

Cytokine Profiling as A Potential Biomarker in Diffuse Large B-Cell Lymphoma

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ABSTRACT

Cytokines are small proteins that mediate and regulate immunity. They are involved in the pathogenesis of many diseases including cancers. The concentration of these proteins in biological fluids (serum or plasma) and tissues in diseases may suggest pathway activation that leads to inflammatory response or disease progression. Therefore, these cytokines may be useful as a tool for screening, diagnosis classification between stages of disease or surveillance for therapy. Enzyme-linked immunosorbent assays (ELISA) and bioassay have been used as a gold standard in cytokine level measurements in clinical practice. However, these methods allow only single cytokine detection at a time and ineffective for screening purposes. Hence, the innovation of multiplexing technology allows measurement of many of these soluble proteins simultaneously, thus allowing rapid, cost-effective and better efficiency by using a minute amount of sample. In this study, we explored the profiles of key inflammatory cytokines from the serum derived from diffuse large b-cell lymphoma (DLBCL, $n=11$) and healthy volunteers ($N, n=11$) using multiplexed bead-based immunoassays. We aimed to evaluate if the levels of these cytokines are significantly different in these two groups and explore the possible application of the cytokine as biomarkers in early-stage screening and/or surveillance. Our results show a significantly high level of IL-17A, IL-10 and IL-6 in DLBCL-derived serum compared to n-derived serum. These preliminary results were obtained from a small sample size and could be further validated with a larger sample size cohort to produce a panel of biomarkers for DLBCL. Our findings might be useful in developing a disease-specific panel for biomarker screening assay. This could be used for early diagnosis and/or treatment surveillance.

INTRODUCTION

Non-Hodgkin lymphoma (NHL) is the 10th most common cancer in the world with 509,590 (0.02%) incidence cases reported globally [1, 2]. In Malaysia, there were 5,830 (0.05%) NHL incidence cases have been reported [3]. The incidence of NHL is variable and affected by age, gender, racial and geographic factors. Diffuse large b-cell lymphoma (DLBCL) is the most common type of adult NHL. DLBCL accounts for approximately 31% of all NHL in western countries and 37% of B-cell tumors worldwide [4]. The median age of DLBCL falls between 60-70 years old. DLBCL corresponds to a group of lymphoid malignancies composed of large cells with vesicular nuclei, prominent nucleoli, basophilic cytoplasm and a usually high proliferation rate. DLBCL is frequently recorded in patients with

inherited immunologic deficiency diseases and in families of patients with immunologic disorders. Treatment for most of DLBCL patients are using R-CHOP (rituximab with cyclophosphamide, doxorubicin, vincristine, and prednisone) with a good survival rate [5]. However, most of the patients suffer many side effects and long-term complications post-treatment. As a result of which, most patients were late-diagnosed. Hence, there is an urgency of developing a panel of reliable biomarkers with high sensitivity and specificity for screening that allows early detection of the disease.

Cytokines are soluble proteins that play a pivotal function in mediating and regulating immune responses. They are involved in the pathogenesis of many diseases including cancers [6, 7]. Under normal circumstances, the concentration of the cytokines

is undetectable or at a very low concentration in biological fluids (serum or plasma) and tissues. Their roles as a biomarker in diagnosis and treatment are often underestimated. An increase in their concentration suggests activation of certain signaling pathways that are usually involved in inflammatory response or disease development. Furthermore, the discrepancies of their concentration levels either locally or systematically in cancer patients may be a result of interactions between cell to cell, cells to the tumor, immune cells or produced by the tumor itself. These suggest that the cytokine may serve as a potential biomarker in diseases including cancer and useful as a screening tool, diagnosis classification between stages of disease or surveillance for therapy. Some examples of the soluble proteins that have been routinely measured in clinics in addition to the standard gold in patients' diagnosis and surveillance are interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α) [8, 9].

In the clinical laboratory, enzyme-linked immunosorbent assays (ELISA), bioassay and flow cytometry are routinely used as a gold standard method in measurements of cytokine level. The limitation of these methods is due to a single protein detection at a time and ineffective for high-throughput screening purposes. Today, with the advancement in technology, multiplexing allows better measurement, high efficiency and good sensitivity in detecting multiple numbers of analytes in a time- and cost-effective manner by using a small volume of samples [10, 11]. Multiplexing bead-based immunoassays were used in monitoring maternal immune status and quantitating the serum cytokine levels during the first half of pregnancy [12]. Several cytokines were shown to decrease in concentration from first to the second trimester with 9 inflammatory cytokines showed the highest intra-group correlation during pregnancy [12]. In prostate cancers, 7 cytokines and chemokines were identified as having significant inflammation markers of possible importance for prostate cancer pathophysiology using multiplexing bead-based immunoassays [13]. Also, in a study with retinitis pigmentosa patients, A significant increase in some cytokines including IL-6 and IL-8 were significantly higher in these patients compared to the control group using this platform [14].

In our study, we explored the profiles of the key inflammatory cytokines in DLBCL using multiplex bead-based technology. Serum derived from DLBCL patients was investigated against the serum of healthy volunteers (N). Concentration levels of these soluble proteins were evaluated if they were any significant differences between these two groups. This approach also allows us to explore the feasibility of the technique for future biomarker screening assays that might be useful in the early diagnosis and/or surveillance of patients. Multiplexing bead-based immunoassay was tested on the key inflammatory cytokines that are associated with tumor development and/or progression: Interleukin-17A (IL-17A), Interferon-gamma (IFN- γ), tumor necrosis factor (TNF), Interleukin-10 (IL-10), Interleukin-6 (IL-6), Interleukin-4 (IL-4) and Interleukin-2 (IL-2).

We propose that there is a correlation of high expression of some of these cytokines in DLBCL that may suggest the pro-inflammatory or pro-tumorigenesis effects. Thus, the results could be useful for the development of a biomarker panel that is specific for DLBCL. This will be useful to support the Immunocore technique which is routinely done in clinical diagnosis. Besides, the cytokines may represent targets for directed therapy approaches and allow discrimination of sera derived from DLBCL patients for early diagnosis.

MATERIALS AND METHODS

DLBCL patients and healthy volunteers

Ethical clearance was released by the UKM Research Ethics Committee (Reference number UKM PPI/111/8/JEP-2016-063). A total of 3ml of peripheral blood was collected in BD Vacutainer® plain tubes containing no anticoagulant (Becton Dickinson), immediately centrifuged for 5min at 4000 rpm). The serum was collected and stored at a -80°C freezer until used. The serum samples were collected from DLBCL patients ($n = 11$) and healthy volunteers (N) ($n = 11$) who were diagnosed in Hospital Canselor Tuanku Muhriz UKM (HCTM). N samples were used as a reference and control. These are the volunteers who underwent annual health screening procedures and were diagnosed as normal. The cases were histologically confirmed by the pathologist and clinically diagnosed. Demographic data including age, gender, disease classification and tumor staging are summarized in Table 1. All donors were free from immune-related disorders i.e. allergies, autoimmune diseases and acute/chronic infections.

Table 1. Clinical data of the patients enrolled in the study.

| Variables | DLBCL ($n=11$) | Healthy controls ($n=11$) |
|-------------|---------------------|-----------------------------------|
| Age (range) | | |
| <50 | 2 | 4 |
| ≥ 50 | 9 | 7 |
| Sex | | |
| Male | 3 | 5 |
| Female | 7 | 6 |
| Race | | |
| Malay | 10 | 7 |
| Chinese | 1 | 4 |

Cytometric beads analysis (CBA) analysis

The list of soluble proteins that were determined by using the BD™ CBA (BD Biosciences, San Diego, CA) is shown in Table 2. Samples preparations were performed according to the manufacturer's instructions. Briefly, 50 μl samples (standard or test) were added to 50 μl of a cocktail of capture beads and 50 μl pre-conjugated detection antibodies. The samples were incubated for 3 hours, at room temperature in the dark. Excess unbound detection antibody was washed off before data acquisition. Sample data were acquired using a BD™ FACSVerse flow cytometer system (BD Bioscience) and BD FacSuite™ software.

Table 2. List of soluble proteins that were detected in CBA analysis.

| No | Types of analyte | CBA kit | Analytes detected |
|----|---------------------|--|---|
| 1 | Cytokine | Human CBA Th1/Th2/Th17 cytokine kit (BD Biosciences, San Diego, CA) | IL-2 IL-4 IL-6 IL-10 TNF IFN- γ IL-17A |

Sandwich enzyme-linked immunosorbent assay (ELISA)

The results from CBA analysis were further validated with sandwich ELISA analysis. The serum samples were from the previously analyzed DLBCL- and N-derived serum used in CBA analysis. The concentrations of IL-6, IL-10, IFN- γ and IL-17a were determined using specific Quantikine® colorimetric sandwich Elisa kits as per the manufacturer's instructions (R & D systems, Ray Biotech) respectively. For each of the Elisa kit, the respective standards were included. The standards were serially diluted to obtain the standard curve for protein concentration determination. The sensitivity for the assay were

0.7pg/ml (IL-6), 3.9pg/ml (IL-10), 5.69pg/ml (IFN- γ), 15pg/ml (IL-17A). Standard and control samples were tested in triplicates. The absorbance was measured with a spectrophotometer at a wavelength of 450nm.

Data analysis

Quantitative data analysis was performed using CBA dedicated analysis software BD FCAP Array™ software (BD Biosciences) which determines the protein concentrations based on the known concentration values of a set of standards (Datasheet 1 in supplementary material S1). The representative results from flow cytometry are included in Datasheet 2 in the supplementary material (S2). The minimum detection levels for each analyte were as recommended by the manufacturer’s protocol. Samples where the concentration of the analyte was below the given sensitivity, were treated as undetectable. Statistical analysis was done using Graph Pad Prism 9.0.0 (version 121). Statistical significance was calculated by T-test with Welch’s correction. P < 0.05 was considered statistically significant.

RESULTS

CBA analysis of serum cytokines concentration

Serum levels of IL-17A, IFN- γ , TNF, IL-10, IL-6 and IL-2 showed measurable concentration which shows some significant difference between the control and disease groups (Fig. 1 and Table 3). Serum cytokine concentrations for IL-6, IL-10, IFN- γ and IL-17A show significantly higher levels in DLBCL patients compared to N volunteers (median: 16.80pg/ml, 45.00pg/ml, 8.46pg/ml and 52.36pg/ml respectively with $p < 0.05$). There was no significant difference in IL-2, IL-4 and TNF.

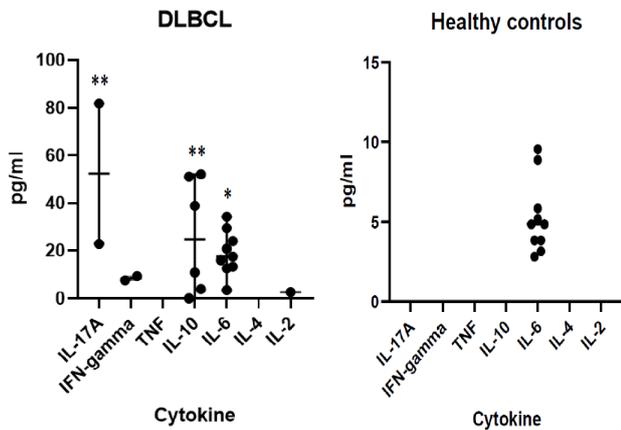


Fig 1. Distribution of human cytokine concentration levels in DLBCL- and N-derived serum. Results are shown as medians with 95% confidence intervals; significant differences between DLBCL- and N- are presented. Level of significance: * $p < 0.05$; ** $p < 0.01$.

Table 3. Concentration level of cytokines from DLBCL- and N-derived serum from CBA analysis. Data show the median and interquartile range.

| Cytokine | DLBCL (median; pg/ml) | N (median/ml) |
|-------------------|-----------------------|--------------------|
| (a) IL-2 | 2.64 (0.00 – 2.64) | 0.00 |
| (b) IL-4 | 0.00 (0.00 – 0.00) | 0.00 |
| (c) IL-6 | 16.80 (3.49 – 34.33) | 4.84 (2.82 – 9.56) |
| (d) IL-10 | 45.00 (3.87 – 52.05) | 0.00 |
| (e) TNF | 0.00 (0.00 – 0.00) | 0.00 |
| (f) IFN- γ | 8.46 (7.58 – 9.33) | 0.00 |
| (g) IL-17A | 52.36 (22.89 – 81.83) | 0.00 |

Sandwich enzyme-linked immunosorbent assay (ELISA)

Validation results were in agreement with the data obtained from CBA analysis (Fig. 4). However, the ELISA showed a lower detection level as expected. IL-2 was not detected in ELISA.

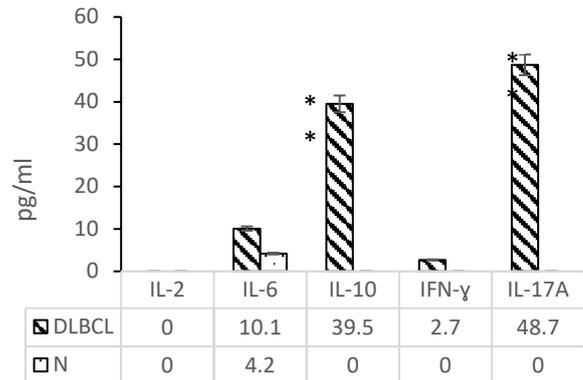


Fig. 4. Sandwich ELISA of human soluble proteins concentration levels in DLBCL- and N-derived serum. Data are mean \pm SD, $n = 11$. Results are presented as absolute values of pg per ml and level of significance * $p < 0.05$.

DISCUSSION

We used BD cytometric bead array (CBA, BD Biosciences, San Diego, CA) for analyzing a total of 7 cytokines in sera of DLBCL patients and N controls. The CBA assay has better sensitivity compared to the conventional ELISA, where it has a higher detection limit up to 0.274pg/ml [15, 16]. Also, CBA allows multiplexing, thus allowing more data to be produced at a time by maximizing the number of markers and less volume of patient samples. This is important especially in clinical settings where samples from patients are often limited. However, the reporter signal from the reacted beads exhibited considerable heterogeneity that was assumed to result primarily from the variable amount of capture antibody bound to each bead. Therefore, even though each bead in a sample tube represented a single assay, a minimum count of 100 beads per cytokine was necessary to obtain a mean or median value that stabilized or did not change with additional counts. [17]. Besides, normal animal sera were used to block or reduce any significant false positivity, just as is done in ELISA to minimize any non-specific bindings such as from potential interference of heterophilic antibodies [17].

In clinical settings, some parameters should be taken into serious considerations to obtain a reliable cytokine measurement such as sample handling, storage and quality control of the platform used for cytokine detection. Timing of sampling is important. Cytokines are under neuroendocrine control and thus have a diurnal rhythm [18]. Many pro-inflammatory cytokines, such as IL-1, IL-6, TNF- α and IFN- γ are linked to melatonin and peak early in the morning. Besides, exercise also impacts cytokine levels in the blood, as after exercise, among others, muscle cells release IL-6 and which will result in temporarily elevated cytokine levels [18]. Our sampling time for DLBCL patients was all done at approximately the same time at midday once diagnosed.

Secondly, proper sample handling, transportation and storage are critical for reliable measurement of circulating cytokines. In this study, our samples were processed immediately and the frozen serum sample was kept at -80°C until analysis. Multiple freeze-thawing cycles were avoided. Any delay of sample

processing containing cellular components may lead to different cytokine expression profiles by either degradation, absorption or cellular production of cytokines [18]. Furthermore, careful consideration should be made for the use of blood collection tubes. During clotting or by centrifugation, platelets can release various cytokines such as IL-6 and CXCL8 [18]. Another critical factor is the quality control of the platform used. In our commercial kit, quality control was done using several dilutions of the recombinant standard curves as a spiked in QC sample. We also use the same antibodies (clones) and lot number to minimize variations. Sample dilution might reduce assay sensitivity especially for detection of cytokines such as IL-4, which is already biologically active in the lower pg/ml range. Thus, optimization of the right dilution for the assay is crucial.

In our study, we identified 4 cytokines that showed an elevated level in DLBCL samples as compared to N-derived serum. 3 of these cytokines (IL-17a, IL-10 and IL-6) showed a significantly higher level ($p < 0.05$) in DLBCL as to normal control. Chronic inflammation can trigger events that would induce the malignant transformation of cells and carcinogenesis. Cytokines play a crucial role and can control the development and multiplication of cancerous cells. However, clinical data present controversy about the participation of these cytokines in the establishment and development of cancer.

IL-17A is a proinflammatory cytokine produced by activated Th-17 cells. Significant high expression of IL-17A in the DLBCL-derived serum in our study may suggest an increase in stimulation of activated B-cell and T-cell-proliferation due to the inflammation in DLBCL patients. This is in agreement with other reports in the breast [19], gastric [20] and prostate cancer [21]. IL-17A function in cancer has been controversial with a double-edged role [22]. It has both the oncogenic role in promoting cancer and also anti-tumor activity in inhibiting tumor progression. In gastric cancer, a significant correlation between IL-17A expression, the number of mast cell tryptase (MCT)-positive cells and the degree of fibrosis was described [23]. IL-17A derived from mast cells contributed to tumor fibrosis in peritoneal dissemination of gastric cancer. In lung cancer studies, direct stimulation of IL-17A/F did not alter lung cancer cell viability or metabolism however, IL-17A/F-stimulated macrophages promoted lung cancer cell progression through an increased migration capacity and enhanced tumor growth, proliferation and angiogenesis [24].

In lymphoma, IL-17A stimulates the proliferation of primary B-NHL cells and neo-angiogenesis via IL-17A-mediated induction of pro-angiogenic gene expression in tumor cells and direct stimulation of endothelial cells [25]. It also enhances tumor growth through the induction of IL-6, which in turn activates oncogenic transcription factor signal transducer and activator of transcription 3 (STAT3) and upregulates pro-survival and pro-angiogenic genes [26]. This supports our results through the elevation of both IL-17A and IL-6. In addition to tumor progression, IL-17A contributes to metastasis through the upregulation of metastasis-associated 1 (MTA1) [21]. With metastasis in place, resistance to therapy is expected. IL-17A promotes rituximab resistance by suppressing p53 expression [27] and anti-VEGF resistance through the recruitment of MDSCs [28, 29].

IL-10 is a potent anti-inflammatory cytokine. Elevated levels of IL-10 as observed in our study are associated with increased tumor growth with poor prognosis and drug resistance [30]. However, IL-10 also inhibits the activation and effector function of T cells, monocytes and macrophages [31]. This might well

explain the poor immune response in these patients. High concentrations of IL-10 in the serum of cancer patients also suggest the expression of IL-10 not only limited to immune cells, but cancer cell is also capable of synthesizing it, which would cause an imbalance in the homeostasis of the immune system. High concentration IL-10 has been shown in many late-stage cancers and correlated with poor prognosis. This includes among others esophageal adenocarcinomas [32], multiple myeloma [30] and thyroid cancer [33]. The correlation between the IL-10 expressions and cancer stage showed that it could be usefully associated with other cytokines as biomarkers to discriminating advanced cancer.

IL-6 acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine. IL-6 is produced by tumors and many other cells in the tumor microenvironment. It facilitates tumor growth and sustenance by influencing and regulating nearly all hallmarks of cancer, besides contributing to therapeutic resistance. IL-6 stimulates tumor cell proliferation and survival by activating the RAS/RAF/MEK/MAPK, PI3K/AKT and JAK/STAT pathways via gp130 tyrosine phosphorylation [34, 35]. These signaling pathways help tumors in the acquisition of unlimited replication potential, which is essentially required to generate large tumors. A significantly high level of IL-6 observed in our study is in accordance with many reports in other cancers including colorectal cancer [36], breast cancer [37] and prostate cancer [38]. This suggests a strong link between IL-6 and cancer. High levels of circulating IL-6 are generally associated with poor prognosis and shorter survival, whilst a lower level of IL-6 is associated with better response to therapy [39]. Apart from cells, cancer treatment such as chemotherapy and radiation can also induce IL-6 expression in tumor and stromal cells [40, 41] through the activation of NF- κ B signaling, leading to therapeutic resistance [42]. Furthermore, other stimuli that are associated with tissue damage or stress (e.g. UV, reactive oxygen species, viruses, microbial products and other pro-inflammatory cytokines) may also activate IL-6 secretion [43, 44]. IL-6 expression has also been found higher in recurrent tumors as compared to primary tumors, as well as in recurrent metastatic lesions as compared to primary metastasis [45]. Therefore, IL-6 is one of the most important biomarkers indicative of tumor development and its prognosis.

CONCLUSION

In summary, the cytokinome approach by multiplexing measurements, although time-dependent, can be very useful to identify cytokine clusters. We have demonstrated some preliminary data on statistically significant concentration levels of cytokines in DLBCL patients' serum as compared to healthy control volunteers. The limitation of this study was the small sample size and could be further validated with a larger sample size cohort. This will eventually produce a panel of biomarkers for DLBCL patients that would consist of a panel of cytokines that is useful for diagnosis, cancer progression and treatment efficiency.

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