Prognostic prediction of the immunohistochemical expression of p16 and p53 in cutaneous melanoma: a comparison of two populations from different geographical regions

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$p16^{INK4a}$ and p53 are tumor-suppressor genes frequently altered in various malignancies, including cutaneous melanoma. The purpose of the study was to establish the prognostic value of immunohistochemical expression of $p16^{INK4a}$ and p53 in sporadic cutaneous melanoma (CM) in two regions with a high-risk for melanoma in Italy and Ecuador. Immunohistochemical staining of p16 and p53 was performed in samples of primary CM from 82 patients with Stage I and II melanoma according to the American Joint Committee on Cancer (AJCC) staging system. Survival differences between categories of p16 or p53 expression were analyzed using the product-limit procedure (Kaplan-Meier method, log-rank test). Clinical variables (gender, age, tumor location, Clark's level, thickness) were correlated with survival and p16 or p53 expression. p16 nuclear immunoreactivity was observed in 85% of Italian patients compared to 48.7% of Ecuadorians; a small number of cases showed p53 immunoreactivity in both populations. Only nuclear p16 expression exhibited a significant correlation with survival (Italians p=0.001, Ecuadorians p=0.017) but did not appear to correlate with any clinicopathological parameter. No significant difference was observed in survival with regard to p53 expression or cytoplasmic p16. Our results demonstrate that nuclear expression of p16 can be considered a molecular prognostic factor in patients with sporadic CM and indicate its importance as a clinical marker.

Key words: cutaneous melanoma, prognosis, biological markers, p16, p53.

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The frequency of melanoma has been reported to be increasing at an alarming rate (Weinstock, 2006). Epidemiological studies have related the rate of melanoma to both ethnographic and geographical factors: the incidence of melanoma varies according to latitude, altitude, sun exposure patterns, and genetic factors, such as skin type and pigmentation (Wang *et al.,* 2001; Tucker and Goldstein, 2003). Malignant melanoma exhibits a variable clinical phenotype, even in patients with apparently localized stages who undergo curative surgical resection. Despite advances in early detection, certain patients are havocked by rapidly occurring disease recurrence and progression. At present, the new AJCC staging system incorporates only tumor thickness, ulceration, satellites, and nodal status as prognostic factors (Balch, 2002), although additional predictors are required when considering the most appropriate surgical and therapeutic approach. In the present report, we evaluated the immunohistochemical expression of the two genes frequently altered in various malignancies, including skin cancer, namely p53 and $p16^{INK4a}$ (Tucker and Goldstein, 2003), in order to assess their clinical utility (Matsumura and Ananthaswamy, 2002). The p53 tumor-suppressor gene, involved in cell cycle regulation, has an anti-proliferative and anti-transforming activity (Giglia-Mari and Sarasin, 2003) and can induce cell arrest in G1 phase and apoptosis. p16, the product of the *CDKN2A/p16INK4a* gene, induces G1 cell cycle arrest by inhibiting the phosphorylation of Rb protein by the cyclin-dependent kinases CDK4 and CDK6 (Ohtani N *et al.,* 2004). The loss of cell cycle regulatory function of p53 and p16 has been previously implicated in the development and/or progression of skin cancers, including melanoma, although conflicting results have been reported (Straume *et al.,* 2000; Basset-Seguin, 2001; Pavey *et al.,* 2002;

Soufir and Ghiorzo *et al.,* 2004; Mihic *et al.,* 2006; Nilsson *et al.,* 2006).

The aim of our study was to correlate the immunohistochemical expression of p53 and p16 in sporadic melanoma with survival and clinicopathological parameters in two different populations coming from two regions potentially with high-risk for melanoma for genetic, geographic factors, and different sun exposure patterns. The first, Azuay, that shows the highest rates of skin cancer among all Ecuadorian regions (NCR, Solca Quito, 1995-2000), is located on the Andes Cordillera at 2500 m above sea level. The second is an Italian island, Sardinia, that is located between 38° and 41° of latitude.

Materials and Methods

All studies were approved by the respective Ethics Committees.

Patients and tissue samples

From January 1995 to January 2001, 115 melanomas (from 44 male and 71 female patients, age range 14-99) were diagnosed at the Department of Pathology, Cancer Center of *Solca,* Cuenca, Ecuador; during the same period, 130 melanomas were diagnosed (from 72 males and 58 females, age range 11-94) at the Department of Pathology, Oncologic Hospital *A. Businco,* Cagliari, Italy. These hospitals are the only oncologic centers in the two regions. All melanomas were obtained from the archives of the respective Departments of Pathology. Information regarding anatomic location, age and gender were available for all cases. There was no history of familial occurrence in any case.

From all patients, cases were selected that had: 1) Stage I and II melanoma according to AJCC staging system (Balch, 2002); 2) original tissue blocks of the primary tumor available for immunohistochemistry; 3) complete clinical data including follow-up, from time of diagnosis of melanoma, until October 2005; 4) time and cause of death. The selected cases included 41 patients from Azuay (17 males, 24 females, mean age 57.6±2.8 years, median follow-up 28 months) and 41 patients from Sardinia (19 males, 22 females, mean age 56.2±2.1 years, median follow-up 54 months). Both patient groups were similar to the total number of patients in the respective regions with respect to tumour location, age, and gender. All specimens from both pathology centers were fixed in 10% buffered formalin (average period of fixation 36 hr) and embedded in paraffin. Melanomas were grouped according to the AJCC staging system (Balch, 2002), Clark's level of invasion (Clark *et al.,* 1969, 1989), and histologic subtype. Among Ecuadorian cases 16 were superficial spreading melanoma (SSM), 9 nodular melanoma (NM), 9 acral lentiginous melanoma (ALM), and 7 were lentigo maligna melanoma (LMM), while in the Sardinian cases 27 were SSM, 10 NM, 1 ALM, and 3 were LMM. The results derived from independent histopathologic review by two pathologists (R.C., J.U.) on separate occasions. The immunohistochemistry was performed in the laboratory of Department of Cytomorphology at Cagliari University at the same time for all specimens.

Immunohistochemical staining

Paraffin sections, 5-6 µm-thick, were treated for the immunohistochemical evaluation of melanoma-associated antigens using the streptavidin-biotin alkaline phosphatase method. Slides were rehydrated in PBS and antigen retrieval was performed by immersion in 0.1% trypsin solution in PBS at 37°C for 5 min or by microwave heating for 20 min in 10 mM citrate buffer solution (pH 6.0). Sections were treated for 45 min with 10% normal goat serum in PBS or normal horse serum in PBS. Rabbit polyclonal antibody to bovine protein S100 (Dako, Glostrup, Denmark; 1:1000), mouse monoclonal anti-human HMB45 (clone HMB45, Dako, Glostrup, Denmark; 1:100), and mouse monoclonal anti-human melan A (clone A103, Dako, Glostrup, Denmark; 1:100) were used as primary antisera for 60 min at room temperature, and biotinylated anti-rabbit and anti-mouse IgG were used as secondary antisera (Vector Laboratories, Burlingame, CA, USA; 1:200) for 30 min at room temperature. The sections were then incubated in alkaline phosphatase streptavidin (Vector Laboratories, Burlingame, CA, USA; 1:1000) for 30 min at room temperature, reacted with Fast Red Substrate System (Dako Glostrup, Denmark) or with Dako® Fuchsin+ Substrate-Chromogen (Dako, Glostrup, Denmark) and then counterstained with Mayer hematoxylin and mounted in glycerol gelatin (Sigma, St. Louis, MO, USA). The sections were thoroughly rinsed in PBS between each step. Additional microtome sections were treated for the

immunohistochemical analysis of $p16^{INK4a}$ and p53, using the alkaline phosphatase streptavidin method. Antigen retrieval was performed by microwave heating for 20 min in 10 mM citrate buffer solution (pH 6.0). Mouse monoclonal antibody to human $p16^{INK4a}$/MTS1 (clone 16P04, NeoMarkers, Union City, CA, USA; 1:50) and mouse monoclonal antibody to human p53 Protein (clone DO-7, Dako Glostrup Denmark, 1:50) were used as primary antisera and biotinylated anti-mouse IgG was used as secondary antiserum. In negative control sections, the specificity of the antisera was tested by replacing the primary antibodies with normal serum and one case of melanoma, which strongly expressed p16, was used as positive control for p16 staining as well as one case of melanoma, which strongly expressed p53, was used as positive control for p53.

Staining interpretation

Immunohistochemical staining of p16 and p53 proteins was assessed using a microscope Zeiss AxioPhot2 (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). The fields selected for analysis were occupied solely by tumor cells. Because p16 cytoplasmic staining is controversial (Talve *et al.,* 1997; Sparrow *et al.,* 1998; Ghiorzo *et al.,* 2004; Evangelou *et al.,* 2004) we decided to evaluate cytoplasmic and nuclear staining separately. A positive immunostaining for p53 was defined as nuclear reactivity of neoplastic cells to the p53 antibody. We evaluated the entire tumor area and selected the fields for the score that were representative of all tumor area. The staining intensity of both p16 or p53 positive tumoral area was graded as follows: no/weak staining, moderate staining and strong staining. The mean percentage of both p16 or p53 positive tumor cells in at least five areas, occupied only by tumor cells, at X400 magnification was determined and assigned to one of the following categories: a) <10% positive cells; b) 10-30%; c) >30% positive cells. As previously described (Straume *et al.,* 2000), cases with moderate or strong staining intensity and with more of 10% of positive cells were defined as positive; all others were defined as negative.

Statistical analysis

Statistical analyses were performed using the SPSS 11.5 statistical software package (SPSS, Inc., Chicago, IL, USA). Survival differences between positive and negative cases for p16 (nuclear or cytoplasmic) or p53 expression in tumoral cells among Azuayan or Sardinian specimens were performed using the product-limit procedure (Kaplan-Meier method) with the date of histological diagnosis as the starting point, and of death from melanoma as the end point. Comparisons were made with use of the log-rank test, and were adjusted for other potential prognostic factors. Patients who died of other causes were censored at the date of death. The influence of p16 and p53 expression on survival was analyzed by Cox proportional hazards model and tested by the likelihood ratio test. Correlation of clinical variables with survival including gender, age, primary tumor location, Clark's level of invasion (Clark *et al.,* 1969, 1989), tumor thickness, was analyzed by the Kaplan-Meier method. Association between different clinical variables and p16 or p53 expression was assessed by Fisher's exact test.

Results

All melanoma specimens showed positive immunostaining for at least two of the melanoma markers (Figure 1A).

Eighty-five percent of samples (95%Confidence Interval, 95%CI, 67.6-92.3) from Sardinian patients and 48.7% of samples from Azuayan patients (95%CI, 32.5-62.7) were positive for p16 nuclear expression, while 73.2% (95%CI, 58.1-84.3) of cases were positive for p16 cytoplasmic staining in Sardinian patients and 34.1% (95%CI, 21.6-49.5) in Ecuadorian patients.

p53 immunoreactivity was found in a low proportion in both populations with 19.1% (95%CI, 7-31) of Azuayan patients and only 5% (95%CI, 0-11.7) of Sardinians showing p53 positive immunostaining. The intensity of p53 staining was from moderate to strong in most cases (Figure 2A, B).

In several specimens p16 immunoreactivity was stronger at the advancing edge of the tumor (Figure 1B). p16 immunostaining reactions showed a distribution and a rate of immunoreactive cells over a wide range of variability (Figure 1C, D, E, F). There was large variation in staining intensity for the cytoplasmic or nuclear compartment and in most cases p16 staining intensity was stronger in the nuclear than in the cytoplasmic compartment.

Only p16 nuclear expression exhibited statistically significant correlation with patient survival

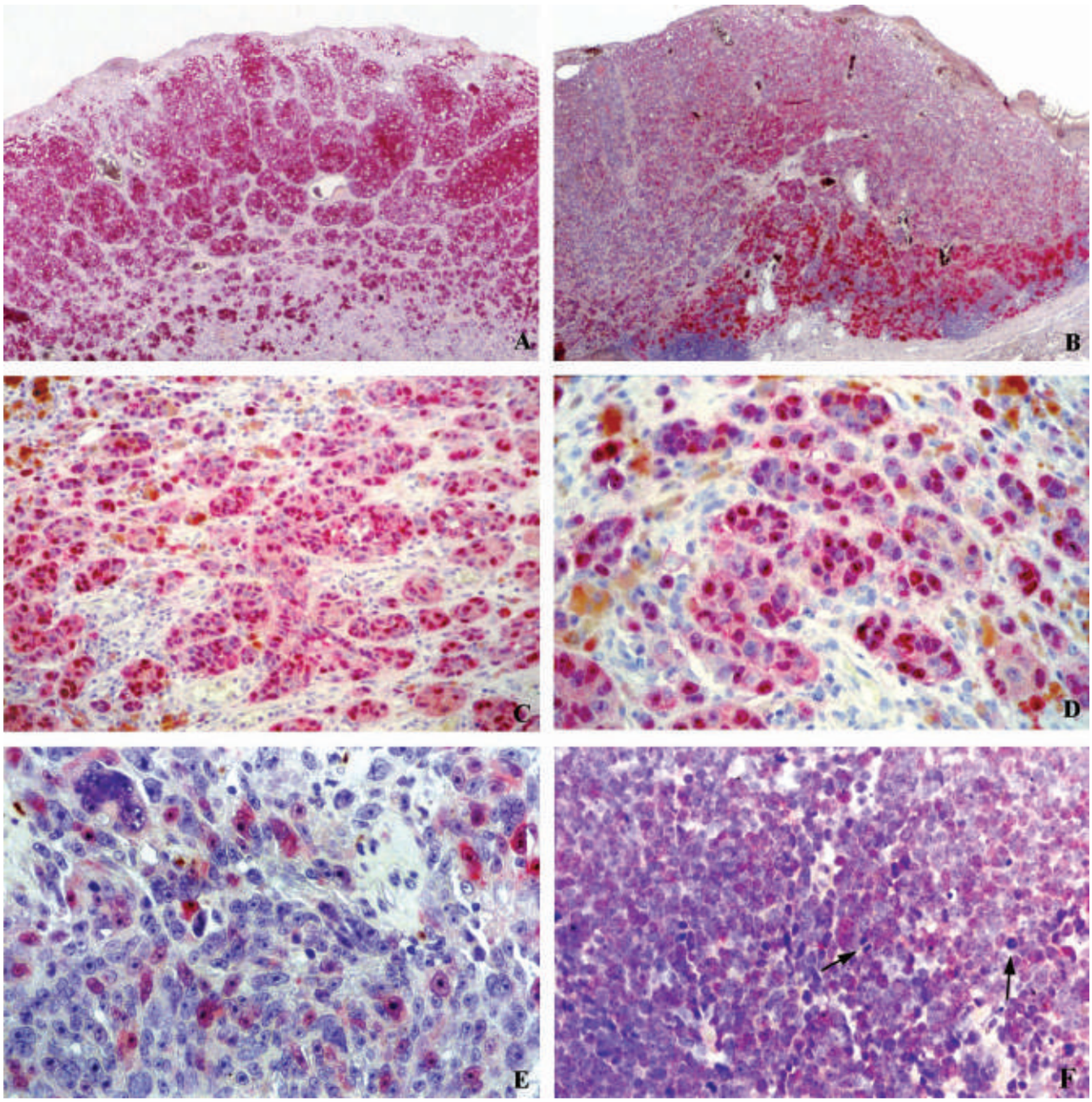

Figure 1. Immunohistochemical expression of p16 protein in cutaneous melanoma. A. tumoral area marked by HMB45. B. the same tumoral area of image A shows a stronger p16 staining at the advancing edge of tumor. The staining was both nuclear and cytoplasmic. C-D. high ratio of positive tumoral cells and strong intensity of the staining. E. moderate ratio of positive tumoral cells. F. moderate intensity of the staining. Mitoses are observable (arrows). A, B, x40; C, D, E, x200; F, x400.

(Kaplan-Meier method) in both Sardinian patients (log-rank test, 10.27; p=0.001; Figure 3) and Azuayan patients (log-rank test, 5.69; p=0.017; Figure 4), while p16 cytoplasmic expression did not correlate with survival. The overall survival was significantly higher in patients with more than 10% of nuclear p16 positive cells and moderate to strong intensity of p16 expression in both Sardinian cases (Table 1) and in Azuayan patients (Table 2). The prognostic relevance of nuclear p16 expression, assessed using the Cox proportional hazard model, suggested that cases negative for p16 nuclear staining had a significant increased risk of death with a Hazard Ratio (HR) of 4,764 (95%CI, 1.640-13.842; p=0.004) for Sardinian patients and a HR of 2,525 (95%CI, 1.137-5.607; p= 0.023) for Ecuadorians. Fisher's exact test demonstrated a correlation between nuclear and cytoplas-

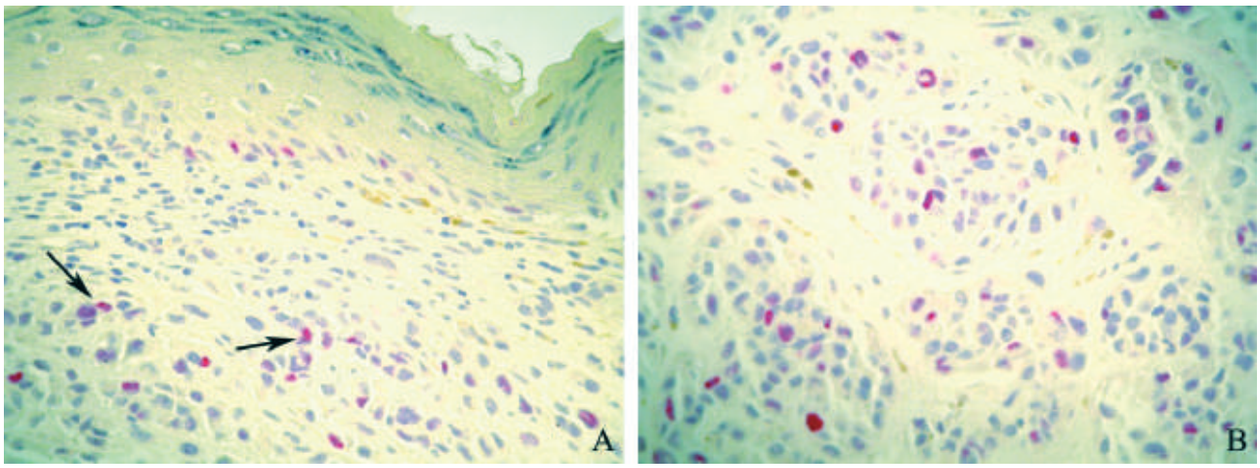

Figure 2. Immunohistochemical expression of p53 protein in cutaneous melanoma. A-B: positive tumoral cells are observable (arrows). A, B, x400.

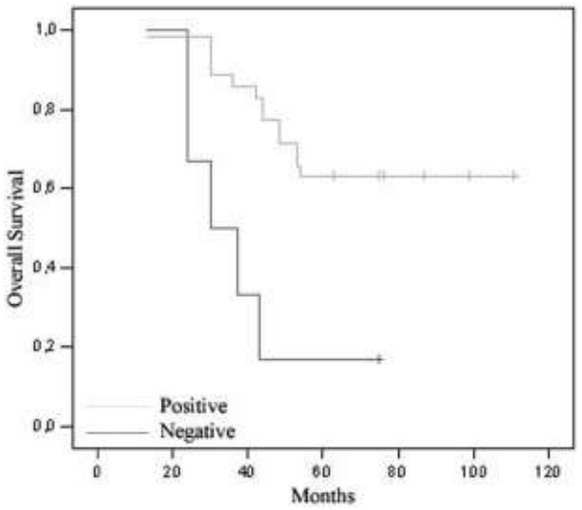

Figure 3. Overall survival, performed by the Kaplan-Meier method, for melanoma Sardinian patients.

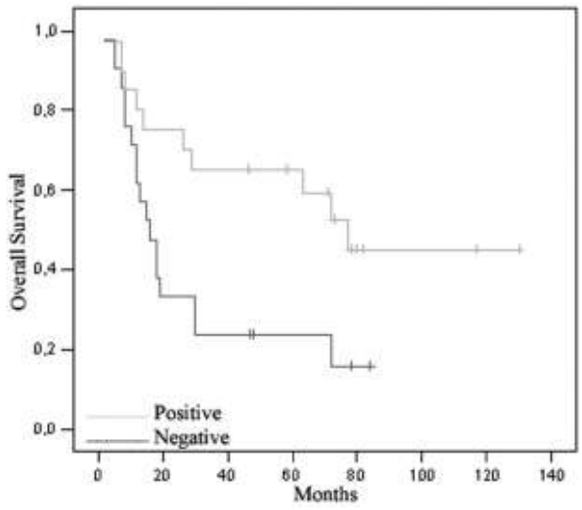

Figure 4. Overall survival, performed by the Kaplan-Meier method, for melanoma Azuayan patients.

mic p16 staining ($p<0.05$). Nuclear p16 positivity was still predictive of better survival after the statistical model was adjusted by cytoplasmic staining in both Sardinian (log rank test, 9.64; $p= 0.001$) and Ecuadorian patients (log rank test, 8.75; $p= 0.003$).

p53 expression had no significant correlation with either survival (Table 1, Table 2) or p16 expression ($p>0.05$). The clinical variables, analyzed in each population, did not show a significant correlation with survival with the exception of Clark's level, tumour thickness in Sardinian cases (Table 1) and gender in Ecuadorian cases (Table 2). No correlation ($p>0.05$) between the expression of p16 (nuclear or cytoplasmic) or p53 and gender, age, primary tumor location, Clark's level of invasion, tumor thickness, was found in either Sardinians or in Ecuadorian patients using the Fisher's exact test. Among the clinicopathologic variables, the anatomic location of the primary tumor more frequently involved in Azuayan patients was the face (12 cases; 7 were LMM), and the foot (14 among 15 cases localized in lower extremities; 7 were ALM), while in Sardinians was the trunk (12 SSM, 5 NM) and the leg (9 among 11 cases localized in lower extremities; 8 were SSM and 1 was NM).

Discussion

We have analyzed the prognostic significance of p53 and p16 protein expression in two populations different for genetic, geographic factors, sun expo-

Table 1. Statistical data of p16, p53 expression, and clinic pathologic variables in Sardinian patients.

Variables	*No. of Patients*	*No. of Events*	*5-yr Survival (%)*	*SE* (%)*	*p***
Sardinian cases	41	18	56.1	7.7	
Nuclear p16					0.001
Negative	6	5	16.7	15.2	
Positive	35	13	62.9	8.17	
Cytoplasmic p16					0.788
Negative	11	4	63.6	14.5	
Positive	30	14	53.3	9.1	
p53 expression					0.573
Negative	39	26	56.4	7.9	
Positive	2	1	50	35.4	
Gender					0.787
Male	19	8	57.9	11.3	
Female	22	10	54.5	10.6	
Age					0.181
< 59***	19	6	68.4	10.7	
≥59	22	12	45.5	10.6	
Anatomic site					0.803
Head and neck	6	3	50	20.4	
Trunk	17	8	52.9	12.1	
Extremities	18	7	61.1	11.5	
Clark's level					0.005
I, II, III	20	4	80	8.94	
IV, V	21	14	33.3	10.3	
Thickness					0.002
T1, T2	27	7	74.1	8.4	
T3, T4	14	11	21.4	10.9	

** Standard error. ** Log-rank test. *** Median age.*

Table 2. Statistical data of p16, p53 expression, and clinicopathologic variables in Azuayan patients.

Variables	*No. of Patients*	*No. of Events*	*5-yr Survival (%)*	*SE* (%)*	*p***
Ecuadorian cases	41	27	43.9	7.7	
Nuclear p16					0.017
Negative	21	17	23.8	9.3	
Positive	20	10	65	10.7	
Cytoplasmic p16					0.435
Negative	27	17	48.2	9.6	
Positive	14	10	35.7	12.8	
p53 expression					0.198
Negative	34	24	38.2	8.3	
Positive	7	3	71.4	17.1	
Gender					0.001
Male	17	15	23.5	10.3	
Female	24	12	58.3	10.1	
Age					0.118
< 61***	20	10	55	11.1	
≥61	21	17	33.3	10.3	
Anatomic site					0.080
Head and neck	17	9	52.9	12.1	
Trunk	3	1	66.7	27.2	
Extremities	21	17	33.3	10.3	
Clark's level					0.144
I, II, III	9	4	55.6	16.6	
IV, V	32	23	40.6	8.7	
Thickness					0.064
T1, T2	10	4	60	15.5	
T3, T4	31	23	38.7	8.8	

** Standard error. ** Log-rank test. *** Median age.*

sure patterns, and life style.

The nuclear proteins p53 and p16 are cell cycle regulators that work in a highly orchestrated manner in cellular regulatory pathways. p53 has been studied extensively, and most normal cells and benign lesions do not show detectable expression. p53 is affected by genetic mutations in almost all types of cancer (Giglia-Mari and Sarasin, 2003; Hussein *et al.,* 2003). It has been reported that 80% of intermediate thickness cutaneous melanoma have detectable p53 protein (Hieken *et al.,* 1999). However, other studies reported that p53 gene is expressed in 18% or less of CM (Whiteman *et al.,* 1998) and, in both cases, the expression of p53 gene was not correlated with any clinicopathological parameters. Straume et al (2000) showed that p53 expression was not correlated with clinicopathological parameters but with survival. The present study revealed positive staining of p53 in only 8 Azuayans and 2 Sardinians and p53 expression did not correlate with survival.

Pavey et al (2002) reported that in previous studies nuclear p16 expression was present in 16% to >90% of CM, and suggested that these large differences are probably related to variabilities in technical approaches and patient bias. The difference in positive staining between Ecuadorian and Italian cases did not derive from methodology, since the same procedure was used for all specimens. Despite the different proportions of positive cases among Ecuadorian and Italian cases, nuclear p16 expression was significantly correlated with survival in both groups.

Our findings are in agreement with previous studies that indicate that loss of nuclear p16 protein expression is independent of clinicopathologic factors and is associated with poor prognosis (Straume *et al.,* 2000; Pavey *et al.,* 2002; Bachmann *et al.,* 2004; Alonso *et al.,* 2004).

Several recent and older studies have shown an additional important cytoplasmic localization of p16. Walker et al already has shown in 1999 an additional perinuclear localization of p16 by immunofluorescence. These authors transfected melanoma cell lines with different CDKN2A cDNAs. They could show that different p16 muta-

tions lead to distinct cytoplasmic or nuclear p16 protein localization. Furthermore, recent studies using laser confocal microscopy and electron microscopy have shown the cytoplasmic localization of p16 and the importance of this localization (Ghiorzo *et al.,* 2004; Evangelou *et al.,* 2004; Mihic *et al.,* 2006). However, in the current study, cytoplasmic localization of p16 protein did not correlate with survival confirming previous studies (Straume *et al.,* 2000; Mihic *et al.,* 2006).

Sun exposure and genetic susceptibility have been identified as the two major predisposing factors to skin cancer, including melanoma (Matsumura and Ananthaswamy, 2002).

We found relevant differences between Azuayan and Sardinian cases for the location sites of skin malignant melanoma. In Azuayans the tumor occurred commonly at chronically sun-exposed sites, such as the face, that was mostly LMM subtype. The tumor also frequently occurred at sites not exposed to the sun, such as the foot, mostly ALM, that probably have nothing to do with sun exposure (Elder *et al.,* 2005).

Instead, in Sardinians it occurred mostly at areas intermittently irradiated by UV rays, such as the trunk, both in men and women, with relative sparing respectively at legs, arms and face, confirming the results of other previous studies (Wang *et al.,* 2001). These melanomas included SSM which is the most common subtype and is particularly associated with intermittent sun exposure (Elder *et al.,* 2005). Numerous data were reported in literature about different characteristics of the lesions, anatomic localization relatively to sun exposure patterns, latitude, altitude, and genetic background of different populations. The lower extremities, with ALM subtype, are the common melanoma site in people with pigmented skin as Hispanics, Asians, and Blacks or white females; while the trunk is common in white people with European origin (Balch *et al.,* 1982; Armstrong and Kricker, 1994; Bulliard *et al.,* 1997; Cress and Holly, 1997; Johnson *et al.,* 2003; Eide and Weinstock, 2005; Tsai *et al.,* 2005).

In the current study, no association was found between the lack of p16 expression and body sites usually exposed to sun in both populations. Based on these findings, it is reasonable that other factors different from sun (UV) exposure can exert an influence on the decreased expression of p16.

Among Azuayans, most cases were at larger thickness and higher Clark's level, while among Sardinians the cases with lower thickness and level were more numerous. The substantial difference in primary tumor thickness for Ecuadorian patients may be explained, at least in part, by a delay in diagnosis. Given the evidence that preventive measures and educational efforts have dramatically impacted the diagnosis and outcome of melanoma patients, similar efforts should be directed at the Ecuadorian population.

At present, the prognosis of melanoma is only related to the thickness, the ulceration, satellites, and nodal status (Balch, 2002). However, understanding the biology of melanoma at the molecular level appears to be a prerequisite to improving the prognosis and to considering the most appropriate surgical and therapeutic approach.

Our results indicate that nuclear p16 immunostaining correlates with survival independently of either clinicopathologic, genetic, or geographic factors, and may be considered a prognostic clinical marker for patients with sporadic cutaneous malignant melanoma.

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