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ORIGINAL RESEARCH

A Deregulated PI3K-AKT Signaling Pathway in Patients with Colorectal Cancer

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Abstract
Background Molecular switches in phosphatidylinositol 3-kinase (PI3K)-AKT signaling pathway may serve as potential targets for the treatment of colorectal cancer (CRC). This study aims to profile the gene alterations involved in PI3K-AKT signaling pathway in patients with CRC.
Methods Tumoral and matched peritumoral tissues were collected from 15 CRC patients who went routine surgery. A human PI3K-AKT signaling pathway polymerase chain reaction (PCR) array, which profiled the transcriptional changes of a total number of 84 genes involved in the PI3K-AKT pathway, was then applied to determine the gene alterations in CRC tumoral tissue with matched peritumoral tissue as a healthy control. Subsequent real-time reverse transcription PCR and western blot (WB) with different subgroups of CRC patients were then performed to further validate the array findings.
Results The PCR array identified 14 aberrantly expressed genes involved in the PI3K-AKT signaling pathway in CRC tumoral tissue, among which 12 genes, CCND1, CSNK2A1, EIF4E, EIF4EBP1, EIF4G1, FOS, GRB10, GSK3B, ILK, PTK2, PTPN11, and PHEB were significantly up-modulated (> two fold) while the remaining two, PDK1 and PIK3CG, were down-regulated (> two fold). These genes involve in the regulation of gene transcription and translation, cell cycle, and cell growth, proliferation, and differentiation. The real-time reverse transcription PCR validation agreed with the array data towards the tested genes, CCND1, EIF4E, FOS, and PIK3CG, while it failed to obtain similar result for PDK1. Interestingly, the WB analyses were further consistent with the PCR results that the protein levels of CCND1, EIF4E, and FOS were apparently up-regulated and that protein PIK3CG was down-modulated.
Conclusion Taken together, the present study identified a deregulated PI3K-AKT signaling pathway in CRC patients, which might serve as therapeutic target(s).

Keywords Colorectal cancer · PI3K-AKT signaling pathway · PCR array

Introduction

Colorectal cancer (CRC) is among the most commonly diagnosed malignancy and the fourth leading cause of cancer-related death all over the world, with approximately 1.4 million new cases and 700 thousand deaths in 2012. Given the temporal profiles and demographic projections, the global burden of CRC is expected to increase by 60% to more than 2.2 million new cases and 1.1 million deaths by 2030 [1]. CRC possesses extremely complicated pathologic courses [2]. CRC development involves multistep processes that result from genetic alterations that underlie the transformation of normal cells to malignant cells, conferring them overwhelming growth advantages [3].

Phosphatidylinositol 3-kinase (PI3K) was first discovered more than two decades ago, and its critical role for oncogenesis and cancer progression is well described [4, 5]. Alterations that lead to increased PI3K signaling are among the most common changes found in human cancers [6]. AKT acts downstream of PI3K to regulate various biological processes, such as cell proliferation and apoptosis as well as

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differentiation [7]. Several components of the PI3K-AKT pathway are dysregulated in a wide spectrum of human cancers, including CRC [8]. In the past decade, the Wnt signaling pathway is one of the best studied pathways in the development of CRC. However, the role of PI3K-AKT signaling pathway is not clearly defined in the development of CRC.

In the present study, we introduced a polymerase chain reaction (PCR) array to profile the expression of a variety of genes that involved in the human PI3K-AKT signaling pathway for CRC patients. We also conducted follow-up real-time PCR as well as western blot (WB) validations.

Materials and Methods

Participants and Samples

Fifteen CRC patients (seven females and eight males), aged from 52 to 67 years old, were recruited from the Affiliated Ruikang Hospital of Guangxi University of Chinese Medicine from 2014 to 2016. Physical examination and TNM staging evaluation confirmed that all the patients were free of any other cancers and that they were at T1N0M0 stage. After routine surgery, we collected tissue samples from the tumor lesion as well as from the peritumoral mucosa, which was defined as tissue that located at 2 cm from the resection margin of the tumor lesion, from each patient. Tissue samples were stored at − 80 °C before use.

Real-Time PCR Array

We performed the real-time PCR array with paired tumoral and peritumoral tissues from a subgroup of six CRC patients (three females and three males) by using the Human PI3K-AKT Signaling Pathway RT^2 Profiler™ PCR Array (PAHS-058Z) obtained from SABiosciences, which profiles the expression of 84 genes involved in PI3K-AKT signaling for each tissue sample. This array contains members of the AKT (protein kinase B) and PI3K families and their regulators. Basically, the experimental protocol involves RNA isolation, assessment of RNA yield and quality, DNase treatment, RNA cleanup, first-strand cDNA synthesis, real-time PCR, and data analysis (detailed protocol is available upon request). The array experiments were conducted with technical assistance from KangChen Bio-tech Inc. (Shanghai, China). Fold change and p value were calculated and used to identify the differentially expressed genes in CRC tumoral tissue when compared to the peritumoral tissue. The genes, which matched $p < 0.05$ and fold change > 2 (up-regulated) or < 0.5 (down-regulated), were considered as differentially expressed and displayed in Table 1.

Real-Time Reverse Transcription PCR Validation

We validated the PI3K-AKT signaling pathway PCR array results by performing real-time reverse transcription PCR with paired tumoral and peritumoral tissues from the other subgroups of six CRC patients (three females and three males). Briefly, total RNA was extracted from homogenized tissues using TRIZOL Reagent (Invitrogen) according the manufacturer's protocol. The 260:230 and 260:280 nm ratios were then calculated by NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies) and used to evaluate the RNA purity as well as concentration. Quality of the extracted RNAs was evaluated by 1% denaturing agarose gel electrophoresis. Three micrograms of total RNA was reversely transcribed

Table 1 Fourteen differentially expressed genes of the PI3K-AKT pathway in CRC tissue

Gene symbol	Gene bank	Description	p value	Fold change
CCND1	NM_053056	Cyclin D1	0.016	9.46
CSNK2A1	NM_001895	Casein kinase 2, alpha 1 polypeptide	0.028	2.27
EIF4E	NM_001968	Eukaryotic translation initiation factor 4E	0.042	3.85
EIF4EBP1	NM_004095	Eukaryotic translation initiation factor 4E binding protein 1	0.000	4.77
EIF4G1	NM_182917	Eukaryotic translation initiation factor 4 gamma, 1	0.003	2.44
FOS	NM_005252	FBJ murine osteosarcoma viral oncogene homolog	0.000	5.68
GRB10	NM_005311	Growth factor receptor-bound protein 10	0.042	5.18
GSK3B	NM_002093	Glycogen synthase kinase 3 beta	0.005	3.69
ILK	NM_004517	Integrin-linked kinase	0.009	2.23
PTK2	NM_005607	Protein tyrosine kinase 2	0.024	2.68
PTPN11	NM_002834	Protein tyrosine phosphatase, non-receptor type 11	0.033	4.81
RHEB	NM_005614	Ras homolog enriched in brain	0.045	3.73
PDK1	NM_002610	Pyruvate dehydrogenase kinase, isozyme 1	0.020	− 2.64
PIK3CG	NM_002649	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	0.022	− 2.51

A positive fold change value indicates up-regulated alteration, while a negative value indicates down-regulation

using SuperScript III Reverse Transcriptase (Invitrogen), and then, the cDNA was amplified by PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Respective primer sequences were showed in Table 2. For real-time reverse transcription PCR, the PCR amplification was performed in a reaction volume of 20 μL on a Bio-Rad CFX96 Touch Real-Time PCR Machine. The reaction mixture contained 1 μL of each cDNA, 10 μL iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 1 μL of each amplification primer (1 pmol/μL), and 7 μL PCR water. Thermal cycling was organized in three repeated steps: the first denaturation step of 10 min at 95 °C, followed by 40 repeated cycles of 95 °C for 15 s and 60 °C for 1 min. Experiments were run in triplicate. The relative expression (fold change) of the target genes was calculated based on the threshold cycle (C_t) as $2^{-\Delta\Delta Ct}$.

Western Blot Validation

In order to further confirm the expression level of part of the identified genes, WB analysis was introduced to determine the protein levels of CCND1, EIF4E, FOS, and PIK3CG between the CRC tumoral and peritumoral tissues (β-actin as an internal control). The WB experiments were conducted for the paired tissues from the remaining three CRC patients. Briefly, total protein was extracted from the tissues by using ReadyPrep™ Protein Extraction Kit (Bio-Rad) according to the manufacturer's instructions, followed by determination of the total protein concentration by BCA Protein Assay Kit (CoWin Bioscience). Equal amounts of total protein solubilized in sample loading buffer were separated applying a 4–12% gradient Bis-Tris PAGE (Invitrogen) and then transferred onto a nitrocellulose membrane, followed by incubation with respective primary and secondary antibodies, which were all obtained from Abcam. The ECL-generated signals were then detected by a ChemiDocXRS$^+$ Chemiluminescence System (Bio-Rad).

Statistical Analyses

STATISTICA 10 (Dell) was used to perform the statistical analyses. MeV version 4.6 software (Dana-Farber) was introduced to perform hierarchical cluster analysis of the differential genes of the PI3K-AKT pathway to reveal distinguished gene expression patterns between the CRC tumoral and peritumoral tissues. Graph preparations were carried out by Microsoft Office Excel 2007, otherwise indicated. Student *t* test was applied to compare data between groups. A *p* value less than 0.05 was considered to be statistically significant.

Results

Gene Alterations of the PI3K-AKT Pathway in CRC Tissue

We determined the mRNA expression profile using a PCR array for 84 genes involved in the PI3K-AKT pathway in CRC tumoral and peritumoral tissues. By array analysis, we observed that *CCND1*, *CSNK2A1*, *EIF4E*, *EIF4EBP1*, *EIF4G1*, *FOS*, *GRB10*, *GSK3B*, *ILK*, *PTK2*, *PTPN11*, and *PHEB* were up-regulated (> two fold), while *PDK1* and *PIK3CG* were down-regulated (> two fold) in the CRC tumoral tissue compared to that in the peritumoral tissue (Table 1). We speculated that the differential genes identified by the PCR array might be able to distinguish the CRC tissue from the peritumoral tissue. Thus, hierarchical cluster analysis was then conducted towards the 14 differentially expressed genes to detect gene expression pattern between them. As showed in Fig. 1, the CRC tissue (CRC-1 to CRC-6) and the peritumoral tissue (HC-1 to HC-6) were well clustered into two separate categories, which indicated that the modulated pattern of these genes might be a good signature for CRC patients.

In Vivo Validations

Because (1) the PCR array signature indicated above might not be well replicated in a different group of CRC patients and/or (2) the array itself might have accidental errors, we next validated the PCR array findings by introducing real-time PCR with a different subgroup of CRC patients (n = 6) for part of the identified genes, namely *CCND1*, *EIF4E*, *FOS*, and *PIK3CG* (*GAPDH* as internal control). We also determined the expression levels of the corresponding proteins by WB with the remaining three CRC patients. Firstly, we extracted total RNA, which was evaluated as qualified by 260:280 ratio (data not shown) as well as by agarose gel electrophoresis (Fig. 2a) for subsequent real-time PCR. Real-time reverse

Table 2 Real-time PCR primers

Gene symbol	Primer forward (5′-3′)	Primer reverse (5′-3′)
CCND1	GCTGCGAAGTGGAAACCATC	CCTCCTTCTGCACACATTTGAA
EIF4E	GAAACCACCCCTACTCCTAATCC	AGAGTGCCCATCTGTTCTGTA
FOS	CCAACTTCATTCCCACGTC	CTCCCTCCTCCGGTTGC
PIK3CG	GGCGAAACGCCCATCAAAAA	GACTCCCGTGCAGTCATCC
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

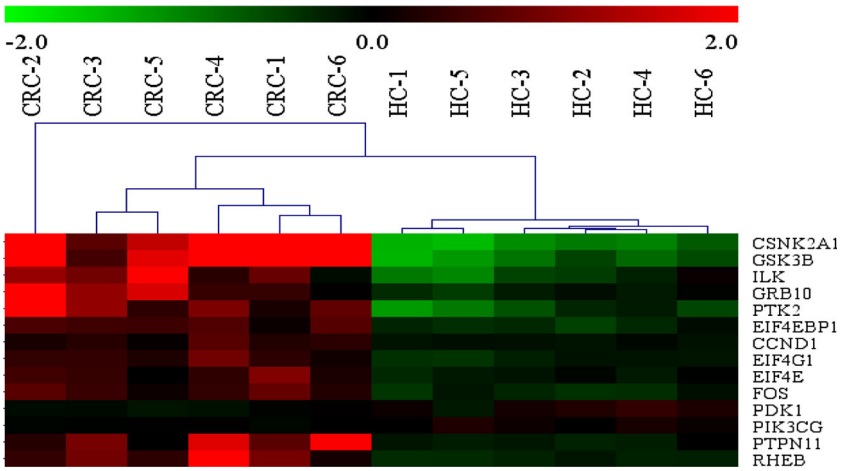

Fig. 1 Heatmap displaying the 14 differentially expressed genes of the PI3K-AKT pathway in CRC tissue. Data of the indicated genes were collected from the PCR array performed with six paired tumoral (CRC) and peritumoral (HC) tissues, and the map was made by MeV software using average linkage and Euclidian distance. The color scale bar (green, dark, red) indicates increasing expression levels (low, medium, high)

transcription PCR was then conducted towards the genes indicated. Table 2 displays the respective primers used (*GAPDH* as internal control), and the $2^{-\Delta\Delta Ct}$ method was applied to calculate the expression levels (fold change) of the genes in CRC tumoral tissue compared with that in the matched peritumoral tissue. We observed that *CCND1*, *EIF4E*, and *FOS* were up-modulated in 6.13 (± 0.63), 3.65 (± 0.49), and 4.08 (± 0.84) folds, respectively, while *PIK3CG* was down-modulated in − 4.62 (± 0.48) folds in CRC tumoral tissue compared with that in the matched peritumoral tissue (Fig. 2b). The findings were consistent with the PCR array results. However, we did not observe a transcriptional change for *PDK1*, which was tested as down-regulated in the tumoral tissue by the PCR array (data not shown). In order to achieve mutual corroboration with the transcriptional level for the indicated genes, we also determined the translational levels of the corresponding proteins by WB for the paired tissues. Interestingly, we found that the protein expression levels of CCND1, EIF4E, and FOS were all apparently up-modulated, while PIK3CG was down-modulated in CRC tumoral tissues (Fig. 2c). These results were also consistent with the PCR array findings as well as with the real-time PCR confirmation.

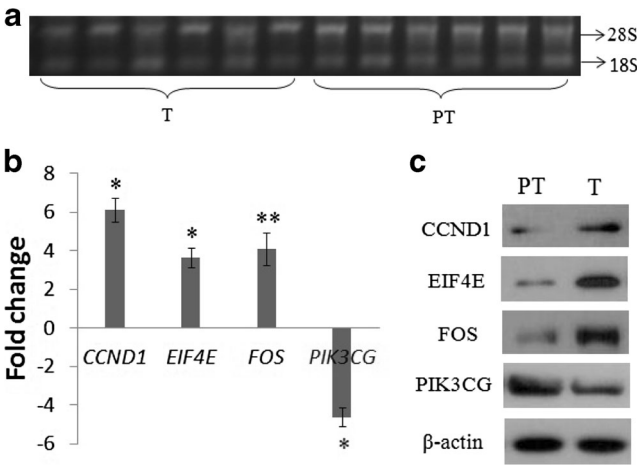

Fig. 2 In vivo real-time PCR and WB validations. **a** Agarose gel electrophoresis of the total RNA extracted from the CRC tumoral (T, $n = 6$) and the matched peritumoral tissues (PT, $n = 6$). 28S and 18S indicate the positions of the corresponding ribosomal RNAs. **b** Real-time PCR validation of the transcriptional levels of the genes in CRC tumoral tissue compared to that in the matched peritumoral tissue. The fold change is calculated by the $2^{-\Delta\Delta Ct}$ method. A positive fold change indicates the indicated gene is up-regulated in CRC tumoral tissue, while a negative value indicates the opposite. Experiments were performed in triplicate. * $p < 0.05$, ** $p < 0.05$ vs. matched peritumoral tissue. **c** WB validation of the expression levels of the corresponding proteins in CRC tumoral tissue (T) compared to that in the matched peritumoral tissue (PT). The graphs shown are representatives from a series of WB experiments ($n = 3$). β-actin is introduced as internal control

Discussion

By PCR array, we identified 14 differentially expressed genes in the PI3K-AKT pathway for CRC patients in the present study (see Table 1 for detailed gene list). The findings were further confirmed by subsequent real-time PCR as well as by WB analysis. However, the transcriptional change of *PDK1* was not consistent between the array data and the real-time PCR validation.

Cell cycle is controlled by a complicated network of regulators. The transformation of normal cell to malignant cell usually involves deregulation of the cell cycle. *CCND1* encodes Cyclin D1 protein that functions as a cell cycle regulator with a critical role in G1-S transition [9]. Mutation, amplification, and overexpression of this gene, which alters cell cycle progression, are observed frequently in a variety of cancers and may contribute to tumorigenesis [10–12]. *CSNK2A1* encodes a serine/threonine protein kinase that phosphorylates acidic proteins, which is involved in various cellular processes, such as cell cycle control. CSNK2A1 has been shown to be involved in tumorigenesis. For example, Bae et al. have recently demonstrated that CSNK2A1 mediates phosphorylation of sirtuins (SIRTs) that might be involved in the progression of breast carcinoma [13]. *RHEB* gene is a member of the small GTPase superfamily [14], and it encodes a cell

membrane protein that is crucial in regulation of cell cycle as well as cell growth [15, 16]. RHEB is frequently overexpressed in human carcinoma [17]. The investigation, which studied the effects of *RHEB* mutant expression on chicken embryonic fibroblasts, further supports the significance of RHEB in oncogenic transformation [18]. In the current study, our results showed that the gene expressions of *CCND1*, *CSNK2A1*, and *RHEB* were significantly up-modulated in CRC tumoral tissue, which were consistent with previous studies. The present findings might represent an accelerated cell cycle in CRC.

Gene transcription is controlled by complicated transcription factors, which widely participate in the regulation of cell proliferation, differentiation, and transformation. Deregulation of the factors has been well documented in human tumors. *FOS* gene encodes a transcription factor of the activating protein-1 (AP-1) family that participates in the regulation of various genes, which partly involve in tumor growth and progression [19]. The oncogenic function of *FOS* has been implicated in a variety of tumors [20–23]. Consistently, our study also observed an up-regulated expression of *FOS* in CRC tumoral tissue, which might implicate an abnormal gene transcription in CRC.

Translational control plays a crucial role in the regulation of gene expression and affects various cellular processes, such as cell proliferation, apoptosis, and differentiation [24]. Molecular alterations with regard to translation control in cancer have been widely addressed. The protein encoded by *EIF4E* is a component of the eukaryotic translation initiation factor 4F complex, which recognizes the 7-methylguanosine cap structure at the 5′ end of mRNAs, and it aids in translation initiation by recruiting ribosomes to the 5′-cap structure [24, 25]. This gene acts as a proto-oncogene, and its overexpression has been observed in various types of human cancers, including CRC [26], and it has been implicated as a therapeutic target for cancer [27]. The protein encoded by *EIF4EBP1* directly interacts with EIF4E that leads to inhibition of complex assembly as well as translation repression. EIF4EBP1 plays a crucial role in the control of protein synthesis as well as cell growth and survival, thus promoting tumorigenesis [28, 29]. A recent investigation indicates that the expression of EIF4EBP1 is associated with clinical survival outcome in CRC [30]. Another translation initiation factor, EIF4G1, functions to recruit ribosomes to the capped end of mRNA to initiate cap-dependent translation [31]. Recent studies have found that EIF4G1 is overexpressed in various cancers [32–34]. Consistent with previous studies in CRC patients, we also observed that the translation initiation factors, *EIF4E*, *EIF4EBP1*, and *EIF4G1*, were aberrantly up-modulated in CRC tumoral tissue, which might implicate a deregulation of translation control in CRC patients.

The epithelial to mesenchymal transition (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells, which involves in the initiation of metastasis for cancer progression. The product of *ILK* gene associates at the cell membrane with the cytoplasmic domain of beta integrins, and it is important for the EMT in human cancers [35, 36]. ILK protein levels are increased in a variety of human cancers, and frequently, the expression level predicts poor clinical outcome [37]. Diverse cellular processes are regulated by tyrosyl phosphorylation, which is controlled by protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). The protein encoded by *PTPN11* is a member of the PTP family, which is known to regulate various cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation. *PTPN11* is implicated as an oncogene that is mutated in several types of leukemia and hyperactivated in many solid tumors [38]. The ability of cells to migrate acquires dramatic implications in the context of cancer. An accumulating body of evidence indicates that PDK1 plays a vital role in the regulation of cell migration [39]. High expression of PDK1 has been shown to be associated with colon cancer [40] and gastric cancer [41]. Up-regulation of PIK3 has been widely observed in various human malignancies [42, 43]. The product of *PIK3CG* gene is a class I catalytic subunit of PI3K, which is divided into two groups (types 1A and 1B). Type 1B PIK3CG is activated by interaction with G-protein-coupled receptors, whereas type 1A PI3Ks are regulated by tyrosine-phosphorylated proteins [44, 45]. Therefore, it is speculated that PIK3CG functions differently from that of type 1A PI3Ks. Interestingly, reduced PIK3CG expression was detected in 85% of human CRC cell lines as well as in primary colon cancers and was closely associated with cell invasion, metastasis, and poor differentiation [46]. In the current study, the transcriptional levels of *ILK* and *PTPN11* were significantly up-regulated in CRC tumoral tissue, which implicated an abnormal EMT as well as a deregulation of cellular processes, such as cell growth, in CRC patients. Interestingly, we detected a down-modulated level of *PIK3CG*, which was consistent with previous study in CRC patients [46]. The finding further supports the idea that PIK3CG may function differently from type 1A PI3Ks. Surprisingly, we failed to detect a consistent change of *PDK1*, which is vital in the regulation of cell migration, in CRC patients. We argued that PDK1 expression profile may not be a suitable signature for CRC because controversial results have been obtained. For example, high expression of PDK1 was showed to be associated with colon cancer [40], while quite opposite results were detected as well [47]. Besides, it has been documented that PDK activities are sensitive to slight changes in ambient pH that may be able to affect its expression profile [48]; thus, the microenvironment of the PDK, especially the pH value, needs to be taken into consideration when profiling the PDK expression.

Taken together, the present study identified a gene signature of the PI3K-AKT signaling pathway in CRC tissue, which might implicate a deregulated PI3K-AKT signaling network for cell cycle, gene transcription and translation, as well as cell growth and proliferation in CRC patients.

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Author Contribution TZ designed the study, analyzed the data, and wrote the paper; CL, JSF, YPM, and LRC collected the specimens, performed the experiments, and collected the data. All authors have read and approved the final version to be submitted.

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Compliance with Ethical Standards

Ethical Approval The study was approved by the Ethics Committee of Ruikang Hospital of Guangxi Traditional Chinese Medical University (Guangxi, China). All procedures were performed according to the principles expressed in the Declaration of Helsinki.

Consent All participants were explained their rights and signed the written informed consent before participation.

Conflict of Interest The authors declare that they have no competing interests.

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