ORIGINAL RESEARCH

β -Tubulin III mRNA expression and docetaxel sensitivity in non-small cell lung cancer

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Abstract

Purpose: Despite the success of docetaxel as an anti-tumour agent, the inter-individual variability in drug response still poses a major impediment to further use of this agent in the treatment of cancer. Current knowledge about predictive biomarkers of docetaxel sensitivity in malignant effusions is poor. The aim of this study was to investigate the association between β -tubulin III mRNA expression and chemosensitivity to docetaxel in metastatic malignant effusions.

Methods: Real-time quantitative PCR was used for analysis of β -tubulin III mRNA expression in 37 malignant effusions collected prospectively. Viable tumour cells obtained from malignant effusions were tested for sensitivity to docetaxel using ATP-TCA assay.

Results: β -tubulin III expression was inversely correlated with sensitivity to docetaxel in pleural effusions of NSCLC patients ($P=0.022$). The lower level of β -tubulin III mRNA expression in malignant effusions was associated with higher chemosensitivity to docetaxel in NSCLC patients *in vitro*. No correlation was found between β -tubulin III mRNA expression and docetaxel sensitivity in malignant effusions of gastric cancer patient.

Conclusion: Our results demonstrated that β -tubulin III mRNA expression level in malignant effusions, in which all cancer cells were metastatic, was correlated with docetaxel sensitivity in NSCLC. This highlights the potential

role of biomarkers in malignant effusions in further customized chemotherapy.

Docetaxel, a semisynthetic taxane, has shown great activity against gastric cancer as well as non-small-cell lung cancer (NSCLC) both as mono-therapy and in combination with other drugs.¹⁻⁴ However, despite the success of docetaxel as an anti-tumour agent, the inter-individual variability in drug response still poses a major impediment on the further use of it in the treatment of cancer. As some patients have inherent or acquired-resistance to this drug, biomarkers are desired to stratify patients into groups of different likelihood of the tumour response before chemotherapy, thus maximizing effects and minimizing toxicities of chemotherapy.

Previous studies have shown that β -tubulin III could be a potential biomarker for taxane resistance in lung⁵⁻⁸, breast^{9, 10}, ovarian¹¹, head and neck carcinoma¹² and in unknown primary carcinomas.¹³ However, in those studies, researchers usually used primary tumour tissues to investigate β -tubulin III

expression and correlate them with clinical response. In the setting of patients with advanced tumours that are unresectable, the unavailability of appropriate tumour tissue for gene expression analysis has become one of the handicaps in the field of customized chemotherapy. The quest to find alternative methods must continue.

Because the sampling of malignant effusions is easy, non-invasive and repeatable, it has been considered an appropriate specimen for acquiring the biological information of cancer.^{14, 15} Few studies have correlated gene mutation or expression in malignant effusions with clinical responses of cancer patients or chemosensitivity to certain drugs.¹⁶⁻¹⁸ The results were in parallel to or contradictory to those based on primary tumour tissues.^{17, 18} Considering the biological heterogeneity between primary and metastatic tumours¹⁹, these differing results demonstrate the urgency to clarify whether β -tubulin III is correlated with docetaxel sensitivity in metastatic effusions before we could further apply this biomarker in clinic.

Thus, we characterized β -tubulin III mRNA expression in metastatic malignant effusions and evaluated the potential value in predicting the chemosensitivity to docetaxel in advanced NSCLC and gastric cancer patients.

Materials and Methods

The protocols for this study were approved by the Human Research Protective Committee of our hospital and informed consents was obtained from all patients.

All specimens and relevant clinical data were obtained from the departments of oncology, respiratory and digestion, Drum Tower Hospital, Nanjing, China, from June 2005 to Dec 2006. Malignant effusions were obtained from 37 patients who had histological or cytological diagnoses of stage IV malignant disease. All samples were collected before the patients received further treatment such as chemotherapy. Clinical characteristics of the patients are summarized

TABLE 1. Demographic and clinical parameters of patients (n=37)

Characteristics	Patients	
	n	%
Age, years		
Median		65
Range		39-87
Sex		
Female	11	30
Male	26	70
ECOG performance status 0-2	37	100
Primary Tumour		
NSCLC	20	54
Gastric	17	46
Sample type		
Pleural effusions	20	54
Peritoneal effusions	17	46

Abbreviation: ECOG, Eastern Cooperative Oncology Group.

in Table 1. In all specimens included in this study, cancer cells comprised at least 50% of the entire cells population based on cytology smears performed by the experienced pathologist.

Sample collection and processing

All patients received an indwelling 16 G single lumen central venous catheter (CVC) (Arrow™ 16 G CVC set Catheter, Arrow, USA) for draining fluid and the further treatment. Effusions were collected in sterile drainage bags with heparin (10,000 U / L effusions), submitted to our laboratory within minutes and processed immediately. Samples were centrifuged at 1000g for 10 min to collect cells. After Ficoll-Hypaque (specific gravity 1.077, Pharmacia) density centrifugation, the interface was collected. The quality and viability of the cell suspension were assessed by trypan blue dye exclusion and cytological examination. Cells were counted, washed and prepared for RNA extraction and ATP-TCA assay.

RNA isolation and cDNA synthesis

Total cell RNA was extracted using Trizol Reagent (Invitrogen, CA, USA) following the manufacture's protocol. cDNA was generated using Exscript™ RT

reagent Kit (TaKaRa) according to manufacture's protocol.

Real time PCR quantification

Quantification of the genes of interest and an internal reference gene (β -actin) was done using a fluorescence based real-time detection method (Mx3000P Real Time PCR System, Stratagene). Briefly, 2 μ l cDNA were used for each RT reaction. The 20 μ l PCR reaction mixture contained 1 $\times$ primers & probe mixture (Applied Biosystems, Foster city, CA. Assay IDs: Hs99999903_1 (β -actin); Hs00964965_m1 (β -tubulin)), 1 $\times$ Absolute QPCR Mix (ABgene, Surrey, UK). The PCR conditions were 95°C for 45 s, followed by 50 cycles at 95°C for 15 s and 60°C for 30 s. Each sample was assayed in triplicate with commercial RNA as positive and RNase-free water as negative control.

Relative gene expression quantifications were calculated according to the comparative Ct method using β -actin as an endogenous control and commercial RNA (Clontech) control as calibrators on each plate. Final results were determined by the formula $2^{-\Delta\Delta C_t}$ [20]

and were analyzed with the Stratagene analysis software.

Evaluation of sensitivity to docetaxel using ATP-TCA

The chemosensitivity test was performed with primary tumour cells from malignant effusions as described before [21]. Briefly, separated cells were resuspended in serum-free medium (CAM; DCS, Innovative Diagnostic Systeme, Hamberg, Germany). The cell suspension was given into 96-well polypropylene microplates (Corning-Costar, Cat No. 3790, 2×10^4 per well) with or without docetaxel at six doubling dilutions in triplicate (0.288, 0.575, 1.15, 2.3, 4.6, and 9.2×10^{-3} g/L). Plates were incubated at 37 °C and 95% humidity in 5% CO₂ atmosphere. After 6 d incubation, intracellular ATP was extracted using CellTiter-Glo Luminescent Kit (Promega, USA) and measured by microplate luminometer (Berthod Diagnostic systeme, Germany). Cell suspensions incubated without anti-cancer drugs were used as reference for 100% tumour cell viability.

Statistics

To investigate the association between the relative expression of a given gene and sensitivity to certain cytotoxic drug tested by the ATP-TCA, statistical evaluation was performed using the Spearman correlation coefficient. The Mann-Whitney U-test was used to test significant associations between the continuous variable 'gene expression' and dichotomous variables (patient's age, sex). All values were based on two tailed statistical analysis. *P* value < 0.05 was considered statistically significant. Statistical analyses were done using SPSS 13.0 (SPSS Inc, Chicago, Illinois, USA)

Results

Real-time quantitative PCR of β -tubulin III

β -tubulin III mRNA expression was detectable by quantitative RT-PCR in all samples. The median β -tubulin III mRNA expression was 0.018 (range 0.001 -

FIGURE 1. Relative β -tubulin III mRNA expression levels in malignant effusions of NSCLC and gastric cancer patients were quantitatively characterized by RT-PCR. The solid lines represent the median for each group (n=20 for lung cancers, n=17 for gastric cancers).

0.366). No correlations between age, sex and β -tubulin III expression were found. β -tubulin III mRNA expression of individual samples in different subsets is shown in Fig 1.

ATP-TCA

ATP-TCA assays were successfully performed on all samples. A sensitivity index ranging from 0 to 600 for each drug was calculated by summing up the percentage of cell viability at the six drug concentrations tested²². Thus, a sensitivity index of 0 indicates maximal drug sensitivity, whereas a sensitivity index of 600 reflects minimal drug sensitivity. Fig. 2 shows the distribution of chemosensitivity to docetaxel in NSCLC and gastric cancer patients. Representative examples of a relatively sensitive and a relatively resistant tumour in NSCLC and gastric cancer group were shown in Figure 3 respectively.

β -tubulin III mRNA expression and docetaxel chemosensitivity

There was an inverse correlation between β -tubulin III mRNA expression and sensitivity to docetaxel in NSCLC patients ($P = 0.022$, $r = 0.510$, $r^2 = 0.260$). However, in gastric cancer patients, there was no association between β -tubulin III mRNA expression and docetaxel chemosensitivity in malignant ascites

Discussion

We found that class III β -tubulin mRNA expression levels were inversely correlated with sensitivity to docetaxel in pleural effusions of NSCLC patients. Tumour cells with lower β -tubulin III expression in effusions were more sensitive to docetaxel than those with higher levels. This is the first study to explore the correlation between β -tubulin III mRNA expression levels and chemosensitivity to docetaxel in malignant pleural and peritoneal effusions of NSCLC and gastric cancer patients.

FIGURE 2. Frequency histograms showing heterogeneity of the sensitivity index for docetaxel in NSCLC (a, n=20) and gastric cancer patients (b, n=17)

Our findings are consistent with previous reports, which have shown that higher β -tubulin III expression was correlated with resistant to taxane-based chemotherapy in preclinical and clinical studies.⁵⁻¹³ Gan et al²³ demonstrated that the silenced expression of β -tubulin III by small interfering RNA increased the sensitivity to paclitaxel in NSCLC cell lines. A clinical study by Tommasi et al¹⁰ in 92 advanced breast cancer patients treated with first line paclitaxel based chemotherapy revealed that high class III β -tubulin expression was correlated with progression of the disease. Potential mechanisms responsible for the association between β -tubulin III

FIGURE 3. Representative results of the in vitro chemosensitivity assay. Tumour L6 is relatively sensitive (Index SUM 206) in contrast to tumour L13 (Index SUM 566) in NSCLC group (a), while tumour G17 is relatively sensitive (Index SUM 382) in contrast to tumor G16 (Index SUM 600) in gastric cancer group (b).

expression and taxanes resistant remain to be defined. The possible reason may be that the overexpression of β -tubulin III reduces the ability of taxanes to suppress microtubule dynamics thus inducing paclitaxel resistance.²⁴ However, in gastric cancer patients, we failed to find the same correlation. There is only one clinical study, based on immunohistochemical method, that demonstrated the association between β -tubulin III and clinical response to docetaxel-based chemotherapy in 20 gastric cancer patients.²⁵ The disagreement between our results and previous research

FIGURE 4. Relationship between relative β -tubulin III mRNA expression levels (logarithmically transformed) and sensitivity to docetaxel.

might be related to small cohorts patients in both studies, different methods being used to evaluate β -tubulin III expression, and different tumour samples (malignant peritoneal effusions vs primary tumour tissues).

Several recent reports have suggested that the association between β -tubulin III and chemosensitivity to taxane was affected by tumour pathology²⁶ and stage.²⁷ Sève et al²⁷ found that patients with higher β -tubulin III expression had greater benefit from adjuvant tubulin-binding agent based chemotherapy in

stage IB-II NSCLC. As most previous studies were based on advanced stage NSCLC, this contradictory result suggests the differential β - tubulin III predictive value might depend on the different tumour stage, early stage versus advanced stage. Tumours often develop the genetic alterations accompanying disease progression. Considering the heterogeneity and biological changes accompanying tumour progression, these results further underlay our hypothesis that it is necessary to verify the predictive value of β - tubulin III in a specified metastases before it can be used in the clinic. Identification of potential biomarkers based on malignant effusions might open a new avenue towards individualized therapy for patients who are not appropriate for tumour resection, e.g. NSCLC patients with pleural effusions. Clearly, these hypothesis-generated results need to be confirmed in large clinical trials.

In conclusion, we demonstrated that β - tubulin III mRNA expression level was inversely correlated with *ex vivo* sensitivity to docetaxel in pleural effusions of NSCLC patients. The data in this study also provided preliminary evidence for using biomarkers in malignant effusions in the further study on the individualized therapy.

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