ORIGINAL RESEARCH

TERT copy gain predicts the outcome of high-dose interferon α-2b therapy in acral melanoma

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Background: Asian populations are more likely to develop acral melanoma (AM) than Caucasians. The high-dose interferon (HD-IFN) α-2b regimen is the main adjuvant treatment for AM. *TERT* encodes the catalytic subunit of telomerase reverse transcriptase, which plays an important role in melanoma. Frequent *TERT* mutation and increased *TERT* gene expression have been described in AM. Our study aimed to investigate the status and the clinical significance of *TERT* copy number in a large cohort of patients with AM and to analyze the relationship between *TERT* copy number gain and the efficiency of HD-IFN.

Patients and methods: A total of 573 melanoma samples were retrospectively collected and analyzed for *TERT* copy number via Sanger sequencing. Clinical data of patients were also collected.

Results: *TERT* copy gain (copy number >2) was detected in 257 of the 573 patients with AM (44.9%). Of the 573 patients, 81 (14.1%) had a high copy gain (copy number >4). Patients with ulceration showed a significantly higher copy gain rate of *TERT* compared to the patients without ulceration ($P=0.028$). Patients with a tumor thicker than 4 mm also had a higher copy number rate of *TERT* than those with <4 mm ($P=0.048$). Our results showed that the overall survival (OS) was not significantly different between patients with and without *TERT* copy gain ($P=0.890$). However, among the 278 patients who received an HD-IFN regimen, Kaplan–Meier survival analysis demonstrated a significant correlation between *TERT* copy gain and relapse-free survival (RFS) ($P=0.008$). In addition, multivariate Cox regression assays validated *TERT* copy gain to be an independent prognostic factor of RFS for patients with AM undergoing HD-IFN therapy (hazard ratio =1.50; $P=0.019$).

Conclusion: The copy number status of *TERT* might be a predictor for HD-IFN efficacy, but it is not a prognostic factor of OS in patients with AM.

Keywords: acral melanoma, *TERT*, gene copy number, interferon α-2b, relapse-free survival

Introduction

Acral melanoma (AM) is the most predominant melanoma subtype among non-Caucasians,[1–3] particularly among Chinese, accounting for almost 50% of all cases of melanomas.[4] Compared with common cutaneous melanoma, AM has a poorer prognosis[2,4,5] and shows a markedly different genomic landscape, with a far lower mutation burden dominated by larger-scale genomic rearrangements.[5–7] Vemurafenib and imatinib are more effective against advanced AM harboring *BRAF* and *C-KIT* mutations than traditional chemotherapies.[8–11] However, the mutation frequencies of *BRAF* and *C-KIT* in AM are only approximately 16%[12,13] and 12%,[13–15] respectively; therefore, a validated targeted therapy is unavailable for the majority of patients. Meanwhile, adjuvant HD-IFN is a year-long treatment modality associated with improved relapse-free survival (RFS)[16] and is currently the primary treatment regimen

for stage II/III AM after surgery.[17] Despite the optimization of this adjuvant treatment, most patients will still develop distant metastases and eventually die. Moreover, biomarkers for identifying patients who would derive significant prognostic benefit from HD-IFN treatment have yet to be determined. To sum up, developing more effective therapeutic strategies and validating new candidate biomarkers for predicting treatment response are urgently needed.

Human telomerase contains two essential components, a functional human telomerase RNA (hTR, also named TERC) and a human telomerase reverse transcriptase (h*TERT*).[18] *TERT* is located at 5p15.33, which regulates telomeric length. *TERT* upregulation plays a critical role in oncogenesis.[19] *TERT* promoter mutations have been reported in up to 50% of all cases of cutaneous melanoma,[20,21] but only in 0%–7% of AM.[22–24] In addition, gene amplification is the most frequent mechanism for *TERT* activation and significantly reduced overall survival (OS) in AM.[25] *TERT* gene amplification has been evaluated via fluorescence in situ hybridization in a series of AM patients, and

such amplification was detected in 6 of 34 cases (17.6%).[26] Another previous study detected *TERT* gene amplification in 5 of 16 (31.2%) patients with AM.[27] However, a small sample size was one of the limitations of these studies.

In the present study, we examined *TERT* copy gain in 573 melanoma samples. To the best of our knowledge, this is by far the largest study of patients with AM focusing on the copy gain of *TERT*, and we determined the association between *TERT* copy gain and the clinicopathological features of AM. Our study demonstrated prognostic value of *TERT* copy gain for different patient subgroups. These findings may also help to discover new potential molecular indicators for patients who may benefit from HD-IFN treatment.

Patients and methods
Study population
A total of 573 patients with AM who were treated at Peking University Cancer Hospital & Institute between 2008 and 2017 were included in this study (Table 1). These samples were evaluated to confirm the diagnosis of melanoma via

Table 1 Correlation of *TERT* copy number to clinical features of AM

Clinical characteristics	Case	*TERT* CNV		
		No gain ≤2	Gain >2	*P*-value[a]
Sex				0.429
Male	307	174 (55.1%)	133 (51.8%)	
Female	266	142 (44.9%)	124 (48.2%)	
Age (years)				0.660
<65	434	242 (76.6%)	192 (75.0%)	
≥65	138	74 (23.4%)	64 (25.0%)	
Anatomic site				0.657
Hand	123	70 (22.2%)	53 (20.6%)	
Foot	450	246 (77.8%)	204 (79.4%)	
Ulceration				0.028
No	159	101 (32.0%)	58 (22.6%)	
Yes	362	191 (60.4%)	171 (66.5%)	
Uncertain	52	24 (7.6%)	28 (10.9%)	
Thickness				0.048
<4 mm	299	178 (56.3%)	121 (47.1%)	
≥4 mm	218	111 (35.1%)	107 (41.7%)	
Uncertain	56	27 (8.6%)	29 (11.2%)	
Stage				0.112
I	68	46 (14.6%)	22 (8.6%)	
II	203	112 (35.4%)	91 (35.4%)	
III	157	79 (25.0%)	78 (27.4%)	
IV	145	79 (25.0%)	66 (25.3%)	
Metastasis site (1)				0.650
With visceral metastasis	140	77 (43.8%)	63 (46.3%)	
Without visceral metastasis	172	99 (46.2%)	73 (53.7%)	
Metastasis site (2)				0.521
Only regional lymph node metastasis	86	46 (26.1%)	40 (29.4%)	
Others	226	130 (73.9%)	96 (70.6%)	

Note: [a]The *P*-value of 573 patients was analyzed by the χ^2 tests.
Abbreviations: AM, acral melanoma; CNV, copy number variation.

H&E staining and immunohistochemistry for melanoma markers (S-100, HMB-45, or MART-1). Clinical data, including age, sex, tumor-node-metastases (TNM) stage, tumor thickness (Breslow), ulceration, and survival period (follow-up persisted until patient was lost to follow-up or death), were collected. Patients who received radiation treatment were excluded from our study. This investigation was performed after approval by the Ethics Committee of Peking University and was conducted according to the Declaration of Helsinki Principles. Written informed consent was obtained from each patient.

Quantification of DNA using QuantiGene Plex 2.0 assay in FFPE tissues

Genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) specimens using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). The QuantiGene Plex 2.0 reagent system (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect the copy number of *TERT* according to the manufacturer's recommended protocols. Briefly, each patient's DNA sample was mixed with an oligonucleotide probe. The mixture was then added to a 96-well plate, and the reagent system captured the target DNA and control DNA. Afterward, working samples were incubated overnight at 54°C. After washing unbound material with 200 μL of wash buffer, sequential hybridization of DNA amplifier molecules, preamplifier hybridization, amplifier hybridization, and label probe hybridization were then performed. The plate was then prepared for analysis by a professional analyzer. After dividing by the sample value, the gene copy numbers of the

patients were calculated as follows: a copy number of 2 or less was considered no gain, while a copy number >2 was considered a copy number gain of *TERT*. In these amplified populations (copy number >2), we further classified the copy numbers as median gain (>2 but <4) or high copy number gain (4 or greater).

Statistical analysis

All statistical analyses were performed using SPSS 21.0 software (IBM Corporation, Armonk, NY, USA). OS was calculated and plotted using the Kaplan–Meier method. Differences between groups were compared via log-rank test. A 2-sided χ^2 test was used to examine the correlations between gains of *TERT* gene and clinicopathological parameters. The Cox proportional hazard regression model was used for multivariate analysis.

Results
Copy gain of *TERT* in patients with acral melanoma

We used pretreatment biopsy tissue samples from 573 patients with AM. The tumor specimens were derived from primary lesions. All the tumor sections were collected at the time of diagnosis before initiating the treatments. The patients' characteristics are shown in Table 1.

To study the copy number variation of *TERT*, we performed QuantiGene Plex 2.0 Assays (Thermo Fisher Scientific) for AM specimens. We found that 257 of the 573 patients (44.9%) displayed *TERT* copy gain (ie, a gain >2-fold change, Figure 1A). Of them, 81 patients (31.5%) had a high
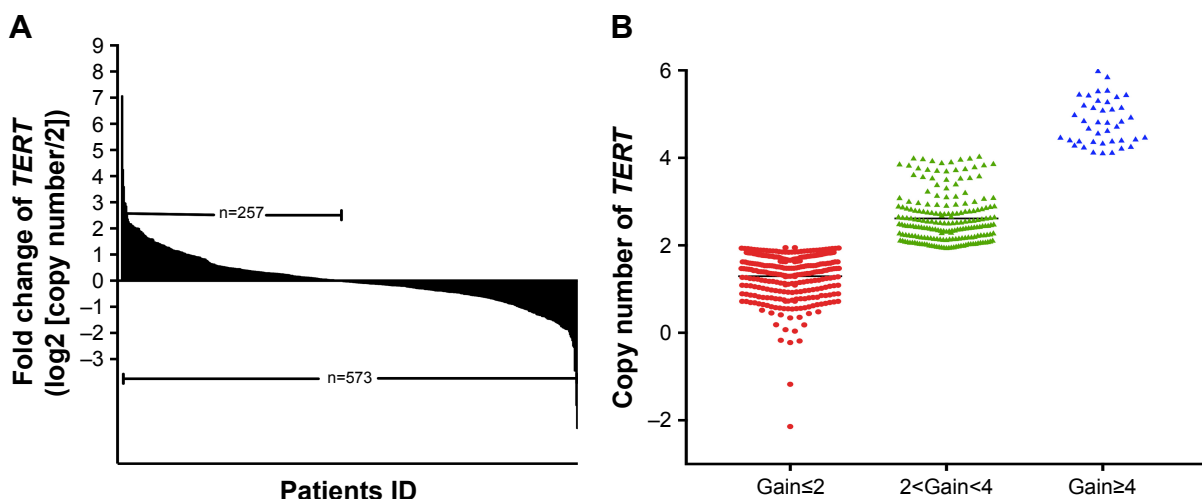
Figure 1 *TERT* copy gain in 573 AM specimens.
Notes: (**A**) A DNA copy number of >2 copies was determined as gene copy gain, and *TERT* copy gain was observed in 44.9% (257/573) of the AM specimens (above the X-axis). (**B**) The scatter plot showed different degrees of copy gain of *TERT* as follows: no copy gain is defined as a copy number of 2 or less, while a copy gain is defined as a copy number >2. In these amplified populations, we further defined <2 copy number and <4 as median copy number gain. A copy number <4 is defined as high copy number gain.
Abbreviation: AM, acral melanoma.

copy number (ie, a gain >4-fold change) (Figure 1B). Patients with ulceration showed a significantly higher copy gain rate of *TERT* than those without ulceration (*P*=0.028). Patients with tumors thicker than 4 mm also had a higher copy gain rate of *TERT* than those with tumors <4 mm thick (*P*=0.048). No relationship was noted between *TERT* copy gain and sex, age, clinical stage, or metastasis site (Table 1).

Relationship between *TERT* copy gain and representative gene of melanoma

The rates of *BRAF* wild-type and mutant genes among the 257 patients with *TERT* copy gain were 88.6% and 11.4%, respectively, while they were 84.4% and 15.6%, respectively, among patients with no *TERT* copy gain. No correlation between *BRAF* mutation and *TERT* copy gain was observed (*P*=0.143). In addition, we also analyzed the relationship between 3 other common mutations (*KIT*, *PDGFRA*, and *NRAS*) and *TERT* copy gain in 573 patients with AM. No correlation was found between *TERT* copy gain and common gene mutation (Table 2).

Influence of *TERT* copy gain on OS of patients

Among the 573 patients with AM, 278 (70.4%) received HD-IFN regimen. Meanwhile, 117 patients underwent different treatment modalities, namely, dacarbazine (PUDEPHARM, Shanxi Datong, China) (n=62 patients), temozolomide (Temodal, Orion Corporation Orion Pharma, Espoo, Finland) (n=14 patients), and other chemotherapeutic drugs or targeted therapy (n=41 patients). Out of the 41 patients, 11 and 1 were treated with imatinib (Glivec, Novartis, Beijing, China) and vemurafenib (Zelbraf, Roche, Basel, Switzerland), respectively.

To evaluate the prognostic value of *TERT* for predicting the OS of patients, Kaplan–Meier survival analysis was performed for all the 573 patients. The median OS

Table 2 Analysis of the relationship of *TERT* copy number status with other gene mutations in AM

Common genes	Mutation	TERT CNV		
		No gain ≤2	Gain >2	P-value[a]
BRAF	WT	280 (88.6%)	217 (84.4%)	0.143
	Mutant	36 (11.4%)	40 (15.6%)	
NRAS	WT	272 (86.1%)	212 (82.5%)	0.239
	Mutant	44 (13.9%)	45 (17.5%)	
PDGFR	WT	308 (99.4%)	253 (99.2%)	0.843
	Mutant	2 (0.6%)	2 (0.8%)	
CKIT	WT	218 (86.2%)	221 (85.9%)	0.956
	Mutant	35 (13.8%)	36 (14.1%)	
Total		316	257	

Note: [a]The *P*-value was analyzed by the χ^2 tests.
Abbreviations: AM, acral melanoma; CNV, copy number variation; WT, wild type.

of patients with and without *TERT* gain was 73.7 months (95% CI: 69.9–78.5 months) and 62.7 months (95% CI: 59.5–67.9 months), respectively. The no *TERT* gain group did not show improved OS relative to the whole population (*P*=0.890, Figure 2A). Among the 278 patients treated with IFN α-2b, those with no *TERT* gain did not demonstrate better OS than those with *TERT* copy gain (*P*=0.389). Among the patients treated with IFN, we analyzed whether *TERT* copy gain affected survival in those with stage II and stage III AM. Our results showed that OS was not significantly different between patients with and without *TERT* gain (n=221, *P*=0.228, Figure 2B).

Influence of *TERT* copy gain on patient response to high-dose interferon treatment and RFS

IFN α-2b is the most commonly utilized adjuvant regimen for patients with AM in China. Thus, we analyzed whether *TERT* copy gain can affect the efficacy of IFN therapy. The median RFS of the patients with and without *TERT* copy gain was 18.2 months (95% CI: 10.6–25.3 months) and 29.1 months (95% CI: 21.7–36.2 months), respectively. We used Kaplan–Meier survival analysis to evaluate the predictive value of *TERT* and RFS for postoperative adjuvant therapy, and the results demonstrated significant correlation between *TERT* copy gain and RFS in cases treated with IFN α-2b. *TERT* copy gain significantly predicted worse RFS (*P*=0.008) (Figure 3A). Then, we further analyzed the efficacy of IFN therapy in patients with stage II and stage III disease. Among these patients, the median RFS was significantly different between patients with and without *TERT* gain (n=221, *P*=0.034, Figure 3B). Collectively, these results indicate that *TERT* copy gain in patients with AM can predict worse RFS among patients treated with HD-IFN, particularly for those with stage II and III disease.

The results of multivariate Cox regression assays showed that TERT copy gain (hazard ratio =1.50; *P*=0.019) and TNM stage (hazard ratio =2.92; *P*<0.001) were independent prognostic factors of RFS for patients with AM undergoing HD-IFN therapy (Table 3).
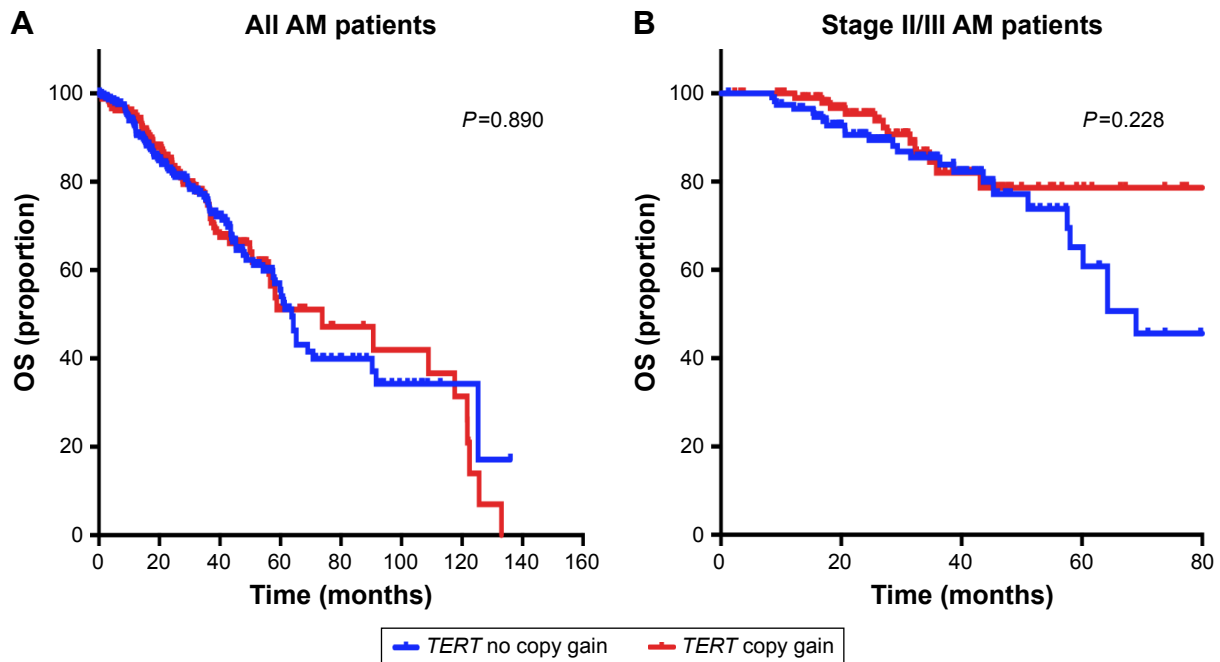
Discussion

AM is distinguishable from other melanoma subtypes by its unique clinical, epidemiological, and genetic features.[1,2,7,28] AM is the predominant melanoma subtype in non-Caucasians and has a notably worse prognosis than common cutaneous melanoma.[4,29] In recent years, AM was found to have a markedly different genomic landscape from other subtypes of melanoma, with a far lower mutation burden dominated

A **All AM patients**

B **Stage II/III AM patients**

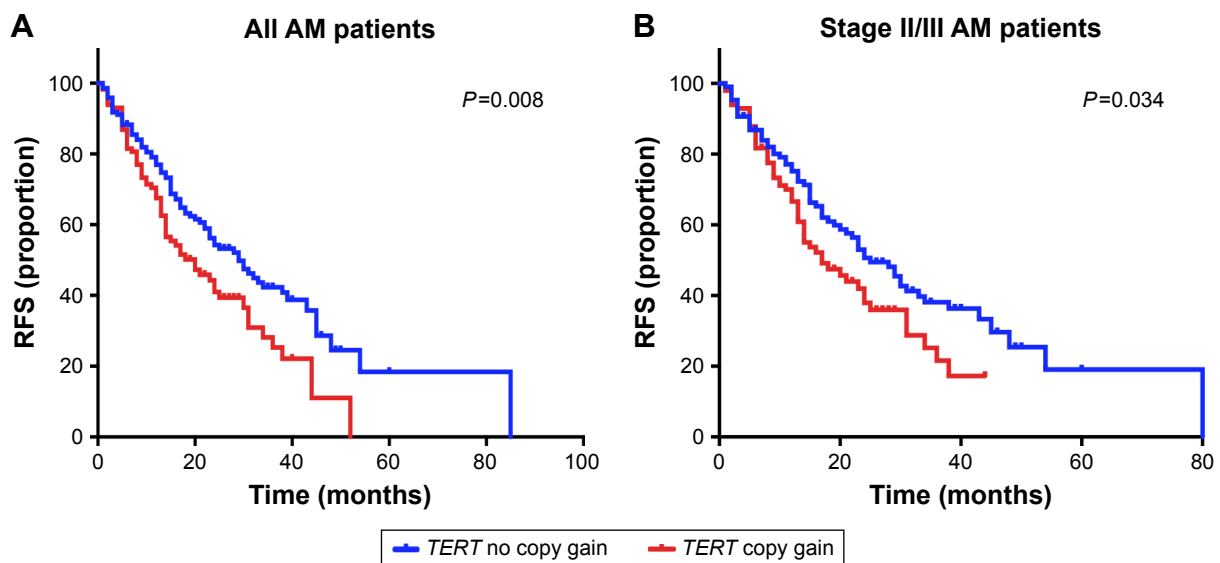**Figure 2** Influence of the *TERT* copy gain on patients' OS.
Notes: (**A**) Kaplan–Meier survival analysis was performed in all the 573 patients. The copy gain of *TERT* did not correlate with the OS of all patients (*P*=0.890). (**B**) Among the 573 patients with AM, 278 received a high-dose IFN regimen. A total of 221 patients had stage II/III disease. Kaplan–Meier survival analysis showed no significant difference in OS between the *TERT* copy gain group and no copy gain group (*P*=0.228).
Abbreviations: AM, acral melanoma; IFN, interferon; OS, overall survival.

by large-scale structural variants[7] and a higher frequency of focal amplifications.[13] In this study, we focused on the gene copy gain of *TERT* on a large scale, with an aim to determine its prognostic significance in patients with AM and to

analyze the relationship between *TERT* and the efficiency of the HD-IFN.

Somatic mutations and amplification are significant sources of genetic diversity. In our study, the copy gain rate

A **All AM patients**

B **Stage II/III AM patients**

Figure 3 Influence of *TERT* copy gain on RFS in patients with AM treated with HD-IFN.
Notes: (**A**) *TERT* copy gain is correlated with short RFS in patients with AM treated with HD-IFN. The curves demonstrated significant correlation between *TERT* copy gain and RFS in cases treated with IFN α-2b (*P*=0.008). (**B**) Among the 221 patients with stage II/III, *TERT* copy gain is correlated with short RFS (*P*=0.034).
Abbreviations: AM, acral melanoma; HD-IFN, high-dose interferon; RFS, relapse-free survival.

Table 3 Cox regression analysis of the correlation between *TERT* copy number and clinicopathologic factors with RFS

Factors	Group	HR	95% CI	*P*-value[a]
Univariate analysis				
Sex	Female vs male	0.930	0.671–1.290	0.660
Age	≥65 vs 65	0.687	0.462–1.023	0.087
TNM stage	III and IV vs I and II	2.893	2.031–4.121	<0.001
Ulceration	Ulceration vs nonulceration	1.092	0.776–1.536	0.612
Thickness	≥4 vs 4	1.142	0.812–1.598	0.422
TERT copy gain	Gain vs no gain	1.465	1.042–2.059	0.018
BRAF mutation	Mut vs WT	1.027	0.631–1.672	0.912
NRAS mutation	Mut vs WT	1.186	0.721–1.95	0.502
CKIT mutation	Mut vs WT	1.239	0.703–2.183	0.409
Multivariate analysis				
TERT copy gain	Gain vs no gain	1.496	1.069–2.094	0.019
TNM stage	III and IV vs I and II	2.922	2.095–4.077	<0.001

Note: [a]The *P*-value was analyzed by the χ^2 tests.
Abbreviations: HR, hazard ratio; Mut, mutation; RFS, relapse-free survival; TNM, tumor-node-metastasis; WT, wild type.

of *TERT* in AM was 44.9% (257 of the 573) (gain >2-fold change). This result was significantly higher than those of previous studies (17.6%–31.2%).[26,30] This is probably due to the small sample size of these studies.

The tumor thickness, ulceration, and stage are known prognostic factors for predicting the outcomes of melanoma.[31] Our results showed that ulceration (*P*=0.028) and thickness (*P*=0.048) were associated with *TERT* copy gain. However, *TERT* copy gain is not correlated with the stage of primary melanomas. In addition, unlike a previous study with a cohort of 43 AM patients,[25] we found no association between *TERT* copy gain and OS in our study. We obtained the same result after stratified analysis according to TNM stage. Our data suggest that the *TERT* copy gain may not play a significant role in the OS of patients with AM.

IFN-α has been the only drug observed to improve RFS in high-risk postoperative patients with melanoma, and the US Food and Drug Administration approved HD-IFN for melanoma in 1996.[32] A randomized phase II trial conducted in Chinese high-risk patients with AM showed that 1-year adjuvant HD-IFN (15×10^6 U/m² for days 1–5/week for 4 weeks + 9×10^6 U twice a week for 48 weeks) may yield clinical benefits in patients with stage IIIb–IIIc AM or those with ≥3 nodal metastases.[16] The median RFS of patients with AM treated with 1-year adjuvant HD-IFN was 17.9 months; however, no specific factor can predict the treatment efficacy.

In our study, the RFS of patients with *TERT* copy gain was significantly shorter than those with normal *TERT* copy number and stage II/III patients treated with HD-IFN. The Cox proportional hazards model also revealed that *TERT* copy gain was an independent adverse prognostic factor of RFS for all patients with AM treated with HD-IFN. This result can be explained as follows. The molecular mechanism of IFN α-2b includes direct and indirect activities. Direct activity occurs through the inhibition of cancer cell growth via cell cycle arrest, apoptosis, or differentiation. Indirect activity occurs through the activation of immune cells, inhibition of vascularization, and induction of cytokines.[33] Many studies have reported that IFN α-2b can regulate more than 300 genes in cellular signal transduction pathways.[34] IFN α-2b can activate JAK-1 and TYK-2 and induce antiproliferative and antiviral activity.[35,36] IFN α-2b can also inhibit the interaction of ERK with MEK or the interaction of MEK with other kinases.[37]

Given the mechanism by which IFN α-2b influences the growth of various cancer cells via the JAK/STAT and MAPK signal transduction pathways, some researchers have also found a relationship between *TERT* and these pathways. They found that the expression levels and enzymatic activity of *TERT* are regulated by multiple signaling molecules and pathways, including the JAK/STAT, RAS/RAF/MEK/MAPK, PI3K/Akt/mTOR, IKK/NFκB, TGFβ/Smads, and PKC pathways.[38] These studies may help to explain why the HD-IFN group with no copy gain of *TERT* exhibited longer RFS than the *TERT* copy gain group. However, the exact mechanism remains unknown; thus, further research is warranted. In addition, to achieve more satisfactory effects, we also suggest that the *TERT* copy number of patients be detected before administering HD-IFN treatment.

Conclusion

In this study, we have shown the status and the clinical significance of *TERT* copy number in a large cohort of patients with AM. We found that the *TERT* copy gain might not be a prognostic factor of OS in patients with AM. However, *TERT* copy gain may predict the outcome of HD-IFN treatment for AM.

Acknowledgments

Author contributions

All authors contributed toward data analysis, drafting, and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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