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Next-generation sequencing reveals lymph node metastasis associated genetic markers in colorectal cancer

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Abstract. Colorectal cancer is the third most prevalent type of cancer in the United States. Early diagnosis of lymph node metastases is essential to improve the prognosis for patients with colorectal cancer. Therefore, the present study aimed to screen genetic markers, including single nucleotide polymorphisms (SNPs), copy number variations (CNVs) and mRNA expression, associated with lymph node metastases in patients with colorectal cancer to enable an early diagnosis. Targeted next-generation sequencing was applied to capture SNPs and CNVs in tumor-related candidate genes within tumor tissues from 39 colorectal cancer patients; reverse transcription-quantitative polymerase chain reaction was used to detect the specific mRNA level of tumor-related candidate genes, including vascular endothelial growth factor C, cyclin-A2, Interleukin-2, ATP-binding cassette sub-family G member 2, epidermal growth factor (EGF) and nuclear factor kappa B subunit 1 (NFKB1) on chromosome 4. The SNPs in solute carrier family 28 member 3 (SLC28A3), breast cancer 1 (BRCA1), ribonucleotide reductase regulators subunit M2 (RRM2), PMS1 homolog 2 (PMS2), cytidine deaminase (CDA), epoxide hydrolase 1 (EPHX1), heterogenous ribonucleoprotein particle-associated with lethal yellow (RALY), Siglec-3 (CD33), B cell lymphoma 10 (BCL10), ETS variant 1 (ETV1), macrophage stimulating 1 receptor 1 (MST1R), lysine methyltransferase 2B (KMT2B), B cell lymphoma 2 (BCL2), U6 small nuclear RNA-associated Sm-like protein 3 (LSM3), thyroid transcription factor 1 (TTF1) and mitogen-activated protein 3 kinase 1 (MAP3K1) were significantly associated with lymphatic metastasis (P<0.05). EGF and NFKB1 were both observed to be significantly downregulated in the lymph node metastases group (P<0.05). Although no association between CNVs and lymph node metastases in patients with colorectal cancer was observed in the present study, SNPs in SLC28A3, BRCA1, RRM2, PMS2, CDA, EPHX1, RALY, CD33, BCL10, ETV1, MST1R, KMT2B, BCL2, LSM3, TTF1 and MAP3K1 were significantly associated with colorectal cancer. Downregulation of EGF and NFKB1 was also identified to be associated with lymph node metastases in colorectal cancer. The findings of the current study provide a scientific basis for the clinical inspection of lymphatic metastasis and prognosis prediction, intervention and guidance therapy for patients with colorectal cancer.

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Introduction

Malignant tumors are a life-threatening disease globally and in China. In 2015, 25% of total mortalities were caused by cancer (1). The morbidity and mortality of cancer has been increasing for a number of years. In 2009, colorectal cancer was the cause of 8% of total mortalities caused by cancer (2). Furthermore, colorectal cancer is one of the most common tumor types. According to data published by the National Cancer Institute in 2016, colorectal cancer was the third most common tumor type (1). In developing countries, the rate of colorectal cancer is also growing rapidly. From 2010-2012 developing countries contributed to 52% of the total number of mortalities caused by colorectal cancer, and limited medical resources meant patients had a poor prognosis and survival rate (3). Treatment for colorectal cancer remains limited to traditional methods, such as surgical operations, radiotherapies and chemotherapies.

The emerging targeted molecular therapies were gradually accepted by doctors and demonstrated particular advantages in clinical treatment for colorectal cancer (4). The combination of emerging and traditional therapies improves the level of disease-free survival, survival rate and prognosis in patients with colorectal cancer. However, disease recurrence following surgery or chemotherapy, drug resistance and deterioration remains inevitable (5,6).

The occurrence and development of colorectal cancer is a process controlled by multiple genes and variable factors. For example, the migration inhibitory factor/cluster of differentiation 74 signalling axis has recently been identified

as a novel therapeutic target for colon cancer (7). Furthermore, loss of periplakin has been demonstrated to be associated with tumorigenesis of colon cancer (8). The lymph node is the primary defence against the metastasis of colorectal tumors, and is also where deterioration of health begins in patients with colorectal cancer.

Lymph node metastasis may worsen the prognosis, reduce the survival rate and even make patients more susceptible to the recurrence of colorectal cancer (3). Furthermore, lymph node metastasis may worsen the postoperative curative effect and enhance drug-resistance to chemotherapies for colorectal cancer. Previous research based on clinical practical experience demonstrated that the 5-year survival rate may reach 60-80% in patients without lymph node metastasis; however, the 5-year survival rate of patients with lymph node metastasis may only reach 30% (9). Surgery is unable to improve the prognosis of patients with colorectal cancer.

With the development of molecular biology, the understanding of colorectal cancer has progressed beyond the cellular level and further to elucidate the role of genetic biomarkers. There are two types of genetic alterations in colorectal cancer, chromosomal instability (CIN) and microsatellite instability (MSI). Aneuploidy and polyploidy are common phenotypes in CIN and contribute to 80-85% of the morbidity of colorectal cancer (10). MSI is primarily caused by errors in the DNA repairing process, and contributes to 15-20% of the total morbidity (10). There are multiple chromosome sites with copy number variation (CNV) in CIN-type colorectal cancer (11). If CNV occurs inside or around the tumor-associated gene sequences, oncogenes may be activated and anti-oncogenes may be inactivated, which eventually induces tumorigenesis (12). A previous study indicated that increased CNVs may be associated with the progression of colitis gravis to colorectal cancer (13). Another study demonstrated that CNVs were able to determine the lymph node metastasis in colorectal cancer (14). Evidence now indicates that the occurrence of CNVs in chromosome 4 may seriously induce lymph node metastasis in colorectal cancer (14-16).

Single nucleotide polymorphisms (SNPs) are a primary cause for variation in the human race. However, the association between SNPs and colorectal cancer remains unclear. In the present study, 1,053 associated genes on chromosome 4 were screened for lymph node metastasis-associated SNPs and CNVs and the mRNA level of lymph node metastasis on these genes was further investigated. The current study aimed to provide a molecular basis for clinical tests and treatment.

Materials and methods

Subjects. A total of 78 tissue samples (39 colorectal tumor and 39 normal tissues) from 39 patients were collected between January 2013 and September 2014 following tumor reduction surgery in Shenzhen Second People's Hospital (Shenzhen, China). The collection of tissues was approved by the Ethics Committee of Shenzhen Second People's Hospital. Written informed consent was provided by all patients. All experiments using human blood samples were conducted in accordance with the Clinical Sample Collection and Treatment Guidelines outlined by the Ethics Committee of Shenzen Second People's Hospital. Among the 39 patients, 19 were female and 20 were male. The age of the subjects ranged from 29-84, with a mean age of 61.4 years. There were 19 cases with tumor diameters ≤5 cm and 20 cases with tumor diameters >5 cm. In the 39 patients, 29 exhibited tumors in the colon, whereas 10 patients exhibited tumors in the rectum. There were 6 cases with highly-differentiated tumors, 25 with medium-differentiated tumors and 8 with low-differentiated tumors, according to a differentiation scale determined by the Pathology Department of Shenzhen Second People's Hospital. There were 17 cases presenting lymph node metastasis, 8 cases were identified with distant metastasis and 14 cases exhibited no tumor metastasis. Pathological staging demonstrated that 19 cases were at phase I-II and 20 cases were at phase III-IV. Pathological staging was assessed according to the TNM colorectal cancer staging system of the American Joint Committee on Cancer (AJCC; AJCC staging system 7th edition 2011; cancerstaging.org) and the Union for International Cancer Control (uicc.org) standards. All patients included in the study were tested to exclude other diseases, such as gastroenteritis.

DNA and RNA extraction. During surgery, 0.2 g tissue was harvested from each patient, pre-treated with liquid nitrogen and processed into a powder. The DNA extraction procedure was performed using a DNeasy Blood & Tissue kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. RNA extraction was performed using TRIzol reagent (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA), following the manufacturer's instructions. The nucleotide concentration was determined using a Qubit 3.0 fluorometer and the integrity of DNA molecules were examined by electrophoresis, using 1% agarose gel stained with 0.01% acridine orange.

Exosome sequencing and bioinformatics analysis. The exosome sequencing was conducted using the Hiesq 2000 System (Illumina, Inc., San Diego, CA, USA) with a NimbleGen 4.6 microarray chip (Roche Diagnostics, Basel, Switzerland). The raw data were acquired and filtered according to signal intensity, gene annotation and sequence clustering. The sequences were then compared and statistically analyzed. Comparative genome hybridization (CGH) data were analyzed using R 3.3.2 rCGH software obtained from Bioconductor (bioconductor.org). Log ratios of CNVs were calculated by comparing normalized data from the sequencing of tumor and normal tissues.

Gene expression analysis via reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from the tumor and normal tissues, as described above. RNA samples were reverse transcribed using a PrimeScript Reverse Transcription kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's instructions. A SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd.) was used for qPCR, according to the manufacturer's instructions. The RT-qPCR primers for vascular endothelial growth factor C (VEGFC), cyclin-A2 (CCNA2), interleukin-2 (IL2), ATP-binding cassette sub-family G member 2 (ABCG2), epidermal growth factor (EGF), nuclear factor kappa B subunit 1 (NFKB1) and glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) are presented in Table I. Blank controls using only primers or templates were included in the RT-qPCR experiment. An Applied Biosystems 7900HT Fast Real-Time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to run the following reaction conditions: 95˚C for 15 min, then 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. Relative gene expression levels were normalized to GAPDH, according to the $2^{-\Delta\Delta Cq}$ method (10,17). Experiments were performed in triplicate.

Statistical analysis. SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA) was used to run data normalization and statistical tests. CGH data were analyzed using R 3.3.2 rCGH software obtained from Bioconductor (bioconductor.org). Fisher's exact test was applied to evaluate the association among SNPs, CNVs and lymph node metastasis in colorectal cancer. Student's t-test was applied to test the statistical significance of gene expression alteration. P<0.05 was considered to indicate a statistically significant difference.

Results

Quality control of sequencing data. Raw data were filtered and adapters were removed. Sequencing results demonstrated that the target sequence was $4.5x10^{12}$ base pairs, with coverage of >99% and the sequencing depth of x250. A quality check was automatically performed on the raw data by the R 3.3.2 rCGH package, and the qualified data were used for subsequent analysis.

Association between SNPs and lymph node metastasis colorectal cancer. All 20,000 SNPs in the 1,053 cancer related genes were analyzed, 10,000 nonsense mutations were filtered out and the remaining SNPs were analyzed by statistical tests. Results indicated that 21 SNPs in 16 genes were significantly associated with colorectal cancer (P<0.05). Patients with lymph node metastasis exhibited higher mutation rates of solute carrier family 28 member 3 (SLC28A3; rs10868138, rs56350726), breast cancer 1 (BRCA1; rs16941, rs16942, rs799917, rs1799966), ribonucleotide reductase regulators subunit M2 (RRM2; rs1130609), PMS1 homolog 2 (PMS2; rs1805323), cytidine deaminase (CDA; rs2072671), epoxide hydrolase 1 (EPHX1; rs2234922), heterogeneous ribonucleoprotein particle-associated with lethal yellow (RALY; rs2281209), Siglec-3 (CD33; rs2455069), B cell lymphoma 10 (BCL10; rs3768235) and ETS variant 1 (ETV1; rs9639168) than patients without lymph node metastasis (P<0.05). Patients with lymph node metastasis had a lower mutation frequency of macrophage stimulating 1 receptor 1 (MST1R; rs1062633), lysine methyltransferase 2B (KMT2B; rs16970649), B cell lymphoma 2 (BCL2; rs1800477), U6 small nuclear RNA-associated Sm-like protein 3 (rs1870134), thyroid transcription factor 1 (TTF1; rs3739914, rs8999) and mitogen-activated protein 3 kinase 1 (MAP3K1; rs702689) than patients without lymph node metastasis (P<0.05). These results are presented in Table II.

Clustering analysis of lymph node metastasis-associated SNPs in colorectal cancer. The mutations present in patients were analyzed via hierarchical clustering. The results divided patients into two groups (G1 and G2) and five sub-groups (1a, 1b, 2a, 2b and 2c). Results demonstrated an association between colorectal cancer stage and mutation frequency, with a higher rate of lymph node metastasis being present in the later stages of colorectal cancer (Fig. 1). Clustering of 21 lymph node metastasis-associated SNPs in colorectal cancer indicated that the mutation frequencies of SLC28A3, BRCA1, RRM2, PMS2, CDA and ETV1 were associated with the G2 group and the mutation frequencies of EPHX1, RALY, CD33 and BCL10 were associated with groups 2b and 2c (Fig. 2). Multiple mutations in BRCA1 were closely clustered, indicating that BRCA1 may be a potential marker for lymph node metastasis in colon cancer. However, a larger sample size is required to verify genetic hallmarks for lymph node metastasis.

Association between CNVs and lymph node metastasis in colorectal cancer. Of all cases, 15 presented with copy

Table I. Primer sequences for polymerase chain reaction.

Gene	Sequence, 5'→3'	Product length, bp
GAPDH	F: GGGTGTGAACCATGAGAAGT R: CAGTGATGGCATGGACTGTG	149
VEGFC	F: TGGGGAAGGAGTTTGGAGTC R: GTTACTGGTTTGGGGCCTTG	181
CCNA2	F: TGCTGACCCATACCTCAAGT R: GGTAGGTCTGGTGAAGGTCC	167
IL2	F: AACTCACCAGGATGCTCACA R: TGCTGATTAAGTCCCTGGGT	159
ABCG2	F: ACGCATCCTGAGATCCTGAG R: CAGGTCATTGGAAGCTGTCG	155
EGF	F: CAGGGAAGATGACCACCACT R: TCTCGGTACTGACATCGCTC	168
NFKB1	F: TGTCCAGCTTCGGAGGAAAT R: CACTACCAAACATGCCTCCG	182

F, forward sequence; R, reverse sequence; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; VEGFC, vascular endothelial growth factor C; CCNA2, cyclin-A2; IL2, Interleukin-2; ABCG2, ATP-binding cassette sub-family G member 2; EGF, epidermal growth factor; NKFB1, nuclear factor kappa B subunit 1.

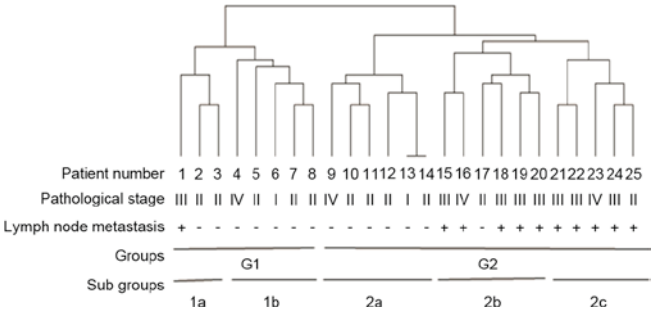

Figure 1. Hierarchical clustering of clinical data from 39 colorectal cancer patients. +, patients with lymph node metastasis; -, patients without lymph node metastasis.

Table II. Association between SNPs and lymph node metastasis colorectal cancer.

			Lymph node metastasis, n (%)		
Gene	SNP, rs ID	Gene type	With lymph node metastasis	Without lymph node metastasis	P-value
MST1R	rs1062633	TC/CC C	3 (27.3)	10 (71.4)	0.047
		TT	8 (72.7)	4 (28.6)	
SLC28A3	rs10868138	TC	6 (54.5)	0 (0)	0.003[a]
		TT	5 (45.5)	14 (100)	
SLC28A3	rs56350726	TA/AA	7 (63.6)	0 (0.0)	0.001[a]
		TT	4 (36.4)	14 (100.0)	
RRM2	rs1130609	TG/GG	8 (72.7)	4 (28.6)	0.047
		TT	3 (27.3)	10 (71.4)	
BRCA1	rs16941	TC/CC	10 (90.9)	7 (50.0)	0.042
		TT	1 (9.1)	7 (50.0)	
BRCA1	rs16942	TC/CC	10 (90.9)	7 (50.0)	0.042
		TT	1 (9.1)	7 (50.0)	
BRCA1	rs799917	GA/AA	10 (90.9)	7 (50.0)	0.042
		GG	1 (9.1)	7 (50.0)	
BRCA1	rs1799966	TC/CC	10 (90.9)	7 (50.0)	0.042
		TT	1 (9.1)	7 (50.0)	
KMT2B	rs16970649	CT	0 (0)	5 (35.7)	0.046
		CC	11 (100)	9 (64.3)	
BCL2	rs1800477	CT/TT	0 (0.0)	5 (35.7)	0.046
		CC	11 (100)	9 (64.3)	
PMS2	rs1805323	GT/TT	10 (90.9)	7 (50.0)	0.042
		GG	1 (9.1)	7 (50.0)	
LSM3	rs1870134	GC/CC	1 (9.1)	8 (57.1)	0.033
		GG	10 (90.9)	6 (42.9)	
CDA	rs2072671	AC/CC	7 (63.6)	3 (21.4)	0.049
		AA	4 (36.4)	11 (78.6)	
EPHX1	rs2234922	AG/GG	4 (36.4)	0 (0.0)	0.026
		AA	7 (63.6)	14 (100.0)	
RALY	rs2281209	GA	4 (36.4)	0 (0.0)	0.026
		GG	7 (63.6)	14 (100.0)	
CD33	rs2455069	AG	4 (36.4)	0 (0.0)	0.026
		AA	7 (63.6)	14 (100.0)	
TTF1	rs3739914	AG/GG	2 (18.2)	9 (64.3)	0.042
		AA	9 (81.8)	5 (35.7)	
TTF1	rs8999	CA/AA	2 (18.2)	10 (71.4)	0.015
		CC	9 (81.8)	4 (28.6)	
BCL10	rs3768235	CT	6 (54.5)	1 (7.1)	0.021
		CC	5 (45.5)	13 (92.9)	
MAP3K1	rs702689	GA/AA	1 (9.1)	9 (64.3)	0.012
		GG	10 (90.9)	5 (35.7)	
ETV1	rs9639168	TC/CC	9 (81.8)	5 (35.7)	0.042
		TT	2 (18.2%)	9 (64.3%)	

[a]P<0.01; all P<0.05. SNP, single nucleotide polymorphism; MST1R, macrophage stimulating 1 receptor 1; SLC28A3, solute carrier family 28 member 3; RRM2, ribonucleotide reductase regulators subunit M2; BRCA1, breast cancer 1; KMT2B, lysine methyltransferase 2B; BCL2, B cell lymphoma 2; PMS2, PMS1 homolog 2; LSM3, U6 small nuclear RNA-associated Sm-like protein 3; CDA, cytidine deaminase; EPHX1, epoxide hydrolase 1; RALY, ribonucleoprotein particle-associated with lethal yellow; CD33, Siglec-3; TTF1, thyroid transcription factor 1; BCL10, B cell lymphoma 10; MAP3K1, mitogen-activated protein 3 kinase 1; ETV1, ETS variant 1.

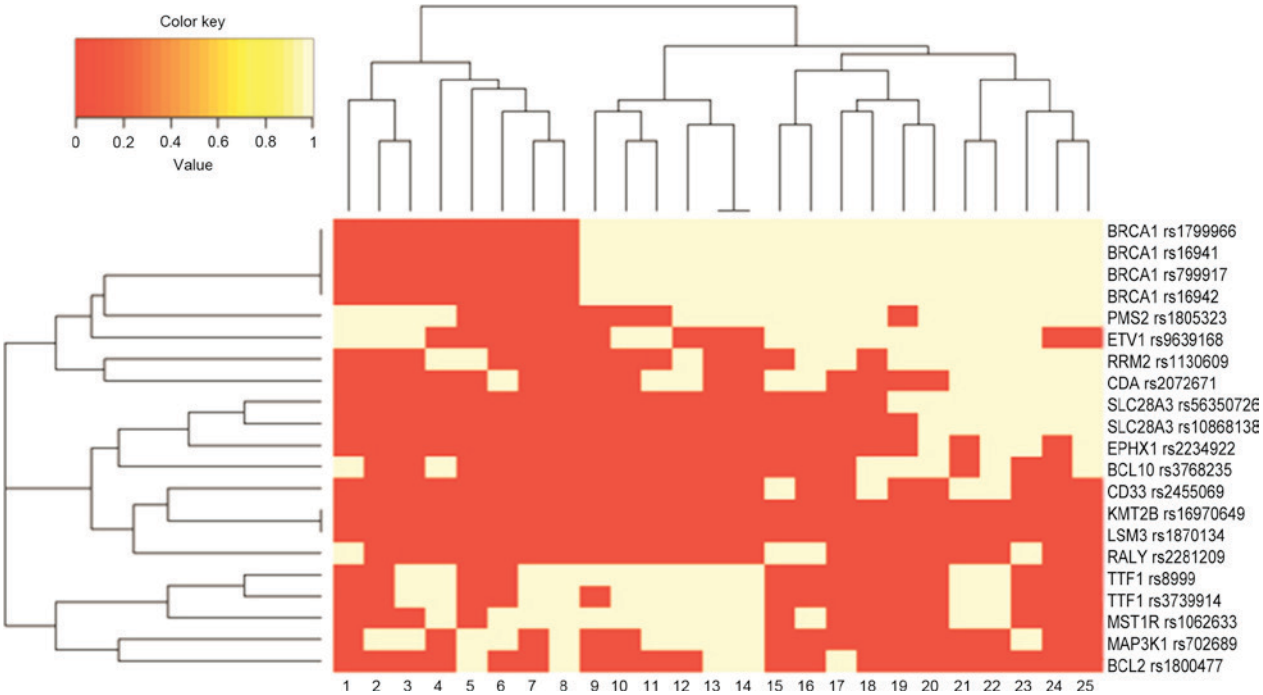

Figure 2. Clustering of correlations between single nucleotide polymorphisms and groups of patients with colorectal cancer. The color key indicates the correlation coefficient, with red being the most-associated. BRCA1, breast cancer 1; PMS2, PMS1 homolog 2; ETV1, ets variant 1; RRM2, ribonucleotide reductase regulators subunit M2; CDA, cytidine deaminase; SLC28A3, solute carrier family 28 member 3; EPHX1, epoxide hydrolase 1; BCL10, B cell lymphoma 10; CD33, Siglec-3; KMT2B, lysine methyltransferase 2B; LSM3, U6 small nuclear RNA-associated Sm-like protein 3; RALY, ribonucleoprotein particle-associated with lethal yellow; TTF1, thyroid transcription factor 1; MST1R, macrophage stimulating 1 receptor 1; MAP3K1, mitogen-activated protein 3 kinase 1; BCL2, B cell lymphoma 2.

number alterations, which accounted for 60% of all subjects. In the 1,503 candidate genes, 80 were identified to have CNVs. However, only one of the CNVs in the 80 genes (DDR1) was associated with lymph node metastasis in colorectal cancer, although this was not statistically significant (P=0.072).

Gene expression alteration in lymph node metastasis of colorectal cancer. The relative mRNA expression level of EGF in tumor tissues (1.00±0.28) was significantly lower than in normal tissues (4.89±1.56; P<0.05). The relative mRNA expression level of NFKB1 in tumor tissues (3.23±0.80) was significantly higher than in normal tissues (1.25±0.25; P<0.05).

Discussion

In the present study, 21 SNPs in 16 genes associated with lymph node metastasis of colorectal cancer were screened. Only 1 CNV in the DDR1 gene was identified to be associated with lymph node metastasis in colorectal cancer, although this difference was not statistically significant. EGF and NFKB1 were abnormally expressed in colorectal tumor tissues.

EGF is a multi-functional growth factor; it is able to bind specific receptors on the cell surface and further induce signal transduction. In tumor cells, EGF may mediate proliferation by activating the EGF receptor pathway which leads to tumor cells survival and metastasis (18). Previous studies demonstrated that EGF is able to induce tumor metastasis through matrix metalloproteinases (19), tyrosine kinase PK2 (20), Podoplanin (21), Rictor binding protein (22), epithelial mesenchymal transition (23) and improving blood vessel (24) and lymph gland growth (25,26). In the present study, the mRNA level of EGF was significantly upregulated in patients with lymph node metastasis, which indicated that EGF may be associated with lymph node metastasis in colorectal cancer. A previous study demonstrated that EGF is able to induce lymph gland metastasis by promoting lymph gland progression (19).

NFKB was initially identified in B lymphocytes, it is associated with a number of transcriptional processes by binding to promoter sites. NFKB is rarely mentioned as being associated with lymph node metastasis. The current study indicates that a significant upregulation of NFKB occurs during lymph node metastasis in colorectal cancer, indicating that NFKB may be associated with lymph node metastasis. The present study may provide a basis for further validation and identification of genetic markers.

In conclusion, the results of the present study indicate a number of potential genetic biomarkers associated with lymph node metastasis which may provide insight into early prognosis of colorectal cancer. However, a limitation of the present study was that the sample size was small. Therefore, a larger sample size is required for further validation of genetic biomarkers for colorectal cancer.

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