anthracycline anticancer agent and a potent TopoII inhibitor [14]. Amrubicin monotherapy with 45 mg/(m^2 day) for 3 consecutive days by intravenous administration produced response rates of 75.8 and 27.9% for previously untreated patients with ES-SCLC and advanced NSCLC, respectively. A phase II study of the combination of 60 mg/m^2 cisplatin and 40 mg/(m^2 day) amrubicin for 3 days has been reported to show response rate of 87.8%, the MST of 13.6 months and 1-year survival rate of 56.1% against ES-SCLC. Based on this result, Japan Clinical Oncology Group (JCOG) is conducting a randomized phase III study to compare the combinations of cisplatin plus amrubicin and cisplatin plus irinotecan for previously untreated ES-SCLC.

To improve clinical outcomes in advanced lung cancer, clinical integration of molecular biomarkers that predict responses to chemotherapeutic agents may be indispensable [16]. Recently, RanBP2 has been reported to act as a small ubiquitin-like modifier (SUMO) ligase for DNA TopoII and play an important role in targeting TopoII to centromeres during mitosis and in maintaining chromosome stability [5]. Embryonic fibroblasts derived from the engineered mutant mice with low expression of RanBP2 have been reported to show formation of chromatin bridges in anaphase, a distinctive feature of cells with impaired DNA decatenation by chemical inhibition of TopoII [4], suggesting that low expression of RanBP2 may have an analogous effect of TopoII inhibitors. In addition, RanBP2 has a tumor suppressor function since these mutant mice succumbed to a range of cancers, primarily lung carcinomas, and were also susceptible to chemically-induced tumorigenesis. Based on these observations, we hypothesized that RanBP2 expression might be involved in chemosensitivity of a TopoII inhibitor, amrubicin.

The identification of molecular biomarkers with the potential to predict treatment outcomes is essential for individualizing the most beneficial chemotherapy. As one of the multiple approaches to establishing predictive biomarkers, we evaluated whether there would be associations between mRNA expression of the RanBP2 gene as well as the TopoII-alpha and beta genes and chemosensitivity to amrubicin using human lung cancer cell lines.

Materials and methods

Cell lines and drug

Fifteen NSCLC and five SCLC cell lines used were described previously [24]. These cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Amrubicin was kindly provided by Sumitomo pharmaceuticals Company, Osaka, Japan.

Cytotoxicity assay

Cytotoxicity was evaluated using an MTT assay as described previously [11]. Suspensions of exponentially growing cells were dispensed into wells of 96-well tissue-culture plates. After incubation at 37°C for 24 h, the solutions of amrubicin at various concentrations were added, and then incubated for 3 days. The effects of treatment were expressed as percent growth inhibition using untreated cells as the uninhibited control and assessed by IC50 (drug concentrations inducing a 50% reduction of cell survival) which was calculated from dose–response curves.

RNA preparation and RT-PCR amplification

Total RNA was extracted and further purified as described previously [24]. The RNAs were stored at −80°C until use. Total RNA (50 ng) extracted from each cell line was subjected to one-step real-time reverse transcriptase-PCR (RT-PCR) for absolute quantitating mRNA levels of the RanBP2, TopoII-alpha, TopoII-beta and beta-actin genes as described previously. The PCR primers used were as follows.

RanBP2-S:	5′-CAATGGAAATGGGGAAGACTTT-3′
-AS:	5′-CATCACTTCAGTCCCACCTGTA-3′
TopoII-alpha-S:	5′-GGTGTGGAACTAGAAGGCCTAA-3′
-AS:	5′-TGAATCAGACCAGGGATTTCTC-3′
TopoII-beta-S:	5′-TTTTTCACCATCATTTGGTCTG-3′
-AS:	5′-GGGCTTAGGGACTGTATCTGAA-3′
Beta actin-S:	5′-TTCTACAATGAGCTGCGTGTG-3′
-AS:	5′-CAGCCTGGATAGCAACGTACA-3′

Linear regression analysis of standard-curves demonstrated a strong correlation for all the genes ($R^2 > 0.99$). The relative gene expression levels were normalized with a house keeping gene, beta-actin.

Western blot analysis

Western blot analysis was done as described previously [11], using the following primary antibodies: anti-RanBP2 (ab2938, Abcam), anti-TopoII-alpha (ab45175, Abcam), anti-TopoII-beta (ab58442, Abcam) and anti-actin (A2268, Sigma-Aldrich) antibodies.

Statistical analyses

The strength of the association between the expression levels of RanBP2, TopoII-alpha and TopoII-beta and chemosensitivity data was calculated by either Pearson's correlation coefficient or linear regression analysis. Correlations were considered significant at $P < 0.05$. For comparison of IC50 values of amrubicin and each gene expression level among histological subtypes, we employed one-way

analysis of variance (ANOVA) followed by Bonferroni post-test. All analysis was performed with the use of Stat View software version 5.0.

Results

Chemosensitivity of amrubicin was examined using 20 human lung cancer cell lines including 15 NSCLC cells and 5 SCLC cells. Cytotoxicity following a 72 h continuous exposure of amrubicin was measured by MTT assay. The IC 50 value of amrubicin in SK-LC-3 was about 9 μM, while the IC 50 values in the other cell lines were less than 1 μM as shown in Table 1. There was no significant difference between histological types (Fig. 1).

The mRNA quantifications of the RanBP2, TopoII-alpha, TopoII-beta genes were carried out in real-time PCR and the expression levels were normalized with beta-actin as an internal control (Table 1). Among 20 cell lines tested, the level of RanBP2 mRNA expression in an H460 cell line was about 20-fold lower than those in non-tumorous lung tissues obtained from two patients with lung cancer. There were statistically significant differences in the RanBP2 expression between SCLC and the other histological subtypes ($p < 0.05$) (Fig. 2a). We checked RanBP2 protein expression in two lung cancer cell lines, SK-LC-2 and H460, representing high and low expression of the RanBP2 gene, and found similar mRNA and protein expression patterns (Fig. 2d). We also found statistically higher expression levels of TopoII-alpha in SCLC and adenocarcinoma cell lines compared with those in normal lung tissues,

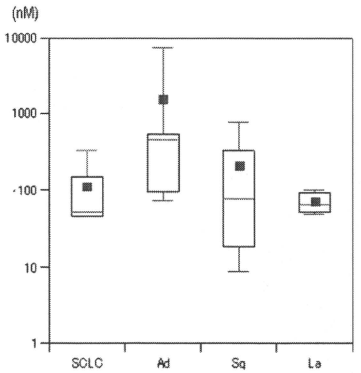

Fig. 1 IC50 values of Amrubicin in lung cancer cell lines. *Box plots* show relationships between IC50 values of Amrubicin and the four histological subtypes of lung cancer. The *horizontal line* within each *box* represents the median value and the closed box shows the mean value, respectively

Table 1 IC50 values for amrubicin and relative mRNA expression for RanBP2, TopoII alpha and TopoII beta in lung cancer cell lines

Cell line	Histology	Amrubicin (μM)	RanBP2	TopoIIa	TopoIIb
ACC-LC-94	Ad	0.0668	0.621	1.324	1.174
ACC-LC-319	Ad	0.579	1.108	1.682	1.437
SK-LC-3	Ad	8.99	2.634	2.009	1.899
A549	Ad	0.131	1.191	1.553	1.665
SK-LU-1	Ad	0.492	1.307	2.930	1.479
VMRC-LCD	Ad	0.0835	4.134	2.660	2.942
RERF-LC-MT	Ad	0.469	0.661	0.8719	0.753
Calu1	Sq	0.203	1.280	2.173	1.750
SK-MES-1	Sq	0.0768	1.160	0.883	0.807
PC-1	Sq	0.009	1.937	1.739	2.888
RERF-LC-A	Sq	0.0222	0.717	1.454	1.036
PC-10	Sq	0.77	0.713	1.049	1.170
NCI-H460	La	0.101	0.043	1.518	2.098
Calu6	La	0.0469	1.467	1.116	1.828
SK-LC-6	La	0.0632	2.362	2.508	4.383
ACC-LC-48	SCLC	0.0512	1.957	1.672	2.044
ACC-LC-49	SCLC	0.0866	2.592	1.975	3.523
ACC-LC-80	SCLC	0.0459	3.953	1.361	1.993
ACC-LC-172	SCLC	0.0439	2.387	4.450	4.207
SK-LC-2	SCLC	0.337	3.662	3.510	4.264
NL 1	Normal lung	NA	1.006	0.158	2.447
NL 2	Normal lung	NA	0.913	0.179	1.937

NL 1 and NL 2: non-tumorous lung tissues obtained from two patients with lung cancer.
Ad adenocarcinoma, *La* large cell carcinoma, *SCLC* small cell lung cancer, *Sq* squamous cell carcinoma.
NA not available

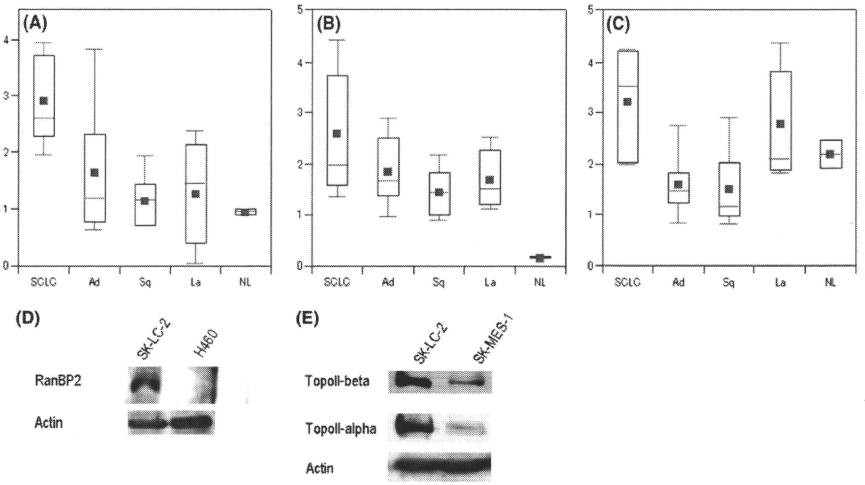

Fig. 2 Relative mRNA expression for (**a**) RanBP2, (**b**) TopoII-alpha and (**c**) TopoII-beta among histological subtypes and normal lung tissues, and protein expression for (**d**) RanBP2 and (**e**) TopoII isoforms in representative cell lines. **a** RanBP2 mRNA expression in SCLC was higher than those in the other histological subtypes of lung cancer. **b** TopoII-alpha mRNA expression levels in lung cancer cell lines were relatively higher compared to those in normal lung tissues. **c** The expression levels of TopoII-beta in lung cancer cell lines were similar to those in normal lung tissues. **d, e** Western blot analyses for RanBP2 and TopoII isoforms in two lung cancer cell lines representing high and low expression, respectively. The expression patterns of protein and mRNA were not different

although there were no significant differences in TopoII-alpha mRNA expression levels among four histological subtypes of lung cancer (Fig. 2b). On the other hand, the expression levels of TopoII-beta in lung cancer cell lines were similar to those of normal lung tissues, although relatively higher expression levels were observed in SCLC and large cell carcinoma (Fig. 2c). In addition, we checked TopoII-alpha and TopoII-beta protein expressions in two lung cancer cell lines, SK-LC-2 and SK-MES-1, representing high and low expression of the two TopoII isoforms, and found that protein expression patterns of these genes were not different with mRNA expression patterns (Fig. 2e).

There were weak but significant positive correlations between RanBP2 and TopoII-alpha mRNA expressions, between RanBP2 and TopoII-beta mRNA expressions and between TopoII-alpha and TopoII-beta mRNA expressions among 20 lung cancer cell lines ($r = 0.532$; $P < 0.05$, Fig. 3a and $r = 0.623$; $P < 0.05$, Fig. 3b, $r = 0.647$; $P < 0.01$, Fig. 3c, respectively). Chemosensitivity data were analyzed in relation to the mRNA expression levels of the RanBP2, TopoII-alpha, TopoII-beta genes using linear regression analysis. No significant associations were observed between the IC50 values of amrubicin and the mRNA expression levels of RanBP2 (Fig. 4a), TopoII-alpha (Fig. 4b) and TopoII-beta (Fig. 4c) among 20 cell lines.

Discussion

RanBP2 has been reported to be involved in both nucleocytoplasmic transport and mitosis and also act as a SUMO ligase for DNA TopoII and play a role in maintaining chromosome stability by recruiting TopoII to centromeres during mitosis [5]. In addition, RanBP2 hypomorphic mice are particularly sensitive to spontaneous and carcinogen-induced lung tumors, indicating that RanBP2 might play a potential tumor suppressor role in human lung cancer. Two previous studies reported that RanBP2 mRNA expression levels are substantially reduced in human non-SCLC [2, 8]. However, the present study showed that RanBP2 transcript levels were infrequently downregulated in human lung cancer cell lines compared with normal lung tissues, although there were statistically significant differences in the RanBP2 expression between SCLC and NSCLC. Consistent with our results, several lines of evidence from publicly available human gene expression data of the Oncomine

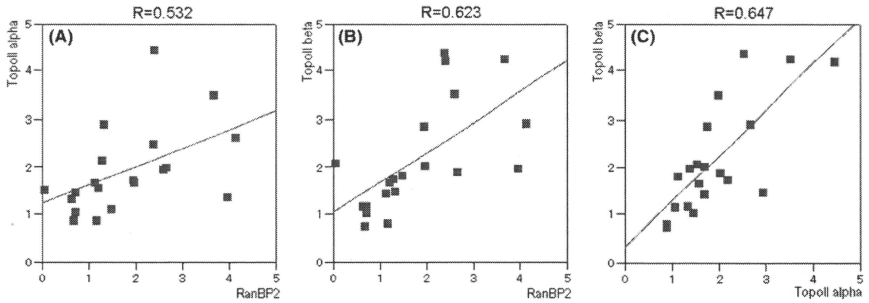

Fig. 3 Correlations between **a** RanBP2 and TopoII alpha mRNA expression, **b** RanBP2 and TopoII beta mRNA expression and **c** TopoII alpha and TopoII beta mRNA expression in lung cancer cell lines

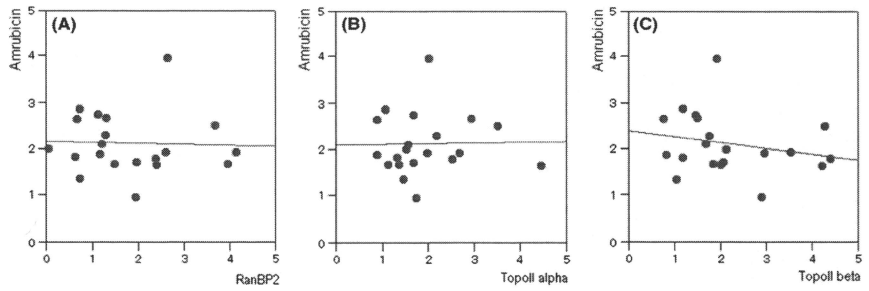

Fig. 4 Associations between relative mRNA expression for (**a**) RanBP2, (**b**) TopoII alpha and (**c**) TopoII beta and chemosensitivity of Amrubicin Log(IC50 in nM)

database (http://www.oncomine.com) and GEO profiles (http://www.ncbi.nlm.nih.gov/geo/) reported that RanBP2 mRNA expression levels are not reduced in NSCLC compared with normal lung tissues [3, 22, 26–28, 30]. In addition, there is a microarray study showing that RanBP2 expression levels are similar to those of our data in four overlapping lung cancer cell lines [9]. The concordance and discordance between our findings and previous works might be caused by the difference between cell lines and resected human lung tumors as well as the different experimental conditions used. Thus, further studies are warranted to establish the role of RanBP2 as a tumor suppressor gene in human lung carcinogenesis.

In RanBP2 hypomorphic murine embryonic fibroblasts (MEFs), formation of chromatin bridges in anaphase, a distinctive feature of cells with impaired DNA decatenation by mutation or chemical inhibition of TopoII-alpha [4], was observed, while spindle structure, kinetochore–microtubule interactions, and localization of kinetochore and spindle assembly checkpoint proteins appeared normal [5]. Therefore, the low expression of RanBP2 may have an analogous effect of TopoII inhibitors, although the inhibitors are able to cause an inevitable consequence of DNA damage at high doses [4, 21]. Then, we speculated that there might be an association between RanBP2 mRNA expression and chemosensitivity of a TopoII inhibitor, amrubicin and tested whether we could see it using human lung cancer cell lines. However, we did not find any associations, suggesting that cytotoxicity of amrubicin might come mainly from DNA damage response induced at high doses and that formation of chromatin bridges in anaphase caused by low expression of the RanBP2 gene might not have additional effects on amrubicin-induced DNA damage response.

The two isozymes, TopoII-alpha and TopoII-beta function to unknot and decatenate covalently closed circles of DNA, although functional differences of these isozymes

and their differential spliced variants as well as precise role of their homodimerization and heterodimerization are unknown [20, 21]. There are several lines of evidence indicating a close relationship between TopoII-alpha levels and drug sensitivity in cell lines made resistant to TopoII inhibitors [7, 17, 25], cell lines with reduced expression of TopoII [1] and a VP-16-resistant breast cancer cell line infected with adenovirus containing TopoII-alpha [32]. Another study has shown the relationship between TopoII expression and multidrug sensitivity including TopoII inhibitors using eight human lung cancer cell lines [10]. There is also some evidence that TopoII-beta may be related with resistance to TopoII inhibitors [6, 15]. However, we did not find any association between expression levels of TopoII isoforms and chemosensitivity of amrubicin. Consistent with our results, a previous report of unselected human lung cancer cell lines also showed no clear association between TopoII-alpha protein expression and in vitro sensitivity to TopoII inhibitors [31]. Another study also failed to show importance of the enzyme using a panel of cell lines [12]. Although the behavior of cell lines in vitro may differ from the in vivo situation, and depend on the experimental conditions, these contradictory findings may require further investigation.

Amrubicin is highly active and one of the most potent anticancer drugs against SCLC and NSCLC [14]. Among the toxicities, hematologic adverse events such as leukopenia and thrombocytopenia are frequent and dose-limiting factors. Although identification of molecular biomarkers with the potential to predict treatment outcomes is essential to eliminate the use of any ineffective agents and to avoid toxic side effects [16], the cellular response to amrubicin is still poorly understood. To predict drug response in lung cancer patients, integrated analyses such as array-based mRNA expression profile, epigenome profiles, proteome analysis would be needed.

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