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Neutrophil-Gelatinase-Associated Lipocalin 2, Krebs Von den Lungen-6, Beta 2 Microglobulin, and Adiponectin: Crosstalk in Early Prediction of Bronchopulmonary Dysplasia in Egyptian Preterm

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Abstract

Bronchopulmonary dysplasia (BPD) is a respiratory distress syndrome caused by chronic lung parenchymal injury, occurring primarily in preterm infants. Therefore, research for early biomarkers for BPD is really important. This study was conducted to evaluate levels of Krebs Von den Lungen-6 (KL-6) and adiponectin in cord blood, neutrophil-gelatinase-associated lipocalin 2 (NGAL2) mRNA gene expression in broncho-alveolar lavage and urinary beta 2 microglobulin (β2MG) for early prediction of lung injury or possible involvement of those molecules in BPD pathogenesis and development.

Method: this study was carried out from September 2012 to December 2013 with 58 preterm neonates of gestational age ≤32 weeks. KL-6, adiponectin and urinary β2MG levels by immunoassay, NGAL2 mRNA level by real-time PCR were determined.

Results: cord blood KL-6, urinary β2MG and broncho-alveolar lavage (BAL) fluid NGAL2 mRNA expression levels were significantly increased, while a non-significant decrease in cord blood adiponectin level in BPD preterm relative to preterm without BPD were observed, with the best sensitivity and specificity were for KL-6 and β2MG relative to preterm without BPD.

Conclusion: NGAL2, KL-6, and urinary β2MG may have role in early prediction and development of BPD in preterm neonates that may help in early prevention and treatment for better prognosis and outcome.

Keywords: Bronchopulmonary dysplasia; Krebs Von den Lungen-6; Neutrophil-gelatinase-associated lipocalin 2; Beta 2 microglobulin

Introduction

Transition from fetal to postnatal life is one of the most important points in fetal developments especially for preterm and very low birth weight (VLBW) infants (birth weight, 1500 g) [1]. Bronchopulmonary dysplasia (BPD) is a syndrome of respiratory distress caused by chronic lung parenchymal injury that occurs primarily in preterm infants [2]. The hallmark of BPD is a requirement for either oxygen therapy or positive pressure ventilation for 4 or more weeks after birth with chest x-ray findings of persistent hazy opacification or a cyst-like pattern of density [2]. It is a common perinatal complication of preterm infants with a significant risk of long-term disability and morbidity that needs specific markers which aid in accurate prediction of disease development with early prevention, early treatment and better prognosis [3].

The pathophysiologic processes that lead to BPD are still incompletely understood, neonatal lung tissue immaturity, mechanical ventilation side effects, oxidative stress and inflammatory events may be claimed [4]. Antenatal/postnatal factors contribute to the release of "pro-inflammatory and anti-inflammatory" cytokines. An imbalance in these mediators leads to activation of the cellular death pathways in the lung, which is followed by healing or repair that is characterized by impaired alveolarization and dys-regulated angiogenesis [5]. Human Krebs Von den Lungen-6 (KL-6) is a circulating high molecular weight mucinous glycoprotein which is expressed by alveolar type 2 pneumocytes and bronchiolar epithelial cells [6]. It was found that KL-6 levels were markedly increased in babies with chronic and interstitial lung disease which is characterized by alveolar type 2 pneumocyte hyperplasia with fibrosis which is the characteristic picture of BPD [7].

Pro-inflammatory cytokines stimulate alveolar type-2 pneumocyte to secrete KL-6 protein, so it is considered as a specific indicator of pulmonary injury affecting the alveolar epithelium and interstitium [8]. Neutrophil gelatinase-associated lipocalin 2 (NGAL2) is a 25 kD lipocalin that is covalently bound to matrix metalloproteinase (MMP)-9 produced by neutrophils, and it is considered as an acceptable marker of infectious/inflammatory processes, cancer monitoring, and induction of apoptotic pathway [9].

Adiponectin is a highly abundant circulating hormone with pleotrophic effects upon diverse pulmonary cell types including alveolar macrophages, epithelium, and vascular endothelium. It has important anti-inflammatory and vascular protective actions in lung [10]. Beta 2 Microglobulin (β2MG) is 11.8 KD a protein present on the surface of most nucleated cells. β2MG is considered as a marker of

inflammatory response, it does not cross the placenta so it is considered as a sensitive marker of inflammatory disorders especially in neonates [11]. Till now trials are go on, hoping to find new biomarkers for the early prediction and prognosis of BPD, so the aim of this study is to know the role played by KL-6, NGAL2, β2MG and adiponectin as novel biomarkers in development, early prediction, and early diagnosis BPD in preterm.

Patients and Methods

This study was approved by the ethical committees of Faculty of Medicine, Tanta University, Egypt (approval code: 2959/12/14). All chemicals unless otherwise described were purchased from sigma (Sigma, St Louis, USA). All chemicals and solvents were of high analytical grade.

Study design

We prospectively enrolled in this study, preterm infants who were admitted to neonatal intensive care unit at Tanta University Hospital with a gestational age ≤32 weeks from September 2012 to December 2013, the recruitment was conducted immediately after birth for whom met the following criteria: endotracheal intubation at delivery room, and gestational age ≤32 weeks. Informed consents of the parents after a complete description of the study were obtained. Preterm infants with congenital anomalies, multiple malformations, and chromosomal abnormalities, failure of urine sampling to be obtained before 48 hours of birth and infants who died of non-respiratory causes or were discharged before reaching a post-conceptional age of 36 weeks were excluded from this study. During the study period, a total of 73 preterm infants were enrolled. Among these preterm infants, 15 infants to whom broncho-alveolar lavage (BAL) was not done due to death or clinical instability and were excluded from this study. Finally 58 preterm infants were included and divided in two groups: group I (control): 28 preterm without BPD and group II: 30 preterm with BPD.

Blood sampling for KL-6 and adiponectin levels assay

Blood samples were aseptically collected from the umbilical vein by needle puncture just after delivery on EDTA tube then immediately centrifuged at 3000×g for 10 min at 4°C to obtain plasma then stored at −80°C. The KL-6 and adiponectin levels in plasma were measured using enzyme-linked immunosorbent assay (ELISA) kits (Kamiya Biomedical Company, USA and Ani Biotech Oy, Organium Laboratories Business Unit, Finland respectively) in accordance with the manufacturer's instructions.

Urine sampling for urinary β2MG level estimation

Spontaneous voided urine samples were obtained using urine collection bags or squeezing urine from cotton balls placed in diapers. Urine samples immediately centrifuged at 3000×g for 10 min at 4°C to obtain the supernatant which stored at -80°C until testing. All pathological samples showing proteinuria, hematuria, bacteriuria or abnormal sediments were excluded. Urine samples were used only when the pH level was >5.5 as β2MG is known to be unstable in acidic urine. Urinary β2MG level was estimated by ELISA using commercial kits (ORGENTEC, ORG 5BM, Germany) in accordance with the manufacturer's instructions.

Broncho-alveolar lavage (BAL) sampling

Endotracheal intubation in the delivery room was done only when clinically indicated according to the neonatal resuscitation program [12]. BAL fluid and cells were obtained by the method described by Choi et al., [13] shortly after birth as soon as the preterm infants became stable, but before surfactant replacement therapy when indicated. Briefly, with the baby supine, two aliquots of sterile saline solution, 1 ml/kg (maximum 2 ml), were instilled via endotracheal tube then immediately sucked back, and the returned BAL fluid was collected in a suction trap. BAL fluid was centrifuged for 10 min at 1,000 g within 10 min after acquisition. The sediment, which is a cell fraction, was collected and stored at -80 till used for RNA extraction.

RNA extraction, cDNA synthesis and real time PCR for NGAL2 mRNA gene expression

Total RNA was extracted from frozen BAL fluid sediment using Magna pure Compact Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions. cDNA synthesis was performed using the Roche LightCycler Fast Start DNA Master PLUS SYBR Green I kits (Roche Diagnostics) according to the manufacturer's instructions. Real-time PCR was carried out with single stranded cDNAs. PCR reactions were performed using Roche LightCycler Fast Start DNA Master PLUS SYBR Green I kits (Roche Diagnostics) following the manufacturer's instructions. Sequence specific primers were designed by Primer3 software: (http://bioinfo.ut.ee/primer3/) as follows: NGAL2 (NO: NM_005564.3) forward primer (5'- ACGGGAGAACCAAGGAGCTGACT -3') and reverse primer (3'- AGGGACAGGGTTAGCTGGTCACA -5'); Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (No: NM_001289746.1) forward primer (5'-AGTGCCAGCCTCGTCTCATAG -3), and reverse primer (5'-CGTTGAACTTGCCGTGGGTAG -3'). GAPDH (housekeeping gene) was used as an internal control. The following conditions were used: pre-denaturation at 95°C for 30 seconds, 40 cycles of denaturation (95°C for 15 seconds), re-annealing (66°C for 15 seconds), and extension (72°C for 15 seconds) [14]. Productions of the expected amplification fragments without unanticipated products and primers were confirmed by melting curve analysis. The final results were automatically calculated from the cross-point values of the target and the reference gene by Light Cycler 4 Relative Quantification Software (Roche Diagnostics).

Statistical Analysis

Statistical analysis was conducted, using SPSS software version.16 (SPSS, Inc., Chicago, IL, USA). Unpaired student t-test was used to evaluate the statistical significance between groups. Data were presented using mean and standard deviation (SD) for all quantitative values and or number of cases (percentage) for qualitative values. The Pearson correlation coefficient (r) was used to identify a correlation between different parameters. P value < 0.05 was considered significant. Receiver operating characteristics (ROC) analysis was used to identify the optimal threshold values of the studied parameters. The area under the curve can range from 0.5 to 1 and diagnostic tests that approach 1 indicate a perfect discriminator.

Results

Demographic character of the studied groups

There were no statistically significant difference between BPD and non BPD groups (p>0.05) as regards to gestational age (weeks), sex, birth weight (grams) and Apgar score (Table 1).

		Preterm with BPD (n=30)	Preterm without BPD (n=28)	T-test or chi-square	
				t or X^2	P-value
Gestational age (weeks)		32.20 ± 1.20	32.77 ± 1.00	1.83	0.07
Birth weight (grams)		1906.00 ± 179.00	1970.00 ± 169.00	1.30	0.20
Apgar score 1 min		4.02 ± 1.03	4.65 ± 1.62	1.64	0.11
Apgar score 5 min		6.54 ± 1.22	6.89 ± 2.05	0.73	0.47
Sex	Female	15 (54%)	24 (48%)	X^2=0.22	0.64
	Male	13 (46%)	26 (52%)		

Table 1: Comparison of gestational age, birth weight Apgar score at 1 and 5 minutes and sex of both studied groups using T-test and chi-square test.

Assessment of alveolar damage, inflammation and matrix remodeling

Cord blood KL-6 and urinary β2MG levels showed significant increase in BPD group when compared to non BPD group (P<0.05) (Table 2). NGAL2 showed significant up-regulation in BPD group when compared to non BPD group (P<0.05). Meanwhile, adiponectin showed non-significant decrease in BPD group relative to non BPD group

Parameters/Groups	Preterm with BPD (n=30)	Preterm without BPD (n=28)	T-test	
			t	P
Cord blood KL-6 (U/ml)	252.68 ± 21.59	78.58 ± 12.44	34.94	0.001*
Urinary B2M (mg/L)	10.39 ± 1.82	2.05 ± 0.57	21.91	0.011*
Adiponectin level (ng/ml)	2.38 ± 0.43	2.62 ± 0.38	7.25	0.398
BAL NGAL2 mRNA expression level	0.63 ± 0.17	0.49 ± 0.09	12.7	0.021*

Table 2: Comparison of cord blood Krebs Von den Lungen-6 (KL-6) (U/ml) and urinary beta 2 Microglobulin (β2MG) (mg/L), adiponectin ((ng/ml) andbroncho-alveolar lavage fluid (BAL) neutrophil-gelatinase-associated lipocalin2 (NGAL2) mRNA gene expression levels between both studied groups using T-test.

Correlation study

β2MG showed significantly positive correlation with BAL NGAL2, Cord blood KL-6, meanwhile insignificant negative correlation was found with adipocectin in BPD group (r=0.46, P<0.05; r=0.9, P<0.05; r=-0.258, p>0.05 respectively).

Data from ROC curve

Area under the ROC curve for KL-6 and β2MG for early diagnosis of BPD was 1, with optimal cutoff values of >109.1 U/ml and 3 mg/L for both respectively. Using those cutoff values, KL-6 and β2MG showed a sensitivity of 100% and a specificity of 100% for diagnosing BPD in preterm neonates (Table 3).

	Cut-off value	Sensitivity	Specificity	Area under the curve (AUC)	Significance (P-value)
Cord blood KL-6 (U/ml)	>109.1	100	100	1	<0.0001*
Urinary β2MG (mg/L)	>3	100	100	1	<0.0001*
Relative NGAL mRNA level	>0.34	92.9	86.7	0.8	<0.0001*

Table 3: Receiver Operating Characteristics (ROC) curve for cord blood Krebs Von den Lungen-6 (KL-6) (U/ml), urinary beta (2) Microglobulin (β2MG) (mg/L) and neutrophil-gelatinase-associated lipocalin2 (NGAL2) gene expression in the studied groups.

Area under the ROC curve for NGAL2 for early diagnosis of BPD was 0.8, with optimal cutoff value of >0.35. Using that cutoff value, NGAL2 showed a sensitivity of 92.9% and a specificity of 86.7% for diagnosing BPD in preterm neonates. KL-6 and β2MG had the best specificity and sensitivity for early diagnosis of BPD in preterm (Table 4).

	Urinary β2MG level	
Relative NGAL2 mRNA level	r	0.46
	p	0.021*
Cord blood adiponectin level	r	-0.258
	p	0.06
Cord blood KL-6 level	r	0.9
	p	0.001*

Table 4: Correlation between urinary beta (2) Microglobulin (β2MG) (mg/L) and neutrophil-gelatinase-associated lipocalin2 (NGAL2) gene expression, adiponectin and KL-6 (U/ml) in BPD group.

Discussions

One of the critical and chronic complications of preterm birth is BPD which is the most common serious pulmonary morbidity in preterm infants with associated social and economic burden [15]. Chest radiograph does not necessarily indicate the extent of lung damage, and their interpretation is subjective [16]. Therefore, a specific and objective marker that accurately reflects lung injury is very much needed. The key ingredient of BPD is low gestational age and its associated lung immaturity which is clearly aggravated by the presence of intrauterine growth retardation, exposure to oxygen supplemention,

pre- and postnatal pro-inflammatory mechanisms and nutritional deficits [16].

KL-6 augments the proliferative effect of fibroblastic growth factor and transforming growth factor-beta with stimulation of epithelial to mesenchymal transition with production of fibroblasts and extracellular matrix, in addition to its anti-apoptotic effect on lung fibroblasts so KL-6 may promote intra-alveolar fibrosis [17]. Circulating KL-6 is markedly higher in patient with interstitial lung diseases, and diseases characterized by type-II alveolar pneomocyte hyperplasia and fibrosis. Similar pathological changes in the lung are predominant in BPD [7]. In this study cord blood KL-6 level were elevated in preterm with BPD than control so that it may has a role in early prediction and future treatment of BPD. These results were in harmony with results obtained by Kim et al., [7] and Shigemura et al., [18] proved the importance of serum KL-6 as a marker of sarcoidosis and other interstitial lung diseases. Ogihara et al., [19] reported that KL-6, a specific lung injury marker, is increased and reflects BPD disease severity.

Pro-inflammatory cytokines are important immune response mediators which are largely secreted from immune cells, including macrophages, cytotoxic T cells and natural killer cells and can induce both acute and chronic inflammation [20]. Interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα), two primary pro-inflammatory cytokines secreted from immune cells, can induce NGAL2 expression [20]. NGAL2 belongs to a family of small proteins that are involved in the transport of steroids and lipids into cells. Recent studies have suggested that NGAL2 serve as mediator in airway inflammation. It is isolated from neutrophils, bone marrow cells, lung, bronchial and colon epithelial cells and could serve as a marker for neutrophil activity. The expression of NGAL2 in epithelial cells and body fluids is increased during inflammations and cancer [21].

This study revealed up-regulation of NGAL2 gene expression level in BAL fluid of BPD preterm than non BPD preterm. These results may be due to increased neutrophil and inflammatory cells infiltration in lungs that reflect lung epithelial cells injuries [22]. Thus, we hypothesized that NGAL2 may be associated with the pathogenesis and could be an early marker for BPD prediction in preterm infants at birth, because their levels were physiologically low. This result was in harmony with Inoue et al., [23].

Various adipo-cytokines have been associated with the occurrence of BPD in preterm neonates. Adiponectin is mainly produced by adipocytes and other cell types such as airway epithelial cells [24]. Adiponectin is considered to be a unique metabolic regulator in normal physiological/pathological conditions, anti-diabetic, anti-atherogenic, anti-inflammatory and has a protectant role against oxidative stress [25]. The results of this study revealed insignificantly decreased adiponectin level in BPD preterm when compared to the control. Animal studies have demonstrated that the onset of acute lung injury was predisposed by decreased adiponectin level [26,27]. Arakawa et al., [28] reported that decreased adiponectin level were associated with a higher incidence of pulmonary fibrosis in patients with scleroderma so that it could be used as a biomarker for interstitial fibrosis.

β2MG, a low-molecular-weight protein released by activated T and B lymphocytes, has been shown to be increased in several inflammatory and hematologic disorders. Increased serum β2MG levels could be used as a good marker of inflammation; however urinary β2MG is a sensitive, simple and non-invasive biomarker [29]. In this study urinary β2MG was significantly increased in BPD group than control which may reflect its importance as a non-invasive biomarker for early prediction of BPD in preterm. This result may be secondary to hypoxic stress that caused subclinical tubular dysfunction [30]. Nishimaki and Shima et al., [31] reported that increased urinary β2MG at birth can indicate fetal inflammatory response and may provide information on the risk of subsequent chronic lung disease. This study also, revealed significantly positive correlation between broncho-alveolar lavage NGAL2, Cord blood KL-6 and urinary β2MG which reflect the importance of urinary β2MG as a non-invasive biomarker that may have an important role in early diagnosis of preterm with BPD in the future. Furthermore, ROC curve results indicated that KL-6 and β2MG had the best specificity and sensitivity for early diagnosis of BPD in preterm. In view of correlation study so it can be concluded that β2MG is anon-invasive, easily assessed with the best specificity and sensitivity. Major limitations of our study remain the small number of preterm neonates included. Further studies involving a larger sample size are needed to further determine the usefulness of urinary β2MG as screening biomarkers for BPD in preterm neonates in addition to its usefulness to follow the course and outcome of BPD.

Conclusion

Based on the findings from this study KL6, adiponectin, NGAL2 and β2MG may provide promise in prediction and early diagnosis of BPD in preterm also the use of urine and serum biomarkers to detect and monitor BPD would aid in easy follow up of these patients. In addition, those biomarkers could be putative candidates for the development of novel drugs against BPD in the future.

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