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RESEARCH

Survivin mRNA Level in Blood Predict the Efficacy of Neoadjuvant Chemotherapy in Patients with Stage IIIA-N2 Non-Small Cell Lung Cancer

Yi-Ming Hu · Jing Li · Li-Chao Yu · Shun-Bing Shi · Yong-Jie Du · Jian-Nong Wu · Wei Lin Shi

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Abstract In a previous study, survivin mRNA expression in non-small cell lung cancer (NSCLC) tissue had been demonstrated to be associated with unfavorable prognosis of patients treated with chemotherapy. In this study, we investigated the survivin mRNA levels in blood of patients with stage IIIA-N2 NSCLC and their association with the efficacy of neoadjuvant chemotherapy (NCT) and disease-free survival (DFS) and overall survival (OS). Blood specimens were collected from 56 patients with stage IIIA-N2 NSCLC before (N0) and after the complete of NCT (N1). Survivin mRNA was measured by real-time quantitative-PCR assay. Receiver operating characteristics curve analysis was undertaken to determine the best cutoff value for survivin mRNA. Results showed that high blood survivin mRNA levels at N0 and N1 were significantly associated with clinical (P=0.01 and P=0.008, respectively) and pathologic response (both P=0.004, respectively). Moreover, the change of blood survivin mRNA levels in these NSCLC patients is associated with the clinical and pathologic response to NCT. Patients with high survivin mRNA levels at N0 and N1 had significantly shorter DFS and OS than those with low survivin mRNA levels (P=0.021 and P=0.014, respectively for DFS; P=0.009 and P=0.005, respectively for OS). Multivariate analysis demonstrated that high blood survivin mRNA level was an independent predictor for worse DFS and OS in the NSCLC patients receiving NCT. In conclusion, survivin mRNA level in blood from stage IIIA-N2 NSCLC patients receiving NCT is predictive of cancer outcome.

Keywords Non-small cell lung cancer · Neoadjuvant chemotherapy · Survivin mRNA · Prognosis · Prediction

Y.-M. Hu · J. Li (✉) · Y.-J. Du · W. L. Shi
Department of Pulmonary Medicine, Affiliated Hospital of Jiangsu University, 438 North Jiefang Street, Zhenjiang 212001, China
e-mail: lijian541226@163.com

L.-C. Yu · S.-B. Shi
Department of Thoracic Surgery, Affiliated Hospital of Jiangsu University, Zhengjian 212001, China

J.-N. Wu
Department of Pathology, Affiliated Hospital of Jiangsu University, Zhengjian 212001, China

Introduction

Stage IIIA non-small cell lung cancer (NSCLC) with involvement of N2 represents a heterogeneous group of patients [1]. Despite continuous progress in therapeutic strategies, locally advanced stage IIIA NSCLC remain of poor prognosis. Although survival benefits of neoadjuvant chemotherapy (NCT) remain controversial [2–7], combined treatment modalities with NCT are currently applied for the management of stage IIIA NSCLC patients [7–9]. Theoretically, NCT may improve the prognosis of patients with stage IIIA NSCLC by increased resectability following the reduction of the tumor burden and by the early systemic control of micrometastases. Moreover, the clinical and pathologic response can be used to select patients who are eligible for further treatment [9–11]. Furthermore, NCT allows for the genetic profiling of tumor prior to treatment coupled with the subsequent assessment of the responsiveness of a particular chemotherapy regimen, thereby providing the potential for individualized therapy for patients with locally advanced stage IIIA NSCLC [12, 13].

Survivin is a special number of the inhibitor of apoptosis protein (IAP) family, which has been implicated in both inhibition of apoptosis and mitosis regulation [14, 15]. Survivin is notable for its high degree of tumor-specific expression, but is either undetectable or expressed at very low levels in differentiated adult tissues [16, 17]. As a result of its selective expression in malignant cells but not in surrounding normal tissue, survivin has generated much interest for its role in modulating apoptosis induced by chemotherapeutic agents, as a target for anticancer drugs and as a marker of prognosis in malignancies [14, 17]. Our previous studies have shown that survivin mRNA expression in NSCLC tissue is correlated with poor prognosis of patients who received platinum-based chemotherapy [18, 19]. Recently, we reported that survivin mRNA is detectable in blood of patients with resectable NSCLC, which was associated with a higher disease relapse and shorter survival after surgery [20].

In the current study, to determine whether survivin mRNA expression in blood is a surrogate marker for predicting efficacy of NCT, we examined the correlation between chemosensitivity to NCT and blood survivin mRNA levels as well as prognosis of patients with stage IIIA–N2 NSCLC after NCT.

Patients and Methods

Patients

A total of 56 patients with NSCLC that was considered to be stage IIIA–N2 were treated with NCT followed by surgery from January 2008 to July 2011. Tumors were confirmed histopathologically by bronchoscopy or transthoracic needle biopsy and were staged by computed tomography (CT) scans of chest and upper abdomen, magnetic resonance imaging (MRI) scan of the brain and nuclear bone scan. Criteria for pretherapy diagnosis of stage IIIA NSCLC with clinical N2 (cN2) were defined as a mediastinal node of short size diameter exceeding 1.5 mm on CT scan. Cases with a diameters between 10 and 14 mm were included only of there was more than one node. In addition, positron emission tomography (PET)/CT scans were used in 18 patients for diagnosis of stage IIIA NSCLC-cN2. Other eligibility criteria included age range of 20–70 years, a performance status (PS, according to the Eastern Cooperative Oncology Group Scale) of 0–1, sufficient bone marrow reserve and adequate liver and kidney function, with a based lung function test allowing lobectomy or pneumonectomy. This study was conducted with the consent of the ethical committee of Affiliated Hospital of Jiangsu University in China, and informed consent was obtained from all subjects.

Treatment and Assessment

All patients received at least two cycles of platinum-based chemotherapy. Treatment regimens consisted of cisplatin (80 mg/m^2 on day one) plus gemcitabine (1,000 mg/m^2 on days one and eight, n=40), or plus vinorelbine (25 mg/m^2 on days one and eight, n=16). After completion of NCT, chest CT scan was repeated for every patient to assess the clinical response and feasibility of surgery. Clinical responses to NCT were categorized as complete response (cCR), partial response (cPR), stable disease (cSD), or progressive disease (cPD) according to the RECIST criteria [21].

Patients who had a clinical response (cCR+cPR) and cSD were eligible for thoracotomy with the aim of complete resection of the primary tumor (i.e. resection margins macro-and microscopically free of tumor) and extensive mediastinal lymph node sampling. The patients who achieved complete resection of the tumor received 2 to 4 cycles of adjuvant chemotherapy with the same regimen as NCT. The patients who had either unresectable disease or incomplete resection with positive margins received further radiation therapy with conventional fractionation 3–4 weeks after surgery.

Pathologic response was assessed using criteria reported by de Bore et al. [22], pathologic complete response (pCR) was defined as absence of any tumor cell in the surgical specimen; partial response (pPR) was defined as the presence of only small residual foci of tumor cells or the presence of significant areas of tumor necrosis, fibrosis, or granulation throughout the surgical specimen; no charge (pNC) was defined as the presence of large areas of identifiable tumor cells in the surgical specimen with minimal evidence of tumor necrosis. N2 status was determined from a postoperative pathologic examination of the lymph nodes in the ipsilateral mediastinum. If the pathologic examination indicated complete fibrosis, scars or the granulation of the lymph nodes, with neither normal nodal formation nor cancer cell, a pCR of the lymph nodes was determined. If the pathologic examination found a normal formation of lymph nodes, without any cancer cell or fibrosis, scar or granulation, the patients were excluded from this study.

Blood Specimen Collection

For all patients with NSCLC, blood specimens were collected at two time points, before (N0) and after NCT and prior to operation (N1). Meantime, blood specimens of 50 patients with benign lung disease (BLD) were used as negative controls, BLD included chronic obstructive pulmonary disease (18), asthma (12), pneumonia (11), tuberculous pleurisy (5) and interstitial lung disease (4).

Six milliliter peripheral blood were collected in tubes containing EDTA as anticoagulant, stored at 4 °C, and processed with 2 h. Peripheral blood mononuclear cells (PBMCs) were firstly isolated by density centrifugation (1,500 rPm for 15 min) with lymphocyte separation median and washed with PBS (1,200 rPm for 10 min), cell pellets were suspended in 1 mL of Isogen (Nippon Gene, Toyama, Japan) and were stored at −80 °C until molecular analysis.

Real-Time Quantitative-PCR Detection

Total RNA was extracted by the guanidium-isothiocyanatephenol-chloroform-based method. Real time quantitative (Rtq)-PCR of the target survivin and β-actin as internal control was carried out on an ABI 7500 thermal cider Real-time PCR System (Applied Biosystems, Foster Cycler, CA, USA), using the SYBR-Green I chemistry. The primer sequences of survivin and β-actin and amplification condition have been described previously in detail [20]. Briefly, a denaturation step at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, annealing for 30 s at 57 °C, and elongation for 30 s at 72 °C. Detection of PCR products was accomplished by measuring the emitting fluorescence (Rn) at the end of each reaction step (reaction cycles). Threshold cycle (C_T) correspond with the cycle number required to detect a fluorescence signal above the baseline. Calculations were made with the use of the comparative C_T ($2^{-\triangle\triangle CT}$) method [23]. According to the method, the mRNA amounts of a target gene were normalized to an endogenous control and relatively to a calibrator. We use the housekeeping gene β-actin as an internal control and the PBMC of healthy volunteer as a calibrator. $\Delta\Delta C_T$ represent the difference between the mean ΔC_T value of a patient blood sample and the mean $\triangle C_T$ value of the calibrator, both calculated after the same PCR run, whereas ΔC_T is the difference between the C_T of the target gene and the C_T of the endogenous reference gene (β-actin) of the same sample. The relative quantitative value was expressed as $2^{-\triangle\triangle CT}$ [23]. Each experiment was performed in triplicate.

Statistical Analysis

Median values with range are used to describe survivin mRNA levels because of its skewed distribution. Mann–Whitney U test was conducted to compare the survivin mRNA levels between two patient groups, or two sampling time points. Receiver operating characteristics (ROC) curve analysis was undertaken to determine the best cutoff value for survivin mRNA levels to achieve 95 % specificity. The Fisher exact test was used to analyze the correlation between the high survivin mRNA levels and clinicopathologic parameters. The log-rank test was used to compare the DFS and OS Kaplan–Meier curves for subgroup of patients. Univariate and multivariate Cox proportional hazards regression analyses were

Table 1 Association between clinicopathologic parameters and survivin mRNA levels

Parameter	Detection rate of high survivin mRNA level (n/n)			
	Before NCT (N0)	*P* value	After NCT (N1)	*P* value
Age (year)				
<58	19/28 (67.9 %)	0.582	12/28 (42.9 %)	0.579
≥58	16/28 (57.1 %)		9/28 (32.1 %)	
Sex				
Male	22/37 (59.5 %)	0.572	15/37 (40.5 %)	0.544
Female	13/19 (68.4 %)		6/19 (31.6 %)	
Smoking status				
Smoker	32/49 (65.3 %)	0.406	19/49 (38.8 %)	0.700
Never smoker	3/7 (42.9 %)		2/7 (28.6 %)	
Performance status				
0	29/46 (63 %)	0.563	18/46 (39.1 %)	0.437
1	6/10 (60 %)		3/10 (33.3 %)	
Histology				
Squamous cell cancer	10/19 (52.6 %)	0.383	6/19 (31.6 %)	0.572
Nonsquamous cell cancer	25/37 (67.6 %)		15/37 (40.5 %)	
Chemotherapy cycles				
2	27/42 (64.3 %)	0.432	17/42 (40.5 %)	0.321
3	8/14 (57.1 %)		4/14 (28.6 %)	
Pathologic T stage				
T1N2	6/11 (54.5 %)	0.071	3/11 (27.3 %)	0.028
T2N2	16/30 (53.3 %)		8/30 (26.7 %)	
T3N2	13/15 (86.7 %)		10/15 (66.7 %)	
Clinical response				
Complete response	1/2 (50 %)	0.01	0/2 (0 %)	0.008
Partial response	9/23 (39.1 %)		4/23 (17.4 %)	
Stable disease	21/26 (80.8 %)		13/26 (50 %)	
Progressive disease	4/5 (80 %)		4/5 (80 %)	
Pathologic response (n=51)				
Complete response	0/4 (0 %)	0.004	0/4 (0 %)	0.004
Partial response	8/15 (53.3 %)		2/15 (13.3 %)	
No change	25/32 (78.1 %)		18/32 (56.3 %)	

NCT neoadjuvant chemotherapy

conducted to determine whether survivin mRNA level was an independent factor for predicting DFS and OS. All values are two sided, $P<0.05$ was regarded as statistical significant.

Results

Patient Clinical Characteristics and Response to NCT

The clinical characteristics of 56 NSCLC patients were showed in Table 1. The median age was 58.4 years (range 47–69). Approximate two thirds of patients were male ($n=37$), and the majority had a PS of 0 ($n=46$). Only 12.5 % of patients were never smokers ($n=7$), and 66 % of patients had nonsquamous histology ($n=37$). Most patients received two cycles of NCT ($n=42$). The cCR rate was 3.6 % (2/56), cPR was 41.1 % (23/56), cSD was 46.4 % (26/56), and cPD was 8.9 % (5/56). Clinical response (cCR+cPR) rate was 44.6 % (25/56). Fifty-one patients who had a clinical response and SD underwent thoracotomy. Five patients experienced PD received further radiotherapy and chemotherapy. Type of surgical resection included lobectomy ($n=40$) biolobectomy ($n=8$), and pneumonectomy ($n=4$). A complete resection (R0) was achieved in

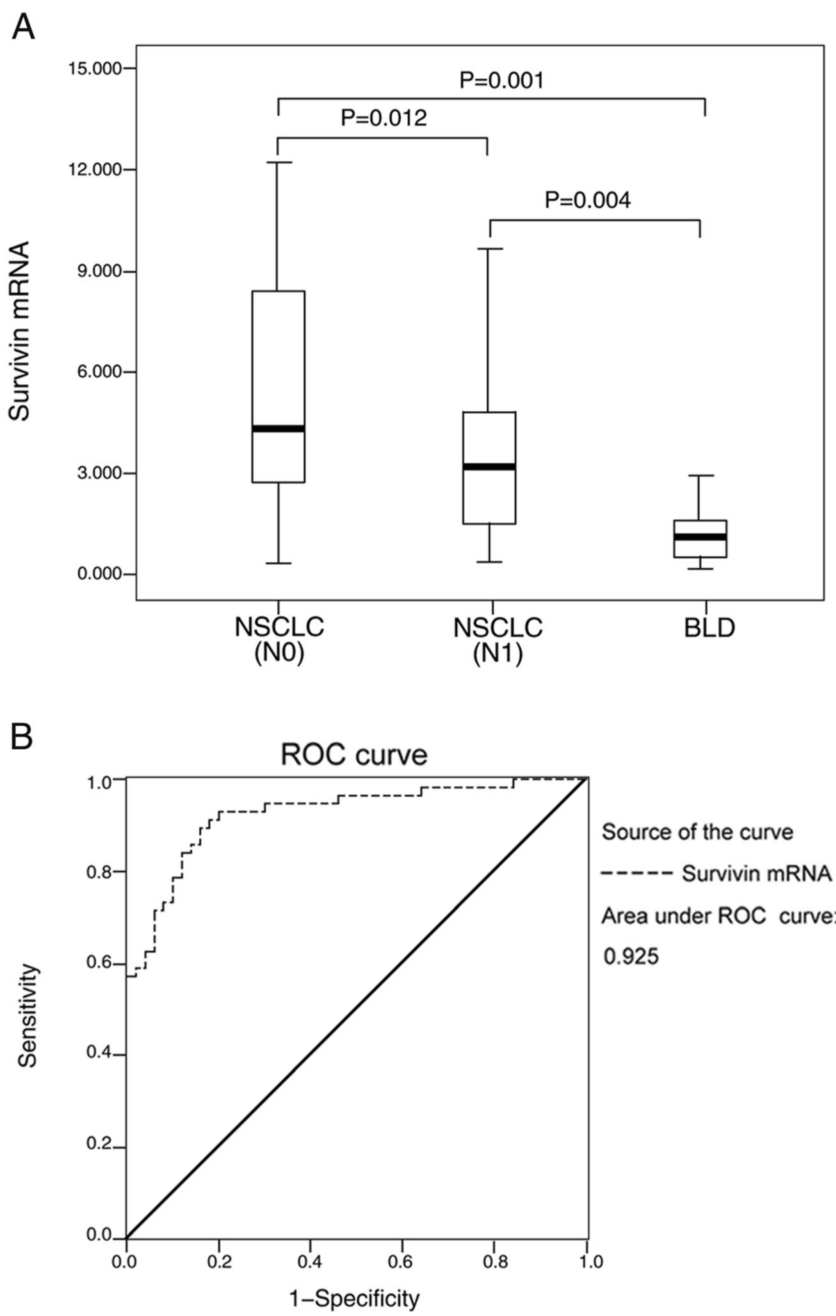

Fig. 1 **a** Distribution of blood survivin mRNA levels in non-small cell lung cancer (NSCLC) patients before (N0) and after neoadjuvant chemotherapy (N1) and benign lung disease (BLD) patients. **b** Receiver-operating characteristic (ROC) curve was conducted to compare NSCLC and BLD patients for survivin mRNA

47 patients (92.2 %). Of the tumor investigated, a pathologic response was seen in 20 (39.2 %) of the 51 patients with surgical resection, with a pCR in 4 patients (7.8 %), a pPR in 16 patients (31.4 %), and pNC in 31 patients (60.8 %).

Blood Survivin mRNA Levels and Response to NCT

Among 56 NSCLC patients, the median of blood survivin mRNA levels at N0 and N1 were 4.438 (range from 0.342 to 14.516) and 3.325 (range from 0.365 to 10.724), respectively, which were significantly higher than 0.958 (range from 0.162 to 3.785) in BLD patients (P=0.001 and P=0.004 respectively Fig. 1a). The level of survivin mRNA in blood of NSCLC patients was decreased dramatically after NCT (P=0.012; Fig. 1a). We analyzed changes in pre-and post-NCT survivin mRNA levels in blood of these 56 patients in terms of cCR, cPR, cSD and cPD. As shown in Fig. 2, post-NCT survivin mRNA levels (at N1) were significantly lower than pre-NCT levels (at N0) in patients with cCP and cPR (P=0.008), but no significant change was found between post- and pre-NSCT in patients with cSD (p=0.116). Whereas post-NCT survivin mRNA levels were markedly elevated in 5 patients with cPD (P=0.002). Meantime, the changes of pre-and post-NCT survivin mRNA levels in the 51 patients who achieved surgical resection were also analyzed. In the patients with pCR and pPR, post-NCT survivin mRNA levels were significantly lower as compared with pre-NCT levels (P=0.001 and P=0.012, respectively). In patients with pNC, post-NCT survivin mRNA levels had marginal increase as compared with pre-NCT (P=0.087).

Association Between Clinicopathologic Parameters and Survivin mRNA Levels

ROC curve was constructed by plotting sensitivity versus 1-specificity. Comparing survivin mRNA levels at N0 in NSCLC patients and BLD patients, the area under the ROC curve (AUC) for survivin mRNA was 0.925. Referring to the ROC curve, the cutoff value of survivin mRNA level for defferentiating high from low blood survivin mRNA level was 3.8. Based on the cutoff value obtained in the ROC curve analysis, detection rates of high survivin mRNA levels at N0

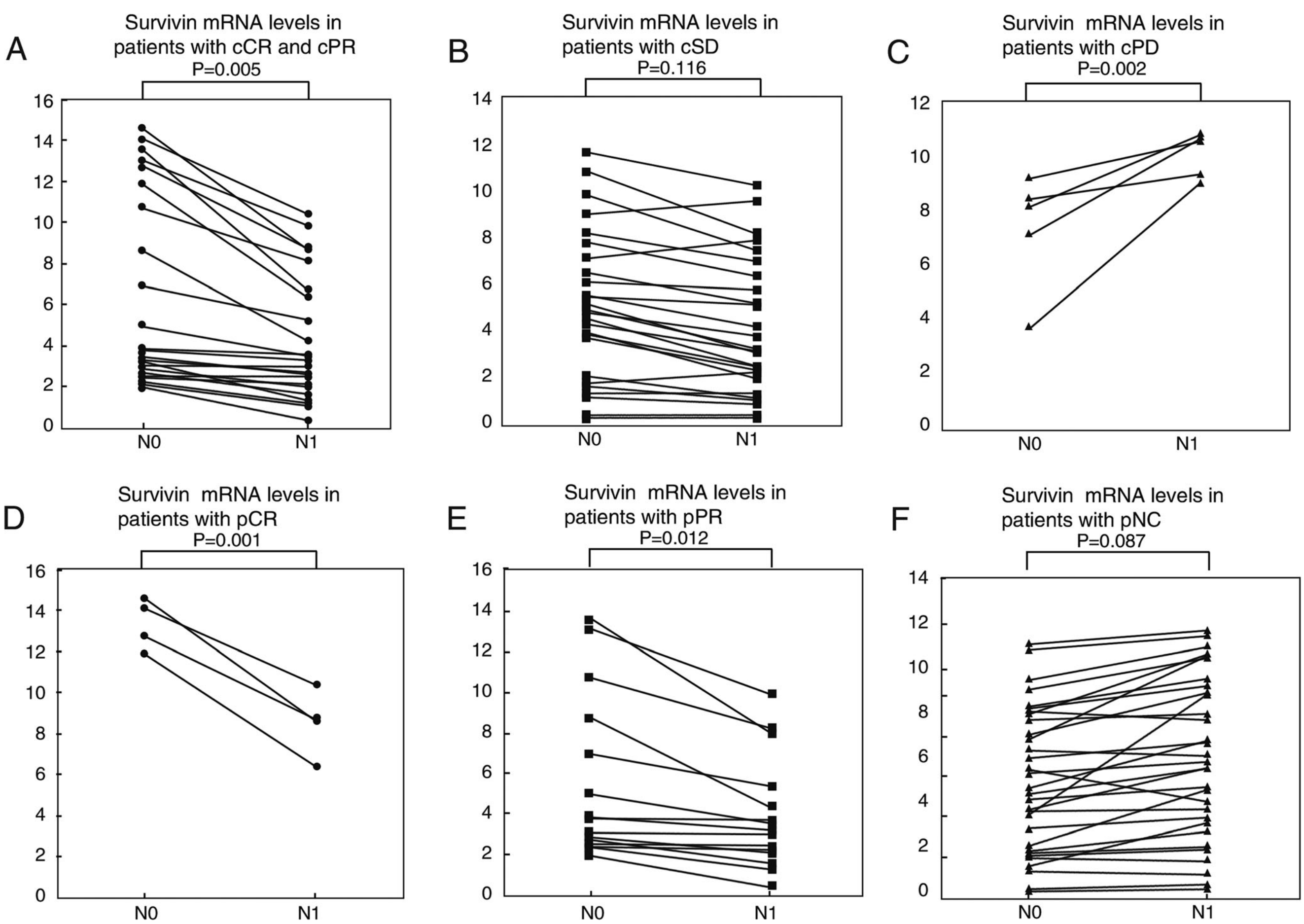

Fig. 2 **a–c** Changes in blood survivin mRNA levels in patients with clinical complete response (cCR), partial response (cPR), stable disease (cSD) and progressive disease (cPD) between before (N0) and after neoadjuvant chemotherapy (N1). **d–f** Changes in blood survivin mRNA levels in patients with pathologic complete response (pCR), partial response (pPR), and no change (pNC) between N0 and N1

and N1 were 62.5 % (35/56) and 37.5 % (21/56, P=0.014), with specificity of 96 %.

The associations between clinicopathologic parameters and high survivin mRNA levels at N0 and N1 from NSCLC patients are shown in Table 1. The patients with T3N2 disease showed higher detection rates of high survivin mRNA levels than those with T2N2 and T1N2 disease (P=0.071 and P=0.028, respectively). Notably, the detection rates of high survivin mRNA levels of patients who achieved a cCR and cPR to NCT were significantly lower than those with cSD and cPD (P=0.01 and P=0.008, respectively). Moreover, the patients who had a pCR and pPR after NCT showed significantly lower detection rates of high survivin mRNA levels as compared with patients with pNC (both P=0.004). No association was observed between the high survivin mRNA levels and other clinicopathologic parameters.

Association Between Survivin mRNA Levels and DFS and OS

With a median follow-up period of 45 months (range 17–68 month), the media DFS and OS for all enrolled NSCLC patients were 28.5 (95 % CI, 15.0–43.5) months and 35.5 (95 % CI, 21.0–51.5) months respectively. Log-rank tests showed that median DFS and OS were significantly shorter for patients with high survivin mRNA level at N0 compared with patients with low survivin mRNA level (P=0.021 and P=0.009, respectively; Fig. 3a and b). Similarly, patients with high survivin mRNA level at N1 time point had a significantly shorter median DFS and OS compared with patients with low survivin mRNA level (P=0.014 and P=0.005, respectively; Fig. 3c and d).

In univariate analyses, factors that correlated with DFS and OS include clinical response to NCT, pathologic response in

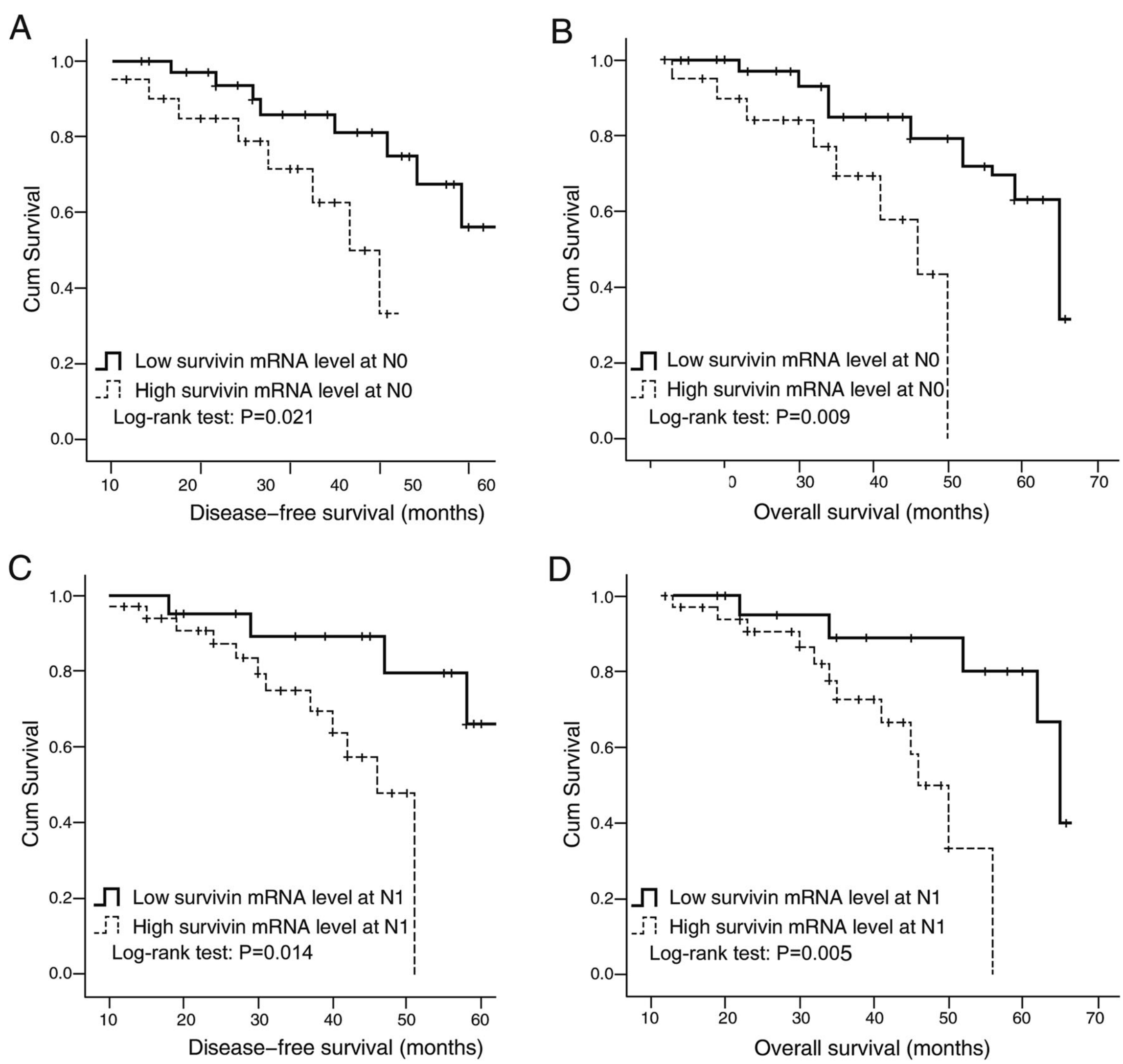

Fig. 3 Kaplan–Meier curves of disease-free survival (DFS) and overall survival (OS) according to high and low blood survivin mRNA level in patients before (N0) and after neoadjuvant chemotherapy (N1). **a–b** DFS and OS curves according to high and low blood survivin mRNA levels at N0. **c–d** DFS and OS curves according to high and low blood survivin mRNA levels at N1

the resected tumor and survivin mRNA levels in blood at N0 and N1 time points. For patients who had undergone tumor resections, a step-by-step multivariate analysis according to the Cox proportional hazard regression model was applied in an explorative approach. Pathologic response and survivin mRNA levels at N0 and N1 were revealed as independent predictors for DFS and OS (Table 2).

Discussion

Patients with locally advanced stage IIIA NSCLC has an unfavorable prognosis. Although complete resection for these patients is technically feasible, 5-year survival rates is only 19 % and 24 % for those using clinical stage prediction and using pathologic stage prediction, respectively [24]. NCT is a therapeutic option that is used in patients with locally advanced resectable NSCLC. For patients receiving NCT, imaging findings are commonly used to evaluate tumor response to chemotherapy and determine eligibility of the patients for subsequent resection. However, the imaging change of tumor size before and after chemotherapy is not always reliable in the prediction of treatment efficacy because of the difficulty in differentiating vital tumor from necrostic tumor tissue or scar formations in an imaging picture [25].

In several survival analyses, it has proven that pCR is an important predictor of survival in patients receiving NCT following by surgical resection [25–28]. However only 6 to 19 % of all locally advanced NSCLC patients achieved pCR following NCT. Thus additional discriminating factors independent of pathologic response would greatly assist clinicians in identifying subgroups of high risk patients to be targeted for either more intensive and/or individually tailored therapy.

Survivin was identified as one of the markers with evidence of it being an independent predictor of NSCLC patient prognosis [29]. In tumor cells, higher survivin expression is associated with enhanced proliferative index, reduced levels of apoptosis, resistance to chemotherapy, and increase rate of tumor recurrence [17]. In previous studies, we had found a high survivin mRNA expression in tumor tissue to be associated with a worse response to platinum-based chemotherapy and a more poor survival of NSCLC patients [18, 19]. In the current study, NCT included cisplatin plus gemcitatine or vinorelbine, which are standard treatment regimens for patients with NSCLC [30]. The clinical response rate (cCR+cPR) was 44.6 % and pCR rate was 7.8 %. We found significant associations between blood survivin mRNA positivity in pre-and post-NCT and clinical and pathologic response to treatment.

The change analysis of survivin mRNA levels before and after treatment may provide further information on the marker that could predict response or resistance to NCT. Thus we analyzed the relationship between NCT-induced changes in survivin mRNA levels and NCT response. The results showed that post-NCT survivin mNRA levels decreased significantly in patients with cCR and cPR, but the decrease was not significant in the patients with cSD. Whereas the patients with cPD had a significant increase in post-NCT survivin mRNA

Table 2 Multivariate Cox proportional hazard regression analysis of disease-free survival and overall survival

Parameter	Overall survival			Disease-free survival		
	Hazard ratio	95 % CI	*P* value	Hazard ratio	95 % CI	*P* value
Smoker status Smoker vs never smoker	0.654	0.312 to 1.334	0.517	0.528	0.244 to 1.219	0.575
Performance status 0 vs 1	0.875	0.433 to 1.625	0.315	0.732	0.352 to 1.427	0.412
Histology SCC vs non-SCC	0.742	0.345 to 1.487	0.422	0.826	0.411 to 1.638	0.356
Pathologic T stage T1 and T2 vs T3	1.239	0.814 to 1.923	0.172	1.353	0.921 to 2.065	0.095
Clinical response CR and PR vs SD and PD	1.453	0.865 to 2.118	0.084	1.377	0.942 to 2.137	0.088
Pathologic response CP and PR vs NC	1.862	1.353 to 2.642	0.009	1.915	1.402 to 2.711	0.007
Survivin mRNA level at N0 High vs low	1.527	1.042 to 2.361	0.022	1.611	1.125 to 2.402	0.015
Survivin mRNA level at N1 High vs low	1.625	1.145 to 2.418	0.018	1.774	1.259 to 2.615	0.011

SCC squamous cell carcinoma, *CR* complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *NC* no change levels (Fig. 2a, b and c). Moreover, our analysis also showed a relationship between the changes of survivin mRNA levels in pre-and post-NCT and pathologic response, which is similar to association between the changes of survivin mRNA levels and clinical response, implying that the changes of pre- and post-NCT survivin mRNA levels might predict response to treatment in both clinical imaging and pathologic examination. Furthermore, in the current study, there were statistically significant DFS and OS advantages in NSCLC patients with low survivin mRNA level in compared with those in NSCLC patients with high survivin mRNA level. These results are reinforced by results of multivariate analysis, in which pre- and post-NCT survivin mRNA levels were demonstrated to be an independent predictor for worse DFS and OS.

Chemotherapy resistance may be a significant contributor to treatment failure in some patients with stage IIIA NSCLC who received NCT. An individualized approach to treatment selection could potentially improve survival in patients with stage IIIA NSCLC treated with NCT. The purpose of our study was to identify the best marker of chemosensitivity and correlate it to survival in a group of patients with stage IIIA–N2 NSCLC. Our data show that patients classified at the same prognostic stage according to classic pathologic criteria and receiving the same therapy modality have very variable prognoses. With this group, we found that blood survivin mRAN measure was a useful tool to characterize patients with a poor outcome. To our best knowledge, this report is the first to demonstrate that a single molecular marker is a predictor of outcome for NSCLC patients receiving NCT followed by surgery.

The limitations of this study are the small sample size, and unreliable N2 staging before NCT. As far as the size of the study group is concerned, we chose only a particular subset of patients (stage IIIA–N2) to avoid bias resulting from different prognoses. In regard to mediastinal staging, we have acknowledged no mediastinoscopy for staging prior to NCT as a drawback with this article. However, the N2 status was determined finally according to the postoperative pathologic examination of the lymph nodes in addition to using a chest CT scan as an initial mediastinal staging. The patients who had a normal nodal formation in their surgical specimens had been excluded from this study. Therefore we can determine N2 status at least in the patients who obtained the resection of tumor. Nevertheless, a future study with the larger number of patients with N2 staging using mediastinoscopy are required to validate the predictive and prognostic significance of blood survivin mRNA for patients with stage IIIA–N2 NSCLC who received NCT.

Conflict of Interest The authors have declared that they have no conflict of interest.

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