Immunoexpression of Ki-67 in pulmonary hypoplasia

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Adequate pulmonary development at birth is the major determinant of postnatal outcome in the perinatal period. Lung hypoplasia is a poorly defined condition. The aim of this study was to investigate expression of Ki-67 in human fetuses with pulmonary hypoplasia compared to fetuses without pulmonary pathology and malformations of other organs used as controls. The analysis comprised 149 formalin-fixed and paraffin-embedded tissue sections from the files of the Clinical Pathology Department of the Research Institute of Polish Mother's Memorial Hospital in Lodz. Tissue sections obtained from lungs during autopsies were divided into two groups. In our studies immunohistochemistry was performed using antibody against Ki-67 as a cell proliferation marker for evaluation of growth fraction in the fetal and neonatal human lungs. The results presented in our study showed higher expression of growth fraction in the control group as compared to study subjects in all stages of lung development. Values of Ki-67 positive cells in the saccular stage of lung development were lower than in the canalicular and alveolar phase in both study and control groups. In conclusion, our results indicate their usefulness to understand better etiology of pulmonary hypoplasia and may be helpful in identifying the most appropriate moment for prenatal interventions.

Key words: pulmonary hypoplasia, growth fraction, Ki-67 immunoexpression.

Introduction

Adequate pulmonary development at birth is the major determinant of postnatal outcome in the perinatal period. Assessment of lung growth is often the most important component of the perinatal autopsy. Despite prenatal diagnosis and new postnatal treatment strategies, the mortality rate of infants with pulmonary hypoplasia is still high.

Pulmonary hypoplasia is a poorly defined condition seen in approximately 10-26% of neonatal autopsies [1-4]. This entity may be either unilateral (mostly related to congenital diaphragmatic hernia) or bilateral. Five independent risk factors for this condition have been described: hydrops fetalis, renal malformations, congenital diaphragmatic hernia/omphalocele, skeletal anomalies and abnormalities of amniotic fluid volume [5]. There is a spectrum of severity of this condition usually proportional to the severity of the underlying lesion. Clinical manifestation of pulmonary hypoplasia in neonates ranges from severe respiratory failure leading to neonatal death to respiratory insufficiency. The radiological recognition of hypoplastic lung is difficult but possible using three-dimensional ultrasonography or magnetic resonance imaging [6-11].

Two histological patterns in pulmonary hypoplasia have been described, although these are not always sharply delineated. In the first pattern, the lungs are poorly grown, but maturation is appropriate for the gestation age of the infant. In the second, the lungs are poorly grown and appear immature for the gestation age (delay in development of blood-air barriers, delay in epithelial maturation, lack of elastic tissue development and low concentration of lung phospholipids). Poor maturation is especially associated with oligohydramnios-related hypoplasia [12-17].

Because histological findings lack specificity, many pathological criteria of lung hypoplasia have

been established. These include: lung weight (LW) [18], lung weight/body weight ratio (LW/BW) [19-22], radial alveolar count [19, 20, 23], mean alveolar length [24], relative arteriolar media thickness [24] and finally DNA content [25]. Numerous authors have attempted to established diagnostic criteria of pulmonary hypoplasia but there is still uncertainty as to which method is more reliable. Lung weight /BW with age-matched reference values established by De Paepe *et al*. seems to be an accurate and objective method of assessment of lung growth at postmortem examination [22]. The RAC tool to evaluate complexity of lung development determined by Askenazi *et al*. is useful in the saccular and alveolar phase of pulmonary growth [19].

The Ki-67 antigen is a nuclear protein which is defined by its reactivity with monoclonal antibody from the Ki-67 clone. This antigen is preferentially expressed during all active phases of the cell cycle. Previous studies showed that Ki-67 is a useful marker for proliferative activity in the fetal and neonatal lung [26-28].

The aim of this study was to investigate expression of Ki-67 in human fetuses with pulmonary hypoplasia compared to fetuses without pulmonary pathology and malformations of other organs used as controls.

Material and methods

The analysis comprised 149 formalin-fixed and paraffin-embedded tissue sections from the files of the Clinical Pathology Department of the Research Institute of Polish Mother's Memorial Hospital in Lodz. Tissue sections obtained from lungs during autopsies were divided into two groups: tissue sections from the lungs of 112 live born and stillborn subjects selected according to recognized pulmonary hypoplasia (Group A) and from 29 so-called "normal" control infants or fetuses with no identifiable risk factors for lung hypoplasia (Group B) selected on the basis of LW/BW ratio. Each group was then divided into three subgroups on the basis of developmental stage of lung (A1/B1 – canalicular stage of lung growth; A2/B2 – saccular stage of lung growth; A3/B3 – alveolar stage of lung growth). Assessment of LW/BW ratio was based on the method established by De Paepe *et al*. [22]. The stage of lung development was evaluated adapting the method described by Langston *et al*. [29]. Immunohistochemistry was performed on formalin-fixed and paraffin-embedded sections mounted on glass slides (4-micrometer thick sagittal sections). The pre-treatment process of deparaffinization, rehydration and epitope retrieval was performed using PT Link (Pre-Treatment Module for Tissue Specimens, DakoCytomation). Immunohistochemistry was performed using En Vision DuoFLEX Doublestain System together with Dako Autostainer Instruments (DakoCytomation). Visualization was based on peroxidase (HRP) using DAB+ as a chromogen. FLEX Monoclonal Mouse Anti-Human Ki-67 Antigen antibody was used (Ki-67 Clone MIB-1, Ready-to-Use antibody, DakoCytomation). Morphometric analysis was performed without previous knowledge of the basic data of the child (gestational age, length of survival, if any, and other anatomical diagnoses obtained during autopsies).

Morphometric analysis was performed at the light microscope level (Nikon Eclipse E-800) using ImageJ software (Java-based image processing program developed at the National Institutes of Health). Slides were evaluated under a microscope connected to the camera (Nikon DS-V2). At least 5 images were taken from each slide (each separated by one visual field, magnification 100×) and stored on the computer's hard drive. Obtained images were selected and some of them were excluded from the analysis. In each obtained image quantitative examinations were carried out automatically using ImageJ's selection tools, working under macroinstructions written for this analysis. Differences in Ki-67 positive proliferating cells were determined in experimental and control groups with regard to developmental stage of lung growth. The results of evaluation were presented as a percentage of positive nuclei.

Student's t-test was used for comparisons between groups. Values were expressed as means ± standard deviation (SD). The significance level was set at $p < 0.05$.

Results

In all cases screened for study the diagnosis of pulmonary hypoplasia was established by assessment of LW/BW ratio (lung weight : body weight ratio). To verify the correlation between proliferative growth fraction and pulmonary development, the percentage of Ki-67 positive stained nuclei in control and experimental groups was analyzed. The results of statistical analysis are presented in Table I. Pulmonary hypoplasia was diagnosed more often in male (62.5%) as compared to female (37.5%) infants or fetuses. Primary lung hypoplasia (without underlying abnormalities) was diagnosed in 15 cases (13%) in this study in which secondary hypoplasia constitutes 87% of cases. In the majority of the secondary hypoplasia cases, renal/urinary tract malformations and congenital diaphragmatic hernia were diagnosed. There were statistically significant differences ($p < 0.05$) between the two investigated groups in the canalicular and saccular stage of lung growth. The mean values in the control group were significantly higher (A1: 9.86%; A2: 5.73%) than in the case group (B1: 7.04; B2: 4.56%). In relation to the alveolar phase of lung development there were higher mean values of growth fraction in the control group, but these differences were not sta-

Table I. Immunoexpression of Ki-67 in study and control groups

	Canalicular phase		Saccular phase		Alveolar phase	
	study group (A1)	control group (B1)	study group (A2)	control group (B2)	study group (A3)	control group (B3)
N	162	69	271	71	20	5
Mean value	7.041	9.861	4.560	5.736	7.506	10.781
Min.	0.354	0.183	0.272	0.309	1.117	9.433
Max.	23.758	25.312	19.952	13.841	18.228	12.589
SD	5.249	5.294	3.585	2.809	4.831	1.269
P	< 0.05		< 0.05		> 0.05	

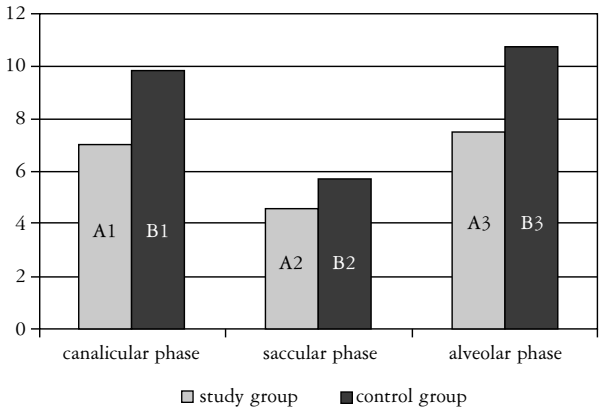

Fig. 1. The graph shows immunoexpression of Ki-67 in study and control groups in relation to lung developmental stage (A1/B1 – canalicular stage of lung growth; A2/B2 – saccular stage of lung growth; A3/B3 – alveolar stage of lung growth)

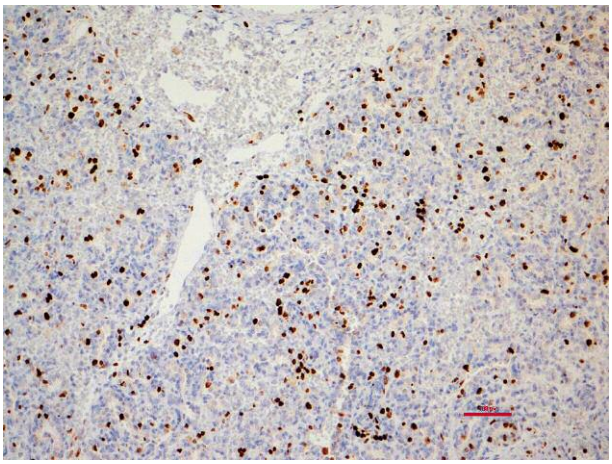

Fig. 2. Ki-67 immunoexpression in the parenchyma of hypoplastic lungs, original magnification 100×

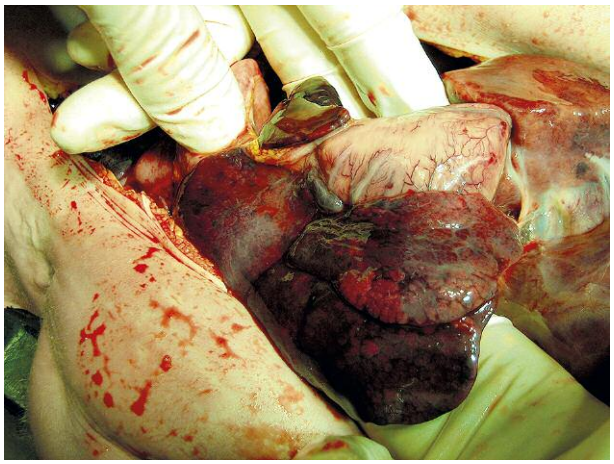

Fig. 3. Postmortem examination. Unilateral, secondary lung hypoplasia related to left-sided congenital diaphragmatic hernia. Pulmonary hypoplasia was diagnosed prenatally at 26 weeks of gestation. Female neonate died 6 hours after delivery

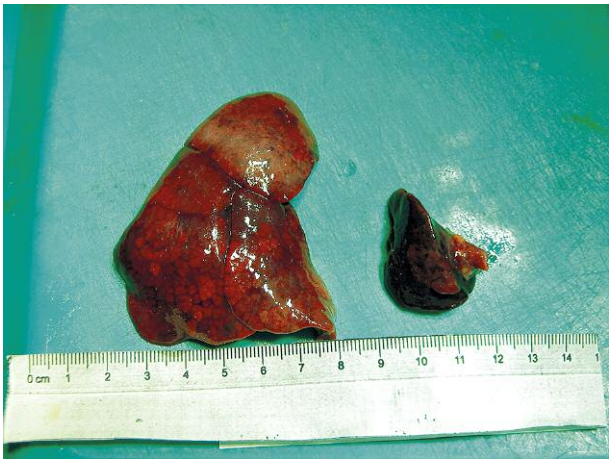

Fig. 4. Unilateral, secondary lung hypoplasia related to congenital diaphragmatic hernia. The same case as presented in Fig. 3

tistically significant. Mean growth fraction values were C1: 10.78% and C2: 7.50% of cases for the control and case group, respectively. Values of Ki-67 positive cells in the saccular stage of lung development were lower than in the canalicular and alveolar phase in both study and control groups.

Discussion

Previously published data showed that quantification of proliferating activity can be applied in studies of lung developmental malformations based on experimental animal models [28]. In our study, immunohistochemistry was performed using an antibody against Ki-67 as a cell proliferation marker for evaluation of growth fraction in the fetal and neonatal human lungs. So far there is only a single published investigation based on human lung examinations [27]. The study conducted by Thomas *et al*. revealed no significant difference in the proliferative ratios between control and hypoplastic lungs when gestational age was under 24 weeks. However, for gestational age above 24 weeks the growth fraction for controls was approximately four times higher than in the group of fetuses with pulmonary hypoplasia. The authors suggest that proliferative potential of the human lungs exists at or before 24 weeks of gestation. In the control group studied by Thomas *et al*. there were included 3 fetuses with conditions resulting in pulmonary hypoplasia (Arnold-Chiari malformation, abruptio placentae, renal agenesis). The previously mentioned study was underpowered as a result of the small sample size (controls: 8 cases; hypoplastic lungs: 12 cases). In addition, the improperly selected control group could affect the results.

Our findings contradict the results obtained by Thomas *et al*. The results presented in our study showed higher expression of growth fraction in the control group as compared to study subjects in all stages of lung development. Our study population consisted of 112 cases with pulmonary hypoplasia and 29 cases of gestational age-matched control subjects. In addition, cases with malformations potentially associated with pulmonary hypoplasia were excluded from the control group.

During histological and morphometric evaluation some interesting results were found. They support the theory that reduction in Ki-67 immunoproliferating cells is a characteristic feature of lung hypoplasia. Moreover, our data suggest that the lung proliferative fraction is reduced in the saccular stage of pulmonary development. The results are explicable. In the saccular phase of lung growth, the basic structure of the gas-exchanging unit is formed. Flattening of the acinar epithelium marks the differentiation of type II pneumocytes, from which type I pneumocytes responsible for gas exchange will be derived. During this period surfactant-producing pneumocytes mature, completing the functional maturity of the respiratory tract. The findings obtained in our investigation prove previously published data showing that in this period of lung development maturation and differentiation are the most intensive [30].

Adequate morphological and functional lung development is a critical determinant of postnatal outcome. It has been shown that advances in prenatal diagnosis of pulmonary hypoplasia have allowed *in utero* interventions. It seems that quantification of pulmonary hypoplasia is a useful tool in the choice of management options, including termination of pregnancy, planned delivery with intensive postnatal therapy, and fetal surgery intervention. Thomas *et al*. suggest that successful prenatal surgery should be performed before 24 weeks of gestation. Our findings enable us to formulate the hypothesis that *in utero* treatment of pulmonary hypoplasia should be performed in the canalicular or alveolar stage of lung development. In these stages the proliferative potential of human lungs for growth is the highest.

Quantification of growth fraction can be performed using manual or automatic counting of the percentage of positively stained nuclei. Previous studies revealed that data obtained using both methods were comparable. Our study was conducted using automatic morphometric analysis. Quantification of the proliferative fraction using this method turned out to be objective and fast.

In conclusion, our results indicate their usefulness to better understand the etiology of pulmonary hypoplasia and may be helpful in identifying the most appropriate moment for prenatal interventions.

The authors declare no conflict of interest.

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