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Short Tandem Repeats Used in Preimplantation Genetic Testing of B-Thalassemia: Genetic Polymorphisms For 15 Linked Loci in the Vietnamese Population

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Keywords: Preimplantation genetic testing (PGT); β-thalassemia; Short tandem repeat (STRs); Microsatellite markers

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Abstract

BACKGROUND: β-thalassemia is one of the most common monogenic diseases worldwide. Preimplantation genetic testing (PGT) of β-thalassemia is performed to avoid affected pregnancies has become increasingly popular worldwide. In which, the indirect analysis using short tandem repeat (STRs) linking with HBB gene to detect different β-globin (HBB) gene mutation is a simple, accurate, economical and also provides additional control of contamination and allele-drop-out ADO.

AIM: This study established microsatellite markers for PGT of Vietnamese β-thalassemia patient.

METHODS: Fifteen (15) STRs gathered from 5 populations were identified by in silico tools within 1 Mb flanking the HBB gene. The multiplex PCR reaction was optimized and performed on 106 DNA samples from at-risk families.

RESULTS: After estimating, PIC values were ≥ 0.7 for all markers, with expected heterozygosity and observed heterozygosity values ranged from 0.81 to 0.92 and 0.53 to 0.86, respectively. One hundred percent of individuals had at least seven heterozygous markers and were found to be heterozygous for at least two markers on either side of the HBB gene.

CONCLUSION: In general, a pentadecaplex marker (all < 1 Mb from the HBB gene) assay was constituted for β-thalassemia PGT on Vietnamese population.

Introduction

β-Thalassemia is one of the most common monogenic diseases, accounting for 1.5% of the population [1], concentrated in Central and Southern Asia, the Middle East, Northern Africa and the Mediterranean including Vietnam with the carrier. *HBB* mutations have more than 200 different types which have been knowns [2] lead to insufficient β-globin synthesis. The frequency of Vietnamese carriers ranged from 1.5 to 25.0% depending on ethnic group [3], [4], [5]. PGT-M (Preimplantation genetic testing for monogenic disease) promises to prevent monogenic disease in children born to at-risk couples by avoiding transferring mutation embryos to women *in vitro* fertilization. In which, PGT-M for β-Thalassemia has become the most common application among monogenic disorders.

There have been various studies that established direct or indirect PGT procedure for β-Thalassemia. Among numerous techniques, microsatellite markers such as STRs have provided many advantages in the indirect analysis. STRs are the repetitive DNA fragments of 2-6 bp which structure is highly conservative, inherited through generations and characteristic for each individual. The STRs is also highly diverse and can be amplified by PCR.

Thus, microsatellite markers linking with *HBB* gene has played an essential role in linkage analysis for β-Thalassemia.

Currently, there have been many typical studies on indirect linkage-based PGT for β-thalassemia published in the world. Wen Wang (2009) combined the Nested-PCR method amplifying STR markers and minisequencing method on nine embryos and concluded five unaffected embryos for transferring [6]. Li Fan (2017) used STR markers to perform PGT on WGA products from 147 day-5 embryos and identified 24 non-mutations, 38 carriers, and 18 mutation embryos [7].

Nevertheless, these direct analysis methods have no probability of controlling contamination and ADO phenomenons, which are considered as the main reasons leading to misdiagnosis in PGT. Thus, β-Thalassemia with wide range of gene and variety of mutations is recommended applying with indirect analysis method. These methods were proved to be sensitive, accurate, reliable and rapid to control the pitfalls of PCR-based PGT, including PCR failure, contamination, and ADO.

Despite the preeminence of STRs, the limited number of researches and available markers for the Vietnamese population, this hampers their utility in linkage-based β-Thalassemia PGT. In this study, we developed a multi-marker panel consisting of 15 STRs for Vietnamese β-Thalassemia patients. The data suggested that the STRs set was qualified to perform PGT-M with high heterozygous values, number of heterozygous markers on each individual and the equal distribution of markers on either side of *HBB* gene.

Materials and methods

Control Samples for Method Optimization

One hundred six genomic DNA samples were extracted from blood and amniotic fluid of at-risk families at the Vietnam National Institute of Hematology and Blood Transfusion (NIHBT), Vietnam from 07/2016 to 6/2018. DNA was used either to pre-screen of microsatellite markers or to determine heterozygosity values of them.

Short tandem repeat

Initial selected STRs were identified based on the STR database and Tandem Repeat Finder provided by Gary Benson (http://tandem.bu.edu/trf/trf.html). DNA sequence within 1 Mb upstream and downstream of the *HBB* gene (11p15.4) (genome assembly GRCh37/hg19, Feb 2009, annotation) was extracted from the UCSC Genome Browser. Initial selection criteria for the STRs followed Machado 2009 [8]. The first microsatellite markers were subsequently compared and selected for Vietnamese population based on report from populations of Malaysia, China, and India [9]. Primers were designed by Primer3 Tool. UCSC In-silico PCR with downloaded reference DNA sequence (genome assembly GRCh37/hg19, Feb 2009, annotation) and BLAST from NCBI were used to determine and exclude the primer complementing with Alu and non-specific sequences.

DNA Extraction

DNA was extracted from blood or amniotic fluid by Blood DNA Extraction QIAamp® DNA Mini Kit (Cat No./ID: 51304, QIAGEN) following the optimal instruction from QIAGEN Producer. Purified DNA was qualified and quantified by *NanoDrop One* Spectrophotometer with criteria: OD A260/A280 between 1.7-2.0 and concentration above 10 ng/µl.

PCR amplification

Single PCR: Followed T_m of 15 primers on http://www.operon.com/tools/oligo-analysis-tool.aspx, determined the average theoretical annealing temperature of these 15 primer pairs which is 60°C. Thus, PCR single primer was conducted according to the temperature range set at 55°C-60°C-65°C, and products were analyzed on the agarose gel. Single PCR amplification was performed in a 50 µL reaction volume consisting of 2 µL genomic DNA (concentration: 10-20 ng/µL), 25 µL 2X QIAGEN Multiplex Master Mix, and 0.2 µM of each primer (Table 1). Thermal cycling involved an initial 15 minutes enzyme activation at 95°C, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute 30 seconds, and extension at 72°C for one minute, and a final extension at 60°C for 30 minutes.

Table 1: Singleplex PCR components (Total volume: 50 µL)

Component	Concentrattion	Volume (µL/tube)
HPLC H_2O		21
2X QIAGEN Multiplex MasterMix	1 X	25
Forward primer	0.2 µM	1
Reverse primer	0.2 µM	1
DNA template		2

Multplex PCR optimization

Multiplex PCR was performed at optimal annealing temperature and primer concentrations initially keeping at the same concentration of 0.2 µM. Then, each primer concentration was adjusted based on product signal strength by increasing or decreasing 0.05 µM. The multiplex PCR amplification was performed in 50 µl reaction consisted of 1X QIAGEN Multiplex PCR Master Mix, 0.05-0.4 µM of each primer and 100 ng DNA template; followed the protocol: an

initial 15 min enzyme activation at 95°C, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 1 min 30 s at the first cycle and 6 additional seconds for every next cycle, and extension at 72°C for 1 min.

Capillary electrophoresis

The PCR products were fluorescently labeled in a 20 µL mixture consisted of 1X QIAGEN Multiplex MasterMix, 0.2 µM each fluorescent primer with M13 sequences as in Table 2, following cycling condition: an initial denaturation step at 95°C for 15 min, followed by 5cycles of denaturation at 98°C for 45 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

One µL aliquot of fluorescent PCR product was mixed with 8.5 µL of Hi-Di Formamide (Applied Biosystems, Foster City, CA, USA) and 0.5 µL of GeneScan 500 LIZ size standard, denatured at 95°C for 5 minutes, cooled to 4°C, and resolved in ABI 3130XL Fragment Analyzer (Applied Biosystems). Post-electrophoresis analysis was performed using GeneMapper 5.0 software (Applied Biosystems).

Statistical analysis

Allele frequency, PIC, expected heterozygosity (He) and Observed Heterozygosity (Ho) of the 15 microsatellite markers were calculated using Microsoft Excel.

Results

Identification of STR

Fifteen (15) STRs were identified within 1 Mb around the *HBB* gene (6 upstream STRs and nine downstream STRs) (Table 2). HBB4506 located farthest to HBB gene (0.74 Mb) and all other markers were comparatively closer. Thus, all STRs had high linkage to the HBB gene.

Table 2: Information on initial selected STR

No.	STR	Repeat	Size (bp)	Location with HBB gene
1	HBB4506	(AC) n	366-398	Downstream
2	D11S988	(TG) n	103-147	Downstream
3	HBB4677	(AC) n	172-214	Downstream
4	D11S2362	(AAT) n	87-123	Downstream
5	HBB5089	(AC) n	241-265	Downstream
6	D11S1243	(TG) n	220-256	Downstream
7	HBB5138	(AC) n	404-428	Downstream
8	HBB5178	(TG) n	158-192	Downstream
9	HBB5205	(AGAT) n	401-449	Downstream
10	D11S1760	(CA) n	195-241	Downstream
11	HBB5576	(AAGG) n	327-369	Upstream
12	HBB5655	(AC)n(AT) n	272-320	Upstream
13	HBB5820	(AC)n(AG) n	311-331	Upstream
14	HBB5859	(ATCT) n	375-417	Upstream
15	D11S1338	(AC) n	137-157	Upstream

Forward primer had an additional sequence for fluorescent primer M13 were shown in Table 3.

Table 3: Information of primers for STR amplification

STR	Primer	Sequence (5'-3')	T_m (°C)
HBB 4506	B1F*	GTAAAACGACGGCCAGTGGTTTGACATATCTGTGAG GAAG	71.9
	B1R	GTTTCAGCAAGTAAATAGGGCACTG	62.9
D11S988	B2F**	GGTTTTCCCAGTCACGACGGACAAGAGAAAGTTGAA CATACTG	72.3
	B2R	GTTTCCACCATTTAAGATGCCAATAAGC	63.2
HBB 4677	B3F*	GTAAAACGACGGCCAGTGTGTAAAAGGGCCTCTAAT CAG	72.1
	B3R	GTTTCACTGATATACAAATGGCAAAGTG	61.7
D11S236 2	B4F*	GTAAAACGACGGCCAGTGCTTCCCTRATCTGGAATG AACTC	73.2
	B4R	GGGTTTCCCAGTCCTTTTAC	60.4
HBB 5089	B5F**	GGTTTTCCCAGTCACGACCAATTTCCTTTTCTTCTCC CTATAC	71.4
	B5R	GTGAGTCTAGCATTTGTCTTGC	60.8
D11S124 3	B6F*	GTAAAACGACGGCCAGTGCTGCCCTAATTCTGTCTAC C	73.3
	B6R	GTTGTGCACYATGAAGATACAC	59.9
HBB 5138	B7F*	GTAAAACGACGGCCAGTGAGAAATGTCCTTTAGAGA AATACCTTC	71.1
	B7R	GTGGAGAGGAATCTRTTACTG	59.6
HBB 5178	B8F**	GGTTTTCCCAGTCACGACCGTAATTGCTTTCAGTACC ATTTATG	71.2
	B8R	GATGTATTCGTCAACAGATAAATGG	59.7
HBB 5205	B9F**	GGTTTTCCCAGTCACGACCCAGGGTAGGTGACATAT AC	73.3
	B9R	GTAACTCAAAAAATGGGACCCAAAC	61.3
D11S176 0	B10F**	GGTTTTCCCAGTCACGACACCCTGAGTGTCTTCAAAA CTC	72.9
	B10R	GTTTCCAAKACTGCTGCATCATGAC	63.8
HBB 5576	B11F**	GGTTTTCCCAGTCACGACTCCTTCAGGTAAGAAGGA GC	73.3
	B11R	CTTGAAGAGGCTAGGTGC	59.9
HBB 5655	B12F**	GGTTTTCCCAGTCACGACTCATTGTTTTGGTAGGTAC TGAAAG	71.4
	B12R	AGTTGTAGTAAGTTTGTCAGGCTA	59.4
HBB 5820	B13F*	GTAAAACGACGGCCAGTGCTGAGATTATTTATACAGC AACACTTG	71.1
	B13R	GTTTCCAGTTATTGGTTGCTTTAGATTAC	63.3
HBB 5859	B14F**	GGTTTTCCCAGTCACGACCTGTCTATTTCATCTGTCA GCTTC	72.5
	B14R	GTTTAAAGTGTTGGCGTGAGC	60.6
D11S133 8	B15F*	GTAAAACGACGGCCAGTGAAGGACACACAGATTCAC TTAAAG	71.5
	B15R	GCTACTTATTTGGAGTGTGAATTTC	59.7
	T_m means		66.6

* Primer fluorescently labeled with HEX; ** Primer fluorescently labeled with FAM.

Multiplex PCR Optimization

Annealing temperature (T_a) and primer concentration optimization were performed. The results of T_a optimization of 15 primer were shown in Figure 1.

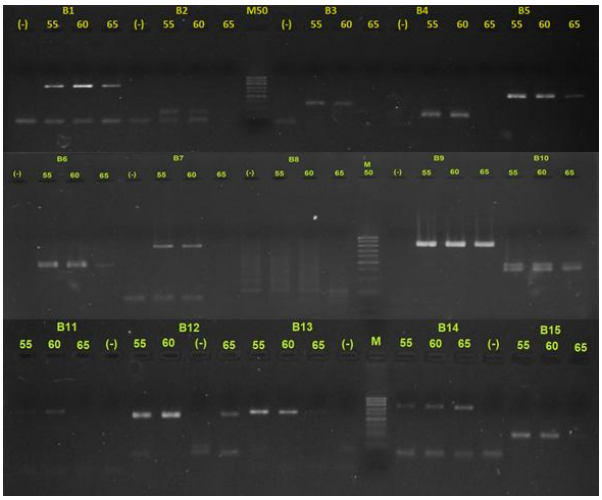

Figure 1: Annealing temperature optimization of 15 STRs. Annealing temperature at 60°C gave clear bands and most uniform signal. The products were electrophoresed on 1% agarose gel, at 120V for 30 minutes. The 10 µl sample was each well

The optimal annealing temperature showed clear bands and fair signals for all 15 primers (Figure 1). We finally concluded that 60°C was the optimal annealing temperature.

After adjusting primer concentrations (0.05-0.4 µM), optimal Multiplex PCR gave clear peaks. The PCR products were fluorescently labeled for capillary electrophoresis (Figure 2).

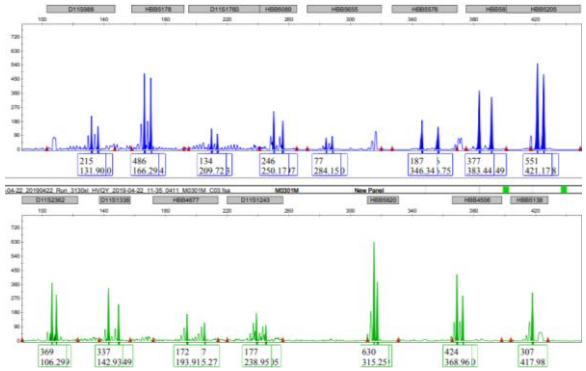

Figure 2: Representative electropherogram of multiplex PCR product after optimization

Polymorphism evaluation

To determine the polymorphism and informativeness of the 15 markers, the allele frequencies, PIC, He, and Ho values of each marker were calculated. Most STRs sequences were successfully amplified in all individuals except D11S1760 (84/106) and HBB5655 (81/106) caused by PCR Failure.

In summary, 270 alleles were observed with 11-28 alleles per marker. Allele frequency ranged from 0.0047 to 0.3255 (Table 4). All marker had PIC values of ≥ 0.7 and among these markers ranged, HBB5178 showed the lowest polymorphism (0.79), and D11S1760 showed the highest polymorphism (0.91).

The data showed that *He* values ranged from 0.81 (HBB5138) to 0.92 (D11S1760), and all markers had *Ho* values of ≥ 0.5, guaranteeing high polymorphic information. The *Ho* values of markers ranged from 0.53 (HBB5655)-0.86 (HBB5205). Thus, HBB5205 was the most informative marker, while HBB5655 was the least informative marker. Furthermore, the number of heterozygous markers of each individual was also counted.

Data showed 100% of individuals had at least seven heterozygous markers (Figure 4). Also, all were observed to be heterozygous for at least two markers on either side of the *HBB* gene (Figure 5). Based on the results, all 15 markers are high in polymorphism and informativeness for the Vietnamese population.

Table 4: Distribution of observed allele frequencies of 15 microsatellite markers

HBB4506		D11S988		HBB4677		D11S2362		HBB5089	
Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency
364	0.0094	115	0.0472	173	0.0047	99	0.0048	242	0.0142
369	0.0283	116	0.0236	179	0.0047	101	0.0095	243	0.0425
371	0.0566	120	0.0047	183	0.0283	103	0.0095	244	0.0189
372	0.0047	121	0.0142	184	0.0047	104	0.0429	248	0.0142
373	0.1651	122	0.0283	185	0.2123	105	0.0048	249	0.0236
375	0.0755	123	0.0189	186	0.0283	106	0.1095	250	0.0991
376	0.0047	124	0.2783	187	0.0047	107	0.2048	251	0.1321
377	0.0094	125	0.0142	188	0.0047	108	0.0048	252	0.0519
380	0.1651	126	0.1038	190	0.0142	109	0.219	253	0.0142
381	0.1462	128	0.1274	191	0.0047	110	0.0857	254	0.0472
382	0.066	129	0.0047	192	0.0094	111	0.0095	255	0.0896
383	0.0425	130	0.0472	193	0.0189	112	0.1	256	0.1274
384	0.1509	131	0.0094	194	0.1085	113	0.1095	257	0.283
385	0.0142	132	0.033	195	0.1179	114	0.0095	258	0.0142
386	0.0142	134	0.0189	196	0.0236	115	0.019	259	0.0094
387	0.0047	136	0.0613	197	0.0236	116	0.0286	260	0.0189
388	0.0377	137	0.1132	198	0.0047	118	0.0048	242	0.0142
390	0.0047	138	0.0189	200	0.0189	119	0.0143	243	0.0425
		139	0.0189	201	0.0047	121	0.0095	244	0.0189
		140	0.0047	203	0.0189				
		141	0.0047	204	0.0755				
		143	0.0047	205	0.0566				
				206	0.1321				
				207	0.0472				
				208	0.0047				
				210	0.0142				
				212	0.0047				
				213	0.0047				

D11S1243		HBB5138		HBB5178		HBB5205		D11S1760	
Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency
224	0.0047	404	0.0802	164	0.1557	392	0.0047	201	0.0833
235	0.0047	405	0.3208	165	0.1698	409	0.0047	202	0.0476
236	0.0094	406	0.0142	166	0.1179	413	0.0094	203	0.0893
237	0.0094	414	0.0189	167	0.1274	417	0.0802	204	0.0179
238	0.0283	415	0.0047	168	0.0094	418	0.0047	205	0.0476
239	0.0943	416	0.0047	169	0.0142	420	0.0047	206	0.0119
240	0.1415	417	0.0189	170	0.0377	421	0.2311	210	0.1607
241	0.0189	418	0.184	171	0.3255	422	0.0142	211	0.0476
242	0.1651	419	0.1557	172	0.0189	425	0.2358	213	0.006
243	0.1085	420	0.1368	173	0.0094	426	0.0047	214	0.119
244	0.2406	421	0.033	177	0.0047	429	0.2217	215	0.0417
245	0.033	422	0.0142	181	0.0047	430	0.0189	216	0.0238
246	0.066	423	0.0094	183	0.0047	431	0.0283	217	0.006
247	0.0189	424	0.0047			433	0.066	218	0.0893
248	0.0283					435	0.0472	219	0.0357
249	0.0047					437	0.0094	221	0.006
250	0.0189					439	0.0047	222	0.0238
254	0.0047					440	0.0047	226	0.006
						442	0.0047	227	0.0298
								228	0.0714
								230	0.0119
								231	0.0119
								277	0.0119

HBB5576		HBB5655		HBB5820		HBB5859		D11S1338	
Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency	Allele	Frequency
330	0.0047	283	0.0123	294	0.0047	374	0.0047	142	0.0047
338	0.0047	284	0.0988	313	0.0047	383	0.283	143	0.1651
339	0.0047	285	0.1049	315	0.1179	384	0.1604	144	0.1274
340	0.0283	286	0.0556	316	0.0991	387	0.0755	145	0.0142
341	0.0094	287	0.0123	317	0.2877	388	0.0472	146	0.0236
342	0.0425	288	0.0679	318	0.0472	391	0.0991	147	0.0047
343	0.1368	289	0.179	319	0.1038	392	0.1226	149	0.033
344	0.0708	290	0.1543	320	0.0094	395	0.0236	150	0.217
345	0.0425	291	0.0247	321	0.0189	396	0.1274	151	0.0377
346	0.0472	292	0.0494	323	0.0047	397	0.0094	152	0.2311
347	0.1415	293	0.0247	324	0.0377	400	0.0472	153	0.0943
348	0.0142	294	0.037	325	0.0472			154	0.033
349	0.0142	315	0.0247	326	0.0142			156	0.0094
350	0.2217	316	0.0864	327	0.0094			157	0.0047
351	0.0047	318	0.0617	328	0.0236				
352	0.0142	325	0.0062	329	0.1274				
353	0.1179			330	0.0283				
354	0.0377			331	0.0094				
357	0.0377			333	0.0047				
360	0.0047								

In general, the study had established a high polymorphic STR panel and relatively close with HBB gene. The panel should be potential to perform PGT for β-thalassemia

Table 5: Observed Heterozygosity, Expected Heterozygous and Polymorphic Information Content values of 15 STRs

Order	Markers	Heterozygous	Total	H_o	He	PIC
1	HBB4506	84	106	0.79	0.883	0.872
2	D11S988	87	106	0.82	0.87	0.86
3	HBB4677	90	106	0.85	0.896	0.888
4	D11S2362	88	105	0.84	0.865	0.851
5	HBB5089	75	106	0.71	0.859	0.847
6	D11S1243	86	106	0.81	0.866	0.853
7	HBB5138	59	106	0.56	0.811	0.789
8	HBB5178	67	106	0.63	0.809	0.785
9	HBB5205	91	106	0.86	0.827	0.805
10	D11S1760	53	84	0.63	0.919	0.914
11	HBB5576	83	106	0.78	0.883	0.873
12	HBB5655	43	81	0.53	0.898	0.89
13	HBB5820	70	106	0.66	0.858	0.846
14	HBB5859	85	106	0.80	0.842	0.825
15	D11S1338	68	106	0.64	0.843	0.824

Discussion

For many years, despite medical advances, the treatment of a β-Thalassemia patient still confronts many obstacles because it is impossible to completely treat the disease. Along with the treatment of individuals with β-Thalassemia, the application of PGT-M to help at-risk couples of having healthy babies is an extensively studied field.

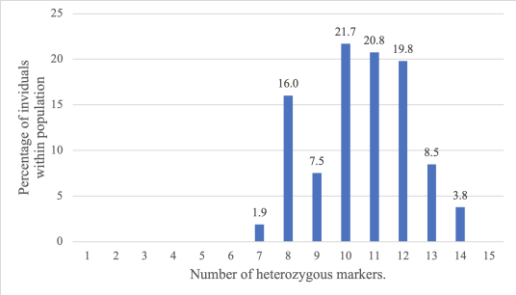

Figure 4: Percentage of individuals for number of heterozygous markers

Direct techniques have been commonly applied in diagnosis. However, for a wide gene such as HBB with a various kind of mutations, direct methods may be ineffective when using Multiplex PCR to simultaneously amplify many mutation sequences, and indirect techniques applying microsatellite markers should be the only option for this situation.

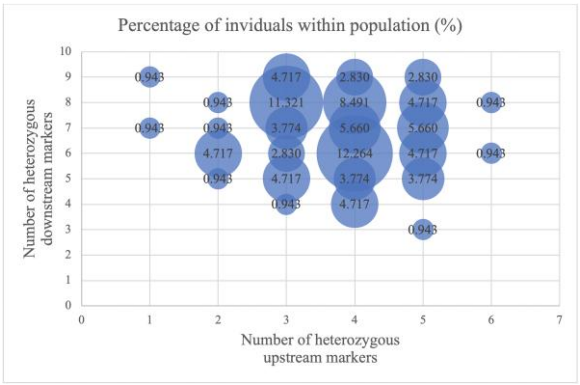

Figure 5: Percentage of individuals heterozygous for different numbers of upstream and downstream flanking microsatellite markers (n = 106)

The application of both direct and indirect techniques will increase the accuracy of the diagnosis. Therefore, linked markers play an essential role in PGT-M β-Thalassemia disease. There have been many studies of microsatellite markers [10], [11], [12], [13], [14], [15], [16], [17], but the number of STRs is still limited, so the detection of new STRs and surveys on different populations are crucial.

In this study, we developed a linked-marker set consisting of 15 STRs used for PGT-M of Vietnamese β-Thalassemia patients. Fifteen published STR located < 1 Mb distance from *HBB* were successfully amplified in one PCR amplification on Vietnamese at-risk individuals with high results in Ho, He and PIC estimation. We observed that 100% individuals were heterozygous for at least 7 of the 15 markers and at least 2 of these heterozygous markers were on either side of *HBB* gene, reliably proving the high polymorphism and informativeness of them in most if not all Vietnamese β-Thalassemia cases.

The studied STRs panel was first established and researched on five populations by Cheng [9]. Our data on Vietnamese cases were consistent with their result and replicated the previous result in the Vietnamese Population Beside the PCR failure happening with D11S1760 (84/106) and HBB5655 (81/106), other STRs were successfully amplified with no contamination and ADO phenomenons. Most previous PGT-M on β-Thalassemia cases included nested-multiplex PCR, however by combining 15 microsatellite markers into a standard multiplex PCR reaction, nested PCR should be removed. For the lack of available published STRs on Vietnamese population, establishment of STR set that compatible with Vietnamese population contributed great support to decline the high rate of Vietnamese β-Thalassemia carriers.

STRs contained different polymorphism and informativeness, but all showed the high values of PIC, Ho, He proving their clinical application. Our method had high sensitivity and specificity along with sufficient contamination and ADO monitoring. Microsatellite markers became a reliable tool when using alone in indirect mutation detection or conjunction with direct mutation detection for more precise diagnosis. Besides, this method required highly trained staffs, accurate instrument, and optimal procedures. Therefore, a particular training program needs carrying out before performing this method.

In addition to the previous study from different populations, the STRs panel continuously showed tremendous potential when applying on various kinds of population. Thus, we suggest applying this STRs panel for PGT in clinical cases.

In conclusion, All 15 STR markers were polymorphic and informative with high Ho, He and PIC. At least 7 of 15 markers were informative for 106 studied individuals, and all were observed to be heterozygous for at least two markers on either side of the HBB gene. Thus, these STRs markerhave significant meaning when applied in PGT-M widely.

Authors' contributions

DTT, NVNM, DPN, HVL, DQ, TNA, TTS, NTT, NTTH, DTPA, NLT, HTH, LH and NDB designed and performed experiments, and collected data and

informed consents. DTT, NVNM, DPN, HVL, DQ, TNA, TTS, NTT, NTTH, DTPA, NLT, HTH, LH, NDB, VTN and DTC analyzed and interpreted the results, and edited and corrected the manuscript. DTT, NVNM, VTN and DTC wrote the manuscript. All authors approved the final manuscript.

Ethical approval

This study is approved by the ethics committee of the Tam Anh General Hospital on 21 April 2018 following the Decision No 59/QĐ/BĐKTA by the director of the Tam Anh General Hospital about the establishment of the ethical committee.

Informed consent

The informed consents were signed by patients and their male partners

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