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RPN2 expression predicts response to docetaxel in oesophageal squamous cell carcinoma

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BACKGROUND: Neoadjuvant chemotherapy – often using docetaxel in various combinatorial regimens – is a standard treatment choice for advanced oesophageal squamous cell carcinoma (ESCC) in Japan. However, no useful markers exist that predict docetaxel's effects on ESCC. Ribophorin II (RPN2) silencing, which reduces glycosylation of P-glycoproteins and decreases membrane localisation, promotes docetaxel-dependent apoptosis. We investigated whether RPN2 expression in ESCC biopsy specimens could be a predictive biomarker in docetaxel-based neoadjuvant chemotherapy.

METHODS: We evaluated RPN2 expression immunohistochemically in biopsy specimens from 79 patients with node-positive ESCC, who received docetaxel-based adjuvant chemotherapy, and compared clinical and pathological responses between the RPN2-positive and RPN2-negative groups. We also studied susceptibility of RPN2-suppressed ESCC cells to docetaxel.

RESULTS: The RPN2-negative group had better clinical and pathological responses to docetaxel than the RPN2-positive group. We also found RPN2 suppression to alter docetaxel susceptibility *in vitro*.

CONCLUSION: Expression of RPN2 in biopsy specimens could be a useful predictive marker for response to docetaxel-based neoadjuvant chemotherapy in ESCC.

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In Japan, prognosis of patients with oesophageal squamous cell carcinoma (ESCC) has improved over several decades, mainly owing to improved surgical techniques, such as three-field lymph node dissection (Akiyama *et al*, 1994; Ando *et al*, 2000). However, survival of patients with node-positive ESCC is still unsatisfactory. Therefore, clinical studies to evaluate the efficacy of adjuvant chemotherapy for resectable ESCC have been conducted. JCOG 9204, which compared postoperative chemotherapy with surgery alone, found that two courses of 5-fluorouracil (5-FU) and cisplatin (FP) prolonged survival of patients with node-positive stage II/III ESCC (Ando *et al*, 2003). JCOG 9907 compared preoperative chemotherapy with postoperative chemotherapy and found the preoperative chemotherapy arm had significantly better overall survival than did the postoperative chemotherapy arm (Ando *et al*, 2012). On the basis of these findings, current standard treatment for resectable stage II/III ESCC in Japan relies on neoadjuvant chemotherapy followed by surgery.

However, an optimal neoadjuvant chemotherapy regimen for ESCC has not been established. Although the FP combination has been a standard regimen for advanced or metastatic ESCC (Kelsen *et al*, 1998; Ancona *et al*, 2001; Ando *et al*, 2012), its response rate is not sufficiently high. Recently, docetaxel combined with FP (DCF) was tested as induction therapy for patients with node-positive ESCC, and had a good result (Overman *et al*, 2010; Watanabe *et al*, 2011; Yamasaki *et al*, 2011). We consider docetaxel to be a key drug for treating patients with ESCC.

Docetaxel-based combination chemotherapy is highly toxic. Therefore, if tumours do not respond to this chemotherapy, its use is not merely pointless, but actually harmful. Worse, as neoadjuvant chemotherapy delays surgical treatment, there is a risk of losing the opportunity to cure non-responders. Therefore, molecular markers that predict response to chemotherapy would be extremely helpful in selecting patients who may benefit from neoadjuvant therapy.

Recently, Honma *et al* (2008) revealed that downregulation of ribophorin II (RPN2), which is part of an *N*-oligosaccharyl transferase complex, efficiently induced apoptosis in docetaxel-resistant human breast cancer cells in the presence of docetaxel. Silencing of RPN2 reduced glycosylation of the P-glycoprotein and decreased membrane localisation, thereby sensitising cancer cells to docetaxel (Honma *et al*, 2008). These findings suggest that RPN2 expression is a candidate predictive marker for resistance to docetaxel-based chemotherapy. There is little current information regarding either RPN2 expression in ESCC or correlation between its expression and resistance to docetaxel. In this study, we examined RPN2 expression immunohistochemically in pretreatment endoscopic biopsy samples from ESCC patients, and assessed the correlation between RPN2 expression and response to neoadjuvant chemotherapy. In addition, we investigated whether RPN2 expression levels affected docetaxel sensitivity in ESCC *in vitro*.

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Table 1 RPN2 expression and the clinicopathological features

Features	Total (n=79)	RPN2 Positive (n=51)	RPN2 Negative (n=28)	P-value
Age (years)				0.100
High (⩾70)	39	29	10	
Low (<70)	40	22	18	
Sex				0.763
Male	66	42	24	
Female	13	9	4	
Depth of tumour invasion				0.383
cT1	6	3	3	
cT2	16	8	8	
cT3	48	33	15	
cT4	9	7	2	
Distant metastasis				0.705
Absent	71	45	26	
Present	8	6	2	
Stage				0.638
II	10	5	5	
III	43	29	14	
IV	26	17	9	
Therapy after DCF				
Esophagectomy	49	34	15	0.573
Continue DCF	11	6	5	
Chemoradiation	18	10	8	
Best supportive care	1	1	0	

Abbreviations: DCF = docetaxel combined with FP; RPN2 = ribophorin II. Analysed by Fisher's exact test.

MATERIALS AND METHODS

Patients and samples

We used paraffin blocks of 79 specimens endoscopically biopsied from patients with node-positive ESCC before treatment with the modified DCF regimen ($60\,mg\,m^{-2}$ docetaxel on day 1; $350\,mg\,m^{-2}$ 5-FU and $6\,mg\,m^{-2}$ cisplatin on days 1–5) at Kumamoto University Hospital for this study from March 2008 to October 2011. Before therapy, all patients underwent upper gastroenterological fiberscope, oesophagography, enhanced CT imaging from neck to abdomen and ^{18}F-fluorode-oxyglucose positron emission tomography (FDG-PET) for tumour staging according to the TNM classification (ver. 6).

After being diagnosed with node-positive ESCC, all patients received combination induction chemotherapy of the DCF regimen given every 3 weeks for two rounds; their clinical response was then evaluated. Imaging by FDG-PET CT, upper gastroenterological fiberscope and oesophagography was conducted in all patients post chemotherapy (2 weeks after the end of therapy). After two rounds of chemotherapy, 49 patients underwent oesophageal resection, 11 patients continued DCF regimen, 18 patients underwent chemo-radiation (DCF + radiation) therapy and 1 patient received the best supportive care. Clinical data are summarised in Table 1. Informed consent was obtained from all patients who participated in this study. This study was approved by the Institute Review Board of the Graduate School of Medical Science, Kumamoto University (approval number: 236; 2 August 2008).

Evaluation of clinical responses to DCF

We evaluated clinical responses to DCF chemotherapy by (1) the Response Evaluation Criteria in Solid Tumors (RECIST) v1.0; (2) World Health Organization (WHO) criteria: upper gastro-enterological fiberscope and oesophagography assessments based on criteria defined by the WHO, including complete response (CR), disappearance of all known disease, partial response (PR), ⩽50% decrease in entire tumour burden, stable disease (SD); <50% decrease or <25% increase in entire tumour burden and progressive disease (PD), ⩾25% increase in the entire tumour burden or appearance of new lesions; and (3) histopathological criteria: for the 49 patients who underwent oesophageal resection, histopathological tumour regression in response to chemotherapy was assessed by evaluating the resected tumours according to a three-grade score established by the Japanese Guidelines for the Clinical and Pathologic Studies on Carcinoma of the Esophagus, with histopathological effects classified into four categories, from grade 0 to 3 (grade definitions shown in Supplementary Table 1).

Response analysis by FDG-PET

We evaluated responses to DCF chemotherapy by changes in standardised uptake value (SUV), which was obtained using FDG-PET values before and after DCF chemotherapy, and calculated the percentage decrease in SUV_{max} rate of primary tumours during chemotherapy using the formula: $((preSUV_{max} - postSUV_{max})/ preSUV_{max}) \times 100$ (Brucher *et al*, 2001).

Immunohistochemical staining for RPN2

Immunostaining was done on 5-μm tissue sections mounted on silane-coated slides. Each paraffin section was deparaffinised with xylene, followed by antigen retrieval. Antigen retrieval was carried out using 0.01 M (pH 9.0) buffer and microwaved for 15 min. Ribophorin II protein expression was evaluated using a polyclonal antibody specific for RPN2 (N-20, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubating overnight, and with the secondary antibody (Histofine MAX PO, Nichirei, Tokyo, Japan) for 30 min. Ribophorin II cytoplasmic expression was assigned intensity grades – no staining: 0, weak staining: 1, moderate staining: 2 and strong staining: 3 (Figure1 shows examples of RPN2 staining). Tumour cells with weaker staining patterns than normal epithelial cells – weak (1), or non-staining (0) – were considered to have negative expression. Expression was independently evaluated by two of the authors (JK and YB) using a blind protocol design; observers had no information on clinical outcome or any other clinicopathological data.

Cell culture

Human oesophageal carcinoma cell lines TE1 and 14 (TE1/14) were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan. All cells were grown in RPMI 1640 (Cambrex, East Rutherford, NJ, USA) supplemented with 10% foetal bovine serum (Sigma-Aldrich, St Louis, MO, USA), and incubated in a humidified chamber supplemented with 5% CO_2.

Transfection of small interfering RNA

Small interfering RNA (siRNA) against *RPN2* and control non-targeting siRNA were obtained from Invitrogen, Inc. (Carlsbad, CA, USA), Stealth RNAi sequences: *RPN2* (5′-GACAUCUCUUCAG GCCUGACAAUUU-3′). The non-silencing control siRNA, which has no sequence homology to any known human gene sequence, was used as a control for non-specific effects in all experiments. Subconfluent human prostate cells were transfected with siRNA using Lipofectamine 2000 transfection regent (Invitrogen) following the manufacturer's instructions. Two days after transfection, the efficacy of siRNA knockdown was assessed using quantitative reverse-transcription PCR (qRT-PCR) and immunoblotting. The optimal amount of siRNA used for transfection was determined to be $20\,nmol\,l^{-1}$, and the siRNA sequence that best reduced >90% of *RPN2* expression was identified.

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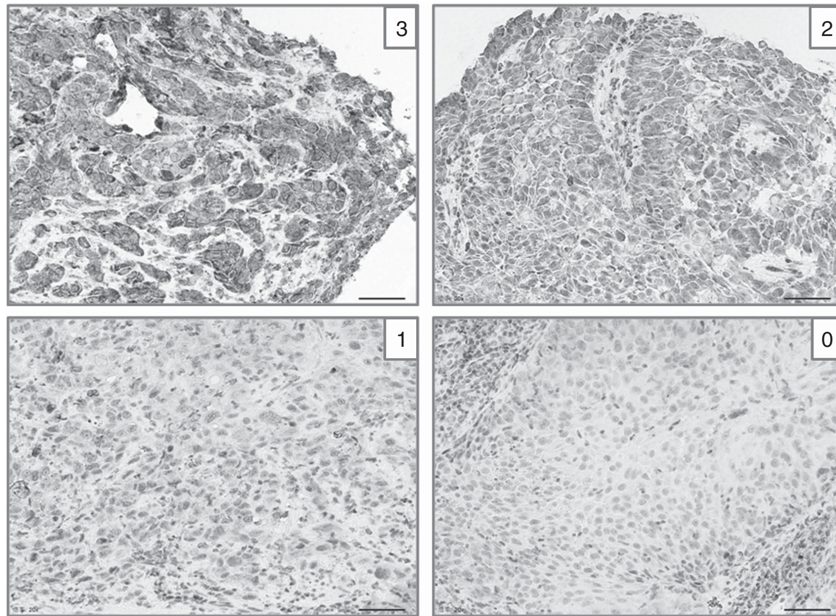

Figure 1 Immunohistochemical staining of RPN2 protein in ESCC tissues. RPN2 protein expression was detected in the cytoplasm. We graded RPN2 protein expression as null (0), weak (1), moderate (2) or strong (3). Tumour cells that exhibited weaker staining patterns than normal epithelial cells – weak (1) or null (0) – were defined as RPN2 negative. Scale bar is 50 μm.

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Chemotherapy dose–response curve

To assess the effect of *RPN2* on docetaxel sensitivity, 3×10^3 cells were seeded onto 96-well microtitre plates. To assess the effect of the combination treatment of *RPN2* silencing plus chemotherapy, TE1/14 cells were transfected with 20 nmol l^{-1} of stealth siRNA against RPN2 for 24 h. Cells were then treated with docetaxel at increasing concentrations (0.5, 1.0, 5.0, 10, 50, 100, 500 or 1000 nM) for 48 h. The cell survival rate was determined using the WST-8 assay with Cell Counting Kit-8 (Dojin Laboratories, Kumamoto, Japan). Absorbance was measured at 450 nm. Cell viability was determined using an MTT assay.

Western blot analysis

To isolate proteins, cells harvested onto six-well plates were washed once in PBS and lysed in lysis buffer (25 mmol l^{-1} Tris-HCl pH 7.4, 100 mmol l^{-1} NaCl, 2 mmol l^{-1} EDTA, 1% Triton X with 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 1 mmol l^{-1} Na_3VO_4, 1 mmol l^{-1} phenylmethylsulfonylfluoride). Each protein sample (15 μg) was resolved on SDS-PAGE, transferred onto a polyvinylidene difluoride membrane and incubated with a polyclonal antibody against RPN2 (N-20, 1:200, Santa Cruz Biotechnology) or β-actin (1:2000; Sigma-Aldrich). The signals were detected using secondary antibodies labelled with HPL and ECL Detection System (GE Healthcare, Little Chalfont, UK).

RNA isolation and real-time qRT-PCR

Total RNA, including miRNA, was isolated from tissue samples and cell lines using RNAeasy (Qiagen, Hilden, Germany), and eluted into 100 μl of heated Elution Solution according to the manufacturer's protocol. The purity and concentration of all RNA samples were quantified using NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA). Expression levels of *RPN2* were quantified using a SYBR Green qRT-PCR with LightCycler 480 SYBR Green I Master (Roche Diagnostics, Basel, Switzerland) and normalised to *GAPDH*. SYBR Green real-time RT-PCR was done using primers specific for *RPN2* (forward: 5′-ATCTAACCTTG ATCCCAGCAATGTG-3′; reverse: 5′-CTGCCAGAAGCAGATCTTT GGTC-3′) and *GAPDH* (forward: 5′-TTGGTATCGTGGAAGGACT C-3′; reverse: 5′-AGTAGAGGCAGGGATGATGT-3′). All qRT-PCR was executed on the LightCycler 480 System II (Roche Diagnostics). Relative amounts of RPN2 were measured using the $2^{-\Delta\Delta CT}$ method. All qRT-PCR reactions were performed in triplicate.

Statistical analysis

All experiments were repeated at least three times. Continuous variables were expressed as medians and ranges. Relationships between RPN2 expression and patient clinicopathological characteristics were analysed using Fisher's exact test. $P<0.05$ was considered to be significant. All statistical analyses were performed using the SPSS v. 13.0 software programme (SPSS, Inc., Chicago, IL, USA).

RESULTS

Patient characteristics and RPN2 expression

Of the 79 patients with ESCC, who were evaluated in this study, we found 64.6% (51 out of 79) of patients belonged in the RPN2-positive group and 35.4% (28 out of 79) belonged in the RPN2-negative group (Figure 1). Expression of RPN2 protein was localised in the cytoplasm. Although we also examined correlations between RPN2 expression and clinicopathological features, such as patient age and sex, tumour depth, presence of distant metastasis and clinical stage, we found no significant correlations between RPN2 expression and clinicopathological factors (Table 1).

Correlation between RPN2 expression and response to chemotherapy

All three criteria used to evaluate clinical responses to DCF chemotherapy showed significant differences between the RPN2-negative and RPN2-positive groups (Table 2). The RECIST v1.0 criteria gave the RPN2-positive group PR 24, SD 25, PD 2 *vs* the RPN2-negative group CR 4, PR 17, SD 7 ($P=0.006$). The WHO criteria gave the RPN2-postive group CR 1, PR 29, SD 20, PD 1 *vs* the RPN2-negative group CR 8, PR 16, SD 4 ($P<0.001$). The histopathological criteria gave the RPN2-positive group grade 2: 2,

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grade 1: 30, grade 0: 2, *vs* the RPN2-negative group grade 3: 5, grade 2: 4, grade 1: 6 ($P<0.001$).

Response analysis by FDG-PET

We also evaluated responses to DCF chemotherapy by SUV changes in primary oesophageal tumour. Median SUV_{max} reduction rate was 55% in all ESCC patients; decreased SUV was observed in 92.4% (73 out of 79) after DCF treatment. Median SUV_{max} reduction rate was 44% (range: −54.1 to 88.1%) in the RPN2-positive group ($n=51$, Figure 2A) and 68% (range: −18.1 to 88.8%) in the RPN2-negative group ($n=28$, Figure 2B). The SUV_{max} reduction rate significantly differed between the RPN2-negative and RPN2-positive groups ($P=0.004$).

RPN2 silencing increases sensitivity to docetaxel

TE1 and TE14 cells expressed *RPN2* mRNA at high levels as evaluated by real-time RT-PCR. We examined whether *RPN2* suppression altered sensitivity to docetaxel. Expression levels of *RPN2* mRNA and protein were suppressed by *RPN2*-specific siRNA, as confirmed by RT-PCR and western blot analyses (Figure 3A and B). At 48 h after treatment with siRNA and docetaxel, there was substantial cell death induced by RPN2 siRNA compared with control siRNA (Figure 3C). We found that *RPN2* suppression increased docetaxel sensitivity in both ESCC cells lines (Figure 3D).

Table 2 The correlation between RPN2 expression and response to chemotherapy

Therapeutic value	Total ($n=79$)	RPN2 Positive ($n=51$)	RPN2 Negative ($n=28$)	*P*-value
RECIST v1.0				0.006
CR	4	0	4	
PR	41	24	17	
SD	32	25	7	
PD	2	2	0	
WHO criteria				<0.001
CR	9	1	8	
PR	45	29	16	
SD	24	20	4	
PD	1	1	0	
Histopathology				<0.001
Grade 3	5	0	5	
Grade 2	6	2	4	
Grade 1	36	30	6	
Grade 0	2	2	0	

Abbreviations: CR = complete response; PD = progressive disease; PR = partial response; RECIST = Response Evaluation Criteria in Solid Tumors; SD = stable disease; WHO = World Health Organization. Analyzed by Fisher's test.

DISCUSSION

In the present study, we have shown the clinical usefulness of RPN2 expression in endoscopic biopsy samples for predicting sensitivity to docetaxel-based chemotherapy. We also found that *RPN2* suppression increases sensitivity to docetaxel *in vitro*. We evaluated responses to neoadjuvant chemotherapy using various methods, including clinical and pathological responses and decrease in SUV by FDG-PET. All the response evaluators demonstrated the efficacy of RPN2 as a response marker.

Reportedly, RPN2 is a key component in modulating docetaxel sensitivity in tumour cells by the glycosylating P-glycoproteins. Honma *et al* (2008) proposed that RPN2 may serve as a predictor for response to anticancer therapy rather than as a prognostic factor, and would be useful for selecting subjects who are likely to benefit for adjuvant chemotherapy in breast cancer. Furthermore, blocking RPN2 expression or function may induce a CR to chemotherapeutic drugs. The *RPN2* gene may therefore represent a promising new target for RNAi therapeutics against multidrug-resistant tumours (Honma *et al*, 2008). Most patients with ESCC, who present with advanced disease stages, are treated with chemotherapy followed by oesophagectomy, which has become a standard treatment option for patients with ESCC in Japan. We previously reported that a DCF regimen is tolerable as induction therapy (Watanabe *et al*, 2011). However, although substantial progress has been made in the treatment of this tumour, relapse or lack of response due to intrinsic or acquired resistance greatly reduces survival rates. Thus, identification of biomarkers that predict treatment response are needed to improve patient care.

This study has some limitations that warrant consideration. First, the sample size is relatively small. A larger independent series with more patients is needed to validate these results; for this reason, we are continuing to collect endoscopic biopsy specimens from ESCC patients. It is unclear whether RPN2 expression carries prognostic significance for ESCC patients who undergo oesophageal resection after docetaxel-based

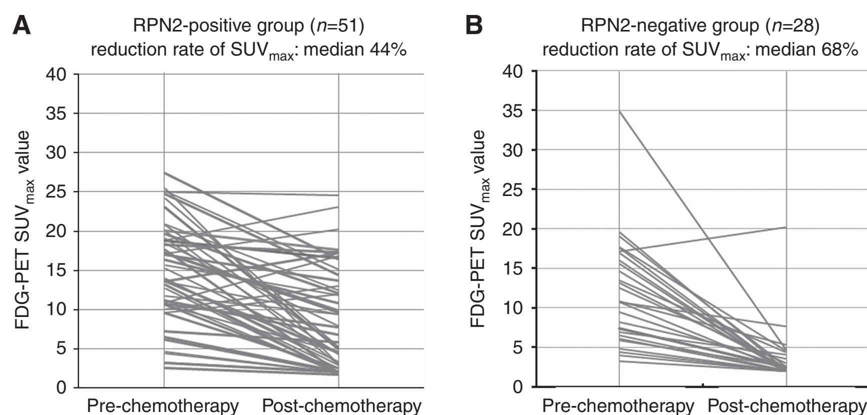

Figure 2 Changes in SUV during neoadjuvant chemotherapy in primary ESCC tumours. (**A**) Median SUV reduction rate was 44% in the RPN2-positive group and (**B**) 68% in the RPN2-negative group. The SUV_{max} reduction rate between the RPN2-negative and RPN2-postive groups was significantly different ($P=0.004$).

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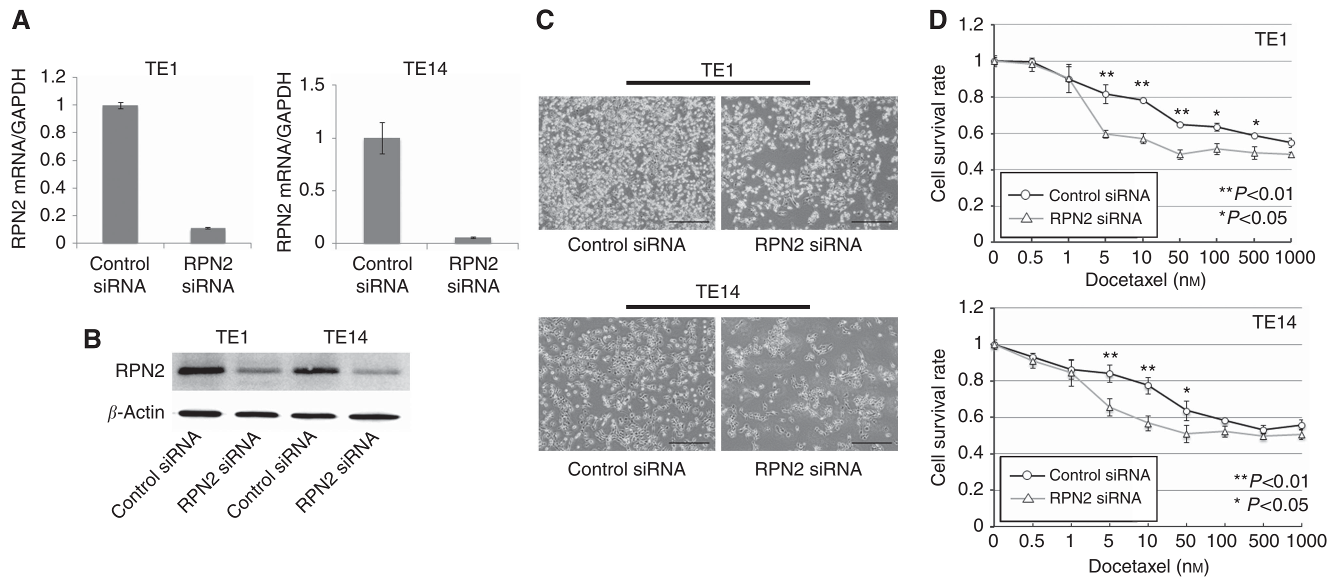

Figure 3 Suppression of *RNP2* by siRNA enhances sensitivity to docetaxel. (**A**) *RPN2* mRNA expression in TE1/14 cells was suppressed by *RPN2* siRNA as confirmed using real-time quantitative PCR. (**B**) RPN2 protein was suppressed by siRNA as confirmed by western blot. (**C**) Phase-contrast micrograph of TE1/14 cells 48 h after treatment with RPN2 siRNAs or control siRNA in the presence of 10 nM docetaxel. Scale bar is 500 μm. (**D**) *RPN2*-suppressed cells were more sensitive to docetaxel than were control cells.

chemotherapy. There is no significant difference in overall survival and disease-free survival between RPN2-positive and RPN2-negative groups currently, because of short follow-up period (data not shown). We are going to present relevant data later, when we have a larger number of samples and longer observed time. Second, as RPN2 induces glycosylation of P-glycoprotein and provokes membrane localisation, our data may indicate sensitivity to other anticancer drugs. However, we had no sufficient number of ESCC patients who received only 5-FU and CDDP regimen, and we could not completely rule out the possibility that RPN2 expression reflects CDDP and 5-FU sensitivity in ESCC cell lines; this too should be tested with a larger sample.

Biopsy under endoscopy is a routine medical examination for gastrointestinal malignancy. Immunohistochemical analysis of biopsy specimens is an easy and safe method of estimating tumour biological characteristics, thus enabling individualised treatment strategies. Ineffective chemotherapy is not only useless, but harmful in the neoadjuvant setting; prediction of chemotherapeutic response, which differ among patients and cancers, is therefore critical.

Previous studies described predictive molecules for therapeutic responses to docetaxel-based neoadjuvant chemotherapy in several cancers. For example, expression of β-tubulin – especially class III β-tubulin – correlated with poor overall survival and reduced response to taxanes, including docetaxel, in patients with advanced non-small-cell lung (Rosell *et al*, 2003), breast (Paradiso *et al*, 2005; Rouzier *et al*, 2005), ovarian (Mozzetti *et al*, 2005; Ohishi *et al*, 2007), gastric cancers (Urano *et al*, 2006), and head and neck squamous carcinoma (Koh *et al*, 2009). MicroRNA-200c regulates class III β-tubulin directly, and thus restores sensitivity to docetaxel in ovarian (Cochrane *et al*, 2010; Leskela *et al*, 2011) and breast cancer (Cochrane *et al*, 2009). CYP3A4 metabolises docetaxel in the liver, and is an important factor in determining docetaxel's efficacy and toxicity. Patients with low CYP3A4 expression showed significantly higher response rates than those with high CYP3A4 expression (Miyoshi *et al*, 2005). These molecules have important implications in docetaxel-induced cell death and can be predictive markers for docetaxel-based chemotherapy. However, no useful predictive markers for docetaxel in ESCC have yet been established. This is the first report that shows the possible use of RPN2 as a predictive marker for docetaxel-based chemotherapy in ESCC.

In conclusion, RPN2 expression in endoscopic biopsy specimens may predict response to docetaxel-based chemotherapy. Although a larger validation study is needed, the findings in this study have important clinical implications for patients receiving neoadjuvant chemotherapy for ESCC.

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