Age-Related Vitamin D Deficiency Is Associated with the Immune Response in Children with Community-Acquired Pneumonia

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Summary  To investigate vitamin D status in children with community-acquired pneumonia (CAP) and explore the association between vitamin D deficiency and the immune response in CAP children, 77 children with CAP were retrospectively analyzed. The baseline characteristics of patients were obtained from medical records. Based on the blood samples collected during diagnosis of CAP, the routine blood examination results and proportions of lymphocyte subsets were assessed. There were 71.4% (55/77) of patients with vitamin D deficiency among CAP children. The serum 25(OH)D level significantly decreased with age. Patients with vitamin D deficiency had a significantly higher neutrophil percentage, but significantly lower lymphocyte percentage and count as well as proportion of CD19 positive lymphocytes (CD19+). Spearman’s rho test further confirmed these positive correlations and negative correlations. Moreover, significant associations of vitamin D deficiency with age and the above immune markers were also confirmed by univariate logistic regression analysis. However, only age entered the backward stepwise regression model in multivariate analysis. Vitamin D status in CAP children was negatively associated with age. Age-related vitamin D deficiency may affect the immune response in children with CAP.

Key Words  25(OH)D, age, vitamin D, community-acquired pneumonia, immune response

Community-acquired pneumonia (CAP) is a common inflammatory process which responds to infection with non-hospital pathogens in lungs (1). Moreover, patients are always co-infected by more than one pathogen (2, 3). Currently, CAP is still the leading cause of death from infectious diseases worldwide, especially in children younger than 5 y of age (4). Globally, it is estimated that the child deaths from pneumonia account for approximately 20% of all deaths in children aged less than 5 y old in the world (5). However, the pathogenesis of CAP is still unclear, which restricts the development of new prevention and treatment strategies.

Recently, Watkins et al. reviewed the association between vitamin D deficiency and common infections (such as sepsis, pneumonia, influenza, methicillin-resistant Staphylococcus aureus, human immunodeficiency virus type-1 (HIV), and hepatitis C virus (HCV)). Some previous studies have reported that vitamin D deficiency is associated with CAP and acute respiratory tract infection (6, 7). Moreover, it was reported that vitamin D played key roles in modulating the immune responses to Streptococcus pneumoniae (8), which is a common pathogen in children with CAP (9, 10). In addition, a great deal of evidence has proved the relationship between vitamin D level and some immunity related markers such as neutrophil/lymphocyte ratio, lymphocyte count and lymphocyte subsets (11–13). Thus, the vitamin D status may be related with the immune ability of patients with CAP and its deficiency-caused dysfunction of the immune system may be the major pathogenesis of CAP. Thus, it is necessary to confirm whether the vitamin D deficiency is associated with immunity in children with CAP.

Vitamin D is an important fat-soluble nutritional component, which includes vitamin D₃ (cholecalciferol) and vitamin D₂ (ergocalciferol) (14). Both of them are converted to 25-hydroxyvitamin D (25(OH)D including 25(OH)D₂ and 25(OH)D₃) in the liver (15) which is the major circulating form of vitamin D and a good indicator of vitamin D status. Thus, we evaluated the vitamin D status in children with CAP based on serum 25(OH)D level in this study. Meanwhile, routine blood examination results and the proportions of lymphocyte subsets (positive expression cells of CD3, CD4, CD8 and CD19, which are markers of immune response to pneumonia (16–18)) were also investigated to explore the association between vitamin D deficiency and immune response in children with CAP. In addition, the dependency of vitamin D status on age has been reported in many previous studies (19, 20). Thus, we also considered the effect of age on vitamin D status as well as the association between vitamin D status and immune markers in children with CAP.

MATERIALS AND METHODS

Patients. This study retrospectively collected children with CAP who were admitted to the Central Hos-
Table 1. The basic characteristics of children with and without vitamin D deficiency and with community-acquired pneumonia.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients with vitamin D deficiency (n=55)</th>
<th>Patients without vitamin D deficiency (n=22)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>4 (2.5–5.0)</td>
<td>2 (1.0–2.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Female</td>
<td>0.113</td>
</tr>
<tr>
<td></td>
<td>24 (43.6%)</td>
<td>14 (63.6%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>31 (56.4%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8 (36.4%)</td>
<td>32 (71.8%)</td>
<td></td>
</tr>
<tr>
<td>Onset season</td>
<td>Spring and summer</td>
<td>Autumn and winter</td>
<td>0.939</td>
</tr>
<tr>
<td></td>
<td>18 (32.7%)</td>
<td>15 (68.2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17 (31.8%)</td>
<td>7 (31.8%)</td>
<td></td>
</tr>
<tr>
<td>Hospitalization (d)</td>
<td>8.8±2.0</td>
<td>8.8±2.2</td>
<td>0.931</td>
</tr>
<tr>
<td>Allergy</td>
<td>Yes</td>
<td>No</td>
<td>0.239</td>
</tr>
<tr>
<td></td>
<td>35 (63.6%)</td>
<td>12 (54.5%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 (36.4%)</td>
<td>10 (45.5%)</td>
<td></td>
</tr>
<tr>
<td>Asthma</td>
<td>Yes</td>
<td>No</td>
<td>0.155</td>
</tr>
<tr>
<td></td>
<td>16 (29.1%)</td>
<td>3 (13.6%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>29 (52.7%)</td>
<td>15 (68.2%)</td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>Yes</td>
<td>No</td>
<td>0.856</td>
</tr>
<tr>
<td></td>
<td>44 (80.0%)</td>
<td>18 (81.8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11 (20.0%)</td>
<td>4 (19.2%)</td>
<td></td>
</tr>
<tr>
<td>Rales</td>
<td>Yes</td>
<td>No</td>
<td>0.503</td>
</tr>
<tr>
<td></td>
<td>33 (60.0%)</td>
<td>15 (68.2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22 (40.0%)</td>
<td>7 (31.8%)</td>
<td></td>
</tr>
</tbody>
</table>

For continuous variables, mean±standard deviation (SD) were used to describe continuous variables when data meet the normal distribution. Otherwise, the median and interquartile range were used. In addition, frequencies and percentages were used to describe categorical variables.

Pneumonia. The CAP was diagnosed and treated according to the guide for treatment of children with CAP (21). The inclusion criteria were: (1) aged 0–14 y old; (2) no clinical signs of rickets; and (3) no use of calcitriol, 1-a-OHD3 or other drugs which could influence the serum 25(OH)D level. Patients were excluded when they simultaneously suffered from other significant medical disorders. This study had approved by the Ethics Committee of Shanghai Xuhui Central Hospital (Approval No.: 2016-08, Supplementary file 1). The study was performed following the Declaration of Helsinki and good clinical practice guidelines.

A total of 77 children with CAP (38 males and 39 females) was included in this study. The median age was 3.5 y old with range of 0.3–12 y. Among them, 61% of patients (47/77) had an allergy and 24.7% (19/77) of patients had asthma. We collected the baseline characteristics of patients from medical records, including age, sex, hospitalization time, and clinical and pathophysiological symptoms.

Measurement of blood markers. Peripheral venous blood samples were collected from all the patients during the diagnosis of CAP. The whole blood samples were used to perform the routine blood examination and detect lymphocyte subsets. Lymphocytes were directly stained using fluorescein isothiocyanate-labeled murine monoclonal anti-CD3, CD4, CD8 and CD19 antibodies (Becton Dickinson, Mountain View, CA) and analyzed on a flow cytometer (FACSscan; Becton Dickinson, FACS Division). Lymphocyte subsets were enumerated as the percentage of positive cells (CD3+, CD4+, CD8+ and CD19+).

In addition, the remaining blood samples were used to test the serum 25(OH)D level for a simultaneously performed prospective study. After 1 h of coagulation at room temperature, serum was separated by centrifugation at 2,000 × g for 20 min at room temperature. The serum samples were stored at −20°C before laboratory experiments. The 25(OH)D level in serum was detected by chemiluminescence immunoassay (Liaison; Diasorin, Saluggia, Italy). The intra- and interassay coefficients of variation were 5.8 and 7.8%, respectively. We defined vitamin D deficiency as 25(OH)D level <30 ng/mL based on the previous studies (22, 23).

Statistical analysis. SPSS 19.0 statistical software was used for analysis in this study. For continuous variables, the Kolmogorov-Smirnov test was applied to test whether data were normally distributed. When data met the normal distribution, mean±standard deviation (SD) was used to describe continuous variables. Otherwise, the median and interquartile range were used. Difference between two groups was compared by Student’s t-test (normally distributed data) or the Mann-Whitney test (non-normally distributed data) (24). Meanwhile, one-way analysis of variance (ANOVA) analysis was used to evaluate difference among three groups. In addition, frequencies and percentages were used to describe categorical variables. The chi-square test was applied for comparison of proportions or distributions. Spearman’s rho test was used to examine the correlations between variables (serum 25(OH)D level as well as factors which were significantly different variables between patients with and without vitamin D deficiency). Furthermore, logistic regression analysis was applied to further evaluate the association between vitamin D deficiency and some factors (which were significantly different variables between patients with and without vitamin D deficiency according to the Student’s t-test, Mann-Whitney test or chi-square test) using the odds ratio (OR) and its 95% confidence interval (CI). A backward stepwise regression model was used in multivariate analysis.
RESULTS

Baseline characteristics of patients

As shown in Table 1, there were 71.4% (55/77) of patients with vitamin D deficiency in children with CAP in this study. Moreover, there were no significant differences between patients with and without vitamin D deficiency in sex, onset season, hospitalization, allergy, asthma, fever, rales or mycoplasma pneumonia infection (p > 0.05). Besides, both groups of patients were well matched with respect to mycoplasma infection and the severity of clinical manifestation. However, the CAP children with vitamin D deficiency (median age = 4 y, interquartile range = 2.5–5.0 y) were significantly older than CAP children without vitamin D deficiency (median age = 2 y, interquartile range = 1.0–2.8 y) (p < 0.001).

Difference between patients with and without vitamin D deficiency in blood markers

Based on routine blood examination results, we found that patients with vitamin D deficiency had a significantly lower lymphocyte count (p = 0.001) and lymphocyte percentage (p = 0.001) than patients without vitamin D deficiency. Meanwhile, the neutrophil count in children with vitamin D deficiency was significantly higher than that in children without vitamin D deficiency (p = 0.002, while no significant difference was found in neutrophil percentage (p = 0.058). In addition, results also showed that the positive expression cells of CD19 in patients with vitamin D deficiency were significantly less than that in patients without vitamin D deficiency (p = 0.008). However, no significant difference was found between patients with and without vitamin D deficiency in other blood markers (Table 2).

Vitamin D deficiency associated with age and some immune markers

According to the univariate logistic regression analysis, results showed that age (OR = 0.413, 95% CI = 0.258–0.661; p < 0.001), lymphocyte count (OR = 1.592, 95%
with the increasing of age (<2 y group: 32.76±7.82; 2–5 y group: 27.16±7.89; ≥5 y group: 19.32±6.24; p<0.05). Based on the definition of vitamin D deficiency, all the patients in the ≥5 y group exhibited vitamin D deficiency. Thus, we only evaluated the differences between the <2 y and 2–5 y groups in factors associated with vitamin D deficiency. The results showed significantly different lymphocyte (Fig. 2B) and neutrophil percentages (Fig. 2C) between the <2 y and 2–5 y groups in patients with vitamin D deficiency, but no significant difference was found in patients without vitamin D deficiency. Moreover, the proportion of CD19+ expression cells in the <2 y group was significantly higher than that in the 2–5 y group in patients without vitamin D deficiency, but this significant difference disappeared in patients with vitamin D deficiency (Fig. 2D). In addition, no significantly different lymphocyte count was found between the <2 y and 2–5 y groups in patients with and without vitamin D deficiency (Fig. 2A). Furthermore, for the above immune markers, there was no significant difference between patients with and without vitamin D deficiency in any age group (Fig. 2).

**DISCUSSION**

Many previous studies have proved the negative correlation between age and vitamin D level (19, 20, 25). Similarly, we confirmed this negative correlation in children with CAP in this study. We also found that vitamin D deficiency in children with CAP was significantly associated with some immune markers (lymphocyte count, lymphocyte percentage, neutrophil percentage and lymphocyte subsets of CD19+). Thus, the age-related vitamin D status might affect the immune response to CAP through mediating these immune markers in children.

Vitamin D binding protein (Gc-globulin) is the primary vitamin D carrier protein, binding 85–90% of total circulating 25(OH)D (26). It was reported that Gc-globulin was associated with the neutrophil chemotactic activity of C5a (27). Moreover, Gc-globulin deficiency could impair the neutrophil recruitment to the lung in both C5a- and CXCL1-induced alveolitis mice (28). Therefore, the vitamin D deficiency in this study may inhibit the neutrophil recruitment and then cumulate the neutrophils in blood. In addition, 1,25(OH)2D was the biologically active form of vitamin D and was converted from vitamin D to its active form of vitamin D.
Vitamin D Deficiency in the Immune Response

25(OH)D in the kidneys (14). It has been reported that 1,25-(OH)2D3 has inhibitory action on lymphocyte proliferation (29), which may be the main reason behind the decrease of lymphocyte percentage and count in children with vitamin D deficiency. CD19, as a response regulator of B lymphocytes surface receptor signaling (30), regulates innate immunity by toll-like receptor RP105 signaling (31). Moreover, the toll-like receptor expression can be impacted by vitamin D deficiency (32, 33). Meanwhile, it was also found that there was a link between toll-like receptors and vitamin D–mediated antimicrobial response (34). Thus, CD19 may play key roles in the vitamin D–mediated immune response in children with CAP. The vitamin D deficiency may cause the dysfunction of the immune system by decreasing the expression of CD19 in B lymphocytes. In addition, it was reported that vitamin D is also a modulator in controlling T cell antigen receptor signaling and activation of human T cells (35). Thus, the decrease of lymphocyte percentage and count in CAP children with vitamin D deficiency may be caused by the dysregulation of vitamin D on both B and T cells in this study. Hence, it was not surprising that there were no significant correlations between CD19+ and lymphocyte percentage and count (which included B and T lymphocytes). However, no significant associations between vitamin D status and CD3+, CD4+ and CD8+ (which were all cell surface glycoproteins found mainly on T lymphocytes) were found in this study. Thus, further studies are required to explore the association between vitamin D status and T lymphocyte-related immune response through the expression of other glycoproteins in T lymphocytes.

In addition, the results also showed the influence of age on these vitamin D-related immune markers in CAP children aged less than 5 y old. Many previous studies have reported that age is always associated with the immune status of patients with infectious diseases (36, 37). CAP, as a respiratory infection disease, also showed an age-related association for its etiology and symptoms (38). The higher incidence of CAP in children may be caused by the poor immune response to bacterial infection, which was commonly associated with the lymphocyte- and neutrophil-related immune response. Evidence has proved that children aged less than 2 y old have significantly higher absolute number of lymphocytes than older children and adults (39). Given the positive correlation between lymphocyte percentage and serum 25(OH)D level as well as negative correlation between lymphocyte percentage and age, the decrease of lymphocyte percentage in CAP children aged 2–5 y compared with children with an age of <2 y may be enhanced by the lower vitamin D level in patients with vitamin D deficiency. The above discussions give a reasonable explanation for the following results of this study: there was a significantly different lymphocyte percentage between the <2 y and 2–5 y groups in patients with vitamin D deficiency, but not in patients without vitamin D deficiency. However, no significant influence...
of age on lymphocyte count was found in patients with or without vitamin D deficiency. Small sample size may cause a higher false negative rate. Therefore, more studies are needed to confirm the effect of age on the vitamin D deficiency-related decrease in lymphocyte count. In addition, age-related decline of neutrophil function (40) may be followed by the decrease of neutrophil recruitment in organs and increase of neutrophil percentage in blood. Given the negative correlation between neutrophil percentage and 25(OH)D level as well as positive correlation between neutrophil percentage and age, the vitamin D deficiency-caused accumulation of neutrophils in blood may enhance the difference of neutrophil percentage between the <2 y and 2–5 y groups in children with vitamin D deficiency. However, there is still no evidence proving the association between neutrophil function and neutrophil percentage in blood. Nor was any significant association between neutrophil count and vitamin D status found despite the consistent trend with results of neutrophil percentage in the present study. Thus, further studies are required to investigate this phenomenon. On the other hand, as the standards of blood neutrophil and lymphocyte data may change in an age-dependent manner, the negative relation between vitamin D level and age suggested that there might be an indirect confounding correlation between vitamin D and neutrophil/lymphocyte coincidently, without immunological mechanisms of vitamin D.

Similarly, according to the correlation analysis results, there was a significant negative correlation between CD19+ and age, but a significant positive correlation between CD19+ and serum 25(OH)D level. Thus, the age-related decrease of vitamin D level may strengthen the decrease of CD19 expression in the 2–5 y group compared with the <2 y group. However, there is still no study considering the effect of age on CD19-related immune response. More studies are needed to verify these results and explore the mechanisms.

Notably, there were some limitations in this study. Firstly, we only retrospectively investigated the association between vitamin D deficiency and immune status from a small sample size of children with CAP, especially for patients without vitamin D deficiency or who aged more than 5 y. Prospective studies with a larger sample size should be performed for further confirmation of the results of this study. Secondly, no patients without vitamin D deficiency were found in the CAP children aged ≥5 y old in this study. Thus, the effect of age on vitamin D deficiency-related immune markers could not be analyzed using the results comparison between patients with and without vitamin D deficiency, so we only evaluated this effect in children aged less than 5 y old. Further studies are required to investigate the effect of age on these vitamin D deficiency-related immune markers in CAP children ≥ 5 y old.

In conclusion, vitamin D deficiency was negatively related with age in children with CAP. The age-related vitamin D status may affect the immune response of children to CAP via lymphocyte-, neutrophil- and CD19-related immune mechanisms.

REFERENCES


