Circulating 25-hydroxyvitamin D in secondary hemophagocytic lymphohistiocytosis

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Abstract

Background: Hemophagocytic lymphohistiocytosis (HLH) is a rare immune disease, accompanied by high mortality. Vitamin D (VD), as a marker of inflammation, plays an important role in the regulation of the immune system by binding to receptors of immune cells (T and B cells). The purpose of this study was to investigate the expression level of vitamin D in HLH and to clarify its relationship with prognosis. Method: We conducted a retrospective analysis of 54 adult sHLH cases from November 2015 to December 2020. Results: Among the cohort, 49 (90.7%) patients with sHLH had VD insufficiency (25-hydroxy vitamin D [25-(OH)D] <50nmol/L) and median level was 23.95 (range: 7.5-90.8) nmol/L. Classified by 30nmol/L as the threshold value, there were no significant differences on baseline characteristics between two groups, except triglycerides (TG). Level of 25-(OH)D was significantly associated with TG (r=-0.34, P=0.011) and ferritin (r=-0.26, P=0.055). Patients with lower 25-(OH)-D level acquired shorter overall survival (28 vs. 125 days P=0.041). After multivariable adjustment, 25-(OH)-D was identified as an independent factor of survival (HR 0.364;95% CI 0.183-0.724; P=0.004). A significant interaction was discovered at 25-(OH)-D with ferritin (P=0.009) through subgroup analysis. Vitamin D is more likely to predict survival in elderly, male, MHLH, EBV infection, hemoglobin<90g/L, platelet<100×109/L, fibrinogen<1.5g/L, triglyceride, ferritin >10000ug/l, sCD25>20000ng/l group. Conclusions: Patients with sHLH almost had vitamin D insufficiency and vitamin D is an important prognostic protective factor for sHLH.

Introduction

Hemophagocytic lymphohistiocytosis (HLH) comprises a heterogeneous spectrum of systemic disorder that are congenital (primary) or acquired (secondary), with the most devastating characteristics and mortality rate more than 50% even with specific treatment[1]. Regardless of specific type, knowledge of the pathogenesis remains deficient. There is a well-established association between immune dysregulation and the development of HLH. Hyperplasia of mononuclear/macrophagocytic system, persistent activation of $CD8^+$ T cells and dis-regulated function of natural killer (NK) cells caused by various triggers, leading to uncontrolled over-secretion of cytokines including interferon gamma (IFN-γ), tumor necrosis factor α(TNF-α), interleukin 6 (IL-6), IL-10, and macrophage-colony-stimulating factor (M-CSF), is widely believed to play a predominant part[2-6]. Therefore, it's of part interest of realizing early diagnosis and judging prognosis, to initiate the most appropriate treatment. Emerging researches showed that low platelet, elevated serum lactic dehydrogenase, higher lymphocyte/monocyte ratio could predict poor survival of HLH patients[7,8]. However, a common limitation of these indicators in improving the overall picture is the inability to be intervened directly. Then, we attempted to explore an index of prognostic importance and therapeutic possiblity.

Vitamin D (VD) is one of the indispensable steroid hormones, with well-documented function of maintaining the metabolism balance of human calcium and the formation of bone[9]. In addition, awareness is growing of the role of VD in immune system optimization on the basis of extrarenal activation by monocytes/macrophages[10]. Combined with receptor expressed on immune cells (B cells, T cells and antigen presenting cells), VD is associated with increased autoimmunity as well as susceptibility to infection. With

a long serum half-life, indistinctive regulation of liver production and dependent on substrate concentration, serum 25-hydroxyvitamin D (25(OH)D) provides the single best assessment of VD status[11]. Much recent works had demonstrated that VD deficiency was closely related to various autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus[12,13.] Also, suboptimal VD levels have been demonstrated to be a significant independent risk factor in chronic graft versus host disease and a promising prognostic predictor in various lymphoma[14,15]. So far, little research has described the characteristic findings of serum VD in patients with HLH. Therefore, the regulating activity of VD in immune processes-key components of HLH pathogenesis- prompted us to explore this role, on this account to extended treatment strategy and even improve overall survival of HLH patients. With our known etiology, we hypothesized that, for secondary HLH (sHLH) patients, vitamin D might possess a prognostic importance and might have the ability in distinguishing the primary triggers.

Materials and Methods

Study patients

A total of 54 adult cases with newly diagnosed sHLH at the First Affiliated Hospital of Nanjing Medical University from November 2015 to December 2020 was enrolled. All the diagnosis of sHLH was based on the 2004 HLH Diagnostic Criteria[16]. The NK cells cytotoxicity assay was not applicable due to the limitation of technology. Excluded criteria included (1) history of rickets; (2) history of parathyroid or metabolic diseases; (3) use of over-the-counter or prescribed vitamin complements; (4) history of severe liver and kidney dysfunction.

Data collection

Baseline patients' characteristics were collected on admission, including age, gender, specific pathogenesis, Epstein-Barr virus (EBV), complete blood cell count (CBC), fibrinogen (FIB), lactate dehydrogenase (LDH), triglycerides (TG), albumin, calcium, β2-microglobulin (β2 -MG), ferritin, serum soluble interleukin-2 receptor (sIL-2R, sCD25), fever, splenomegaly and serum 25-(OH)D. Our previous studies have revealed clinical significance of PET-CT in diagnosing lymphoma-associated HLH (LHLH) [17,18]. Therefore, in this study, lymphoma with lymph node or bone marrow biopsy and suspected by PET/CT were regarded as LHLH.

The evaluation of baseline vitamin D level was performed in the clinical laboratory of our hospital and assayed by electrochemiluminescence immunoassay (Rochee 170, Roche Co. Ltd. Shanghai, China). 25-(OH)-D levels were categorized according to a cut-off value of a published literature:30nmol/L[19].

Follow-up

Overall survival was regarded as the outcome of this study. It was calculated as the interval between days from sHLH diagnosis and death from any cause or the last follow-up. The survival status of all participants was assayed through inpatient medical records and telephone call.

Treatment

Specific treatment for sHLH was administrated to patients. Of 32 cases in 25-(OH)-D <30nmol/L, 24 patients (75%) received HLH-94 or HLH-04 orsystemic combination chemotherapy like EPOCH, CHOP, DEP; 6 patients (18.8%) received GC± Ivig ± CsA alone; and 2 patients (6.3%) received only supportive therapy due to progressive multiple organ dysfunction or patients' aspiration. Among 22 (40.7%) patients with 25-(OH)-D[?]30nmol/L, 18 (81.8%) were treated with chemotherapy or HLH-94/04; 3 (13.6%) were treated with GC+- Ivig +- CsA alone; and 1 patient (4.5%) was administrated with supportive therapy. No statistically significant difference was observed in treatment regimen between two 25-(OH)-D groups (P =0.839).

Statistical Analysis

All statistical analyses were performed using R software (version 3.6.3; The R Foundation for Statistical Computing), SPSS (version 23, Chicago) and STATA/MP(version 14, USA). Patients' continuous charac-

teristics were summarized as median+-quartile for non-normally distributed variables and mean standard+-deviations (SDs) for normally distributed variables. Categorical variables were presented as numbers and percentages. Differences between VD groups were analyzed using wilcox and chi-square tests, where appropriate. We conducted Kaplan-Meier method, univariable Cox proportional hazards models and multivariable Cox model (variables with P <0.05) to estimate hazard ratios (HRs) and 95% confidence interval to discover independent prognostic variables on OS. All subgroup analyses were presented with a COX test for interaction. A two-sided $P < 0.05$ was considered statistically significant.

Results

Baseline characteristics

Table 1 displayed patients' clinical characteristics and laboratory examination. The median age at diagnosis of total study population was 56 years and 23 patients (42.6%) were defined old patients (over 60 years). Male occupied a large proportion (70.4%), with ratio of 2.4. More than half (53.7%) suffered EBV infection. To be specific in terms of clinical manifestations, 54 patients (100%) presented with fever; 38 patients (70.4%) manifested with splenomegaly. Other laboratory findings of diagnostic criteria included hypofibrinogenemia (34/54), hypertriglyceridemia (26/54), hyperferritinemia (52/54) and hemophagocytosis (40/54).

The median level of 25-(OH)-D was 23.95 (range:7.5-90.8)ng/ml and 49 patients (90.7%)had vitamin D deficiency (25-(OH)-D<50nmol/L, i.e., 20ng/ mL). Then, we separated total cases into two groups according to a published research: low 25-(OH)-D level (n=32, <30 nmol/L) and relatively high 25-(OH)-D level (n=22, [?]30 nmol/L). Participants with lower 25-(OH)-D had higher TG (3.28*vs.* 2.45mmol/L P =0.032). There was no statistical difference on other characteristics. Patients in the high 25-(OH)-D group had a higher rate of hemophagocytosis, with a statistical trend (86.4% *vs.* 65.6% P =0.056).

According to the correlation analysis, the level of 25-(OH)-D was opposite to triglycerides and ferritin, with significant importance (Figure 1).

Association with 25-(OH)-D and prognosis

Upon final follow-up (February 1, 2021), the mortality rate was 83.3%. Median OS of the cohort group was 42 days (range: 1-669 days). As shown in Figure 2, patients with low vitamin D level acquired lower overall survival compared to those with high level (28 *vs.* 125 days$P$ =0.041). In table 2, univariate KM or Cox analysis demonstrated that hemoglobin, platelet, albumin, TG, β2 -MG, ferritin, $\log_e$ (sCD25) was significantly predictive for survival (P <0.05). Then, we transferred those indexes with$P < 0.05$ into multivariate Cox regression analysis to screen out independent risk factors of OS. The result demonstrated that vitamin D was an independent factor for OS HR=0.364 ;95% CI 0.183-0.724; $P = 0.004$). Also, inferior survival engaged other four factors: $\log_e$(sCD25)(HR=2.291;95%CI=1.186-4.426;P =0.014); ß2-MG (HR=1.093;95%CI=1.017-1.174;P =0.015); platelet (HR=0.983; 95%CI=0.971-0.996; P =0.008) .

Significance of 25-(OH)D deficiency through subgroup analysis

Stratified subgroup analyses were conducted according to age ([?] 60 years, > 60 years), gender (male, female), EBV infection status (negative, positive), pathogensis(MHLH,non-MHLH), hemoglobin (<90g/L, [?]90g/L), platelet (<100x10^9/L, [?]100x10^9/L), Fib (<1.5 g/l, [?]1.5g/l), TG (<3mmol/L, [?]3mmol/L), ferritin ([?]10000 ug/L, >10000 ug/L), sCD25 ([?]20000ng/l, >20000ng/l). 25-(OH)-D had a significant interaction with ferritin (P =0.009), and vitamin D was an important protective factor for ferritin>10000ug/L (HR=0.052(0.005-0.590),P =0.017). Although there were no significant interactions for the associations between 25-(OH)-D and survival across strata by other variables, vitamin D was more likely to predictive of survival in older, male, MHLH, EBV infection, hemoglobin<90g/L, platelet<100x10^9/L, Fib <1.5g/L, triglyceride, sCD25>20000ng/l group (Table 3).

Discussion

To the best of our knowledge, this was the first cohort study assessing the prognostic role of vitamin D in secondary HLH. We discovered that 49 (90.7%) sHLH suffered VD insufficiency (25-(OH)-D<50nmol/L).

Low 25-(OH)-D level was associated with survival of sHLH after adjusting for other prognostic factors.

Vitamin D, one of the indispensable elements of health and disease prevention, is mainly synthesized by the skin during sun exposure, or ingested in daily diet. Measurement of serum 25(OH)D was capable of represent the overall level of vitamin D in vitro [20]. According to previous research, vitamin D insufficiency is a widespread healthy issue, more common in Asia[21]. A large multi-center study (2173 healthy adults in Dalian, Beijing, Hangzhou, Guangzhou, and Urumqi) showed that 55.9% populations had insufficient vitamin D—serum 25(OH)D levels were less than 20 ng/ml[22]. In immune-related diseases, the proportion of VD insufficiency is higher or more serious. Schundeln et al[23] reported that the incidence in hemolytic anemia was 80.5%. 80% of patients with multiple myeloma have 25-(OH)-D<50nmol/L[24]. Consistent with those studies about vitamin D in different diseases, VD insufficiency incidence rate was over 90% in HLH and 71.1% patients were assigned to vitamin D deficiency (<30nmol/L), suggesting vitamin D may be the cause or rather a consequence of the disease. The result of a meta-analysis showed circulating 25-(OH)-D contributed to the occurrence of lung cancer, with increasing 10 nmol/L dose associated with 8% reduction in the risk[25].

The biological effects of vitamin D ranged from maintaining serum calcium and skeletal homeostasis to modulating immune processes[26]. The latter may be one of the reasons related to the prognosis of VD in sHLH. So far, the specific mechanisms of HLH remains no consensus, but more researches considered that congenital and acquired immunity contributed to the occurrence and development. The excessive activation of $CD8^+$lymphocytes and macrophages resulted in wild secretion of pro- and anti-inflammatory cytokines, like IFN-γ, IL-6, IL-12, IL-16, IL-18, TNF-a, and IL-10; those in turn to trigger persistent activation and cause the organ dysfunction characteristics[27]. Reichel et al [28] showed that the biologically active metabolite of vitamin D3 participated in modulating lymphocyte function via suppressing IL-2 secretion and decreasing production of IFN-γ by normal human peripheral blood lymphocytes. In addition, vitamin D is known to exert several immunomodulatory effects on cytokines synthesis in vitro, as it inhibits TNF-α, IL-12, and IL-17 and stimulates IL-10, promoting a shift from a Th1 to a TH2 phenocyte[29]. Furthermore, it increased the expression of regulatory T lymphocytes with a skewing away from the Th17 phenotype. It's noteworthy that 25-(OH)-D show a deeper immunoregulatory effect after lymphocyte activation[30]. Therefore, 25-(OH)-D was considered to exert the beneficial effect on protecting the risk of immuneregulated diseases. In our study, a negative correlation between 25-(OH)-D and TG levels was observed (r=-0.34, P =0.011), while the increase in TG in sHLH was mostly attributed to IFN-γ. High levels of IFN-γ increase very low-density lipoprotein and decrease lipase activity in the posterior liver, leading to TG clearance defects and hypertriglyceridemia[31]. 25-(OH)-D was relatively associated with ferritin (P =0.055). Therefore,we speculated that VD may represent the level of IFN-γ. Overall survival was shorter in low 25-(OH)-D level group than that in high level group (28 *vs.* 125 days P =0.041). After adjusting for hemoglobin, platelet, albumin, TG, β2-microglobulin, ferritin, $\log_e$(sCD25), 25-(OH)-D was proved to be an independent protective factor for overall survival (HR=0.364, 95%CI=0.183-0.724, P=0.004). Fattizzo et al[30] reviewed that vitamin D deficiency is correlated with disease severity/activity in autoimmune cytopenias. Another plausible pathway reasonable for the relationship between 25-(OH)-D and HLH maybe the mediation of iNK cells. Vitamin D receptor (VDR), the mediator of VD activity, contributed to the development of invariant natural killer (iNK) cells, a subset of lymphoid cells which participates in the host immune response and limits autoimmunity in negative feedback [32].

Second HLH is triggered by various pathologies, lymphoma, infections, autoimmune disorders[16]. Subgroup analysis showed that in MHLH, vitamin D displayed remarkably ability in predicting survival (HR=0.379, 95%CI=0.187-0.769, P =0.004). Lymphoma accounts for 98.1% of malignant tumors in this study. In diffuse large B-cell lymphoma (DLBCL) and T-cell lymphoma (TCL), Drake et al[33] found that the level of 25(OH)D and 1,25-(OH)-D was directly predictive for OS (HR=1.99 95% CI =1.27-3.13 for DLBCL; HR=2.38 95% CI=1.04-5.41 for TCL) and EFS (HR=1.41 95%CI=0.98-2.04 for DLBCL; HR=1.94 95% CI=1.04-3.61 for TCL). Vitamin D can regulate several key cellular processes, including inhibiting carcinogenesis by inducing cell differentiation, inhibiting proliferation and angiogenesis, and promoting apoptosis[34]. VD also can inhibit the proliferation of lymphoma cell lines and induce their differentiation in vitro experiments[35]. 25-(OH)-D was proven to influence tumors microenvironment, where chemokines, chemokine receptors, adhesion

molecules interacted[36]. Those may explain the prognostic role of vitamin D in HLH.

Some limitations were present in our study. First, there was a retrospective cohort study and the sample size was small. Then, further clinical researches with more samples are needed to confirm our result. Second, index including proinflammatory cytokines and/or inflammation markers were absent to allow for the assessment of severity. Sfeir et al[37] conducted a study about supplying vitamin D and found the improvement of prognosis in lymphoma patients. However, this study did not conclude the supplement of VD and answer whether 25-(OH) D supplement improve the therapeutic effect.

Conclusion

In general, vitamin D was significantly associated with survival outcome and was adopted as a prognostic factor of HLH. We hope that clear oral 25-(OH)D supplement can receive more attention from clinicians.

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Figure 1: Correlation between 25-(OH)-D and neutrophil/lymphocyte ratio (NLR), lymphocyte/monocyte ratio (LMR), hemoglobin (HB), platelet (PLT), triglycerides (TG), albumin (ALB), calcium (Ca), ß2- microglobulin (ß2-MG), serum ferritin (SF), sCD25. R is the correlation coefficient.

Figure 2: Survival curves of different groups of 25-(OH)-D.

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