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Prognostic significance of tyrosinase expression in sentinel lymph node biopsy for ultra-thin, thin, and thick melanomas

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Abstract. – BACKGROUND: Investigate if the tyrosinase mRNA expression may be predictive of the outcome on ultra-thin, thin, and thick melanoma patients.

AIM: In our study, we sought to correlate tyrosinase mRNA expression to the outcome in a group of 71 patients with thick, thin and ultra-thin melanomas.

MATERIALS AND METHODS: 71 patients with melanomas underwent a SLNB (sentinel lymph node biopsy) at the "Sapienza" University of Rome. Among these, 38 patients had thin melanomas, while the other 33 patients had thick melanomas. In every patient's sample histology, immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) was completed. We then correlated tyrosinase mRNA expression to the statistical analysis of the outcome of patients.

RESULTS: Positivity of histology was found in one patient (1.4%), immunohistochemistry in five patients (7%), and tyrosinase in 52/71 (73.2%). Thickness and tyrosinase positivity were predictive for disease progression ($p < 0.05$). The median follow-up was 58.24 months. There were recurrences and/or deaths in both groups of patients.

CONCLUSIONS: Nodal metastasis in melanoma is uncommon, especially in patients with thin melanomas. In this study, histology and immunohistochemistry were found to be non predictive for the risk of nodal metastases, while instead, tyrosinase m-RNA expression appeared to play a role in highlighting those patients with a risk of disease progression. Moreover, no differences among the thin melanoma groups of patients (0.30-0.75 mm and 0.76-1.00 mm) were observed.

Key Words:
Sentinel lymph node, Thin melanoma, Melanoma, Tyrosinase, RT-PCR.

Introduction

In the United States, the incidence of thin melanoma is increasing more rapidly than the incidence of all cutaneous melanoma, including those with rare location[1-4]. The American Joint Committee on Cancer (AJCC)[5], in 2001, established a staging system that classified tumours with a thickness ≤ 1.00 mm, as a thin melanoma, thus changing the threshold for a T1 melanoma from 0.75 to 1 mm. Thin melanoma generally indicates low-risk disease and good prognosis. Nevertheless, the presence of ulceration (the Clark's level III-IV) have been recognised as (an adverse histological characteristic that may lead to a different prognosis)[6]. Since that time, the thin melanoma subset of patients is currently under investigation in order to find any additional risk factor that may allow a sub staging of this group.

However, long-term follow-up studies have shown thin melanomas to be associated with disease progression; 9.4% of these patients recur in a median follow-up of 11 years, whereas the disease-free survival at 20 years was reported at 59%[7].

Nevertheless, the American Joint Committee on Cancer (AJCC) data of survival at 10 years of follow-up for patients with thin primary melanoma is 87.9% for stage IA, and 83.1% for stage IB[8], and as underlined by Halpern and Marghoob: "*the qualitative description of the progression of thin melanoma has accordingly shifted from excellent to good*"[9].

Recently, intraoperative lymphatic mapping and sentinel lymph node biopsy (SLNB), the first sites of melanoma metastases[10], were developed

*The contribution of A. Gradilone and P. Gazzaniga must be considered equal.

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and have been widely accepted as a precise tool to classify early-stage-melanoma, and in most cases, the sentinel node is the only lymph node invaded by tumour cells[11]. The SLNB in these patients may be useful for the detection of tumoral cells and for a good prognosis of patients. The SLNB is routinely used for patients with lesions of >1.0 mm, however with selected patients, those with thin melanomas (≤1 mm), SLNB has been introduced to study the prognosis of these patients. The indication for the use of SLNB in patients with thin melanoma is not well established, and some questions remain unresolved. The controversial benefit, the low percentage of positive SLN, the good prognosis for most of them and the associated cost are discussed by scientists[12].

To date, the Breslow's tumour thickness is one of the most powerful predictor factors of survival in melanoma patients with stage I and II of the disease[5]. To detect the presence of melanoma cells in sentinel lymph nodes (SLNs) and in peripheral blood, the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) assay was used for the first time in 1991, as a sensitive method to identify tyrosinase mRNA[13-15]. To date, many groups of work have focused their studies on the molecular profile of SLNs in melanoma patients, by using techniques of molecular biology, such as the RT-PCR assay, to obtain a better analysis of the specimens, even if very few data concerning the molecular pattern of SLN in thin melanoma, are present[16].

In our study, we sought to correlate tyrosinase mRNA expression to the outcome in a group of 71 patients with thick, thin and very-thin melanomas, by the statistical analysis, in a median follow-up period of 58.24 months.

Materials and Methods

Patients and Samples

Seventy-one patients (35 males and 36 females), between 17 to 81 years (mean age 54.5), affected by malignant melanoma, were enrolled in this study at the Department of Dermatology and Plastic Surgery of the "Sapienza" University of Rome. An informed consent was obtained from all patients. Patients with primary tumours of Breslow thickness <.75 mm were offered SLNB, only if they had one of the following criteria: Clark level III or IV, ulceration, regression or patient demand.

Tumour thickness and level of invasion were documented, as was the tumour type and stage of disease, according to TNM classification of the International Union Against Cancer[5]. The Breslow thickness ranged between 0.3 mm and 9.00 mm (mean Breslow thickness 1.77 mm). The patients with stage IA of disease were 34/71 (47.8%), those with stage IB were15/71 (21.1%), whereas the patients with stage of disease II A, IIB and IIIA were 13/71 (18.3%), 3/71 (4.2%) and 6/71 (8.45%), respectively. After surgical excision, SLNs were frozen in liquid nitrogen and stored at –80°C, until used for the reaction of RT-PCR. The presence of capsular nevus cells in SLNs was tested by histologic examination, and the positive samples were excluded from the study. A total of 71 SLNs excised from an equal number of patients were collected. In order to verify, by the statistical analysis, the usefulness of the tyrosinase RT-PCR assay in SLN of melanoma patients, we divided our specimens into three different groups of samples according to the Breslow thickness. The first comprised 33 SLN, obtained from patients with a melanoma thickness >1.00 mm; a second group of 26 SLN, from patients with melanoma's thickness included between 0.76 mm and 1.00 mm, which we identified as "normal Thin" (nThin); and the last group of samples with 12 SLN from patients with a melanoma with a Breslow thickness <0.76 mm, which we called "very thin" (vThin) melanoma. The characteristics of patients and samples are included in Table I.

Lymphatic Mapping and SLN Biopsies

Lymphatic mapping and SLN biopsies were successfully performed at the "Sapienza" University of Rome, Department of Plastic and Reconstructive Surgery, as previously described[17]. Each SLN was examined by Hematoxylin-Eosin (H&E), and Immunohistochemistry (IHC), and RT-PCR molecular assay.

Histology and Immunohistochemistry

The sentinel nodes were analysed histologically in serial sections of paraffin-embedded samples, using Haematoxylin and Eosin (H&E) staining, and Immunohistochemistry (IHC), by using antibodies against HMB-45 antigen and S-100 protein (Dakopatts, Hamburg, Germany), and detected with the avidin-biotin-peroxidase technique. Negative controls were obtained if normal animal serum was used instead of specific primary antibodies.

Table I. Features of melanoma patients and results.

Follow-up			Tumor		
OS months	DFS months	OS	Site	Clark	Breslow
135.2	24	#	3	2	0.3
36.3	36.3	d.f.	1	3	0.3
58	58	d.f.	1	3	0.35
44.9	44.9	d.f.	1	2	0.4
18	18	d.f.	1	3	0.5
36.9	36.9	d.f.	1	3	0.54
24	24	d.f.	1	2	0.55
41.3	41.3	d.f	1	3	0.6
82.56	82.56	d.f.	1	3	0.6
27	27	d.f.	2	4	0.6
91.19	91.19	d.f.	3	2	0.65
21	21	d.f.	2	3	0.65
24.56	24.56	d.f.	1	3	0.8
60	60	d.f.	1	3	0.84
49.1	49.1	d.f.	2	3	0.85
83.86	83.86	d.f.	3	3	0.85
48	38	#*	1	2	0.9
109	109	d.f.	1	3	0.9
77.53	77.53	d.f.	1	3	0.9
44	44	d.f.	1	3	0.93
27	27	d.f.	1	3	0.95
24.2	24.2	d.f.	2	4	0.98
83	83	d.f.	0	3	1
58.73	58.73	d.f.	1	3	1
20.56	20.56	d.f	1	3	1
84.06	84.06	d.f.	1	3	1
126	126	d.f.	2	3	1
64	64	d.f.	2	3	1
48.73	48.73	d.f.	1	3	1
48.96	48.96	d.f.	1	3	1
52.167	52.167	d.f.	2	3	1
77.96	77.96	d.f.	2	3	1
78.39	78.39	d.f.	2	3	1
70	70	d.f.	3	3	1
32.6	32.6	d.f.	1	4	1
55.06	55.06	d.f.	2	4	1
57.96	57.96	d.f.	3	4	1
12	12	d.f.	1	3	1
89.43	89.43	d.f.	1	3	1.11
88.26	88.26	d.f.	2	4	1.21
86.93	86.93	d.f.	3	3	1.3
83.8	83.8	d.f.	1	3	1.32
94	90	#*	1	3	1.4
17.667	9.6667	#*	2	4	1.5
102.13	102.13	d.f.	2	4	1.5
49.267	48.267	#*	3	3	1.5
43.167	34.4	#*	2	4	1.5

Table continued

Table I *(Continued)*. Features of melanoma patients and results.

Follow-up			Tumor		
OS months	DFS months	OS	Site	Clark	Breslow
116.43	116.43	d.f.	2	2	1.7
87.63	87.63	d.f.	2	4	1.89
77.433	77.433	d.f.	2	4	2
14.8	7.8667	#*	1	3	2
36.067	25.333	#*	1	3	2
121.73	121.73	d.f.	1	3	2
93.1	93.1	d.f.	1	3	2.1
69.4	69.4	d.f.	2	4	2.25
21.067	1.5	#*	1	4	2.75
42.333	6.4667	#*	1	4	3
82.467	64.033	#*	1	4	3
11.333	8.1667	#*	1	4	3
10.567	5.8333	#*	2	4	3
45.333	44.833	#*	2	4	3
42.267	28.433	#*	2	4	3
19.167	17.9	#*	1	4	3.25
15.367	1.1	#*	1	4	3.4
41.9	24.1	#*	1	4	3.75
61.433	56.433	#*	2	4	4
65.533	65.533	d.f.	3	4	4.5
45.433	23.433	#*	1	5	5
124.06	24.067	#	2	4	6
11.8	11.333	#*	1	5	7
90.39	90.39	d.f.	0	5	9

Abbreviations: P: patient; OS: Overall survival; DFS: disease free survival; d.f.: patients disease free; *Patient died; #Patient with disease progression; Primary melanoma site: 0 = Head and neck, 1 = Trunk, 2 = Lower extremities, 3 = upper extremities; Tyr, Tyrosinase; H&E, Hematoxylin-Eosin; IHC, Immunohistochemistry.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA extracted from the frozen tissues was reverse transcribed and amplified with the tyrosinase upstream and downstream primers as previously described[17]. All the recommended precautions were taken to avoid the possibility of false-positive results. The preparation of the reaction mixture and the analysis of amplified products were carried out in separate rooms. Each RT-PCR experiment included a sample without RNA as a negative control. The analysis of the RT-PCR products was performed by electrophoresis on 2% agarose gel of 20 µl of the amplification products, and only samples that showed the specific amplification product, were considered positive.

Follow-up

Patients were examined prospectively for recurrent or metastatic disease at three-month intervals. The evaluation consisted of a physical examination and routine blood investigations during a median follow-up of 58.24 months; the minimum follow-up time was 10.567 month and the maximum follow-up time was 135.2 months. An ultrasound examination of the regional lymph nodes basins and the abdomen, and a chest X-ray, were performed at least once a year. Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) were also performed in patients with findings suggestive of metastatic melanoma.

Statistical Analysis

To establish those variables that were statistically significant for death and/or progression of the disease, all data were entered into a Microsoft Excel spreadsheet and analysed as follows: contingency tables were evaluated by the χ^2 test. The overall survival curves were compared by the Kaplan-Meier method, with the log-rank test; a p

value of < 0.05 was considered statistically significant. A statistical analysis was performed through MedCalc® version 8.0.0.0 Copyright 1993-2005 Frank Schoonjans[18].

Results

Histology and Immunohistochemistry

A simultaneous analysis of histology and IHC was performed on 71 SLNs of melanoma patients.

One patient was positive at the histologic examination (IHC assay as well). This patient died due to disease. Another five SLNs obtained from five patients showed a negative result by the histologic examination, and a positive result at the IHC assay. Four of these five patients are currently disease free, and one patient had progression of disease.

RT-PCR

In order to investigate the suitability of samples, all RNAs were subjected to RT-PCR using glyceraldehyde-3-phosphate deydrogenase (GADPH) specific primers. They were all found suitable for PCR analysis. Tyrosinase expression was found positive in 52/71 (73.23%) specimens, and negative in 19/71 (26.77%) of examined samples. Concerning the RT-PCR-positive-group, 18/52 (34.5%) patients with a SLN positive for tyrosinase showed progression of disease and died, 2/52 (4%) had progression of disease, and the remaining 32/52 (61.5%) are currently disease free. Among the 19 patients with SLN that showed negative for tyrosinase, 2/19 (10.5%) had progression of disease and died because of disease, whereas 17/19 (89.5%) are still disease free.

In the stage I disease group of patients (49 of 71 SLNs) 32 SLNs were positive for tyrosinase expression while 17 were negative; within the 32 positive patients 26/32 are, at moment, disease free, one patient had progression of disease and 5/32 of patients had disease progression and then died of melanoma. The follow-up of tyrosinase negative patients in disease-stage I (16/17) showed one progression and death due to the disease while 16 are disease free.

In the stage II disease group of patients (16 of 71 SLNs) 15 SLNs were positive for tyrosinase expression and only one patient with a SLN negative for tyrosinase expression is, at moment, disease free; of the 15 RT-PCR assay positive patients, 3/15 are disease free and 12/15 had progression of disease and then died.

In this work only 6 SLNs were analysed from patients with stage III of disease. One SLN was negative for tyrosinase, and the patient was disease free at follow-up. The remaining 5 SLNs were found to be positive for tyrosinase expression. 3 patients were disease free, 1 patient had progression of disease and one died for melanoma. In Figure 1 are summarized the results obtained from RT-PCR assay analyzed respect to disease stage and to follow-up. Agarose gel electrophoresis of RT-PCR products is shown in Figure 2 (Panel a). The characteristics of patients, samples, outcome of patients and results obtained from Histology, IHC and RT-PCR assay, are shown in Table II.

Statistical Analysis

The median follow-up in our series was 58.24 months (range 10.567-135.2).

Table II shows patient characteristics and, according to the univariate analyses (χ^2 test), the risk of death or disease progression. The death risk is related to Breslow's thickness (p trend < 0.0001), Clark's level (p trend = 0.0007) and age (p = 0.0145 p trend = 0.0358), while sex, primary melanoma site, drainage site, histotype and tyrosinase positivity showed no statistical significance. The disease progression is correlated to Breslow's thickness (p trend < 0.0001), Clark's level (p trend = 0.0023), age (p = 0.0031 p trend = 0.0106) and tyrosinase positivity (p = 0.0496) while sex, primary melanoma site, drainage site and histotype showed no statistical significance.

The Kaplan Meier survival curve (Figure 1 Panel b) showed that tyrosinase positivity was statistically significant (p = 0.0485) as a prognostic factor for melanoma patients.

Discussion

Recently, many studies have been published concerning the prognostic value of SLN in thin melanoma. In the first meta analysis focused on SLNB in this subset of patients recently published, has been underlined as, to date, the available data are not adequate to establish criteria for patients selection and to draw conclusions regarding SLNB in thin melanoma patients[19]. Very little literature is available on the molecular research of thin melanoma, mainly performed by techniques such as the histology, IHC[20,21] and rarely the RT-PCR assay[15]. The RT-PCR method can detect one tumour cell out of 10^6 to 10^7 non-

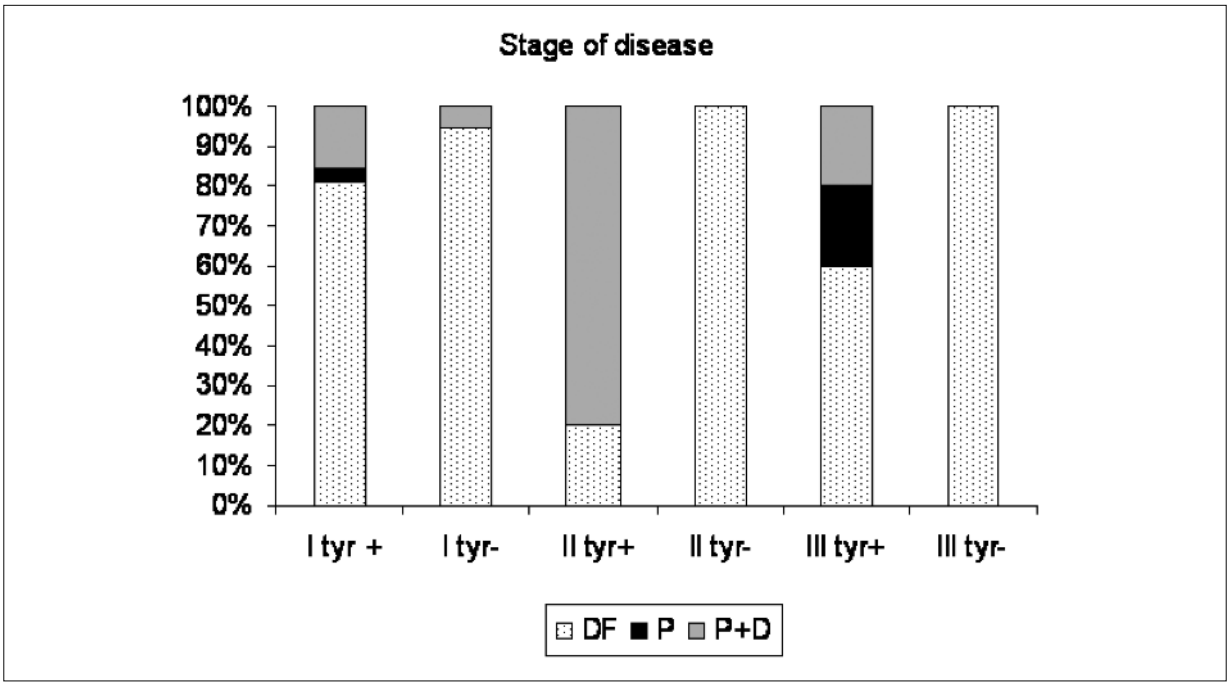

Figure 1. The figure shows the results obtained by RT-PCR essay from LNSs patients, divided by stage. Disease free patients (DF), patients in progression (P), and patients died after progression (P+D).

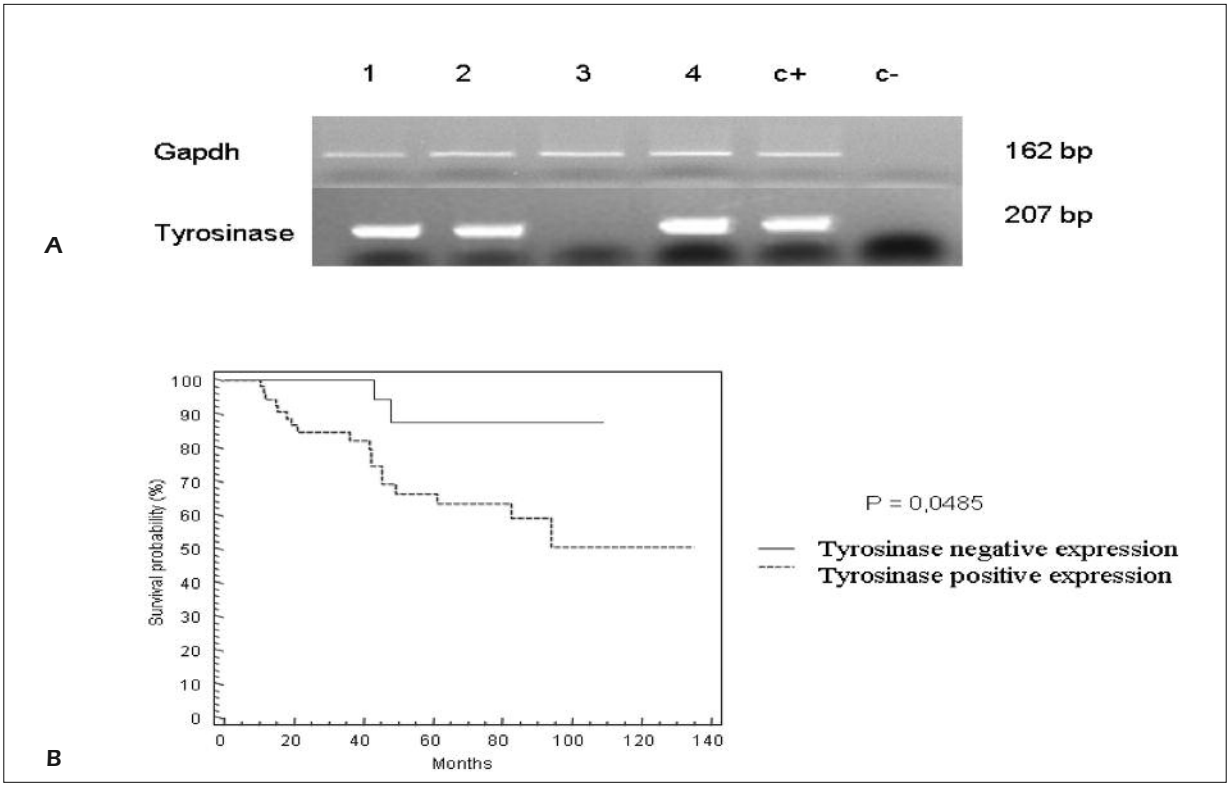

Figure 2. ***A,*** Lanes 1-4 Samples from Sentinel Lymph-Nodes analyzed for the expression of GAPDH and Tyrosinase. Lane 5 Positive control (RNA from M14 cell line). Lane 6 Negative control (sample without RNA) bp (Base pair). ***B,*** Kaplan-Meyer disease free survival rate. The probability of disease free survival is shown for patients with SLN positive for Tyrosinase expression and for patients with SLN negative for Tyrosinase expression.

Table II. Patients characteristic (n° 71).

Characteristic	N° of patients	%	χ^2 test endpoint: death *p* value	χ^2 test endpoint: progression *p* value
Tyrosinase				
Positive	52	73.23	$p = 0.0892$	$p = 0.0496$
Negative	19	26.77		
Sex				
Male	35	49.29	$p = 0.1634$	$p = 0.3956$
Female	36	50.71		
Age				
≤ 40	13	18.32	$p = 0.0145$	$p = 0.0031$
41-60	27	38.02	p trend = 0.0358	p trend = 0.0106
> 60	31	43.66		
Primary melanoma site				
Head and neck	2	02.81	$p = 0.4771$	$p = 0.7482$
Trunk	38	53.52		
Upper extremities	8	11.28		
Lower extremities	23	32.39		
Drainage site				
Axillary	39	54.92	$p = 0.2317$	$p = 0.2543$
Groin	30	42.27		
Cervical	2	02.81		
Breslow's thickness				
≤ 0.75	12	16.90	p trend < 0.0001	p trend < 0.0001
0.76-1.00	26	36.61		
1.01-4.00	28	39.43		
> 4.00	5	07.06		

tumour cells, thus identifying a population of patients at risk of recurrence, who are not identified by routine assay. Nevertheless, to date the real utility of the detection of tyrosinase mRNA by RT-PCR assay is not well defined for the research of occult melanoma cells both in SLN and in circulating bloodstream[22,23].

In the last few years, we focused our attention on the research of a molecular markers profile in SLN, which may be useful as prognostic factors for melanoma patients[17,24,25]. In this study, we sought to investigate the tyrosinase expression of SLNs from 71 patient with a Breslow range from 0.3 mm to 9.0 mm, in order to verify if its expression could be a useful approach to predict the outcome of patients in very-Thin, nThin, and thick melanoma patients in a median follow-up time of 58.24 months.

Even though the sentinel lymph node mapping and dissection is now regularly performed for primary melanomas > 1 mm, this procedure is not well accepted for thinner lesions, in consideration of the low incidence of lymph node metastases; only few research centres have considered a lower cut off point (> 0.75 mm) to perform the SLNB[26-29].

The detection of a subset of patients with disease spread, allows for additional therapies such as surgery, adjuvant systemic interferon therapy, adjuvant radiation therapy, or enrolment in adjuvant experimental clinical trials for chemotherapy, autologous vaccine[30] or monoclonal antibodies[31].

The advantage of highlighting a small subgroup of node-positive patients has to be correlated to the potential unnecessary morbidity of the surgical procedure and the increased costs of the entire thin-group treated by SLNB.

Even though adjuvant trials have not sufficiently detected survival differences by tumour thickness, the adjuvant surgery seems to be more effective in thinner lesions, presumable for a lower risk of distant metastatic disease, already being present at the time of surgery[32]. Unfortunately, due to the small number of patients with very-thin lesions we could not statistically compare the results obtained by SLNs excised from this group of patients with the results obtained on SLNs belonging to patients with thin and thick melanomas.

Among the 38/71 patients enrolled with a lesion of Breslow ≤ 1.00 mm, two showed progression of disease, whereas the other 36 patients are currently disease free. The first patient, a female of 62 years, had a melanoma with a Breslow thickness of 0.3 mm and the SLN analysed, re-

sulted as tyrosinase positive. This patient had a recurrence of disease 24 months after diagnosis and to date, with an overall survival of 135.2 months, is disease free. The second patient, a male of 40 years, with a melanoma with a Breslow of 0.9 mm had recurrence of disease 38 months after the diagnosis, and died 10 months later. The SLN excised from this patient resulted negative for the expression of tyrosinase. The statistical analysis of all data showed as the tyrosinase positive expression identify a subset of melanoma patients at risk of disease progression ($p = 0.0496$) by χ^2 test; in addition, the Kaplan Meier exact test highlights in the tyrosinase positive expression a negative prognostic factor with a $p = 0.0485$ in SLNs from melanoma patients with a Breslow range from 0.3 to 9 mm.

These preliminary results may lead to some considerations: (1) It should be auspicable, in our opinion, to perform the SLNB on patients with nThin and very-Thin melanoma, although some literature studies suggest that these groups of patients were overtreated by undergoing SLNB[32,34]. Our data are in agreement with long-term and mid-term follow-up studies that show recurrences for thin melanoma patients[35]. (2) The results obtained by the Kaplan-Meier exact test in an adequate median follow-up period (58.24 months) are encouraging. We believe that the detection of tyrosinase expression in SLNs may add additional prognostic information to other validated prognostic factors such as the Breslow's thickness and Clark level, even if its role in the staging of melanoma patients must be further investigated; in addition, we believe that the tyrosinase negative expression may identify a subset of patients at lower risk of progression of disease. We obtained 19 SLNs negative for tyrosinase expression belonging to patients at different stage of disease and among these 18/19 (95%) are disease free and only one patients died due to the disease. (3) The possibility of having disease progression in both groups of nThin and vThin melanoma patients suggests that the disease has already spread at the time of diagnosis, also for < .75 mm melanoma. The extension of SLNB to patients with a melanoma thickness < .75 mm has to be valuated and the molecular profile of SLNs further investigated, as well as the SLNs from nThin and the thick melanoma patients. The lower limit of the class of patients to be treated with SLNB will probably be defined by statistical analysis on the results obtained by molecular studies performed to elucidate the differences in the molecular profile during the tumour progression on large numbers of patients with vThin and nThin melanomas with more than 10 years of follow-up.

For this reason the enrolment of thin melanoma patients for SLNB has to be considered still investigational (the majority of our very-Thin patients were volunteer demanding for the surgery) because no differences have been showed by the analysis of this two subset of patients. The majority of our samples resulted negative at H&E and IHC and the result obtained by the Kaplan Maier test (borderline for statistical significativity) probably is of comparable to a typical group of patients with melanoma > 1 mm.

Conclusions

From the results obtained emerges as tyrosinase positive expression is predictive of disease progression and not for death; we can speculate that as demonstrated by Orlow et al[36] the pigmentary change in melanoma progression require variations in expression of pigment genes may explain the high numbers of SLNs positive for tyrosinase expression obtained by an assay much more sensitive respect to H&E and IHC. Although other work performed on a larger number of sample are present in literature on the real utility of SLNB for thin melanoma, we conclude that this work, performed for the first time with RT-PCR on very thin melanoma group of patients, could be one of the first report in constructing a large study on thin melanoma.

In future, more refinements in molecular staging for melanoma would provide additional information of prognostic value for the most part for patients with vThin and nThin melanoma.

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