Abstract. The deregulation of apoptosis is characteristic of human carcinogenesis. Survivin, an inhibitor of apoptosis, p53 and p16, two tumour suppressor proteins involved in cell cycle control, play a central role in apoptosis. The aim of this study was to investigate, in primary cutaneous melanoma from 68 patients, the expression of survivin with respect to p53 or p16; the association of these proteins, alone or in combination with clinicopathological features; and, most importantly, to elucidate the role of these markers in predicting survival. The level of survivin expression was significantly higher in the p53 positive group of melanomas compared with the p53 negative one, suggesting a cooperative effect in favouring the progression of melanoma, while no correlation was found between survivin and p16. Moreover, the altered expression of nuclear survivin, p53 and p16 were all associated with poor survival, as demonstrated by univariate analysis. However, these biomarkers have been shown to have superior predictive value when studied in combination (P<0.0001) rather than alone, while the risk of mortality grew progressively with increasing the number of altered biomarkers. These data suggest that the assessment of the combined marker status and number of altered markers in patients with melanoma provides important additional prognostic information that may help in patient selection for adjuvant therapies.

Introduction
Survivin is a member of the inhibitor of apoptosis protein (IAP) family that is almost absent in normally differentiated cells, but can be recognized in developing fetal tissues (1,2). It is overexpressed in almost all types of human malignancies and such overexpression is associated with poor prognosis in affected individuals, an increased rate of tumour recurrence and resistance to certain anticancer agents and radiation (3). Survivin has been implicated in multiple essential functions, including cell division, programmed cell death or apoptosis, the cellular stress response and checkpoint mechanisms of genomic integrity (4). The expression of survivin is regulated in a cell cycle-dependent manner and is most abundant in cells at the G2-M phase of the cell cycle. The literature uniformly shows that survivin expression protects normal or transformed cells from apoptosis (5-7) although the mechanism through which survivin antagonizes cell death is controversial. Apoptosis is induced by p53, a protein involved in cell cycle checkpoint mechanisms, in response to DNA damage, thereby preventing cell cycle progression (8). Following its induction in response to DNA damage, p53 up-regulates the expression of various genes that contribute to cell cycle arrest, DNA repair or apoptosis. Since survivin and p53 are critical modulators of the opposing cellular processes of proliferation and apoptosis, the analysis of the interaction between mechanisms regulating the expression of each of these proteins appears to be particularly attractive. Another cell cycle suppressor, p16INK4a, seems to interact with survivin. It has been demonstrated, in human hepatoma cell lines, that survivin competitively interacts with p16INK4a to remove it from the Cdk4/p16INK4a complex. The resultant Cdk4/survivin complex activates the Cdk2/cyclin E complex leading to Rb phosphorylation for the S-phase progression (9).

In primary and metastatic cutaneous melanoma, survivin overexpression has been detected (10-13) and demonstrated to be an independent biomarker for disease recurrence and overall survival in affected patients (14-16). As regards p53, its role in melanoma has been controversial. Several studies showed that p53 immunoreactivity is significantly and independently associated with a decreased survival (17), but the relation between mutations and protein overexpression is not clear. Numerous surveys have found either rare or absent TP53 point mutations or allelic loss in surgical specimens
from primary and metastatic melanomas (18), raising questions as to the pathogenetic relevance of the p53 pathway in melanoma. p16\textsuperscript{INK4a} gene alterations, that were originally associated with the development of familial melanoma, frequently occur in sporadic melanoma. Loss of p16 protein expression has been associated with increased tumour cell proliferation and a decreased survival of melanoma patients (17,19).

On the basis of these findings, the aim of this study was to investigate i) the expression of survivin with respect to p53 or p16 proteins in 68 stage I and II primary cutaneous melanomas according to the American Joint Committee on Cancer (AJCC) staging system (20); ii) the association of these proteins, alone or in combination with the clinicopathological features of patients; and, iii) most importantly, to elucidate the role of these markers in the prediction of survival.

Materials and methods

Patients and tissue specimens. Archival tissue blocks of sporadic primary skin melanoma from 68 patients, who underwent observation at the Oncologic Hospital ‘Businco’, Cagliari, Italy, and at the Department of Pathology, Cancer Center of Solca, Cuenca, Ecuador, between November 1995 and May 2006, were selected for further study according to the following criteria: melanoma with vertical growth phase, no evidence of metastasis at the time of diagnosis and complete clinical data, including the follow-up, until January 2007. Lymph node status and the presence of metastases were verified by a clinical and pathological examination. The patients included 38 women and 30 men, ranging in age from 12 to 100 years (mean 67.2±17.6). The anatomic location of the primary tumour included 16 tumours located in the trunk, 14 head and neck, 11 in the upper extremities and 27 in the lower extremities. According to Clark's classification (21), 10 tumours were level II, 16 level III, 24 level IV and 18 level V. According to the AJCC staging system, 19 tumours were stage IА, 8 IB, 19 IІА, 20 IІB and 2 IІC.

Each tumour, after surgical resection, was fixed in formalin and completely embedded in multiple paraffin blocks. The sections taken from the block with the largest tumour thickness were evaluated. Tumoral areas were identified on haematoxylin and eosin-stained sections and on adjacent sections stained immunohistochemically for melanoma-associated antigens, including S-100 protein, melan A and HMB-45 (see below). An independent histopathological analysis was performed by two pathologists (C.F and J.U.) on separate occasions. Informed consent was obtained from all the patients involved in the study.

Immunohistochemistry. Serial microtome sections, 5-μm thick, were treated for the immunohistochemical staining of survivin, p53, p16\textsuperscript{INK4a} and melanoma-associated antigens S100, melan A and HMB-45, using the alkaline phosphatase-streptavidin method. Heat-induced antigen retrieval was performed at 95°C for 40 min in 10 mM citrate buffer solution (pH 6.0), for survivin, p53, p16\textsuperscript{INK4a} and melan A, and by immersion in 0.1% trypsin solution in PBS, at 37°C for 10 min, for S-100 protein and the HMB-45 antigen. Non-specific binding was blocked with 10% normal goat or normal horse serum for 45 min. Rabbit polyclonal antibodies against recombinant human survivin protein (Novus Biologicals, Littleton, CO, 1:2000 dilution), and to bovine S-100 protein (Dako, Glostrup, Denmark, 1:1000 dilution), mouse monoclonal antibodies to human p53 protein (clone DO-7, Dako Glostrup, Denmark, 1:50), to human p16INK4a/MTS1 (clone 16P04, NeoMarkers, Union City, CA, USA, 1:50), to human melan A (clone A103, Dakopatts, 1:100 dilution), and to human HMB-45 (clone HMB-45, Dakopatts, 1:100 dilution) were used as primary antisera. Biotinylated anti-rabbit and anti-mouse immunoglobulin G were used as secondary antisera (Vector Laboratories, Burlingame, CA, 1:1000 dilution) and reacted with the fast red substrate system (Dakopatts). Negative controls were established by replacing the primary antibodies with normal serum. Melanoma specimens, which strongly expressed survivin, p53 or p16 were used as positive controls.

Evaluation of immunoreactivity. Since survivin appears to have different functions correlated with its subcellular location (22), we evaluated nuclear and cytoplasmic staining separately and in combination (total survivin). The entire tumour of each case was microscopically evaluated to find areas with the strongest survivin immunoreactivity. In at least five x400 magnification fields of these areas survivin positivity was graded, for each tumour, on the basis of the intensity and the average number of positive cells, as follows: cases with >10% of cells showing a moderate/strong intensity of nuclear or cytoplasmic staining were considered positive. In adjacent sections, microscopic fields corresponding to those selected for a count of survivin-positive cells, were found to evaluate p53- or p16-positive cells. Regarding p16 expression, we evaluated the nuclear and cytoplasmic staining separately. Cases with a moderate/strong intensity of p53 or nuclear/cytoplasmic p16-positive cells and with >10% of positive cells were defined as positive. All markers were placed in one of two categories: altered or normal. Positive cases for survivin or p53 and negative cases for p16 expression were considered as altered.

Statistical analysis. Data were computed with the SPSS 15.0 statistical software package. The association of survivin with p53 or p16 expression and the correlation of these molecular markers, alone or combined with the clinicopathological variables were assessed by Fisher’s exact test. Overall survival was calculated from the date of histological diagnosis to the date the patients succumbed due to melanoma, or the last follow-up, until January 2007. Survival information was unavailable for 4 patients. Data on patients who succumbed to other causes were censored at the time of death. The Kaplan-Meier method was used to calculate survival functions. Comparisons were made using the log-rank test and were adjusted for specified prognostic factors. We undertook univariate and multivariate survival analyses with the Cox proportional hazard model and the likelihood ratio test. The 95% confidence intervals for survival were calculated and reported. Differences were considered significant at P<0.05.
Results

Immunohistochemistry. Total survivin was detected in 81% of cases. Positive cells were distributed homogeneously throughout the tumour of some cases, while others showed a heterogeneous staining pattern with groups of positive and negative cells side by side. In 29 cases, survivin was observable in the nucleus and cytoplasm (Fig. 1A). In 12 cases, the nuclear reaction was prominent with a low cytoplasmic reaction (Fig. 1B), while 14 cases exhibited staining confined exclusively to the cytoplasm (Fig. 1C). The arrows indicate mitotic figures.

Table I. Comparison of survivin expression in relation to p53 and p16 status.

<table>
<thead>
<tr>
<th></th>
<th>Total patients</th>
<th>Total survivin</th>
<th>P</th>
<th>Nuclear survivin</th>
<th>P</th>
<th>Cytoplasmic survivin</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>38</td>
<td>35</td>
<td>0.012</td>
<td>30</td>
<td>0.001</td>
<td>27</td>
<td>0.205</td>
</tr>
<tr>
<td>Negative</td>
<td>30</td>
<td>20</td>
<td>0.759</td>
<td>21</td>
<td>0.323</td>
<td>22</td>
<td>0.801</td>
</tr>
<tr>
<td>Nuclear p16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>34</td>
<td>28</td>
<td>1</td>
<td>20</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>34</td>
<td>27</td>
<td>0.323</td>
<td>21</td>
<td>0.801</td>
<td>22</td>
<td>0.801</td>
</tr>
<tr>
<td>Cytoplasm p16</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>40</td>
<td>33</td>
<td>0.759</td>
<td>22</td>
<td>0.323</td>
<td>26</td>
<td>0.801</td>
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<tr>
<td>Negative</td>
<td>28</td>
<td>22</td>
<td>1</td>
<td>19</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Fisher's exact test.

Statistical analysis. Survivin expression in relation to p53 or p16 status is presented in Table I. The total survivin expression was significantly higher in the p53 positive group of mela-
nomas compared with the p53 negative. When the nuclear and cytoplasmic expression was separately analyzed, a significant correlation resulted between nuclear survivin and p53 staining. No association was demonstrated between survivin and p16 expression.

Table II shows the expression of the three markers and their associations with clinicopathological characteristics. The total survivin expression was significantly associated with tumour thickness, AJCC stage and anatomical location. The significance of these associations increased when nuclear staining was considered. Nuclear staining also correlated with the Clark level, while no relationship was found between cytoplasmic survivin and the examined clinicopathological parameters. P53 staining was correlated with tumour thickness and the Clark level. No significant correlation was found between the nuclear or cytoplasmic p16 expression and other clinicopathological variables. The Kaplan-Meier univariate analysis demonstrated that an altered expression of nuclear survivin, p53 and nuclear p16 was associated with increased melanoma mortality, even if the significance for p16 was borderline (P<0.0001, P=0.013 and P=0.052, respectively). The predictive value of nuclear survivin and p53, but not of nuclear p16 expression (P=0.06), maintained the significance.

![Figure 3. Overall survival analysis, performed using the Kaplan-Meier method, is illustrated for patients with melanoma who had a number of altered biomarkers.](image)
after adjusting the model by independent prognostic factors (P<0.05) such as tumour thickness, the Clark level and AJCC stage. An altered cytoplasmic survivin and p16, and the clinicopathological factors considered did not correlate with overall survival (P>0.05).

On the basis of the evidence that only an altered expression of nuclear survivin, p53 and nuclear p16 showed a prognostic value, they were analyzed in combination. Table III shows associations of the number of altered markers with the clinical and pathological characteristics of patients. There was a significant association with the tumour thickness, Clark level and AJCC stage. The Kaplan-Meier univariate analysis showed that the risk of melanoma mortality rose progressively with an increasing number of altered biomarkers (P<0.0001, Fig. 3). The multivariate analysis demonstrated that the prognostic value was retained after adjusting for tumour thickness, Clark level and AJCC stage (P<0.05). This analysis was also conducted using the Cox proportional hazard model. The results are shown in Table IV.

### Discussion

Cancer cells resist programmed cell death with an altered expression of pro-apoptotic and anti-apoptotic proteins, and with loss of tumour suppressor genes, thereby bypassing internal surveillance checkpoints and acquiring an invasive phenotype. Survivin and p53 play a central role in the regulation of apoptosis, and p16, an inhibitor of cell proliferation, is involved in pathways strongly associated with tumours. However, the mechanism of survivin up-regulation in tumours is only partially understood. Survivin is slowly expressed in fast dividing normal cells in a cell cycle-dependent manner (23), while other non-cell cycle-dependent mechanisms, that can drive survivin gene transcription independently of mitosis, are proposed as dominant in tumours (4) and this explains the up-regulation of this protein in nearly every tumour independently of cell-cycle periodicity (5). It has been reported that a transcriptional factor, such as p53, can regulate survivin expression in various human cancer cells (24,25). P53 appears able to repress transcription of the survivin gene by directly binding the p53 element that is present in the survivin promoter. It is possible that p53 acts alone or in combination with other proteins, such as E2F (a transcriptional activator) or sin3 and HDAC. These factors have an affinity with p53 and can form a complex to bind the survivin promoter and repress it (26).

Herein, we demonstrated that 81% of human melanoma specimens overexpress survivin, while p53 immunostaining has been found in 56% of cases. Accumulation of the p53 protein in tumour cells, enabling its immunohistochemical detection, has been regarded as a marker for p53 mutation.
as a result of nuclear translocation. A relationship between the Cdk4/p16INK4a complex and initiated the cell-cycle entry into S-phase shift which coincided with its nuclear translocation. (9). Overexpression of survivin resulted in an accelerated proliferation, a process representing the balance between cell birth and death as influenced by the activity of up-regulated mitotic proteins, such as nuclear survivin, and by the functional loss of apoptotic proteins, such as p53. However, although pathological characteristics, such as tumour thickness, Clark level and AJCC stage, have previously been recognized as significant associations between these molecular markers and tumour thickness or the Clark level, because they reflect the degree of cellular proliferation and tumour thickness (31,32). In the present study, nuclear survivin staining, as well as p53, were found to be significantly associated with tumour cell proliferation and tumour thickness (31,32).

In the present study, expression of survivin was significantly higher in the p53 altered group of melanomas. Of note is the association between the accumulation of p53 and nuclear survivin expression reported in laryngeal squamous cell carcinoma (30). On the basis of this evidence, we analyzed the presence of survivin or p16 staining in the nucleus and cytoplasm in corresponding tumoral areas. However, no significant association was found between these proteins.

Molecular markers have the potential to further identify individuals who are likely to have tumour progression. Other studies on stage I cutaneous melanoma have suggested that increased p53 expression may be associated with tumour cell proliferation and tumour thickness (31,32). In the present study, nuclear survivin staining, as well as p53, were found to be significantly associated with tumour thickness, Clark level and AJCC stage I and II melanomas. Of note is the association between these molecular markers and tumour thickness or the Clark level, because they reflect the degree of cellular proliferation, a process representing the balance between cell birth and death as influenced by the activity of up-regulated mitotic proteins, such as nuclear survivin, and by the functional loss of apoptotic proteins, such as p53. However, although pathological characteristics, such as tumour thickness, Clark level and AJCC stage, have previously been recognized as prognostic factors, the ability of a molecular marker to predict survival independently of the specified prognostic factors is important for treatment planning. Multivariate analyses showed that an altered expression of p53 and nuclear survivin was still associated with poor survival after adjusting for specified prognostic factors, whereas an altered expression of p16 correlated with survival with a borderline significance.

The ubiquitous survivin expression and change of its subcellular localization during the cell cycle has been reported (9). Overexpression of survivin resulted in an accelerated S-phase shift which coincided with its nuclear translocation. Furthermore, a competitive interaction between survivin and p16 has been suggested. Survivin competitively interacted with the Cdk4/p16complex and initiated the cell-cycle entry as a result of nuclear translocation. A relationship between expression of survivin gene and p16 protein was recently found in laryngeal squamous cell carcinoma (30). On the basis of this evidence, we analyzed the presence of survivin or p16 staining in the nucleus and cytoplasm in corresponding tumoral areas. However, no significant association was found between these proteins.

Table IV. Univariate and multivariate Cox regression analyses of survivin, p53 and p16 expression for the prediction of survival in melanoma patients.

<table>
<thead>
<tr>
<th></th>
<th>Nuclear survivin</th>
<th>p53</th>
<th>Nuclear p16</th>
<th>Combined biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR: 95% CI</td>
<td>HR: 95% CI</td>
<td>HR: 95% CI</td>
<td>HR: 95% CI</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>Univariate analyses</td>
<td>9.67</td>
<td>4.07</td>
<td>0.32</td>
<td>4.52</td>
</tr>
<tr>
<td></td>
<td>2.1-44.5</td>
<td>1.22-13.58</td>
<td>0.097-1.077</td>
<td>2.26-9.01</td>
</tr>
<tr>
<td></td>
<td>0.004</td>
<td>0.022</td>
<td>0.066</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Multivariate analyses</td>
<td>9.50</td>
<td>3.83</td>
<td>0.35</td>
<td>4.77</td>
</tr>
<tr>
<td>Thickness</td>
<td>9.37</td>
<td>3.70</td>
<td>0.36</td>
<td>2.14-10.59</td>
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<tr>
<td>T1+T2 vs.</td>
<td>1.80-48.63</td>
<td>1.09-12.52</td>
<td>0.11-1.22</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>T3+T4</td>
<td>0.008</td>
<td>0.035</td>
<td>0.10</td>
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<tr>
<td>Clark level</td>
<td>9.50</td>
<td>3.83</td>
<td>0.35</td>
<td>4.77</td>
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<td>II+III vs.</td>
<td>1.87-48.33</td>
<td>1.14-12.88</td>
<td>0.10-1.17</td>
<td>2.16-10.57</td>
</tr>
<tr>
<td>IV+V</td>
<td>0.007</td>
<td>0.030</td>
<td>0.088</td>
<td>&lt;0.0001</td>
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<tr>
<td>AJCC stage</td>
<td>9.60</td>
<td>3.68</td>
<td>0.36</td>
<td>4.75</td>
</tr>
<tr>
<td>IA+IB vs.</td>
<td>1.85-49.84</td>
<td>1.09-12.44</td>
<td>0.11-1.23</td>
<td>2.14-10.55</td>
</tr>
<tr>
<td>IIA+IIB+IIC</td>
<td>0.007</td>
<td>0.036</td>
<td>0.104</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*aHazard risk; bconfidence interval; Cox proportional hazard model and models adjusted for tumour thickness, Clark level and AJCC stage.

since wild-type p53 is generally not detected by immuno-histochemistry because of its short half-life (27). However, it is known that positive staining for the p53 protein may reflect either gene alterations or aberrant stabilization of the wild-type (non-mutant) protein. In melanoma, numerous studies have found either rare or absent TP53 point mutations (18). In contrast, support for the role of p53 in the pathogenesis of melanoma has been suggested by Mintz and colleagues, who showed that the SV40 T antigen (which inactivates RB and p53) generates a highly penetrant and aggressive melanoma phenotype (28).

In the present study, the expression of survivin was significantly higher in the p53 altered group of melanomas, with 35 cases showing survivin overexpression, compared with the p53 normal group. Since p53 may be a transcription inhibiting factor of survivin, a functional loss of the p53 protein, either for gene alterations or aberrant stabilization of the wild-type protein, may be responsible for the survivin up-regulation in melanoma. A correlation between the accumulation of p53 and survivin expression resulted in other tumours, such as gastric, pancreatic, prostate, lung, epidermoid and laryngeal squamous cell carcinoma (26,29). Our findings show a significant association between the presence of survivin expression and the presence of p53 staining, also in melanoma.

The ubiquitous survivin expression and change of its subcellular localization during the cell cycle has been reported (9). Overexpression of survivin resulted in an accelerated S-phase shift which coincided with its nuclear translocation. Furthermore, a competitive interaction between survivin and p16 has been suggested. Survivin competitively interacted with the Cdk4/p16complex and initiated the cell-cycle entry as a result of nuclear translocation. A relationship between
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