ABSTRACT

Background: Recent reports have suggested that lower vitamin D serum levels are associated with susceptibility to and severity of asthma in different white populations, which may be due to a lack of sunlight exposure, genetic polymorphism of vitamin D pathway genes, and dietary intake. We investigated the associations between vitamin D concentration, genetic polymorphism of the vitamin D receptor (VDR), and asthma traits in Mongolian and Taiwanese populations that inhabited two different geographical areas.

Methods: In total, 328 Han Taiwanese subjects and 381 Mongolian subjects were enrolled, and their vitamin D serum levels assayed. Genomic DNA of 178 Han Taiwanese subjects and 90 Mongolian subjects was obtained from blood samples. Single-nucleotide polymorphisms (SNPs) of VDR, Apal (rs7975232), TaqI (rs731236), BsmI (rs1544410) and FokI (rs2228570), were selected for genotyping. Logistic regression analyses were performed to detect an association between allergic asthma status and the interaction of the VDR SNP and serum vitamin D concentration in the case-control samples.

Results: We observed a significantly lower vitamin D level in the Mongolian subjects as compared with the Taiwanese population. In particular, in the population under 14 years of age, the serum vitamin D level was significantly higher in the Taiwanese population, in both non-asthmatic and asthmatic subjects, than in the Mongolian non-asthmatic and asthmatic subjects, respectively (P < 0.01). Moreover, the vitamin D level in the asthmatic children was significantly lower than that in the non-asthmatic children in both the Taiwanese and Mongolian populations (P < 0.01, respectively). Furthermore, we found that the rs2228570 genotype (OR, 3.763) of the VDR SNP and the vitamin D concentration (lower than 40 ng/ml, OR: 38.938) both contribute to increased susceptibility to bronchial asthma.

Conclusion: Our results demonstrated an association between vitamin D concentration and the risk of asthma in two populations of differing ethnicity living in different geographical areas. This...
INTRODUCTION

Bronchial asthma is one of the most common chronic respiratory diseases, of increasing prevalence and financial burden, with more than 300 million sufferers worldwide. This disease affects individuals in all countries and all ethnic groups; however, the prevalence rate of asthma has been reported to vary significantly among different regions. A number of epidemiologic studies have been performed to compare the burden of asthma, and these studies have reported striking differences in asthma prevalence between countries and geographical areas. Without doubt, in addition to racial differences, environment and lifestyle, such as dietary preference, also play significant roles in the development of asthma. Despite these confounding factors, few comparison studies have been conducted on the prevalence of bronchial asthma according to ethnicity, geographical area, and cultural differences across various countries and populations.

Vitamin D is a nutrient and hormone that is key to the metabolism of calcium and phosphorus. Vitamin D is primarily acquired and produced in the skin upon sunlight exposure or absorbed from dietary intake (e.g., from oily fish and dairy products), and from supplements as secondary sources. Several studies have found that low cord blood vitamin D levels are associated with increased risks of wheezing and recurrent lung symptoms in young children, but not with asthma. A recent systematic review found that several prospective studies that measured the 25-hydroxyvitamin D [25(OH)D] level in cord blood at birth or during pregnancy did not identify any association between the 25(OH)D level and asthma in children aged 4-8 years. However, higher serum levels of 25(OH)D were found to be associated with a reduced risk of asthma exacerbation. In the Childhood Asthma Management Program (CAMP) study, a higher risk of severe asthma exacerbation leading to an emergency room (ER) visit or hospitalization was associated with vitamin D insufficiency [25(OH)D < 30 ng/mL]. As the vitamin D serum level is not only affected by diet and supplements, but also by climate and lifestyle, whether or not vitamin D can be considered a good biomarker for asthma susceptibility across different geographical regions and ethnic populations is still unproved.

The gene encoding the vitamin D receptor (VDR), being a transcription factor that alters the transcription of target genes responsible for a wide spectrum of biologic responses, is the most widely-studied target along the vitamin D pathway. VDR is primarily activated by the binding of its primary ligand, 1,25-dihydroxyvitamin D 3 (1,25(OH)₂D₃), and it is expressed in the majority of immune cells, including B and T lymphocytes, monocytes, macrophages, and dendritic cells. Among the known VDR polymorphisms, the most common single-nucleotide polymorphisms (SNPs) that influence VDR expression within the immune system include BsmI (rs1544410), Apal (rs7975232), TaqI (rs731236) and FokI (rs10735810). BsmI, Apal and TaqI have been shown to be in strong linkage disequilibrium (LD) in several populations.

Mongolia and Taiwan, with different ethnic backgrounds, are located at different latitudes of the Asian northern hemisphere, with markedly different degrees of sunshine, temperature variation, and dietary habits; hence, it is interesting and of academic importance to compare factors including the vitamin D serum level and genetic polymorphisms of VDR that are related to increased susceptibility to asthma in both countries. We believe the results of this study may indicate a potential role of vitamin D in the prevention, or in the future, the treatment of bronchial asthma in different ethnic populations and geographical regions.
MATERIALS AND METHODS

Study population and clinical evaluation

Our study population consisted of asthmatic children ranging from 5 to 18 years of age. The non-asthma subjects included children and adults without a history of asthma. The study protocol was approved by the Ethical and Clinical Trial Committee of National Cheng-Kung University Hospital, Tainan, Taiwan, and the School of Medicine, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia. A signed informed consent form was required from all participants or their guardians after completing a modified British Medical Society respiratory questionnaire, which was identical to the European Community Respiratory Health Survey (ERCHS). This survey presented similar results to those of the ISAAC and ERCHS, pertinent to the diagnosis and assessment of asthma. Pulmonary function was evaluated using standard methods, including spirometry before and after the administration of two puffs of inhaled salbutamol (200 μg/puff). To diagnose the presence of asthma, the following criteria had to be met: (1) a history of wheezing and experiencing shortness of breath during or without concurrent respiratory infections; (2) chronic coughing for more than one month, as well as the presence of wheezing, as observed by a physician; and (3) a bronchodilator test confirming a 15% increase in the FEV1. Non-asthma controls were defined as neither having a history of asthma, as per criteria (1) above, nor being diagnosed with asthma, as per criteria (2). Other evaluations included skin prick tests to examine responsiveness to 6 common aeroallergens, a differential blood count (including total eosinophil count), the level of total serum IgE, and the levels of IgE specific to house dust and mixed pollens, using the Unicap system (Pharmacia, Diagnostic, Sweden). A positive skin test was defined as the presence of ≥1 reaction with a wheal diameter ≥5 mm. Total serum IgE was measured by solid-phase immunoassay (Pharmacia IgE EIA; Pharmacia Diagnostics). Non-allergy subjects were defined as having a total serum IgE <200 and negative skin tests. All Taiwanese subjects were Han-Taiwanese and lived in Taiwan. The Mongolian study subjects, enrolled from an outpatient department, included asthmatic children and adults, and their clinical status was assessed in the same way as for the Taiwanese study group.

Measurement of total IgE and allergen-specific IgE

Serum samples were collected from the study subjects to obtain the total IgE and allergen-specific IgE sensitization profile using multiplex in vitro allergen sensitization diagnostic kits, BioIC®. Serum total IgE levels was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s protocols (IgE ELISA kits; cat. no. BMS2097; eBioscience).

Measurement of 25-hydroxyvitamin D [25(OH)D] concentration

The serum level of 25(OH)D was measured by sandwich EIA using a commercial kit (cat.no. AC57SF1; Immunodiagnostic Systems, Fountain Hills, AZ, USA).

DNA preparation

Genomic DNA was extracted from blood samples of 178 Han Taiwanese subjects and 90 Mongolian subjects using a QIAamp DNA blood kit (QIAGEN, Valencia, CA, USA) according to the manufacturer’s instructions. The extracted genomic DNA was analyzed by agarose gel electrophoresis, quantified by spectrophotometry, and stored at −80 °C until use.

SNP genotyping

SNPs of the VDR gene, Apal (rs7975232), TaqI (rs731236), BsmI (rs1544410) and FokI (rs2228570), were selected for genotyping. All selected SNPs were genotyped using the high-throughput, 384-microtiter plate MassARRAY™ System, SEQUENOM®, according to the manufacturer’s protocol. In brief, DNA containing the SNP site of interest was amplified, followed by performance of the homogenous MassEXTEND™ (hME) assay, in which label-free primer extension chemistry was used to generate allele-specific diagnostic products. Each allele-specific diagnostic product had a unique molecular weight, and this could be distinguished through the application of matrix-assisted laser desorption ionization time-of-flight mass spectrometry. SNPs with a call
rate lower than 90% were excluded from the statistical analysis.

**Statistical analysis**

The quality of the genotype data was evaluated by testing for Hardy-Weinberg equilibrium (HWE) proportions and Mendelian inheritance consistency. The χ² test was used to detect associations between allergic asthma and each SNP. Logistic regression analyses were performed to detect an association between allergic asthma status and interaction of the VDR SNP and the serum vitamin D concentration in the case–control samples. In the logistic regression models, the SNP genotype was coded 0 and 1, indicating whether the risk genotype was carried or not carried by individual subjects.

**RESULTS**

**Total IgE and vitamin D levels in the Mongolian and Taiwanese study subjects**

In total, 323 Han Taiwanese subjects (194 asthma patients and 129 non-asthma controls) and 371 Mongolian subjects (115 asthma patients and 256 non-asthma controls) were enrolled, and blood samples were collected from all subjects for conduction of a serum Total IgE and vitamin D assay. Of the enrolled subjects, genomic DNA was extracted from blood samples from 178 Han Taiwanese and 90 Mongolians. The demographic data and clinical information of the study subjects are presented in Table 1. There were more male asthma patients in the Taiwanese subjects than in the Mongolian subjects. The average age of the non-asthma subjects was higher than that of the asthma subjects in both the Taiwanese and

|                                 | Taiwanese (323) | Mongolian (371) | \( P \) value (between Asthma groups) |
|---------------------------------|-----------------|-----------------|-------------------------------------|
|                                 | Non-asthma      | Asthma          | Non-asthma                          | Asthma |                                |
| Sex (M/F)                       | 21/108          | 129/64          | 140/116                             | 79/36  | 0.730                          |
| Age (years; mean ± SD)          | 28.11 ± 9.67    | 7.77 ± 4.09     | 27.75 ± 13.16                       | 11.94 ± 10.28 | <0.0001                      |
| FEV₁ (pred%)                    | N/A             | 92.2 ± 9.3      | N/A                                 | 89.3 ± 11.5 | <0.0001                      |
| ICS used (%)                    | N/A             | 68.1            | N/A                                 | 23.5   | <0.0001                      |
| Total IgE(ng/ml)                | 297.9 ± 36.9    | 8376 ± 712.9    | 2540 ± 385.7                        | 3813 ± 329.8 | <0.0001                      |
| HDM (SPT%)                      | N/A             | 69.1            | N/A                                 | 13.0   | <0.0001                      |
| Mugwort (SPT%)                  | N/A             | 0.01            | N/A                                 | 17.4   | <0.0001                      |
| Sensitization to:               |                 |                 |                                     |        |                               |
| 1. Any mite or cockroach        | N/A             | 77.3            | N/A                                 | 24.3   | <0.0001                      |
| 2. Any pollen                   | N/A             | 19.1            | N/A                                 | 51.3   | <0.0001                      |
| 3. Any mold                     | N/A             | 31.9            | N/A                                 | 13.0   | <0.0001                      |
| 4. Any animal                   | N/A             | 18.1            | N/A                                 | 26.9   | <0.0001                      |
| Vitamin D blood concentration   | 68.88 ± 2.57    | 31.13 ± 1.77    | 14.34 ± 1.59                        | 12.56 ± 0.75 | <0.0001                      |
| (mean ± SD)                     |                 |                 |                                     |        |                               |

Table 1. Demographic data and clinical information of the study subjects *One outlier with a vitamin D blood concentration of 155 ng/ml in the Mongolian sample was excluded*
Mongolian groups. Non-asthma subjects of a greater age (than the asthma subjects) were enrolled in order to decrease the false negative rate of asthma status. Fig. 1 shows that the level of total IgE was significantly higher in the asthmatic subjects than non-asthmatics in the Taiwanese (p < 0.001) as well as in Mongolian populations (p < 0.05). Moreover, the total IgE level was significantly higher in the non-asthmatics in Mongolian populations as compared with the non-asthmatic subjects in Taiwan (p < 0.01). The serum vitamin D concentration of the non-asthma subjects was significantly higher than that of the asthma subjects in the Taiwanese group. Almost all Mongolian subjects, including asthma patients and non-asthma controls, had a serum vitamin D concentration lower than 20 ng/ml. As shown in Fig. 2, in the study population over 14 years of age, the vitamin D level was significantly higher in the non-asthmatic Taiwanese subjects than in the same Mongolian population (P < 0.01); it was also higher than in the asthmatic Taiwanese subjects (p < 0.01). In the study population under 14 years of age, the serum vitamin D level was significantly higher in the Taiwanese population, both non-asthmatic and asthmatic subjects, than in the Mongolian non-asthmatic and asthmatic subjects, respectively (P < 0.01). Moreover, the vitamin D level was significantly lower in the asthmatic children as compared with the non-asthmatic children in both the Taiwanese and Mongolian populations (P < 0.01, respectively). Mean vitamin D levels in each groups were shown in the tables under the respective figures.

**Fig. 1** Comparison of the total IgE levels in the Taiwanese and Mongolian study populations. The level of total IgE was significantly higher in the asthmatic subjects than non-asthmatics in the Taiwanese (p < 0.001) as well as in Mongolian populations (p < 0.05). Moreover, the total IgE level was significantly higher in the non-asthmatics in Mongolian populations as compared with the non-asthmatic subjects in Taiwan (p < 0.01).

| Total IgE (ng/ml) | Mongolian | Taiwanese |
|------------------|-----------|-----------|
| Non-asthma       | 2540 ± 385.7 | 297.9 ± 36.9 |
| asthma           | 3813 ± 329.8 | 8376 ± 712.9 |

**Fig. 2** Comparison of the serum vitamin D levels in the asthmatic Taiwanese and Mongolian subjects over 14 years of age (A) and under 14 years of age (B). In the study population aged over 14, the level of vitamin D was significantly higher in the non-asthmatic subjects in the Taiwanese population than in the Mongolian population (P < 0.01), and was also higher than that in the asthmatic Taiwanese subjects (P < 0.01). In the study population under 14, the serum vitamin D level was significantly higher in the Taiwanese population, both non-asthmatic and asthmatic subjects, than in the Mongolian non-asthmatic and asthmatic subjects, respectively (P < 0.01). Moreover, the vitamin D level was significantly lower in the asthmatic children as compared with the non-asthmatic children in both the Taiwanese and Mongolian populations (P < 0.01, respectively). Mean vitamin D levels in each groups were shown in the tables under the respective figures.
asthmatic and asthmatic subjects, than in the Mongolian non-asthmatic and asthmatic subjects, respectively \((P < 0.01)\). Moreover, the vitamin D level in the asthmatic children was significantly lower than that in the non-asthmatic children in both the Taiwanese and Mongolian populations \((P < 0.01, \text{respectively})\).

**VDR genotypic associations with asthma traits**

The genotype distributions of the selected SNPs are presented in Table 2. Only rs2228570 (VDR missense polymorphism) showed marginal significance in the Taiwanese group, and the rs2228570 GG genotype frequency was slightly higher in the Mongolian group (41.1%) than in the Taiwanese group (27.0%) (Table 2). Table 3 presents the VDR polymorphism rs2228570 and vitamin D concentrations in the Taiwanese subjects. The recessive model of the rs2228570 GG genotype (vs. AG + AA) is associated with a risk of asthma \((P < 0.02)\). Moreover, a lower vitamin D blood concentration, below the cut-off point of 40 ng/ml, was also found to be related to a high risk of asthma \((P < 0.0001)\) (Table 3). According to multifactorial analysis by logistic regression, the rs2228570 genotype (GG genotype, OR: 3.763, 95%CI: 13.8–109.9) and the

| SNP ID       | Genotype | Taiwanese |          |          | Mongolian |          |          | P value |          |          |          | P value |
|--------------|----------|-----------|----------|----------|-----------|----------|----------|---------|----------|----------|----------|---------|
|              |          | Asthma    | Non-Asthma |          | Asthma    | Non-Asthma |          |         |         |         |         |         |
| rs7975232a   | CC       | 31 (61%)  | 63 (51%)  | 0.426    | 35 (54%)  | 13 (52%)  | 0.981    |         |         |         |         |         |
|              | AC       | 15 (29%)  | 49 (40%)  |          | 23 (35%)  | 9 (36%)   |          |         |         |         |         |         |
|              | AA       | 5 (10%)   | 11 (9%)   |          | 7 (11%)   | 3 (12%)   |          |         |         |         |         |         |
| rs731236a    | TT       | 44 (86%)  | 109 (89%) | 0.666    | 54 (83%)  | 21 (84%)  | 0.916    |         |         |         |         |         |
|              | CT       | 7 (14%)   | 14 (11%)  |          | 11 (17%)  | 4 (16%)   |          |         |         |         |         |         |
| rs1544410    | CC       | 44 (86%)  | 105 (82%) | 0.620    | 54 (83%)  | 21 (84%)  | 0.916    |         |         |         |         |         |
|              | CT       | 7 (14%)   | 20 (16%)  |          | 11 (17%)  | 4 (16%)   |          |         |         |         |         |         |
|              | 0        | 2 (2%)    | 3 (2%)    |          | 9 (14%)   | 3 (12%)   |          |         |         |         |         |         |
| rs2228570    | GG       | 20 (39%)  | 28 (23%)  | 0.065    | 28 (43%)  | 9 (36%)   | 0.748    |         |         |         |         |         |
|              | AG       | 20 (39%)  | 63 (50%)  |          | 28 (43%)  | 13 (52%)  |          |         |         |         |         |         |
|              | AA       | 11 (22%)  | 36 (27%)  |          | 9 (14%)   | 3 (12%)   |          |         |         |         |         |         |

Table 2. Association of VDR polymorphism with allergic asthma 

| SNP ID       | Genotype | Taiwanese |          |          | Mongolian |          |          | P value |          |          |          | P value |
|--------------|----------|-----------|----------|----------|-----------|----------|----------|---------|----------|----------|----------|---------|
|              |          | Asthma    | Non-Asthma |          | Asthma    | Non-Asthma |          |         |         |         |         |         |
| rs2228570    | GG       | 28        | 99        |          | 20        | 31        |          |         |         |         |         |         |
|              | AG + AA  | 99        | 20        |          | 31        | 20        |          |         |         |         |         |         |

Table 3. VDR polymorphism rs2228570 and vitamin D concentration in the Taiwanese population
vitamin D concentration (lower than 40 ng/ml, OR: 38.938, 95%CI: 1.34–10.58) both contributed to increased susceptibility to allergic asthma in the Taiwanese group. Table 4 shows the joint effects of VDR polymorphism rs2228570 and the serum vitamin D concentration in the Taiwanese group. Subjects with the GG genotype of rs2228570 combined with a serum vitamin D concentration <40 ng/ml were at greatest risk of bronchial asthma (OR: 147.3, 95%CI: 27.4–793.8).

DISCUSSION

In this study, we investigated the serum vitamin D concentration and genetic variations of VDR, and their associations with bronchial asthma, in Taiwanese and Mongolian populations. We found that a low serum vitamin D concentration (<40 ng/ml) and VDR polymorphism of the GG genotype of rs2228570, conferred a high risk of bronchial asthma in the Taiwanese population. The susceptibility to bronchial asthma conferred by the serum vitamin D concentration was found to be stronger than the effect conferred by VDR genetic polymorphism. Almost all the Mongolian subjects had a very low serum vitamin D concentration (<20 ng/ml). Genetic variants of VDR were not found to be associated with asthma in the Mongolian population.

Vitamin D deficiency (<20 ng/ml) and insufficiency (21–29 ng/ml) are currently on the rise globally, and are associated with increased asthma morbidity. Epidemiological data suggested that low levels of vitamin D during pregnancy and early life contribute to the development of childhood wheezing and asthma. Studies have shown that vitamin D plays a role in fetal lung development and maturation, as well as maintaining lung structure and function. In a US nationwide study that examined vitamin D insufficiency, asthma and lung function among US children and adults, it was found that vitamin D insufficiency was associated with current asthma and wheezing, a lower FEV1, and the forced vital capacity (FVC) in both adults and children. Interestingly, this National Health and Nutrition Examination Survey also showed that over years in which vitamin D insufficiency decreased, the prevalence of asthma also decreased.

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In addition to genetic factors, the main factors that determine the 25(OH)D serum level are skin pigmentation, sun exposure, age, gender, latitude of residence (higher latitudes result in decreased opportunity for vitamin D skin synthesis), diet and vitamin D fortification. As compared with the Taiwanese group, our results showed very low vitamin D serum levels in the Mongolian group, who were mainly enrolled from Ulaanbaatar; this result is consistent with previous reports on populations from the same area. In fact, it has been reported that vitamin D supplementation improved winter-related atopic dermatitis among Mongolian children. Effective vitamin D intervention has been strongly recommended for the Mongolian adult population, particularly among women and residents of Ulaanbaatar.

Based on our study and aforementioned studies, the Mongolian population is likely to suffer generalized vitamin D deficiency, regardless of individual genetic variants of VDR, and therefore, it will be at greater risk of susceptibility to allergic diseases and bronchial asthma.

| Interaction term                      | Odds ratio (95% confidence interval) | P value |
|---------------------------------------|--------------------------------------|---------|
| rs2228570* vitamin D concentration    |                                      |         |
| GG *Vit. D < 40 ng/ml                 | 147.33 (27.35, 793.77)               | <0.0001 |
| AG + AA * Vit. D < 40 ng/ml           | 34.67 (9.60, 125.24)                 | <0.0001 |
| GG *Vit. D ≥ 40 ng/ml                 | 3.12 (0.59, 16.45)                   | 0.180   |
| AG + AA * Vit. D ≥ 40 ng/ml           | 1                                    |         |

Table 4. Joint effects of VDR polymorphism rs2228570 and vitamin D concentration in the Taiwanese population
In contrast, in the Taiwanese group, the vitamin D serum level and genetic variants of VDR played significant roles in terms of conferring a risk of asthma. In our study, an association between the VDR FokI (rs2228570) genetic variant and allergic asthma was identified in the Taiwanese population, but not in the Mongolian population. A meta-analysis was conducted by Zhao et al. to examine the relationships between childhood asthma and VDR gene polymorphisms Apal (rs7975232), BsmI (rs1544410), FokI (rs2228570) and TaqI (rs731236). Of the four SNPs identified, the Apal polymorphism was found to play a role in childhood asthma in Asians, the FokI polymorphism may be related to pediatric asthma in Caucasians, and BsmI polymorphism was identified as marginally contributing to susceptibility to childhood asthma. Recently, a report showed TaqI and Apal polymorphisms were associated with asthma in Irish children. Though our genetic data from Mongolian controls may contain less information due to the small sample size, the genotype frequency of the FokI polymorphism in the control group was similar to that reported in a previous study examining brick-tea type fluorosis. The FokI polymorphism genotype frequencies of GG vs. AG + AA in the controls in our study were 36% vs. 64% in the Mongolian control subjects (Table 2), and were 34.5% vs. 65.5% in the Mongolian control subjects in the previous brick-tea type fluorosis study.

In this study, we found that the effect of serum vitamin D concentration on susceptibility to bronchial asthma was stronger than that of VDR polymorphism in the Taiwanese group (Table 4). In the Mongolian population, who suffered vitamin deficiency, VDR polymorphism played a minor role as compared with the vitamin D serum concentration, which confers a risk of bronchial asthma.

In conclusion, our results indicated that the vitamin D serum concentration and variants of the VDR gene are major risk factors for the development of bronchial asthma in the subtropical region of Taiwan, while the vitamin D level is the major determinant of the risk of asthma in the high-altitude, temperate zone of Mongolia. Effective vitamin D intervention for Taiwanese and Mongolian populations is warranted, and more extensive studies will have to be carried out in relation to vitamin D supplementation in the Mongolian population.

Ethics approval and consent to participate
The study protocol was approved both by the Ethical and Clinical Trial Committee of National Cheng-Kung University Hospital, Tainan, Taiwan, and the School of Medicine, Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia.

Consent for publication
All authors have seen and approved the last version.

Authors’ contributions
SM, HFK, JYW and LSHW designed the study, supervised experiments and wrote and edited the manuscript; SM, NT, BB-U and JYW collected the study subjects; YIH, LN and LSHW was responsible for the experiments including measurement of vitamin D and genotyping; JYW, HFK and LSHW conducted the statistical analysis; SM and JYW was responsible for the ethical approval and the handling of the human samples for in vitro testing; All authors read and approved the final version of the manuscript.

Declaration of competing interest
The authors declare that they have no competing interests.

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