ORIGINAL ARTICLE

Epigenetic vs Proteomic Biomarkers in Preterm Prediction: A Prospective Study

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Abstract

Background: Preterm birth (PTB) forms the prime etiology of mortality and morbidity in neonates worldwide. Our study compares serum epigenetic [microRNA-miRNA:150-5p, 223-3p, 302b-3p, 548ai] and proteomic profiling [interleukin-6 (IL-6), alpha-fetoprotein (AFP)] in prediction of preterm birth.

Materials and methods: Blood was drawn from 88 pregnant women at 19–26 weeks of gestation who were followed until delivery. The concentrations of miR-150-5p, miR-223-3p, miR-302b-3p, and miR-548ai (Real-time polymerase chain reaction-RT-PCR) were compared with IL-6 and AFP [enzyme-linked immunosorbent assay (ELISA)].

Results: Our study had 75 term and 13 preterm deliveries. A "*p*" value of 0.003 for birth weight and preterm delivery; statistically noteworthy was appreciated. Upregulation of miR-150-5p, miR-223-3p, miR-302b-3p was seen in preterm patients with *p*-value of 0.021, 0.060, and 0.062, respectively. The area under the ROC curve (AUC-ROC) analysis for miR-150-5p (0.739) showed 46.15% sensitivity with 100% specificity and positive predictive value (*p*-value = 0.0042). miR-302b-3p had the highest sensitivity and negative predictive value of 84.6 and 96.1%, respectively. miR-223-3p defined a 100% positive predictive value and specificity. miR-548ai had 69.23% sensitivity, 44% specificity and *p*-value = 0.6884 (AUC-ROC). The IL-6 and AFP levels were not significantly different between two delivery groups (*p*-value = 0.466 and 0.399).

Conclusion: miR-150-5p is an effective epigenetic biomarker for prediction of preterm labor compared to IL-6 and AFP. miR-223-3p, miR 302b-3p levels are upregulated in preterm women.

Keywords: Alpha-fetoprotein, Biomarker, Interleukin-6, MicroRNA, Preterm labor.

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Introduction

The WHO estimates the annual international burden of preterm birth (PTB) at 15 million.[1] In 2014, third-world countries (Asia and sub-Saharan Africa) contributed to 81% of global preterm births, wherein India, China, Nigeria, Bangladesh, and Indonesia added to 6 million preterm births (44.6%). India alone contributes to 23.4% of the global preterm birth rate.[2]

More than a third of the world's annual neonatal mortality (3.1 million) is due to preterm birth and associated complications; which also ranks as the second most common cause of mortality in children under 5 years.[3] In India, prematurity was the major contributor of neonatal mortality in 2015 (43.8%).[4] Most of the preterm births are spontaneous; have no identifiable etiology or history as compared to 40% of iatrogenic cases;[5] a vast majority occurring in the first pregnancy.[6]

The prediction of spontaneous preterm birth is important because:

- It enables the early identification of women at high risk of preterm birth, wherein judicious use of resources and obstetric care toward prevention could be aimed and improve maternal and fetal outcomes.[7]
- Studies on predictors of spontaneous preterm birth may improve our understanding of the mechanisms of biological and disease pathways leading to spontaneous preterm birth, and possibly to better innovations in therapy.
- It identifies women at low risk in whom, the use of unnecessary and costly interventions can be avoided.

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The successful prediction, prevention, and treatment of preterm labor has a significant influence on the perinatal outcome, health care expenditure, and quality of life.[8]

MicroRNAs (miRNAs) are short, single-stranded, non-coding, 19–25 nucleotide molecules regulating mRNA stability, transcription, and 80% of human genes expression.[9,10] miRNA expression in every cell, tissue, and body fluid is distinctive, which varies with disease, inflammation, and pathological processes. In response to fetal signals, the placenta releases miRNAs into maternal blood circulating in the uterine environment during pregnancy.[11]

Plasma miRNAs are readily quantifiable and relatively unwavering, and are altered in infection, inflammatory, and immune conditions,[12,13] which forms the crux of preterm labor etiology.

Elovitz et al.[14] and Sanders et al.[15] have shown that cervical miRNAs can be utilized as predictors of preterm delivery. Only a few studies have scrutinized the role of plasma miRNAs in prediction of preterm birth.[5,16–18]

Our study aims to determine the prediction of preterm birth comparing profiling of epigenetic (miR-150-5p, miR-223-3p, miR-302-3bp, and miR-548ai) and proteomic [interleukin-6 (IL-6) & alpha-fetoprotein (AFP)] biomarkers.

Materials and Methods

Our study was conducted on 88 women with singleton uncomplicated pregnancy of 19–26 weeks gestational age who were followed up to delivery spanning 2 years. Maternal demographic details, socioeconomic status, obstetric history, body mass index (BMI), blood pressure (BP), gestational age were noted.

Sample Collection for miRNAs

A total of 6 ml blood was collected per subject, 3 ml each for serum and plasma separation using BD plain and spray coated K2 EDTA vacutainer tubes separately; which underwent centrifugation at 3500 rpm at 4°C to separate serum and plasma. The distinct serum and plasma were collected in 2 ml sterile tubes and stored at −80°C.

miRNA Quantification

About 200 µL of plasma was used to isolate microRNA using kit method (miRNeasy Serum/Plasma Kit Qiagen). The microRNA was transformed to cDNA by using advanced cDNA synthesis kit (Applied Biosystems). The synthesized cDNA was used for quantification of selected microRNAs: hsa-miR-150-5p, hsa-miR-223-3p, hsa-miR-302b-3p, and hsa-miR-548ai using TaqMan assay probes (Applied Biosystems). The experiment was normalized using two endogenous controls (hsa-miR-24-3p, hsa-let-7d-5p) and one exogenous control (Cel-miR-39-3p). The target microRNAs were quantified by using Step One real-time polymerase chain reaction (RT-PCR) (ABI - Step One; USA) and the expressions was normalized using endogenous control hsa-let-7d-5p microRNA target.

Cycle threshold (Ct) values are inversely proportional to the levels of microRNA. It denotes the number of amplifications cycles, a sample is subjected for detection of a miRNA threshold. The raw Ct value data calculation based on the endogenous microRNA control are copied to excel spreadsheets and the expression is calculated by using $2^{-\Delta\Delta CT}$ method. The $2^{-\Delta\Delta CT}$ generated expression data was subjected to heat map to see differential expression in targets vs samples. The final fold change gene expression was tabulated for all the microRNA targets.

Sample Collection for AFP, IL-6

Blood for AFP, IL-6 was collected in 2 ml sterile vacutainer, centrifuged at 3000 rpm for 15 minutes, serum separated and stored at −80°C. The biomarkers were analyzed using the enzyme-linked immunosorbent assay (ELISA) (Multiskan Go, Thermo, USA) method - kit protocol (R&D systems). The human AFP (catalogue number: DY1369) and human IL-6 (catalogue numbers: DY206-06) were estimated in pregnant women using serum samples. A seven-point standard curve with two-fold serial dilutions in reagent diluent was used.

Statistical Analysis

The Chi-square test or Fischer's exact test (for 2 × 2 tables only) was used as a test of significance for qualitative data.

The independent *t*-test was used as a test of significance to identify the mean difference between two quantitative variables.

For the calculation of sensitivity, specificity, positive and negative predictive values, the receiver operating characteristic (ROC) and optimal cut-off points were chosen. A test that predicts an outcome no better than chance has an area under the ROC curve (AUC) of 0.5. An area under the ROC curve above 0.8 indicated good prediction. p-value < 0.05 was considered statistically significant.

Statistical Software

MS Excel, SPSS version 22 (IBM SPSS Statistics, Somers NY, USA).

Results

Thirteen (14.7%) of 88 women had preterm delivery. The majority (92%) of subjects were under 30 years. Women over 30 years accounted for 7.95%. About 68.18% of patients were multigravida. No statistically significant difference was found between age group, gravidity, sex of baby, and the pregnancy outcome. Low birth weight babies accounted for 21.59% of total deliveries as shown in Figure 1. Preterm accounted for 72.72% of low birth babies. A p-value of 0.003 for birth weight and preterm delivery; statistically noteworthy was appreciated.

Our study demonstrated higher levels of miR-150-5p in women with preterm birth (Fig. 2) with a statistically significant p-value of 0.021. The miRNA has an AUC of 0.739 as shown in Figure 3. The positive predictive value of miR-150-5p is 100%.

Expression of miR-223-3p was categorically increased in preterm in contrast to term subjects with p-value of 0.060. The specificity and positive predictive value of miR-223-3p was 100%.

Higher levels of miR-302b-3p levels in women were detected in preterm as against term cases as seen in Figure 2 ($p = 0.062$). miR-302b-3p had the highest sensitivity and negative predictive value of 84.6 and 96.1%, respectively among the miRNAs in our study.

miRNA-548ai showed a downward trend of expression in preterm patients (16.1055) as compared to term cases (60.5680) with a p-value of 0.552 as shown in Figure 2. It has the lowest positive predictive value of 17.6% among the miRNAs in our study.

Area under the ROC curve for IL-6 and AFP were 0.507 and 0.571, respectively. Figures 4 and 5 show that there is no statistically paramount difference in IL-6 and AFP concentration between two delivery groups, respectively. The p-values for IL-6 and AFP were 0.466 and 0.399 at 450 nm, respectively. Interleukin-6 and AFP have a positive predictive value of 18.8 and 19.6%, respectively.

Discussion

The syndrome of preterm labor is the amalgamation of multiple etiologies. miRNAs determine gene expression through progesterone receptors and modulate uterine contractility. The distinctive attribute of miRNA is that an individual miRNA has the ability to synchronize expression of multiple genes. Likewise, a single gene can be modulated by various miRNAs.[19]

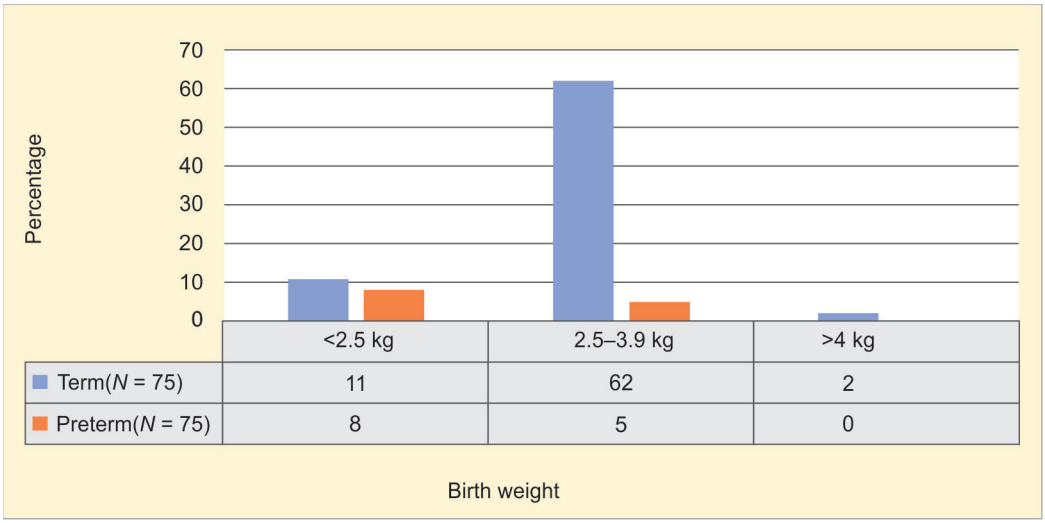

Fig. 1: Birth weight in term and preterm

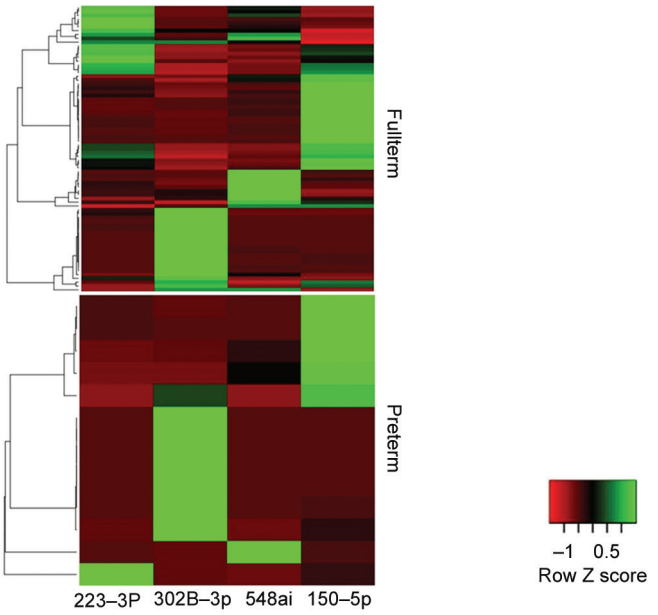

Fig. 2: Heat map of miRNA expression in term (n = 75) and preterm (n = 13). Each column represents one miRNA with row Z score: green = upregulated, red = downregulated, and black = unknown

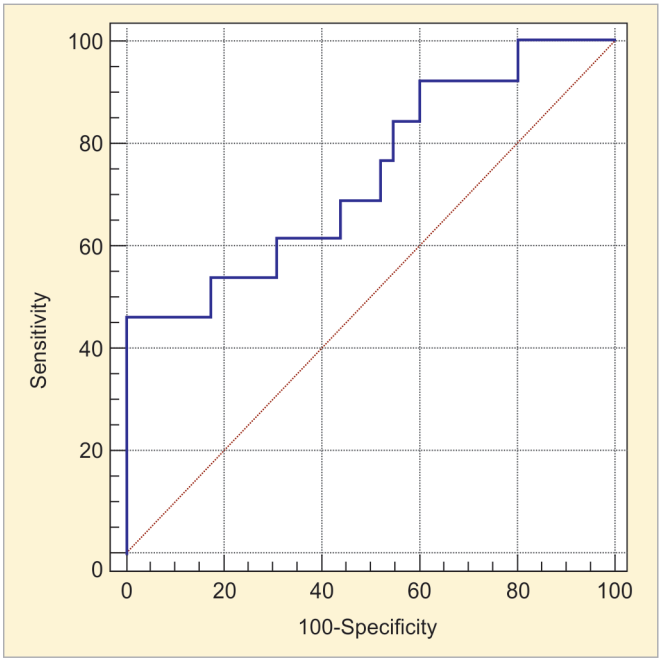

Fig. 3: miR 150-5p ROC curve

150-5p

Our study demonstrates increased levels of miR-150-5p with significant p-value of 0.021 and AUC of 0.739 [95% confidence interval (CI), 0.542–0.750] as compared to Cook et al.[16] [p = 0.005, AUC = 0.8725 (95% CI, 53.08–74.45%)]. The specificity and mean relative expression of miR-150-5p in preterm women were 100% and 58.399 in our study as compared to Cook et al.[16] (64.29% and 15.4).

Hsa miR-150-5p is coordinated by NF-kB, an inflammation associated transcription factor instrumental in initiating labor.[20,21] It ripens the cervix by activating membrane-type-1 MMP.[22,23]

223-3p

Increase in levels of Hsa miR-223-3p was seen in preterm women in comparison, with p-value of 0.060 in our study; though not statistically significant. In studies by Cook et al.,[16] miR-223-3p also was expressed in human plasma above background level, but did not qualify as a biomarker. miR-223-3p had an AUC of 0.651 as compared to 0.77 in studies by Winger et al.[18] It was proficiently escalated in plasma from preterm women (p < 0.001; control = 1731 ± 223 vs preterm = 2945 ± 304) in studies by Gray et al.[5] Similar differences between the two groups were seen in our study; term – 2.9387 vs preterm – 14.6322.

IKKα is acted upon by hsa-miR-223-3p during monocyte-macrophage differentiation, activating the noncanonical and canonical NF-kB pathways paramount to parturition.[24] microRNA-223 synchronizes genes regulating inflammation and immune modulation in pregnancy.[25] Increased expression of hsa-miR-223 in women delivering preterm has been demonstrated in studies by Gray et al.,[5] Sanders et al.,[15] Enquobahrie et al.,[26] Menon et al.[27] (p = 0.012), Sanders et al.[15] (p < 0.05), and Hassan et al.[28]

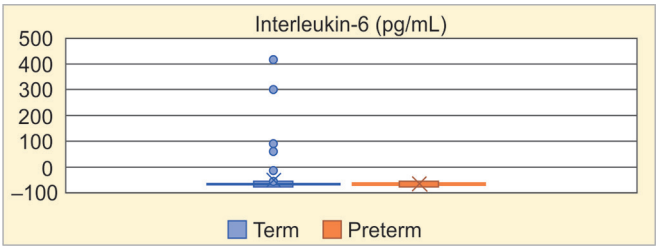

Fig. 4: IL-6 scattered graph - term vs preterm

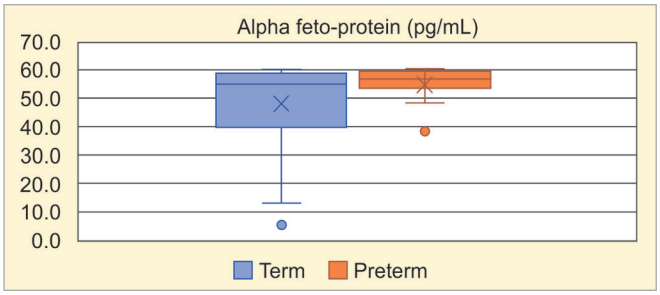

Fig. 5: AFP scattered graph - term vs preterm

302b-3p

Our study demonstrated accentuated levels of miR-302b-3p levels in preterm cases in comparison to term cases, contrary to Gray et al.[5] The mean relative expression in term and preterm patients were 140.2 and 3670.3, respectively, depicting an upward trend. miR-302b-3p showed AUC of 0.753, the highest for miRNAs in our study.

548ai

In our study, with a *p*-value of 0.552, expression of miR-548ai in term and preterm patients was not remarkably dissimilar. However, Gray et al.[5] and Son et al.[29] (*p*-value < 0.01) illustrated diminution of mi-548 congregate in patients with spontaneous preterm birth.

IL-6

In our study, AUC and *p*-value for IL-6 were 0.507 and 0.466; which were not significant. Similarly, Wei SQ et al.[30] failed to attribute plasma IL-6, unlike the cytokine found in cervicovaginal secretions and amniotic fluid to increased incidence of preterm birth.

AFP

The AUC and *p*-value for AFP were 0.571 and 0.399, respectively and found statistically insignificant. Yuan et al., described that maternal AFP levels only in conjunction with aberrant pregnancy markers are proficiently affiliated to preterm birth rather than as an individual biomarker.[31] In contrary, Wang et al.[32] attributed positive correlation of maternal AFP with preterm birth.

Hsa-miR-150-5p, hsa-miR-223-3p, and hsa-miR-302b-3p perform better than IL-6 and AFP in prediction of preterm labor.

List of Abbreviations

PTB = Preterm birth; IL-6 = Interleukin-6; AFP = Alfa-fetoprotein; IKKα = Inhibitor of nuclear factor-κB (IκB) kinase (IKK) complex-α; NF-κB = Nuclear factor-Kb; MMP = Matrix metalloproteinases.

Conclusion

The forecasting of preterm birth formulated on risk factors and pertinent laboratory investigations remains imprecise at large. An absolute predictor for preterm birth should be able to identify subclinical pathological changes prior to appearance of clinical symptoms and signs; capable of being administered in the initial phases of pregnancy with noninvasive testing.

Our study is the first epigenetic preterm screening study in Indian subcontinent. Our findings suggest that epigenetic biomarkers miRNAs, i.e., 150-5p, 223-3p, and 302b-3p perform better than proteomic markers, i.e., IL-6 and AFP in prediction of preterm delivery in asymptomatic pregnant women at risk. This enables development of a miRNA panel which is a minimally invasive bedside screening test, offering effective but invasive therapies to pregnant women at risk who are likely to benefit. In future, with evolving larger scale epigenetic studies and innovative drug trials, novel interventions could be designed to impede the barrage of molecular events that initiate preterm labor and reduce the burden of neonatal and infant mortality and morbidity.

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