TERT Promoter Mutations are Associated with Visceral Spreading in Melanoma of the Trunk

Simona Osella-Abate 1,†, Luca Bertero 1,†, Rebecca Senetta 2, Sara Mariani 1, Francesco Lisa 3, Vittoria Coppola 1, Jasna Metovic 1, Rebecca Pasinetti 4, Susana Puig S 5, Maria Teresa Fierro 3, Esperanza Manrique-Silva 6, Rajiv Kumar 7, Eduardo Nagore 6, Paola Cassoni 1,* and Simone Ribero 3

1 Department of Medical Sciences, Pathology Unit, University of Torino, 10126 Torino, Italy; simona.osellaabate@unito.it (S.O.-A.); luca.bertero@unito.it (L.B.); sara.mariani@unito.it (S.M.); vittoria.coppola@gmail.com (V.C.); jasna.metovic@unito.it (J.M.)

2 Pathology Division, “Città della Salute e della Scienza di Torino” University Hospital, 10126 Torino, Italy; rsenetta@cittadellasalute.to.it

3 Department of Medical Sciences, Dermatology Unit, University of Torino, 10126 Torino, Italy; francesco.lisa@unito.it (F.L.); mariateresa.fierro@unito.it (M.T.F.); simone.ribero@unito.it (S.R.)

4 Department of Medical Sciences, Medical Genetics Unit, University of Torino, 10126 Torino, Italy; barbara.pasini@unito.it

5 Melanoma Unit, Dermatology Department, Hospital Clinic, Universitat de Barcelona & Institut d’investigacions biomèdiques August Pi i Sunyer (IDIBAPS), 08036 Barcelona, Spain; susipuig@gmail.com

6 Servicio de Dermatologia, Instituto Valenciano de Oncologia, 46009 Valencia, Spain; emanriques19@gmail.com (E.M.-S.); eduardo_nagore@ono.com (E.N.)

7 Division of Molecular Genetic Epidemiology, German Cancer Research Center, 69120 Heidelberg, Germany; r.kumar@dkfz.de

* Correspondence: paola.cassoni@unito.it
† S.O.-A. and L.B. contributed equally to this article.

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Abstract: Survival predictions are currently determined on the basis of NRAS/BRAF mutations, even though TERT promoter mutations have been recently associated with a poor prognosis in stage I-II melanomas. Usually, it is not recommended to perform a mutational test on primary melanoma, as the results do not always reflect the mutational status of metastases. In particular, trunk melanomas have been reported to have an unfavourable prognosis. A series of 105 advanced melanoma patients were analysed by TERT promoter Sanger sequencing. Univariate/multivariate binary logistic regression models were performed using progression to a visceral site as the dependent variable and patient/tumour characteristics as covariates. Performance of the model was assessed in an external independent primary melanoma patients’ dataset. Male gender (odds ratio (OR), 344; 95% CI, 1.12–10.6; \( p = 0.031 \)), AJCC (American Joint Committee on Cancer) classification (OR, 022; 95% CI, 0.07–0.67; \( p = 0.008 \)), SLNB (Sentinel Lymph Node Biopsy) status (OR, 3.05; 95% CI, 1.06–8.78; \( p = 0.039 \)) and TERT-mutated trunk lesions (OR, 3.78; 95% CI, 1.35–10.6; \( p = 0.011 \)) were significantly associated with the risk of developing a visceral spreading as first site of progression using multivariate logistic regression analysis. These results were confirmed in the external validation control group. Therefore, in trunk primary melanomas, due to their high risk of progression to visceral sites, we encourage somatic TERT mutation analysis at diagnosis to identify those patients who would potentially benefit from a more intensive follow-up protocol and a prompt initiation of therapy.

Keywords: TERT promoter; trunk; melanoma; visceral metastases
1. Introduction

Genetic testing for targetable somatic mutations is considered mandatory by the European Guidelines in the context of diagnosis, treatment and follow-up of cutaneous melanomas in patients with advanced disease (unresectable stage III or stage IV), and highly recommended in high-risk resected disease (stage IIC or stages IIIB–IIIC) [1]. In BRAF wild type tumours, alternative mutations occurring at NRAS and c-Kit genes must be tested. Tumour heterogeneity in advanced stage melanoma has important implications for molecular testing and treatment. Mutation load increases with progression and unique patterns of genetic changes termed ‘evolutionary trajectories’, have been observed in different melanoma subtypes [2,3]. In particular, as reported by Shain et al., melanomas mutated at NRAS or BRAF (V600K or K601E) are more commonly associated with intermediate lesions or melanomas in situ that have already accumulated other pathogenic mutations [2]. In clinical practice, it is not recommended to test for BRAF mutations in primary cutaneous melanoma unless these data are required to guide systemic therapy in advanced stages. Several studies suggest that detection of a wild type BRAF (B-Raf proto-oncogene) in the primary tumour may not necessarily reflect the BRAF mutation status of metastases [4,5]. Therefore, clinicians should consider repeating the test in the most recent metastasis (if available) to determine the BRAF mutation status [4]. In daily clinical practice, patients often come to our attention with advanced stages, progressing either after a positive or a negative sentinel lymph node (SLN), or directly from a primary to a distant metastatic site [6,7]. Moreover, when metastasis sampling is not feasible due to the anatomical site or to other patient clinical comorbidities, mutation analysis is performed on the primary lesion.

Recently, Nagore et al. provided preliminary evidence that in stage I/II melanoma patients TERT promoter status in combination with BRAF/NRAS mutations can be used to identify patients at risk of aggressive disease and the possibility of further prognostic refinement by assessing the rs2853669 polymorphism within TERT promoter [8].

Based on these assumptions, we analysed a consecutive series of patients with advanced melanoma who underwent mutational testing preliminary to treatment with specific targeted therapies at the tertiary Dermo-Oncologic Centre of our Hospital. In particular, in this study we retrospectively analysed advanced melanomas that had directly progressed from stage IB/II AJCC (American Joint Committee on Cancer) 2017 [9], whose mutational status of BRAF, KRAS, NRAS and PIK3CA genes had already been tested. In these specimens, TERT promoter status and its rs2853669 single nucleotide variant (SNV) were then investigated to ascertain associations between the molecular profile and the pattern of progression with the aim of determining the prognostic value of TERT promoter mutations in a subset of IB/II stage melanoma patients with poorer prognosis.

2. Results

2.1. Clinical Characteristics

A total of 105 patients (Table 1) satisfied the inclusion criteria of our study protocol. The average Breslow thickness of primary melanomas was 3.7 mm ± 2.8, while the median follow-up after melanoma diagnosis was 5.8 years (3.2–12.2). The median age at diagnosis was 70 years (range 22–88). Overall, 63 patients (60.0%) were male. The majority of melanomas appeared on the trunk (n = 41, 39.1%). The Nodular melanoma (NM) histotype was observed in 16 out of 105 (15.2%). Ulceration was present in 49 out of 105 patients (46.7%). SLNB (Sentinel Lymph Node Biopsy) was performed in 41 out of 105 patients (39.0%). In the remaining 64 patients SLNB was not performed: in 27 patients after collegial decision, in six patients whose melanoma thickness was above 4 mm and between 0.8–1 mm respectively (SLNB not indicated, ante 2018), in 16 patients older than 75 years, in seven patients with other comorbidities, in two patients whose SLN were not traced, in four patients refusing the procedure and in two patients diagnosed ante-SLNB technique.
Table 1. Clinical characteristics based on the pattern of first relapse.

| Patients                          | Pattern of First Relapse | p ** |
|-----------------------------------|--------------------------|------|
|                                   | Total (105)              | Regional (A) (58; 55.2%) | Regional + Distant (B) (15; 14.3%) | Visceral Only (C) (32; 30.5%) |
| Median time to relapse (years. 25th–75th) | 1.9 (0.7–3.8) | 1.8 (0.7–3.8) | 1.8 (0.5–5.7) | 2.2 (0.8–3.8) | 0.956 |
| Gender                           |                          |                  |                  |                  |
| F                                | 42 (40%)                 | 27 (70.5%)       | 8 (53.3%)        | 7 (21.9%)        | 0.038 |
| M                                | 63 (60%)                 | 31 (29.5%)       | 7 (46.7%)        | 25 (78.1%)       | (C vs. A 0.06) |
| Age at diagnosis                 |                          |                  |                  |                  |
| Median (interval)                | 70 (22–88)               | 71 (28–87)       | 72 (34–86)       | 66 (22–88)       | 0.249 |
| Primary site                     |                          |                  |                  |                  |
| Head/neck a                      | 16 (15.2%)               | 8 (13.8%)        | 5 (33.3%)        | 3 (9.4%)         | 0.030 |
| Trunk b                          | 41 (39.1%)               | 16 (27.6%)       | 5 (33.3%)        | 20 (62.5%)       | (d vs. b 0.021) |
| Upper extremities c              | 12 (11.4%)               | 8 (13.8%)        | 2 (13.4%)        | 2 (6.2%)         | |
| Lower extremities d              | 36 (34.3%)               | 26 (44.8%)       | 3 (20.0%)        | 7 (21.9%)        | |
| Histotype                        |                          |                  |                  |                  |
| Nodular                          | 16 (15.2%)               | 8 (13.8%)        | 4 (26.7%)        | 4 (12.5%)        | 0.407 |
| Other                            | 89 (84.8%)               | 50 (86.2%)       | 11 (73.3%)       | 28 (87.5%)       | |
| Breslow mm ± DS                  | 3.7 ± 2.8                | 4.0 ± 2.8        | 4.8 ± 2.8        | 2.7 ± 1.9        | 0.005 |
| Ulceration                       |                          |                  |                  |                  |
| Absent                           | 59 (56.2%)               | 33 (56.9%)       | 4 (26.7%)        | 22 (68.7%)       | 0.025 |
| Present                          | 46 (43.8%)               | 25 (43.1%)       | 11 (73.3%)       | 10 (38.3%)       | (C vs. B p = 0.02) |
| SLNB                             |                          |                  |                  |                  |
| Not performed                    | 64 (60.9%)               | 39 (67.2%)       | 11 (73.3%)       | 14 (43.7%)       | 0.052 |
| Negative                         | 41 (39.1%)               | 19 (32.8%)       | 4 (26.7%)        | 18 (56.2%)       | <0.001 |
| AJCC 2017                        |                          |                  |                  |                  |
| IB                               | 24 (22.9%)               | 9 (15.5%)        | 2 (13.3%)        | 13 (40.6%)       | (C vs. A p = 0.03) |
| IIA                              | 31 (29.5%)               | 19 (32.8%)       | 5 (33.3%)        | 7 (21.9%)        | (C vs. B p = 0.12) |
| IIB                              | 29 (27.6%)               | 18 (31.0%)       | 0 (0.0%)         | 11 (34.4%)       | |
| IIC                              | 21 (20.0%)               | 12 (20.7%)       | 8 (53.4%)        | 1 (3.1%)         | |
Table 1. Cont.

| Patients                          | Pattern of First Relapse | p **          |
|-----------------------------------|--------------------------|--------------|
|                                   | Total (105)              | Regional (A) (58; 55.2%) | Regional + Distant (B) (15; 14.3%) | Visceral Only (C) (32; 30.5%) |
| Mutational status (Sequenom)      |                          |              |                              |                            |
| WT*                              | 32 (30.5%)               | 15 (25.9%)   | 6 (40.0%)                    | 11 (34.4%)                 | 0.470                     |
| BRAF MUT                         | 44 (41.9%)               | 25 (43.1%)   | 8 (53.3%)                    | 11 (34.4%)                 |
| NRAS MUT                         | 27 (25.7%)               | 17 (29.3%)   | 1 (6.7%)                     | 9 (28.1%)                  |
| KRAS MUT                         | 1 (0.9%)                 | 1 (1.7%)     | 0 (0%)                       | 0 (0%)                     |
| PIK3CA MUT                       | 1 (0.9%)                 | 0 (0%)       | 0 (0%)                       | 1 (3.1%)                   |
| Mutational status site           |                          |              |                              |                            |
| Primary                          | 42 (40.0%)               | 14 (24.1%)   | 8 (53.4%)                    | 20 (62.5%)                 | <0.001 (B vs. A, C vs. B <0.001) |
| Regional mts                     | 46 (43.8%)               | 39 (67.3%)   | 5 (33.3%)                    | 2 (6.3%)                   |
| Distant mts                      | 17 (16.2%)               | 5 (8.6%)     | 2 (13.3%)                    | 10 (31.2%)                 |
| TERT promoter mutations          |                          |              |                              |                            |
| WT                               | 31 (29.5%)               | 20 (34.5%)   | 4 (26.7%)                    | 7 (21.9%)                  | 0.440                     |
| −146 or −124 C > T              | 74 (70.5%)               | 38 (65.5%)   | 11 (73.3%)                   | 25 (78.1%)                 |
| TERT rs2853669                   |                          |              |                              |                            |
| Absent                           | 51 (48.6%)               | 28 (48.3%)   | 8 (53.3%)                    | 15 (46.9%)                 | 0.916                     |
| Present                          | 54 (51.4%)               | 30 (51.7%)   | 7 (46.7%)                    | 17 (53.1%)                 |
| TERT/Trunk site                  |                          |              |                              |                            |
| WT/no trunk a                    | 24 (22.9%)               | 15 (25.9%)   | 3 (20.0%)                    | 6 (18.8%)                  | 0.0045 (D vs. B p = 0.01) |
| −146 or −124 C > T mut/no trunk b | 40 (38.1%)               | 27 (46.5%)   | 7 (46.7%)                    | 6 (18.8%)                  |
| WT/trunk c                       | 7 (6.7%)                 | 5 (8.6%)     | 1 (6.7%)                     | 1 (3.1%)                   |
| −146 or −124 C > T mut/trunk d   | 34 (32.4%)               | 11 (18.9%)   | 4 (26.7%)                    | 19 (59.4%)                 |

* One case D816H c-kit mutated; ** Bonferroni correction has been reported when significative. a: Head/neck; b: Trunk; c: Upper extremities; d: Lower extremities Bonferroni correction groups.
Metastases included in the study appeared after median follow-up of 1.9 years from primary melanoma diagnosis (0.7–3.8). Fifty-eight patients developed regional metastases only (24 skin, 29 lymph nodes, and five skin and lymph nodes), 15 had concomitant regional and distant metastases and 32 exhibited visceral metastases as first and unique sites of relapse. In detail, among the latter with visceral metastases, 19 patients had a single site of involvement (eight lung, seven brain, two liver, one spleen and one peritoneum), nine patients exhibited two metastatic sites and four patients had more than two sites involved. The visceral pattern of relapse seems to be related to the trunk site of primary melanoma but not with NM histotype. No differences in median time to relapse were observed to be associated with the site of disease relapse (log-rank test \( p = 0.9564 \)) (Table 1, Figure 1).

![Figure 1. Time to relapse based on type of site of disease relapse (log-rank test \( p = 0.9564 \)).](image)

### 2.2. Mutational Assessment

Mutational assessment to guide targeted therapy was performed in 42 primary lesions: 20 patients (48.8%) with visceral metastases were surgically inaccessible, 8 had regional lymph node and visceral metastases without indications for lymph node dissection and 14 had only regional metastases that did not undergo surgical resection due to other clinical conditions (older age, comorbidities). In the remaining patients, mutational status was assessed on the most recently available metastases: 46 regional metastases and 17 visceral metastases, respectively (Table 1). All patients were tested with Sequenom (32 WT (Wild-Type), 44 \( \text{BRAF} \)-mutated, 27 \( \text{NRAS} \)-mutated, 1 \( \text{KRAS} \)-mutated and 1 \( \text{PIK3CA} \)-mutated), among these 4 out of 6 acral melanomas (4 WT, 1 \( \text{NRAS} \)-mutated and 1 \( \text{BRAF} \)-mutated) were also tested for \( \text{KIT} \) mutations: only 1 out of 4 WT lesions carried a D816H \( \text{KIT} \) mutation. Moreover, 74 out of 105 samples showed the most frequent \( \text{TERT} \) promoter mutations: −124 C > T or −146 C > T (minor mutations were reported in Table 2).

No association between \( \text{BRAF} \), \( \text{KRAS} \), \( \text{NRAS} \), \( \text{PIK3CA} \) and \( \text{TERT} \) promoter mutations or rs2853669 was observed with first relapse site. Conversely, the \( \text{TERT} \) promoter mutation detected in any tested site was associated with the trunk site of primary lesion (34 out 41 patients, 82.9%) (Tables 1 and 3).
Table 2. TERT promoter mutations details based on pattern of first relapse.

| Patients | Total (105) | Other Site of Progression (73; 55.4%) | Only Visceral (32; 44.6%) |
|----------|-------------|--------------------------------------|---------------------------|
| TERT Promoter mutations | | | |
| −124 C > T | 37 | 24 (32.9%) | 13 (40.6%) |
| −146 C > T | 37 | 25 (34.2%) | 12 (37.5%) |
| −57 A > C | 2 | 2 (2.7%) | 0 (0%) |
| −125_124 CC > TT | 2 | 2 (2.7%) | 0 (0%) |
| −139_138 CC > TT | 2 | 1 (1.4%) | 1 (1.4%) |
| WT | 25 | 19 (26.0%) | 6 (18.8%) |

Table 3. Clinical characteristics based on TERT promoter mutations.

| Patients | TERT Promoter | Total | WT or Minor Mutations (31) | −146 or −124 C>T Mutation (74) |
|----------|---------------|-------|---------------------------|---------------------------------|
| Gender | | | | |
| F | 42 (40%) | 13 (41.9%) | 29 (39.2%) | 0.793 |
| M | 63 (60%) | 18 (58.1%) | 45 (60.8%) | |
| Age at diagnosis | | | | |
| Median (interval) | 70 (22–88) | 72 (22–85) | 68 (26–88) | 0.950 |
| Primary site | | | | |
| Head/neck a | 16 (15.2%) | 5 (16.1%) | 11 (14.9%) | 0.010 |
| Trunk b | 41 (39.0%) | 7 (22.6%) | 34 (45.9%) | (d vs. b 0.014 c vs. d 0.010) |
| Upper extremities c | 12 (11.4%) | 1 (3.2%) | 11 (14.9%) | |
| Lower extremities d | 36 (34.3%) | 18 (58.1%) | 18 (24.3%) | |
| Histotype | | | | |
| Nodular | 16 (15.2%) | 3 (9.7%) | 13 (17.6%) | 0.305 |
| Other | 89 (84.8%) | 28 (90.3%) | 61 (82.4%) | |
| Breslow mm ± DS | 3.7 ± 2.8 | 4.1 ± 3.2 | 3.6 ± 2.6 | 0.362 |
| Ulceration | | | | |
| Absent | 59 (56.2%) | 16 (51.6%) | 43 (58.1%) | 0.541 |
| Present | 46 (43.8%) | 15 (48.4%) | 31 (41.9%) | |
| SLNB | | | | |
| Not performed | 64 (60.9%) | 19 (61.3%) | 45 (60.8%) | 0.963 |
| Negative | 41 (39.1%) | 12 (38.7%) | 29 (39.2%) | |
| AJCC 2017 | | | | |
| IB | 24 (22.8%) | 3 (9.7%) | 21 (28.4%) | 0.175 |
| IIA | 31 (29.5%) | 12 (38.7%) | 19 (25.7%) | |
| IIB | 29 (27.6%) | 10 (32.3%) | 19 (25.7%) | |
| IIC | 21 (20.0%) | 6 (19.3%) | 15 (20.3%) | |
| Mutational status (Sequenom) | | | | |
| WT * | 32 (30.5%) | 12 (38.7%) | 20 (27.0%) | 0.187 |
| BRAF MUT | 44 (41.9%) | 8 (25.8%) | 36 (48.7%) | |
| NRAS MUT | 27 (25.7%) | 11 (35.5%) | 16 (21.6%) | |
| KRAS MUT | 1 (0.95%) | 0 (0%) | 1 (1.35%) | |
| PIK3CA MUT | 1 (0.95%) | 0 (0%) | 1 (1.35%) | |
| Mutational status site | | | | |
| Primary | 42 (40%) | 13 (41.9%) | 29 (39.2%) | 0.496 |
| Regional mts | 46 (43.8%) | 15 (48.4%) | 31 (41.9%) | |
| Distant mts | 17 (16.2%) | 3 (9.7%) | 14 (18.9%) | |
| TERT rs2853669 | | | | |
| Absent | 51 (48.6%) | 16 (51.6%) | 35 (47.3%) | 0.687 |
| Present | 54 (51.4%) | 15 (48.4%) | 39 (52.7%) | |

* One case D816H KIT mutated; ** Bonferroni corrections were reported when significative. a: Head/neck; b: Trunk; c: Upper extremities; d: Lower extremities Bonferroni correction groups.
In consideration of the limited sample size due to a real-life study setting, the two most represented mutations (−124 C > T and −146 C > T) have been combined together for the analyses. Indeed, to analyse the variants separately it would have been necessary a minimum of 230 melanoma cases in order to detect a significant 5% difference in outcomes between −124 and −146 TERT mutations according to a power calculation analysis using visceral specific recurrence as the primary end point with a 80% power and a two-sided α = 0.05 test. Moreover, we have not stratified patients according to −57A > C because only two cases were observed (Table 2).

2.3. Association Between Trunk Site and TERT Mutation

Considered that visceral spread seemed to be associated with a trunk primary melanoma and that the TERT promoter mutation frequently occurred in patients whose primary lesion developed at this site, we analysed the progression pattern by evaluating the association between the trunk site and the TERT promoter mutation. In the group in which the mutational status was performed on the primary lesion, data showed that 12 out of 18 cases (66.7%) with a −124 or −146 C > T mutation and the primary site on the trunk had developed visceral spreading as the first site of progression. At univariate analysis (Table 4), logistic regression highlighted a significant association between the TERT promoter mutation and trunk site with visceral spreading (OR 5.33, CI 1.02–27.7) when considering cases in which only the primary lesion was analysed. This finding was consistent even when considering the cases in which both the primary lesion and the metastasis were analysed. In summary, 19 out of 34 patients (55.9%) who had a TERT promoter mutation in the primary or metastatic lesion associated with a primary melanoma trunk site, developed visceral spreading as the first site of progression.

| Variable | Only Visceral Site |
|----------|--------------------|
| Age (linear) | 0.98 (0.95–1.00) 0.141 |
| Gender (F vs. M) | 3.28 (1.26–8.55) 0.015 |
| Breslow (linear) | 0.74 (0.57–0.94) 0.015 |
| Ulceration (absent vs. present) | 0.46 (0.19–1.12) 0.089 |
| AJCC 2017 (IB vs. IA, IIB, IIC) | 0.26 (0.09–0.67) 0.006 |
| NM versus other histotype | 0.72 (0.21–2.45) 0.606 |
| Trunk vs. other site | 3.41 (1.43–8.11) 0.006 |
| Mutational status (Mut vs. WT) | 0.77 (0.32–1.87) 0.566 |
| SLNB status (negative vs. not performed) | 2.79 (1.18–6.57) 0.019 |
| −146 or −124 C > T TERT promoter mutations | 1.75 (0.66–4.61) 0.259 |
| TERT rs2853669 | 1.10 (0.48–2.53) 0.818 |
| TERT status and trunk association only in primary lesions |  |
| WT/no trunk | 1 |
| −146 or −124 C > T mut/no trunk | 1.52 (0.25–9.29) 0.648 |
| WT/trunk | 2.67 (0.12–57.6) 0.532 |
| −146 or −124 C > T mut/trunk | 5.33 (1.02–27.7) 0.047 |
| TERT status and trunk association in all |  |
| WT/no trunk | 1 |
| −146 or −124 C > T mut/no trunk | 0.68 (0.21–2.28) 0.538 |
| WT/trunk | 0.50 (0.05–5.03) 0.556 |
| −146 or −124 C > T mut/trunk | 3.64 (1.14–11.66) 0.029 |
| TERT mut + trunk site vs. other only in primary | 4.00 (1.09–14.62) 0.036 |
| TERT mut + trunk site vs. other | 4.78 (1.93–11.8) 0.001 |
Multivariate logistic regression analyses (Table 5) confirmed the significant association between TERT promoter mutation and trunk site with visceral spreading also when adjusting for age, gender, AJCC staging and SLNB, suggesting that preferential visceral spreading as the first site of progression is related to TERT-mutated lesions in patients whose primary lesion is located on the trunk. Moreover, the Hosmer–Lemeshow goodness-of-fit statistics (\( p\)-value = 0.5460) indicated that the model adequately describes the data.

| Multivariable Only Visceral Site | OR  | CI       | \( p \) |
|---------------------------------|-----|---------|--------|
| Age (linear)                    | 0.99| 0.96–1.03| 0.684  |
| Gender (F vs. M)                | 3.44| 1.12–10.6| 0.031  |
| AJCC 2017 (IB vs. IIA, IIB, IIC) | 0.22| 0.07–0.67| 0.008  |
| SLNB status (negative vs. not performed) | 3.05| 1.06–8.78| 0.039  |
| \( TERT \) mut + trunk site vs. other | 3.78| 1.35–10.6| 0.011  |

2.4. Validation in the Independent Cohort of the Instituto Valenciano de Oncología Data Set

To test the reproducibility of the association between TERT promoter mutation in trunk site with visceral metastases as first site of progression, we investigated its performance in data collected from 83 stage II primary melanoma patients all progressed to a metastatic stage recruited in Valencia by the Instituto Valenciano de Oncología (Prof. E Nagore) (Table 6). Data on the variables used to define association were extracted from dataset and equally classified as for the pooled data described above. Multivariate logistic regression analyses performed in the Valencia dataset (Table 7) confirmed the significant association between TERT promoter mutation and trunk site with visceral spreading (OR 4.81, CI 1.01–22.9; Hosmer–Lemeshow goodness-of-fit statistics, \( p = 0.8986 \)).

| Patients Total (83) | Other Site of Progression (46; 55.4%) | Only Visceral (37; 44.6%) | \( p \) |
|--------------------|--------------------------------------|--------------------------|-------|
| Gender             |                                      |                          |       |
| F                  | 31 (37.3%)                           | 21 (45.6%)               | 10 (27.0%) | 0.081 |
| M                  | 52 (62.7%)                           | 25 (54.4%)               | 27 (73.0%) |       |
| Age at diagnosis   |                                      |                          |       |
| Head/neck          | 64 (21–87)                           | 65 (26–87)               | 60 (21–84) | 0.404 |
| Trunk              | 22 (26.5%)                           | 9 (19.6%)                | 13 (35.1%) | 0.059 |
| Upper extremities  | 9 (10.8%)                            | 2 (13.0%)                | 2 (8.1%)  |       |
| Lower extremities  | 27 (32.5%)                           | 3 (43.5%)                | 7 (18.9%)  |       |
| SLNB               |                                      |                          |       |
| Not performed      | 18 (21.7%)                           | 10 (21.7%)               | 8 (21.6%)  | 0.990 |
| Negative           | 65 (78.3%)                           | 4 (78.3%)                | 18 (78.4%) |       |
| AJCC 2017          |                                      |                          |       |
| IB                 | 24 (28.9%)                           | 15 (32.6%)               | 9 (24.3%)  | 0.408 |
| II, IIB, IIC       | 59 (71.1%)                           | 31 (67.4%)               | 28 (75.7%) |       |
| TERT/Trunk site    |                                      |                          |       |
| WT/no trunk        | 34 (40.9%)                           | 20 (43.5%)               | 14 (37.8%) | 0.149 |
| \( \geq 146 \) or \( \geq 124 \) C > T mut/no trunk | 24 (28.9%) | 15 (32.6%) | 9 (24.3%)  |       |
| WT/trunk           | 13 (15.7%)                           | 8 (17.4%)                | 5 (13.5%)  |       |
| \( \geq 146 \) or \( \geq 124 \) C > T mut/trunk | 12 (14.5%) | 3 (6.5%)  | 9 (24.3%)  |       |
Table 7. Multivariate logistic regression in Istituto Valenciano de Oncologia dataset.

| Multivariable Logistic Regression | Only Visceral Site |
|----------------------------------|--------------------|
|                                  | OR   | CI          | p   |
| Age (linear)                    | 0.99 | 0.97–1.02  | 0.856 |
| Gender (F vs. M)                | 2.49 | 0.92–6.70  | 0.071 |
| AJCC 2017 (IB vs. IIA, IIB, IIC) | 0.99 | 0.32–3.07  | 0.997 |
| SLNB status (negative vs. not performed) | 1.26 | 0.41–3.86 | 0.682 |
| TERT mut + trunk site vs. other | 4.80 | 1.01–22.9  | 0.049 |

3. Discussion

Our experience in daily practice highlights that mutational status assessment of progressed Stage IB/II melanoma patients is conditioned not only by the progression site, but also by patient comorbidities that can influence surgical management. All these conditions affect the available tissue type for molecular testing. In particular, disease progression in not surgically accessible anatomical sites often (40% of cases) makes it necessary to use the primary lesion instead of the most recently developed distant metastasis. Guidelines for follow-up of stage I–II patients (correctly staged through SLNB or not) differ around centres and usually do not envisage serial screening of visceral sites in the low risk of progression group [10,11] although many patients with an intermediate Breslow thickness melanoma risk progress directly to a visceral site [6,12]. In stage IB/II patients, a more individualised follow-up taking into account the risk factors associated with relapse in visceral sites, which include male gender, trunk site and different SLNB proposal and management [6,7,13–16], would be desirable.

In our consecutive case series of metastatic melanomas, stage IB was more commonly associated with visceral site than higher stages (IIA, IIB and IIC). At the moment, no reliable biomarkers except SLN status have proven to be associated with the real risk of primary melanoma progression, and no information can be specifically ascertained based upon the hypothesis (not supported by biological data, so far) of a predisposition of trunk melanoma to progress to visceral sites [14,17].

In this context, new markers are needed for discriminating patients at higher risk of visceral relapse. Despite the heterogeneity of our cohort, our results showed that the TERT promoter mutations in primary trunk melanoma are related to visceral spreading as the first site of progression, independently of SLNB management and AJCC stage, thus suggesting that −124 or −146 C > T mutations may be considered as risk factors of visceral spreading in trunk lesions. This finding has been validated in an independent Valencia cohort of primary melanoma patients all progressed to a metastatic phase, confirming the reliability of this association.

Furthermore, the association between TERT promoter mutations and trunk site is supported by evidence that melanomas occurring in intermittently sun-exposed skin as trunk site displayed an increased prevalence of TERT promoter mutations compared with melanomas occurring in sun-protected areas [18].

It is well known that TERT mutations enhance the expression of the TERT gene by creating de novo binding motifs for different transcription factors [19] involved in tumorigenic mechanisms, but its prognostic role is debated. Several authors have reported a prognostic significance of TERT promoter mutations in primary melanomas [18,20,21], whereas other studies [22,23] found no impact in primary and metastatic melanoma. In our experience, TERT promoter mutations have been identified both in primary and metastatic visceral lesions of patients with a trunk primary. These data are in agreement with Shain et al. [2], who also identified TERT promoter mutations in intermediate lesions and melanomas in situ, in addition to invasive and metastatic melanomas, thus confirming that TERT mutations are selected in an early stage of the neoplastic progression and maintained during the entire metastatic process [2], in variance with other mutations like BRAF which can show a discordance between primary and metastatic tumours in 11% of cases [4,24].
A meta-analysis indicates that TERT promoter mutations are associated with patient age, gender and distant metastases in individuals with cancers [25]. In particular promoter mutations were found to be independent risk factors for distant metastases in thyroid carcinoma. Moreover, in thyroid carcinoma TERT inhibition has been related to reduced cell growth, invasion, migration and angiogenesis [26,27]. Trunk melanomas have been associated in many studies to visceral involvement [15–17], TERT promoter mutation can be the biological explanation of trunk melanoma ability to skip regional metastases by promoting visceral spreading.

Our data reflect daily clinical practice, which often deviates from optimal conditions. We are aware of the limitations of this study:

1) The cohort is not homogeneous in term of SLN management, but in order to keep this fact in consideration, the model had been adjusted for this feature (SLNB performed vs. not performed). The association between visceral progression and TERT promoter mutation in trunk site patients should be considered even more reliable due to the fact that it maintained its prognostic role after this adjustment.

2) The relative low sample of our study did not allow us to perform stratification on the basis of TERT promoter mutation variant.

3) It was not possible to confirm the preservation of TERT promoter mutation along the disease course (from primary tumour to metastases in the same patient) due to incomplete specimen availability.

4) Conventional Polymerase Chain Reaction (PCR) followed by Sanger sequencing can underestimate the wild type/mutated allele ratio of TERT promoter compared to other methods [28].

However, our results and the external validation in a group of progressed primary melanomas support the previously reported finding of a worse outcome in TERT-mutated trunk melanomas. Therefore, we encourage TERT promoter mutation analysis at diagnosis in the primary melanomas of the trunk since their progression will likely occur in a visceral site in the majority of cases and patients would potentially benefit from early detection and prompt treatment.

4. Material and Methods

4.1. Patients

A series of 105 progressed stage IB/II AJCC melanoma patients (24 stage IB, 31 stage IIA, 29 stage IIB and 21 stage IIC) with complete clinicopathological annotated features underwent mutational analyses for therapeutic purposes between April 2014 and December 2017 at the Pathology Unit and were followed up at the Dermatologic Clinic of “Città della Salute e della Scienza” University Hospital (Torino, Italy).

The study was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans and within the guidelines and regulations defined by the Research Ethics Committee of the University of Turin. This study was approved by the Research Ethics Committee of the University of Turin (DSM-ChBU 5/2016). Considered the retrospective nature of the research protocol and the lack of clinical impact on patient care, no specific written informed consent had been required.

Clinical, epidemiological and histological data were collected from the medical history of patients, whose tumours were diagnosed, treated and followed up according to previously reported protocols [6,7,25].

At our institution, SLN biopsy (SLNB) has been performed since 1998 and the criteria adopted for SLNB inclusion have been previously reported [13–15]. Age above 75 years and significant comorbidities are exclusion criteria for this procedure. Moreover, due to the lack of specific guidelines, in thick (>4 mm) melanomas [15,16], a multidisciplinary team discusses each case analysing the risk/benefit ratio before proceeding with SLNB. All decisions are based upon the physicians’ experience and clinical situation, considering that no evidence-based recommendations are available in this
setting. All patients gave their written consent before undergoing ultrasound or CT scan to exclude the presence of regional of distant metastases before SLNB or at diagnosis according to regional guidelines (http://www.reteoncologica.it).

4.2. Mutational Status and TERT Promoter Assessment

Primary or metastatic tumour tissue sections were obtained for DNA extraction as previously described [29]. Mutational detection was performed using the Sequenom MassARRAY® system (Sequenom, San Diego, CA, USA) in conjunction with the Myriapod Colon Status kit that identifies 58, 54, 23 and 66 nucleotide substitutions in the KRAS, NRAS, BRAF and PIK3CA genes, respectively. Mutant and wild type alleles were discriminated with the Sequenom MassARRAY® Analyser 4 platform. When warranted, the KIT mutational status was assessed by Sanger sequencing in BRAF wild type samples (primary acral or mucosal lesions) [30]. Mutational status of the TERT core promoter was determined in samples by PCR and Sanger sequencing between nucleotides −27 and −286 from the starting region (ATG) of the coding site, which includes the polymorphic site represented by rs2853669.

In detail, we amplified the TERT promoter (located on chromosome 5) target region with a touch-down PCR scheme using TaqGold 360 + GC enhancer (Applied Biosystems, Waltham, MA, USA) and the following primer pair; TERT promoter forward 5′-CTCCAGTGATTCCGGCGGC-3′ and reverse 5′-CCCACGTGCGCAGCAGGAC-3′, as described by Heidenreich et al. [31]. With 2% agarose gel electrophoresis, we verified that the PCR products were of the expected size (260 bp) and free of aspecific amplicons. PCR products were then purified and used as templates for the sequencing reactions, which were performed in both directions (forward and reverse) with the same primers using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). After purification, the sequences were analysed by Sanger direct sequencing using the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems), with the Sequencing Analysis 5.0 Software (Applied Biosystems). The two most frequently identified variations within the TERT promoter gene region at positions 1295228 and 1295250 are known as C228T and C250T, respectively. These mutations are located at −124 and −146 bp upstream of the ATG start codon and were considered for analyses.

4.3. Statistical Analyses

All analyses were performed using Stata/MP 15.0 Statistical Software (STATA, College Station, TX, USA). Continuous variables were summarised as the mean and standard deviation (SD), whereas for categorical variables the frequency was provided. The patients’ characteristics were compared using the chi-squared test for categorical variables and the T-test or ANOVA test for continuous variables, according to Bonferroni corrections. Univariate/multivariate binary logistic regression models were performed using progression to a visceral site as the dependent variable (yes or no) and patient/tumour characteristics as covariates. Odds ratios and 95% CIs were estimated. The Hosmer–Lemeshow goodness-of-fit statistics were used to determine whether the model adequately described the data.

Disease-free interval (DFI) was calculated from the date of primary lesion diagnosis to the date of tumour progression/recurrence or last follow-up. Survival curves between different groups were plotted using the Kaplan–Meier method and the statistical comparisons were performed with log-rank test.

The final association (TERT promoter mutation and Trunk site towards visceral progression) has been validated in an external independent cohort of primary melanoma patients.

5. Conclusions

Our findings encourage TERT mutation analysis at diagnosis in primary melanomas arising on the trunk since they are more likely progress to visceral site. TERT screening would help in selecting
patients who will potentially benefit from a more intensive follow-up protocol and a prompt initiation of therapy.

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