Up-regulation of Survivin mRNA Might Be a Marker for Non-invasive Detection of Non-small Cell Lung Cancer Rather than for Prognosis

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Abstract. *Background: Survivin suppresses programmed cell death and regulates cell division. To evaluate the prognostic importance of the apoptosis inhibitor survivin for non-small cell lung cancer (NSCLC), a study was performed in 64 patients with R0 resections for NSCLC and histopathological stages I-IIIA. Patients and Methods: Analysis of survivin mRNA expression was performed on 64 paired tumor and normal tissues by quantitative real-time RT-PCR. Results: Survivin expression in tumors (median 3.68, min. 0.19, max. 28.63) was significantly higher (p<0.04) than in corresponding normal tissues (median 1.68, min. 0.03, max. 155.59). Survivin mRNA was up-regulated in tumors of 42 patients (66%). However, survivin overexpression did not correlate with survival of lung cancer patients. There was no significant association of survivin mRNA levels with histological type (p=0.29), pT (p=0.41) and pN categories (p=0.57), grading of the primary tumor (p=0.45), or histopathological stage (p=0.87). Conclusion: Overexpression of survivin mRNA in NSCLC might be a marker for noninvasive tumor detection, but has no prognostic importance.*

Despite improvements in its detection and therapy, modest progress has been made in the outcome for patients diagnosed with lung cancer. In fact, the relative 5-year survival rate for all stages of lung cancer combined was 13% from 1974-1976 and 15% from 1995-2000 (1). The World Health Organization describes three major histological subtypes of non-small cell lung cancer (NSCLC): squamous cell carcinoma, adenocarcinoma and large cell carcinoma (2). These entities represent approximately 75% of all lung cancer cases. Radical surgery (R0 resection) offers the only chance for cure in patients with NSCLC and survival probabilities are mainly dependent on tumor stage (3, 4). Only 15% of lung cancer patients are diagnosed at an early stage (5). This places a high priority on elucidating the molecular mechanisms underlying this disease with the aim of ultimately developing novel and effective therapeutic strategies to target this malignancy. Substantial efforts have been made to identify prognostic factors in order to individualize treatment and improve prognosis (6, 7). A variety of molecular markers has been implicated both in the pathogenesis and prognosis of NSCLC (8, 9).

Survivin is a member of the inhibitor of apoptosis protein (IAP) gene family containing one baculovirus IAP repeat (BIR) domain. As an inhibitor of programmed cell death, survivin is expressed in most fetal and proliferating adult tissues, whereas almost no transcripts are detected in resting cells. It mediates suppression of apoptosis by inhibition of the caspases 3 and 7, the terminal effectors in apoptotic protease cascades (10). Survivin is expressed in the G2-M-phase of the cell cycle and its interaction with the mitotic spindle apparatus has been reported to be essential for antiapoptotic function (11, 12). Depletion of survivin in human cells has been observed to cause apoptosis and pleiotropic defects in cell division. Furthermore, it has been described that anti-sense targeting against survivin induced apoptosis and sensitized lung cancer cells to chemotherapy (13).

A large analysis of human transcripts revealed survivin as the fourth most highly expressed protein in human cancer tissues compared to normal tissues (14). This overexpression of survivin in human tumors was shown to be associated with an unfavourable prognosis in several carcinomas reviewed by Li (15), including lung cancer (16, 17). However, other groups could not confirm these results (18-20). We examined the expression of survivin mRNA in R0

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resected non-small lung cancer patients to conclusively evaluate its prognostic importance.

Patients and Methods

Patients. Paired normal and tumor tissues from 64 patients with histopathologically confirmed NSCLC were included in this study. These patients from a previous prospective trial (21) did not receive a pre-treatment therapy and underwent R0 resection. The clinical data are summarized in Table I. The collective comprised 52 men (81.2%) and 12 (18.8%) women with a median age of 66 years (range 34-80 years), 56 (87.5%) were smokers, 8 (12.5%) were non-smokers. Patients with histopathological stage IIIa received postoperative radiotherapy. The median follow-up was 85.9 months (range 63-105 months). Patients were seen at 3-month intervals during the first postoperative year, every 6 months in the second and third year and once a year thereafter. Evaluation consisted of physical examination, biochemical profile, chest radiograph, CT scan of brain, chest and abdomen, abdominal ultrasound and technetium bone scan. Data on recurrence and cause of death were obtained for all patients. Data and tissue collection were in accordance with the regulations of the local Ethics Committee. Informed consent was obtained from each patient.

Tissue acquisition. For the evaluation of gene expression, tumor samples and corresponding normal tissues were obtained immediately after lung resection and snap-frozen. The histologically normal appearing lung tissues were taken at the greatest distance from the tumor. Samples were chosen after control staining with hematoxylin and evaluation by a pathologist (S.E.B.).

RNA isolation. Total cellular RNA was isolated using Trizol reagent (Life Technologies/GIBCO, Grand Island, NY, USA) according to the manufacturer's recommendation and quantified at $A_{260/280\ nm}$ (Smart Spec; Biorad, Hercules, CA, USA).

Quantitative real-time-reverse transcriptase (RT)-PCR. Total cellular mRNA (0.5 μg) was reverse-transcribed as reported elsewhere (22) using an oligo$(dT)_{18}$ primer and MMLV (Moloney murine leukemia virus) reverse transcriptase Advantage™ RT-for-PCR kit (Clontech Lab., Palo Alto, USA). Real-time RT-PCR was performed applying 25 ng of cDNA (TaqMan ABI PRISM 7900HT Sequence Detection System; Applied Biosystems, Darmstadt, Germany). By means of fluorescence emission, this technique allows the cycling point to be found when PCR product is detectable (threshold cycle). To normalize the amount of total RNA present in each reaction, the housekeeping gene *β-actin* was amplified as described elsewhere (23).

Primers and probes were designed for full length cDNA sequences blasted against human genomic sequences to identify exon-exon junctions (NCBI), using the Primer Express software (Applied Biosystems). The *β-actin* primers and probe were obtained from Applied Biosystems, the *survivin* primers and probe from Eurogentec, Seraing, Belgium. The sequences have been reported elsewhere (23). Briefly, the PCR reaction mixture contained 300 nM of each primer and 200 nM probe in a final volume of 20 μl. PCR conditions were 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 s and 60˚C for 1 min. Gene expression levels were calculated using standard curves generated by serial dilutions of placenta cDNA (Clontech Lab.). All reactions were performed in triplicates.

Table I. *Survival in NSCLC based on clinical parameters*.

Parameter	N=64	%	5-year survival (%) ± SD	Median survival (months)	*P*-value
Histology					
SCC	35	54.7	60.0±8	n.r.	<0.02
AC	19	29.7	42.1±11	46.8	
LC	10	15.6	30.0±14	20.9	
pT-category[a]					
T1	12	18.8	75.0±13	n.r.	n.s.
T2	44	68.8	47.7±8	46.8	
T3	8	12.5	25.0±15	26.7	
pN-category[b]					
N0	35	54.7	71.4±8	n.r.	<0.0001
N1	17	26.6	41.2±12	34.0	
N2	12	18.8	0	12.6	
Grading					
G2	14	21.9	50.0±13	59.0	n.s.
G3	50	78.1	50.0±7	51.8	
UICC Stage[c]					
I	32	50	71.9±8	n.r.	<0.0001
II	13	20.3	53.8±14	n.r.	
IIIA	19	29.7	10.5±7	16.7	

[a]Histopathological tumor category; [b]histopathological lymph node category; [c]histopathological stage according to UICC, Union Internationale Contre Le Cancer, 5th edition, 1997. n, Number of patients; n.r., not reached; n.s., not significant; SCC, squamous cell carcinoma; AC, adenocarcinoma; LC, large cell carcinoma.

Statistical analysis. Gene expression analyses yield values which are expressed as ratios between two absolute measurements: the gene of interest and the reference gene *β-actin* (*survivin*/*β-actin*) in tumor and normal tissues, respectively. Relative mRNA expression levels (ratio tumor/normal) were calculated as (*survivin*/*β-actin* in tumor) / (*survivin*/*β-actin* in paired normal tissue).

Associations between gene expression levels and clinico-pathological parameters were evaluated using the χ^2 test for dichotomised variables applying exact testing for significance. Nonparametric tests were used for paired variables and the Mann-Whitney test for independent variables.

Partitioning of gene expression levels to construct prognostic groups was performed according to LeBlanc and colleagues (24). Kaplan-Meier plots (25) were used to describe the survival distribution and the log-rank test was used to evaluate survival differences (26). The level of significance was set to $p<0.05$ unless otherwise specified, *p*-values were given for 2-sided testing. Statistical tests were performed using the Software Package SPSS for Windows, Version 12.0, Chicago, IL, USA.

Results

Clinical data. Median survival rates depending on various clinical variables are summarized in Table I. Histopathological UICC tumor stage ($p<0.0001$), pN category ($p<0.0001$) and histology ($p<0.02$) were of significant prognostic importance using the log-rank test. Gender, age and grading of the primary tumor, as well as pT category, had no prognostic impact.

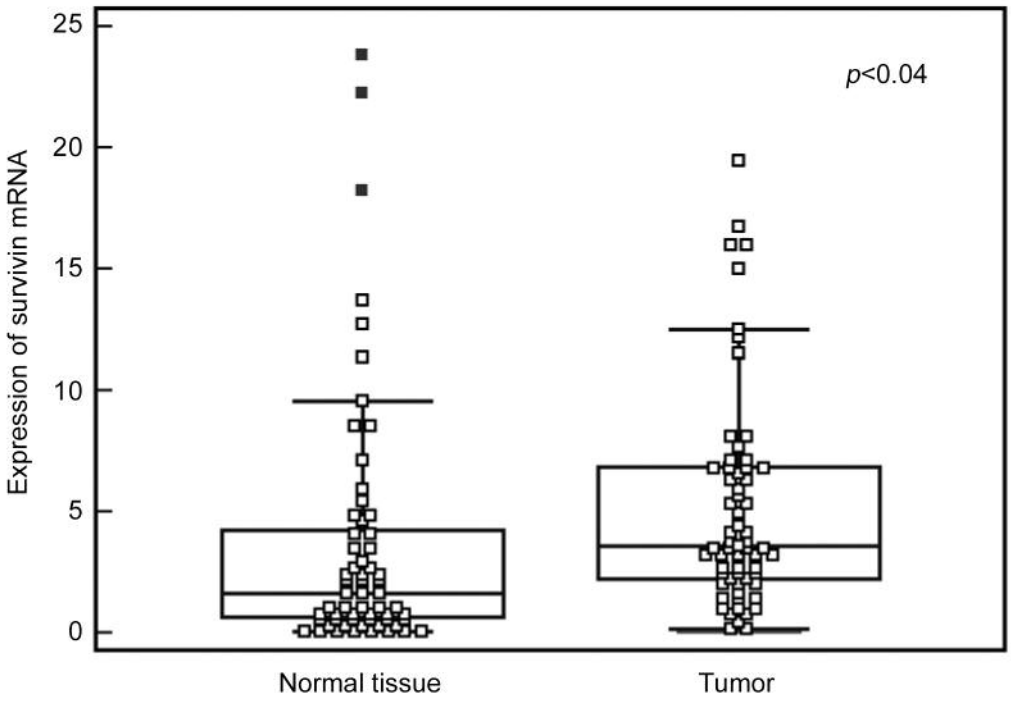

Figure 1. *Survivin mRNA expression in NSCLC and corresponding normal tissues. Box plot showing expression levels of survivin mRNA in tumor and paired normal tissues.*

Distribution of survivin m-RNA expression in tumors and normal tissues. Survivin mRNA expression was detected by quantitative real-time RT-PCR in all 64 tumors and adjacent normal epithelium specimens (Figure 1). Median absolute survivin mRNA expression levels standardized for β-actin were 3.68 (min. 0.19, max. 28.63) in tumors and 1.68 (min. 0.03, max. 155.59) in normal tissues, and this difference was significant ($p<0.04$). Survivin expression was frequently up-regulated in tumors, in 42 of 64 patients (66%). The median relative expression level (ratio tumor/normal) was 2.35 (min. 0.05, max. 443.18). However, survivin overexpression did not correlate with survival. There was no significant association of relative survivin mRNA expression levels with histological type ($p=0.29$), pT ($p=0.41$), and pN categories ($p=0.57$) or grading of the primary tumor ($p=0.45$), and histopathological stage of the tumor ($p=0.87$).

Discussion

A variety of molecular markers have been implicated both in the pathogenesis and prognosis of NSCLC (27). Survivin was identified as one of the markers with evidence of it being an independent predictor of patient outcome (9). The aim of our study was to evaluate survivin as a prognostic tumor marker for patients with NSCLC.

Survivin is expressed in a vast majority of human cancers (28) and is one of the key factors conferring and maintaining resistance to apoptosis (11). Our data obtained from 64 lung cancer patients show a significant up-regulation of *survivin* gene expression in tumors. These results confirm genome-wide searches, which indicated survivin as the fourth top "transcriptome" in cancers of various histologies (14).

The human *survivin* gene is located on chromosome 17 (29, 30). Exon 1 of *survivin*, which is silenced by methylation in normal ovarian epithelium, becomes demethylated and consequently transcriptionally active in most cases of ovarian cancer (31). The *survivin* promoter was highly active in human tumor cells, but not in normal cells, and up-regulated by hypoxia in tumor (32). Several polymorphisms were identified in the *survivin* gene promoter, with one located at repressor elements, and were correlated with increased survivin expression at both the mRNA and protein levels (33). Despite these approaches, the mechanism of up-regulation of *survivin* mRNA in lung cancer still requires further investigation.

Since survivin is frequently overexpressed in lung cancer, it might play a role in oncogenesis and progression of the tumor. It inhibits apoptosis and promotes mitosis. However, clear evidence for an association of survivin expression with survival is still missing. Although survivin overexpression in cancer has been associated with poor prognosis in some

reports (17, 20, 34), we could not find such an association. One reason for these contradictory results might be the detection of survivin either in the nuclear or cytoplasmatic compartment by immunochistochemistry. Two multivariate survival analyses described only nuclear expression of survivin as an independent prognostic factor, whereas cytoplasmic survivin had no prognostic importance (34, 35). Vischioni and co-workers predicted a longer relapsefree survival for nuclear survivin levels only (36). Consistent with our data Hofmann *et al*. (18) described a strong survivin mRNA up-regulation in NSCLC without significant relations to any clinico-pathological parameter. In accordance with our results another study with 83 patients performed by Falleni and co-workers (19) could not detect the proclaimed association of survivin expression with patient survival. In this study, both survivin mRNA levels and protein expression were analyzed.

However, due to the frequent overexpression in tumor cells, survivin might be a target for early diagnosis of cancer, for example by noninvasive analysis of sputum and blood (37). The use of survivin as a diagnostic tool for cancer has been reviewed (38). Our group has recently demonstrated that survivin mRNA expression can be detected in peripheral blood of gastrointestinal cancer patients (39). Recently Dong *et al*. (40) reported on survivin mRNA detection in sputum samples and pleural effusions as a new diagnostic approach for lung cancer.

Conclusion

Our data confirm frequent overexpression of survivin mRNA in NSCLC. Consistent with other reports, a correlation between survivin mRNA-expression and survival could not be detected. Because of its role as a promoter of lung cancer, survivin might be a marker for noninvasive detection of lung cancer rather than for prognosis.

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