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Establishing and validating non-invasive prenatal testing procedure for fetal aneuploidies in Vietnam

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Keywords:NIPT, trisomy, aneuploidy,

Abstract

Objective: Non-invasive prenatal testing (NIPT) for fetal aneuploidies has been widely adopted in developed countries. Despite the sharp decrease in the cost of massively parallel sequencing, the technical know-how and skilled personnel are still one of the major limiting factors for applying this technology to NIPT in low-income settings. Here, we present the establishment and validation of our NIPT procedure called triSure for detection of fetal aneuploidies.

Methods: We established the triSure algorithm based on the difference in proportion of fetal and maternal fragments from the target chromosome to all chromosomes. Our algorithm was validated using a published data set and an in-house data set obtained from high-risk pregnant women in Vietnam who have undergone amniotic testing. Several other aneuploidy calling methods were also applied to the same data set to benchmark triSure performance.

Results: The triSure algorithm showed similar accuracy to size-based method when comparing them using published data set. Using our in-house data set from 130 consecutive samples, we showed that triSure correctly identified the most samples (overall sensitivity and specificity of 0.983 and 0.986, respectively) compared to other methods tested including count-based, sized-based, RAPIDR and NIPTeR.

Conclusion: We have demonstrated that our triSure NIPT procedure can be applied to pregnant women in low-income settings such as Vietnam, providing low-risk screening option to reduce the need for invasive diagnostic tests.

Introduction

Non-invasive prenatal testing (NIPT) for fetal aneuploidies (trisomy 13, 18 and 21) has been widely adopted in developed countries in recent years [1,2]. Since its introduction into the clinics, NIPT has showed high sensitivity and specificity across multiple clinical validation studies [1,3–7] and received endorsement of key organizations including the American College of Obstetrics and Gynecology (ACOG), the Society of Maternal and Fetal Medicine (SMFM) [8], the National Society of Genetic Counselors (NSGC) [9] and the International Society of Prenatal Diagnosis (ISPD) [10]. A recent study suggested that universal application of NIPT would increase fetal aneuploidy detection rates and might be economically justified [11] but genetic counselors' opinions are evenly split on the merits of expanding the use of NIPT to the general population [12]. Despite rapid decrease in the cost of massively parallel sequencing (MPS) as the technology reaches maturity, the adoption rate of MPS-based testing such as NIPT remains low in low- and middle-income countries due to several reasons including large capital investment of such platform as well as high running costs and limited expertise available locally [2]. Therefore, a successful establishment of NIPT protocol in low-income settings would be beneficial to these countries.

Chiu et al (2008) reported the count-based (CB) method for detection of trisomy 21 using percentage of unique count for chromosome 21 over total unique count and comparing it with the percentage from a group of euploid samples as reference by mean of z-score [13]. To increase sensitivity and specificity, various methods were used including GC-content correction [14,15] and mappability correction [15]. Several analysis packages were also published that can combine different correction methods and improvements in z-score calculations such as RAPIDR [16] and NIPTeR [17]. Attempts to build reference-free detection algorithm have also been made, such as WISECONDOR [18] and COFFEE [19]. An interesting development in detection algorithm was the use of fragment size to separate fetal and maternal derived fragments within a sample [20]. Specifically the size-based (SB) method calculates differences in the proportions of short DNA (#150 bp) between the target and reference chromosomes. This SB algorithm provides a simple alternative to CB method without employing complicated correction procedures.

The SB algorithm is simpler and faster in comparison to CB with GC and mappability correction whilst maintaining good performance. However, it uses only a small portion of the sequence data, ie. the small fragments # 150 bp. Thus, we devised triSure method that make use of both small (# 150 bp) and large fragments (# 170 bp) in the data to improve the senstitivity and specificity of the test. We aimed to demonstrate that the performance of our triSure NIPT protocol was comparable to, if not better than, that of SB method in calling fetal aneuploidy for high-risk pregnant women in Vietnam.

Materials and Methods

The triSure algorithm

The fetal derived cell-free DNA fragments have been shown to exhibit shorter length than maternal DNA. This characteristic was previously used by Yu et al. to develop a size-based algorithm for testing of aneuploidy in NIPT samples [20]. This size-based algorithm works by determining the size-based z-score of the difference between the proportion of small (fetal) fragments from the target chromosome (either chromosome 13, 18 or 21) and the proportion of small fragments from all autosomes except chromosomes 21, 18, and 13. Our triSure algorithm also works by separating fetal-derived (small) fragments from maternal (large) fragments but instead calculate the difference in proportion (DP) of fragments from the target chromosome to all chromosomes between fetal-derived and maternal-derived fraction.

$$DP_{chrN} = \frac{N_{fetal}}{T_{fetal}} - \frac{N_{maternal}}{T_{maternal}}$$

Where N_{fetal} denotes number of fetal fragments (# 150bp) from target chromosome (either chromosome 21, 18, or 13) and T_{fetal} denotes number of fetal fragments from all chromosomes; $N_{maternal}$ denotes number of maternal fragments (# 170bp) from target chromosome (either chromosome 21, 18, or 13) and $T_{maternal}$ denotes number of maternal fragments from all chromosomes.

This DP is then used to determine the z-score by comparing it with DP from a reference set of euploid samples. A z-score ≥ 3 is classified as trisomy.

$$z - score_{chrN} = \frac{DP_{chrN} - mean(DP_{chrN}^{RefSet})}{SD(DP_{chrN}^{RefSet})}$$

Data collection

In this study, we used two data sets, a previously published data set of 144 samples from Yu et al. (Table S1 from Yu et al.) [20] and our in-house sequence data collected from 130 prospective, consecutive high-risk Vietnamese women with singleton pregnancies in the first or second trimester recruited from Hung Vuong hospital, Ho Chi Minh City, Vietnam with written consent during the period of 11 months (2/2017 - 1/2018). The high-risk criteria (eligibility criteria) were defined as having any of the following: (i) age > 40 years, (ii) nuchal translucency (NT) > 3mm, (iii) combined test (bhCG and PAPP-A combined with NT) or triple test (AFP, bhCG and estriol) with risk > 1/250, or (iv) any abnormalities detected by ultrasound scan. All patients were subjected to karyotype tests performed by Genetic laboratory of Hung Vuong hospital and the karyotype results were used as reference standard for validating of triSure method. Karyotype results were blinded to the staffs performing triSure. The study was approved by institutional ethics committees of University of Medicine and Pharmacy at HCM city and Hung Vuong hospital. Cell-free DNA (cfDNA) in plasma was extracted using MagMAX Cell-Free DNA Isolation Kit from Thermo Fisher Scientific (Waltham, MA, USA). Library preparation was done using NEBNext Ultra II DNA Library Prep Kit from New England BioLabs (Ipswich, MA, USA) and sequenced on the MiniSeq platform using paired-end 2x75bp Reagent Kit from Illumina (San Diego, CA, USA).

Calculation of z-score by other methods

To compare triSure with other methods, the z-scores of count-based and size-based methods were calculated based on the formula reported previously [13,20]. The z-scores of RAPIDR was calculated using the RAPIDR package version 0.1.1 [16] with GC-correction and masked bin filtering. NIPTeR package version 1.0.2 [17] was used with bin weighted GC-correction, Chi-squared-based variation reduction for pre-processing and Z-score as the trisomy prediction method.

Statistical calculation and plotting

Sensitivity and specificity were calculated using the caret package version 6.0-76. All plots were drawn with ggplot2 (version 2.2.1) and ggpubr packages (version 0.1.5). All analysis codes were run using the open source programming language and software environment R (version 3.4.3).

Results

Differences in the chromosome-wise proportion of fetal and maternal fragments

Our triSure algorithm works by calculating the differences in proportion of fragments from target chromosome (either chromosome 21, 18, or 13) between fetal-derived and maternal-derived fraction. To demonstrate that the value of DP could be used to separate trisomy from euploid samples, we calculated and plotted DP for each chromosome using the previously published data from Yu et al. The distributions of DP from euploid samples were different in both their mean and range of values (Fig 1 and Fig S1) for all chromosomes. However, in the cases of chromosomes13, 18 and 21, the trisomy samples were clearly separated from euploid ones. Therefore, standardisation of DP using a set of reference controls would produce z-scores that would be useful for detection of aneuploidies.

Comparison of triSure with count-based and size-based algorithms

We first tested our triSure method against two other algorithms: count-based [13] and size-based [20] on a data set published previously (see Methods) [20]. This data set contains 144 samples, of which 60 samples are euploid, 21 are trisomy 13, 27 trisomy 18 and 36 trisomy 21. Because all three methods are reference-based and as such need a set of euploid samples as reference controls, we randomly selected 20 euploid samples to be used as reference and the remaining samples as testing set. This random selection was repeated 1000 times and the sensitivity and specificity of each method in calling of trisomy 13, 18 and 21 were compared. In general, the sensitivity and specificity of the three methods for trisomy 21 were similar (Table 1). The biggest difference among these three methods was highest sensitivity for trisomy 13 by triSure (0.814) in comparison to count-based (0.060) and size-based (0.780, Table 1). This comparison demonstrated that both triSure and size-based methods outperformed count-based method for calling of trisomy 13 and 18, and triSure was comparable to size-based method using the original data set that was used to develop size-based algorithm.

Performance of triSure on in-house data

Next we performed a comparison between triSure, count-based and size-based methods using our in-house data set of 130 pregnant women who have undergone amniotic testing. The median age of the participants in this study is 33 years (range 19 – 46 years) and the median gestation age is 17 weeks (range 12 – 29 weeks). This data set includes 72 euploid, 4 trisomy 13, 11

trisomy 18 and 43 trisomy 21 samples. Our results showed that size-based method had the lowest performance of the three methods for trisomy 21 (sensitivity = 0.953 and specificity = 0.954), followed by triSure (sensitivity = 0.977 and specificity = 0.989) and CB being the best of the three (Table 2, Table S1, Figure 2). For trisomy 18, triSure method achieved 100% accuracy while CB specificity drop slightly to 0.983 and SB again had lowest sensitivity of 0.818 (Table 2, Table S1, Figure 2). The sensitivity of both CB and SB method for trisomy 13 dropped dramatically to 0.75 while triSure still maintain 100% accuracy.

There are several methods employing read counts (count-based z-score) in combination with various correction steps to increase the sensitivity and specificity of their results. Here we chose two such methods, RAPIDR and NIPTeR, which are available as free program packages, to compare against our triSure algorithm using the same set in-house data as above. Comparing among triSure, RAPIDR and NIPTeR, RAPIDR showed the lowest sensitivity for trisomy 21 (0.628), followed by triSure then NIPTeR being the best (Table 2, Table S1, Figure 2). NIPTeR and triSure were comparable for trisomy 13 and 18 with triSure achieved slightly better specificity. RAPIDR exhibited lowest sensitivity and specificity for trisomy 18 (0.818 and 0.941, respectively).

Discussion

NIPT is a safe and highly accurate screening test for targeted fetal aneuploidies and it has been rapidly adopted in the clinics among developed countries. However, several limiting factors are hindering the rate of adoption of NIPT in developing countries, including, but not limited to, technical expertise, MPS costs and MPS data analysis. In this study, we demonstrated our capacity to establish NIPT protocol and deliver NIPT in Vietnam, a low-income setting, with comparable sensitivity and specificity to those achieved in developed countries. Our own triSure method is a simple yet robust and accurate algorithm to detect fetal aneuploidies from MPS sequencing data. This method improves on SB method by using the difference in proportions of fetal-derived fragments (# 150 bp) and maternal-derived fragments (# 170 bp) in each target chromosome to detect anomalies in chromosomal copy number.

We have rigorously tested triSure against CB and SB methods using a published data set that was originally used to build SB method [20] and showed that our method performed consistently

well across one thousand sets of reference controls. This outcome confirmed the robustness of our method in dealing with sequencing data obtained from others. Using our own sequence data set obtained from 130 high-risk pregnant women, our method still achieved high sensitivity and specificity. The triSure algorithm achieved higher sensitivity than SB for calling all three types of aneuploidies. Although CB and triSure performed similarly for trisomy 21 and 18, triSure outperformed both CB and SB at sensitivity for trisomy 13. Thus of the three methods, triSure achieved the best overall performance for all three types of aneuploidies.

Furthermore, two publicly available packages for calling of fetal aneuploidies, the first one was RAPIDR currently used by the United Kingdom National Health Service (NHS) [16] and the second method was NIPTeR newly published in 2017 [17], were also used to compare the performance on our sequence data set. Unexpectedly, RAPIDR lost its sensitivity for trisomy 21 (only 0.628). This low sensitivity was also found in several trisomy prediction approaches offered by NIPTeR including “Regression based Z-score” and “Normalized Chromosome Value” (Table S2). The most likely explaination for this under-performance was that these methods were optimised on high coverage sequence data on HiSeq platforms and thus were less robust on our data which were of lower coverage on MiniSeq platform. However, NIPTeR’s standard z-score approach (essentially CB z-score with GC-correction and chi-squared based variation reduction) showed superior performance (Table 2), proving the effectiveness of NIPTeR corrections in improving trisomy calling compared to CB. The NIPTeR’s standard z-score approach exhibited 100% accuracy for trisomy 21 but returned 4 false positives for trisomy 18 and 3 false postives for trisomy 13, resulted in 123/130 (94.6%) samples correctly identified. In comparison, triSure correctly called 128 samples (98.5%), with only one false positive and one false negative for trisomy 21 and was 100% accurate for trisomy 13 and trisomy 18. In summary, triSure and NIPTeR both gave superior performance on this data set.

Our current data set included only 4 cases of trisomy 13 and 11 cases of trisomy 18, which was a reflection of the low prevalence of these cases during our sampling period. Therefore, while it is encouraging to see triSure method detected trisomy 13 and 18 cases with 100% accuracy, we acknowledge that this low number of cases would not give us good estimation of sensitivity

and specificity of triSure for trisomy 13 and 18. To further monitor the accuracy of triSure, we are continuously adding more cases as well as performing follow-up investigation on the population that have been tested.

The accuracy and robustness of triSure algorithm were the reflection of a complete NIPT protocol established locally in Ho Chi Minh City, Vietnam, from blood sample collection, cell-free DNA extraction, library preparation, sequencing and triSure data analysis. Beside triSure, currently all NIPT services available in Vietnam require transportation of samples to another country for processing, resulted in total cost of more than $500 per sample and 7-14 days of waiting time. With the ability to perform triSure locally, we believe that triSure protocol could be a suitable solution for low-income settings. To make NIPT, especially our triSure protocol, affordable for women in low-income settings, future work will be needed to further minimize cost per sample and shorten waiting time for results.

Conclusion

In conclusion, we have demonstrated that our triSure algorithm performed robustly with high accuracy. Thus, by employing triSure, NIPT can be made accessible to more patients in low- and middle-income countries such as Vietnam.

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Conflict of interest statement:MDP, HG are current employees of Gene Solutions, a company that performs noninvasive prenatal screening.

Figure legends

Figure 1. Distribution of the differences in proportion of fetal and maternal fragments in chromosome 13, 18 and 21. The samples were divided into 4 groups: euploid, trisomy 13 (T13), trisomy 18 (T18) and trisomy 21 (T21) and the DPs of each group were plotted respectively to their chromosomes.

Figure 2. Z-scores of samples from in-house data set for chr13, chr18 and chr21 calculated by count-based, size-based, triSure NIPTer, and RAPIDR methods. The z-scores above the threshold of 3 were considered trisomy for the respective chromosome.

Figure S1. Distribution of the differences in proportion of fetal and maternal fragments all chromosomes. The samples were divided into 4 groups: euploid, trisomy 13 (T13), trisomy 18 (T18) and trisomy 21 (T21) and the DPs of each group were plotted respectively to their chromosomes.

Table 1: Sensitivity and specificity (mean ± sd)of count-based, size-based and triSure methods from 1000 iterations of random sampling for reference set.

	Count-based		Size-based		triSure	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Trisomy 13	0.060 (±0.083)	0.771 (±0.306)	0.780 (±0.112)	0.996 (±0.009)	0.814 (±0.096)	0.999 (±0.004)
Trisomy 18	0.789 (±0.139)	0.954 (±0.047)	0.953 (±0.018)	1.000 (±0.002)	0.962 (±0.008)	0.997 (±0.007)
Trisomy 21	1.000 (±0.000)	0.997 (±0.005)	0.994 (± 0.011)	1.000 (± 0.003)	0.995 (± 0.010)	1.000 (± 0.002)

Table 2: Comparing sensitivity and specificityof several aneuploidy calling algorithms against triSure method using in-house data set.

							NIPTeR		RAPIDR	
							Sens	Spec	Sens	Spec
	0.750	0.960	0.750	1.000	**1.000**	**1.000**	1.000	0.976	1.000	0.992
	1.000	0.983					1.000	0.966	0.818	0.941
	1.000	0.989					1.000	1.000	0.628	1.000
	0.983	0.917					1.000	0.931	0.707	0.931

* Overall sensitivity and specificity measure the ability of a method to distinguish between euploid and aneuploid (any of the three trisomy types).

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							NIPTeR		RAPIDR	
							Sens	Spec	Sens	Spec
	0.750	0.960	0.750	1.000	**1.000**	**1.000**	1.000	0.976	1.000	0.992
								E^R T^R		E^R T^R
							E	123 0	E	125 0
							T	3 4	T	1 4
	1.000	0.983					1.000	0.966	0.818	0.941
								E^R T^R		E^R T^R
							E	115 0	E	112 2
							T	4 11	T	7 9
	1.000	0.989					1.000	1.000	0.628	1.000
								E^R T^R		E^R T^R
							E	87 0	E	87 16
							T	0 43	T	0 27
	0.983	0.917					1.000	0.931	0.707	0.931
								E^R T^R		E^R T^R
							E	67 0	E	67 17
							T	5 58	T	5 41

Note: For each sensitivity/specificity pair, a cross tabulation table is provided. E: Euploid, T: trisomy, ^{R}: reference count

Table S2: The sensitivity and specificityof NIPTeR using different calling parameters.

				rbz.3		rbz.4		z	
				Sens	Spec	Sens	Spec	Sens	Spec
0.750	0.937		0.968	1.000	0.968	0.750	0.984	1.000	0.976
					E^R	T^R		E^R	T^R		E^R	T^R
				E	122	0	E	124	1	E	123	0
				T	4	4	T	2	3	T	3	4
1.000	0.941			0.818	0.992	0.909	0.992	1.000	0.966
					E^R	T^R		E^R	T^R		E^R	T^R
				E	118	2	E	118	1	E	115	0
				T	1	9	T	1	10	T	4	11
0.860	0.977			0.651	0.989	0.628	0.989	1.000	1.000
					E^R	T^R		E^R	T^R		E^R	T^R
				E	86	15	E	86	16	E	87	0
				T	1	28	T	1	27	T	0	43

Notes:

- For each sensitivity/specificity pair, a cross tabulation table is provided. E: Euploid, T: trisomy, ^{R}: reference count
- NIPTeR analysis was performed on in-house data set with GC-correction (“bin” method), Match control group (to determine a subset of reference samples that fits the test samples), Chi-squared based variation reduction before applying one of the three trisomy prediction functions to calculate the following z-scores: ncvz (Normalized Chromosome Value, Sehnert et al., 2011), rbz (Regression based Z-score from 4 models: rbz.1, rbz.2, rbz.3 and rbz.4) and z (Z-score approach, introduced by Chiu et al in 2008). For more details on NIPTeR parameters and methods, please refer to Dirk de Weerd and Lennart Johansson (2016).

Table 1: Sensitivity and specificity (mean ± sd)of count-based, size-based and triSure methods from 1000 iterations of random sampling for reference set.

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	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
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Trisomy 18	0.789 (±0.139)	0.954 (±0.047)	0.953 (±0.018)	1.000 (±0.002)	0.962 (±0.008)	0.997 (±0.007)
Trisomy 21	1.000 (±0.000)	0.997 (±0.005)	0.994 (± 0.011)	1.000 (± 0.003)	0.995 (± 0.010)	1.000 (± 0.002)

Table 2: Comparing sensitivity and specificityof several aneuploidy calling algorithms against triSure method using in-house data set.

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							Sens	Spec	Sens	Spec
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	1.000	0.983					1.000	0.966	0.818	0.941
	1.000	0.989					1.000	1.000	0.628	1.000
	0.983	0.917					1.000	0.931	0.707	0.931

* Overall sensitivity and specificity measure the ability of a method to distinguish between euploid and aneuploid (any of the three trisomy types).

Table S1: Comparing sensitivity and specificityof several aneuploidy calling algorithms against triSure method using in-house data set

							NIPTeR		RAPIDR	
							Sens	Spec	Sens	Spec
	0.750	0.960	0.750	1.000	**1.000**	**1.000**	1.000	0.976	1.000	0.992
								E^R T^R		E^R T^R
							E	123 0	E	125 0
							T	3 4	T	1 4
	1.000	0.983					1.000	0.966	0.818	0.941
								E^R T^R		E^R T^R
							E	115 0	E	112 2
							T	4 11	T	7 9
	1.000	0.989					1.000	1.000	0.628	1.000
								E^R T^R		E^R T^R
							E	87 0	E	87 16
							T	0 43	T	0 27
	0.983	0.917					1.000	0.931	0.707	0.931
								E^R T^R		E^R T^R
							E	67 0	E	67 17
							T	5 58	T	5 41

Note: For each sensitivity/specificity pair, a cross tabulation table is provided. E: Euploid, T: trisomy, ^{R}: reference count

Table S2: The sensitivity and specificityof NIPTeR using different calling parameters.

				rbz.3		rbz.4		z	
				Sens	Spec	Sens	Spec	Sens	Spec
0.750	0.937		0.968	1.000	0.968	0.750	0.984	1.000	0.976
					E^R	T^R		E^R	T^R		E^R	T^R
				E	122	0	E	124	1	E	123	0
				T	4	4	T	2	3	T	3	4
1.000	0.941			0.818	0.992	0.909	0.992	1.000	0.966
					E^R	T^R		E^R	T^R		E^R	T^R
				E	118	2	E	118	1	E	115	0
				T	1	9	T	1	10	T	4	11
0.860	0.977			0.651	0.989	0.628	0.989	1.000	1.000
					E^R	T^R		E^R	T^R		E^R	T^R
				E	86	15	E	86	16	E	87	0
				T	1	28	T	1	27	T	0	43

Notes:

- For each sensitivity/specificity pair, a cross tabulation table is provided. E: Euploid, T: trisomy, ^{R}: reference count
- NIPTeR analysis was performed on in-house data set with GC-correction (“bin” method), Match control group (to determine a subset of reference samples that fits the test samples), Chi-squared based variation reduction before applying one of the three trisomy prediction functions to calculate the following z-scores: ncvz (Normalized Chromosome Value, Sehnert et al., 2011), rbz (Regression based Z-score from 4 models: rbz.1, rbz.2, rbz.3 and rbz.4) and z (Z-score approach, introduced by Chiu et al in 2008). For more details on NIPTeR parameters and methods, please refer to Dirk de Weerd and Lennart Johansson (2016).