RESEARCH ARTICLE

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BRAF and *NRAS* Mutations and the Association with Prognosis of Acral Lentiginous and Nodular Melanomas in Indonesia

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Abstract

Background: Melanomas are rare yet the most aggressive skin cancer among Asians. Clinical presentation, risk factors, and the underlying molecular mechanisms are strikingly different from cutaneous melanoma in Caucasians. **Methods:** Mutation patterns of *BRAF* and *NRAS* genes were examined from DNAs derived from primary melanoma tumor tissues (fresh tissues or formalin-fixed paraffin-embedded samples) using pyrosequencing. **Results:** A total of 63 patients consisting of acral lentiginous melanoma (N=22, 34.9%) and nodular melanoma (N=41, 65.1%) were included in this study. Most patients were diagnosed at Stage III-IV (N=49, 77.8%), Breslow thickness more than 4 mm (N=51, 80.9%), presence of ulceration (N=35, 55.6%), diameter larger than 6 mm (N=61, 96.8%), regional node infiltration (N=41, 77.8%). *BRAF* and *NRAS* mutations were found in 28 (44.4%) and 8 (12.7%), respectively. *BRAF* and *NRAS* mutations were significantly associated with older melanoma patients (OR = 6.075, 95%CI = 2.013-18.333 dan OR = 13.263, 95%CI = 1.518-115.901, respectively). *BRAF* mutations were associated with lower overall survival (Median survivals were 16.5 vs 31.4 months, Log-rank test P=0.001). *NRAS* mutations were not significantly associated with lower overall survival. **Conclusion:** In this study, melanoma patients are largely diagnosed at the late stages with ulceration and involvement of regional lymph nodes. *BRAF* mutations are associated with lower survival of cutaneous melanoma patients.

Keywords: Melanoma- *BRAF*- *NRAS*- survival- acral lentiginous- nodular melanoma

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Introduction

Melanoma arises from transformed melanocytes that clinicially represent the most aggressive cutaneous cancer with high frequency of distant metastases and premature mortality [1]. Although the prevalence is only 1% of all skin cancers, melanoma has been associated with 47% mortality due to skin cancers [2]. Risk factors, clinical presentation, and response to treatment of patients with cutaneous melanoma are known to be immensely different between Caucasians and Asians [3, 4]. Almost 90% of melanoma among Caucasians is diagnosed in sun-exposed skin and 60% of them present as superficial spreading melanomas [4]. Among Asians, around 50% of cutaneous melanomas are acral lentiginous melanomas that present in body areas with little sun exposure and often with less pigment entity [5]. In addition, a higher proportion of cutanous melanoma in Asia is manifested in the acral or extremity with nodular subtype and more often with ulceration [6].

Molecular characteristics and landscape of actionable mutations differ among melanoma subtypes [7]. In the superficial spreading melanoma, *BRAF* mutations account for almost 60% of patients and more frequently in younger patients [7]. In the primary melanoma tissues, *CDKN2A*, *NRAS*, and *TP53* are also more frequently mutated among Caucasians than Asians [8, 9]. *BRAF*, *NRAS*, *NF1*, and *KIT* are mutated with lower frequency in acral melanoma [8, 9]. In a large systematic review [10], *BRAF* mutations were associated with superficial spreading melanoma, *NRAS* mutations were associated with nodular melanoma, and *KIT* mutations were associated with mucosal melanoma. In addition, cutaneous melanoma in Asians is often diagnosed at late stages at diagnosis causing particular obstacles in the complete surgical resection [5, 8]. As a rare cancer that presents in body areas that are not

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routinely examined during general check-up, cutaneous melanoma among Asians is often delayed in the diagnosis and treatment initiation [3]. In addition, responses to immunotherapy and targeted therapy among Asians seem to be lower than melanoma in Caucasians.

BRAF and *NRAS* mutations play a significant role in the development and progression of melanoma [1]. In addition, *BRAF* and *NRAS* mutations are associated with almost a 3-fold higher risk of mortality compared to wildtype melanoma patients after adjusting other prognostic variables [11]. However, there is still limited information regarding the frequency and correlation of *BRAF* and *NRAS* mutations in melanoma patients from different populations. Current literature for melanoma [8, 12], the therapeutic responses, and clinical guidelines mostly derive from studies among Caucasians. The association of various ethnicities to the histological and clinical manifestation and outcome is also lacking. In addition, actionable mutations in melanoma including *BRAF* and *NRAS* carry potential options for further treatment. Therefore, information regarding the mutations might also be beneficial in the policy making to improve clinical management for melanoma patients.

Materials and Methods

Study participants

All cutaneous melanoma patients treated at our department were recruited in this study. Inform consent was obtained from all participants. Primary tumor tissues were collected from fresh tissues or paraffin-embedded tissues. Fresh tumor tissues were directly submerged in RNAleter® Solution (Thermo Fisher). Deoxyribose nucleic acid (DNA) was extracted using QIAwave® Tissue Kit (Qiagen) or Quick-DNATM FFPE Kit (Zymo Research). Melanoma patients with a minimun follow-up of 3 months were included in the survival analysis. Patients with unresectable lesions and loss of follow-up were not included. Cancer staging was determined using the American Joint Committee on Cancer (AJCC) system [13]. Sentinel lymph node biopsy (SLNB) for early-stage melanomas was not routinely performed in this study cohort. Lymph node dissection was performed in Stage III patients or the presence of positive regional lymph nodes. Categorization of high-risk melanomas was determined for patients with Breslow depth > 4 mm, positive regional lymph nodes, or lesions with ulceration [13, 14].

BRAF and NRAS mutation analyses

Mutation analysis for *BRAF* was performed to detect hotspot mutation in the Exon 15 at codon V600. For *NRAS* mutation analysis was targeted at the Codon 59/61. DNAs from primary tissue samples (25ng) were amplified in polymerase chain reaction (PCR) using PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.5 U HotStart Taq-Polymerase (Platinum TaqTM DNA polymerase, ThermoFisher Scientific), and a mixture of 3 primers. Primers used for *BRAF* mutation detection were a forward primer (5'-GAAGACCTCACAGTAAAAATAG-3'), a reverse primer (5'-ATAGCCTCAATTCTTACCATCC-3'), and a universal biotinylated primer (5'-GGGACACCGCTGATCGTTTA-3'). For NRAS mutation detection, a forward primer (5`-GAAACCTGTTTGTTGGACATACTGG-3`), a reverse primer (5`-GGATTGTCAGTGCGCTTTTC-3`), and a universal biotinylated primer (5'-GGGACACCGCTGATCGTTTA-3') were used. Thermocycling was performed in Biometra® Personal Thermocyler (Analytic Jena) with the following program: preheating of 95°C in 5 minutes and 45 cycles of 95°C for 30 seconds, 95°C for 45 seconds, 95°C for 30 seconds, and 95°C for 5 minutes. PCR products were 122 bp and 82 bp for *BRAF* and *NRAS*, respectively. Pyrosequencing was performed using 10µL of PCR products for separation of biotinylated fragments using 3µL of Streptavidin Sepharose (Amersham Bioscience) and binding buffer (10 mmol/L tris-HCl, 2 M NaCl, 1 mmol/L EDTA, 1 ml/L Tween 20) in the PyroMark Q96 Vacuum workstation (Qiagen). For sequencing, 5'-GGTGATTTTGGTCTAGCTAC-3' and 5`-TTGGACATACTGGATACA-3` were used as primers. Pyrograms were analyzed using PyroMark Q96 Software ® (Qiagen). More detailed procedures for pyrosequencing and analysis were described in a previous study [15]. All methods were performed according to the standard guidelines and regulations. Clinicovariables were presented according to age, sex, Breslow thickness, and location according to previous report [14]. Occupation was determined into outdoor (agriculture, construction, fisherman, and merchant) or indoor (public sector, teacher, and factory). Residence was classified according to the official address in the identity card, smoking was determined as ever or active if participants had consumed more that 100 cigarettes. Education was categorized into elementary or less and highschool or college.

Statistical analyses

The mutational status of *BRAF* and *NRAS* were compared with clinical and pathological variables using univariable and multivariable regression analysis tests. Overall survivals were compared using the Kaplan-Meier curve and Log-rank (Mantel-Cox) tests. All statistical analyses were performed using the SPSS v17.0 software (SPSS Inc, Chicago). Significant differences were determined if P-values were less than 0.05.

Results

Mutations of BRAF and NRAS in cutaneous melanoma patients

We included 63 cutaneous melanoma patients with a median age of 63 years consisting of acral lentiginous melanomas (N=22, 34.9%) and nodular melanomas (N=41, 65.1%), Table 1. Most patients were diagnosed at late stages (Stages III and IV, N=49, 77.8%), with Breslow thickness of more than 4 mm (N=51, 80.9%), with ulceration (N=35, 55.6%), positive regional lymph nodes (N=49, 77.8%), the largest diameter of lesions more than 6 mm (N=61, 96.8%), Table 1. The primary cutaneous melanomas were found mostly in the acral (N=28, 44.4%), extremity (N=32, 50.8%), and a small proportion in the body axis (N=1, 1.6%), and the head and neck (N=2,

Table 1. Clinicopathological Characteristics of Cutaneous Melanoma Patients

Variable	Category	Frequency (%)
Median age	63 years	
Age	≤ 50 years	8 (12.8)
	51-70	45 (71.4)
	>70	10 (15.8)
Sex	Male	27 (42.9)
	Female	36 (57.1)
Histological subtype	Acral lentigenous melanoma	22 (34.9)
	Nodular melanoma	41 (65.1)
Breslow thickness	T1	1 (1.6)
	T2	1 (1.6)
	T3	10 (15.9)
	T4	51 (80.9)
Ulceration	Yes	35 (55.6)
	No	28 (44.4)
Diameter	≤ 6 mm	2 (3.2)
	> 6 mm	61 (96.8)
Lymph node status	N0	14 (22.2)
	N1	7 (11.1)
	N2	15 (42.9)
	N3	27 (42.9)
Stage	I	0 (0)
	II	14 (22.2)
	III	47 (74.6)
	IV	2 (3.2)
Location of primary tumor	Acral	28 (44.4)
	Head and neck	2 (3.2)
	Body axis	1 (1.6)
	Extremity	32 (50.8)
Occupation	Mostly indoor	11 (17.5)
	Mostly outdoor	52 (82.5)
Residence	Rural	57 (90.5)
	Urban	6 (9.5)
Education	Elementary	58 (92.1)
	Highschool and college	5 (7.9)
Smooking	Never	38 (60.3)
	Ever or active	25 (39.7)

3.2%). Patients were mostly from rural area (N=57, 92%), non smokers (N=25, 39.7%), lower education status (elementary or lower, N=58, 92%), and outdoor worker (N=52, 82%). *BRAF V600E* mutations were detected in 28 of 63 (44.4%) and *NRAS* mutations were detected in 8 of 62 (12.7%) among cutaneous melanoma patients in our cohort. Using semiquantitative pyrosequencing, signal detection of mutation more than 1% was determined as positive. We found 3 primary melanoma tumors in this cohort had both *BRAF* and *NRAS* mutations.

Association of BRAF and NRAS mutations with clinicopathological variables

BRAF V600 mutations were associated with several clinical and pathological variables. In this study, *BRAF* mutations were significantly found in patients older than 65 years both using univariate and multivariate regression tests (OR= 6.075, 95%CI = 2.013-18.333, P = 0.001 and OR = 15.15, 95%CI = 2.683-90.90, P = 0.002, Table 2). *BRAF* mutations were more frequently detected in primary tumors of patients diagnosed with advanced stages and located in the acral and extremity (Table 2).

NRAS mutations were also associated with melanomas diagnosed in older age patients (OR = 13.263, 95% CI = 1.518-115.901, P = 0.019 and OR = 19.11, 95% CI = 1.296-281.9, P = 0.032 for univariate and multivariate regression tests, respectively). Other clinicopathological variables including sex, regional lymph node involvement, tumor diameter, and presence of ulceration were not significantly associated with mutations of *BRAF* and *NRAS* genes, Table 2 and Table 3. Occupation, residence, education levels, and smoking behavior were also not significantly associated with *BRAF* and *NRAS* mutation status

Association of BRAF and NRAS mutations with overall survival of cutaneous melanoma patients

Cutaneous melanoma patients with *BRAF* mutations were significantly associated with poor overall survival in comparison to those with wild-type (mean survivals were 14.4 vs 26.4 months, respectively; Log-rank test P = 0.001). *NRAS* mutations were not significantly associated with the overall survival of melanoma patients in our cohort (Mean survivals were 22.5 vs 30.5 months, respectively; Log-rank test P = 0.390). Patients with either *BRAF* or *NRAS* mutations were significantly associated with lower overall survivals than those with wild-type tumors (Mean survivals were 21.9 vs 36.1 months, respectively; Log-rank test P = 0.001).

Discussion

Most scientific literature on genomic landscapes in melanoma derives from studies among Caucasians [16, 17]. Clinical characteristics and treatment recommendations are also developed mostly from studies in this population [8, 18]. Therefore, differences in the oncogenic alteration during melanoma carcinogenesis in other populations including Asians need to be delineated. More importantly, actionable mutations have significant impacts on the treatment options and prognosis determination. *BRAF* mutations are found in more than 50% of melanoma patients that collectively promote MAPK activaltion and cell proliferation pathways [19]. *BRAF* mutations are detected in the early carcinogenesis of melanoma. However, the mutations are also associated with regional lymph node infiltration as well as hepatic and brain metastasis [20]. *NRAS* mutations account for 15-20% of all melanoma patients. The *NRAS* mutation activates MAPK signaling and cell cycle pathways [21]. Melanoma patients with *NRAS* mutations had poorer survival compared to patients with *BRAF* mutations [21]. *BRAF* and *NRAS* mutations and their association with clinicopathological and clinical outcomes among melanoma patients in Indonesia have not yet been comprehensively reported.

Table 2. Association of *BRAF* Mutation Status with Clinicopathological Variables Using Univariate and Multivariate Regression Analyses

Variable	Category	Reference	Univariable regression		Multivariable regression	
			OR(95%CI)	P-value	OR(95%CI)	P-value
Age	>65 years	≤65 years	6.075 (2.013-18.333)	0.001	15.15 (2.638-90.90)	0.002
Sex	Female	Male	0.769 (0.282-2.100)	0.609	0.178 (0.004-7.963)	0.374
Histology	ALM	Nodular	1.412 (0.498-4.000)	0.516	2.121 (0.477-10.05)	0.344
Breslow	>4mm	≤4 mm	0.781 (0.145-4.207)	0.774	7.299 (0.486-199.1)	0.137
Lymph node	Positive	Negative	1.592 (0.466-5.441)	0.458	5.787 (0.157-213.7)	0.34
Ulceration	Yes	No	1.460 (0.533-3.997)	0.462	1.274 (0.302-5.386)	0.741
Stage	Advanced(III-IV)	Early(I-II)	2.469 (0.588-10.360)	0.217	166.6 (1.700-1754)	0.029
Location	Acral, extremity	Axis, Head and neck	-	0.047	-	0.996
Diameter	>6 mm	≤ 6mm	0.794 (0.047-13.289)	0.873	0.343 (0.068-1.733)	0.998
Occupation	Outdoor	Indoor	4.500 (0.885-22.870)	0.07	0.076 (0.004-1.612)	0.098
Residence	Rural	Urban	4.500 (0.494-40.983)	0.182	2.114 (0.362-38.25)	0.334
Education	Highschool/college	Elementary	0.821 (0.127-5.284)	0.835	0.902 (0.114-5.512)	0.901
Smoking	Ever or active	Never	1.296 (0.460-3.502)	0.645	5.649 (0.121-249.8	0.377

Cutaneous melanoma among Asians is often diagnosed in advanced stages with relatively poorer outcomes [6, 22]. In our study, only two subtypes of melanoma were presented i.e. acral lentiginous and nodular melanomas with 77.8% of them diagnosed at Stage III-IV (Table 2). More than 80% of patients were diagnosed with Breslow thickness more than 4 mm and more than half of patients had ulceration (Table 2). Acral lentiginous and nodular melanomas account for almost 85% of cutaneous melanoma among Asians [23, 24]. In addition, multiple studies on Asians also showed that melanoma patients are often diagnosed in advanced stages [6, 23, 24]. In our study, *BRAF* mutations were found in 44.4% of melanoma patients and *NRAS* mutations were detected in 12.7% of patients. The frequency of both *BRAF* and *NRAS* mutations in our population was lower than in studies both in Caucasians and Asians [10, 25]

BRAF mutations have been significantly associated with younger melanoma patients [26]. On contrary, our study showed that *BRAF* mutations were associated with melanoma patients older than 65 years. Meckbach et al. [26] included early-stage melanoma patients with a majority subtype of superficial spreading melanoma. In a large meta-analysis that includes a population of Caucasians and Asians, *BRAF* mutations are associated with age, body sites, subtypes, and advanced stages [27]. We did not find an association of *BRAF* mutations with larger tumor size, advanced stages, anatomical body sites, and the presence of ulceration. Another study also did not find an association of *BRAF* mutations with Breslow thickness and ulceration [28]. The conflicting association of clinicopathological characteristics with *BRAF* mutation status is also reported by other studies [11, 26, 29].

NRAS mutations are more frequently detected in nodular melanoma particularly those with more aggressive clinical behavior [28]. A significant association between

Table 3. Association of *NRAS* Mutation Status with Clinicopathological Variables Using Univariate and Multivariate Regression Analyses

Variable	Category	Reference	Univariable regression		Multivariable regression	
			OR (95%CI)	P-value	OR (95%CI)	P-value
Age	>65 years	≤65 years	13.263 (1.518-115.901)	0.019	19.11 (1.296-281.9)	0.032
Sex	Female	Male	1.290 (0.280-5.934)	0.744	2.121 (0.477-10.054)	0.435
Histology	ALM	Nodular	1.137 (0.245-5.279)	0.87	0.486 (0.050-4.739)	0.486
Breslow	>4mm	≤4 mm	0.235 (0.035-1.568)	0.135	0.300 (0.100-1.035)	0.051
Lymph node	Positive	Negative	-	0.106	5.787 (0.157-213.7)	0.34
Ulceration	Yes	No	1.389 (0.302-6.392)		1.274 (0.302-5.386)	0.741
Stage	Advanced (III-IV)	Early (I-II)	-	0.164	166.7 (1700-1754)	0.029
Location	Acral, extremity	Axis, Head and neck	0.264 (0.021-3.305)	0.302	0.292 (0.011-7.721)	0.461
Diameter	≤ 6 mm	>6mm	-	0.584	-	
Occupation	Outdoor	Indoor	0.587 (0.102-3.386)	0.551	0.076 (0.04-1.612)	0.098
Residence	Rural	Urban	0.700 (0.71-6.899)	0.76		
Education	Highschool/college	Elementary	0.549 (0.053-8.547)	0.614	0.423 (0.031-9-712)	0.498
Smoking	Ever or active	Never	0.900 (0.195-4.155)	0.893	0.177 (0.004-8.251)	0.377

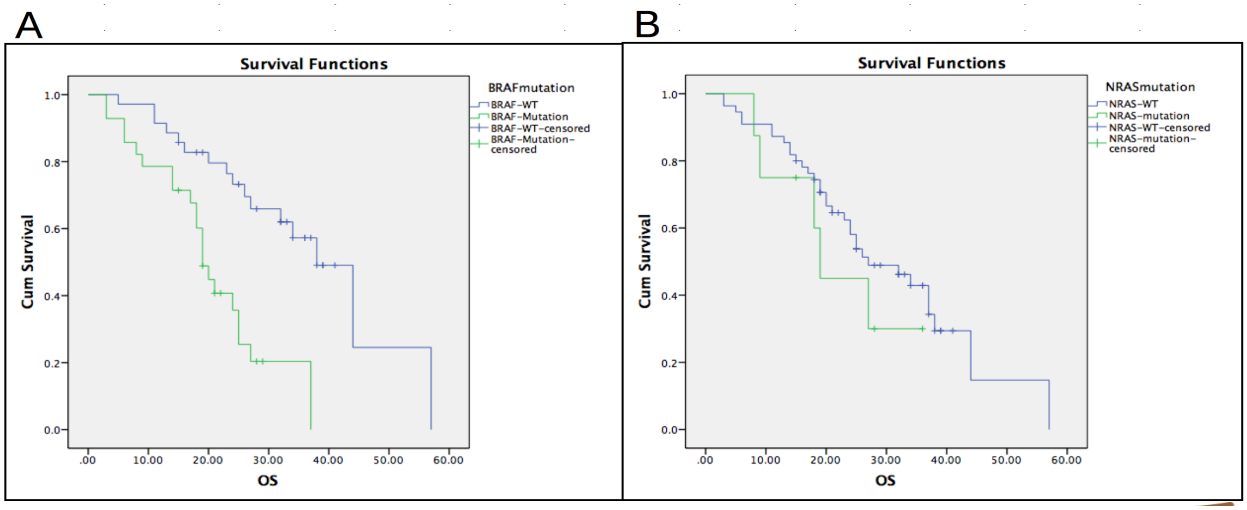

Figure 1. Association of *BRAF* and *NRAS* Mutations with Overal Survival of Patients with Cutaneous Melanoma. (A) *BRAF* mutations were significantly associated with poor overall survival compared to wild-type (mean survival rates were 14.4 vs 26.4 months, respectively; Log-rank test P = 0.001). (B) *NRAS* mutations were not associated with the overall survival of melanoma patients compared to wild-type (mean survival rates were 22.5 vs 30.5 months, respectively; Log-rank test P = 0.390).

NRAS mutations and older age melanoma patients was found in this study. Other studies showed no association between *NRAS* mutations and age at melanoma diagnosis [26, 28]. By other studies [26–28], we also did not find an association of *NRAS* mutations with Breslow's depth, ulceration, lymph node involvement, and body sites. Although *BRAF* and *NRAS* mutations are mutually exclusive, we found 3 patients harbor both mutations. Concurrent *BRAF* and *NRAS* mutations in a small proportion of melanoma patients were also previously reported [28, 30].

Mutational statuses of *BRAF* and *NRAS* genes were not consistently associated with the survivals of cutaneous melanomas in different settings. We showed that *BRAF* mutations were associated with worse overall survival among high-risk cutaneous melanoma patients (Figure 1). Patients with *BRAF* or *NRAS* mutations are associated with 3-times the risks of mortality in high-risk melanoma [11]. *NRAS* mutations in melanoma have been associated with lower tumor-infiltrating lymphocytes and a more immunosuppressive microenvironment causing more aggressive clinical behavior [11]. Other studies show that *BRAF* mutations are not associated with disease-free progression [31, 32]. Additionally, Heppt et al. [28] showed that *BRAF* mutations were associated with better prognosis in cutaneous melanoma patients. *NRAS* mutation are frequently associated with adverse outcomes of cutaneous melanoma patients (lower overall or progression survival and time to distant metastases) [28, 30]. Among Asian patients, *NRAS* mutations were also associated with a worse prognosis compared to those with wild-type [33]. Another study in Asian melanoma patients demonstrated that both *BRAF* and *NRAS* mutations were not associated with overall- and disease-free survivals [34]. Both in European and Asian populations using a wide-range of clinical settings, the association between *BRAF* and *NRAS* mutations and the prognosis of cutaneous melanoma has shown mixed results. However, the mutations are recommended to be routinely examined to determine a treatment plan for selected patients [8].

The primary strength of this study is the mutation analysis of *BRAF* and *NRAS* using sequencing analysis. The mutation analysis is required to further forecast the need for targeted therapy for selected patients to improve the clinical management of melanoma patients in Indonesia. Limitations of this study are single institution with small study participants and retrospective clinical follow-up in the survival analyses. The patients involved in this study also did not receive tyrosine kinase inhibitors regarding the mutational statuses. The future collaborative study is required to determine the burden of melanoma and the projection of the need for targeted therapy to advocate the improvement of clinical management in Indonesia.

In conclusion, the frequency of *BRAF* and *NRAS* mutations were 44.4% and 12.7% respectively in high risk cutaneous melanoma patients. *BRAF* and *NRAS* mutations are significantly associated with older melanoma patients at diagnosis. *BRAF* mutations were associated with worse overall survival in melanoma patients in Indonesia.

Author Contribution Statement

SLA conceived the study and performed the mutation analyses. SLA, PR, RB, WS, and GDP summarized and analyzed the clinical data. SLA initially wrote the manuscript draft with feedback from PR. All authors approved the final version of the manuscript draft.

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Ethics approval and consent

The study protocol has been evaluated and approved by the Medical and Health Research Ethics Commitee

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Competing interest

All authors declared for no competing interest.

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