Evaluation of P53 immunostaining in patients with cutaneous melanoma

JIRAROCH MEEVASSANA[1,2], CHANYA MITTRAKULKIJ[1,3], PONTEERA TOWORRAKUL[1,3], WANTAMOL SAENSUK[1,3], SUPITCHA KAMOLRATANAKUL[4], TIPPAWAN SIRITIENTONG[1,5], KOMKRIT RUANGRITCHANKUL[6] and NAKARIN KITKUMTHORN[7]

[1]Center of Excellence in Burn and Wound Care, Chulalongkorn University; [2]Department of Anatomy; [3]Division of Plastic and Reconstructive Surgery, Department of Surgery, Faculty of Medicine, Chulalongkorn University, Bangkok 10330; [4]Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400; [5]Department of Food and Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences; [6]Department of Pathology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330; [7]Department of Oral Biology, Faculty of Dentistry, Mahidol University, Bangkok 10400, Thailand

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Abstract. P53 is a tumor suppressor gene that is mutated in numerous types of cancer. The aim of the present study was to determine the frequency of this mutation in cutaneous melanomas and to conduct clinicopathological characteristics and clinical outcome association analyses with the P53 mutation. P53 immunohistochemical staining was used as a surrogate marker for P53 mutation analysis to assess P53 status. In the present study, 50 pathological samples of cutaneous melanoma from 2012 to 2018 at Chulalongkorn University (Bangkok, Thailand), were subjected to anti-P53 immunohistochemistry, followed by an examination of the association between P53 statuses and clinical and pathological characteristics, along with clinical outcomes. A positive staining for anti-P53 antibody was detected in 30% of patients (15/50) with cutaneous melanomas. Positivity was significantly associated with female sex, nodular histological subtype and Breslow level 4. Cox regression analysis revealed that an age >65.5 years and Breslow grade 4 disease were associated with mortality. The Kaplan-Meier curve revealed a shorter duration of recurrence time in the P53 mutation than P53 wild type. In the present study, P53 mutations in specific cases of cutaneous melanoma were identified. Notably, patients who were older and/or had a Breslow score of 4 exhibited an increased risk of mortality. These findings suggested the potential involvement of P53 mutations in cutaneous melanoma, highlighting the necessity for further investigations to improve understanding of their roles.

Correspondence to: Dr Nakarin Kitkumthorn, Department of Oral Biology, Faculty of Dentistry, Mahidol University, 272 Rama VI Road, Ratchathewi, Bangkok 10400, Thailand
E-mail: nakarinkit@gmail.com

Abbreviations: IHC, immunohistochemistry; DFS, disease-free survival; OS, overall survival; HR, hazard ratio

Key words: tumor protein P53, immunostaining, cutaneous melanoma, IHC, clinicopathological features, OS

Introduction

Cutaneous melanoma is the most common type of melanoma and is a malignant tumor. Several subtypes that are classified according to histological criteria, include superficial spreading, nodular, lentigo maligna and acral lentiginous melanomas (1). Although the histological criteria are widely accepted, histological subtyping plays a very small role in predicting the behavior and prognosis of the disease (2). Cutaneous melanomas arise from melanocytes, which are melanin-producing cells found in the basal layer of the epidermis. Melanin prevents injury due to ultraviolet exposure, which is the most important risk factor for cutaneous melanoma (3). Besides ultraviolet exposure, genetic mutations are another factor involved in cutaneous melanoma-genesis (4). Although cutaneous melanoma accounts for <5% of all skin cancers, it can develop in individuals of all ages and colors and is responsible for >70% of skin cancer-related deaths (5). Countries with a high incidence of cutaneous melanoma are those with Caucasian populations and are mostly located near the equator, where UV levels are the highest. Despite its location, Thailand has lesser instances of melanoma, but a high mortality rate (6).

P53 is a transcription factor that suppresses tumor growth through cell cycle arrest and apoptosis (7). Mutations in BRAF, CDKN2A and MDMX are frequently observed in cutaneous melanoma (5,8). BRAF causes melanoma by dysregulating the activation of downstream MEK/ERK effectors (9). CDKN2A, a tumor suppressor gene, encodes p14ARF that inhibits MDM2 mediated degradation of P53 (4,10). Accordingly, the loss of CDKN2A can inactivate the P53 pathway. In addition, missense mutations are common in TP53. Because of their predominant

occurrence in the DNA-binding domain, P53 cannot bind to DNA and regulate transcription (11). Consequently, mutations in P53 can disable tumor suppressor activity and confer oncogenic potential, thereby enhancing cancer cell proliferation.

Since P53 mutations are commonly found in human cancers, researchers are attempting to identify chemotherapeutics to target this mutation precisely. For example, there are strategies that involve the development of small molecular compounds and short peptides to restore normal functionality of the gene (12).

Currently, there is scant data on the frequency of P53 mutations in cutaneous melanoma, particularly in the Thai population. A cohort of Thai patients with cutaneous melanoma was examined. Owing to its simplicity of implementation and widespread adoption by pathologists, immunohistochemistry (IHC) technique was utilized as it serves as a widely accepted and reliable surrogate marker for P53 mutational analysis (13,14). The clinicopathological features and prognostic variables were assessed in association with the prevalence of P53 mutations.

Materials and methods

Patient recruitment. The present study was approved (approval no. 1643/2564) by the Ethics Committee of the Faculty of Medicine of Chulalongkorn University (Med Chula IRB; Bangkok, Thailand). Written informed consent was signed by all participants. The exclusion criteria are low amounts of pathologic tis-sue, a lack of clinical data and poor follow-up. The population of the present study consisted of 50 patients admitted to the Plastic and Reconstructive Surgery Unit, Department of Surgery, King Chulalongkorn Memorial Hospital (Bangkok, Thailand) between 2012 and 2018 (Table I). The median age was 65.5 years (range, 28-95 years) and 26 patients (52%) were women. According to the AJCC Cancer Staging Manual, Eighth Edition (15), 28 of the 50 patients (56%) were in the advanced stage (III-IV) at initial diagnosis, and 22 (44%) were in the early stage (I-II). Among them, 31 (62%) presented with ulcers and 19 (38%) did not. Pathological records were manually screened to record the histological characteristics of the primary tumor and to identify recurrences and deaths. Histological subtype analysis revealed that superficial spreading accounted for 10% (five cases) of the diagnoses, nodular subtype accounted for 48% (24 cases) and acral lentiginous subtype accounted for 42% (21 cases). The majority were at Breslow level 4, followed by Breslow levels 3, 2 and 1 (56, 20, 16 and 8%, respectively). For survival analysis, all patients were followed-up from the day of surgery to the day of death, recurrence, or final follow-up. The median follow-up period for disease-free survival (DFS) was 14 months (range: 2-53 months), despite the median follow-up period for overall survival (OS) being 24.5 months (range: 4-96 months). Owing to the authors' concern about the relationship between the variables of interest, the possible variable factors of interest were examined and used in the crude and adjusted hazard ratio analyses.

IHC staining. The anti-P53 monoclonal DO7 mouse antihuman antibody (cat. no. GA61661-2; Dako; Agilent Technologies, Inc.) was applied to 3-μm-thick paraffin-embedded tissue slides. IHC was performed using an automated immunostainer (BenchMark XT, Ventana Medical Systems; Roche Tissue Diagnostics; Roche Diagnostics, Ltd.), following the manufacturer's instructions. Based on the previous studies, positive IHC staining was considered to be the mutated form of P53, whereas negative IHC staining for P53 was determined to be the wild-type (13,14). A total of >10% of the strong and homogeneous nucleus-stained tumor cell samples were considered positive, whereas the samples with absent nuclear staining of tumor cells were classified as negative. Representative images from each category are shown in Fig. 1. Tumor cells with scattered melanin pigments were identified and not counted for P53 staining.

Statistical analysis. Chi-square or Fisher's exact tests were used (as appropriate) to assess significant differences in the distribution of mutated P53 staining. For comparisons between more than two groups, Two way analysis of variance (ANOVA) was used. Kaplan-Meier analysis was used to investigate the DFS and OS. Crude and adjusted hazard ratios were calculated using a Cox regression model. The significance level was set at a two-tailed P-value of less than 0.05. SPSS Statistics ver. 23.0 (IBM Corp.) was used for all statistical analyses.

Results

Association of the mutated P53 with clinicopathological characteristics. IHC staining for P53 was positive in 15 cases (30%), which were considered to be mutant P53 (Fig. 1A). Negative P53 IHC staining was determined to be the wild-type (Fig. 1B). The association between clinicopathological characteristics and P53 IHC status is summarized in Table I. Among the 9 variables, only 3 showed statistical significance for association with the P53 mutation: sex (P=0.048), Breslow level (P=0.006), and histological subtype (P=0.001). Cases with mutated P53 were considerably more frequent in female patients (11 of 26 cases, 42.31%) than in male patients (4 of 24 cases, 16.67%). Furthermore, Breslow level 4 cases had a higher prevalence of mutated P53 compared with Breslow level 1-3 cases. Of the samples from 28 Breslow level 4 cases, 14 (50%) exhibited positive P53 IHC results, whereas only 1 of 22 cases (4.55%) with Breslow level 1-3 was positive for P53 staining. As for histological subtype, positive P53 IHC was mostly observed in the nodular subtype (13 of 24 cases, 54.2%), followed by superficial spreading (1 of 5 cases, 20%) and acral lentiginous (1 of 21 cases, 4.76%). Other clinicopathological variables, including age, tumor stage and clinical presence of ulcers, were not significantly associated with mutated P53. Normal tissues adjacent to melanoma were negative for P53 using IHC staining (Fig. S1).

DFS. During the follow-up period, 60.4% patients with cutaneous melanoma experienced disease recurrence. Moreover, mutated P53 and advanced tumor stage demonstrated higher crude hazard ratios (HR) of 1.140 [95% Confidence Interval (CI): 0.527-2.469] and 1.249 (95% CI: 0.548-2.851), respectively (Table II). Although the Kaplan-Meier curve revealed that patients with mutated P53 had a shorter DFS time, the difference was not statistically significant (P=0.739, Fig. 2A).

Table I. Clinicopathological characteristics of patients with cutaneous melanoma and P53 status.

Clinicopathological characteristics	n	P53 wild-type	Mutated P53	P-value
Sex				0.048[a]
Male	24	20	4	
Female	26	15	11	
Age				0.355
≤65.5	25	19	6	
>65.5	25	16	9	
Histological subtype				0.001[b]
Superficial spreading	5	4	1	
Nodular	24	11	13	
Acral lentiginous	21	20	1	
Breslow level				0.006[a]
1	4	4	0	
2	8	8	0	
3	10	9	1	
4	28	14	14	
Tumor stage				0.804
Early stage (I-II)	22	15	7	
Advanced stage (III-IV)	28	20	8	
Ulcer				0.086
Presence	31	19	12	
Absence	19	16	3	
Staging node				0.308
0	24	16	8	
1	13	8	5	
2	6	4	2	
3	7	7	0	
Recurrence[c]				0.217
Recurrent	29	18	11	
Not Recurrent	19	15	4	
Death[c]				0.869
Dead	27	19	8	
Alive	22	15	7	
Total	50	35	15	

[a]P<0.05 and [b]P<0.001. [c]Remarks for missing data.

OS. The crude and adjusted HR analyses for mortality in patients with cutaneous melanoma are listed in Table III. The overall mortality rate in the present study was 55.1%. The two significant variables were age (P=0.023) and Breslow level (P=0.035). Patients over the age of 65.5 years had significantly longer survival times than younger patients, with a crude HR of patient mortality of 0.368 (95% CI: 0.156-0.869). Remarkably, this result was very close to the adjusted HR of 0.383 (95% CI, 0.156-0.937). Another significant discovery in patients with Breslow level 4 was that their primary tumor had a significantly lower HR for mortality of 0.073 (95% CI: 0.006-0.827) with a P-value of 0.035. After two years of follow-up, the Kaplan-Meier curve revealed no difference between the OS time associated with mutated P53 and wild type P53 (P=0.796, Fig. 2B).

Discussion

Wiriyakulsit *et al* (6), reported the incidence and mortality rates of melanoma to be 0.52 and 0.25 per 100,000 individuals, respectively (6). Although there were few melanoma cases in Thailand, the death rate was significant at 48.08%. This is in concurrence with the findings of the present study, indicating the overall death rate as 55.1%.

The prevalence of P53 mutation in cutaneous melanoma has received little attention, and there has been no prior research on Thai patients. A study from Europe found that 17 out of 81 (21%) cutaneous melanoma specimens harbored a P53 mutation (16). On similar lines, a systematic review of reported cases in the United States found 68 of the 575 cases

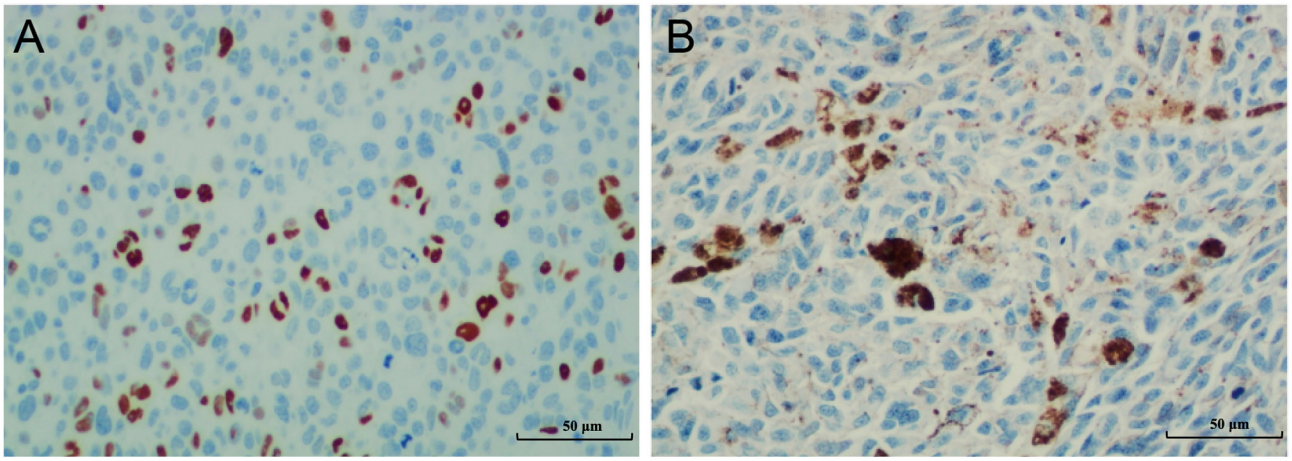

Figure 1. IHC of P53 mutation. (A) IHC staining of cutaneous melanoma with a P53 mutation (original magnification, x200) shows the brown color of the positive P53 signal in tumor cells. (B) IHC staining of P53 wild-type cutaneous melanoma (original magnification, x200) showing only the blue color of Hematoxylin I counterstaining in tumor cells. Melanin pigment levels were remarkable. IHC, immunohistochemical.

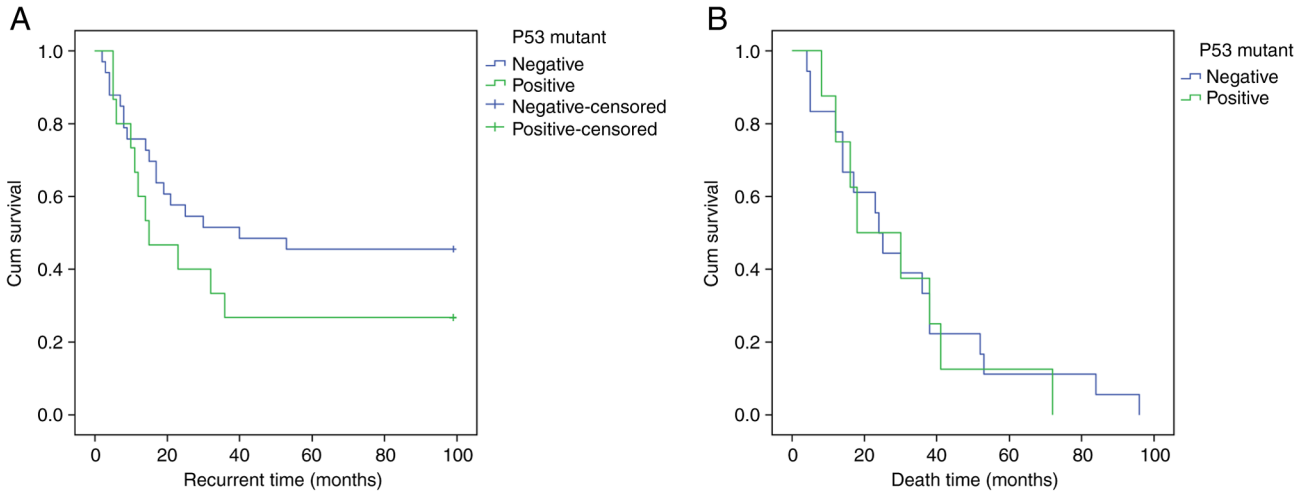

Figure 2. Kaplan-Meier curve of (A) disease-free survival (P=0.736) and (B) overall survival (P=0.796) compared between P53 wild-type and P53 mutated cutaneous melanoma patients.

(11.9%) analyzed were with the P53 mutation in cutaneous melanoma (17). The present study revealed that, P53 mutations were discovered in 30% of the participants, which is higher than that previously reported. This higher occurrence of cutaneous melanoma P53 mutation in Thailand demonstrated the relationship between the two and could potentially lead to the development of effective and optimum targeted treatment.

Since melanoma originates from melanocytes, the present study did not involve a direct comparison of melanoma cells with normal melanocytes. This decision was primarily due to the small proportion of melanocytes present in normal skin and the difficulty in identifying melanocytes in H&E sections, which typically require special staining techniques. However, during the present's study inspection of the normal epithelium next to the melanoma, no cells were identified that tested positive for the anti-P53 antibody, as illustrated in Fig. S1. This particular finding confirms the absence of P53 mutations in normal skin.

Furthermore, it was revealed that the P53 mutation had a significant association with some clinicopathological characteristics including female sex, nodular subtype and Breslow level 4. However, it was not associated with age, tumor stage, ulcer presentation and node stage. These clinicopathological results have not been widely reported and varied across individual studies. To the best of the authors' knowledge, a study in the United States reported that P53 mutation is associated with older age (18), which contrasts with the present study. However, in line with the findings of the present study, another study suggested no association between P53 mutation and ulceration (18). These controversies could be resolved in an improved way with larger sample sizes. Further molecular testing of other methods, such as next-generation sequencing could also be useful, as a report of inter-method discrepancy exists (19).

Although the prevalence of mutated P53 in cutaneous melanoma was moderate (30%), the rates of recurrence and death in patients with P53-mutated cutaneous melanoma in the present study were relatively high (60.5 and 55.1%, respectively). Furthermore, the duration for recurrence in cases with the P53 mutation was lower than that for wild-type P53. This poor clinical outcome may stem from the dysfunction of mutated P53 itself and its interaction with other oncogenic genes, especially

Table II. Univariate and multivariate analysis of DFS and P53 status in cutaneous melanoma patients.

		Crude hazard ratio		
Characteristics	Recurrence, N (%)	Hazard ratio	95% Confidence interval	P-value
Sex				
Male	11 (22.4)	1		
Female	18 (37.5)	1.080	0.503-2.316	0.844
Age				
≤65.5	12 (25)	1		
>65.5	17 (35.4)	0.696	0.326-1.489	0.351
Histological subtype				
Superficial spreading	4 (8.3)	1		
Nodular	16 (33.3)	0.961	0.315-2.931	0.944
Acral lentiginous	9 (18.8)	0.431	0.123-1.513	0.189
P53				
P53 wild type	18 (37.5)	1		
Mutated P53	11 (22.9)	1.140	0.527-2.469	0.739
Breslow level				
1	1 (2.1)	1		
2	3 (6.3)	1.000	0.000-49486.161	1.000
3	5 (10.4)	1.000	0.000-47365.258	1.000
4	20 (41.7)	1.000	0.000-46358.619	1.000
Tumor stage				
Early stage (I-II)	9 (18.8)	1		
Advanced stage (III-IV)	20 (41.7)	1.249	0.548-2.851	0.597
Ulcer				
Absence	8 (16.7)	1		
Presence	21 (43.75)	1.342	0.565-3.185	0.505
Staging node				
0	11 (22.9)	1		
1	8 (16.7)	1.388	0.545-3.539	0.492
2	5 (10.4)	0.916	0.307-2.734	0.875
3	5 (10.4)	2.669	0.870-8.195	0.086
Total	29 (60.4)			

BRAF, which was shown in a study by Celesia *et al* (20) to be preferentially interacting with mutated P53 than the wild-type. In the present study, it was also revealed that the mortality rate associated with mutated P53 significantly increased with advanced age and Breslow level 4. This is consistent with previous studies that found that older age was strongly associated with higher mortality rates (21,22).

This association between mutated P53 and the prognosis of cutaneous melanoma needs to be studied further to elucidate the underlying mechanisms and develop an effective and ideal targeted therapy to counter the resistance of melanoma to current regimens. Accumulating evidence supports the idea that P53 plays an important role in the tumor suppression in multiple types of cancer, including skin cancer (5). Stabilization and activation of P53, known as the guardian of the genome, results in cell cycle arrest, DNA repair, and/or apoptosis in severely and persistently damaged tumor cells (5,11). Understanding the relationship between P53 and other proteins such as MDMX, MDM2, BRAF, and melanoma, is important. Thus, targeted therapy aimed at P53 is currently promising, but also challenging, for the treatment of cutaneous melanoma and other skin cancers in future (23).

In addition to BRAF, P53-targeted therapy is novel and promising in this new era, and a number of researchers are attempting to uncover its secrets. P53 is a key target of numerous novel chemotherapeutics but is generally ineffective as a stand-alone agent (24). Therapeutic outcomes can be achieved by combining a drug with P53 activation and MDMX-MDM2, BRAF, or MEK inhibitors (5,11). The drug currently under study, known as PRIMA-1^{MET}, activates P53, thereby leading to tumor suppression, and helps sensitize melanoma cells to other targeted therapies. Furthermore, the combination of PRIMA-1^{MET} and pimasertib noticeably promoted apoptosis in melanoma cells (5).

Table III. Univariate analysis of overall survival and P53 status in cutaneous melanoma patients.

		Crude hazard ratio			Adjusted hazard ratio		
Characteristics	Death (N, %)	HR	95% CI	P-value	HR	95% CI	P-value
Sex							
Male	9 (18.4)	1					
Female	18 (36.7)	1.003	0.441-2.277	0.995	-	-	-
Age							
≤65.5	12 (24.5)	1			1		
>65.5	15 (30.6)	0.368	0.156-0.869	0.023[a]	0.383	0.156-0.937	0.036[a]
Histological subtype							
Superficial spreading	4 (8.2)	1					
Nodular	15 (30.6)	1.141	0.342-3.807	0.830	-	-	-
Acral lentiginous	8 (16.3)	0.625	0.173-2.260	0.474	-	-	-
P53							
P53 wild type	19 (38.8)	1					
Mutated P53	8 (16.3)	1.119	0.478-2.620	0.796	-	-	-
Breslow level							
1	1 (2.0)	1			1		
2	4 (8.2)	0.097	0.008-1.254	0.074	0.161	0.012-2.121	0.165
3	5 (10.2)	0.104	0.008-1.343	0.083	0.130	0.010-1.672	0.117
4	17 (34.7)	0.073	0.006-0.827	0.035[a]	0.112	0.010-1.299	0.080
Tumor stage							
Early stage (I-II)	9 (18.4)	1					
Advanced stage (III-IV)	18 (36.7)	1.910	0.775-4.709	0.160	-	-	-
Ulcer							
Absence	9 (18.4)	1					
Presence	18 (36.7)	1.592	0.673-3.766	0.290	-	-	-
Staging node							
0	11 (22.5)	1					
1	5 (10.2)	1.960	0.587-6.544	0.274	-	-	-
2	6 (12.2)	1.585	0.557-4.513	0.388	-	-	-
3	5 (10.2)	1.814	0.587-5.608	0.301	-	-	-
Total	27 (55.1)						

[a]P<0.05. HR, hazard ratio; CI, confidence interval.

The limited sample size of the present study is a key limitation because it restricts the extent to which the results can be applied, including in terms of ethnicity. Thailand is a multiethnic society; however, due to a strong homogenizing culture, most Thai nationals identify as ethnic Tai, including in the patient registry, regardless of their actual ethnic background. This predicament obscures the genetic diversity in the research of the present study. Therefore, further research with a larger sample size is crucial to validate and reinforce these results. It is also suggested to collect additional information on more lifestyle factors related to melanoma for analysis, such as sunlight exposure and the use of sun protective products.

In conclusion, the findings of the present study revealed that cutaneous melanomas corresponded to P53 mutation, recurrence rates and mortality rates. The addition of cancer cells to mutated P53 still makes it an attractive target for melanoma therapy. It is considered that these findings will be one of the initial steps towards understanding disease incidence and prognostic factors and promoting the innovation of future curative therapies for melanoma. Consequently, additional studies with larger populations should be conducted to investigate novel targeted therapies.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors conceptualized the present study and performed formal analysis. JM, KR and NK acquired funding. WS, SK, TS, KR and NK developed methodology. JM and NK supervised the study, performed project administration, provided resources and conducted software analysis. JM, TS, KR and NK validated data. JM, CM, PT, WS and SK performed data visualization. JM, CM, PT, WS, KR and NK conducted investigation. JM, KR and NK confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved approval no. 1643/2564 by the Ethics Committee of the Faculty of Medicine of Chulalongkorn University (Med Chula IRB; Bangkok, Thailand). Written informed consent was signed by all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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