Gene Expression Analysis of Peripheral Blood Cells Reveals Toll-Like Receptor Pathway Deregulation in Colorectal Cancer

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Abstract

Colorectal cancer is the leading cause of cancer-related deaths worldwide. The disease is curable when detected at an early stage. However, the compliance rate with current screening recommendations remains poor. An accurate, minimally invasive blood test that has the potential for greater patient compliance would be a welcome addition to the current methods. Recent data have shown that gene expression profile of peripheral blood cells can reflect disease states and thus have diagnostic value. In this study, genome-wide gene expression profiling of peripheral blood cells from 20 healthy controls and 20 colorectal cancer patients were performed using PAXgene™ technology and Affymetrix GeneChip® microarrays. We identified a list of 1,469 genes that were differentially expressed between the healthy controls and cancer patients. Gene annotation and functional enrichment analysis revealed that those genes are mainly related to immune functions. Particularly, a set of genes belonging to the Toll-Like Receptor pathways were up-regulated in the colorectal cancer patients. These findings provide a new understanding of blood gene expression profile in colorectal cancer. Our result may serve as the basis for further development of blood biomarkers for the diagnosis and treatment of colorectal cancer.

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Introduction

Colorectal cancer (CRC) is the third most common cancer in men and the second most common cancer in women worldwide. In 2008, more than 1,234,000 cases were newly diagnosed, and more than 608,000 people died from the disease [1]. Given its slow development from removable precancerous lesions and from the curable early stages, screening for CRC has the potential to reduce both the incidence and mortality of the disease [2]. The available screening tools include fecal occult blood test (FOBT), stool DNA test, flexible sigmoidoscopy, CT colonography and colonoscopy. Different screening strategies are in place in various countries. However, the compliance with current CRC screening recommendations remains poor. The low rate of participation in CRC screening is due to a number of factors, including low accuracy of current stool based screening methods, patient discomfort and poor acceptability for endoscopy based methods. An accurate, minimally invasive blood test that has the potential for greater patient compliance would be a welcome addition to the current methods. When an abnormality has been detected by the blood test, further tests involving colonoscopy and pathological examination would be recommended to confirm whether the detected abnormality is CRC.

We and others have previously showed the potential use of gene expression profiling of whole blood samples for cancer detection and diagnosis [3–9]. Prior to the clinical manifestation of CRC, which usually takes several years, the host reacts on the implantation of cancer cells via the activated immune system [10]. The activation of immune system is reflected in changes in gene expression profiles of immune competent blood cells, and these changes are detectable in peripheral blood [11,12]. In this study, we performed gene expression profiling of peripheral blood cells using PAXgene™ technology and Affymetrix GeneChip® microarrays. A large number of genes differentially expressed between controls and CRC patients were identified. Particularly, we reported the overexpression profiles of Toll-Like Receptor (TLR) signaling pathways related genes in CRC to pave the way for further functional studies.
Materials and Methods

Patients and Sample Collection

This study was carried out at the Fudan University Shanghai Cancer Center (FDUSCC), Shanghai, China. The study was approved by the Ethical Committee of FDUSCC for clinical research. Written informed consent was obtained from all participants. Twenty CRC patients were recruited in the Department of Colorectal Surgery, FDUSCC. No patient received preoperative radiotherapy or chemotherapy. Patients suffering from hereditary CRC or inflammatory bowel diseases (Crohn’s disease or ulcerative colitis) were excluded from this study. Twenty healthy volunteers without any gastrointestinal symptoms (diarrhea and abdominal pain) were recruited through FDUSCC. All participants had blood collection at least seven days after the colonoscopy examination. For each collection, 2.5 ml of peripheral blood was drawn into a PAXgene™ Blood RNA tube (PreAnalytiX GmbH, Hombrechtikon, CH) and stored at −80°C.

RNA Extraction and Microarray Experiments

Total RNA was extracted with the PAXgene™ Blood RNA System (PreAnalytiX GmbH). The quantity of total RNA was measured with a spectrophotometer at 260 nm wavelengths, and the RNA integrity was assessed using an RNA 6000 Nano LabChip® Kit on a BioAnalyzer Agilent 2100 (Agilent Technologies, Palo Alto, CA, U.S.A.). All samples met the quality criterion: RNA Integrity Number >7.0 [13]. Fifty nanograms of total RNA were reversely transcribed and linearly amplified as single stranded cDNA using Ribo-SPIATM technology with the WT-OvationTM reversely transcribed and linearly amplified as single stranded. Integrity Number

RNA was assessed using an RNA 6000 Nano LabChip® Kit on a BioAnalyzer Agilent 2100 (Agilent Technologies, Palo Alto, CA, U.S.A.). Total RNA was extracted with the PAXgene™ Blood RNA tube (PreAnalytiX GmbH, Hombrechtikon, CH) and stored at −80°C.

Statistical Analysis

Gene expression data analyses were performed using the R software and packages from the Bioconductor project [15–17]. The raw data were collected from CEL files and preprocessed using the Robust Multi-chip Average (RMA) algorithm for background correction, quantile normalization and median polish summarization [18,19]. The probe-set-level data were log2-transformed. In addition, we applied a bioinformatics-based filtering approach using information in the Entrez Gene Database [20]. Probe sets without Entrez Gene ID annotation were removed. For multiple probe sets mapping to the same Entrez Gene ID, only probe sets showing the largest inter quantile range were kept, and the rest were excluded.

Significance Analysis of Microarrays (SAM) method [21] was used to identify genes differentially expressed between the Control and CRC groups. For gene expression studies involving microarrays, it has become common practice to focus on control of the false discovery rate (FDR), which estimates the expected proportion of incorrect rejections among the rejected hypotheses [22,23]. To minimize false positives, we set the threshold of FDR at 0.01 for all the comparisons. Gene Ontology (http://www.geneontology.org) [24] and Panther pathway analysis (http://www.pantherdb.org/pathway) [25] were performed using the GeneCodis bioinformatics tool [26] and MetaCore™ software (GeneGo Inc., USA).

Results

Our study included 20 CRC patients and 20 healthy controls. All the participants were Chinese, including 18 males and 22 females with a median age of 58 years (range, 42–69 years). The age and gender distributions were balanced between the Control and CRC groups. The tumors were staged according to the Tumor-Node-Metastasis (TNM) system. Two of the CRC patients were stage I, 7 were stage II, 6 were stage III, and 5 were stage IV. Detailed patient specifications are described in Table 1 and Table S2.

The recent release of the HG-U133plus2 microarray offers 54,000 probe sets for screening 38,500 human genes. Confronted

Table 1. Clinical characteristics of Controls and CRC Patients.

| Characteristics | CRC | Control |
|-----------------|-----|--------|
| Age - yr        | 56.6| 57.1   |
| range           | 42–69| 42–68 |
| Sex - no. (%)   |     |        |
| male            | 9 (45.0) | 9 (45.0) |
| female          | 11 (55.0) | 11 (55.0) |
| Tumor site - no. (%) |   |       |
| colon           | 9 (45.0) | –      |
| rectum          | 11 (55.0) |       |
| Tumor stage - no. (%) | |       |
| I               | 2 (10.0) | –      |
| II              | 7 (35.0) | –      |
| III             | 6 (30.0) | –      |
| IV              | 5 (25.0) | –      |

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with such an overwhelming amount of information, it was necessary to reduce the total number of genes analyzed to a manageable number of genes with verified biological annotation and use visualization schemes to facilitate the recognition of patterns in the data [29]. We thus performed a bioinformatics-based filtering procedure to summarize the probe sets at the gene level and exclude those probe sets with low-grade biological annotations. After filtering, the expression profiles of 9,529 unique genes in 20 CRC patients and 20 controls were retained for downstream analysis.

Differential Expressed Genes (DEGs) between the Control and CRC groups were identified with the SAM analysis (FDR = 0.01; Type = “Two class unpaired”; test statistic = “t-statistic”; number of permutations = 1,000). In total, 481 and 588 genes were found to be up- and down-regulated in the CRC patients. Functional enrichment analysis of Gene Ontology and the Panther pathway were carried out with a significance threshold of 0.05 for the adjusted P value. The Panther pathway analysis revealed a list of 22 canonical pathways that were significantly enriched in the DEG list. As expected, pathways associated with specific immune functions were well represented and highly significant, including the B cell activation, T cell activation, Interferon-gamma signaling pathway, and Interleukin signaling pathway. In parallel, several angiogenesis-related pathways including the PDGF, VEGF and FGF signaling pathways were also significantly overrepresented. The top ten associated molecular pathways and relevant genes are shown in Table 2. In addition to identifying the significant canonical pathways, we also checked genes associated with functional categories. The Gene Ontology analysis revealed a total of 74 Biological Process categories that were significantly overrepresented, including innate immune response, signal transduction, protein transport, apoptotic process, protein phosphorylation and viral reproduction. The top ten associated Biological Process categories are listed in Table 3.

Remarkably, the TLR signaling pathways were the most significantly enriched item in both the Panther pathway and GO analysis (GO0002224: toll-like receptor signaling pathway, \( P = 1.7E^{-8} \); GO0002755: MyD88-dependent toll-like receptor signaling pathway, \( P = 1.1E^{-7} \); GO0034142: toll-like receptor 4 signaling pathway, \( P = 1.1E^{-7} \) and Panther00054: Toll receptor signaling pathway, \( P = 1.1E^{-06} \). The TLR signaling pathways from the MetaCore™ software are graphically represented in Figure 1. As observed from the graph, multiple TLRs (TLR1, TLR2, TLR4, TLR6 and TLR8), as well as their downstream targets, were significantly up-regulated in the CRC patients. In addition, endogenous ligands for TLRs were also identified, including HSP70 and HMGB1, which have been shown to up-regulate TLR2 and TLR4 on tumor cell surfaces and induce tumor progression and metastasis [30,31]. The activated TLRs then recruit MyD88, leading to subsequent activation of downstream targets, including NF-\( \kappa \)B, mitogen-associated protein (MAP) kinase and interferon regulatory factors [32].

Quantitative real-time PCR is generally considered as the “gold-standard” assay for measuring gene expression and is often used to confirm the findings of microarray studies [33]. We thus selected six TLR signaling pathways related genes (JIRAK3, MD2, TLR1, TLR2, TLR4 and TLR8) for quantitative real-time PCR validation. The results, as shown in Table 4, indicated that gene expression profiles determined by microarray hybridization and quantitative real-time analysis were highly comparable. The gene overexpression profiles in the CRC patients were confirmed in real-time PCR data.

**Discussion**

Early detection of CRC is crucial for successful treatment and patient survival. However, the lack of compliance remains the greatest challenge currently limiting CRC screening effectiveness. The rich content of diverse cellular and molecular elements in blood, which provide information about the health status of an individual, make it an ideal compartment to develop noninvasive tests for CRC detection [34]. In this study, we performed global gene expression profiling of peripheral blood samples collected from 20 controls and 20 CRC patients. We identified a list of 1,469 consensus genes that differentially expressed between the controls and CRCs. Our results are consistent with previous studies [3,5,9] and show that most DEGs are involved in immune responses, as well as cellular apoptosis, signal transduction, protein transport and gene expression regulation.

| Panther id | Molecular pathway | No. of genes | Adjusted P value | Genes |
|------------|-------------------|--------------|-----------------|-------|
| P00054     | Toll receptor signaling pathway | 11 | 1.1E-06 | TLR2, TLR4, TANK, NF kBIA, MAP3K8, IL1B, TLR1, TLR6, TLR8, MYD88, IRAK3 |
| P00036     | Interleukin signaling pathway | 10 | 2.0E-03 | ELK4, SOS2, MAPK6, MKNK1, IRS2, MAPK1, MAP3K2, RPS6KA3, IL1RA1, IL5RA |
| P00010     | B cell activation | 8 | 2.1E-03 | NFATC3, SOS2, NFkBIA, GRAP, PPP3CA, MAPK1, MAP3K2, NFATC2 |
| P00053     | T cell activation | 9 | 2.3E-03 | PLCG1, B2M, NFATC3, SOS2, NFkBIA, PPP3CA, CD86, MAPK1, NFATC2 |
| P00047     | PDGF signaling pathway | 12 | 2.5E-03 | PLCG1, ELK4, SOS2, MAPK6, MKNK1, GRAP, JAK2, MAPK1, MAP3K2, RPS6KA3, PKN2, ETS1 |
| P00056     | VEGF signaling pathway | 8 | 3.7E-03 | PLCG1, PRKCH, SH2D2A, MAPK6, MAPK1, HIF1A, LPXN, ETS1 |
| P00021     | FGF signaling pathway | 10 | 4.8E-03 | PLCG1, PRKCH, SOS2, PPP2R5A, GRAP, MAPK1, MAP3K2, PPP2R1B, MAP2K4, PPP4R1 |
| P00005     | Angiogenesis | 12 | 5.0E-03 | PLCG1, PRKCH, SH2D2A, SOS2, MAPK6, TC17L2, GRAP, MAPK1, MAP2K4, HIF1A, LPXN, ETS1 |
| P00018     | EGF receptor signaling pathway | 10 | 5.3E-03 | TGFA, PLCG1, PRKCH, SOS2, PPP2R5A, GRAP, MAPK1, MAP3K2, MAP2K4, RHOQ |
| P00035     | Interferon-gamma signaling pathway | 5 | 5.4E-03 | IFNGR2, PIAS1, JAK2, MAPK1, IFNGR1 |

Table 2. The top ten molecular pathways enriched in the DEG list.
Perhaps the most striking result to emerge from the data is the overexpression of the TLR signaling pathway related genes in the CRC patients. TLRs, the mammalian homologues of the drosophila toll protein, are the best-characterized family of pattern-recognition receptors (PRRs) [35]. To date, TLRs 1–10 have been identified in humans [36]. TLRs play a crucial role in the innate immune response and the subsequent induction of adaptive immune responses against microbial infection or tissue damage.

![Figure 1. Differentially expressed genes associated with Toll-Like Receptor signaling pathways.](https://example.com/figure1.png)

**Figure 1. Differentially expressed genes associated with Toll-Like Receptor signaling pathways.** The pathway illustration was generated and linked with available experimental data in the MetaCore™ suite. Twelve genes (TLR1, TLR2, TLR4, TLR6, TLR8, MYD88, MD-2, Beclin 1, TAB2, TAK1, IRAKM and IκB) that up-regulated in the CRC patients are linked to Toll-Like Receptor signaling pathways and can be visualized on the map as thermometer-like figures. Upward thermometers have a red color and indicate up-regulated levels of gene expression in the CRC patients. doi:10.1371/journal.pone.0062870.g001

| GO id      | Biological process                                      | No. of genes | Adjusted P value |
|------------|---------------------------------------------------------|--------------|------------------|
| GO:0045087 | innate immune response                                  | 38           | 2.09E-12         |
| GO:0007165 | signal transduction                                     | 78           | 1.18E-11         |
| GO:0015031 | protein transport                                       | 39           | 1.18E-09         |
| GO:0006915 | apoptotic process                                       | 48           | 1.42E-09         |
| GO:0002224 | toll-like receptor signaling pathway                    | 16           | 1.71E-08         |
| GO:0006468 | protein phosphorylation                                 | 36           | 1.88E-08         |
| GO:0016032 | viral reproduction                                       | 31           | 9.98E-08         |
| GO:0002755 | MyD88-dependent toll-like receptor signaling pathway    | 15           | 1.07E-07         |
| GO:0034142 | toll-like receptor 4 signaling pathway                  | 15           | 1.12E-07         |
| GO:0043123 | positive regulation of I-kappaB kinase/NF-kappaB cascade | 20           | 1.12E-07         |

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injury [37,38]. Recent studies show that functional TLRs are expressed not only on immune cells but also on cancer cells, thus implicating a role of TLRs in tumor biology [39,40]. A growing body of evidence have suggested that TLRs act as a double-edged sword in cancer cells [41]. On one hand, TLRs play pivotal roles in the activation of antitumor immune responses in order to inhibit tumor progression. On the other hand, deregulated TLR signaling may provide a microenvironment that is necessary for tumor cells to proliferate and evade the immune response [42].

In particular, there have been several studies reporting an association between TLRs and colorectal neoplasia. Fukata et al. showed that TLR4 was overexpressed in mouse inflammation-associated colorectal neoplasia. The TLR4-deficient mice were significantly protected from colon carcinogenesis [43]. Wang et al. reported high expression levels of TLR4 and MyD88 associated with liver metastasis and poor prognosis in CRC patients [44]. Additionally, TLR4 and IL-6 expression in the tumor microenvironment were associated with the presence of adenocarcinoma, and higher levels of TLR4 expression in the tumor stroma were noted with disease progression [45].

As the first line of immune defense, peripheral blood cells have been shown to express all TLRs and exhibit higher levels of TLR mRNAs compared with other tissues [46]. We postulate that the up-regulation of TLR signaling-related genes in the peripheral blood is likely due to both the infiltration of TLR-expressing inflammatory cells and the up-regulation of receptor expression on these cells that occurs in response to tumor growth stimuli. Further research will be needed to understand the mechanistic relationship and the biological meanings of the overexpression of TLR pathways related genes in CRC patients. Given the therapeutic roles in the activation of antitumor immune responses [41], the systemic study of TLRs’ functions may contribute substantially to the development of new targets for the diagnosis and treatment of CRC.

In conclusion, we show that monitoring gene expression in blood results in distinct transcriptional profiles between controls and CRC patients. Thus, microarray-based blood gene expression profiling holds great promise for developing novel biomarkers for CRC detection. Future studies should include more samples for biomarker identification and validation. Furthermore, given that CRC is considered to be a genetically and epigenetically heterogeneous disease [47], it would also be interesting to investigate the blood gene expression profile of different subtypes, which may provide a new understanding of CRC.

Supporting Information

Table S1 Primer sequences of TLR-related genes and CSNK1G2 reference gene.

Table S2 Detailed clinical information of Controls and CRC Patients.

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Author Contributions

Conceived and designed the experiments: XM YX SJC XD. Performed the experiments: XY FL LY. Analyzed the data: QHX LY. Contributed reagents/materials/analysis tools: LY YX GXC SJF FY CT. Wrote the paper: XM XY QHX.

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Table 4. Quantitative real-time PCR validation of TLR pathways related gene expression data.

| Affy id    | Symbol  | Description               | Microarray Fold change | Microarray P value | Real-time PCR Fold change | Real-time PCR P value |
|------------|---------|---------------------------|------------------------|--------------------|----------------------------|-----------------------|
| 210176_at  | TLR1    | toll-like receptor 1      | 1.43                   | 0.001              | 1.57                       | 0.002                 |
| 204924_at  | TLR2    | toll-like receptor 2      | 1.37                   | 0.001              | 1.32                       | 0.03                  |
| 232068_s_at| TLR4    | toll-like receptor 4      | 1.57                   | 0.001              | 1.37                       | 0.04                  |
| 229560_at  | TLR8    | toll-like receptor 8      | 1.50                   | 0.001              | 1.64                       | <0.001               |
| 213817_at  | IRAK3   | interleukin-1 receptor-associated kinase 3 | 1.92                 | <0.001            | 1.68                       | 0.003                 |
| 206584_at  | MD2     | myeloid differentiation protein 2 | 1.82               | <0.001            | 3.02                       | <0.001               |
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