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A Method of Screening for Trisomy 18 by Detecting Amniotic Fluid Punctures

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Abstract

Objective: We aimed to develop a new method of screening for Trisomy 18 by detecting amniotic fluid punctures to complement the current methods.

Method: Two commercially available genomic DNA extracted from the amniotic fluid puncture of the pregnant woman with the Trisomy 18 fetus, two genomic DNAs extracted from two healthy male and four genomic DNAs extracted from four healthy female were used as the qPCR template DNAs and the commercially available Sybr green qPCR mater mix were used; we designed and synthesized 5 pairs of qPCR primers respectively corresponding to IL-10 gene on 1# chromosome, STAT1 gene on 2# chromosome, CXCR3 gene on X chromosome, TSPY1 gene on Y chromosome and LINC01910 gene on 18# chromosome. We then performed Sybr green qPCR measurement.

Results: We processed the qPCR data by mathematical calculation and finally formed a new algorithm. Using the new algorithm, we easily distinguished the Trisomy 18 female samples out of the normal female samples.

Conclusion: We developed a new method of screening for Trisomy 18 for the female fetus by detecting amniotic fluid punctures to complement the current methods.

Keywords: Trisomy 18; Amniotic fluid puncture; Sybr green qPCR; Algorithm; Index for TRISOMY 18

Introduction

Trisomy 18 syndrome, also known as Edwards syndrome, mainly includes abnormalities in the mesoderm and its derivatives (such as bone, urogenital system, the heart is most obvious) [1-3]. In addition, the ectoderm close to the mesoderm (such as skin folds, cuticle cristae and hair) and the endoderm (such as Meckel's diverticulum, lungs and kidneys) are also abnormal. It is reported that for Trisomy 18, the embryo develops normally before 5 weeks, and begin to appear abnormal at the 6th to 8th week of gestation [4].

There is no effective treatment for Edwards syndrome. The best course of action is to terminate the pregnancy before delivery. Prenatal "screening" methods for Edwards syndrome include serological screening and noninvasive prenatal testing [5,6]. Prenatal "diagnosis" of Edwards syndrome includes: karyotype analysis, chromosome microarray or gene chip testing of amniotic fluid, chorionic tissue, or fetal cord blood. Amniocentesis tests (including karyotype analysis, chromosomal microarrays and gene microarrays) are considered the "gold standard tests".

For the "gold standard test" to confirm Trisomy 18, specific detection techniques include karyotype analysis, chromosome microarray, gene chip, etc. However, the above "gold standard tests" have some obvious drawbacks: (1) the material cost of these tests is relatively high, all more than $80 per person by rough calculation; (2) the time cost of karyotype analysis is high (at least 12 days). In addition, the cells may change during proliferation due to the status of chromosomes, and the detection requires skilled operators; (3) although the time cost of chromosomal microarray and gene microarray detection is relatively low, their sample waiting time is longer because they need to complete the minimum sample size required for chromosomal microarray or gene microarray detection due to cost consideration.

In this study, we completed a pilot study which aimed at establishing a new complementary method for the identification of Trisomy 18 by amniocentesis. The method was based on Sybr Green qPCR and its characteristics were: (1) the genomic DNA of fetal cells in amniotic fluid was directly extracted for detection without cell proliferation and culture, which greatly shortened the detection time; (2) single sample detection is not limited by the minimum number of samples and does not require waiting time; (3) the result judgment is based on the preset threshold, which has good objectivity; (4) the new method is positioned as a supplementary detection method of karyotype analysis, chromosome microarray and gene microarray detection. The new method is not to replace the "gold standard detection", but to strengthen the "gold standard detection", which has potential promotion potential. The idea frame of this study has already been successfully fulfilled in the detection of Trisomy 21 [7] and Trisomy 13 [8] before, and it would be a novel try to apply this idea frame in the detection of Trisomy 18.

Materials and Methods

The DNA templates for Sybr green qPCR and ethics

Two healthy man and four healthy women had oral epithelial cells collected as health samples by mouth swabs and signed informed consent; after collection, the corresponding genomic DNA was rapidly extracted using BIOG DNA Swab kit (Catalog No.: 51029, Lot No.: 202301, Changzhou, China). The extracted DNA was used as the template DNA for normal samples in Sybr green qPCR detection.

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The genomic DNA of Trisomy 18 fetus was obtained by purchasing the commercial human chromosome polyploid assay kit (Product No.: YST0015-01, Lot No.: 202301001) from GIMT Co.Ltd, Guizhou, China). This genomic DNA of the Trisomy 18 fetus was extracted from amniotic fluid puncture of the pregnant woman with Trisomy 18 fetus, which was used as a positive sample for subsequent Sybr green qPCR detection.

Primers for Sybr green qPCR

Five pairs of primers were designed and synthesized, corresponding to IL-10 gene on chromosome 1, STAT1 gene on chromosome 2, CXCR3 gene on chromosome X, TSPY1 gene on chromosome Y and LINC01910 gene on chromosome 18. The sequence and amplicon information were shown in Table 1.

qPCR instrument and qPCR reaction conditions

The qPCR instrument was Stepone Plus (ABI Co.Ltd, USA). Sybr green qPCR solution was purchased from China Qingke Biotechnology Co., LTD. (Product No.: TSE201, Lot No.: 202212). The setting of qPCR detection was: (1) 4 repeated reactions for each gene of each sample; (2) a total of 8 samples were used: normal samples (labeled as Female-1, Female-2, Female-3, Female-4, Male-1, Male-2) and 2 Trisomy 18 (labeled as 18#-1, 18#-2); (3) Sybr green qPCR was used to detect the contents of the following five genes: IL-10 gene on chromosome 1, STAT1 gene on chromosome 2, CXCR3 gene on chromosome X, TSPY1 gene on chromosome Y, and LINC01910 gene on chromosome 18#; (4) totally, 160 reactions were performed: 8 samples × 5 genes × 4 repeats = 160 reactions; (5) the total reaction volume of each reaction was 20 μL, 2 ng template DNA was used for normal samples, and 2 μL positive solution was used for Trisomy 18 sample. Because the positive solution is a commercially available product with a small volume (totally 5 μL), its amount was difficult to accurately control. Therefore, we chose 2 μL volume of Trisomy 18 positive solution as a compromise; (6) reaction conditions: 1 cycle: 95°C, 3 min; 69 cycles: 95°C 20 s, 55°C 30 s (this step to collect fluorescence signals); melting point curve detection: 65°C to 95°C, temperature rise gradient: 0.1°C, 10 s (this step to collect fluorescence signals).

Results

The first step to establish the new method was the preliminary evaluation and processing of the raw data

After obtaining the original data of Sybr green qPCR (Table 2), the data were preliminatively evaluated and processed: (1) calculate the mean value of the four experimental replicates (Table 3 was the example); (2) calculate the mean deviation (Table 3); (3) calculate the precision index: precision index = mean deviation/mean value of 4 experimental replicates (Table 3 was the example); (4) for data sets with precision index greater than 5%, carry out detailed data inspection (Pauta criterion, Chauvenet criterion, Grubbs criterion, Dixon criterion and Romanovsky criterion would be used to identify the data outlier for the data sets with precision index greater than 5%) [9]. for the entire data, we found that the data precision accuracy index of all samples for all genes was less than 5%. Therefore, there was no significant data

Table 1: Primer information (the suffix "-F" stands for "forward primer"; the suffix "-B" stands for "backward primer").

Chromosome number	Primer name	Primer sequence	Amplicon size (number of base pairs)
1	IL10-1#-F	tgagctgcat gctggggaga	120
1	IL10-1#-B	gtatttgtat gtgcacattt	120
2	STAT1-2#-F	gatattccta aatgctttgg	120
2	STAT1-2#-B	tgtttaacca ttaatttggc	120
21	Sumo3-21#-F	agaagggcag cgtctttgag	120
21	Sumo3–21#-B	taaaaaagaa aaaaaagttt	120
X	CXCR3-X-F	acatagttca tgccacccag	120
X	CXCR3-X-B	ccgacaggaa gatgaagtct	120
Y	TSPY1-Y-F	agctttctcc accttgtcct	120
Y	TSPY1-Y-B	tattccttgg taatcacttt	120
18	LINC01910-18#-F	attgcaattc ccctggcttg	120
18	LINC01910-18#-B	gctggaatgc agtggtacaa	120

Table 2: Partial original data of Sybr green qPCR reaction: 4 samples × 5 genes × 4 replicates.

	IL10-1#	STAT1-2#	CXCR3-X	TSPY1-Y	LINC01910-18#
Female-1	28.896	32.017	28.443	36.721	27.926
Female-1	28.13	30.254	28.537	36.743	27.79
Female-1	28.258	30.419	28.861	35.992	27.962
Female-1	28.41	30.074	28.449	37.31	28.004
Female-2	27.884	30.005	28.933	34.954	27.702
Female-2	27.548	29.96	28.841	36.242	27.772
Female-2	27.728	29.915	28.859	34.816	27.772
Female-2	27.743	30.28	28.886	35.728	27.73
Male-1	29.283	35.295	30.254	26.56	28.816
Male-1	29.409	34.811	30.09	26.689	28.663
Male-1	28.988	32.688	29.925	26.506	28.929
Male-1	29.123	36.004	29.757	26.796	28.919
18#-1	34.525	37.728	32.9	43.768	33.804
18#-1	33.823	36.578	33.083	41.96	33.204
18#-1	33.92	36.448	33.486	37.957	32.973
18#-1	35.114	39.638	33.909	44.799	33.128

Table 3: Preliminary analysis and processing of partial raw data of Sybr green qPCR.

		IL10-1#	STAT1-2#	CXCR3-X	TSPY1-Y	LINC01910-18#
Female-1	Average	28.424	30.691	28.573	36.691	27.921
	Standard deviation	0.335	0.895	0.197	0.54	0.093
	Precision index	1.18%	2.92%	0.69%	1.47%	0.33%
Female-2	Average	27.726	30.04	28.88	35.435	27.744
	Standard deviation	0.138	0.164	0.04	0.671	0.034
	Precision index	0.50%	0.55%	0.14%	1.89%	0.12%
Male-1	Average	29.201	34.7	30.006	26.638	28.832
	Standard deviation	0.184	1.428	0.214	0.13	0.124
	Precision index	0.63%	4.12%	0.71%	0.49%	0.43%
18#-1	Average	34.346	37.598	33.345	42.121	33.277
	Standard deviation	0.599	1.476	0.449	3.014	0.364
	Precision index	1.74%	3.93%	1.35%	7.16%	1.09%

Table 4: Judgment on the gender of the samples.

	$2^{-(CXCR3-TSPY1)}$	Sex type
Female-1	277.999	Female
Female-2	94.032	Female
Female-3	14.333	Female
Female-4	13.851	Female
Male-1	0.097	Male
Male-2	0.023	Male
18#-1	438.522	Female
18#-2	16.096	Female

Table 5: Judgment of existence of trisomy of chromosome 18.

	Index for Trisomy 18	Existence of Trisomy 18
Female-1	15.19	Not exist
Female-2	10.6	Not exist
Female-3	0.81	Not exist
Female-4	7	Not exist
18#-1 (female positive control)	44	Exist
18#-2 (female positive control)	51	Exist

outlier in all the data, which could be used for further data processing in the next step.

The second step to establish the new method was to determine the sex of the sample

After preliminary processing of the data, the sex type of the sample was determined: the value $2^{-(CT\ of\ CXCR3-CT\ of\ TSPY1)}$ (quantity ratio of CXCR3 to TSPY1) was calculated, and if the value was greater than 1, the sample sex type was judged as female, and if the value was less than 1, the sample sex type was judged as male. This criterion was based on the basic fact that CXCR3 is on the X chromosome and TSPY1 is on the Y chromosome. The detailed principles of this sex determination method were published before [10]. Based on the criteria and data, it was easy to distinguish male samples from female samples. In addition, the sex type of two trisomy chromosome 18 samples was judged to be female (Table 4).

The third step to establish the new method was to calculate the index of Trisomy 18 and make a judgment based on that index

After determining the sex type of the sample, the trisomy index of chromosome 18 was calculated: (1) for the male sample, the trisomy index of chromosome 18 in males was calculated as $2^{-(CT\ of\ LINC00458-CT\ of\ IL-10)}\times 2^{-(CT\ of\ LINC00458-CT\ of\ STAT1)}\times 2^{-(CT\ of\ LINC00458-CT\ of\ CXCR3)}\times 2^{-(CT\ of\ LINC01910-CT\ of\ TSPY1)}$, which, in fact, was the multiplying value of the amount ratio between LINC01910 and IL-10, the amount ratio between LINC01910 and STAT1, the amount ratio between LINC01910 and CXCR3, and the amount ratio between LINC01910 and TSPY1 (Table 5); (2) for the female sample, the trisomy index of chromosome 18 in females was calculated as $2^{-(CT\ of\ LINC00458-CT\ of\ IL-10)}\times 2^{-(CT\ of\ LINC00458-CT\ of\ STAT1)}\times 2^{-(CT\ of\ LINC00458-CT\ of\ CXCR3)}$, which, in fact, was the multiplying value of the amount ratio between LINC01910 and IL-10, the amount ratio between LINC01910 and STAT1, the amount ratio between LINC01910 and CXCR3. The female index missed the amount ratio between LINC01910 and TSPY1 because TSPTY1 was on the Y chromosome, while the female sample had no Y chromosome; (3) because the sex of the two Trisomy 18 were female, this study focused on the comparison of "female Trisomy 18 index" between the four normal female samples and the Trisomy 18 samples. It could be seen from Table 5 that for the four female samples, the indexes of Trisomy 18 were 15.19, 10.6, 0.81, 7.0 (average: 8.42; standard deviation: 6.08); meanwhile, the index for the two Trisomy 18 positive samples was 44.0 and 51.0 (average: 47.5; standard deviation: 4.95), which showed a very significant difference ($P < 0.001$), indicating that the index could effectively distinguish the Trisomy 18 female sample from the normal female sample.

Discussion

The newly established method had two main highlights. The first highlight was that the multiplying value of several ratios was used, which accumulates and magnifies the small increase of chromosome 18#, so that the examiner could easily identify the difference. The second highlight was that some of the random errors could be offset by the multiplying value of several ratios.

Because our Trisomy 18 positive samples were by chance female, we at this time could only establish the screening method for triomy 18 for the female fetus. In the future, if we could obtain the DNA samples from the Trisomy 18 male fetus, we would be able to establish the screening method for triomy 18 for the male fetus. Additionally, another drawback of our study was that this new method could not deal with the situation that the pregnant woman has the twin babies. So, further exploration should be continued [11].

Conclusions

Based on the above exploration, we originally created the trisomy index for chromosome 18 for the female fetus and finally, we established the new screening method or procedure for detecting Trisomy 18 for the female fetus: (1) obtain the amniotic fluid from the pregnant woman; (2) extract the genomic DNA from this amniotic fluid; (3) perform the Sybr green qPCR using the extracted genomic DNA as the template

and using the primers listed in Table 1; (4) according to the qPCR data, judge the sex type of sample and calculate the Trisomy 18 index for the female, which was: the trisomy index of chromosome 18 is calculated as $2^{-(\text{CT of LINC00458-CT of IL-10})} \times 2^{-(\text{CT of LINC00458-CT of STAT1})} \times 2^{-(\text{CT of LINC00458-CT of CXCR3})}$; (5) if the sex type for the sample was female and the calculated Trisomy 18 index of the female was calculated bigger or near 47.5, there was high possibility for Trisomy 18 for this female fetus.

In summary, we initiated a pilot study to establish a simple, rapid and low-cost method for screening Trisomy 18 female fetuses from amniotic fluid puncture. The method established in this study could enhance and complement existing methods. This study opened up some new directions in the field of detection.

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