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Profiling of cytokines, chemokines and other soluble proteins as a potential biomarker in colorectal cancer and polyps

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ABSTRACT

Soluble proteins including cytokines, chemokines and growth factors are small proteins that mediate and regulate immunity. They 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 soluble proteins 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 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, chemokines and other soluble proteins from the serum derived from colorectal carcinoma (CRC, $n = 20$), colorectal polyps (P, $n = 20$) and healthy volunteers (N, $n = 20$) using multiplexed bead-based immunoassays. We aimed to evaluate if the levels of these soluble proteins can classify these groups of populations and explore the possible application of the soluble proteins as biomarkers in early stage screening and/or surveillance. We observed significant high IL-4, MIP-1β, FasL and TGF-β1 levels but lower levels for RANTES in P-derived serum as compared to N-derived serum. Significant high IL-8, VEGF, MIP-1β, Eotaxin and G-CSF observed in CRC-derived serum when compared to N-derived serum. Between CRC- and P-derived serum, significantly higher levels of IL-8, Eotaxin and G-CSF but lower levels for TGF-β1 were detected in CRC-derived serum. These preliminary results were obtained from small sample size and could be further validated with larger sample size cohort to produce a panel of biomarkers for CRC and P patients. 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.

1. Introduction

Colorectal carcinoma (CRC) is one of the commonest cancer worldwide with 1.4 million (9.7%) cases reported globally [1]. There were 2246 CRC cases (12.3%) have been reported in Malaysia [2]. Risk factor such as genetic is known to contribute to the CRC development and progression. However, it was not until 2011, other risk factors such as evading immune response were also recognized as contributors to cancer progression [3]. Since then, many pieces of evidence have shown that immune systems do have a protective role in tumorigenesis [4–6]. In fact, the correlation between serrated polyps (P) and CRC have been also reported [7–9]. It is hypothesized that P could be the precursor lesions for CRC because 15–20% of all sporadic CRCs arise via the serrated pathway [10,11]. According to the World Health Organization (WHO) criteria, P are classified pathologically as hyperplastic polyps (HPs), sessile serrated adenoma/polyps (SSA/Ps) with or without cytological dysplasia and traditional serrated adenomas (TSAs) [12]. The problems often arise when CRC only gives vague and non-specific symptoms and thus leads to difficulty in detecting CRC at an early stage. As a result of which, most patients are 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.

Soluble proteins including cytokines, chemokines and growth factors are small proteins that mediate and regulate immunity. They play a role in the pathogenesis of many diseases including cancers [13,14]. Normally, the concentration of these proteins in biological fluids (serum or plasma) and tissues are undetectable or very low. In fact,

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their roles as a biomarker often are underestimated. However, an increase in their concentration may suggest pathways activation that is involved in inflammatory response or disease development. Furthermore, the variability of their concentration levels either locally or systematically in cancer patients may be a result of the interaction between tumor, immune system and the production of these soluble proteins by the tumor itself. These suggest that the soluble proteins may serve as a potential biomarker of various diseases including cancer. Indeed, this could be used as a tool for screening, diagnosis classification between stages of disease or surveillance for therapy [15]. Interleukin-6 (IL-6), Tumor Necrosis Factor Alpha (TNF-α) and C-reactive Protein (CRP) are some examples of the soluble proteins that have been routinely measured in clinics in addition to the standard gold in patients' diagnosis and surveillance [16,17].

In the clinical laboratory, enzyme linked immunosorbent assays (ELISA), bioassay and flow cytometry are routinely used as a gold standard method in cytokine level measurements. However, these methods allow only single protein detection at a time and ineffective for high-throughput screening purposes. Today, with the advance in technology, multiplexing allows better measurement and efficiency in detecting multiple numbers of analytes in a time- and cost-effective manner by using a small volume of samples [15,18].

In this study, we explored the profile of key inflammatory cytokines, chemokines and other soluble proteins using multiplexing technology. Serum derived from CRC and P patients were used as the test samples against the control samples from the serum of the healthy volunteers (N). Concentration levels of these soluble proteins were evaluated if they were significantly different between the groups. This approach also allows us to explore the feasibility of the technique for future biomarker screening assay that might be useful in the early diagnosis and/or surveillance of patients. Multiplexing was tested on the key inflammatory cytokines, chemokines and growth factors 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), Interferon gamma-induced protein 10 (IP-10), monocyte chemotactic protein (MCP-1), monokine induced by gamma interferon (MIG), regulated on activation, normal T cell expressed and secreted (RANTES), Interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), Lymphotoxin-alpha (LT-α), macrophage inflammatory protein 1a (MIP-1α), macrophage inflammatory protein 1a 1β (MIP-1β), basic fibroblast growth factor (FGF), Eotaxin, granulocyte colony-stimulating factor (G-CSF), Angiogenin, Fas ligand (FasL), granulocyte macrophage colony stimulating factor (GM-CSF) and transforming growth factor-β1 (TGF-β1).

We propose that the different groups of soluble proteins may have pro-inflammatory or pro-tumorigenesis effects. Thus, the results could be useful for the development of a biomarker panel that is specific for CRC and/or P. This will be useful to support the Immunoscore technique which is routinely used in clinical diagnosis. In addition, the soluble proteins may represent targets for directed therapy approaches and allow discrimination of sera derived from CRC and P in early diagnosis.

2. Method

2.1. Patients and healthy volunteers

Ethical clearance was released by the UKM Research Ethics Committee (Reference number UKM 1.5.3.5/244/FRGS/2/2013/SKK01/UKM/03/3). A total of 3 ml of peripheral blood was collected in BD Vacutainer® Plain tubes containing no anticoagulant (Becton Dickinson), immediately centrifuged for 5 min at 4000 rpm). Serum was collected promptly and stored at −80 °C freezer until used. The serum was collected from CRC patients (n = 20), P patients (n = 20) and N volunteers (n = 20) who were diagnosed in UKM Medical Center, Kuala Lumpur (UKMMC) from 2014 to 2015. N samples were used as a reference and control. These are the volunteers who underwent endoscopy procedures as part of their annual health screening and were diagnosed as normal. Patients with polyps were diagnosed with primary polyps including serrated adenoma, adenoma polyps and dysplasia. The hyperplastic polyps were not included in the study. For the CRC cases, the serum samples were collected from the Dukes' B and C stages. The serum collected from CRC and P samples were confirmed by a panel of the pathologist, primary diagnosed and free from any treatments before blood sampling. Demographic data are summarized in Table 1. All donors were free from immune related disorder i.e. allergies, autoimmune diseases, and acute/chronic infections.

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

Variables	N (n = 20)	P (n = 20)	CRC (n = 20)
Age (range)	55–77	56–80	49–81
Median	62 ± 12	68 ± 10	72 ± 11
Sex			
Male	10	9	11
Female	10	11	9
Race			
Malay	11	10	9
Chinese	9	10	11
Classification			
Serrated adenoma		8	
Tubular adenoma		12	
Dukes' B			9
Dukes' C			11

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

No	Types of analyte	CBA Kit	Analytes detected
1	Cytokine	(1) Human CBA Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Diego, CA)	(a) IL-2 (b) IL-4 (c) IL-6 (d) IL-10 (e) TNF (f) IFN-γ (g) IL-17A
2	Chemokine	BD™ CBA Human Chemokine Kit (BD Biosciences, San Diego, CA)	(a) IL-8/CXCL8, (b) RANTES/CCL5 (c) MIG/CXCL9 (d) MCP-1/CCL2 (e) IP-10/CXCL10
3	Soluble proteins	(1) BD™ CBA Human Soluble Protein (BD Biosciences, San Diego, CA)	(a) VEGF (b) LT-α (c) MIP-1α (d) MIP-1β (e) FGF (f) Eotaxin (g) G-CSF (h) Angiogenin (i) FasL (j) GM-CSF
		(2) BD™ CBA Human TGF-β1 Single Plex Flex Set BD (Biosciences, San Diego, CA)	(k) TGF-β1

2.2. 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