Overexpression of *Survivin* Levels in Circulation and Tissue Samples of Lung Cancer Patients

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Abstract. *Background: Survivin, an apoptosis inhibitor protein, has multiple functions that favor cancer cell survival. We sought to determine survivin levels in blood samples and biopsies from patients with lung cancer compared to normal individuals and healthy lung tissues respectively. Patients and Methods: Blood samples were obtained from 32 patients with non-small cell lung cancer (NSCLC) and 49 healthy individuals. Tissue samples were also collected, 15 NSCLC biopsies and 15 histopathologically normal lung tissues. For quantitative evaluation of survivin mRNA expression levels, the hybridization polymerase chain reaction (PCR) method was used. Results: Overexpression of survivin was detected in all malignant samples. In spindle carcinomas survivin expression levels were higher than in adenocarcinomas (p=0.009) and squamous carcinomas (p=0.026). In moderately-differentiated tumors, survivin levels were higher compared to poorly differentiated ones. (p=0.0054). Disease's stage was not associated with survivin expression in blood and biopsies from patients with NSCLC. Conclusion: Survivin is overexpressed in blood and tissue of patients with NSCLC and is associated with histological type and tumor grade.*

Survivin is a protein which belongs to the family of inhibitors of apoptosis (IAP) (1, 2). It is considered that it mainly supports the indirect inhibition of caspases, which are involved in the extrinsic and intrinsic pathways of apoptosis (3). Moreover, survivin plays an important role in cell division, especially in the G_2/M phase (2). Survivin is normally expressed in embryonic and fetal tissues, but is almost undetectable in differentiated adult ones (2, 4). However, its overexpression has been reported in several human malignancies including, lung cancer (5).

In non-small cell lung cancer (NSCLC), survivin is overexpressed both in malignant tissue biopsies and in blood (6, 7). However, there are several controversies regarding its role in these patients. Some studies reported that survivin overexpression is associated with worse prognosis in NSCLC (6), while other investigators questioned this finding (8-10). Secondly, there is conflicting evidence regarding correlation of survivin expression with histological type and TNM stage. Finally, levels of survivin have not been simultaneously determined in tissue and blood samples of patients with NSCLC.

To address the above controversies and gaps in evidence, we studied the expression of survivin mRNA both in malignant and healthy tissue biopsies, as well as in blood samples in patients with NSCLC in comparison with healthy individuals.

Patients and Methods

We studied 32 consecutive patients with NSCLC, aged 70 (range=61-78) years, treated in our Institution from January 2011 until February 2012. We obtained blood samples from all patients on the day of the operation for NSCLC, before surgery. In 15 patients of the initial sample, we also obtained lung tissue samples both neoplastic and non-neoplastic during the operation. Moreover, we obtain blood samples from 49 age-matched healthy individuals with no history of cancer, active inflammation or other chronic diseases, such as heart failure, connective tissue diseases and chronic inflammation. All patients provided informed written consent for their participation in the study.

Both tissues and blood were assessed for survivin as described in detail below. The biopsies were stored immediately in RNA later at –80˚C. Ethical permission provided for the study with Scientific Council protocol number 793, 13/07/2011.

RNA Extraction. Total RNA was extracted from frozen (–80˚C) lung specimens and blood samples (leucocytes) using a commercial kit (RNeasy MiniKit; Qiagen Austin, Texas USA), according to the

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Table I. *Main demographics and clinical characteristics of the study population.*

Characteristic	No. patients (%)
Total	32
Gender	
Male	31 (97%)
Female	1 (3%)
Histological subtype	
Adenocarcinoma	15 (47%)
Squamous cell carcinoma	13 (41%)
Spindle cell	4 (12%)
Type of surgery	
Lobectomy	18 (56%)
Other	14 (44%)
T-factor	
T1	8 (25%)
T2	15 (47%)
T3	9 (28%)
Differentiation grade	
Moderately-differentiated	17 (53%)
Poorly-differentiated	15 (47%)
Lymph node metastasis	
Absent	18 (60%)
Present	14 (40%)
Disease stage	
IA	2 (6%)
IB	4 (13%)
IIA	2 (6%)
IIB	2 (6%)
IIIA	9 (28%)
IV	13 (41%)

manufacturer's protocol. RNA was quantified spectrophotometrically and its quality was checked by electrophoresis through denaturing 1.5% agarose gels.

cDNA synthesis. For each sample, 1 μg of quality-checked RNA was used to synthesize cDNA with 2000 units Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen) and 100 pmol/μl hexamer random primers (Invitrogen). RT reaction mix was incubated for 50 min at 37˚C. cDNA yield was assessed after 1:10 dilution by a regular PCR using Abelson Murine Leukemia (ABL) primers. No genomic contamination was detected. Quantification analysis and real time quantitative PCR (qRT-PCR). *Survivin* mRNA expression levels in blood samples and lung tissues biopsies were analyzed by real-time quantitative RT-PCR.

In this study, qRT-PCR was carried out in duplicate for each individual gene for each sample and all reactions were performed twice by means of specific fluorescence dye labeled hybridization probes (Molecular Probes, Eugene, Oregon, USA) which are suitable for high sensitive quantification (11-13). Probe-based detection formats are based on externally-standardized real time RT-PCR using fluorescence resonance energy transfer (FRET) (14) on the thermocycler Light Cycler (Roche, Mannheim, Germany). Primers and probe for Abelson Murine Leukemia (ABL) (15) and *survivin* mRNAs (16) were chosen as described by the literature. The primers, placed in different exons, were tested as not amplifying genomic DNA.

Table II. *Blood and tissue survivin mRNA levels in lung cancer patients and controls.*

	Lung cancer patients	Controls	*p*-Value
Survivin in blood	4.8±7.87	0.15±0.09	0.0001
	1.60 (3.96)	0.12 (0.11)	
Tissue	24±43.94	1.93±3.89	0.0002
	4.09 (27.16)	0.25 (1.64)	

Data are expressed as mean±SD, median (interquartile range).

Reaction volume was 20 μl and carried out with 2x mastermix (QuantiTect Multiplex PCR Master Mix, Qiagen) 10 μl, 10 pmol/μl of each 3' and 5' primer (TIB MolBiol, Berlin, Germany) 0.5 μl, 5 pmol/μl of each probe (TIB MolBiol) 0.25 μl, 2 μl cDNA (from 1 μg RNA) and adjusted to 20 μl reaction final volume with dd.H_2O. Mixtures were then incubated in the Light Cycler instrument. Forty-five cycles of PCR amplification were run at 95˚C for 15 s for denaturation, 65˚C for annealing 10 s, and at 72˚C for 20 s for extension. Melting curve experiments had previously established that the fluorescence signal for each amplicon was derived from the products only, and no primer dimers were found.

Absolute quantification was carried out by calculating the ratio of the number of the transcripts of the target gene to that of the reference gene, Abelson Murine Leukemia (ABL). The external standards consisted of 10-fold serial dilutions of K562 cell line mRNA. The real-time PCR efficiencies were calculated from the slope of -3.361 Amplification efficiencies were similar (1,984-1,986) between target and reference gene. RNA samples from 15 lung cancer biopsies and 15 normal lung biopsies, as well as their respective blood samples were analyzed. Values of normal lung tissue mRNA expression to lung cancer mRNA ratio were calculated providing fine comparison.

Statistical analysis. Categorical variables are expressed as the number of patients (percentages of the corresponding population). Continuous variables were tested for normal distribution using the Kolmogorov-Smirnov test; as none was distributed normally, they are all expressed both as mean±SD and as median (interquartile range). Continuous variables were compared using the Mann-Whitney *U* and Kruskal-Wallis tests. *Post-hoc* comparisons were performed using the Tukey test. Bivariate correlation was performed using the Spearman test. All results presented are statistical significant at $p<0.05$.

Results

Table I summarizes the patients' demographics and tumor characteristics. Overexpression of survivin was detected in all malignant tissues and blood samples of patients with NSCLC. More specifically, *survivin* mRNA expression levels in blood were significantly higher in patients with NSCLC compared to healthy individuals (Table II). In addition, there was a correlation between survivin levels and histological type. In spindle carcinomas, survivin expression was higher

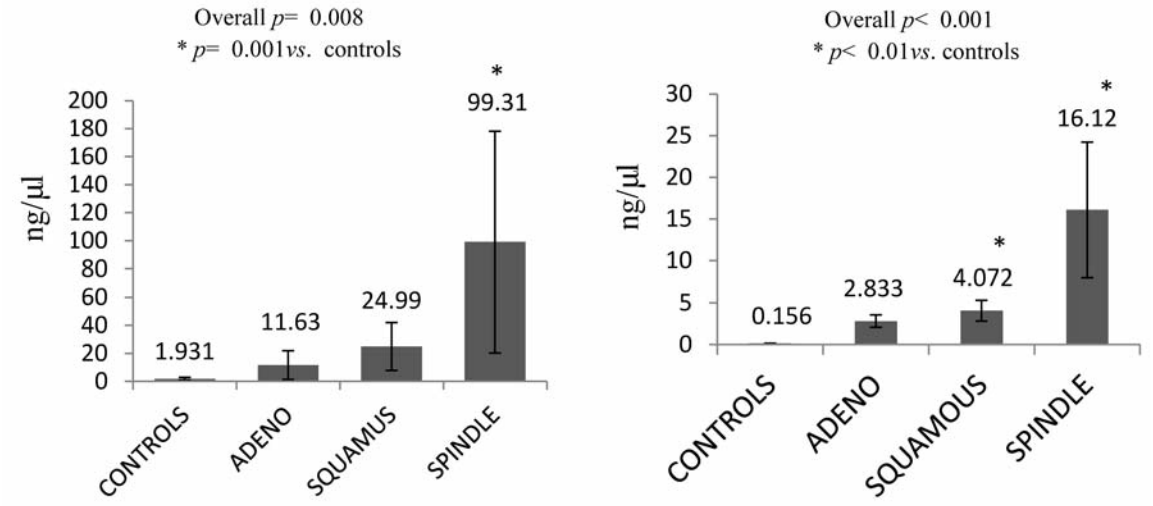

Figure 1. *Levels of survivin RNA in tissue (left panel) and blood (right panel) according to histological type in patients with non-small cell lung cancer.*

than in adenocarcinomas and squamous carcinomas, both in tissue and in blood samples (Figure 1).

Regarding biopsies, *survivin* mRNA levels were higher in samples *obtained* from neoplastic than in those *obtained* from non-neoplastic sites (Table II). Likewise, a correlation was observed between tumor grade and histological type for survivin expression levels. Spindle carcinoma exhibited the highest level of survivin expression. In moderately differentiated tumors, the levels of survivin were higher compared to poorly differentiated ones. The stage of the disease was not associated with survivin expression levels, neither in blood nor in biopsies of patients with NSCLC.

Discussion

The innovation of the present study is that we studied a subgroup of 15 patients from the NSCLC group, for whom *survivin* mRNA levels were determined in blood and malignant and non-malignant lung tissues in each patient. Survivin expression was higher in blood and malignant tissues of patients with NSCLC than in the controls. In addition, survivin levels were correlated with histological type both in blood and in tissue; spindle carcinoma had the highest levels compared to squamous and adenocarcinomas. Interestingly, no correlation was observed between survivin levels and stage of the disease, while survivin levels were higher in moderately-differentiated tumors than in poorly-differentiated ones.

Survivin gene expression is up-regulated in different types of cancer, including pancreatic (17, 18), melanoma (19, 20), colorectal (21), breast (22), ovarian (23), and esophagial (24, 25). Up-regulation is correlated with more aggressive disease (26), with resistance to chemotherapy or radiation therapy (4, 27) and is linked to poor prognosis, suggesting that cancer cell survival involves survivin (4, 28, 29).

Overexpression of survivin is present not only in proliferating cells in several types of cancer but also in inflammatory diseases such as acute appendicitis, cystic fibrosis, ulcerative colitis (30). In addition, expression of survivin is observed in osteoarthritis. In non-arthritic cartilages showed none or very weak expression, whereas survivin overexpression was detectable in arthritic cartilage (31).

In accordance with previous reports, we recorded higher survivin levels both in blood and tissue samples of patients with NSCLC. Falleni *et al*. also reported an increase in survivin levels in biopsies (8), while Yie *et al*. found an increase of survivin expression in blood (7).

A number of studies have shown a correlation between survivin levels and histological type of NSCLC. However there is a discrepancy among them regarding levels of survivin in different types. Viscioni *et al*. identified squamous cell carcinoma as the histological type with the lowest levels of survivin compared to other types (32). On the other hand, Falleni *et al*. concluded that survivin levels were higher in squamous carcinomas (8). In the present study, spindle carcinoma was the type with the highest levels of expression compared to adenocarcinomas and squamous cell. In contrast, other researchers reported that there was no association between histological type and survivin levels (7). As a result, no firm conclusions on this issue can be drawn and further research is needed with larger samples in order to determine with precision whether there is a correlation between survivin expression levels and histological type in NSCLC.

Stage of the disease was not associated with survivin levels, neither in blood nor in biopsies in the present study, which is in agreement with previous reports concerning biopsies of patients with lung cancer (8, 9). In contrast, Xu *et al*. (33) found a correlation of survivin levels with the stage of the disease in lung tissues as well as Yie *et al*. (7) who found a correlation, between stage and survivin levels but it must be pointed out that blood sample was evaluated.

There is also a controversy regarding the correlation between survivin's expression levels and tumor grade. Although previous studies found no correlation (6), our findings revealed an association in biopsy samples. We detected higher mRNA levels in moderately-differentiated tumor biopsies compared to poorly-differentiated ones. Similar findings were observed in bladder tumors (34). In blood samples, our findings agree with the literature on the absence of any association between survivin levels and tumor grade (6) and the correlation observed in biopsies may result from the smaller tissue sample compared to blood samples.

The source of increased *survivin* mRNA levels in blood may originate either from circulating cancer cells or from T-lymphocytes. Studies support the hypothesis that circulating cancer cells are responsible for increased mRNA levels in blood in patients with lung cancer (35), while others found an increased expression of survivin levels in T-lymphocytes in patients with leukemia (36). This question falls outside the aims of the present study. Although a correlation would be expected between survivin levels in blood and tissue of the same patient, in the present study this could not be proved statistically. A possible explanation might be the sample's origin. Levels of Survivin mRNA extracted directly from the primary tumor were 5-fold higher compared to those from blood.

Survivin, as an inhibitor of apoptosis, has been implicated in many functions such as inhibition of apoptosis (37, 38), cell proliferation (39), and angiogenesis (40). IAPs strongly associated with the pathophysiology of cancer and its progression (41). Many studies have revealed that overexpression of survivin is correlated with resistance to chemotherapy and radiation (4, 25). In addition, high survivin levels are a negative prognostic factor for the overall survival of patients (6). Accordingly, other researchers mentioned that low survivin levels are a positive prognostic factor for the progress of the cancer and response to therapy (42).

The detection of survivin mRNA levels especially in blood samples may play an important role for the assessment of lung cancer, especially in early disease stages, monitoring the disease, or in selecting and modifying therapeutic strategies. As a result, survivin could be an attractive marker of cancer and a useful tool to evaluate severity, progression and relapse of the disease. Further research is required to reach to firm conclusions and better-investigate the possibilities for the clinical application of survivin as a novel cancer biomarker.

A potential limitation of our study is the over-representation of spindle cell carcinoma; it is known that it generally represents 0.3-1.3% of all lung tumors (43, 44), while in this population, it was present in 20% of cases, despite the fact that patients were consecutively enrolled. This may be a result of the relatively small sample size.

Conclusion

Survivin is overexpressed in blood and malignant tissues of patients with NSCLC. In addition survivin, both in blood and tissue is correlated with histological type, with spindle carcinoma having the highest levels. No correlation was observed with disease stage while survivin levels were higher in moderately-differentiated tumors than in poorly-differentiated ones. Further research is necessary with larger tissue and blood samples, in order to reach to safe conclusions considering whether survivin could be used as a prognostic biomarker and how clinicians could be guided to monitoring therapy strategy.

Conflicts of Interest

None.

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