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Title

Molecular detection of CTG repeat lengths in the *DMPK* gene in healthy individuals and diagnosis of myotonic dystrophy type 1 using triplet-primed PCR in Malaysia

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Abstract

Objective The lack of epidemiological data and molecular diagnostic services in Malaysia has hampered the setting-up of a comprehensive management plan for myotonic dystrophy type 1 (DM1) patients, leading to delayed diagnosis, treatment and support for patients and families. The aim of this study was to estimate the prevalence of DM1 in the three major ethnic groups in Malaysia and evaluate the feasibility of a single tube triplet-primed polymerase chain reaction (TP-PCR) method for diagnosis of DM1 in Malaysia.

Design, **setting and participants** We used PCR to determine the size of CTG repeats in 377 healthy individuals and 11 DM1 suspected patients, recruited from a tertiary hospital in Kuala Lumpur. Triplet-primed PCR was performed on selected samples, followed by Southern blotting to confirm and estimate the size of CTG expansion.

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Outcome measures The number of healthy individuals with $(CTG)_{>18}$ was determined according to ethnic group and as a whole population. Chi-squared test was performed to compare the distribution of $(CTG)_{>18}$ with 12 other populations. Additionally, the accuracy of TP-PCR in detecting CTG expansion in 11 DM1 patients was determined by comparing the results with that from Southern Blot testing.

Results Of the 754 chromosomes studied, $(CTG)_{>18}$ frequency of 3.60%, 1.57% and 4.00% in the Malay, Chinese and Indian sub-populations respectively, was detected, showing similarities to data from Thai, Taiwanese and Kuwaiti populations. We also successfully detected CTG expansions in nine patients using the TP-PCR method followed by the estimation of CTG expansion size via Southern blot.

Conclusions The results show a low DM1 prevalence in Malaysia with the possibility of underdiagnosis and demonstrates the feasibility of using a clinical and TP-PCR-based approach for rapid and cost effective DM1 diagnosis in developing countries.

Strengths and limitations of this study

- This is the first DM1 epidemiological study on healthy individuals from the three major ethnic groups in Malaysia.
- To date molecular diagnostic testing for DM1 is not performed in any hospital in Malaysia. This study describes the feasibility of a cost and time-effective TP-PCR based method for rapid screening and diagnosis of DM1.
- The number of DM1 samples analysed is small as DM1 is a rare disease in Malaysia.

Key Words

CTG repeats/genetic counselling/myotonic dystrophy type 1/molecular diagnosis/TP-PCR/prevalence

Introduction

The myotonic dystrophies (DM) are the most prevalent adult muscular dystrophy worldwide, with an estimated prevalence of 1 in 8000.[1] They are classified into two main sub-groups, myotonic dystrophy type 1 (DM1) and type 2 (DM2). These are caused by nucleotide repeat expansions, which are inherited

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in an autosomal dominant manner, and manifest as clinically heterogeneous diseases. DM1 is due to CTG nucleotide repeats beyond the normal length of five to 49, in the 3' untranslated region (UTR) of the Dystrophia Myotonica Protein Kinase (*DMPK*) gene, located on chromosome 19q 13.3.[2, 3] It is a progressive disease and categorised into several subtypes. The congenital form of DM1 is maternally transmitted more frequently, although the disease occurs equally in males and females.[4] The general consensus is that the larger the CTG repeat in an individual, the more severe the disease and the earlier the age of onset. It is however, difficult to classify individual DM1 cases into distinct categories based merely on the size of CTG repeats, as genotype-phenotype correlation often overlap and are not clearly defined. In addition, the repeat sizes have shown variation, both between tissues, and over time in the same tissue.[5, 6] This has made disease prognosis difficult. The genetic phenomenon of anticipation can also be observed in the inheritance of the disease, resulting in a more severe form of the disease coupled with an earlier age of onset in subsequent generations.[7, 8]

The prevalence of DM1 varies greatly across populations—it is pre-dominantly seen amongst the Europeans and Japanese.[9] A study also estimated a high disease frequency in the Finnish population.[10] In Quebec, Canada, a particularly high DM1 prevalence of 1 in 500 has been recorded due to founder effects.[11] In contrast, it is a rare disease amongst ethnic sub-Saharan populations,[12] being almost unheard of with the exception of one case reported in Nigeria[13] and two more recent cases amongst African Americans.[14] In view of this disparity, a study was undertaken to determine the distribution of CTG repeats in normal African individuals. It was found that there was a highly significant difference in the distribution of normal CTG alleles larger than 18 between the African population and the European and Japanese populations.[12] This reiterates a previous theory that CTG alleles between 19 and 30 act as a source of DM1 mutations in subsequent generations.[15] These findings have formed the basis for the estimation of DM1 incidence within a population.[9, 12, 16-25]

Prior to the establishment of molecular diagnostic tests, DM1 was diagnosed in clinics mainly by observing clinical symptoms and conducting electromyography (EMG) tests, with confirmation by muscle biopsy.[26] At present, there are several molecular techniques that can be utilised in making a DM1

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diagnosis, rendering little use for the invasive and painful EMG test and muscle biopsy. [27] However, a single test that is able to detect all ranges of expansion sizes is yet to be established. Laboratories often employ a combination of methods depending on mutation dynamics in the population and available equipment. Conventional PCR can detect the normal range of CTG repeats as well as premutated alleles. Optimised PCR conditions can detect alleles up to $(CTG)_{85}$, whereas those beyond that rely on Southern Blot for detection. Recently, a novel TP-PCR method was developed to detect the presence of large expanded alleles, thus reducing the number of reflex Southern Blot tests.[28]

As a Southeast Asian country, Malaysia has a population consisting mainly of ethnic Malay, Chinese and Indian. There is also a large group of indigenous people belonging to various tribes. While DM1 is not commonly seen in this country, there is a possibility of underdiagnosis or misdiagnosis due to the lack of awareness about this condition with its diverse presentations. No study has been performed on the prevalence and incidence of the disease in the predominant ethnic groups, and to the best of our knowledge, diagnostic tests for this disease at the molecular level is not available anywhere in the country. Given the multisystemic and variable phenotypic manifestations in patients, it is therefore important for a simple standard confirmatory diagnostic test to be available, especially when trying to rule out different diagnoses. Here we report the use of PCR and Southern Blotting methods for the molecular analysis of healthy individuals from the Malay, Chinese and Indian sub-populations, where we studied the length of the CTG alleles in order to predict the prevalence of DM1 in these subpopulations. We also describe the use of a single-tube TP-PCR method for the screening and confirmation of DM1 amongst Malaysian patients, with the aim of reducing the number of Southern Blot tests that need to be performed.

Materials and Methods

Ethics Statement

Ethical approval to conduct this study was obtained from the University of Malaya Medical Centre (UMMC) ethics committee (Reference numbers 577.17 & 800.6). The ethics board required that all human subjects recruited in the study were briefed on the nature of the study, and provided with an

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information sheet describing the study. Subjects were also assured that their privacy will be protected, and all personal information provided will be kept confidential. Participation in the study was on a voluntary basis, and had no bearing on the quality of care patients received at the hospital.

Sample collection

Blood samples from 377 anonymous healthy blood donors of Malay, Chinese and Indian descent were obtained from the UMMC blood bank following oral consent to participate in the study. In addition, 11 patients displaying DM1 symptoms were recruited to this study. Written consent, clinical and familial history were obtained from these patients. The ethnicity of subjects was determined to be Malay, Chinese or Indian based on their own admission.

Molecular analysis

Genomic DNA was extracted from the blood samples using the QIAamp DNA Blood Mini kit according to manufacturer's protocol (QIAGEN, Hilden, Germany).

Conventional PCR

Analyses of the samples were carried out according to techniques described by Surh et al.[29] PCR was performed in a final volume of 30µL utilising the Perkin Elmer GeneAmp PCR system. The forward, 103, 5' – CCA GTT CAC AAA CCG CTC CGA GCG TG – 3' and reverse, 96, 5' – GGT GCG TGG AGG ATG GAA CAC GGA C – 3' primers were used. The PCR conditions were set as follows: initial denaturation at 96°C for 5 minutes, followed by 25 cycles of denaturation, annealing and extension at 96°C, 62°C and 72°C respectively, for a period of one minute for each step. Final extension was performed at 72°C for seven minutes. The PCR products were sized by gel electrophoresis on 1.5% agarose gel, at 100V for 45 minutes. The separated products from a number of the total samples were cut out from the gel, purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and sent to a service lab for sequencing to determine the exact number of CTG repeats.

Triplet-primed-PCR

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Thirteen samples were subjected to TP-PCR analysis—11 individuals with DM1 symptoms and two healthy controls. Testing was performed with 100 ng of genomic DNA from blood samples in a reaction volume of 25µl. The primers FAM-P1-Forward 5'FAM – GGG GCT CGA AGG GTC CTT GT – 3' and P2-Reverse 5' – GTG CGT GGA GGA TG AAC ACG – 3' flanked the CTG repeat region, with the forward primer labeled with FAM fluorescence. The third primer P3 5' – AGC GGA TAA CAA TTT CAC ACA GGA – 3' was designed to bind to the complement of the tail of the fourth primer P4-$(CAG)_6$ –Reverse 5' – AGC GGA TAA CAA TTT CAC ACA GGA CAG CAG CAG CAG CAG CAG – 3'. The primer combination was prepared in a ratio of FAM-P1-Forward: P4-$(CAG)_6$ –Reverse:P3:P2 = 1.5:1:1.5:1.5. The TP-PCR conditions were set as follows: initial denaturation at 95°C for five minutes, followed by 10 cycles each of denaturation (97°C) for 35 seconds, annealing (65°C) for 35 seconds and extension (68°C) for four minutes. Subsequently, 20 cycles of denaturation, annealing and extension were performed, with the extension time increased by 20 seconds per cycle to allow for increased yield of PCR product. The products were separated on an ABI PRISM 3130 x 1 genetic analyser (Life Tech, New York, USA) and fragment size determined using GeneMarker V2.6 (Softgenetics, State College, USA).

Southern Blotting

Southern Blotting was carried out in samples that only showed single peaks in the electropherograms, which indicated a CTG expansion. The conventional PCR products were transferred overnight from the agarose gel to a positively charged nylon membrane by capillary transfer and fixing of the DNA to the membrane done via the UV cross-linking method. The membrane was hybridised overnight in a hybridization buffer with the addition of 20µl alkaline phosphatase-conjugated $(CTG)_{10}$ oligonucleotide at 50°C. The membrane was then removed and the excess liquid drained off, prior to being washed using pre-heated wash buffers. Following hybridisation and washing of the membrane, development of the signals was carried out by exposing the blot to an autoradiography film. The presence of smears as opposed to distinct bands in the autoradiogram confirmed that the samples analysed were true DM1 patients.

Statistical analysis

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The frequency of each of the allele present in the 754 chromosomes from the healthy individuals was calculated. Statistical analysis was performed by administering the chi-squared (χ^2) test with Yates' correction to compare the distribution of normal large repeats, $(CTG)_{>18}$, with 12 other populations.

Results

Estimation of DM1 prevalence

The distribution of $(CTG)_{>18}$ alleles in the Malay, Chinese and Indian sub-populations all point towards a low prevalence of DM1. Figure 1 shows the breakdown of all alleles present in the three sub-populations. A bimodal allelic distribution was noted—this was in alignment with patterns observed in other populations with low DM1 frequencies. The first peak came from the $(CTG)_5$ alleles, which totaled to 33.7% of all alleles, while the second peak consisted of three alleles, 11 to 13 that accounted for a majority of 51.1% of the total alleles. The frequencies for $(CTG)_{>18}$ alleles were 9/250 = 3.60% (95% CI = 0.0166–0.0672) in the Malay subpopulation, 4/254 = 1.57% (95% CI = 0.0043–0.0398) in the Chinese subpopulation, and 10/250 = 4.00% (95% CI = 0.0193–0.0723) amongst the Indians. Heterozygosity was measured at 79.9%, 77.0%, and 76.2% in the three subpopulations, respectively, averaging at 77.7%. This result is aligned to those reported in other populations, which ranged from 73.0% in Europeans[9] to 92% in Iranians.[24]

Tables 1 and 2 show the comparison and χ^2 analysis of the frequency of $(CTG)_{>18}$ alleles in healthy individuals from the three subpopulations in this study, and in those from 12 worldwide populations, respectively. The $(CTG)_{>18}$ frequency for the Malay, Chinese and Indian subpopulations were significantly different when compared to frequencies in European, German and Chilean populations. All three Malaysian subpopulations showed frequencies similar to Thai,[20] Taiwanese[21] and Kuwaiti[23] populations. It is also interesting to note that the Han-Chinese show similarity with the Malaysian Chinese, the population that the majority of Malaysian Chinese trace their ancestry to. This allows for our speculation that the DM1 frequency among Chinese Malaysians is low, similar to that observed in the Han-Chinese,[22] Taiwanese[21] and South African negroids.[12]

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Diagnostic testing for DM1

Samples from 11 individuals with DM1-like symptoms and two healthy controls were analysed for CTG expansion using TP-PCR followed by confirmation by Southern Blot. Triplet-primed PCR testing showed single peaks in nine of the samples, and double peaks in the remaining four. The samples with single peaks also showed a clear laddering pattern indicating the presence of CTG expansion (Figure 2). Southern Blot testing confirmed the diagnosis of DM1 in the nine samples, with the detection of expanded alleles ranging from a size of 97 to 690 CTG repeats, as shown in Figure 3. Table 3 shows a summary of the characteristics of the disease exhibited by each patient. Figure 4 shows the pedigree diagram and the CTG repeat size of the families and individuals we studied. It is important to note that apart from those diagnosed (dark squares/circles), none of the other family members were examined or tested for DM1. Hence, there is a possibility that there may be family members showing very mild symptoms who have not presented in our clinics, contributing to the apparent under transmission of the disease in the families.

Discussion

In order to obtain a better understanding of the burden of DM1, we estimated the prevalence of the DM1 using the distribution of CTG alleles larger than 18 in the Malaysian population. The result of $(CTG)_{>18}$ of 3.05% (23/754) was observed in the Malaysian population. By comparing with the results of studies performed in other populations, we predict that DM1 is a rare disease in Malaysia. A larger study is needed to verify these findings, due to the fact that the subjects in this study were recruited from a major hospital in the capital city of Malaysia, therefore may not be representative of the whole country. It is likely that DM1 in the local community is underdiagnosed due to a lack of awareness amongst the public and healthcare professionals. There are also other contributing factors such as social stigma, and reduced access to major hospitals where specialised consultation and testing are available.

It is interesting to note that the frequency of $(CTG)_{>18}$ was the lowest in the Chinese subpopulation, although they account for the most number of DM1 patients seen in our hospital (including those not

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reported here). The Indians on the other hand show the highest frequency of $(CTG)_{>18}$ in agreement with the findings that DM1 is highly prevalent in India[30]. However, the number of Indian DM1 patients seen in our study was the lowest among the three subpopulations. This may reflect socio-economic and demographic reasons, as well as misdiagnosis/underdiagnosis of DM1 in the respective subpopulations.

Our study also provides for the first time, data on the $(CTG)_{>18}$ allele frequency in a Malay population. The Malay ethnic group is genetically more similar to the Chinese compared to the Indians. Comparison of the $(CTG)_{>18}$ distribution of the three ethnic groups however, shows a closer similarity between the Malays and the Indians (p=0.8151) compared to the Chinese (p=0.249). It would be interesting to see this same analysis done on other modern Malay populations in the region, such as the Singapore Malays and the Indonesians, as well as the aboriginal Malays.

The usage of the single tube TP-PCR allows for the rapid identification of large pathogenic CTG repeats, thus reducing the need for reflex Southern Blot testing. Southern Blot requires large amounts of DNA, the use of radioactive materials and is time consuming. In addition, this procedure is also less sensitive and may be difficult to replicate. Hence, any method that reduces the number of Southern Blot that needs to be performed, while demonstrating high sensitivity and specificity is advantageous in a clinical setting. However, the TP-PCR test used requires a highly specialized equipment, the genetic analyser, which may not yet be widely available and is unable to estimate the size of CTG expansions beyond 85 repeats.

Genotype-phenotype correlation studies in DM1 patients have thus far given conflicting results, with various underlying mechanisms, associations and theories proposed[31-34]. In our study, a disparity in the genotype-phenotype correlation in the Chinese family was seen, whereby Patient 3 is largely asymptomatic although she carries 350 repeats. Her disease status was only suspected and diagnosed following the birth of her children who exhibited symptoms. Both her children were congenitally affected, which is consistent with findings in previous studies that showed that the majority of congenital cases were maternally transmitted. Patients 2 and 7 on the other hand paternally inherited their pathogenic alleles, resulting in the classic/adult onset DM1. The same disease phenotype is seen in patients 8 and 9. We were not able to determine whether their diseases were inherited, as their parents have never been tested. However, these patients were given genetic counselling and in accordance with ethical principles,

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have the autonomy of deciding whether or not to disclose their disease status to family members at risk, for future counselling and testing. It was also observed that congenitally affected patient 5 showed a comparable expansion size to those who were classically affected. The only symptoms he has shown, however is neonatal hypotonia and a mild cognitive dysfunction. The comparable repeat size is most likely due to the younger age of patient 5 compared to the classically affected adults, and suggest that a larger repeat size would be observed, as the patient grows older. Apart from these disease dynamics, there have also been findings of contraction of allele sizes upon transmission reported elsewhere[25]. All these factors point towards the high complexity of DM1 and illustrate the important need for genetic counselling services to be offered to affected families.

Molecular testing is generally established as the gold standard in diagnosing genetic disorders such as DM1. This is because a molecular test is rapidly able to eliminate differential diagnoses, confirm the DM1 diagnosis, and estimate the size of CTG expansion in a patient, thus avoiding the need for invasive procedures such as muscle biopsies. Hilbert et al[35] who studied a large cohort of DM patients enrolled in the US National Registry, explored their diagnostic journeys, which on average took seven years for a correct DM1 diagnosis to be made. This delay brought about many implications to the patients and their families, ranging from lack of appropriate disease management to missed opportunities for genetic counselling. The situation in many developing countries is much similar or even worse as molecular diagnostic testing for DM1 is not easily available. Potentially, there could be a large number of patients who are undiagnosed/misdiagnosed, as well as those who have been unnecessarily subjected to various investigations for a definitive diagnosis to be made.

The findings from our preliminary study can aid the structuring of a rare disease management framework in Malaysia, using DM1 as a disease model. The data presented here adds to the scarce literature of DM1 in the South East Asian region. The information on CTG repeat lengths of the *DMPK* gene in healthy individuals, and DM1 patients, together with proper clinical assessment as well as a cost-effective molecular approach, carry implications for earlier diagnosis of DM1 and genetic counselling in a low resource setting.

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Contributorship statement

All authors were involved in the conception and design of the work as well as the final approval of the submitted manuscript.

KA and TI were involved in the acquisition and analysis of data and drafting the manuscript.

LLH, GKJ, KTW, AAA and MKT contributed to the critical evaluation of the manuscript.

Competing interests

The authors state no competing interests

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Data sharing statement

None available

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Figure Legends

Figure 1. Frequency of CTG repeats in healthy individuals from the Malay, Chinese and Indian sub-populations. *The frequency for large normal alleles, $(CTG)_{>18}$ was 9/250 or 3.60% in the Malays, 4/254 or 1.57% in the Chinese, and 10/250 or 4.00% in the Indians. A bimodal allelic distribution was observed in the Malaysian population, in alignment with patterns observed in other populations with low*

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DM1 frequency. The most frequently seen allele was $(CTG)_5$ in all three sub-populations, whereas $(CTG)_{10-13}$ was the most common allele group.

Figure 2. Electropherogram results of TP-PCR. *The X-axis represent the CTG repeat size and the Y-axis represents the allele peak height. (A) The electropherogram shows a DM1 patient sample with a single peak corresponding to $(CTG)_{11}$ and a laddering pattern indicating an expanded allele. (B) Two normal heterozygous alleles with sizes 5 and 11 and no laddering pattern observed.*

Figure 3. **Expanded CTG repeats of DM1 patients following PCR-Southern blotting as seen on an autoradiography film.** *Expanded alleles in patients ranging from a size of 97 to 690 CTG repeats have been detected. A sample of the bands are shown here, ranging from 270 repeats (1045bp) to 690 repeats (2305bp). Normal alleles of four sizes were seen amongst the patients, 5 (332bp), 11 (350bp), 12(356bp) and 13 (356bp). Due to somatic heterogeneity, the expanded alleles usually appear as smears. A 1Kb DNA ladder as well as samples from healthy individuals were run alongside patient samples as controls.*

Figure 4. Pedigree diagrams of DM1 patients studied including the size of their CTG alleles. *Members of three families and two individuals had their CTG repeat size analyzed. The sizes of the allele pairs for each patient are as stated in the pedigree diagrams. The phenomenon of anticipation was clearly observed in the three families, whereby with the increased CTG expansion in successive generations, a decreasing age of onset is noted.*

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Tables

Table 1: Comparison and χ^2 analysis of the frequency of $(CTG)_{>18}$ alleles in healthy individuals from the Malay, Chinese and Indian sub-populations.

Population	$(CTG)_{>18}$ alleles / Total alleles analyzed (%)	Comparison of Malay data with other populations χ^2 (p value)	Comparison of Chinese data with other populations χ^2 (p value)	Comparison of Indian data with other populations χ^2 (p value)
Malay	9/250 (3.60)	-	1.329 (0.249)	0.055 (0.8151)
Chinese	4/254 (1.57)	1.329 (0.249)	-	1.919 (0.166)
Indian	10/250 (4.00)	0.055 (0.8151)	1.919 (0.166)	-

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Table 2: Comparison and χ^2 analysis of the frequency of $(CTG)_{>18}$ alleles in healthy individuals from the three Malaysian sub-populations to those in twelve worldwide populations.

Population	$(CTG)_{>18}$ alleles / Total alleles analyzed (%)	Comparison of Malay data with other populations χ^2 (p value)	Comparison of Chinese data with other populations χ^2 (p value)	Comparison of Indian data with other populations χ^2 (p value)
[a] European[9]	15/130 (11.54)	7.817 (0.005**)	16.094 (<0.0001***)	6.729 (0.009**)
German[16]	22/104 (21.20)	26.17 (<0.0001***)	39.141 (<0.0001***)	24.239 (<0.0001***)
Mexican[17]	51/800 (6.38)	2.232 (0.135)	8.037 (0.005**)	1.553 (0.213)
Brazilian[18]	24/312 (7.69)	3.497 (0.062)	9.88 (0.002**)	2.334 (0.127)
Chilean[19]	30/272 (11.00)	9.354 (0.002**)	17.887 (<0.0001***)	8.131 (0.004**)
Japanese[9]	9/106 (8.50)	2.760 (0.097)	8.386 (0.004**)	2.149 (0.143)
Thai[20]	11/400 (2.75)	0.142 (0.706)	0.505 (0.477)	0.421 (0.516)
Taiwanese[21]	7/499 (1.40)	2.867 (0.090)	0.018 (0.893)	3.962 (0.050)
Han Chinese[22]	6/600 (1.00)	5.463 (0.019*)	0.134 (0.714)	7.052 (0.008**)
Kuwaiti[23]	14/370 (3.78)	0.010 (0.920)	1.894 (0.169)	0.006 (0.938)
Iranian[24]	29/400 (7.25)	3.090 (0.079)	9.292 (0.002**)	2.334 (0.127)
South African[12]	3/420 (0.71)	5.869 (0.015*)	0.457(0.499)	7.249 (0.007*)

*P < .05 (significant); ** P < .01 (highly significant); *** P < .001(very highly significant)

[a] Includes British, German, Belgian, Swedish and Finnish subjects

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Table 3: A summary of the disease characteristics exhibited by DM1 patients in this study

Family	Patient	Gender	[a] Age	[b] Disease Onset	[c] Phenotype	CTG Repeat Size
1	1	Male	54	Late adult	Classical	330
	2	Male	30	Early adult	Classical	690
2	3	Female	30	Early adult	Mild	350
	4	Female	31	Early adult	Mild	97
	5	Male	5	Birth	Congenital	596
3	6	Male	60	Late adult	Classical	270
	7	Male	30	Early adult	Classical	570
4	8	Male	44	Early adult	Classical	550
5	9	Male	32	Early adult	Classical	520

[a] Age of patient at time of molecular testing

[b] Early adulthood: 20 – 49 years old; Late adulthood: >50 years old

[c] Phenotype classification as described by Kamsteeg et al

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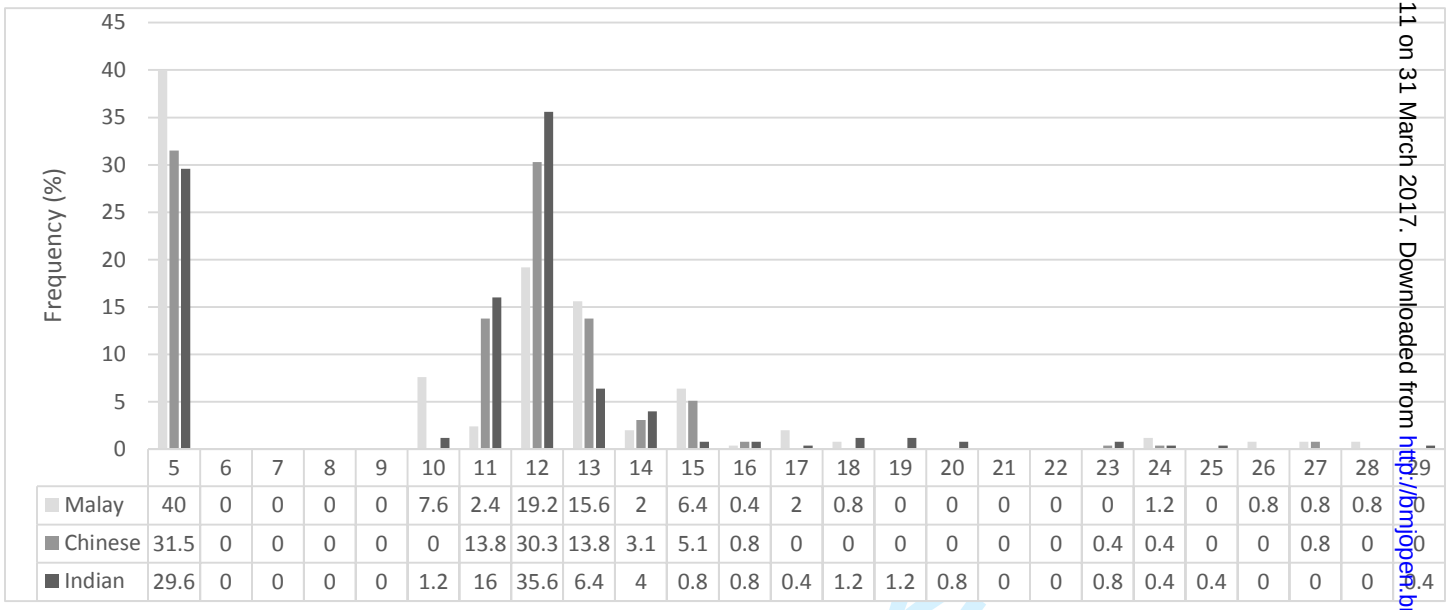

	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Malay	40	0	0	0	0	7.6	2.4	19.2	15.6	2	6.4	0.4	2	0.8	0	0	0	0	0	1.2	0	0.8	0.8	0.8	0
Chinese	31.5	0	0	0	0	0	13.8	30.3	13.8	3.1	5.1	0.8	0	0	0	0	0	0	0.4	0.4	0	0	0.8	0	0
Indian	29.6	0	0	0	0	1.2	16	35.6	6.4	4	0.8	0.8	0.4	1.2	1.2	0.8	0	0	0.8	0.4	0.4	0	0	0	0.4

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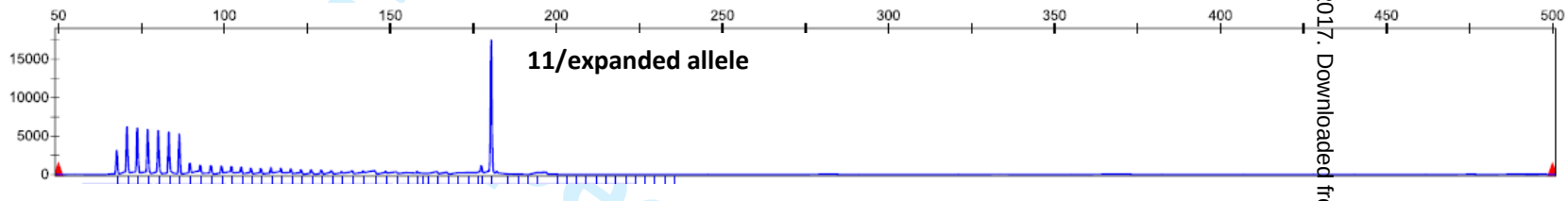

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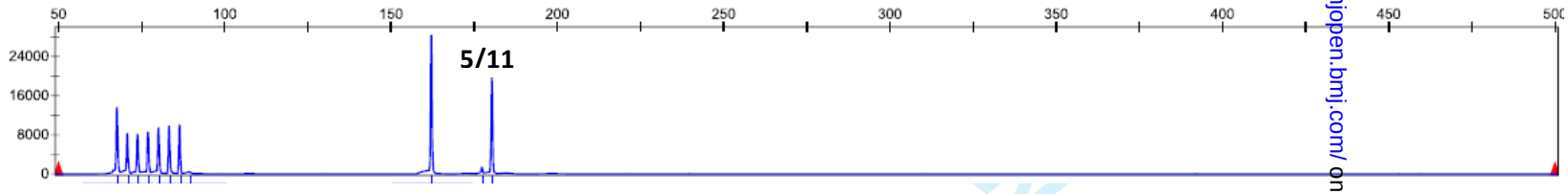

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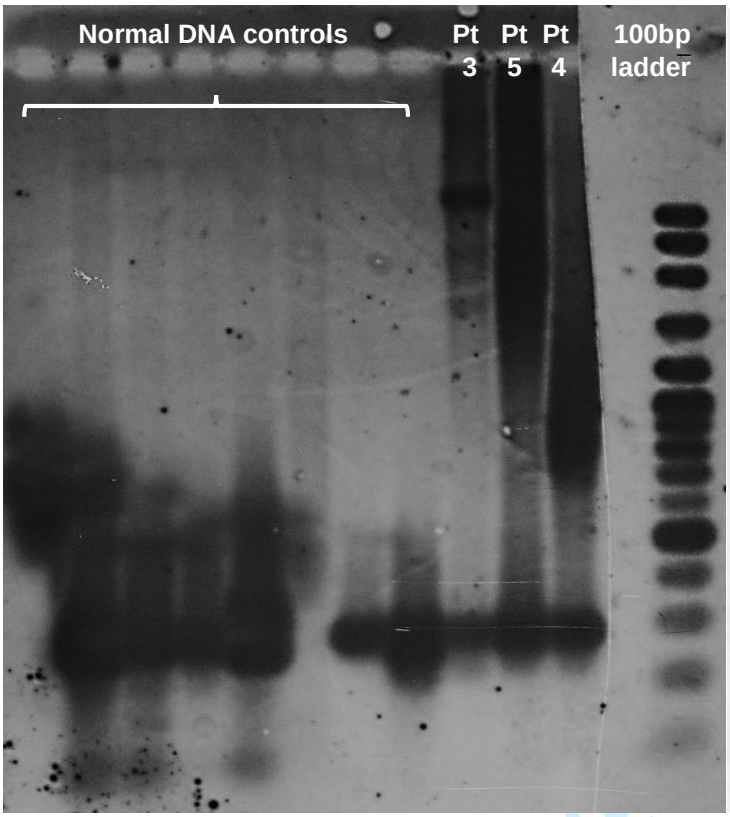

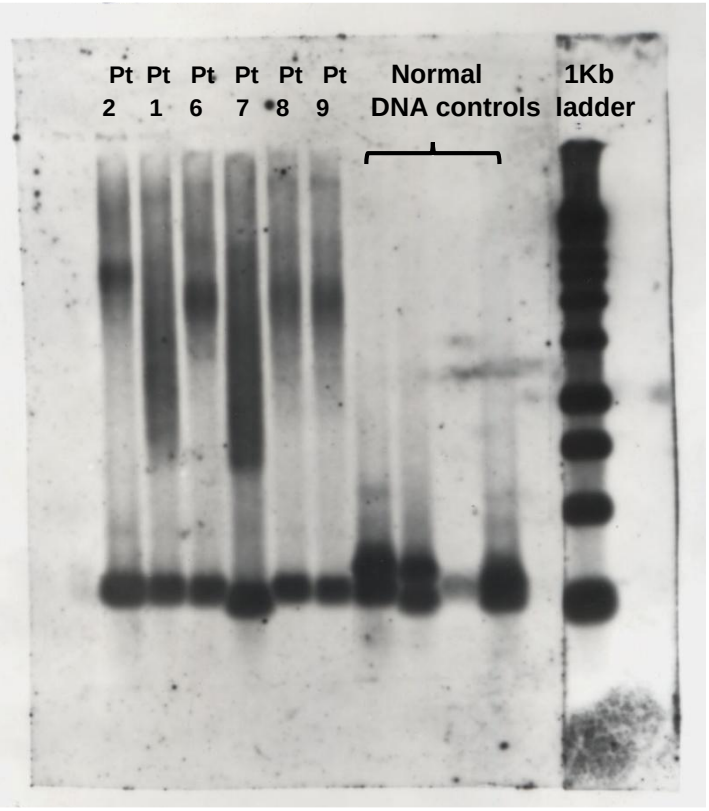

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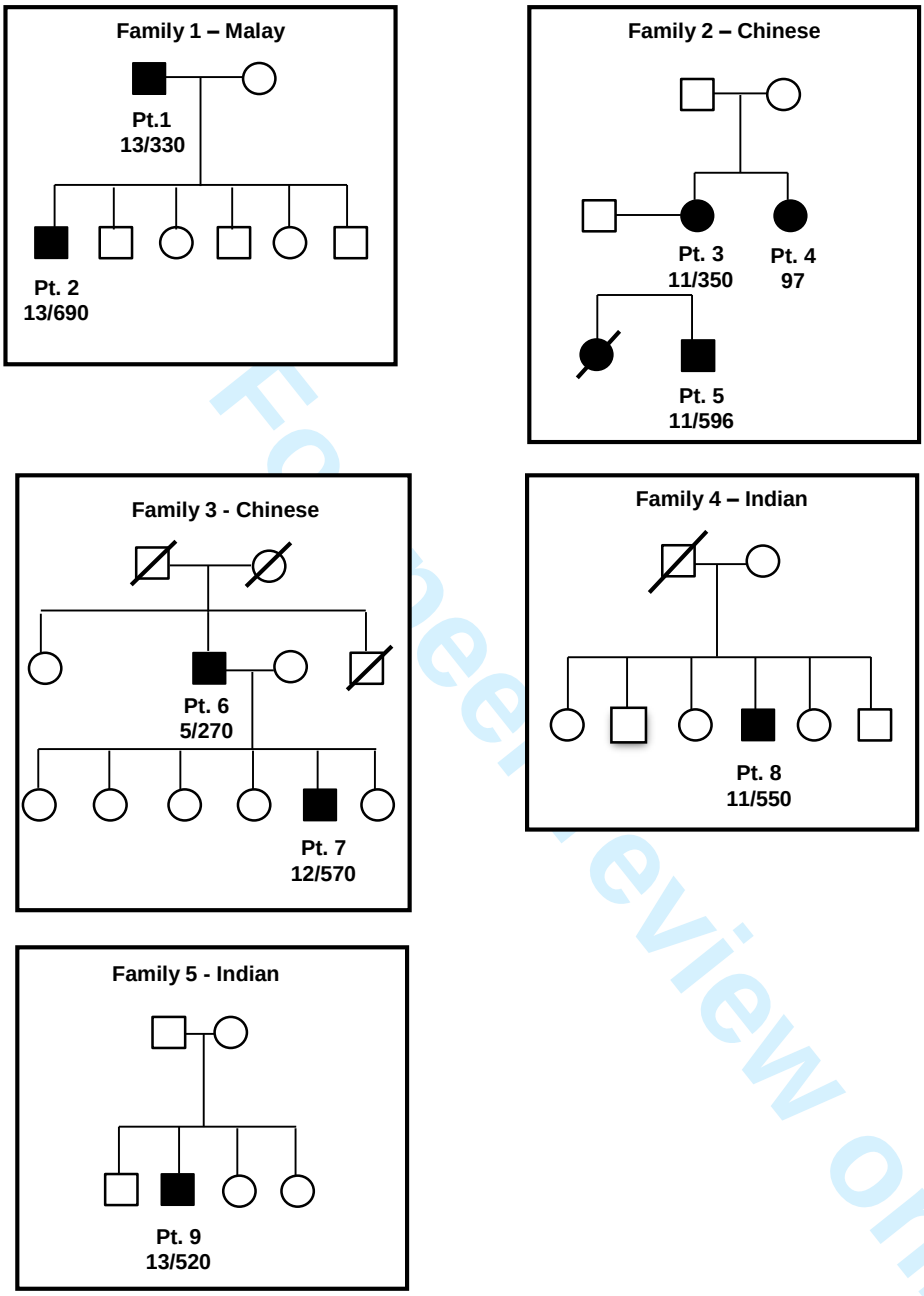

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Reporting Checklist

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	1
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	2–4
Methods			
Study design	3	Present key elements of study design early in the paper	4–7
Setting	4	Describe the setting, locations, and relevant dates, including periods of recruitment, and data collection	4–7
Participants	5	(*a*) *Cohort study*—Give the sources and methods of selection of participants.	5
Statistical methods	6	(*a*) Describe all statistical methods, including those used to control for confounding	7
Results			
Participants	7	(a) Report numbers of individuals at each stage of study	7–8
Descriptive data	8	(a) Give characteristics of study participants (eg demographic, clinical, social	7–8
Outcome data	9	*Cohort study*—Report numbers of outcome events or summary measures over time	7–8
Main results	10	(*a*) Give unadjusted estimates and their precision (eg, 95% confidence interval)	7–8
Discussion			
Key results	11	Summarise key results with reference to study objectives	8 & 9
Interpretation	12	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	8–10
Other information			
Funding	13	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	11

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Analysis of CTG repeat length variation in the DMPK gene in the general population and the molecular diagnosis of myotonic dystrophy type 1 in Malaysia

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Keywords:	CTG repeats, genetic counselling, myotonic dystrophy type 1, molecular diagnosis, TP-PCR, prevalence

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Title

Analysis of CTG repeat length variation in the *DMPK* gene in the general population and the molecular diagnosis of myotonic dystrophy type 1 in Malaysia

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Abstract

Objective The lack of epidemiological data and molecular diagnostic services in Malaysia has hampered the setting-up of a comprehensive management plan for myotonic dystrophy type 1 (DM1) patients, leading to delayed diagnosis, treatment and support for patients and families. The aim of this study was to estimate the prevalence of DM1 in the three major ethnic groups in Malaysia and evaluate the feasibility of a single tube triplet-primed polymerase chain reaction (TP-PCR) method for diagnosis of DM1 in Malaysia.

Design, **setting and participants** We used PCR to determine the size of CTG repeats in 377 individuals not known to be affected by DM and 11 DM1 suspected patients, recruited from a tertiary hospital in Kuala Lumpur. Triplet-primed PCR was performed on selected samples, followed by Southern blothybridisation of PCR amplified fragments to confirm and estimate the size of CTG expansion.

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Outcome measures The number of individuals not known to be affected by DM with $(CTG)_{>18}$ was determined according to ethnic group and as a whole population. Chi-squared test was performed to compare the distribution of $(CTG)_{>18}$ with 12 other populations. Additionally, the accuracy of TP-PCR in detecting CTG expansion in 11 DM1 patients was determined by comparing the results with that from Southern blot testing.

Results Of the 754 chromosomes studied, $(CTG)_{>18}$ frequency of 3.60%, 1.57% and 4.00% in the Malay, Chinese and Indian sub-populations respectively, was detected, showing similarities to data from Thai, Taiwanese and Kuwaiti populations. We also successfully detected CTG expansions in nine patients using the TP-PCR method followed by the estimation of CTG expansion size via Southern blot hybridisation.

Conclusions The results show a low DM1 prevalence in Malaysia with the possibility of underdiagnosis and demonstrates the feasibility of using a clinical and TP-PCR-based approach for rapid and cost effective DM1 diagnosis in developing countries.

Strengths and limitations of this study

- This is the first DM1 epidemiological study on individuals not known to be affected by DM from the three major ethnic groups in Malaysia.
- To date molecular diagnostic testing for DM1 is not performed in any hospital in Malaysia. This study describes the feasibility of a cost and time-effective TP-PCR based method for rapid screening and diagnosis of DM1.
- The number of DM1 samples analysed is small as DM1 is a rare disease in Malaysia.

Key Words

CTG repeats/genetic counselling/myotonic dystrophy type 1/molecular diagnosis/TP-PCR/prevalence

Introduction

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The myotonic dystrophies (DM) are the most prevalent adult muscular dystrophy worldwide, with an estimated prevalence of 1 in 8000.[1] They are classified into two main sub-groups, myotonic dystrophy type 1 (DM1) and type 2 (DM2). These are caused by nucleotide repeat expansions, which are inherited as an autosomal dominant trait, and manifest as clinically heterogeneous diseases. DM1 is due to CTG nucleotide repeats beyond the normal length of five to 49, in the 3' untranslated region (UTR) of the dystrophia myotonica protein kinase (*DMPK*) gene, located on chromosome 19q 13.3.[2, 3] It is a progressive disease and categorised into several subtypes. The congenital form of DM1 is maternally transmitted more frequently, although the disease occurs equally in males and females.[4] The general consensus is that the larger the CTG repeat in an individual, the more severe the disease and the earlier the age of onset. It is however, difficult to classify individual DM1 cases into distinct categories based merely on the size of CTG repeats, as genotype-phenotype correlation often overlap and are not clearly defined. In addition, the repeat sizes have shown variation, both between tissues, and over time in the same tissue.[5, 6] This has made disease prognosis difficult. The genetic phenomenon of anticipation can also be observed in the inheritance of the disease, resulting in a more severe form of the disease coupled with an earlier age of onset in subsequent generations.[7, 8]

The prevalence of DM1 varies greatly across populations—it is pre-dominantly seen amongst the Europeans and Japanese.[9,10] A study also estimated a high disease frequency in the Finnish population.[11] In Quebec, Canada, a particularly high DM1 prevalence of 1 in 500 has been recorded due to founder effects.[12] In contrast, it is a rare disease amongst ethnic sub-Saharan populations,[13] being almost unheard of with the exception of one case reported in Nigeria.[14] Two more recent cases amongst African Americans have also been observed, most likely representing recent population admixture .[15] In view of this disparity, a study was undertaken to determine the distribution of CTG repeats in normal African individuals. It was found that there was a highly significant difference in the distribution of normal CTG alleles larger than 18 between the African population and the European and Japanese populations.[13] This reiterates a previous theory that CTG alleles between 19 and 30 act as a source of DM1 mutations in subsequent generations.[16] These findings have formed the basis for the estimation of DM1 incidence within a population.[17, 13, 18-6-27]

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Prior to the establishment of molecular diagnostic tests, DM1 was diagnosed in clinics mainly by observing clinical symptoms and conducting electromyography (EMG) tests, with confirmation by muscle biopsy.[28] At present, there are several molecular techniques that can be utilised in making a DM1 diagnosis, rendering little use for the invasive and painful EMG test and muscle biopsy. [29] However, a single test that is able to detect all ranges of expansion sizes is yet to be established. Laboratories often employ a combination of methods depending on mutation dynamics in the population and available equipment. Conventional PCR can detect the normal range of CTG repeats as well as premutated alleles. Optimised PCR conditions can detect alleles up to $(CTG)_{85}$, whereas those beyond that rely on Southern blot for detection. The TP-PCR method was developed to detect the presence of large expanded alleles, thus reducing the number of reflex Southern blot tests.[30]

As a Southeast Asian country, Malaysia has a population consisting mainly of ethnic Malay, Chinese and Indian. There is also a large group of indigenous people belonging to various tribes. While DM1 has not been frequently diagnosed in this country, there is a possibility of underdiagnosis or misdiagnosis due to the lack of awareness about this condition with its diverse presentations. No study has been performed on the prevalence and incidence of the disease in the predominant ethnic groups, and to the best of our knowledge, diagnostic tests for this disease at the molecular level is not available anywhere in the country. Given the multisystemic and variable phenotypic manifestations in patients, it is therefore important for a simple standard confirmatory diagnostic test to be available, especially when trying to rule out different diagnoses. Here we report the use of PCR and Southern blothybridisation methods for the molecular analysis of individuals not known to be affected by DM from the Malay, Chinese and Indian sub-populations, where we studied the length of the CTG alleles in order to predict the prevalence of DM1 in these subpopulations. We also describe the use of a single-tube TP-PCR method for the screening and confirmation of DM1 amongst Malaysian patients, with the aim of reducing the number of Southern blot tests that need to be performed.

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Materials and Methods

Ethics statement

Ethical approval to conduct this study was obtained from the University of Malaya Medical Centre (UMMC) ethics committee (Reference numbers 577.17 & 800.6). The ethics board required that all human subjects recruited in the study were briefed on the nature of the study, and provided with an information sheet describing the study. Subjects were also assured that their privacy will be protected, and all personal information provided will be kept confidential. Participation in the study was on a voluntary basis, and had no bearing on the quality of care patients received at the hospital.

Sample collection

Blood samples from 377 randomly selected anonymous blood donors not known to be affected by DM of Malay, Chinese and Indian descent were obtained from the UMMC blood bank following oral consent to participate in the study. In addition, 11 patients displaying DM-like symptoms were recruited to this study. Written consent, clinical and familial history were obtained from these patients. The ethnicity of subjects was determined to be Malay, Chinese or Indian based on their own admission.

Molecular analysis

Genomic DNA was extracted from the blood samples using the QIAamp DNA Blood Mini kit according to manufacturer's protocol (QIAGEN, Hilden, Germany).

Conventional PCR

Analyses of the samples were carried out according to techniques described by Surh et al.[31] PCR was performed in a final volume of 30µL utilising the Perkin Elmer GeneAmp PCR system. The forward, 103, 5' – CCA GTT CAC AAA CCG CTC CGA GCG TG – 3' and reverse, 96, 5' – GGT GCG TGG AGG ATG GAA CAC GGA C – 3' primers were used. The PCR conditions were set as follows: initial denaturation at 96°C for 5 minutes, followed by 25 cycles of denaturation, annealing and extension at 96°C, 62°C and 72°C respectively, for a period of one minute for each step. Final extension was performed at 72°C for

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seven minutes. The PCR products were sized by gel electrophoresis on 1.5% agarose gel, at 100 V for 45 minutes. The separated products were cut out from the gel, purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and sent to a service lab for sequencing to determine the exact number of CTG repeats.

Triplet-primed-PCR

Analysis of the samples were done according to techniques described by Singh et al.[32] Thirteen samples were subjected to TP-PCR analysis—11 individuals with DM1 symptoms and two controls not known to be affected by DM. The subjects recruited were all adults between the ages of 30 and 60, and one child aged 5. Testing was performed with 100 ng of genomic DNA from blood samples in a reaction volume of 25 µl. The primers FAM-P1-Forward 5'FAM – GGG GCT CGA AGG GTC CTT GT – 3' and P2-Reverse 5' – GTG CGT GGA GGA TG AAC ACG – 3' flanked the CTG repeat region, with the forward primer labeled with FAM fluorescence. The third primer P3 5' – AGC GGA TAA CAA TTT CAC ACA GGA – 3' was designed to bind to the complement of the tail of the fourth primer P4-$(CAG)_6$ –Reverse 5' – AGC GGA TAA CAA TTT CAC ACA GGA CAG CAG CAG CAG CAG CAG – 3'. The primer combination was prepared in a ratio of FAM-P1-Forward: P4-$(CAG)_6$ –Reverse:P3:P2 = 1.5:1:1.5:1.5, with a final working concentration of 0.6 µM:0.4 µM:0.6 µM:0.6 µM. The TP-PCR conditions were set as follows: initial denaturation at 95°C for five minutes, followed by 10 cycles each of denaturation (97°C) for 35 seconds, annealing (65°C) for 35 seconds and extension (68°C) for four minutes. Subsequently, 20 cycles of denaturation, annealing and extension were performed, with the extension time increased by 20 seconds per cycle to allow for increased yield of PCR product. The products were separated on an ABI PRISM 3130 x 1 genetic analyser (Life Tech, New York, USA) and fragment size determined using GeneMarker V2.6 (Softgenetics, State College, USA).

Southern blothybridisation of PCR amplified fragments

Southern blot hybridisation of amplified PCR fragments was carried out in samples that only showed single peaks in the electropherograms, which indicated a CTG expansion or homozygosity for a non-expanded allele. The conventional PCR products were transferred overnight from the agarose gel to a

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positively charged nylon membrane by capillary transfer and fixing of the DNA to the membrane done via the UV cross-linking method. The membrane was hybridised overnight in a hybridisation buffer with the addition of 20 µl alkaline phosphatase-conjugated $(CTG)_{10}$ oligonucleotide at 50°C. The membrane was then removed and the excess liquid drained off, prior to being washed using pre-heated wash buffers. Following hybridisation and washing of the membrane, the CDP-Star Detection Reagent is applied and the development of the signals was subsequently carried out by exposing the blot to an autoradiography film. Identification of DM1 positive samples were done by comparing the size of the bands or smears obtained with DNA molecular weight markers.

Statistical analysis

The frequency of each of the allele present in the 754 chromosomes from the individuals not known to be affected by DM was calculated. Statistical analysis was performed by administering the chi-squared (χ^2) test with Yates' correction to compare the distribution of normal large repeats, $(CTG)_{> 18}$, with 12 other populations.

Results

Analysis of DMPK CTG repeat length variation in the general population

The distribution of $(CTG)_{>18}$ alleles in the Malay, Chinese and Indian sub-populations all point towards a low prevalence of DM1. Figure 1 shows the breakdown of all alleles present in the three sub-populations. A bimodal allelic distribution was noted—this was in alignment with patterns observed in other populations with low DM1 frequencies. The first peak came from the $(CTG)_5$ alleles, which totaled to 33.7% of all alleles, while the second peak consisted of three alleles, 11 to 13 that accounted for a majority of 51.1% of the total alleles. The frequencies for $(CTG)_{>18}$ alleles were 9/250 = 3.60% (95% CI = 0.0166–0.0672) in the Malay subpopulation, 4/254 = 1.57% (95% CI = 0.0043–0.0398) in the Chinese subpopulation, and 10/250 = 4.00% (95% CI = 0.0193–0.0723) amongst the Indians. Heterozygosity was measured at 79.9%, 77.0%, and 76.2% in the three subpopulations, respectively, averaging at 77.7%.

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This result is aligned to those reported in other populations, which ranged from 73.0% in Europeans[17] to 92% in Iranians.[26]

Tables 1 and 2 show the comparison and χ^2 analysis of the frequency of $(CTG)_{>18}$ alleles in individuals not known to be affected by DM1 from the three subpopulations in this study, and in those from 12 worldwide populations, respectively. The $(CTG)_{>18}$ frequency for the Malay, Chinese and Indian subpopulations were significantly different when compared to frequencies in European, German and Chilean populations. All three Malaysian subpopulations showed frequencies similar to Thai,[22] Taiwanese[23] and Kuwaiti[24] populations. It is also interesting to note that the Han-Chinese show similarity with the Malaysian Chinese, the population that the majority of Malaysian Chinese trace their ancestry to. This allows for our speculation that the DM1 frequency among Chinese Malaysians is low, similar to that observed in the Han-Chinese,[24] Taiwanese[23] and South African negroids.[13]

Diagnostic testing for DM1

Samples from 11 individuals with DM1-like symptoms and two controls not known to be affected by DM were analysed for CTG expansion using TP-PCR followed by confirmation by Southern blot. Triplet-primed PCR testing showed single peaks in nine of the samples, and double peaks in the remaining four. The samples with single peaks also showed a clear laddering pattern indicating the presence of CTG expansion (Figure 2). Southern blot testing confirmed the diagnosis of DM1 in the nine samples, with the detection of expanded alleles ranging from a size of 97 to 690 CTG repeats, as shown in Figure 3. Table 3 shows a summary of the characteristics of the disease exhibited by each patient. Figure 4 shows the pedigree diagram and the CTG repeat size of the families and individuals we studied. It is important to note that apart from those diagnosed (dark squares/circles), none of the other family members were examined or tested for DM1. Hence, there is a possibility that there may be family members showing very mild symptoms who have not presented in our clinics, contributing to the apparent under transmission of the disease in the families.

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Discussion

In order to obtain a better understanding of the burden of DM1, we estimated the prevalence of the DM1 using the distribution of CTG alleles larger than 18 in the Malaysian population. The result of $(CTG)_{>18}$ of 3.05% (23/754) was observed in the Malaysian population. By comparing with the results of studies performed in other populations, we predict that DM1 is a rare disease in Malaysia. A larger study is needed to verify these findings, due to the fact that the subjects in this study were recruited from a major hospital in the capital city of Malaysia, therefore may not be representative of the whole country. It is likely that DM1 in the local community is underdiagnosed due to a lack of awareness amongst the public and healthcare professionals. There are also other contributing factors such as social stigma, and reduced access to major hospitals where specialised consultation and testing are available.

It is interesting to note that the frequency of $(CTG)_{>18}$ was the lowest in the Chinese subpopulation, although they account for the most number of DM1 patients seen in our hospital (including those not reported here). The Indians on the other hand show the highest frequency of $(CTG)_{>18}$ in agreement with the findings that DM1 is highly prevalent in India[33]. However, the number of Indian DM1 patients seen in our study was the lowest among the three subpopulations. This may reflect socio-economic and demographic reasons, as well as misdiagnosis/underdiagnosis of DM1 in the respective subpopulations.

Our study also provides for the first time, data on the $(CTG)_{>18}$ allele frequency in a Malay population. The Malay ethnic group is genetically more similar to the Chinese compared to the Indians.[34] Comparison of the $(CTG)_{>18}$ distribution of the three ethnic groups however, shows a closer similarity between the Malays and the Indians ($p=0.8151$) compared to the Chinese ($p=0.249$). It would be interesting to see this same analysis done on other modern Malay populations in the region, such as the Singapore Malays and the Indonesians, as well as the aboriginal Malays.

The usage of the single tube TP-PCR allows for the rapid identification of large pathogenic CTG repeats, thus reducing the need for reflex Southern blot testing. Southern blot requires large amounts of DNA, the use of radioactive materials and is time consuming. In addition, this procedure is also less sensitive and may be difficult to replicate. Hence, any method that reduces the number of Southern blot that needs to be performed, while demonstrating high sensitivity and specificity is advantageous in a clinical setting.

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However, the TP-PCR test used requires a highly specialized equipment, the genetic analyser, which may not yet be widely available and is unable to estimate the size of CTG expansions beyond 85 repeats.

Genotype-phenotype correlation studies in DM1 patients have thus far given conflicting results, with various underlying mechanisms, associations and theories proposed[35-1-38]. In our study, a disparity in the genotype-phenotype correlation in the Chinese family was seen, whereby patient 3 is largely asymptomatic although she carries 350 repeats. Her disease status was only suspected and diagnosed following the birth of her children who exhibited symptoms. Both her children were congenitally affected, which is consistent with findings in previous studies that showed that the majority of congenital cases were maternally transmitted. Patients 2 and 7 on the other hand paternally inherited their pathogenic alleles, resulting in the classic/adult onset DM1. The same disease phenotype is seen in patients 8 and 9. We were not able to determine whether their diseases were inherited, as their parents have never been tested. However, these patients were given genetic counselling and in accordance with ethical principles, have the autonomy of deciding whether or not to disclose their disease status to family members at risk, for future counselling and testing. It was also observed that congenitally affected patient 5 showed a comparable expansion size to those who were classically affected. The only symptoms he has shown, however is neonatal hypotonia and a mild cognitive dysfunction. The comparable repeat size is most likely due to the younger age of patient 5 compared to the classically affected adults, and suggest that a larger repeat size would be observed, as the patient grows older. Apart from these disease dynamics, there have also been findings of contraction of allele sizes upon transmission reported elsewhere[27]. All these factors point towards the high complexity of DM1 and illustrate the important need for genetic counselling services to be offered to affected families.

Molecular testing is generally established as the gold standard in diagnosing genetic disorders such as DM1. This is because a molecular test is rapidly able to eliminate differential diagnoses, confirm the DM1 diagnosis, and estimate the size of CTG expansion in a patient, thus avoiding the need for invasive procedures such as muscle biopsies. Hilbert *et al*[39] who studied a large cohort of DM patients enrolled in the US National Registry, explored their diagnostic journeys, which on average took seven years for a correct DM1 diagnosis to be made. This delay brought about many implications to the patients and their

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families, ranging from lack of appropriate disease management to missed opportunities for genetic counselling. The situation in many developing countries is much similar or even worse as molecular diagnostic testing for DM1 is not easily available. Potentially, there could be a large number of patients who are undiagnosed/misdiagnosed, as well as those who have been unnecessarily subjected to various investigations for a definitive diagnosis to be made.

The findings from our preliminary study can aid the structuring of a rare disease management framework in Malaysia, using DM1 as a disease model. The data presented here adds to the scarce literature of DM1 in the Southeast Asian region. The information on CTG repeat lengths of the *DMPK* gene in individuals not known to be affected by DM, and DM1 patients, together with proper clinical assessment as well as a cost-effective molecular approach, carry implications for earlier diagnosis of DM1 and genetic counselling in a low resource setting.

Contributorship statement

All authors were involved in the conception and design of the work as well as the final approval of the submitted manuscript.

KA, MKT and IT were involved in the acquisition and analysis of data and drafting the manuscript and were joint senior authors.

LLH, GKJ, KTW and AAA contributed to the critical evaluation of the manuscript.

Competing interests

The authors state no competing interests

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Data sharing statement

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None available

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Figure Legends

Figure 1. Frequency of CTG repeats in individuals not known to be affected by DM from the Malay, Chinese and Indian sub-populations. *The frequency for large normal alleles, $(CTG)_{>18}$ was 9/250 or 3.60% in the Malays, 4/254 or 1.57% in the Chinese, and 10/250 or 4.00% in the Indians. A bimodal allelic distribution was observed in the Malaysian population, in alignment with patterns observed in other populations with low DM1 frequency. The most frequently seen allele was $(CTG)_5$ in all three sub-populations, whereas $(CTG)_{10-13}$ was the most common allele group. The genotyping data for each individual is provided in the supplementary files 1–3.*

Figure 2. Electropherogram results of TP-PCR. *The X-axis represents the CTG repeat size and the Y-axis represents the allele peak height. (A) The electropherogram shows a DM1 patient sample with a*

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single peak corresponding to $(CTG)_{11}$ and a laddering pattern indicating an expanded allele. (B) Two normal heterozygous alleles with sizes 5 and 11 and no laddering pattern observed.

Figure 3. **Expanded CTG repeats of DM1 patients following PCR-Southern blotting as seen on an autoradiography film.** *Expanded alleles in patients ranging from a size of 97 to 690 CTG repeats have been detected. A sample of the bands are shown here, ranging from 270 repeats (1045 bp) to 690 repeats (2305 bp). Normal alleles of four sizes were seen amongst the patients, 5 (332 bp), 11 (350 bp), 12(356 bp) and 13 (356 bp). Due to somatic heterogeneity, the expanded alleles usually appear as smears. A 1 Kb DNA ladder as well as samples from individuals not known to be affected by DM were run alongside patient samples as controls.*

Figure 4. Pedigree diagrams of DM1 patients studied including the size of their CTG alleles. *Members of three families and two individuals had their CTG repeat size analyzed. The sizes of the allele pairs for each patient are as stated in the pedigree diagrams. The phenomenon of anticipation was clearly observed in the three families, whereby with the increased CTG expansion in successive generations, a decreasing age of onset is noted.*

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Tables

Table 1: Comparison and χ^2 analysis of the frequency of $(CTG)_{>18}$ alleles in individuals not known to be affected by DM from the Malay, Chinese and Indian sub-populations.

Population	$(CTG)_{>18}$ alleles / Total alleles analyzed (%)	Comparison of Malay data with other populations χ^2 (p value)	Comparison of Chinese data with other populations χ^2 (p value)	Comparison of Indian data with other populations χ^2 (p value)
Malay	9/250 (3.60)	-	1.329 (0.249)	0.055 (0.8151)
Chinese	4/254 (1.57)	1.329 (0.249)	-	1.919 (0.166)
Indian	10/250 (4.00)	0.055 (0.8151)	1.919 (0.166)	-

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Table 2: Comparison and χ^2 analysis of the frequency of $(CTG)_{>18}$ alleles in individuals not known to be affected by DM from the three Malaysian sub-populations to those in twelve worldwide populations.

Population	$(CTG)_{>18}$ alleles / Total alleles analyzed (%)	Comparison of Malay data with other populations χ^2 (p value)	Comparison of Chinese data with other populations χ^2 (p value)	Comparison of Indian data with other populations χ^2 (p value)
[a] European[17]	15/130 (11.54)	7.817 (0.005**)	16.094 (<0.0001***)	6.729 (0.009**)
German[18]	22/104 (21.20)	26.17 (<0.0001***)	39.141 (<0.0001***)	24.239 (<0.0001***)
Mexican[19]	51/800 (6.38)	2.232 (0.135)	8.037 (0.005**)	1.553 (0.213)
Brazilian[20]	24/312 (7.69)	3.497 (0.062)	9.88 (0.002**)	2.334 (0.127)
Chilean[21]	30/272 (11.00)	9.354 (0.002**)	17.887 (<0.0001***)	8.131 (0.004**)
Japanese[17]	9/106 (8.50)	2.760 (0.097)	8.386 (0.004**)	2.149 (0.143)
Thai[22]	11/400 (2.75)	0.142 (0.706)	0.505 (0.477)	0.421 (0.516)
Taiwanese[23]	7/499 (1.40)	2.867 (0.090)	0.018 (0.893)	3.962 (0.050)
Han Chinese[24]	6/600 (1.00)	5.463 (0.019*)	0.134 (0.714)	7.052 (0.008**)
Kuwaiti[25]	14/370 (3.78)	0.010 (0.920)	1.894 (0.169)	0.006 (0.938)
Iranian[26]	29/400 (7.25)	3.090 (0.079)	9.292 (0.002**)	2.334 (0.127)
South African[13]	3/420 (0.71)	5.869 (0.015*)	0.457(0.499)	7.249 (0.007*)

*P < .05 (significant); ** P < .01 (highly significant); *** P < .001(very highly significant)

[a] Includes British, German, Belgian, Swedish and Finnish subjects

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Table 3: A summary of the disease characteristics exhibited by DM1 patients in this study

Family	Patient	Gender	[a] Age	[b] Disease Onset	[c] Phenotype	CTG Repeat Size
1	1	Male	54	Late adult	Classical	330
	2	Male	30	Early adult	Classical	690
2	3	Female	30	Early adult	Mild	350
	4	Female	31	Early adult	Mild	97
	5	Male	5	Birth	Congenital	596
3	6	Male	60	Late adult	Classical	270
	7	Male	30	Early adult	Classical	570
4	8	Male	44	Early adult	Classical	550
5	9	Male	32	Early adult	Classical	520

[a] Age of patient at time of molecular testing

[b] Early adulthood: 20 – 49 years old; Late adulthood: >50 years old

[c] Phenotype classification as described by Kamsteeg *et al*

Supplementary file 4 is a record of the responses to the comments by reviewers and revisions done to the manuscript.

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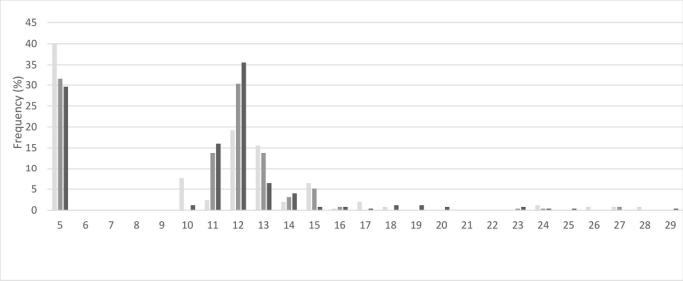

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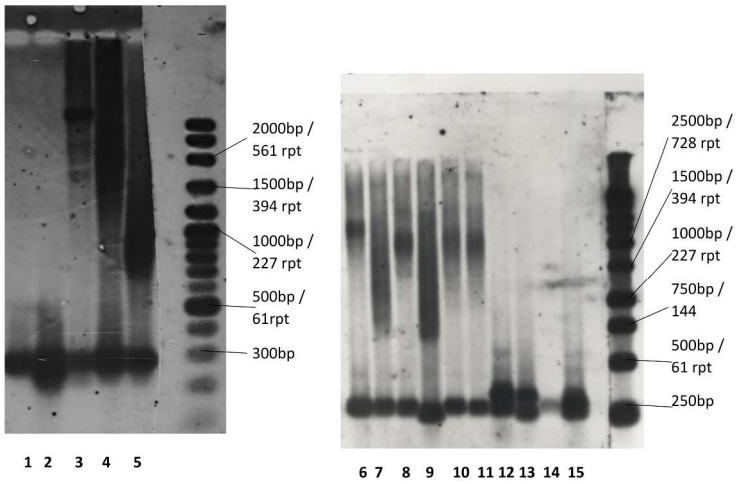

Figure 2. Electropherogram results of TP-PCR. The X-axis represents the CTG repeat size and the Y-axis represents the allele peak height. (A) The electropherogram shows a DM1 patient sample with a single peak corresponding to (CTG)11 and a laddering pattern indicating an expanded allele. (B) Two normal heterozygous alleles with sizes 5 and 11 and no laddering pattern observed.

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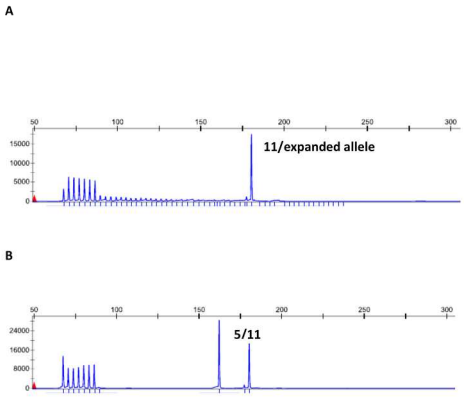

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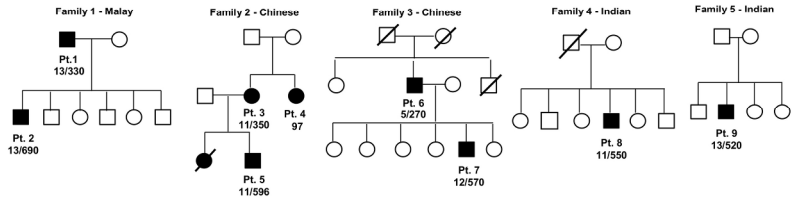

Figure 4. Pedigree diagrams of DM1 patients studied including the size of their CTG alleles. Members of three families and two individuals had their CTG repeat size analyzed. The sizes of the allele pairs for each patient are as stated in the pedigree diagrams. The phenomenon of anticipation was clearly observed in the three families, whereby with the increased CTG expansion in successive generations, a decreasing age of onset is noted.

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Complete list of allele distribution in healthy individuals from the Malay subpopulation

No.	Code	Allele 1	Allele 2
1	N1	10	5
2	N2	10	5
3	N3	11	5
4	N5	12	12
5	N6	12	12
6	N7	5	5
7	N10	13	11
8	N25	10	10
9	N26	5	5
10	N31	5	5
11	N32	10	5
12	N33	5	5
13	N35	13	13
14	N36	13	5
15	N37	11	5
16	N38	12	12
17	N40	5	5
18	N45	13	13
19	N46	13	13
20	N47	12	5
21	N51	13	10
22	N52	10	5
23	N59	15	5
24	N60	17	5
25	N63	5	5
26	N68	5	5
27	N73	12	5
28	N78	12	5
29	N80	5	5
30	N82	13	5
31	N84	14	5
32	N87	13	13
33	N89	11	11
34	N90	15	5
35	N92	12	5
36	N95	5	5
37	N97	28	11
38	N99	13	5
39	N101	24	5
40	N105	13	13
41	N108	12	12
42	N110	13	13
43	N116	14	5
44	N118	18	5

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45	N120	13	13
46	N125	5	5
47	N130	12	12
48	N132	12	12
49	N133	10	5
50	N134	12	5
51	N137	5	5
52	N139	15	5
53	N144	10	5
54	N147	12	5
55	N148	13	5
56	N157	13	5
57	N159	5	5
58	N160	13	13
59	N165	12	5
60	N171	18	10
61	N173	12	5
62	N177	13	10
63	N178	12	5
64	N179	5	5
65	N185	13	5
66	N188	14	5
67	N189	15	5
68	N191	17	5
69	N193	17	5
70	N195	17	12
71	N196	16	5
72	N198	14	5
73	N199	15	13
74	N206	17	5
75	N207	17	5
76	N208	17	13
77	N209	16	5
78	N211	28	12
79	N212	27	12
80	N213	17	12
81	N214	5	10
82	N215	13	5
83	N217	15	5
84	N218	26	10
85	N219	13	5
86	N220	13	5
87	N223	15	12
88	N228	5	15
89	N229	24	24
90	N230	5	5
91	N232	12	5

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92	N233	12	5
93	N234	12	5
94	N235	12	5
95	N236	13	5
96	N239	12	12
97	N240	26	12
98	N242	15	12
99	N242	15	10
100	N243	5	5
101	N244	5	5
102	N245	13	13
103	N247	13	13
104	M1	12	5
105	M2	5	5
106	M3	5	5
107	M4	12	12
108	M5	12	5
109	B12	12	5
110	B14	5	5
111	B18	14	14
112	B20	5	5
113	B21	15	15
114	B34	15	15
115	B35	13	13
116	B36	13	5
117	B37	5	5
118	B38	5	5
119	B40	10	10
120	B48	15	5
121	B49	12	12
122	B50	12	12
123	B53	13	13
124	B54	12	5
125	B56	27	10

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Complete list of allele distribution in healthy individuals from the Chinese subpopulation

No.	Code	Allele 1	Allele 2
1	N024W	11	5
2	N029W	11	11
3	N039W	13	5
4	N041W	13	13
5	N042W	11	5
6	N043W	12	12
7	N044W	12	12
8	N048W	12	5
9	N049W	12	5
10	N050W	11	5
11	N053W	13	5
12	N054W	12	12
13	N055W	12	5
14	N056W	13	5
15	N057W	14	5
16	N058W	12	5
17	N061W	13	5
18	N062W	12	5
19	N064W	12	12
20	N065W	13	5
21	N066W	24	5
22	N071W	11	5
23	N072W	12	5
24	N074W	13	13
25	N075W	12	5
26	N076W	14	14
27	N077W	13	5
28	N079W	12	5
29	N081W	12	12
30	N083W	12	12
31	N085W	14	5
32	N086W	27	5
33	N088W	5	5
34	N093W	11	11
35	N094W	11	5
36	N096W	12	12
37	N098W	15	5
38	N100W	13	5
39	N103W	12	12
40	N104W	12	5
41	N106W	12	5
42	N107W	12	12
43	N111W	14	5
44	N112W	15	15

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45	N113W	5	5
46	N114W	13	5
47	N115W	12	12
48	N117W	13	13
49	N119W	13	5
50	N122W	5	5
51	N124W	12	12
52	N127W	12	12
53	N128W	13	5
54	N138W	5	5
55	N142W	11	11
56	N143W	5	5
57	N146W	13	13
58	N149W	12	12
59	N150W	13	5
60	N151W	13	5
61	N152W	11	11
62	N153W	11	11
63	N154W	27	16
64	N155W	13	5
65	N156W	11	5
66	N158W	13	5
67	N161W	15	15
68	N162W	12	5
69	N163W	12	5
70	N164W	15	15
71	N166W	12	5
72	N167W	12	12
73	N168W	5	5
74	N169W	15	5
75	N170W	11	11
76	N172W	11	5
77	N174W	12	5
78	N175W	13	5
79	N176W	15	15
80	N180W	11	5
81	N181W	12	5
82	N182W	11	11
83	N183W	13	13
84	N184W	5	5
85	N186W	23	14
86	N187W	12	12
87	N190W	12	12
88	N194W	12	12
89	N197W	11	11
90	N200W	12	5
91	N221W	12	12

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92	N222W	11	11
93	N224W	13	13
94	N227W	15	5
95	N237W	13	5
96	C1	11	5
97	C2	5	12
98	C3	11	11
99	C4	13	13
100	C5	12	12
101	C6	11	5
102	C7	12	12
103	C8	12	5
104	C9	11	5
105	C10	12	5
106	C11	12	5
107	C12	13	13
108	C13	15	15
109	C15	13	13
110	C16	11	5
111	C19	12	5
112	C20	12	12
113	C22	12	5
114	C24	12	5
115	C25	12	12
116	C27	13	13
117	C29	11	11
118	C30	12	5
119	C31	12	5
120	C32	14	14
121	C33	12	12
122	C34	12	12
123	C35	12	12
124	C36	5	5
125	C37	12	5
126	C38	11	5
127	C39	16	5

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Complete list of allele distribution in healthy individuals from the Indian subpopulation

No.	Code	Allele 1	Allele 2
1	I001	14	14
2	I005	5	5
3	I007	15	5
4	I008	11	11
5	I009	12	5
6	I010	5	5
7	I011	23	11
8	I012	12	12
9	I014	11	5
10	I015	5	5
11	I016	13	13
12	I017	14	5
13	I018	11	11
14	I019	12	12
15	I020	24	12
16	I021	12	12
17	I022	12	12
18	I023	12	12
19	I025	12	5
20	I026	12	12
21	I029	20	12
22	I030	10	12
23	I031	12	12
24	I032	12	12
25	I033	14	14
26	I034	11	11
27	I035	15	11
28	I036	13	5
29	I037	19	11
30	I038	11	5
31	I039	12	12
32	I040	17	5
33	I041	11	5
34	I042	14	12
35	I043	12	5
36	I044	13	5
37	I045	5	5
38	I046	12	12
39	I047	12	5
40	I048	11	11
41	I049	12	12
42	I050	12	12
43	I051	12	12
44	I052	5	5

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45	I053	14	5
46	I055W	11	5
47	I056W	12	5
48	I057W	12	5
49	I059W	12	5
50	I060W	11	5
51	I061W	5	5
52	I062W	12	12
53	I064W	12	12
54	I066W	16	5
55	I067W	12	5
56	I068W	10	10
57	I069W	11	11
58	I070W	18	5
59	I071W	11	5
60	I073W	11	11
61	I074W	20	11
62	I075W	11	11
63	I077W	13	13
64	I078W	12	12
65	I079W	5	5
66	I085W	12	12
67	I086W	12	5
68	I087W	11	5
69	I088W	5	5
70	I089W	5	5
71	I090W	12	12
72	I091W	11	11
73	I093W	12	12
74	I094W	23	11
75	I095W	18	5
76	I096W	12	12
77	I097W	12	5
78	I098W	29	12
79	I099W	12	12
80	I102W	12	12
81	I103W	11	5
82	I104W	25	5
83	I105W	11	5
84	I106W	11	11
85	I107W	11	5
86	I108W	12	5
87	I109W	13	13
88	I110W	12	12
89	I111W	12	5
90	I112W	12	5
91	I113	12	5

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92	I114	5	5
93	I116	11	5
94	I117	14	11
95	I118	11	5
96	I119	5	5
97	I120	11	5
98	I121	13	13
99	I123	12	12
100	I124	19	12
101	I125	12	12
102	I126	18	11
103	I127	13	5
104	I129	12	12
105	I130	12	5
106	I131	19	11
107	I133	12	12
108	I137	13	13
109	I138	5	5
110	I140	12	12
111	I144	12	5
112	I145	12	5
113	I146	5	5
114	I150	12	12
115	I151	14	14
116	I153	12	5
117	I156	12	12
118	I158	12	5
119	I159	13	13
120	I160	12	5
121	I162	12	5
122	I163	16	5
123	I164	11	5
124	I167	13	5
125	I168	12	12

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No.	Reviewers' Comments	Response
1.	Please remove the research checklist from the submission- we do not feel that the checklist is very appropriate for this kind of study	Checklist removed
Reviewer: 1		
1.	Although correlation between prevalence of CTG repeat >18 in DMPK gene and prevalence of DM1 is relatively well established, to conclude that prevalence of DM1 is low in Malaysia may be a bit over-claimed. The conclusions should be toned down.	Our conclusion states that the results show a low DM1 prevalence in Malaysia with the possibility of underdiagnosis. We also state that DM1 as a rare disease in Malaysia is only a prediction and acknowledge that a larger study is needed to verify these findings. Based on these justifications, we would like to keep our current conclusions.
2.	Since there were only 9 DM1 cases; sensitivity of TP-PCR might not be well tested.	We acknowledge that due to the small sample size the sensitivity of TP-PCR cannot be ascertained in our study. However, TP-PCR is an established method and is recommended as a molecular diagnostic test for DM1, hence the aim of our study was to determine its feasibility in the local healthcare setting rather than determining sensitivity.
3.	Did the authors allow healthy controls to be from the same family? If not, this point should be clarified in the methods.	The healthy controls were anonymous blood donors who were randomly selected. This point is now clarified in the methods section.
Reviewer: 2		
1.	Santoro et al ever reported that the presence of CCG, CTC or GGC interruptions could potentially lead to the drop-out of the abnormal allele from TP-PCR. In order to rule out the false negative results due to these interruptions, TP-PCR is recommended to be repeated with a Hex labeled Reverse primer. Protocol can be founded from Santoro's work (2013) or Singh's work (2015).	The omission of this step is one of the limitations of this study. It is taken note of and will be incorporated in other future work.
2.	11 individuals were included as positive DM-1. Two of eleven were tested using PCR amplification without Southern Blot. The genotyping data should be provided to indicate the largest repeat length can be amplified by PCR.	All 11 with DM1-like symptoms were tested by Southern blot hybridisation. Two out of the 11 samples showed negative results for DM1.

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3.	The reference of the TP-PCR methodology part should be provided.	The methodology is now referenced to Singh et al, 2014
4.	Reference 29 was published in 2001 not 1998.	The year of publication is now corrected
Reviewer: 3		
1.	Title. “Molecular detection of CTG repeat lengths in the DMPK gene in healthy individuals and diagnosis of myotonic dystrophy type 1 using triplet-primed PCR in Malaysia” is OK, but I don't consider the use of triplet-primed to be that significant and suggest instead: “Analysis of CTG repeat length variation in the DMPK gene in the general population and the molecular diagnosis of myotonic dystrophy type 1 in Malaysia”.	Title changed as suggested
2.	Prevalence. At several points the authors talk about the “prevalence” of DM1 in Malaysia. However, they provide little data that directly address this question. For instance, having now identified 11 patients with DM1 it should be possible to provide a minimum point estimate of the countrywide prevalence of DM1 and to provide a better estimate based on the approximate catchment size of the hospital at which these patients were identified. Notably, the first section of the results is entitled “Estimation of DM1 prevalence” yet again this section contains no direct estimates of the actual prevalence of DM1 in Malaysia. This section would be far more accurately described as “Analysis of DMPK CTG repeat length variation in the general population”. The authors go on to compare the proportion of alleles >18 CTG repeats that is observed in other populations. Such alleles are deemed to be the pool of alleles from which new disease causing expansions arise and the frequency of such alleles likely is correlated to the actual prevalence of DM1. In making these comparisons it would be useful for the reader to be provided with some indication of the actual prevalence that might be predicted from these population level comparisons and compare them with those directly observed. Inevitably, any such estimate from both of these approaches will be rather approximate, but in the absence of any other data it would be the useful for the authors to at least provide some insight into credible prevalence beyond the even more vague “low” that is currently reported.	The nine patients identified in this study were diagnosed in the years 2011 and 2012. During these two years, the number of patients who were seen at UMMC were 957,418 and 964,497 respectively, totaling to 1,921,915. Using these figures as guide, we estimate the prevalence of DM1 in Malaysia to be less than 1 in 200,000. This estimate is similar to estimates of DM1 prevalence in Thai and Taiwanese populations. Additionally, the prevalence of 1 in 200,000 is low when compared to the Finnish prevalence of 1 in 2,760 and that in the Quebec founder population at 1 in 500. The first section of the results is retitled as suggested.
3.	Repeat length estimates of non-disease associated alleles in the general population. The authors use conventional PCR to amplify the CTG repeat and resolve alleles by electrophoresis on 1.5% agarose gels. I am rather surprised that the resolution of such a system is sufficient to size alleles to one repeat accuracy, including the detection of	The separated products were cut out from the gel, purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and sent to a service lab for sequencing to determine

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	heterozygotes with closely spaced alleles. It would be useful for the authors to provide some indication as to whether they believe, and if so what evidence they have to support such a contention, that alleles were indeed sized with such an accuracy. The authors should also provide the genotyping data for each individual as a supplementary Excel file and confirm the genotypes match Hardy-Weinberg expectations.	the exact number of CTG repeats. The genotyping data for each individual is provided as a supplementary Excel file. The distribution of the allele frequency follows the bimodal distribution with the first peak contributed by the $(CTG)_5$ alleles, and the second comprised of $(CTG)_{11-13}$ alleles The bimodal allelic distribution is in alignment with patterns observed in other populations with low DM1 frequency.
4.	Southern blotting. In order to detect and size large expansions, the authors use Southern blot hybridisation of PCR amplified DM1 alleles. This they describe rather loosely as Southern blotting. Indeed their method does employ Southern blotting as part of the process. However, it must be made clear throughout that they have used Southern blot hybridisation of PCR amplified fragments. This must be done to ensure that this approach is clearly distinguished from the more traditional, but still gold standard method of DM1 diagnosis via the Southern blot hybridisation of restriction digested genomic DNA.	Southern blotting is now rephrased to Southern blot hybrisation of PCR amplified fragments
5.	The two cases of DM1 observed in African Americans most likely represents recent admixture as opposed to independent cases of DM1 in sub-Saharan Africa as is implied in the introduction.	The sentence is rephrased to reflect population admixture as the probable cause of DM1 in the two cases of African Americans
6.	The authors should note the work of Morales et al., that refines the genotype-phenotype correlation in DM1. In this regard the authors should also make it clear how they have determined the number of CTG repeats reported in expanded alleles. Assuming this is derived from the middle of the smear, they should make it clear that the value reported is an estimate of the average repeat number and is age at sampling dependent.	The work of Morales et al is described by the following additions to the discussion: In their study on the somatic instability of expanded CTG repeats in DM1, Morales *et al* showed that there was no evidence to indicate that pathogenesis of the disease is constrained to threshold above which repeat length does not contribute toward age at onset. Additionally, they showed that age at onset is further modified by the level of somatic instability, which is a highly heritable trait.

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		The estimation of the CTG repeat size in the expanded alleles was done as follows: The lengths of the 100 bp/1Kb size marker fragments were first converted into logarithmic values. These values were then graphed on the y axis against their migration distance on the x axis. Using linear regression, a line of best fit was drawn through these points and an equation describing that line was derived. The unknown fragment's migration distance derived from the most intense region of the expanded allele was placed into the equation for the regression line to determine a log value for the fragment's size. Taking the antilog of this value will yield the unknown fragment's size in bp. This value is an estimate of the average repeat number and is dependent on age at sampling.
7.	TP-PCR was not "recently" described. The First application of TP-PCR to DM1 diagnosis was reported by Warner et al., back in 1996.	The word 'recently' is now removed
8.	"While DM1 is not commonly seen in this country…" would be better as "While DM1 has not been [previously or frequently?] diagnosed in this country…"	Sentence changed as suggested
9.	"Reflex Southern blots"?	Reflex Southern blot tests refer to tests that are performed as a result of the PCR amplification falling outside the normal range
10.	"In addition, 11 patients displaying DM1 symptoms were recruited" would be better as "In addition, 11 patients displaying DM-like symptoms were recruited". Related to this, what was the phenotype of the two patients who were DM1 negative? Might they be DM2?	Sentence changed as suggested. Both patients displayed symptoms of myotonia, distal and proximal weakness. It is entirely possible that they may have DM2.
11.	What was the actual concentration of primers used for TP-PCR?	The final working concentration of the TP-PCR primers are now included.

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12.	"Southern Blotting was carried out in samples that only showed single peaks in the electropherograms, which indicated a CTG expansion". A single peak is consistent with a large non-amplifiable expansion, but may also reflect homozygosity for a non-expanded allele.	The sentence is now amended to include 'homozygosity for a non-expanded allele'.
13.	The authors need to provide details on how they detected their alkaline phosphatase labelled probe.	The probe was detected using the CDP-Star Detection Reagent.
14.	"The presence of smears as opposed to distinct bands in the autoradiogram confirmed that the samples analysed were true DM1 patients." This is not true. The smear represents somatic mosaicism, but if the patients are sampled early in life, or have relatively small expansions, there will be no somatic mosaicism and expanded alleles my present as distinct bands. Critical in these analyses is the size of the bands detected, not their compactness.	The sentence is now revised to: Identification of DM1 positive samples were done by comparing the size of the bands or smears obtained with DNA molecular weight markers
15.	Figure 1 should be presented as a standalone properly labelled histogram. The raw data on allele frequencies (numbers, not percentages) should be presented as a supplementary Excel file.	Figure 1 is changed as suggested.
16.	Figure 2. The X-axis does not represent CTG repeat size, but presumably fragment length in bases. However, the authors should indeed translate this scale into CTG repeat number for both the TP-PCR and conventional PCR products. The authors also need to provide a zoom of the TP-PCR trace that convincingly shows that the 3 base ladder does indeed extend beyond 50 CTG repeats. Assuming the signal fades out not much after 50 repeats there seems little point in showing the electropherogram signal out to 500 bases. I am not convinced that the electropherograms show alleles of 11 repeats. The repeat primer contains 6 CAGs, thus the smallest fragment observed should contain six repeats. Counting up from this I make the two "11" repeat alleles as 12.	The X-axis is labeled as fragment length in base and the electropherogram is cropped to show only up to 300 bases.
17.	Figure 3. The autoradiographs should be better cropped and informative labelling placed around the outside of autoradiographs in black text. The size of the molecular weight markers in base pairs and converted into CTG repeats should also be provided on the figure.	Figure 3 is changed as suggested.
18.	Figure 4. There is no need for the boxes around each family. These families could also be better arranged into a rectangular layout to avoid excessive white space.	Figure 4 is changed as suggested
19.	'Healthy' and 'normal'. The authors refer to individuals as being either affected with myotonic dystrophy, or being 'healthy' or 'normal'. I am not sure that there are that many people who may be described as completely 'healthy' and even less who may be regarded as 'normal'. In the absence of a clinical evaluation of these individuals,	Control individuals are now referred to as 'individuals not known to be affected by DM'

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	and to avoid pejorative implications of abnormality, control individuals are probably better described as 'unaffected [by myotonic dystrophy] individuals from the general population'. It should also be made clear if these individuals have been clinically assessed to be sure they do not display any symptoms of myotonic dystrophy, or whether they may better be described, as 'not known to be affected by myotonic dystrophy'.	
20.	Italics. Gene names and Latin text should be italicised (e.g. DMPK, dystrophia myotonica, et al., etc.). This includes in the references.	All mentions of DMPK and et al have been italicized
21.	Inappropriate capitalisation. Only proper nouns should be capitalised (e.g. Southern, as in Southern blot). Disease names (e.g. dystrophia myotonica) and other words such as 'blot', 'protein' are not proper nouns and should not be capitalised even when defining an abbreviation.	All inappropriate capitalisations have been corrected
24.	Space between a number and its unit. There should be a space between a number and its units, except for % and °C.	Corrected
25.	Inappropriate referencing. Many of the references are inappropriate. Using recent research papers to support background concepts in the introduction that are only themselves covered in the introduction of the reference is simply not acceptable. The authors should use either recent reviews, or better still, quote the original papers that actually demonstrate the point in question. For example, reference 9 contains no real data on the relative prevalence of DM1 in Japan and Europe relative the rest of the world.	All references have been rechecked and appropriately cited.
Reviewer: 4		
1.	Though the authors do mention that the severe neonatal or infantile form of myotonic dystrophy is more severe, they should emphasize more the much larger CTG expansion in this infantile form of the disease, and it would be useful also to discuss the clinical genetic phenomenon of "anticipation" in autosomal dominant traits, in which each successive generation is more severely involved, in this case because of increasing numbers of repeats in the genome.	The large CTG expansion in congenital DM1 and the phenomenon of anticipation is only briefly mentioned as the focus of the study was directed towards determining the prevalence of DM1 in Malaysia and studying the feasibility of the diagnostic techniques in the local healthcare setting.
2.	Neither the Materials and Methods section of the text, nor the Abstract, states that patients studied were adults. The age range should be stated. Table 3 is a tabular list of patients and distinguishes diagnosis in young vs. older adults and also lists one congenital case symptomatic at birth.	The materials and methods section has been revised to include the age of the patients
3.	The English grammar is generally satisfactory. A few items can be improved; for example, in the Introduction, on line	Corrected

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	4, "…which are inherited in an autosomal dominant manner,…" might better be stated, "…which are inherited as an autosomal dominant trait,…" References are appropriate in number and selection. The tables are useful summaries for comparison with future studies. The Discussion is focussed and appropriate.	

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Analysis of CTG repeat length variation in the DMPK gene in the general population and the molecular diagnosis of myotonic dystrophy type 1 in Malaysia

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Title

Analysis of CTG repeat length variation in the *DMPK* gene in the general population and the molecular diagnosis of myotonic dystrophy type 1 in Malaysia

Authors

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Abstract

Objective The lack of epidemiological data and molecular diagnostic services in Malaysia has hampered the setting-up of a comprehensive management plan for myotonic dystrophy type 1 (DM1) patients, leading to delayed diagnosis, treatment and support for patients and families. The aim of this study was to estimate the prevalence of DM1 in the three major ethnic groups in Malaysia and evaluate the feasibility of a single tube triplet-primed polymerase chain reaction (TP-PCR) method for diagnosis of DM1 in Malaysia.

Design, **setting and participants** We used PCR to determine the size of CTG repeats in 377 individuals not known to be affected by DM and 11 DM1 suspected patients, recruited from a tertiary hospital in Kuala Lumpur. Triplet-primed PCR was performed on selected samples, followed by Southern blot hybridisation of PCR amplified fragments to confirm and estimate the size of CTG expansion.

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Outcome measures The number of individuals not known to be affected by DM with $(CTG)_{>18}$ was determined according to ethnic group and as a whole population. Chi-squared test was performed to compare the distribution of $(CTG)_{>18}$ with 12 other populations. Additionally, the accuracy of TP-PCR in detecting CTG expansion in 11 DM1 patients was determined by comparing the results with that from Southern blot hybridisation.

Results Of the 754 chromosomes studied, $(CTG)_{>18}$ frequency of 3.60%, 1.57% and 4.00% in the Malay, Chinese and Indian sub-populations respectively, was detected, showing similarities to data from Thai, Taiwanese and Kuwaiti populations. We also successfully detected CTG expansions in nine patients using the TP-PCR method followed by the estimation of CTG expansion size via Southern blot hybridisation.

Conclusions The results show a low DM1 prevalence in Malaysia with the possibility of underdiagnosis and demonstrates the feasibility of using a clinical and TP-PCR-based approach for rapid and cost effective DM1 diagnosis in developing countries.

Strengths and limitations of this study

- This is the first DM1 epidemiological study on individuals not known to be affected by DM from the three major ethnic groups in Malaysia.
- To date molecular diagnostic testing for DM1 is not performed in any hospital in Malaysia. This study describes the feasibility of a cost and time-effective TP-PCR based method for rapid screening and diagnosis of DM1.
- The genotyping data does not give allele size accurate to one trinucleotide repeat, but rather is a close approximation of the allele size.
- The number of DM1 samples analysed is small as DM1 is a rare disease in Malaysia.

Key Words

CTG repeats/genetic counselling/myotonic dystrophy type 1/molecular diagnosis/TP-PCR/prevalence

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Introduction

The myotonic dystrophies (DM) are the most prevalent adult muscular dystrophy worldwide, with an estimated prevalence of 1 in 8000.[1] They are classified into two main sub-groups, myotonic dystrophy type 1 (DM1) and type 2 (DM2). These are caused by nucleotide repeat expansions, which are inherited as an autosomal dominant trait, and manifest as clinically heterogeneous diseases. DM1 is due to CTG nucleotide repeats beyond the normal length of five to 49, in the 3' untranslated region (UTR) of the *dystrophia myotonica* protein kinase (*DMPK*) gene, located on chromosome 19q 13.3.[2, 3] It is a progressive disease and categorised into several subtypes. The congenital form of DM1 is maternally transmitted more frequently, although the disease occurs equally in males and females.[1,4] The general consensus is that the larger the CTG repeat in an individual, the more severe the disease and the earlier the age of onset. It is however, difficult to classify individual DM1 cases into distinct categories based merely on the size of CTG repeats, as genotype-phenotype correlation often overlap and are not clearly defined. In addition, the repeat sizes have shown variation, both between tissues, and over time in the same tissue.[5, 6] This has made disease prognosis difficult. The genetic phenomenon of anticipation can also be observed in the inheritance of the disease, resulting in a more severe form of the disease coupled with an earlier age of onset in subsequent generations.[7, 8]

The prevalence of DM1 varies greatly across populations—it is pre-dominantly seen amongst the Europeans and Japanese.[9,10] A study also estimated a high disease frequency in the Finnish population.[11] In Quebec, Canada, a particularly high DM1 prevalence of 1 in 500 has been recorded due to founder effects.[12] In contrast, it is a rare disease amongst ethnic sub-Saharan populations,[13] being almost unheard of with the exception of one case reported in Nigeria.[14] Two more recent cases amongst African Americans have also been observed, most likely representing recent population admixture .[15] In view of this disparity, a study was undertaken to determine the distribution of CTG repeats in normal African individuals. It was found that there was a highly significant difference in the distribution of normal CTG alleles larger than 18 between the African population and the European and Japanese populations.[13] This reiterates a previous theory that CTG alleles between 19 and 30 act as a source of DM1 mutations in

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subsequent generations.[16] These findings have formed the basis for the estimation of DM1 incidence within a population.[17, 13, 18-6-27]

Prior to the establishment of molecular diagnostic tests, DM1 was diagnosed in clinics mainly by observing clinical symptoms and conducting electromyography (EMG) tests, with confirmation by muscle biopsy.[28] At present, there are several molecular techniques that can be utilised in making a DM1 diagnosis, rendering little use for the invasive and painful EMG test and muscle biopsy. [29] However, a single test that is able to detect all ranges of expansion sizes is yet to be established. Laboratories often employ a combination of methods depending on mutation dynamics in the population and available equipment. Conventional PCR can detect the normal range of CTG repeats as well as premutated alleles. Optimised PCR conditions can detect alleles up to $(CTG)_{85}$, whereas those beyond that rely on Southern blot for detection. The TP-PCR method was developed to detect the presence of large expanded alleles, thus reducing the number of reflex Southern blot tests.[30]

As a Southeast Asian country, Malaysia has a population consisting mainly of ethnic Malay, Chinese and Indian. There is also a large group of indigenous people belonging to various tribes. While DM1 has not been frequently diagnosed in this country, there is a possibility of underdiagnosis or misdiagnosis due to the lack of awareness about this condition with its diverse presentations. No study has been performed on the prevalence and incidence of the disease in the predominant ethnic groups, and to the best of our knowledge, diagnostic tests for this disease at the molecular level is not available anywhere in the country. Given the multisystemic and variable phenotypic manifestations in patients, it is therefore important for a simple standard confirmatory diagnostic test to be available, especially when trying to rule out different diagnoses. Here we report the use of PCR and Southern blot hybridisation methods for the molecular analysis of individuals not known to be affected by DM from the Malay, Chinese and Indian sub-populations, where we studied the length of the CTG alleles in order to predict the prevalence of DM1 in these subpopulations. We also describe the use of a single-tube TP-PCR method for the screening and confirmation of DM1 amongst Malaysian patients, with the aim of reducing the number of Southern blot tests that need to be performed.

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Materials and Methods

Ethics statement

Ethical approval to conduct this study was obtained from the University of Malaya Medical Centre (UMMC) ethics committee (Reference numbers 577.17 & 800.6). The ethics board required that all human subjects recruited in the study were briefed on the nature of the study, and provided with an information sheet describing the study. Subjects were also assured that their privacy will be protected, and all personal information provided will be kept confidential. Participation in the study was on a voluntary basis, and had no bearing on the quality of care patients received at the hospital.

Sample collection

Blood samples from 377 randomly selected anonymous blood donors not known to be affected by DM of Malay, Chinese and Indian descent were obtained from the UMMC blood bank following oral consent to participate in the study. In addition, 11 patients displaying DM-like symptoms were recruited to this study. Written consent, clinical and familial history were obtained from these patients. The ethnicity of subjects was determined to be Malay, Chinese or Indian based on their own admission.

Molecular analysis

Genomic DNA was extracted from the blood samples using the QIAamp DNA Blood Mini kit according to manufacturer's protocol (QIAGEN, Hilden, Germany).

Conventional PCR

Analyses of the samples were carried out according to techniques described by Surh et al.[31] PCR was performed in a final volume of 30µL utilising the Perkin Elmer GeneAmp PCR system. The forward, 103, 5' – CCA GTT CAC AAA CCG CTC CGA GCG TG – 3' and reverse, 96, 5' – GGT GCG TGG AGG ATG GAA CAC GGA C – 3' primers were used. The PCR conditions were set as follows: initial denaturation at

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96°C for 5 minutes, followed by 25 cycles of denaturation, annealing and extension at 96°C, 62°C and 72°C respectively, for a period of one minute for each step. Final extension was performed at 72°C for seven minutes. The PCR products were sized by gel electrophoresis on 1.5% agarose gel, at 100 V for 45 minutes. The separated products were cut out from the gel, purified using the QIAquick gel extraction kit (QIAGEN, Hilden, Germany) and sent to a service lab for sequencing to determine the exact number of CTG repeats.

Triplet-primed-PCR

Analysis of the samples were done according to techniques described by Singh et al.[32] Thirteen samples were subjected to TP-PCR analysis—11 individuals with DM1 symptoms and two controls not known to be affected by DM. The subjects recruited were all adults between the ages of 30 and 60, and one child aged 5. Testing was performed with 100 ng of genomic DNA from blood samples in a reaction volume of 25 µl. The primers FAM-P1-Forward 5'FAM – GGG GCT CGA AGG GTC CTT GT – 3' and P2-Reverse 5' – GTG CGT GGA GGA TG AAC ACG – 3' flanked the CTG repeat region, with the forward primer labeled with FAM fluorescence. The third primer P3 5' – AGC GGA TAA CAA TTT CAC ACA GGA – 3' was designed to bind to the complement of the tail of the fourth primer P4-$(CAG)_6$ –Reverse 5' – AGC GGA TAA CAA TTT CAC ACA GGA CAG CAG CAG CAG CAG CAG – 3'. The primer combination was prepared in a ratio of FAM-P1-Forward: P4-$(CAG)_6$ –Reverse:P3:P2 = 1.5:1:1.5:1.5, with a final working concentration of 0.6 µM:0.4 µM:0.6 µM:0.6 µM. The TP-PCR conditions were set as follows: initial denaturation at 95°C for five minutes, followed by 10 cycles each of denaturation (97°C) for 35 seconds, annealing (65°C) for 35 seconds and extension (68°C) for four minutes. Subsequently, 20 cycles of denaturation, annealing and extension were performed, with the extension time increased by 20 seconds per cycle to allow for increased yield of PCR product. The products were separated on an ABI PRISM 3130 x 1 genetic analyser (Life Tech, New York, USA) and fragment size determined using GeneMarker V2.6 (Softgenetics, State College, USA).

Southern blot hybridisation of PCR amplified fragments

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Southern blot hybridisation of amplified PCR fragments was carried out in samples that only showed single peaks in the electropherograms, which indicated a CTG expansion or homozygosity for a non-expanded allele. The conventional PCR products were transferred overnight from the agarose gel to a positively charged nylon membrane by capillary transfer and fixing of the DNA to the membrane done via the UV cross-linking method. The membrane was hybridised overnight in a hybridisation buffer with the addition of 20 µl alkaline phosphatase-conjugated $(CTG)_{10}$ oligonucleotide at 50°C. The membrane was then removed and the excess liquid drained off, prior to being washed using pre-heated wash buffers. Following hybridisation and washing of the membrane, the CDP-Star Detection Reagent is applied and the development of the signals was subsequently carried out by exposing the blot to an autoradiography film. Identification of DM1 positive samples were done by comparing the size of the bands or smears obtained with DNA molecular weight markers. The size of the expansion was determined as the point of highest band intensity on the autoradiograph. To estimate the size of the expansion, the lengths of the 100 bp/1Kb size marker fragments were first converted into logarithmic values. These values were then graphed on the y axis against their migration distance on the x axis. Using linear regression, a line of best fit was drawn through these points and an equation describing that line was derived. The unknown fragment's migration distance derived from the most intense region of the expanded allele was placed into the equation for the regression line to determine a log value for the fragment's size. Taking the antilog of this value yielded the unknown fragment's size in base pairs. This value is an estimate of the average repeat number and is dependent on age at sampling.

Statistical analysis

The frequency of each of the allele present in the 754 chromosomes from the individuals not known to be affected by DM was calculated. Statistical analysis was performed by administering the chi-squared (χ^2) test with Yates' correction to compare the distribution of normal large repeats, $(CTG)_{> 18}$, with 12 other populations.

Results

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Analysis of DMPK CTG repeat length variation in the general population

The distribution of $(CTG)_{>18}$ alleles in the Malay, Chinese and Indian sub-populations all point towards a low prevalence of DM1. Figure 1 shows the breakdown of all alleles present in the three sub-populations. (Individual genotyping data is provided in supplementary files 1–3) A bimodal allelic distribution was noted—this was in alignment with patterns observed in other populations with low DM1 frequencies. The first peak came from the $(CTG)_5$ alleles, which totaled to 33.7% of all alleles, while the second peak consisted of three alleles, 11 to 13 that accounted for a majority of 51.1% of the total alleles. The frequencies for $(CTG)_{>18}$ alleles were 9/250 = 3.60% (95% CI = 0.0166–0.0672) in the Malay subpopulation, 4/254 = 1.57% (95% CI = 0.0043–0.0398) in the Chinese subpopulation, and 10/250 = 4.00% (95% CI = 0.0193–0.0723) amongst the Indians. Heterozygosity was measured at 79.9%, 77.0%, and 76.2% in the three subpopulations, respectively, averaging at 77.7%. This result is aligned to those reported in other populations, which ranged from 73.0% in Europeans[17] to 92% in Iranians.[26]

Tables 1 and 2 show the comparison and χ^2 analysis of the frequency of $(CTG)_{>18}$ alleles in individuals not known to be affected by DM1 from the three subpopulations in this study, and in those from 12 worldwide populations, respectively. The $(CTG)_{>18}$ frequency for the Malay, Chinese and Indian subpopulations were significantly different when compared to frequencies in European, German and Chilean populations. All three Malaysian subpopulations showed frequencies similar to Thai,[22] Taiwanese[23] and Kuwaiti[24] populations. It is also interesting to note that the Han-Chinese show similarity with the Malaysian Chinese, the population that the majority of Malaysian Chinese trace their ancestry to. This allows for our speculation that the DM1 frequency among Chinese Malaysians is low, similar to that observed in the Han-Chinese,[24] Taiwanese[23] and South African negroids.[13]

Diagnostic testing for DM1

Samples from 11 individuals with DM1-like symptoms and two controls not known to be affected by DM were analysed for CTG expansion using TP-PCR followed by confirmation by Southern blot hybridisation of PCR amplified fragments. Triplet-primed PCR testing showed single peaks in nine of the samples, and

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double peaks in the remaining four. The samples with single peaks also showed a clear laddering pattern indicating the presence of CTG expansion (Figure 2). Southern blot hybridisation of PCR amplified fragments confirmed the diagnosis of DM1 in the nine samples, with the detection of expanded alleles ranging from a size of 97 to 690 CTG repeats, as shown in Figure 3. Table 3 shows a summary of the characteristics of the disease exhibited by each patient. Figure 4 shows the pedigree diagram and the CTG repeat size of the families and individuals we studied. It is important to note that apart from those diagnosed (dark squares/circles), none of the other family members were examined or tested for DM1. Hence, there is a possibility that there may be family members showing very mild symptoms who have not presented in our clinics, contributing to the apparent under transmission of the disease in the families.

Discussion

In order to obtain a better understanding of the burden of DM1, we estimated the prevalence of the DM1 using the distribution of CTG alleles larger than 18 in the Malaysian population. The result of $(CTG)_{>18}$ of 3.05% (23/754) was observed in the Malaysian population. By comparing with the results of studies performed in other populations, we predict that DM1 is a rare disease in Malaysia. A larger study is needed to verify these findings, due to the fact that the subjects in this study were recruited from a major hospital in the capital city of Malaysia, therefore may not be representative of the whole country. It is likely that DM1 in the local community is underdiagnosed due to a lack of awareness amongst the public and healthcare professionals. There are also other contributing factors such as social stigma, and reduced access to major hospitals where specialised consultation and testing are available.

Population studies done previously have showed evidence for the association of $(CTG)_{>18}$ allele frequency and DM1 prevalence. In European populations the frequency of DM1 is estimated to be 1 in 8000 which corresponded to $(CTG)_{>18}$ of approximately 10%. On the other end of the spectrum, DM1 has only been reported in one Southern African Negroid family where the prevalence of $(CTG)_{>18}$ is reported to be 0.7%. In the absence of epidemiological data on real cases of DM1, other populations such as the Brazilian, Mexican, Thai, Taiwanese and Han Chinese report the prevalence of DM1 as either higher or lower than populations with known prevalence.

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The nine patients identified in this study were diagnosed in the years 2011 and 2012. During these two years, the number of patients who were seen at UMMC were 957,418 and 964,497 respectively, totaling to 1,921,915. Using these figures as guide, we estimate the prevalence of DM1 in Malaysia to be less than 1 in 200,000. This estimate is similar to the low estimates of DM1 prevalence reported in Thai[22],Taiwanese[23] and Kuwaiti[25] populations, where the authors reported the observed frequencies of alleles >18 and correlated them to the prevalence of DM1 in their respective countries. This was also in concordance with the results of our χ^2 analysis.

It is interesting to note that the frequency of $(CTG)_{>18}$ was the lowest in the Chinese subpopulation, although they account for the most number of DM1 patients seen in our hospital (including those not reported here). The Indians on the other hand show the highest frequency of $(CTG)_{>18}$ in agreement with the findings that DM1 is highly prevalent in India[33]. However, the number of Indian DM1 patients seen in our study was the lowest among the three subpopulations. This may reflect socio-economic and demographic reasons, as well as misdiagnosis/underdiagnosis of DM1 in the respective subpopulations.

Our study also provides for the first time, data on the $(CTG)_{>18}$ allele frequency in a Malay population. The Malay ethnic group is genetically more similar to the Chinese compared to the Indians.[34] Comparison of the $(CTG)_{>18}$ distribution of the three ethnic groups however, shows a closer similarity between the Malays and the Indians (p=0.8151) compared to the Chinese (p=0.249). It would be interesting to see this same analysis done on other modern Malay populations in the region, such as the Singapore Malays and the Indonesians, as well as the aboriginal Malays.

The usage of the single tube TP-PCR allows for the rapid identification of large pathogenic CTG repeats, thus reducing the need for Southern blot based approaches to detect or exclude the presence of a large expansion. Southern blot may require large amounts of DNA, the use of radioactive materials and is time consuming. In addition, this procedure is also less sensitive and may be difficult to replicate. Hence, any method that reduces the number of Southern blot that needs to be performed, while demonstrating high sensitivity and specificity is advantageous in a clinical setting. However, the TP-PCR test used requires a highly specialised equipment, the genetic analyser, which may not yet be widely available and is unable to estimate the size of CTG expansions beyond 85 repeats.

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Genotype-phenotype correlation studies in DM1 patients have thus far given conflicting results, with various underlying mechanisms, associations and theories proposed[35--38]. In particular, in their study on the somatic instability of expanded CTG repeats in DM1, Morales *et al*[39] showed that there was no evidence to indicate that pathogenesis of the disease is constrained to a threshold above which repeat length does not contribute toward age at onset. Additionally, they showed that age at onset is further modified by the level of somatic instability, which is a highly heritable trait. In our study, a disparity in the genotype-phenotype correlation in the Chinese family was seen, whereby patient 3 is largely asymptomatic although she carries 350 repeats. Her disease status was only suspected and diagnosed following the birth of her children who exhibited symptoms. Both her children were congenitally affected, which is consistent with findings in previous studies that showed that the majority of congenital cases were maternally transmitted. Patients 2 and 7 on the other hand paternally inherited their pathogenic alleles, resulting in the classic/adult onset DM1. The same disease phenotype is seen in patients 8 and 9. We were not able to determine whether their diseases were inherited, as their parents have never been tested. However, these patients were given genetic counselling and in accordance with ethical principles, have the autonomy of deciding whether or not to disclose their disease status to family members at risk, for future counselling and testing. It was also observed that congenitally affected patient 5 showed a comparable expansion size to those who were classically affected. The only symptoms he has shown, however is neonatal hypotonia and a mild cognitive dysfunction. The comparable repeat size is most likely due to the younger age of patient 5 compared to the classically affected adults, and suggest that a larger repeat size would be observed, as the patient grows older. Apart from these disease dynamics, there have also been findings of contraction of allele sizes upon transmission reported elsewhere[27,36]. All these factors point towards the high complexity of DM1 and illustrate the important need for genetic counselling services to be offered to affected families.

Molecular testing is generally established as the gold standard in diagnosing genetic disorders such as DM1. This is because a molecular test is rapidly able to eliminate differential diagnoses, confirm the DM1 diagnosis, and estimate the size of CTG expansion in a patient, thus avoiding the need for invasive procedures such as muscle biopsies. Hilbert *et al*[40] who studied a large cohort of DM patients enrolled in the US National Registry, explored their diagnostic journeys, which on average took seven years for a

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correct DM1 diagnosis to be made. This delay brought about many implications to the patients and their families, ranging from lack of appropriate disease management to missed opportunities for genetic counselling. The situation in many developing countries is much similar or even worse as molecular diagnostic testing for DM1 is not easily available. Potentially, there could be a large number of patients who are undiagnosed/misdiagnosed, as well as those who have been unnecessarily subjected to various investigations for a definitive diagnosis to be made.

The findings from our preliminary study can aid the structuring of a rare disease management framework in Malaysia, using DM1 as a disease model. The data presented here adds to the scarce literature of DM1 in the Southeast Asian region. The information on CTG repeat lengths of the *DMPK* gene in individuals not known to be affected by DM, and DM1 patients, together with proper clinical assessment as well as a cost-effective molecular approach, carry implications for earlier diagnosis of DM1 and genetic counselling in a low resource setting.

Contributorship statement

All authors were involved in the conception and design of the work as well as the final approval of the submitted manuscript.

KA and TI were involved in the acquisition and analysis of data and KA, TI and MKT were involved in the drafting of the manuscript.

LLH, GKJ, KTW, AAA and MKT contributed to the critical evaluation of the manuscript.

Competing interests

The authors state no competing interests

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Data sharing statement

None available

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Figure Legends

Figure 1. Frequency of CTG repeats in individuals not known to be affected by DM from the Malay, Chinese and Indian sub-populations. *The frequency for large normal alleles, $(CTG)_{>18}$ was 9/250 or 3.60% in the Malays, 4/254 or 1.57% in the Chinese, and 10/250 or 4.00% in the Indians. A bimodal allelic distribution was observed in the Malaysian population, in alignment with patterns observed in other*

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populations with low DM1 frequency. The most frequently seen allele was $(CTG)_5$ in all three sub-populations, whereas $(CTG)_{10-13}$ was the most common allele group. The genotyping data for each individual is provided in the supplementary files 1–3.

Figure 2. Electropherogram results of TP-PCR. *The X-axis represents the size in base pairs and the Y-axis represents the allele peak height. (A) The electropherogram shows a DM1 patient sample with a single peak corresponding to $(CTG)_{11}$ and a laddering pattern indicating an expanded allele. (B) Two normal heterozygous alleles with sizes 5 and 12 and no laddering pattern observed.*

Figure 3. Expanded CTG repeats of DM1 patients following Southern blot hybridisation of PCR amplified fragments as seen on an autoradiography film. *Expanded alleles in patients ranging from a size of 97 to 690 CTG repeats have been detected. A sample of the bands are shown here, ranging from 270 repeats (1045 bp) to 690 repeats (2305 bp). Normal alleles of four sizes were seen amongst the patients, 5 (332 bp), 11 (350 bp), 12(356 bp) and 13 (356 bp). Due to somatic heterogeneity, the expanded alleles usually appear as smears. A 1 Kb DNA ladder as well as samples from individuals not known to be affected by DM were run alongside patient samples as controls.*

Figure 4. Pedigree diagrams of DM1 patients studied including the size of their CTG alleles. *Members of three families and two individuals had their CTG repeat size analyzed. The sizes of the allele pairs for each patient are as stated in the pedigree diagrams. The phenomenon of anticipation was clearly observed in the three families, whereby with the increased CTG expansion in successive generations, a decreasing age of onset is noted.*

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Tables

Table 1: Comparison and χ^2 analysis of the frequency of $(CTG)_{>18}$ alleles in individuals not known to be affected by DM from the Malay, Chinese and Indian sub-populations.

Population	$(CTG)_{>18}$ alleles / Total alleles analyzed (%)	Comparison of Malay data with other populations χ^2 (p value)	Comparison of Chinese data with other populations χ^2 (p value)	Comparison of Indian data with other populations χ^2 (p value)
Malay	9/250 (3.60)	-	1.329 (0.249)	0.055 (0.8151)
Chinese	4/254 (1.57)	1.329 (0.249)	-	1.919 (0.166)
Indian	10/250 (4.00)	0.055 (0.8151)	1.919 (0.166)	-

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Table 2: Comparison and χ^2 analysis of the frequency of $(CTG)_{>18}$ alleles in individuals not known to be affected by DM from the three Malaysian sub-populations to those in twelve worldwide populations.

Population	$(CTG)_{>18}$ alleles / Total alleles analyzed (%)	Comparison of Malay data with other populations χ^2 (p value)	Comparison of Chinese data with other populations χ^2 (p value)	Comparison of Indian data with other populations χ^2 (p value)
[a] European[17]	15/130 (11.54)	7.817 (0.005**)	16.094 (<0.0001***)	6.729 (0.009**)
German[18]	22/104 (21.20)	26.17 (<0.0001***)	39.141 (<0.0001***)	24.239 (<0.0001***)
Mexican[19]	51/800 (6.38)	2.232 (0.135)	8.037 (0.005**)	1.553 (0.213)
Brazilian[20]	24/312 (7.69)	3.497 (0.062)	9.88 (0.002**)	2.334 (0.127)
Chilean[21]	30/272 (11.00)	9.354 (0.002**)	17.887 (<0.0001***)	8.131 (0.004**)
Japanese[17]	9/106 (8.50)	2.760 (0.097)	8.386 (0.004**)	2.149 (0.143)
Thai[22]	11/400 (2.75)	0.142 (0.706)	0.505 (0.477)	0.421 (0.516)
Taiwanese[23]	7/499 (1.40)	2.867 (0.090)	0.018 (0.893)	3.962 (0.050)
Han Chinese[24]	6/600 (1.00)	5.463 (0.019*)	0.134 (0.714)	7.052 (0.008**)
Kuwaiti[25]	14/370 (3.78)	0.010 (0.920)	1.894 (0.169)	0.006 (0.938)
Iranian[26]	29/400 (7.25)	3.090 (0.079)	9.292 (0.002**)	2.334 (0.127)
South African[13]	3/420 (0.71)	5.869 (0.015*)	0.457(0.499)	7.249 (0.007*)

*P < .05 (significant); ** P < .01 (highly significant); *** P < .001(very highly significant)

[a] Includes British, German, Belgian, Swedish and Finnish subjects

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Table 3: A summary of the disease characteristics exhibited by DM1 patients in this study

Family	Patient	Gender	[a] Age	[b] Disease Onset	[c] Phenotype	CTG Repeat Size
1	1	Male	54	Late adult	Classical	330
	2	Male	30	Early adult	Classical	690
2	3	Female	30	Early adult	Mild	350
	4	Female	31	Early adult	Mild	97
	5	Male	5	Birth	Congenital	596
3	6	Male	60	Late adult	Classical	270
	7	Male	30	Early adult	Classical	570
4	8	Male	44	Early adult	Classical	550
5	9	Male	32	Early adult	Classical	520

[a] Age of patient at time of molecular testing

[b] Early adulthood: 20 – 49 years old; Late adulthood: >50 years old

[c] Phenotype classification as described by Kamsteeg *et al*

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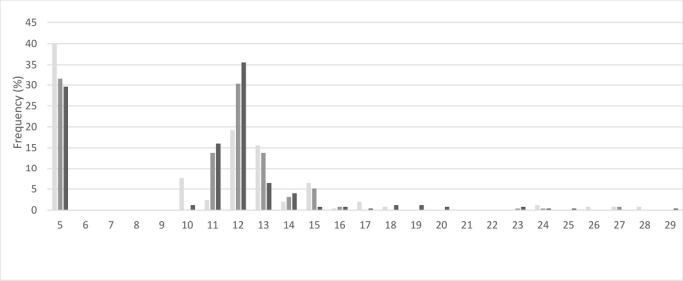

Figure 1. Frequency of CTG repeats in individuals not known to be affected by DM from the Malay, Chinese and Indian sub-populations. The frequency for large normal alleles, (CTG)>18 was 9/250 or 3.60% in the Malays, 4/254 or 1.57% in the Chinese, and 10/250 or 4.00% in the Indians. A bimodal allelic distribution was observed in the Malaysian population, in alignment with patterns observed in other populations with low DM1 frequency. The most frequently seen allele was (CTG)5 in all three sub-populations, whereas (CTG)10-13 was the most common allele group. The genotyping data for each individual is provided in the supplementary files 1–3.

215x166mm (300 x 300 DPI)

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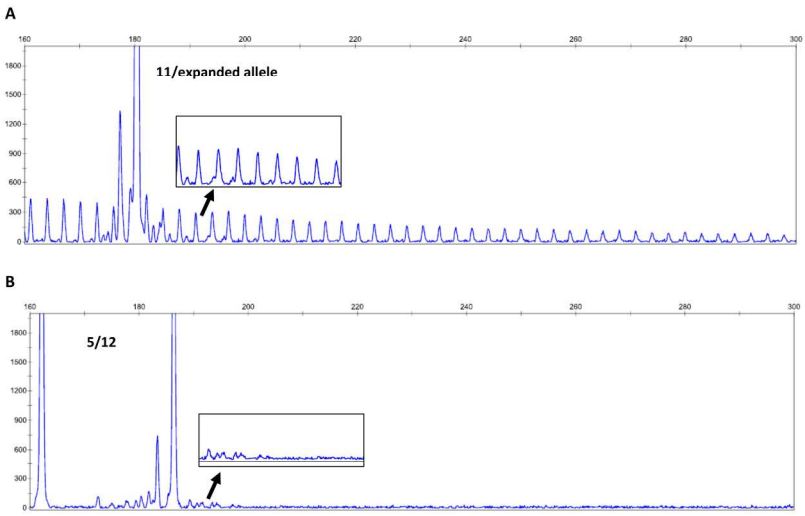

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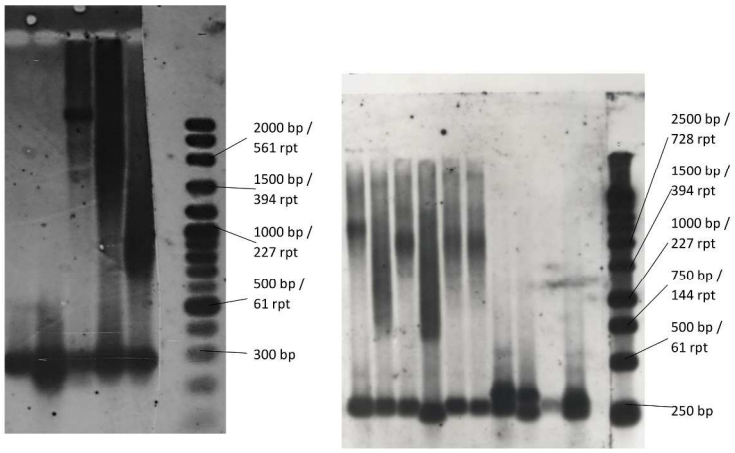

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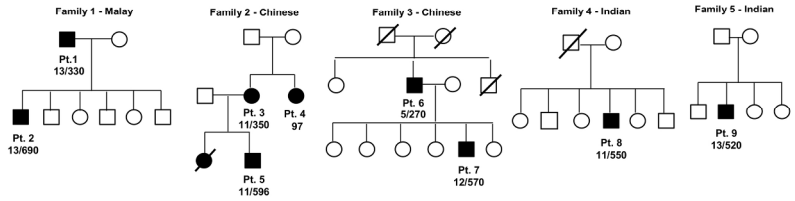

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215x166mm (300 x 300 DPI)

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Complete list of allele distribution in healthy individuals from the Malay subpopulation

No.	Code	Allele 1	Allele 2
1	N1	10	5
2	N2	10	5
3	N3	11	5
4	N5	12	12
5	N6	12	12
6	N7	5	5
7	N10	13	11
8	N25	10	10
9	N26	5	5
10	N31	5	5
11	N32	10	5
12	N33	5	5
13	N35	13	13
14	N36	13	5
15	N37	11	5
16	N38	12	12
17	N40	5	5
18	N45	13	13
19	N46	13	13
20	N47	12	5
21	N51	13	10
22	N52	10	5
23	N59	15	5
24	N60	17	5
25	N63	5	5
26	N68	5	5
27	N73	12	5
28	N78	12	5
29	N80	5	5
30	N82	13	5
31	N84	14	5
32	N87	13	13
33	N89	11	11
34	N90	15	5
35	N92	12	5
36	N95	5	5
37	N97	28	11
38	N99	13	5
39	N101	24	5
40	N105	13	13
41	N108	12	12
42	N110	13	13
43	N116	14	5
44	N118	18	5

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45	N120	13	13
46	N125	5	5
47	N130	12	12
48	N132	12	12
49	N133	10	5
50	N134	12	5
51	N137	5	5
52	N139	15	5
53	N144	10	5
54	N147	12	5
55	N148	13	5
56	N157	13	5
57	N159	5	5
58	N160	13	13
59	N165	12	5
60	N171	18	10
61	N173	12	5
62	N177	13	10
63	N178	12	5
64	N179	5	5
65	N185	13	5
66	N188	14	5
67	N189	15	5
68	N191	17	5
69	N193	17	5
70	N195	17	12
71	N196	16	5
72	N198	14	5
73	N199	15	13
74	N206	17	5
75	N207	17	5
76	N208	17	13
77	N209	16	5
78	N211	28	12
79	N212	27	12
80	N213	17	12
81	N214	5	10
82	N215	13	5
83	N217	15	5
84	N218	26	10
85	N219	13	5
86	N220	13	5
87	N223	15	12
88	N228	5	15
89	N229	24	24
90	N230	5	5
91	N232	12	5

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92	N233	12	5
93	N234	12	5
94	N235	12	5
95	N236	13	5
96	N239	12	12
97	N240	26	12
98	N242	15	12
99	N242	15	10
100	N243	5	5
101	N244	5	5
102	N245	13	13
103	N247	13	13
104	M1	12	5
105	M2	5	5
106	M3	5	5
107	M4	12	12
108	M5	12	5
109	B12	12	5
110	B14	5	5
111	B18	14	14
112	B20	5	5
113	B21	15	15
114	B34	15	15
115	B35	13	13
116	B36	13	5
117	B37	5	5
118	B38	5	5
119	B40	10	10
120	B48	15	5
121	B49	12	12
122	B50	12	12
123	B53	13	13
124	B54	12	5
125	B56	27	10

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Complete list of allele distribution in healthy individuals from the Chinese subpopulation

No.	Code	Allele 1	Allele 2
1	N024W	11	5
2	N029W	11	11
3	N039W	13	5
4	N041W	13	13
5	N042W	11	5
6	N043W	12	12
7	N044W	12	12
8	N048W	12	5
9	N049W	12	5
10	N050W	11	5
11	N053W	13	5
12	N054W	12	12
13	N055W	12	5
14	N056W	13	5
15	N057W	14	5
16	N058W	12	5
17	N061W	13	5
18	N062W	12	5
19	N064W	12	12
20	N065W	13	5
21	N066W	24	5
22	N071W	11	5
23	N072W	12	5
24	N074W	13	13
25	N075W	12	5
26	N076W	14	14
27	N077W	13	5
28	N079W	12	5
29	N081W	12	12
30	N083W	12	12
31	N085W	14	5
32	N086W	27	5
33	N088W	5	5
34	N093W	11	11
35	N094W	11	5
36	N096W	12	12
37	N098W	15	5
38	N100W	13	5
39	N103W	12	12
40	N104W	12	5
41	N106W	12	5
42	N107W	12	12
43	N111W	14	5
44	N112W	15	15

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45	N113W	5	5
46	N114W	13	5
47	N115W	12	12
48	N117W	13	13
49	N119W	13	5
50	N122W	5	5
51	N124W	12	12
52	N127W	12	12
53	N128W	13	5
54	N138W	5	5
55	N142W	11	11
56	N143W	5	5
57	N146W	13	13
58	N149W	12	12
59	N150W	13	5
60	N151W	13	5
61	N152W	11	11
62	N153W	11	11
63	N154W	27	16
64	N155W	13	5
65	N156W	11	5
66	N158W	13	5
67	N161W	15	15
68	N162W	12	5
69	N163W	12	5
70	N164W	15	15
71	N166W	12	5
72	N167W	12	12
73	N168W	5	5
74	N169W	15	5
75	N170W	11	11
76	N172W	11	5
77	N174W	12	5
78	N175W	13	5
79	N176W	15	15
80	N180W	11	5
81	N181W	12	5
82	N182W	11	11
83	N183W	13	13
84	N184W	5	5
85	N186W	23	14
86	N187W	12	12
87	N190W	12	12
88	N194W	12	12
89	N197W	11	11
90	N200W	12	5
91	N221W	12	12

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92	N222W	11	11
93	N224W	13	13
94	N227W	15	5
95	N237W	13	5
96	C1	11	5
97	C2	5	12
98	C3	11	11
99	C4	13	13
100	C5	12	12
101	C6	11	5
102	C7	12	12
103	C8	12	5
104	C9	11	5
105	C10	12	5
106	C11	12	5
107	C12	13	13
108	C13	15	15
109	C15	13	13
110	C16	11	5
111	C19	12	5
112	C20	12	12
113	C22	12	5
114	C24	12	5
115	C25	12	12
116	C27	13	13
117	C29	11	11
118	C30	12	5
119	C31	12	5
120	C32	14	14
121	C33	12	12
122	C34	12	12
123	C35	12	12
124	C36	5	5
125	C37	12	5
126	C38	11	5
127	C39	16	5

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Complete list of allele distribution in healthy individuals from the Indian subpopulation

No.	Code	Allele 1	Allele 2
1	I001	14	14
2	I005	5	5
3	I007	15	5
4	I008	11	11
5	I009	12	5
6	I010	5	5
7	I011	23	11
8	I012	12	12
9	I014	11	5
10	I015	5	5
11	I016	13	13
12	I017	14	5
13	I018	11	11
14	I019	12	12
15	I020	24	12
16	I021	12	12
17	I022	12	12
18	I023	12	12
19	I025	12	5
20	I026	12	12
21	I029	20	12
22	I030	10	12
23	I031	12	12
24	I032	12	12
25	I033	14	14
26	I034	11	11
27	I035	15	11
28	I036	13	5
29	I037	19	11
30	I038	11	5
31	I039	12	12
32	I040	17	5
33	I041	11	5
34	I042	14	12
35	I043	12	5
36	I044	13	5
37	I045	5	5
38	I046	12	12
39	I047	12	5
40	I048	11	11
41	I049	12	12
42	I050	12	12
43	I051	12	12
44	I052	5	5

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45	I053	14	5
46	I055W	11	5
47	I056W	12	5
48	I057W	12	5
49	I059W	12	5
50	I060W	11	5
51	I061W	5	5
52	I062W	12	12
53	I064W	12	12
54	I066W	16	5
55	I067W	12	5
56	I068W	10	10
57	I069W	11	11
58	I070W	18	5
59	I071W	11	5
60	I073W	11	11
61	I074W	20	11
62	I075W	11	11
63	I077W	13	13
64	I078W	12	12
65	I079W	5	5
66	I085W	12	12
67	I086W	12	5
68	I087W	11	5
69	I088W	5	5
70	I089W	5	5
71	I090W	12	12
72	I091W	11	11
73	I093W	12	12
74	I094W	23	11
75	I095W	18	5
76	I096W	12	12
77	I097W	12	5
78	I098W	29	12
79	I099W	12	12
80	I102W	12	12
81	I103W	11	5
82	I104W	25	5
83	I105W	11	5
84	I106W	11	11
85	I107W	11	5
86	I108W	12	5
87	I109W	13	13
88	I110W	12	12
89	I111W	12	5
90	I112W	12	5
91	I113	12	5

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92	I114	5	5
93	I116	11	5
94	I117	14	11
95	I118	11	5
96	I119	5	5
97	I120	11	5
98	I121	13	13
99	I123	12	12
100	I124	19	12
101	I125	12	12
102	I126	18	11
103	I127	13	5
104	I129	12	12
105	I130	12	5
106	I131	19	11
107	I133	12	12
108	I137	13	13
109	I138	5	5
110	I140	12	12
111	I144	12	5
112	I145	12	5
113	I146	5	5
114	I150	12	12
115	I151	14	14
116	I153	12	5
117	I156	12	12
118	I158	12	5
119	I159	13	13
120	I160	12	5
121	I162	12	5
122	I163	16	5
123	I164	11	5
124	I167	13	5
125	I168	12	12

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