Intronic hexanucleotide repeat expansion in *TYMS* in monozygotic twins with congenital progressive universal melanosis

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Abstract. Hyperpigmentation presents a diverse clinical spectrum, largely influenced by genetic factors that remain incompletely understood. The present study describes a case of monozygotic twin girls aged 15 years with congenital progressive universal melanosis (CPUM) born to non-consanguineous unaffected parents. CPUM represents a novel clinical entity characterized by progressive widespread hyperpigmentation beginning at birth, without other accompanying symptoms. Skin biopsy and histopathological analysis were performed, followed by long-read whole-genome sequencing and short tandem repeat analysis. Gene expression was evaluated using reverse transcription-PCR, and protein levels were assessed by western blotting in cultured skin fibroblasts from the twins and unaffected controls. Long-read genome sequencing revealed a biallelic GATGGT repeat expansion of 210-259 repeat units within the third intron of the thymidylate synthase (*TYMS*) gene in both twins, whereas their parents were heterozygous. Controls (n=236), derived from the in-house long-read sequencing database at Excellence Center for Genomics and Precision Medicine, King Chulalongkorn Memorial Hospital, Bangkok, Thailand, carried the GATGGT repeat in the 42-172 range. No single nucleotide or structural variants or copy number variations were present in both affected individuals and absent in the unaffected parents. RNA and protein levels of *TYMS* using cultured skin fibroblasts from both twins showed no discernible differences compared with controls. Fibroblasts were used due to their accessibility via skin biopsy and their role in skin pigmentation through paracrine signaling to melanocytes. More relevant cells, such as melanocytes or keratinocytes, may be required to exhibit such changes, as these cells are directly involved in melanin production and skin pigmentation. *TYMS* is implicated in skin pigmentation in normal physiological process and in disorders manifesting abnormal skin pigmentation, such as dyskeratosis congenita. The present findings imply a connection between the identified repeat expansion in *TYMS* and CPUM, underscoring the need for further investigations to elucidate its causal association.

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Introduction

Hyperpigmentation disorders are conditions characterized by the darkening of the skin due to excessive melanin production or deposition. Such disorders are categorized into three types: Diffuse, linear and reticulated (1). Diffuse hyperpigmentation refers to a condition characterized by the widespread

darkening of the skin, often affecting multiple areas of the body, including acquired universal melanosis (AUM), erythema dyschromicum perstans (EDP), lichen planus pigmentosus (LPP), familial progressive hyperpigmentation (FPH), post-inflammatory hyperpigmentation and drug-induced hyperpigmentation (2-4). Linear hyperpigmentation presents darkened streaks or lines on the skin that follow a linear pattern. This includes flagellate pigmentation, pigmentary demarcation lines, linear and whorled nevoid hypermelanosis and incontinentia pigmenti (5). Reticulated hyperpigmentation refers to a pattern of darkened patches on the skin that form a net- or lace-like appearance and includes dyskeratosis congenita, prurigo pigmentosa, dermatopathia pigmentosa reticularis, Dowling-Degos disease and reticulate acropigmentation of Kitamura (6). The causes of hyperpigmentation are diverse, encompassing genetic factors, hormonal changes, sun exposure, inflammation, certain medications, and underlying medical conditions (7,8).

Although many hyperpigmentation disorders are rare, available epidemiological data provide varying estimates of prevalence across the three clinical types: diffuse, linear, and reticulated. Among diffuse hyperpigmentation disorders, Addison's disease, an acquired condition caused by adrenal insufficiency, has a reported prevalence of 100-140 cases per million in Europe and North America (9,10). In contrast, genetic causes of diffuse hyperpigmentation are much rarer. For example, dyschromatosis universalis hereditaria, which may be inherited in an autosomal dominant or recessive manner and is associated with mutations in ATP binding cassette subfamily B member 6 (*ABCB6*), SAM and SH3 domain containing 1 (*SASH1*), or KIT ligand (*KITLG*), has an estimated prevalence of approximately 0.3 per 100,000 individuals (11,12). Another rare diffuse condition, FPH, has been reported in only 9 cases worldwide (13). Reticulated hyperpigmentation is seen in disorders such as DC, which is associated with mutations in genes involved in telomere maintenance, including dyskerin pseudouridine synthase 1, telomerase RNA component (*TERC*), and regulator of telomere elongation helicase 1 and is estimated to occur in fewer than 1 per 1,000,000 individuals (14). DPR has only 20 cases reported globally, while reticulate acropigmentation of Kitamura has 130 documented cases worldwide (13). Linear hyperpigmentation disorders are also rare. IP, a well-characterized example, is an X-linked dominant disorder caused by pathogenic variants in the inhibitor of nuclear factor kappa B kinase regulatory subunit gamma (*IKBKG*) gene. It primarily affects females and is typically lethal in males. In European populations, the estimated birth prevalence of IP is ~1.2 per 100,000 live births (13). Given the rarity and genetic complexity of these conditions, especially those with suspected Mendelian inheritance, advanced genomic approaches are essential for elucidating their molecular basis.

Next-generation sequencing technologies have revolutionized genomic research by enabling high-throughput sequencing and comprehensive DNA sequence analysis. Among these, long-read whole-genome sequencing is advantageous owing to its ability to generate long contiguous sequences of DNA (15). This technology excels in resolving complex genomic regions, including structural variants (SVs), repetitive sequences and intricate genomic rearrangements that short-read sequencing methods often miss or inaccurately characterize (16,17). The present study used PacBio long-read whole-genome sequencing to ensure a complete and accurate genome representation.

The aim of the present study was to investigate the genetic basis of a rare congenital hyperpigmentation disorder in monozygotic twins.

Materials and methods

Skin biopsy and histopathological study. The present study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (Bangkok, Thailand (approval no. 264/62) and conducted in accordance with the 1964 Helsinki Declaration and its later amendments, as well as all relevant ethical guidelines and regulations. The rights and interests of all participants were fully protected. Fifteen-year-old monozygotic twin girls with generalized hyperpigmentation presented in April 2019 at Khanu Woralaksaburi Hospital, Kamphaeng Phet, Thailand were referred to Naresuan University Hospital, Phitsanulok, Thailand. Both parents of the patients were aged 56 years. Skin biopsy was performed on the abdomen of both twins for histopathological study, diagnosis and fibroblast culture. Written informed consent was obtained from the parents of the patients. Tissue was fixed in 10% formaldehyde at room temperature for 24 h, followed by tissue processing which included dehydration through a graded ascending ethanol series, clearing in xylene, and embedding in paraffin. Paraffin-embedded tissues were then sectioned at 4-5 μm thickness for histological analysis. Hematoxylin and eosin staining was performed at room temperature following deparaffinization in xylene, rehydration through graded descending ethanol, staining with Harris's hematoxylin for 7.5 min, bluing with lithium carbonate, counterstaining with eosin for 1 min, followed by dehydration and clearing. Sections were imaged using an Olympus BX50 light microscope (Olympus Corporation), with image capture via Olympus cellSens Standard software v1.3 (evidentscientific.com/en/products/software/cellsens).

Clinical laboratory tests. As part of the clinical work-up, both twins underwent a physical examination, electrocardiogram (ECG), and routine laboratory testing, including a complete blood count (CBC) and morning serum cortisol assessment, performed at Naresuan University Hospital, Phitsanulok, Thailand. A standard-dose (250 μg) adrenocorticotropic hormone (ACTH) stimulation test was performed in the morning between 08:00 and 09:00 to account for diurnal variation in cortisol secretion. A baseline blood sample was collected for measurement of serum cortisol and 17-hydroxyprogesterone prior to ACTH administration. Subsequently, 250 μg of cosyntropin was administered intravenously, followed by blood sampling at 30 and 60 min post-injection to assess stimulated cortisol and 17-OHP levels. Serum cortisol levels were analyzed using the electrochemiluminescence immunoassay method. The reference range was 6.2-19.4 μg/dl in the morning and 2.3-11.9 μg/dl in the afternoon. Serum 17-hydroxyprogesterone levels were analyzed using the radioimmunoassay (RIA) method, with a reference range of 0.10-1.50 ng/ml.

Whole genome long-read sequencing and data processing. Genomic DNA was obtained from both twins and their parents, extracted from leucocytes using a Gentra Puregene Blood kit (Qiagen GmbH) and sheared using a Megaruptor 2 long hydropore to obtain DNA fragments of 15 kb. DNA fragments were analyzed using a Qubit fluorometer and pulsed-field gel electrophoresis. DNA fragments (2-5 μg) were prepared using the SMRTbell Express Template Prep kit 2.0 (cat. no. P/N 100-938-900) and SMRTbell Enzyme Clean-up kit 2.0 (cat. no. P/N 101-932-600; both Pacific Biosciences). Fragments <10 kb were eliminated using BluePippin (Sage Science). SMRTbell libraries were sequenced on the Sequel II system using Sequel II sequencing kit 2.0 (cat. no. P/N 101-820-200; Pacific Biosciences) and raw subreads were processed through the circular consensus sequencing (CCS) workflow using PacBio SMRTLink version 10.0 (pacb.com/products-and-services/analytical-software/smrt-analysis) to generate high fidelity (HiFi) long reads, in which both DNA strands were sequenced multiple times due to the circular nature of the template, achieving a minimum estimated quality value of 20 (phred-scaled, corresponding to an accuracy of 99%). All samples were aligned to the GRCh38 human reference genome using pbmm2 v1.9.0 (github.com/PacificBiosciences/pbmm2). The aligned CCS binary alignment map data were used for small variant, SV and copy number variant (CNV) detection.

Short tandem repeat (STR) analysis. FASTQ files, generated from long-read whole-genome sequencing, were aligned to GRCh38 using LAST v1418 tool (18), yielding a MAF file. The MAF file was then used by tandem-genotypes v1.1.0 (19) to predict STR unit change of each read in relative to repeat unit in the reference genome. The repeat unit change was estimated in the twins, parents and 236 unaffected controls from the in-house database at Excellence Center for Genomics and Precision Medicine, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. This database contains long-read sequencing data from patients with various genetic conditions, as well as from parents or siblings of certain probands. Importantly, the 236 samples designated as controls did not include individuals with pigmentation disorder. Next, STRs were identified using an in-house algorithm. Repeat patterns with 2-6 bases were selected to calculate distributions of repeat unit using kernel density estimation. Gaussian kernel with a bandwidth of five was selected to smooth the estimated density curve. Minimum points were used at the curve to cluster the data. For autosomal dominance mode, STRs exhibiting expansion only in the twins but not in their parents and controls, were searched. For autosomal recessive mode, expansion in both twins and parents but not in controls were searched. STRs were further reviewed for their relevance to the disease by examining genomic context, literature on disease association and predicted effects on gene function or expression. Analysis of single nucleotide variants (SNVs), insertions and deletions (Indels), SV, CNV and methylation profile is described in the Data S1.

Kinship analysis. Kinship coefficient was estimated using kinship-based inference from the KING package v2.3.2, based on genotype data derived from whole-genome sequencing (20). Relatedness was inferred based on estimated kinship coefficients using the following classification: Kinship coefficient >0.354 indicates duplicates or monozygotic twins, where (0.177, 0.354), (0.0884, 0177) and (0.0442, 0.0884) indicate first-second- and third-degree relatives, respectively.

Cell culture. Fibroblast cells were acquired from skin biopsies of two affected twins carrying the *TYMS* variant and four unaffected individuals who were not in the in-house database. The controls included three males and one female, all pediatric donors aged 1-5 years, with no history of pigmentation disorder or related skin conditions. The cells were cultured in DMEM supplemented with 10% fetal bovine serum (both Cytiva; HyClone) and 1X antibiotic-antimycotic (Gibco; Thermo Fisher Scientific, Inc.) and then incubated at 37˚C with 5% CO_2 (21). The use of primary human cells in cell culture experiments was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (approval no. 813/63).

Reverse transcription-quantitative (RT-q)PCR-based mRNA quantification. RNA was extracted from fibroblasts and peripheral blood mononuclear cells (PBMCs) using the QIAamp RNA Blood Mini kit (Qiagen GmbH). PBMC controls included both parents of the twins and one unrelated 45-year-old adult female, all without any history of pigmentation disorders or related skin conditions. These PBMC controls were independent from the aforementioned fibroblast controls. cDNA was prepared from 1 μg RNA using the ImProm-II™ RT System (Promega Corporation) according to the manufacturer's protocol. The input RNA from fibroblasts was primed with an equal mix of anchored dT oligonucleotides for reverse-transcribing RNA to cDNA. TaqMan probes specific for *TYMS* (cat. no. Hs00426586_m1) and β-actin (Assay ID: Hs01060665_g1) were used, and all reactions were set up with Luna® Universal Probe qPCR Master Mix (New England Biolabs). A total of three replicates/sample were run for both *TYMS* and β-actin on the Applied Biosystems StepOnePlus Real-Time PCR system (Thermo Fisher Scientific, Inc.) and the results were analyzed using StepOne Software v2.3 (Thermo Fisher Scientific, Inc.). qPCR thermocycling conditions were as follows: Initial denaturation at 95˚C for 60 sec, followed by 40 cycles of denaturation at 95˚C for 15 sec and extension at 60˚C for 30 sec. The expression of the gene of interest (*TYMS*) was normalized against the control gene (β-actin), and the relative change between affected and controls was analyzed using the $2^{-\Delta\Delta Cq}$ method (22).

Protein expression analysis by western blotting. To assess *TYMS* expression, western blot analysis was performed. Briefly, protein lysate was prepared from fibroblast cells using radioimmunoprecipitation assay buffer supplemented with protease inhibitors (Cell Signaling Technology, Inc.). The protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein (50 μg/lane) were separated by 12% SDS-PAGE. The proteins were transferred onto a PVDF membrane using iBlot™ 2 Transfer Stacks (Invitrogen; Thermo Fisher Scientific, Inc.). The membrane was blocked with 5% non-fat dry milk and 1% BSA in TBS for 1 h at room temperature to prevent non-specific binding. Subsequently, the

membrane was incubated overnight at 4˚C with the primary antibody against TYMS (1:500; cat. no. HL1236; Invitrogen; Thermo Fisher Scientific, Inc.). After washing with 0.1% TBST, the membrane was incubated with the corresponding anti-rabbit IgG, horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 3 h at room temperature. TYMS protein bands were visualized at 34 kDa using an enhanced chemiluminescence detection SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc.), and chemiluminescent signals were visualized and quantified using the ImageQuant™ LAS 4000 system (version 1.3; GE Healthcare Life Sciences). The relative protein expression levels were normalized to the housekeeping gene β-actin (13E5) Rabbit mAb (1:1,000, Cat. No. 7087; Cell Signaling Technology, Inc.).

Results

Clinical characterization of twins. The twins had a kinship coefficient of 0.4934, confirming their monozygosity and ensuring that the sequencing data accurately corresponded to them. Both twins exhibited progressive skin darkening since birth, with strikingly similar pigmentation patterns. Both twins were healthy without any other symptoms. They were born to non-consanguineous parents with normal skin color and no family history of hyperpigmentation disorder. No history of drug intake or urine discoloration or photosensitivity was reported. The date of admission was April 2019 for adrenal insufficiency workup, which yielded a negative result. The patients were referred to a dermatologist. The first skin biopsy was performed in September 2019 for histopathological examination, followed by a second biopsy ~6 months later to obtain fibroblasts for cell culture. There was no subsequent follow-up.

On examination, both twins had generalized, diffuse, dark-black hyperpigmentation of the skin on the face, neck, body, hands and feet (Fig. 1A-D) and some reticulate hypopigmentation and hyperpigmentation were found on the abdomen. Hyperpigmentation was prominent on the distal finger and feet. Tongue and buccal mucosa also exhibited some hyperpigmentation. The hair and nails were normal.

Skin biopsy from the right trunk revealed increased melanin pigmentation in basal and suprabasal layers and presence of melanophages in upper dermis. These melanophages were identified as large, round or oval cells with abundant cytoplasmic melanin granules, giving them a dark brown/black appearance under the microscope. Additionally, no melanocyte proliferation was observed, based on the absence of increased melanocyte density or atypical melanocytic nests in the basal epidermis (Fig. 1E). Physical examination of both twins revealed no abnormality aside from hyperpigmentation. Electrocardiogram results were normal. Complete blood count showed mild anemia with hematocrit 31.7% and no macrocytosis. Cortisol levels in the morning and ACTH stimulation test results were normal, thus, excluding Addison's disease.

Sequencing output. HiFi reads were mapped on the GRCh38 human reference genome. The mean breadth of coverage of the four samples was 97.19% (range, 95.08-98.94%). The average sequencing depth of the four samples was 28X (range, 26-31X).

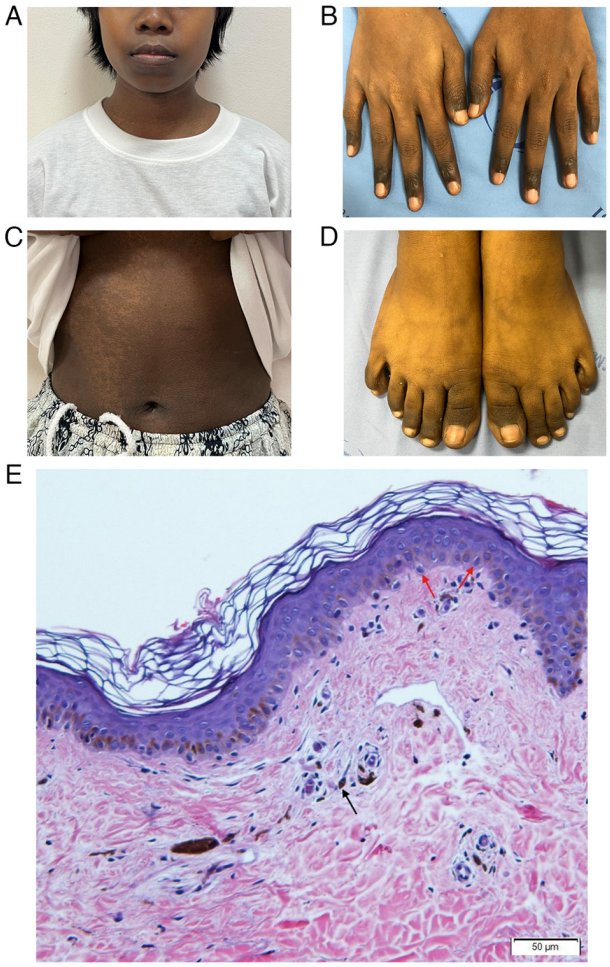

Figure 1. Patient with congenital progressive universal melanosis. Hyperpigmentation of skin on (A) face and neck, (B) hands, (C) abdomen and (D) foot of twin A. (E) Histopathological results from the lesion shows basal hyperpigmentation and superficial dermal melanophages. Red arrows indicate melanocytes, the pigment-producing cells found in the basal layer of the epidermis. Black arrow indicates melanophage, which is characterized by large, darkly pigmented granules and is located in the dermis.

Variant analysis

SNV/Indel analysis. Variants were filtered based on inheritance pattern. The number of concordant SNVs (twins have the same genotype) and Indels between twins from DeepVariant was 7,030,746. There were 29,137 autosomal dominant *de novo* variants, 236,591 autosomal recessive variants and 28,108,264 pairs of compound heterozygous variants. In the first approach, two *de novo* and two pairs of compound heterozygous variants were classified as likely pathogenic and frameshift mutations, according to Franklin by Genoox. However, those variants were regarded as false positives after visually inspecting BAM files with Integrative Genomics Viewer v2.15.4 (23).

In the second approach, one autosomal recessive was identified in phosphatidylinositol glycan anchor biosynthesis class B (*PIGB)* (c.981T>G), which was a missense variant. This variant was absent in the in-house database but present in controls in gnomAD database. The gene constraint score of the c.981T>G variant indicated a tolerance to variation. Based on the guidelines of the American College of Medical Genetics and Genomics (ACMG), *PIGB* (c.981T>G) variant was classified as benign. Moreover, eight compound heterozygous

variants which were missense variants in myosin heavy chain 13 (*MYH13)* (c.53G>A and c.791A>G), serpin family H member 1 (*SERPINH1)* (c.293G>A and c.1066G>A), myosin VIIA *(MYO7A)* (c.781G>A and c.4349A>G), UDP glucuronosyltransferase family 1 member A1 (*UGT1A1)* (c.1459C>T and c.964A>G), keratin 36 (*KRT36)* (c.122G>A and c.677G>T), tectonin β-propeller repeat containing 1 (*TECPR1)* (c.2270C>A and c.577G>A), tripartite motif containing 10 (*TRIM10)* (c.842G>A and c.977C>T), and missense and intronic variants in cholinergic receptor nicotinic epsilon subunit *(CHRNE)* (c.1375T>C and c.917+562T>C) were identified (Table SI). Despite their absence in the in-house database and controls in gnomAD database, the gene constraint score of all variants indicated tolerance to variation. Based on ACMG guidelines, compound heterozygous variants were classified as variants of uncertain significance.

SV analysis. The number of concordant SVs between twins from pbsv was 73,948 variants, of which 69,106 passed quality control and were filtered based on inheritance pattern, yielding 157 *de novo* and 2,267 autosomal recessive variants. No compound heterozygous variants were retained. In the first approach, no pathogenic or likely pathogenic variants were found. In the second approach, two autosomal recessive variants in the solute carrier family 22 member 31 (*SLC22A31*) and a region from sialic acid binding Ig like lectin 14 (*SIGLEC14*) to *SIGLEC5* were obtained. After inspecting BAM files, these SVs were true positives; however, they were not rare (frequency >1% in the in-house genome database) in Thai individuals. Therefore, no SVs were considered to be associated with the phenotype.

CNV analysis. No CNVs were phenotype-associated. Copy numbers that were different from both parents were identified in 13 regions. After annotating the regions with ClassifyCNV, eight regions were predicted as uncertain significance (four of which were located within or overlapped protein-coding genes) and five were predicted as benign. After checking the copy number in these regions in the in-house database, all regions were ruled out since allele frequency was >1%.

Methylation profile analysis. The present study identified 211 DMRs, of which 161 resided in 143 genes and 50 DMRs were not in genes. The annotation showed that 51.66, 18.01, 12.32, 10.90, 5.69 and 1.42% were in the promoter (≤2kb), distal intergenic, intron, exon, promoter (2-3 kb) and 3' untranslated region. respectively. After performing biological function prediction, 35 significant terms were identified (Table SII). However, the significant enrichments were not related to skin pigmentation or melanogenesis.

STR analysis. After starting with 718,245 repeat patterns, filtering for STR motifs 2-6 base pairs in length, resulting in 240,677 patterns. Results from the in-house algorithm returned eight candidate patterns. Only one pattern (GATGGT) was consistent with autosomal recessive inheritance, whereas other patterns were not consistent between phenotype and mode of inheritance. There was a biallelic GATGGT repeat expansion with a range of 210-259 repeats in intron 3 of *TYMS* in both twins (Fig. 2A), whereas the parents exhibited a heterozygous expansion. The father had 106 repeat units in one allele and 230-245 repeat units expanded in the other allele. The mother had 93 repeat units in one allele and 217-224 repeat units expanded in the other allele compared with the reference genome (data not shown). The 236 controls had ≤172 repeat units (Fig. 2B). The majority of controls (47%; 112/236) had 106 repeat units. All controls with 172 repeat units carried the expansion on a single allele only. No homozygous controls were identified, unlike in the twins.

Determination of the TYMS mRNA and protein expression. RT-qPCR was used to investigate the expression levels of *TYMS* in identical twin patients. RNA was extracted from cultured skin fibroblasts and PBMCs. The mean *TYMS* expression levels in the patients (PBMCs, 0.74 and 0.76; skin fibroblasts, 0.91 and 1.03) were within the range observed in control samples (PBMCs, 0.30-1.00; skin fibroblasts, 0.47-1.41; Figs. 3A and S1). No apparent differences were observed in either cell type. Protein was extracted from cultured skin fibroblasts. The western blot analysis showed that TYMS protein levels varied between the two probands, with twin A showing lower expression than twin B. Overall, the levels observed in both twins were within the range seen in control samples (Fig. 3B, S2-3).

Discussion

The present study reported monozygotic twin girls with distinct types of diffuse hyperpigmentation. Both had hyperpigmentation on the trunk, although some areas showed reticulated hypo- and hyperpigmentation, which differs from the pigmentation of Addison's disease, which is most intense in the flexures, at sites of pressure and friction and in the creases of the palms and soles (9,24). Moreover, the investigation for Addison's disease showed negative results. Unlike other diffuse hyperpigmentation disorders, the present condition was characterized by a congenital onset, progressive maturation and absence of accompanying symptoms. By contrast with the acquired onset in AUM (25), localized ashy-gray patches in EDP (26), pruritus and sun-exposed areas in LPP (27) and irregular patches and specific area involvement in FPH (28), the twins exhibited symptom-free, whole-body pigmentation. This distinct presentation suggests a novel entity in the spectrum of diffuse hyperpigmentation disorders.

Single gene disorders, such as X-linked recessive hyperpigmentation, Dowling-Degos disease, dyschromatosis universalis hereditarian and Kitamura disease, have reticulate lesions and other signs and symptoms (29-32). For example, a case report of hyperpigmentation in a Taiwanese child demonstrated an inborn error of vitamin B12 metabolism with identification of a homozygous mutation in ATP binding cassette subfamily D member 4 (c.423C>G; p.Asn141Lys) (33). Due to these distinguished features, the present condition was termed congenital progressive universal melanosis (CPUM).

In the present case, the condition was similar to AUM, also known as carbon baby syndrome (diffuse hyperpigmentation subtype). To the best of our knowledge, only a few reports of AUM have been published (25,34-46). In patients with AUM, pigmentation of basal and suprabasal layers is increased without melanocyte proliferation (44). Histopathological examination of the present patients also showed these traits but hyperpigmentation was congenital. The present patients

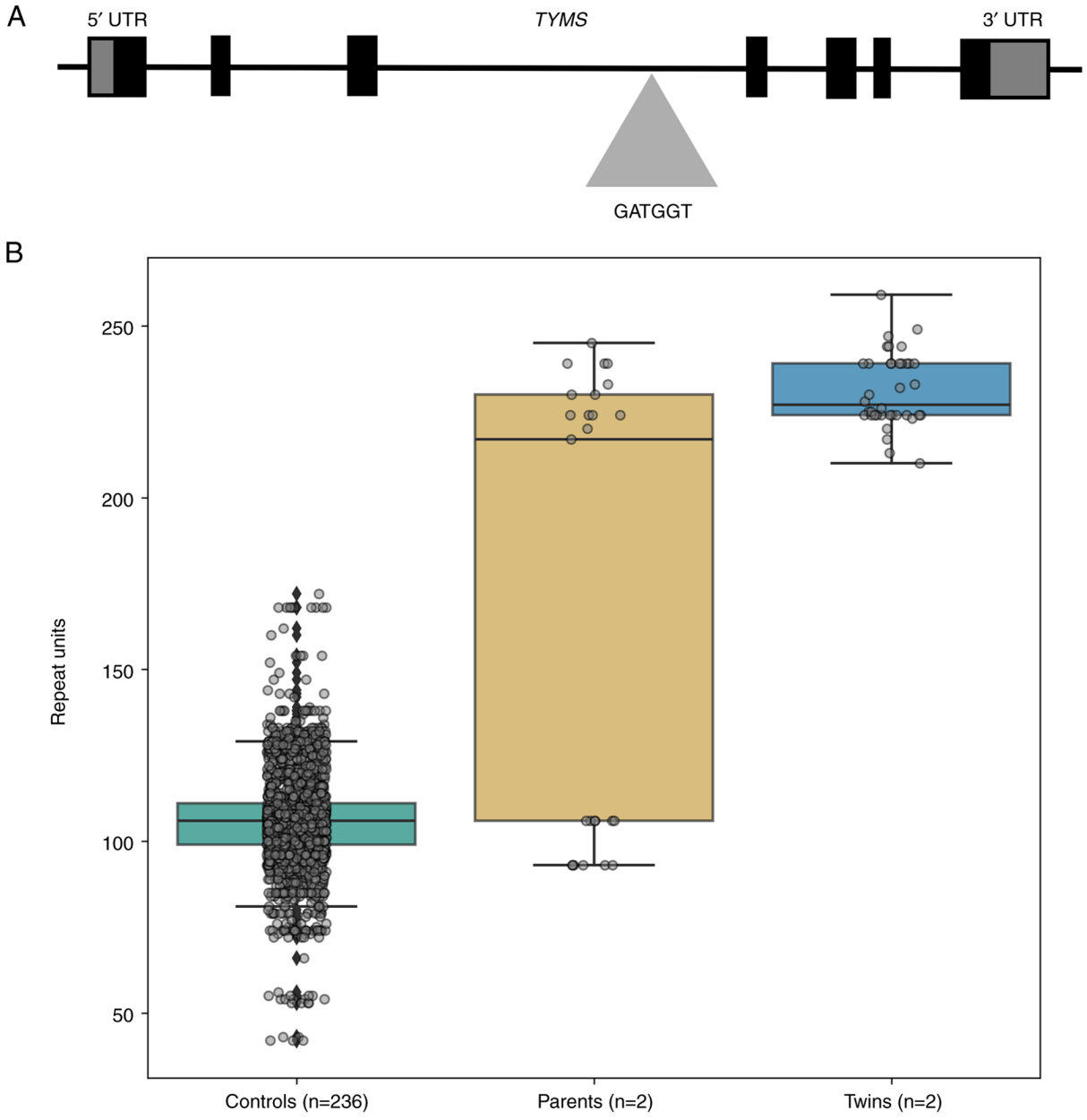

Figure 2. GATGGT repeat expansion in the *TYMS* gene. (A) Schematic diagram showing GATGGT repeats expansion position (chr18, position:666,891-667,632 of intron 3 in *TYMS*; hg38 version). (B) Repeat units in affected twins compared with carrier parents and controls. TYMS, thymidylate synthase; UTR, untranslated region.

presented with heavily pigmented areas especially on the lips, tongue, knuckles and feet, with generalized hypopigmented patches around the abdomen. Electrocardiogram test, serum cortisol, ACTH stimulation and physical examination results were within normal limits and no retinitis pigmentosa was detected. Patients had no history of drug intake and no family history of hyperpigmentation or associated conditions. While melanin production regulation, excessive production of β melanocyte-stimulating hormone, abnormal sensitivity of melanocytes or neural stimuli may be involved in AUM (46), the actual etiology of AUM is yet to be elucidated.

The present study performed long-read genome sequencing owing to its ability to provide a more comprehensive and accurate representation of the genome than short-read sequencing. SNV/Indel, SV, CNV, methylation profile and STR filtering were performed, focusing on variants present in both affected individuals and absent in the unaffected parents. A total of eight variants from the SNV/Indel analysis and a novel unreported biallelic repeat expansion in the third intron of the *TYMS* gene from the STR analysis were found. No pathogenic or likely pathogenic variants were identified in other variant classes.

The eight SNV/Indel pairs of variants were found in eight genes. Mutations of the five genes (*UGT1A1*, *SERPINH1*, *CHRNE*, *TRIM10* and *MYO7A*) have been linked to human disorders, but none of the disorders manifest as abnormal skin pigmentation. Specifically, *UGT1A1* mutation causes Crigler-Najjar syndrome (47), whereas *SERPINH1* mutations result in osteogenesis imperfecta due to abnormal collagen biosynthesis (48). *CHRNE* mutations lead to congenital

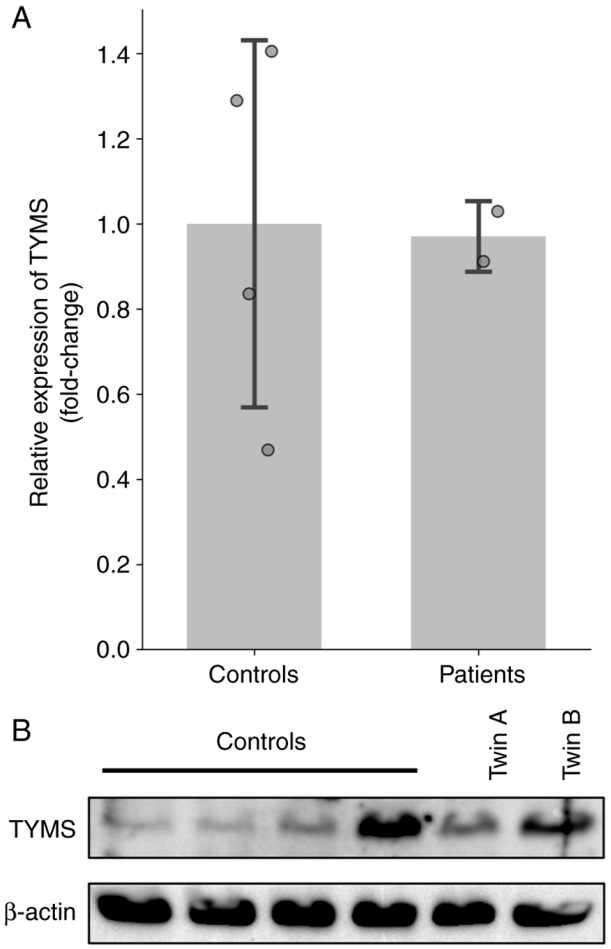

Figure 3. Expression of *TYMS* mRNA and protein in fibroblast cells. Mean *TYMS* (A) mRNA and (B) protein expression in controls (n=4) and patients (n=2). The signal intensity was normalized to β-actin. The blots were obtained from the same PVDF membrane, with *TYMS* and β-actin detected in separate experiments. TYMS, thymidylate synthase.

myasthenic syndrome (49) and *TRIM10* mutation is associated with nephronophthisis (50). Additionally, a mutation in *MYO7A* causes non-syndromic hearing loss (51). To the best of our knowledge, for the remaining three genes, *MYH13, KRT36* and *TECPR1*, no disease has yet been directly associated. However, *MYH13* is associated with muscle contraction (52). *KRT36* is a member of the keratin gene family involved in hair and nail formation (53) and *TECPR1* serves a role in autophagy (54). Given the functions and associated conditions of these genes, no candidate gene among the SNV/Indel variants appeared to be responsible for causing CPUM.

An unreported biallelic GATGGT hexanucleotide repeat expansion in the third intron of the *TYMS* gene was identified from the STR analysis in the twins, with 210-159 repeat units (124 repeat units of the hexanucleotide repeats are present in the human reference genome hg38). The father and mother each had one non-expanded allele, with the other allele measuring 230-245 units in the father and 217-224 units in the mother. A total of 206 controls carried the GATGGT repeat in a range of 42 to 172 and almost half of the controls had 106 units. The repeat expansion was present in both alleles of the affected twins and heterozygous state in each parent, consistent with an autosomal recessive mode of inheritance.

The *TYMS* gene catalyzes the methylation of deoxyuridine monophosphate to form deoxythymidine monophosphate by transferring a methyl group from 5,10-methylenetetrahydrofolate. This conversion is key for maintaining the balance of nucleotides necessary for DNA replication and repair (55). Dysregulation of *TYMS* expression causes DNA damage, leading to defective DNA synthesis. This triggers cell stress responses, including activation of the tumor suppressor protein p53 and potentially increasing microphthalmia-associated transcription factor (*MITF*) expression (56). *MITF* activation upregulates both tyrosinase and associated proteins, resulting in increasing melanin synthesis (57,58). Therefore, *TYMS* may indirectly influence skin pigmentation via its involvement in DNA repair mechanisms. Hyperpigmentation can arise from genes involved in DNA repair or early senescence (58). A total of >150 DNA damage repair genes, including *TYMS*, have been identified in the hallmark gene set of the Molecular Signatures Database as playing a role in DNA repair mechanisms (59).

Digenic germline variants in both *TYMS* and enolase superfamily member 1 result in dyskeratosis congenita (DC) (60). In the aforementioned study, RT-qPCR analysis revealed a significantly reduced expression of *TYMS* in lymphoblastoid cells derived from affected probands compared with controls. TYMS deficiency due to loss-of-function missense mutations disrupts the nucleotide metabolism resulting in genome instability, which was measured by the level of protein associated with DNA damage. Clinical manifestations of DC include abnormal skin pigmentation, nail dystrophy and oral leucoplakia. This suggests that the abnormal skin pigmentation in DC is a result of genome instability. Certain mutations in *TYMS* causing a dysmorphic syndrome may lead to non-syndromic anomaly. For example, p63 mutations cause Mendelian clefting syndromes, including ectrodactyly-ectodermal dysplasia-clefting syndrome [Online Mendelian Inheritance in Man (OMIM no. #129900)], ankyloblepharon-ectodermal dysplasiaclefting syndrome (OMIM #106260) and RappHodgkin syndrome (OMIM #129400), while some missense mutations can lead to non-syndromic cleft lip and palate (61). Therefore, it was hypothesized that since DC, a syndrome with hyperpigmentation, can be caused by certain forms of genetic variants in *TYMS*, another form of genetic variant in the same gene may lead to non-syndromic hyperpigmentation.

The present study attempted to identify the molecular mechanism underlying GATGGT repeats in non-coding regions of the *TYMS* gene which lead to hyperpigmentation. Non-coding repeat expansions can lead to disease through three major mechanisms: Epigenetic silencing resulting in loss-of-function of the expanded allele, sequestration of RNA-binding splicing factors and repeat-associated non-AUG translation (62). For example, Friedreich ataxia disorder exhibits GAA repeat expansion within the first intron of frataxin (*FXN*) gene (63), which leads to increases in DNA methylation in the upstream region of the *FXN* repeats, resulting in changes of chromatin structures. Benign adult familial myoclonic epilepsy type 1 (BAFME1) exhibits TTTTA repeats expansion with TTTCA repeats insertion in the intron 4 of sterile alpha motif domain containing 12 (*SAMD12*) (64,65). BAFME3, BAFME4, BAFME6, BAFME7 and BAFME8 also have the same pattern of repeat expansion in the intron of membrane associated ring-ch-type finger 6

(*MARCH6*) (66), yeats domain containing 2 (*YEATS2*) (67), trinucleotide repeat containing adaptor 6A (*TNRC6A*) (68), rap guanine nucleotide exchange factor 2 (*RAPGEF2*) (68) and retinoic acid induced 1 (*RAI1*) (69). BFAME2 is caused by ATTTC repeats expansion within the first intron of StAR related lipid transfer domain containing 7 (70). To the best of our knowledge, however, the exact mechanisms underlying BAFME have not yet been explained.

It was hypothesized that the intronic repeat expansions in the present twins decreased *TYMS* RNA levels and subsequently lowered TYMS protein levels, leading to genome instability. This, in turn, activates p53, similarly to DNA damage caused by UV exposure (71), increasing MITF expression and resulting in increased melanin synthesis and hyperpigmentation. RNA and protein levels in fibroblasts were assessed because of the possible role of fibroblasts in skin pigmentation. According to previous studies, fibroblasts produce paracrine factors that bind receptors on melanocytes, leading to melanogenesis (72,73). In addition, fibroblast-derived growth factors including hepatocyte growth factor (HGF), neuregulin 1 (NRG-1) and corticotropin-releasing hormone (CRH) have been shown to increase pigment contents in melanocytes (74). However, RT-qPCR to quantify *TYMS* RNA levels and immunoblot analyses to determine TYMS protein levels showed no differences between the twins and controls. It is important to note that statistical analysis was not performed due to the limited sample size (n=2).

Based on the present results, it is hypothesized that the GATGGT repeats in non-coding regions of the *TYMS* gene may reduce *TYMS* expression specifically in melanocytes or keratinocytes as no decrease was observed in PBMCs or cultured skin fibroblasts. This decreased expression could lead to the disruption of nucleotide balance in DNA repair and skin hyperpigmentation. Future studies should investigate melanocytes and keratinocytes to verify the role of repeat expansion in *TYMS* associated with CPUM. The present study did not directly assess *TYMS* expression in melanocytes or keratinocytes, the primary cells responsible for pigmentation. Moreover, the present study was based on a single rare case of monozygotic twins with CPUM, and no additional cases were available for validation. Independent replication studies with additional cases are needed to confirm the pathogenic relevance of *TYMS* repeat expansion in CPUM. Despite these limitations, the present findings provide novel insights into a potential genetic mechanism underlying CPUM and offer a foundation for future functional studies.

In summary, the present study suggested a association link between intronic hexanucleotide repeat expansion in *TYMS* and CPUM. However, further studies are necessary to confirm this association and understand its pathogenesis.

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Availability of data and materials

The data generated in the present study are not publicly available due to privacy constraints but may be requested from the corresponding author.

Authors' contributions

VS conceptualized and supervised the study. VS, ST, SP and MP designed the experiments and edited the manuscript. SC, TK, CV and PW collected clinical data and biological samples. CS performed the sequencing analysis. ST and KS performed RT-qPCR analysis and western blotting. SK and MP analyzed the data. SK and MP confirmed the authenticity of all the raw data. SK, SP, KS and MP drafted the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (approval nos. 264/62 and 813/63) and was conducted in accordance with the 1964 Helsinki Declaration and its later amendments, as well as all relevant ethical guidelines and regulations. The rights and interests of all participants were fully protected. Written informed consent was obtained from the parents of the patients.

Patient consent for publication

The authors confirm that the parents of the patients provided written informed consent for publication of potentially identifying images.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

ChatGPT (OpenAI) was used to refine the clarity and readability of English sentences in the manuscript. The AI-assisted revisions were limited to grammar, syntax and phrasing, without altering the scientific content, interpretation or originality of the work. All final edits were reviewed and approved by the authors.

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