Effectiveness of alpha-fetoprotein variants L2 and L3 as substitutes of alpha-fetoprotein in screening for fetal Trisomy 18

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\textbf{ABSTRACT}

\textbf{Objective}: To evaluate the effectiveness of alpha-fetoprotein variants (AFP-L2, AFP-L3) in fetal screening for Trisomy 18 in place of alpha-fetoprotein (AFP).

\textbf{Methods}: A retrospective case-control study was conducted. Collectively, 39 pregnant women bearing Trisomy 18 fetuses and 48 pregnant women with clinically normal and healthy fetuses were included. The serum AFP-L2 and AFP-L3 concentrations were detected by enzyme-linked immunosorbent assays. The likelihood ratio method and Python software were used to construct the risk model with AFP, free β-hCG, AFP-L2, and AFP-L3 to predict Trisomy 18. Receiver operating characteristic (ROC) curves were used to determine the optimal cutoff value, while the area under the curve (AUC) was used to assess the screening performance of AFP-L2 and AFP-L3 for fetal Trisomy 18.

\textbf{Results}: Compared to values observed for the control group, AFP-L2 and AFP-L3 concentrations, which were significantly higher (both \(p < .001\)) in pregnant women with Trisomy 18 fetuses were 7.95 ± 3.57 ng/mL and 2.53 ± 1.80 ng/mL, respectively. Comparisons across multiple modeling methods showed that the highest AUC of screened Trisomy 18 fetuses (0.992, 0.986, and 0.976) was yielded by AFP-L2 + AFP-L3 + free β-hCG, AFP-L2 + free β-hCG, and AFP-L3 + free β-hCG, with a sensitivity of 1.000 indicated in both instances. In different modeling methods, the order of AUC values was AFP-L2 + AFP-L3 + free β-hCG > AFP-L2 + free β-hCG > AFP-L3 + free β-hCG > AFP + free β-hCG.

\textbf{Conclusions}: AFP-L2 and AFP-L3 showed higher sensitivity and specificity as substitutes for AFP in screening Trisomy 18. These two markers indeed improved the screening efficiency and reduced the false positive rate, when compared with AFP only.

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\textbf{Introduction}

Trisomy 18, also known as Edwards syndrome (ES), is an autosomal trisomy syndrome whose incidence is second only to Trisomy 21 (Down’s syndrome, DS). While its prevalence in live births is between 1/6000 and 1/8000, Trisomy 18 often precedes spontaneous abortion. With the development of prenatal diagnostic techniques in recent years, most Trisomy 18 fetuses are naturally eliminated or artificially terminated, so the incidence of Trisomy 18 is higher than its prevalence in live births reaching about 1/2500–1/2600 [1]. Most children with Trisomy 18 die within 1 year of being born, and only 5–10% survive beyond this age [2]. The diagnosis of Trisomy 18 cases is currently based on maternal age, maternal serum marker screening (alpha fetoprotein (AFP)+free beta human chorionic gonadotropin (free β-hCG)) [3], or ultrasound imaging [4,5]. These three bands are called alpha-fetoprotein variants L1, L2, and L3 (AFP-L1, AFP-L2, and AFP-L3), with AFP-L1 and AFP-L2 representing the lens culinaris agglutinin (LCA) non-binding types and AFP-L3 associated with LCA binding. In addition, different configurations of AFP are usually called AFP heterosomes [6]. AFP-L3, AFP, and CEA are effective biomarkers for the diagnosis of hepatocellular carcinoma (HCC). Their combinations could improve the diagnostic performance compared to the capacity of...
individual markers in detecting HCC [7]. The AFP and AFP-L3 combination, adopting cutoff values (5 ng/mL and 4%, respectively), significantly improved the sensitivity for detecting HCC at a very early stage [8]. It has been reported that AFP-L3 is superior to AFP in screening for Trisomy 21 [9–12], but there is no report on the combined use of AFP-L2 and AFP-L3 as markers for Trisomy 18 screening. This case-control study selected 48 pregnant women with healthy fetuses and 39 pregnant women with a prenatal diagnosis of Trisomy 18 in the fetus to investigate the relationship between maternal serum AFP-L2 and AFP-L3 levels and Trisomy 18 fetuses. Additionally, as a potential replacement for AFP screening, the effectiveness of AFP-L2 and AFP-L3 combined with free β-hCG concentrations in screening for Trisomy 18 fetuses was tested.

**Materials and methods**

**Research object**

Between October 2007 and September 2016, we used a retrospective case-control study method to analyze women pregnant with single fetuses that were 15–20 weeks and six days of age. These women attended prenatal clinics associated with one of two prenatal screening centers (Hangzhou Women’s Hospital (Hangzhou Maternity and Child Health Care Hospital), or the Maternity and Child Health Care Hospital, Yuhang District, Hangzhou, China). We analyzed a total of 463,298 cases, 366,355 of which derived from the Hangzhou Women’s Hospital and 96,943 cases from the Maternity and Child Health Care Hospital. Per the presence or absence of fetal Trisomy 18, the study subjects were divided into case and control groups, respectively. By strict screening criteria, the case group included 39 pregnant women diagnosed by karyotype analysis of amniotic fluid as carrying Trisomy 18 fetuses, while the control group included 48 pregnant women with normal fetal development. The latter cases were determined by choosing the next two consecutive cases after each Trisomy 18 case. All cases had an AFP and free β-hCG screening. The study was approved by the Hangzhou Women’s Hospital Ethics Committee, and the approval number was [2018] medical ethics (004) no. 01.

Diagnosis and exclusion criteria: diagnostic criteria based on the China Birth Defects Monitoring Network [13,14].

The typical clinical manifestations of Trisomy 18 include physical abnormalities (including occipital kyphosis, a narrow forehead, small head, and wide frontal suture), growth retardation, and heart and kidney defects. In order to be considered as being Trisomy 18, the fetus needed to have an additional copy of all, or part of, chromosome 18. Children with chimeric Trisomy 18 have milder clinical manifestations, varying degrees of developmental delay, and longer survival time. Children with a three-body malformation in the short arm of chromosome 18 show unobvious deformity which may manifest as stunting. While the above characteristics can be used to perform a preliminarily screen for Trisomy 18, a chromosome examination is required for the clinical diagnosis. The typical karyotypes are 47, XX, +18 or 47, XY, +18.

Exclusion criteria applied to both groups, included: (1) twins or multiple births, (2) the coexistence of other medical conditions such as insulin-dependent diabetes and severe pregnancy complications, (3) smoking, (4) pregnancy via in vitro fertilization; (5) follow-up results showing neural tube defects or other congenital birth defects, (6) cases with incomplete clinical or molecular data, and (7) a mismatch between maternal information and serum specimens.

**Reagents and instruments**

The 1235 automatic time-resolved fluorescence immunoassay analyzer (DELFIA; PerkinElmer, Waltham, MA) was used with double protein labeling kit (AFP/free β-hCG), enhancer, rinse solution and reference standards (Wallac Oy, Turku, Finland) in both hospitals involved in our study. Human AFP-L2 and AFP-L3 were detected using a RT-6100 (Rayto, Shenzhen, China) microplate reader, 988 plate washers (Tianshi, Beijing, China), and AFP-L2 and AFP-L3 reagent (BIM, San Francisco, CA, batch numbers: B170658 and B160785, respectively).

**Detection method**

A total of 2–3 mL of peripheral venous blood was taken from fasting patients. Serum samples were separated and stored in a refrigerator at 2–8°C within 30 min of collection. The samples were sent for analysis within 1 week of collection. Samples were tested using two screening markers: AFP and the free β-hCG. The DELFIA method was utilized and conducted in accordance with the manufacturer's instructions. Residual serum was stored at −80°C after the prenatal screening test was performed. A double-antibody one-step ELISA method was used to detect AFP-L2 and AFP-L3 concentrations.
Establishment of risk models

Python 3.7 software (Google, Mountain View, CA) was used alongside the likelihood ratio method to construct the AFP, free β-hCG, AFP-L2, and AFP-L3 risk models associated with Trisomy 18. The concentration values for AFP, free β-hCG, AFP-L2, and AFP-L3 demonstrated a multivariate normal distribution. The risk calculation model [15] was used to calculate the distribution of each indicator, as well as Trisomy 18 likelihood risk. Eight models were constructed. Model 1: constructed using the free β-hCG; model 2: constructed using the AFP; model 3: constructed using the AFP-L2; model 4: constructed using the AFP-L3; model 5: constructed using the AFP + free β-hCG values; model 6: constructed using the AFP-L2 + free β-hCG values; model 7: constructed using the AFP-L3 + free β-hCG values; and model 8: constructed using the AFP-L2 + AFP-L3 + free β-hCG values.

Establishment of risk models with AFP, free β-hCG, AFP-L2, and AFP-L3 levels to predict Trisomy 18

The following was used for the life cycle-like risk calculation method [15,16].

The calculation to determine the likelihood ratio:

\[
LR_{\text{Multinorm}} = \frac{\text{Likelihood of Trisomy 18}}{\text{Likelihood of controls}}
\]

The Trisomy 18 risk value calculation [17]:

\[
\text{Risk}_{\text{Trisomy18}} = \frac{1}{LR_{\text{Multinorm}} \times \text{Risk}_{\text{Maternal age}}}
\]

Statistical analyses

Microsoft® Excel 2007 (Redmond, WA) was used to establish a test result database, which was processed by SPSS version 21.0 software (SPSS, Armonk, NY). A data normality test was performed using the one-sample Kolmogorov–Smirnov’s test, and the normally distributed data measured by the mean ± standard deviation ( \( \bar{x} \pm s \) ). AFP-L2 and AFP-L3 concentrations were compared between the case and control groups using two independent sample t-tests. The Trisomy 18 risk value was calculated by multiplying the age risk of Trisomy 18 [16] by the corresponding normal distribution likelihood [15]. The joint risk threshold is the log value of the corresponding risk value. Receiver operating characteristic (ROC) curves were plotted and the optimal cutoff, area under the curve (AUC), and Youden index values for AFP-L2 and AFP-L3 concentrations were calculated accordingly. \( p < .05 \) was considered significant.

Results

Comparison of baseline demographic data between each group

As shown in Table 1, there was no significant difference in maternal age, gestational age, and maternal weight between the case and control group women.

Comparison of serum AFP-L2 and AFP-L3 levels in the two groups

As reflected in Table 2, the AFP-L2 and AFP-L3 concentrations in pregnant women with Trisomy 18 fetuses were 7.95 ± 3.57 ng/mL and 2.53 ± 1.80 ng/mL, respectively, which was significantly higher than those of the control group (both \( p < .001 \)).

The value of AFP-L2 and AFP-L3 as independent indicators of Trisomy 18

Use of AFP-L2 for the screening of Trisomy 18 fetuses had an AUC of 0.848. Per the ROC curve, the optimal value of AFP-L2 for fetal Trisomy 18 screening was 6.340 ng/mL. The sensitivity, specificity, and Youden’s index values were 0.615, 1.000, and 0.615, respectively. When screening Trisomy 18 fetuses using AFP-L3, the AUC was 0.806 with the optimal AFP-L3 value determined to be 1.705 ng/mL following ROC analysis. The corresponding sensitivity, specificity, and Youden’s index values were 0.641, 0.958, and 0.599, respectively. The data are summarized in Table 3 and Figure 1(A).
The diagnostic value of AFP, free β-hCG, AFP-L2, and AFP-L3 as individual or combined markers for Trisomy 18

Following comparisons between different modeling methods, it was shown that AFP-L2 + AFP-L3 + free β-hCG, AFP-L2 + free β-hCG, and AFP-L3 + free β-hCG had the highest AUC for screening Trisomy 18 fetuses, reaching 0.992, 0.986, and 0.976, respectively. The sensitivity of both models was 100%. In different modeling methods, the order of AUC value was AFP-L2 + AFP-L3 + free β-hCG > AFP-L2 + free β-hCG > AFP-L3 + free β-hCG > AFP + free β-hCG (Table 3 and Figure 1(B)).

Comparison of screening Trisomy 18 fetuses using AFP-L2, AFP-L3, and AFP

Results showed that the sensitivity of AFP, AFP-L2, AFP-L3, and AFP-L2 + AFP-L3 in detecting Trisomy 18 was all 100%. In the control group, the four models showed 22.92%, 8.33%, 12.50%, and 12.50% false positive rates (Table 4).

Discussion

Studies have shown that AFP-L2 and AFP-L3 are superior to AFP in detecting Trisomy 21 [18,19], but the joint use of AFP-L2 and AFP-L3 in screening for

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**Table 2.** Comparison of serum AFP-L2 and AFP-L3 levels in the two groups.

| Group       | n   | AFP (U/mL)       | free β-hCG (ng/mL) | AFP-L2 (ng/mL) | AFP-L3 (ng/mL) |
|-------------|-----|-----------------|--------------------|----------------|----------------|
| Trisomy 18  | 39  | 21.57 ± 9.86 (18.38–24.77) | 2.73 ± 1.34 (2.29–3.16) | 7.95 ± 3.57 (6.79–9.10) | 2.53 ± 1.80 (1.94–3.11) |
| Control     | 48  | 34.16 ± 14.69 (29.89–38.42) | 46.20 ± 23.56 (39.36–53.04) | 3.73 ± 1.63(3.26–4.20) | 0.84 ± 0.60 (0.66–1.01) |

**t** – 4.576

| p           | <.001 |
|-------------|-------|

**Table 3.** Value of AFP, AFP-L2, AFP-L3 separate screening and joint screening for the Trisomy 18.

| Screening method | AUC | 95%CI       | p  | Cut-off | Sensitivity | Specificity | Youden index |
|------------------|-----|-------------|----|---------|-------------|-------------|--------------|
| Free β-hCG       | 0.962 | 0.923–1.000 | <.001 | 11.325 | 0.917 | 1.000 | 0.917 |
| AFP              | 0.826 | 0.739–0.914 | <.001 | 22.600 | 0.813 | 0.692 | 0.505 |
| AFP-L2           | 0.848 | 0.767–0.930 | <.001 | 6.340  | 0.615 | 1.000 | 0.615 |
| AFP-L3           | 0.806 | 0.707–0.905 | <.001 | 1.705  | 0.641 | 0.958 | 0.599 |
| AFP + free β-hCG | 0.969 | 0.932–1.000 | <.001 | 5.138  | 1.000 | 0.917 | 0.917 |
| AFP-L2 + free β-hCG | 0.986 | 0.967–1.000 | <.001 | 4.150  | 1.000 | 0.938 | 0.938 |
| AFP-L3 + free β-hCG | 0.976 | 0.948–1.000 | <.001 | 4.976  | 1.000 | 0.929 | 0.929 |
| AFP-L2 + AFP-L3 + free β-hCG | 0.992 | 0.981–1.000 | <.001 | 3.021  | 1.000 | 0.933 | 0.992 |

**AFP:** α-fetoprotein; free β-hCG: free beta-human chorionic gonadotropin; AFP-L2, AFP-L3: alpha-fetoprotein variants L2, L3.

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**Figure 1.** ROC curve of AFP, free β-hCG, AFP-L2, and AFP-L3 corresponds to single-indicator and Multi-indicator joint screening Trisomy 18. (A) Trisomy 18 ROC curve corresponds to single-indicator; (B) Trisomy 18 ROC curve corresponds to multi-indicator. AFP: α-fetoprotein; free β-hCG: free beta-human chorionic gonadotropin; AFP-L2, AFP-L3: alpha-fetoprotein variants L2, L3.
Trisomy 18 has not been investigated yet. Consequently, in order to explore the relationship between maternal serum AFP-L2 and AFP-L3 levels prior to and subsequently to combining these proteins with free β-hCG and their screening efficiency for Trisomy 18, this case-control study included 39 pregnant women with a prenatal diagnosis of Trisomy 18, and 48 pregnant women with healthy fetuses.

The results of this study showed that AFP-L2 and AFP-L3 serum levels were significantly higher in Trisomy 18 fetuses than in the control group (both p < .01). AFP-L2 and AFP-L3 screenings in this study had AUCs of 0.848 and 0.806, respectively. Per the ROC curves, the optimal cutoff values of AFP-L2 and AFP-L3 for Trisomy 18 fetuses were 6.340 ng/mL and 1.705 ng/mL, respectively. The sensitivity, specificity, and Youden’s index values were 0.615, 1.000, 0.615 and 0.641, 0.958, 0.599, respectively. The results of this study showed that both AFP-L2 and AFP-L3 were effective differentiators between pregnant women who had healthy or Trisomy 18 fetuses.

Our previous laboratory study has shown that AFP-L2 and AFP-L3 are good markers for the screening of Trisomy 21 fetuses in the second trimester of pregnancy due to high sensitivity and specificity. Screening with combined markers is better than screening with individual markers, while screening with AFP-L2 + AFP-L3 + free β-hCG showed the most effective results [19]. In the current study, different modeling methods were used to test Trisomy 18 screening results. The highest AUC for Trisomy 18 fetuses was yielded by AFP-L2 + AFP-L3 + free β-hCG, AFP-L2 + free β-hCG, and AFP-L3 + free β-hCG, all of which had values of 0.992, 0.986, and 0.976, respectively, with sensitivity scores of 1.000. This indicated that maternal serum AFP-L2 and AFP-L3 were good markers for Trisomy 18 as they have high sensitivity and specificity for screening of Trisomy 18 fetuses in the second trimester of pregnancy.

In this study, AFP-L2 and AFP-L3 were used as substitutes for AFP to screen for fetal Trisomy 18. Results of the two combined tests were compared. The sensitivity of the four screening combinations in detecting Trisomy 18 was 100%. In the control group, false positive rates of 22.92%, 8.33%, 12.50%, and 12.50% were observed. AFP-L3 has been shown to be an effective alternative to AFP in prenatal Trisomy 21 screening. With high sensitivity and specificity, the results of this study indicated that AFP-L2 and AFP-L3 can also be used in combination with AFP for joint screening of Trisomy 18 fetuses.

The detection of AFP-L2 and AFP-L3 in this study was based on the ELISA method. The need for serum sample batch testing is, however, inconvenient for normal work. Nevertheless, in 2005, the US Food and Drug Administration approved the clinical use of AFP-L3-associated reagents and detection methods for early stage liver cancer. In China, the affinity adsorption centrifugation method (lectin capture micro-spin column method) and AFP-L3 detection kit were the first to obtain China Food and Drug Administration clearance. For clinical use, Japan’s Wako’s fully automated microfluidic immunofluorescence method was launched in China in September 2016 [20], and recently developed high sensitivity chemiluminescence enzyme immunoassay kit products [21–23]. The development of the AFP-L3 kit will provide automated detection of AFP-L3 in Trisomy 18 screening, and provide conditions for the development of Trisomy 18 screening. The AFP-L2 and AFP-L3 cases detected in this study are still too few. This may affect the accuracy of the modeling results and will need to be verified by large sample numbers in the future.

In summary, with high sensitivity and specificity, AFP-L2 and AFP-L3 were good joint markers for Trisomy 18 screening when replacing the AFP marker. Compared to AFP, they significantly improved the screening efficiency and indeed reduced the false positive rate.

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The study was approved by Hangzhou Women’s Hospital (Hangzhou Maternity and Child Health Care Hospital) ethics committee, in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The approval number was [2018] medical ethics (004) No. 01. Consent obtained from study participants was written.

Disclosure statement
The authors have no conflicts of interest to declare.

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