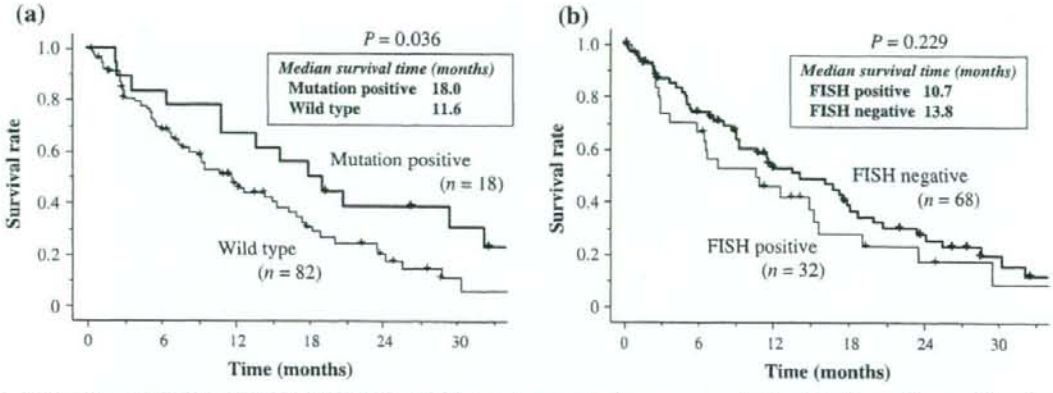

Fig. 2. Kaplan–Meier plots of overall survival in patients with advanced non-small cell lung cancer and either (a) with or without epidermal growth factor receptor (*EGFR*) mutations or (b) with or without a high *EGFR* copy number. FISH, fluorescence *in situ* hybridization.

to EGFR TKI (44.4 vs 29.0% for FISH-positive vs FISH-negative patients, respectively, $P = 0.437$).

Discussion

We have analyzed both *EGFR* mutation and *EGFR* copy number in paired tumor specimens as well as the relationship between these two types of *EGFR* alterations in advanced NSCLC. We used two methods to detect *EGFR* mutations, direct sequencing and Scorpion-ARMS, which identified eight and 16 mutations, respectively. Direct sequencing failed to detect 10 of the 16 mutations identified by Scorpion-ARMS. Of the 10 patients with *EGFR* mutations detected by Scorpion-ARMS alone, seven were assessable for an objective response to EGFR TKI, with five exhibiting a partial response and two having stable disease. Consistent with previous observations,[28–30] our data thus indicate that Scorpion-ARMS is more sensitive than direct sequencing for detection of the two major types of *EGFR* mutation that reflect responsiveness to EGFR TKI. It should be noted, however, that most polymerase chain reaction-based systems for mutation analysis, including Scorpion-ARMS, are able to detect only known *EGFR* mutations targeted by the designed primers. Indeed, two minor variants of deletion mutation in exon 19 were not identified by Scorpion-ARMS in the present study. Given the exclusion of recurrence after surgical resection in our study, most tumor specimens analyzed were obtained either by transbronchial lung biopsy or by percutaneous needle lung biopsy. The amount of tumor tissue obtained by these procedures is limited, but our results suggest that it is sufficient both for histopathological analysis and for the detection of *EGFR* mutations by Scorpion-ARMS in patients with advanced NSCLC.

Scorpion-ARMS identified three E746_A750 deletion mutations in exon 19 and 13 L858R point mutations in exon 21 in the present study. The frequency of the E746_A750 mutation detected by Scorpion-ARMS thus appeared low compared with that of the L858R mutation. Previous studies have shown that the incidence of the E746_A750 deletion is approximately the same as that of the L858R mutation.[10,12] The sensitivity of Scorpion-ARMS for detection of the E746_A750 deletion is equivalent to that for detection of the L858R point mutation. The low frequency of the E746_A750 deletion mutation in the present study is thus likely due to the small number of samples.

Previous studies have revealed a higher prevalence of *EGFR* mutations in East Asians than in Caucasians.[4,10–12,20,22,24,26,27,32–36] The prevalence of *EGFR* mutations in our Japanese cohort was low (18%) compared with values determined previously for East Asian populations. Given that most previous studies examined only individuals treated with EGFR TKI, patient selection based on clinical predictors might have led to an increase in the proportion of subjects with adenocarcinoma histology, a factor known to be associated with *EGFR* mutations. In contrast, our study was carried out with consecutive cases irrespective of EGFR TKI treatment. The relatively low proportion of patients with adenocarcinoma histology (61%) in our cohort is therefore consistent with the low prevalence of *EGFR* mutations. However, the FISH positivity of 32% in our study is similar to that in previous studies that adopted the same criteria, with values ranging from 31 to 48%.[22–24,26,27] Consistent with previous

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results,[1,7–9,12] *EGFR* mutations were significantly more frequent among women, never-smokers, and patients with adenocarcinoma in the present study. In contrast, neither *EGFR* amplification (analysis not shown) nor FISH positivity was associated with any such clinicopathological factor in our study, although the relationship between *EGFR* amplification and never-smoking status approached statistical significance ($P = 0.090$).

The relationship between *EGFR* mutation and FISH positivity (gene amplification or high polysomy) in NSCLC patients has remained unclear.[22–24,26,27] In the present study, we have demonstrated a significant relationship between *EGFR* mutation and *EGFR* amplification, but not between *EGFR* mutation and FISH positivity, in tumor specimens from patients with advanced NSCLC. *EGFR* mutant alleles were previously found to be amplified selectively, resulting in a high *EGFR* copy number, as detected by quantitative real-time polymerase chain reaction analysis.[12] *EGFR* amplification has also been shown to be acquired during invasive growth of lung adenocarcinoma with *EGFR* mutations.[37] Furthermore, recent studies have found that an increase in *EGFR* copy number is a relatively late event in NSCLC pathogenesis[38] and that *EGFR* mutation precedes *EGFR* amplification but not necessarily high polysomy.[37,39] These observations thus support the existence of a close association between *EGFR* mutation and *EGFR* amplification. We previously showed that *EGFR* mutation was significantly associated with *EGFR* amplification in human NSCLC cell lines and that endogenous EGFR expressed in such cell lines that manifested both of these *EGFR* alterations were activated constitutively as a result of ligand-independent dimerization.[25] However, the biological consequences of high polysomy for *EGFR* have not been elucidated. We did not find any cut-off value of high polysomy that was associated with *EGFR* mutation. We therefore propose that *EGFR* amplification, but not high polysomy, plays a key role in the pathogenesis of NSCLC and correlates with *EGFR* mutation.

We sought to determine whether *EGFR* mutation or *EGFR* copy number might affect overall survival of NSCLC patients. Previous studies of EGFR TKI have suggested that *EGFR* mutation is a favorable prognostic indicator for patients with NSCLC.[35,36] We also found that the survival time of patients with *EGFR* mutations was longer than that of those without them (18.0 vs 11.6 months, $P = 0.036$) in the univariate analysis. However, interpretation of this result requires that the effect of EGFR TKI on survival be taken into account, given that 83% (15/18) of patients with *EGFR* mutations were treated with EGFR TKI compared with only 46% (38/82) of those without such mutations. Indeed, analysis of survival after initiation of EGFR TKI treatment as a second-line or subsequent therapy revealed a survival time of 15.6 months for mutation-positive patients vs 6.0 months for mutation-negative patients in our study. It was therefore not possible to determine the prognostic significance of *EGFR* mutation for NSCLC patients. To clarify whether *EGFR* mutation is a predictor of sensitivity to EGFR TKI or a prognostic indicator for NSCLC patients, we are currently carrying out a phase III randomized study comparing platinum-based chemotherapy with gefitinib in chemotherapy-naive NSCLC patients with *EGFR* mutations. Patients with FISH-positive tumors tended to have a shorter survival time than did those with FISH-negative tumors (10.7 vs 13.8 months), although this difference was not statistically significant. This result is consistent with previous observations indicative of an association between high *EGFR* copy number and poor prognosis for certain malignancies, including NSCLC.[1,40]

In conclusion, we have analyzed both *EGFR* mutation and *EGFR* copy number in paired tumor specimens from patients with advanced NSCLC. We found that Scorpion-ARMS is more sensitive than direct sequencing for detection of *EGFR* mutations in small tumor specimens. Furthermore, we showed that *EGFR* mutation was significantly associated with *EGFR* amplification but not with FISH positivity. These observations warrant confirmation in further studies as well as exploration of the biological mechanisms of the relationship between *EGFR* mutation and *EGFR* amplification. The effects of *EGFR* mutation and *EGFR* copy number on clinical outcome in individuals with advanced NSCLC also warrant investigation in a prospective study.

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