Association of ATP7A expression and *in vitro* sensitivity to cisplatin in non-small cell lung cancer

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Abstract. Expression of copper-transporting P-type adenosine triphosphatase A (ATP7A) is reportedly associated with platinum drug resistance in various types of solid tumors. However, the impact of ATP7A expression on platinum drug resistance in non-small cell lung cancer (NSCLC) has yet to be adequately elucidated. *In vitro* cisplatin (CDDP) sensitivity was investigated using the collagen gel-droplet embedded culture drug sensitivity test, and the ATP7A mRNA expression levels were assessed by real-time polymerase chain reaction in surgically resected specimens of NSCLC. The relationship between the ATP7A expression levels and the *in vitro* CDDP sensitivity was then evaluated. The ATP7A mRNA expression levels in the CDDP-resistant tumors were significantly higher than those in the CDDP-sensitive tumors (p=0.0167, Mann-Whitney U test). In conclusion, the results suggest that evaluation of ATP7A expression is useful as a marker for cisplatin chemoresistance.

Introduction

Cisplatin (CDDP), an anticancer drug containing platinum, is widely used in the treatment of solid tumors, such as testicular, ovarian, cervical, bladder, head and neck, and non-small cell lung cancers (NSCLC) (1). CDDP has been used as a key drug for chemotherapy against NSCLC for more than 20 years.

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Abbreviations: NSCLC, non-small cell lung cancer; CDDP, cisplatin; CD-DST, collagen gel-droplet embedded culture drug sensitivity test; PCR, polymerase chain reaction; GIR, growth inhibition rate

Key words: non-small cell lung cancer, cisplatin, drug resistance

However, the overall response rate to cisplatin used as a single agent is reported to be no more than 20% in patients with NSCLC (2). Furthermore, development of resistance to CDDP is common during treatment of NSCLC and is therefore a significant factor to be considered by clinical oncologists. Subsequently, exploration of a chemoresistance marker is crucial. The mechanisms underlying the development of CDDP resistance include decreased drug accumulation, enhanced detoxification and increased DNA repair efficiency. However, previous studies have been limited mainly to the *in vitro* or *in vivo* level. Additionally, no study has shown factors that have been validated as common contributors to clinical CDDP resistance and prognosis in NSCLC patients (1).

Copper-transporting P-type adenosine triphosphatase A (ATP7A) plays a significant role in copper distribution within cells. ATP7A is expressed in the intestinal epithelium as well as in most other tissues apart from the liver (3-7). Binding of Cu to ATP7A triggers highly regulated subcellular relocalization that involves movement from the basal position in the trans-Golgi network to the plasma membrane (3,8). Defective function of ATP7A causes Menkes disease, and the pathology of Menkes disease reflects the inadequate mobilization of Cu from a number of tissues. Previous studies suggested that the copper export system also functions as an efflux transporter for platinum drugs. These studies also showed an association between ATP7A expression and resistance to platinum drugs in malignancies (9-11). However, the ATP7A expression level and its impact on CDDP resistance in NSCLC has yet to be adequately elucidated.

The present study aimed to investigate the predictive value of ATP7A gene expression for the *in vitro* chemosensitivity of NSCLC to CDDP using surgically resected specimens from patients with NSCLC.

Materials and methods

Study population. Eligible patients included those with a histologic diagnosis of NSCLC, and who had undergone

Table I. Relationship between *in vitro* CDDP sensitivity and patient characteristics.

Characteristics	In vitro CDDP sensitivity[a]		P-value[b]
	R	S	
Age (mean ± SD, years)	69±7	68±8	0.60
Gender			
Male	7	9	0.30
Female	4	1	
Histology			
Adenocarcinoma	7	2	0.08
Non-adenocarcinoma[c]	4	8	

[a]*In vitro* CDDP sensitivity: R, resistant and S, sensitive. [b]P-value evaluated by the Mann-Whitney U test or Fisher's exact test as appropriate. [c]Non-adenocarcinoma (i.e., squamous cell carcinoma, large cell carcinoma and poorly differentiated NSCLC).
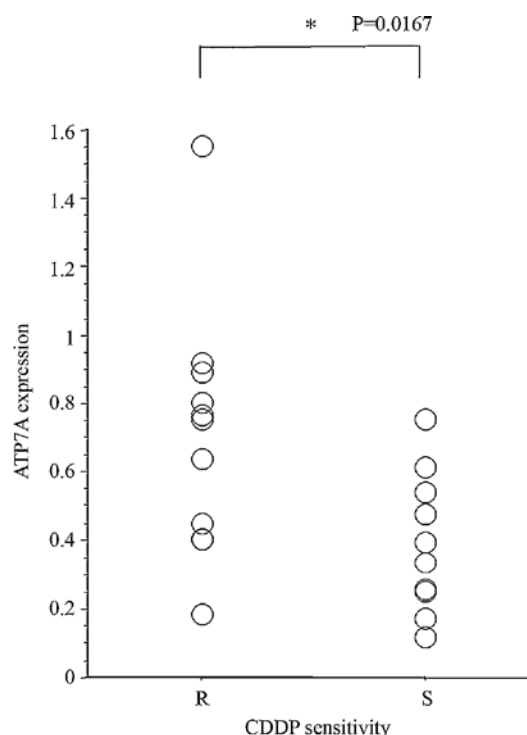
Table II. Relationship between the ATP7A mRNA expression level and patient characteristics.

Characteristics	ATP7A gene expression level (mean ± SD)	P-value[a]
Age		
>70	0.61±0.38	0.90
≤70	0.54±0.30	
Gender		
Male	0.87±0.42	0.04
Female	0.46±0.25	
Histology		
Adenocarcinoma	0.74±0.38	0.03
Non-adenocarcinoma[b]	0.42±0.22	

[a]P-value evaluated by Mann-Whitney U test. [b]Non-adenocarcinoma (i.e., squamous cell carcinoma, large cell carcinoma and poorly differentiated NSCLC).

Figure 1. ATP7A mRNA expression level and *in vitro* cisplatin sensitivity.

surgical resection but had received no previous chemotherapy or radiotherapy. Patient specimens were subjected to a chemosensitivity test between 2005 and 2007 at the Teikyo University Hospital and Tokai University Hospital. Eligible patients who gave informed consent were recruited for the study. The patients consisted of 16 males and 5 females, ranging in age from 54 to 79 years (mean 68). A total of 9 tumors were confirmed to be adenocarcinomas, 8 were squamous cell carcinomas, 3 were large-cell carcinomas and 1 was confirmed to be poorly differentiated NSCLC.

In vitro chemosensitivity test. *In vitro* chemosensitivity was examined using the collagen gel-droplet embedded culture drug sensitivity test (CD-DST) method, as described by Kobayashi *et al* (12,13) and Kawamura *et al* (14), with minor modifications. Briefly, surgically resected specimens were finely minced using a scalpel and digested in cell dispersion enzyme solution EZ (Nitta Gelatin Inc.). The dispersed cancer cells were then washed and filtered through a nylon mesh with a pore size of 200 mm, collected by centrifugation, suspended in PCM-1 medium (Nitta Gelatin Inc.) and incubated in a CO_2 incubator at 37°C for 24 h. Viable cells were collected and re-suspended in reconstituted type I collagen solution with a final cell density of $1x10^5$ cells/ml. Three drops of the collagen-cell mixture (30 ml/drop) were placed in each well of a 6-well multiplate and allowed to gel overnight at 37°C. Cisplatin (CDDP) (Bristol-Myers Squibb Inc.) was then added at a final concentration of 2 mg/ml, and the plates were incubated for 24 h. Following removal of the medium containing the anticancer drugs, each well was rinsed twice, overlaid with PCM-2 medium (Nitta Gelatin Inc.) and incubated for 7 days. At the end of the incubation period, the colonies were stained with neutral red (50 mg/ml, 3 h). Each collagen droplet was then fixed with 10% neutral formalin buffer, washed in water, air-dried and quantified using imaging analysis. The *in vitro* sensitivity was expressed as the growth inhibition rate (GIR): (1 - T/C) x 100 (%), where T is the total volume of the treated group and C is the total volume of the control group.

In this study, a tumor was judged as 'resistant' when the GIR was <61% and 'sensitive' when the GIR was ≥61%.

Cell line. The human colorectal cancer HCT8 cell line was obtained from Riken (Saitama, Japan). The cell line was cultured at 37°C in Dulbecco's modified Eagle's medium (containing 10% bovine serum) in a 5% CO_2 atmosphere.

Quantitative evaluation of ATP7A mRNA expression. Total RNA was extracted from the tumor samples and the cell line using the acid guanidinium thiocyanate-phenol-chloroform extraction method. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA as previously described (15-17).

Real-time PCR assays were run on an ABI PRISM 7000 Sequence Detection system (Perkin-Elmer Applied Biosystems) in accordance with the manufacturer's instructions and those of previously published studies (15-17). Briefly, a total volume of 50 μl of a reaction mixture containing 1 μl of the cDNA template, 25 μl of TaqMan Universal PCR Master Mix (Perkin-Elmer Applied Biosystems) and 2.5 μl of a primer probe mixture for ATP7A and β-actin was amplified using the following protocol: after initial denaturation (2 min at 95°C), amplification was performed for 50 cycles of 15 sec at 95°C and 60 sec at 60°C. The primer probe mixture for ATP7A (TaqMan® Gene Expression Assays, assay ID: Hs00163707_m1) and β-actin (human ACTB, 4310881E) (both from Perkin-Elmer Applied Biosystems) were purchased as part of the commercial provider's kit.

To precisely quantify the ATP7A gene transcripts, β-actin transcripts were used as a quantitative control, and each sample was normalized based on its β-actin transcript content. Standard curves for ATP7A and β-actin mRNA were generated using serially diluted solutions (1/5, 1/25, 1/125 and 1/625) of HCT8 cDNA. After determination of the threshold cycle (Ct) which was defined as the PCR cycle number at which point the fluorescent intensity exceeded the threshold, the target gene expression level was calculated using the standard curve, and the quantitative normalization of cDNA in each sample was performed using the expression of the β-actin gene as an internal control. Finally, the ATP7A mRNA levels were represented as a ratio to the β-actin mRNA levels. Real-time PCR assays were conducted in duplicate on one dish for each sample, and the mean value was used to calculate the mRNA expression levels.

Statistical analysis. Categorical variables were analyzed using the Fisher's exact test. Continuous variables were compared using the Mann-Whitney U test. All reported P-values were two-sided. P<0.05 was considered to be statistically significant.

Results

In vitro chemosensitivity test. The GIR values ranged from 0 to 68.6% (mean ± standard deviation, 38.0±18.4%). According to the protocol described in Materials and methods, 11 tumors were judged as 'resistant (R)' to CDDP, while the remaining 10 tumors were judged as being 'sensitive (S)' to CDDP.

No significant correlation was observed between the *in vitro* CDDP sensitivity and any of the clinical patient characteristics, including age, gender and histology (Table I).

ATP7A mRNA expression. The ATP7A mRNA expression levels varied from 0.12 to 1.55 (0.56±0.33). Table II shows the relationship between the ATP7A expression levels and the clinical patient characteristics, including age, gender and histology. No significant correlation was observed between the ATP7A mRNA expression level and age (p=0.90, Mann-Whitney U test).

The ATP7A mRNA expression level in the male group was significantly higher than that in the female group (p=0.04, Mann-Whitney U test), and the ATP7A mRNA expression level in the adenocarcinoma group was significantly higher than that in the non-adenocarcinoma group (p=0.03, Mann-Whitney U test).

Relationship between ATP7A expression and CDDP sensitivity. The difference in the ATP7A mRNA expression level between the CDDP-resistant (R group, n=11) and CDDP-sensitive tumors (S group, n=10) was evaluated. The ATP7A mRNA expression levels in the R group were significantly higher than those in the S group (p=0.017, Mann-Whitney U test; Fig. 1).

Discussion

ATP7A expression was reported to be associated with the sensitivity of ovarian carcinoma cells to platinum drugs (9,10). Overexpression of ATP7A in ovarian carcinoma cells has been shown to be associated with higher levels of platinum drug accumulation, Additionally, ATP7A-overexpressing cells exhibit a significantly enhanced degree of resistance to platinum drugs (10). Analysis of the expression of ATP7A in ovarian carcinoma patients before and after treatment with a platinum drug-containing regimen demonstrated that in certain patients, treatment caused an enrichment of ATP7A-expressing cells in the tumors, resulting in a poor patient outcome (9). However, the ATP7A expression levels and their clinical significance in NSCLC have yet to be adequately elucidated.

The present study examined the association between the ATP7A mRNA expression levels and *in vitro* chemosensitivity to CDDP, using surgically resected specimens of NSCLC. The results revealed significantly higher expression levels of ATP7A mRNA in the CDDP-resistant tumors than in the CDDP-sensitive tumors.

Little is known regarding the differences in the ATP7A expression levels according to gender and histological subtype in NSCLC patients. In this study, significant correlations between the ATP7A expression levels and gender and histological subtypes were observed. Further study is needed to confirm and clarify the clinicopathological significance of this observation.

In conclusion, although the data presented in this study were based only on an *in vitro* assay, our results suggest that the ATP7A gene expression level is correlated with resistance to CDDP in NSCLC and that the ATP7A expression level may be a useful marker of chemoresistance to cisplatin.

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