

ORIGINAL ARTICLE

Significance of survivin mRNA blood levels in patients with melanoma

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Summary

Purpose: Survivin represents a key anti-apoptotic molecule that is highly expressed in the vast majority of tumors. The aim of the study was to examine the significance of survivin mRNA blood levels in melanoma patients.

Methods: In this prospective translational research study, survivin mRNA blood levels were measured in melanoma patients treated with adjuvant interferon or systemic treatment for advanced disease.

Results: Sixty-four patients with melanoma and 40 healthy controls were included. The majority of them had tumor stages III and IV. The upper 95% confidence interval (95%CI) of survivin levels in controls was set as normal cut-off. Fifty-two (81.3%) patients had survivin levels above normal cut-off. Melanoma patients had higher survivin levels than controls ($p < 0.0001$). Survivin levels were non-significantly higher in stage III compared to stage IV patients. Patients

with survivin levels above vs. below median had median progression-free survival (PFS) 19.5 months vs. 7.4 months ($p = 0.045$), but median overall survival (OS) not reached vs. 18.4 months ($p = 0.091$). Cox proportional hazard models showed that only tumor stage was associated with PFS and OS. There was no statistically significant change in survivin levels between baseline and during treatment ($p = 0.845$) or during follow-up ($p = 0.101$).

Conclusion: Although melanoma patients had significantly higher survivin levels than controls, the study showed that survivin mRNA blood levels did not represent an independent prognostic factor for patients with melanoma. The role of circulating survivin should be further examined in larger studies.

Key words: biomarker study, interferon alpha, melanoma, prognosis, survivin mRNA blood levels

Introduction

Survivin is an anti-apoptotic molecule that belongs to a family of proteins called inhibitors of apoptosis proteins (IAP) and is encoded by *BIRC5* gene [1]. Baculovirus IAP repeat (BIR) is a common domain for all IAP members, which is responsible for their anti-apoptotic activity. Apart of survivin wild-type molecule, alternative splicing leads to the formation of 5 different isoforms, survivin Δ Ex3, survivin 2a, survivin 2B, surviving 3a and survivin 3B. Survivin can be found within normal

cell as a homodimer which seems to ensure mitotic integrity by binding to tubulin, as well as in monomer form which most likely exerts its main anti-apoptotic activity. There is evidence that survivin isoforms can form heterodimers with wild-type survivin, thus influencing its action [1]. The role of these heterodimers is under research. Survivin is widely expressed in almost every tumor, whereas it is virtually absent in normal mature tissues, with the exception of actively proliferating cells [2].

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Due to its relative specificity for cancer tissues, survivin has been recognized as an oncoprotein. Apart from its anti-apoptotic effects, also other tumorigenic effects have been described. Some data suggest that survivin mediates cancer cell invasion and migration and promotes tumor metastasis by upregulating NF- κ B. In addition, survivin overexpression might activate Aurora B kinase, thus reducing cell maturation and promoting proliferation and tumorigenesis. Finally, survivin was shown to decrease p53-mediated NF- κ B down-regulation, thus leading to the upregulation of drug transporter proteins. Survivin expression in cancer has been associated with adverse prognosis and resistance to treatment. Also, survivin blood levels as well as the presence of survivin expressing circulating tumor cells were linked to poor survival [2,3].

Notably, survivin was described to be constantly expressed in virtually all melanomas but much less in normal melanocytes [4]. Additionally, survivin expression in melanoma was shown to be predictive of poor disease outcome [5]. Therefore, this protein could represent a good candidate biomarker for melanoma. Survivin protein and mRNA blood levels have been studied in several tumors as potential biomarkers [2]. However, limited data exist about the significance of measuring survivin blood levels in patients with melanoma.

The aim of the present study was to assess the levels of survivin mRNA in peripheral blood of a cohort of melanoma patients and to determine their potential role in predicting prognosis.

Methods

Study population

This prospective translational research study was conducted at the Oncology Unit of the 1st Department of Medicine, Laikon Hospital, from November 2010 to April 2012. Inclusion criteria were melanoma diagnosis, age \geq 18 years and availability of blood samples for translational research. Participants were recruited from the cohort of melanoma patients of the Unit, during routine care. All patients gave written informed consent to participate.

Staging was performed according to the 7th Edition of the American Joint Committee on Cancer (AJCC) staging system [6]. All patients were regularly followed-up by medical history, clinical examination, blood tests and imaging studies. Imaging included computed tomography or magnetic resonance exams repeated approximately every 3 months. Response and tumor progression were assessed according to RECIST1.1 criteria.

Survivin mRNA measurement

Survivin mRNA blood levels were measured before starting treatment with adjuvant interferon or with sys-

temic treatment for advanced unresectable disease (time point 1) and also were serially measured approximately 2 months after starting adjuvant interferon (time point 2) and after completion of interferon during follow-up before relapse (time point 3). Survivin levels were also measured in a cohort of normal controls. These were selected from health professionals without active illnesses and current medication history.

Survivin mRNA was measured by qRT-PCR according to the method described by Kapellos et al. [7]. We chose Abelson murine leukemia (*ABL*) as a reference gene and we constructed the standard curve using mRNA from the cell line K562.

Ethics

The informed consent form was in accordance to the guidelines of the Greek Bioethics Committee and the World Medical Association Declaration of Helsinki [8]. The study protocol was approved by the General Assembly of the National and Kapodistrian University of Athens Medical School on 28 July 2010. Informed consent was obtained from all patients enrolled in the study.

Statistics

For statistical analyses we have used the IBM SPSS Statistics v24.0 software (IBM, New York, USA). The aim of the present study was to assess the role of survivin as a biomarker in patients with recent diagnosis of melanoma. Survivin expression levels were studied as continuous as well as categorical variable. Cut-off was the median value as well as the upper 95% confidence interval (95%CI) of the distribution of survivin levels in normal controls. The association of survivin levels with baseline patient characteristics was examined by parametric and non-parametric tests. Categorical variables were compared by chi-square test, while continuous variables were examined for normality of the distribution by D'Agostino & Pearson omnibus normality test, and were compared by parametric (t-test) and non-parametric (Mann-Whitney or Kruskal-Wallis) tests. Survivin levels at different time points were compared by Wilcoxon test. Survival estimates analyzed were progression-free (PFS) and overall survival (OS). PFS was defined as the time from the date of diagnosis to the date of tumor progression or to the date of death without documented tumor progression. Non-progressing patients were censored at the date of last follow-up or death. OS was defined as the time from the date of diagnosis to the date of death from any cause. Alive patients were censored at the last date of documentation of their survival status. Survival curves were created by the Kaplan-Meier method and were compared by Log-rank test. Cox-proportional hazard models were used to assess the prognostic significance of cofactors. All comparisons were two-tailed with level of significance $p < 0.05$.

Results

Baseline patient characteristics

Sixty-four patients with melanoma and 40 healthy controls were included in the study. Base-

line demographic and tumor characteristics, as well as follow-up data, were recorded in 60 patients. Baseline characteristics of melanoma patients are shown in Table 1. The majority of the patients were men, with median age above 50 years, while the most common tumor stages were III and IV.

Survivin in healthy controls

We measured survivin expression in 40 healthy controls. Survivin values ranged from 0.054 to 0.459. The distribution of the values failed D'Agostino & Pearson omnibus normality test. Me-

dian value was 0.163 and the interquartile range (IQR) was 0.116. The upper 95% confidence interval (95%CI) of the distribution of survivin levels was 0.348 and we have set this value as a cut-off for normal range.

Survivin in melanoma patients

We measured survivin mRNA blood levels in 64 patients with melanoma. Median survivin level was 2.30 (range, 0.01-843.0). The distribution of the values failed D'Agostino & Pearson omnibus normality test. There were 52 (81.3%) patients with

Table 1. Baseline patient characteristics and their association with survivin mRNA blood levels

Characteristics	Total		Survivin levels		p value	High survivin		Low survivin		p value
	n	(%)	Median	(range)		n	(%)	n	(%)	
Age					0.102					0.507
Median (range), Years	54	(15-85)				56.5	(23-85)	52	(15-85)	
<60	38	(63.3)	0.98	(0.01-260)		29	(60.4)	9	(75.0)	
≥60	22	(36.7)	2.98	(0.18-843)		19	(39.6)	3	(25.0)	
Sex					0.824					0.735
Male	41	(68.3)	1.58	(0.01-843)		32	(66.7)	9	(75.0)	
Female	19	(31.7)	2.40	(0.13-441)		16	(33.3)	3	(25.0)	
Stage (AJCC7)					0.467					0.558
I	2	(3.3)	6.21	(5.43-6.98)		2	(4.2)	0	(0)	
II	3	(5.0)	2.30	(1.98-2.34)		3	(6.3)	0	(0)	
III	24	(40.0)	4.43	(0.08-441)		20	(41.7)	4	(33.3)	
IV	31	(51.7)	1.35	(0.01-843)		23	(47.8)	8	(66.7)	
Total	60	(100)	2.17	(0.01-843)		48	(100)	12	(100)	

AJCC7: American Joint Committee on Cancer staging 7th Edition; High survivin: survivin mRNA blood levels above normal cutoff; Low survivin: survivin mRNA blood levels within normal range.

Table 2. Baseline patient characteristics and their association with survivin mRNA blood levels above or below median

Characteristics	Survivin levels below median		Survivin levels above median		p value
	n	(%)	n	(%)	
Age, Years					0.425
<60	22	(68.7)	16	(57.1)	
≥60	10	(31.3)	12	(42.9)	
Sex					0.586
Male	23	(71.9)	18	(64.3)	
Female	9	(28.1)	10	(35.7)	
Stage (AJCC7)					0.280
I	0	(0)	2	(7.1)	
II	2	(6.3)	1	(3.6)	
III	11	(34.4)	13	(46.4)	
IV	19	(59.3)	12	(42.9)	
Total	32	(100)	28	(100)	
Total	60	(100)	2.17	(0.01-843)	

AJCC7: American Joint Committee on Cancer staging 7th Edition.

survivin levels above normal range cut-off. All patients grouped together had higher survivin levels than controls (Mann-Whitney test, $p < 0.0001$).

Survivin levels were higher in stage III melanoma at baseline in comparison with stage IV melanoma patients (Table 1). However, the difference did not reach statistical significance (Table 1). Furthermore, patients with stage III and patients with stage IV (as distinct groups) had significantly higher survivin levels in comparison to controls (Kruskal-Wallis test, $p < 0.001$). Notably, 4/24 (16.7%) patients with stage III and 8/31 (25.8%)

with stage IV melanoma had normal survivin levels (Table 1). Also, there was no difference in survivin levels, either as continuous variable or as categorical (normal vs. high), based on the cut-off for normal values that we have determined, between males and females and different age groups (Table 1).

Finally, we also examined survivin levels as a dichotomous variable using the median as cut-off (i.e. above or below median), as shown in table 2. In this case, again there was no difference between age, sex or tumor stage (Table 2).

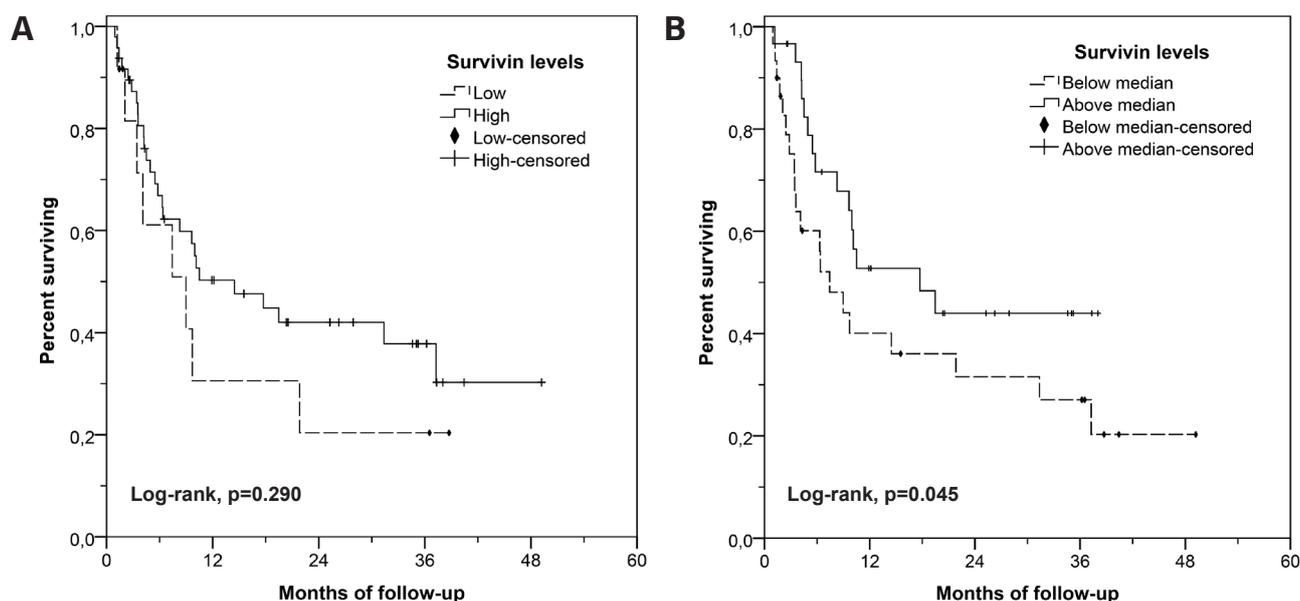


Figure 1. Progression-free survival curves for patients with survivin mRNA blood levels (A) within normal limits (low) or above normal limits (high) and (B) below or above median value of 2.30.

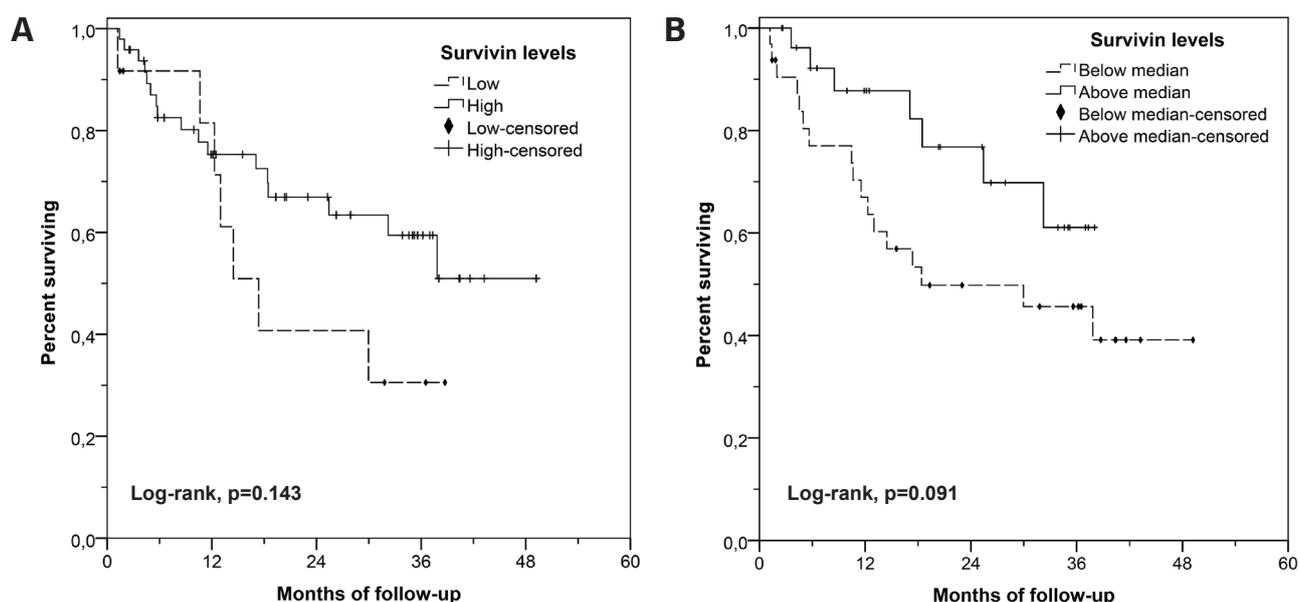


Figure 2. Overall survival curves for patients with survivin mRNA blood levels (A) within normal limits (low) or above normal limits (high) and (B) below or above median value of 2.30.

Table 3. Prognostic significance of baseline survivin mRNA blood levels adjusted for other covariates by Cox proportional hazard model

Covariates	Values	HR	95%CI	p value
<i>Progression-free survival</i>				
Age	≥60 vs. <60 years	1.060	0.499-2.254	0.880
Sex	male vs. female	0.651	0.284-1.492	0.311
Stage (AJCC7)	IV vs. I-III	5.079	2.290-11.262	<0.001
Survivin	above vs. below median	0.777	0.372-1.621	0.501
<i>Overall survival</i>				
Age	≥60 vs. <60 years	0.554	0.204-1.503	0.246
Sex	male vs. female	0.882	0.362-2.144	0.781
Stage (AJCC7)	IV vs. I-III	3.088	1.252-7.617	0.014
Survivin	above vs. below median	0.658	0.265-1.633	0.366

AJCC7: American Joint Committee on Cancer staging 7th Edition.

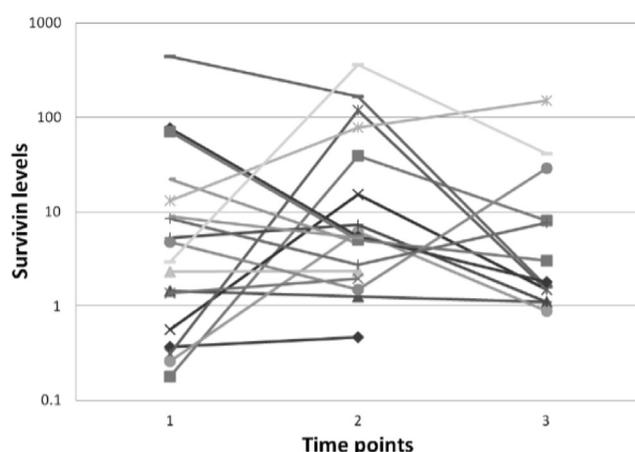


Figure 3. Survivin mRNA blood levels at different time points during treatment with adjuvant interferon. Time point 1: before starting treatment; time point 2: approximately 2 months after starting treatment; time point 3: after completion of treatment.

Prognostic significance

After a median time of follow-up of 34.6 months (range, 1.2-49.2), 35 (58.3%) patients had disease progression, while 25 (41.7%) died. Median PFS was 10.1 months (95%CI 3.9-16.4), while OS was 37.8 months (95%CI not assessable).

The prognostic significance of survivin mRNA blood levels for PFS was examined by using different cutoffs, as described in Methods. Patients with survivin levels above normal had better PFS (median 14.5 months, 95%CI 3.1-25.8), than those who had survivin levels within normal limits (median 8.7 months, 95%CI 1.7-16.3). However, this difference did not reach statistical significance (Log-rank test, $p=0.290$). Similarly, patients with survivin levels above the median had median PFS 19.5 months (95%CI not assessable), while those with survivin

levels below median had a median PFS 7.4 months (95%CI 3.0-11.8), with a statistically significant difference (Log-rank test, $p=0.045$). Respective Kaplan-Meier survival curves are shown in Figure 1. Cox proportional hazard model showed that only tumor stage was independently prognostic for PFS (Table 3).

Overall survival

Median OS of patients with survivin levels above normal cutoff was not reached, while patients with survivin levels within normal limits had median OS 17.4 months (95%CI 10.8-23.9). There was no statistically significant difference in OS between these 2 groups (Log-rank test, $p=0.143$). Also, patients with survivin levels above median had marginally longer OS (median not reached) than those with survivin levels below median (18.4 months, 95%CI 0-42.1, $p=0.091$). Respective survival curves are shown in Figure 2. Cox proportional hazard model showed again that only tumor stage was associated with OS (Table 3).

The effect of interferon on survivin levels

Among patients treated with adjuvant interferon, survivin levels were serially measured in 18 patients at time point 2 and in 13 patients at time point 3. Median survivin levels at baseline (time point 1) were 3.85 (range, 0.18-441), during interferon treatment (time point 2) were 5.38 (range, 0.47-365) and during follow-up (time point 3) were 1.8 (median, 0.89-150.70). In 10 patients survivin levels increased with interferon treatment, while in 8 patients they decreased between time points 1 and 2. Wilcoxon test did not show statistically significant change in survivin levels between time points 1 and 2 ($Z=-0.196$, $p=0.845$). Survivin levels

increased in 3 patients and decreased in 10 patients between time points 2 and 3 (Wlcoxon $Z=-1.642$, $p=0.101$). Figure 3 demonstrates survivin levels at different time points for each patient.

Discussion

In the present study, we demonstrated that survivin mRNA blood levels above median in patients with melanoma were associated with prolonged PFS but not with longer OS. However, this association was lost in multivariate analysis. Survivin levels in blood have been extensively studied in many tumor types, mostly non-small cell lung cancer [7,9-13], as well as in pancreatic [14,15], esophageal [16], gastric [17] and ovarian cancer [18], mesothelioma [19], gall bladder cancer [20], hepatocellular carcinoma [21] and acute lymphoblastic leukemia [22]. In the majority of these studies, high survivin levels were demonstrated to have adverse prognostic significance, when measured as protein levels by ELISA [14,15,22] or as mRNA levels by qRT-PCR [11-13,16,17].

The prognostic significance of survivin has also been studied in patients with melanoma. Only few studies were conducted so far and most of them demonstrated that survivin expression in melanoma is associated with poor clinical outcome [5,23-25]. Gradilone et al. [23] showed that all patients lacking survivin expression in sentinel lymph nodes were progression-free after a median follow-up of 52.9 months, while among those with sentinel lymph nodes positive for survivin, 61.5% relapsed. Takeuchi et al. [24] demonstrated that survivin mRNA expression was increased in almost all patients with metastatic melanoma. Patients with higher survivin mRNA expression in their melanoma had statistically shorter survival compared to those with lower survivin levels. Piras et al. [5] found a correlation between survivin and p53 expression in melanoma and their combined use had the strongest prognostic value. Additionally, Conway et al. [26] described a strong correlation of survivin and osteopontin mRNA with the former having independent poor prognostic significance. Simonetti et al. [27] studied the role of survivin immunohistochemical expression in a small sample of patients with oral mucosal melanoma and found a significant positive correlation with adverse clinical outcome. In contrast, survivin immunohistochemical expression was not associated with OS in a study of 70 melanoma patients [28]. Overall, a systematic review of high quality translational research studies indicated survivin as one of the most promising prognostic biomarkers among 254 genes associated with outcome [25].

However, the role of circulating survivin is less studied in melanoma patients. The advantage of examining survivin levels in blood is that they do not require the collection of tumor tissue, they can be measured at any time in a blood sample and also they can be serially measured and correlated with tumor outcome. However, to our knowledge, only 2 studies assessed the prognostic significance of survivin levels in blood in melanoma patients [29,30]. Both studies measured circulating protein levels by ELISA. Tas et al. [29] examined 42 patients with dissected and metastatic melanoma. Surprisingly and in contrast to our findings, they did not detect a significant difference in survivin levels between melanoma patients and controls. Also, survivin levels did not correlate with any prognostic parameter. Finally, survivin levels did not change significantly in patients who received adjuvant interferon-alpha (IFN- α), while they significantly increased in those treated with dacarbazine for metastatic disease [29]. Jovic et al. [30] examined tumor tissue as well as circulating levels of survivin protein in 84 patients with melanoma. Tissue survivin expression was positively correlated with more aggressive and advanced disease characteristics, with the presence of metastasis and with shorter disease-free interval. Serum survivin levels were significantly higher in melanoma patients than in controls. However unexpectedly, serum survivin levels were inversely correlated with survivin tumor expression. Also, serum survivin levels were significantly higher in patients with melanoma AJCC stage IA, Clark II depth of invasion, superficial phase of spread, no lymphovascular infiltration, higher mitotic activity or higher lymphocyte infiltration. However, serum survivin levels did not correlate with Breslow stage, tumor regression or ulceration and the presence of metastasis or clinical outcome.

To our knowledge, the present study examined for the first time survivin mRNA blood levels in patients with advanced unresectable melanoma before starting systemic treatment as well as in patients who underwent recently melanoma dissection and were starting adjuvant IFN- α . Although our study failed to demonstrate the independent prognostic significance of survivin mRNA levels, it provided several interesting findings. Firstly, we showed that melanoma patients had significantly higher survivin mRNA blood levels than normal controls. However, no difference was observed between patients with advanced unresectable disease and those with completely resected melanoma. Unexpectedly, a subgroup of postoperative patients had significantly elevated survivin mRNA in blood, despite the absence of macroscopic residual disease. This finding is important because it confirmed

previous findings [30] that in melanoma patients circulating survivin is not always proportional to tumor survivin, in contrast to other tumor settings as described above.

Several explanations can be speculated for this discordance. It has been shown that survivin might have a role in normal cell and organ physiology and in non-cancerous disease settings as well, such as brain, liver, pancreas, gastrointestinal, urogenital, gynecological and autoimmune diseases [31,32]. However, we could not identify any obvious associations of survivin blood levels with concurrent illnesses, as reflected by the lack of association between advanced age and higher survivin levels. Another potential explanation might be the potential role of survivin in wound healing. However, we could not identify any compelling evidence in the literature supporting this association. Nevertheless, many investigators described a possible role for survivin in keloid formation [33]. However, even if we accept the hypothesis that wound healing increases survivin blood levels, this does not explain why Tang et al. [13] demonstrated a significant decrease in survivin levels post surgery. Finally, Jovic et al. [30] gave another potential explanation for the discordance between survivin tumor expression and blood levels; they pointed out a study showing that survivin is excreted by cancer cells within exosomes [34]. Thus, exosomal survivin might not necessarily reflect intracellular survivin concentration.

A second interesting finding is that in the present study survivin mRNA blood levels did not change significantly with IFN- α adjuvant treatment. In fact, we observed large fluctuations in survivin levels in some cases, but without any significant trend toward an increase or a reduction. We did not examine the prognostic significance of the changes in survivin levels, because only few patients had serial survivin measurements. A possible explanation for these fluctuations in survivin levels is that IFN- α might stimulate normal cells

to secrete survivin. IFN- α influences many different cell functions and among them the immune system [35]. As described above, survivin might have a role in autoimmunity, thus it is plausible to suggest that IFN- α might affect survivin levels. However, to our knowledge, this hypothesis has not been demonstrated yet.

The present study has strengths and weaknesses. To our opinion, the strengths are that we assessed for the first time survivin mRNA blood levels in melanoma, that we included a healthy control group as well as adjuvant patients without postoperative evidence of residual melanoma and, finally, that we measured serially survivin levels in patients receiving adjuvant IFN- α , which is completely novel. In contrast, the weaknesses are the relatively small sample size, the heterogeneity of patient and treatment characteristics and the lack of data regarding survivin expression in the tumor.

In conclusion, this study examined for the first time the significance of survivin mRNA levels in blood of patients with advanced unresectable melanoma and those who underwent complete melanoma surgical dissection. Patients with melanoma had significantly higher survivin mRNA levels than normal controls. Also, high survivin levels were paradoxically associated with better outcome, but this was not confirmed in multivariate analysis, probably confirming previous reports that circulating survivin might not reflect its expression in the tumor. Moreover, we examined for the first time how adjuvant IFN- α treatment influences survivin levels in patients without evidence of active disease post surgery. The role of circulating survivin in melanoma patients should be studied more thoroughly and in larger cohorts.

Conflict of interests

All authors declare that they have no conflict of interest related to the study. This research did not receive funding from any source.

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