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Screening and identification of a tumor specific methylation phenotype in the colorectal laterally spreading tumor

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Abstract. – OBJECTIVE: We screened and identified the differential expression of the methylation phenotype in the whole genome of colorectal laterally spreading tumor (LSTs).

MATERIALS AND METHODS: 3 tissue samples of colorectal polypoid adenomas (PAs), 3 tissue samples of LSTs and 3 tissue samples of colon cancer were analyzed with a high-density gene chip, and about 450,000 methylation sites were detected covering approximately 95% of the CpG islands. The Delta Data screening was taken through a cluster analysis of methylation phenotype differential expression. 50 tissue samples each of PAs patients, LSTs patients, and colorectal cancer patients were selected.

Methylation-specific PCR (MSP) was used to detect RASSF1A and WIF-1 methylation levels. He RT-PCR method was used to detect the relative mRNA expression levels for methylation expression identification.

RESULTS: The degree of LST methylation was higher than that of PAs, and 1234 genes were found to have a lower expression when compared to colorectal cancer samples. 764 genes had a higher expression when compared to colorectal cancer, and 559 genes lower expression when compared to PAs. The average methylation level of LSTs was higher than that of PAs, and lower than that of colorectal cancer. The chromosomal location was taken on these 1234 genes, which were higher than that of PAs, and lower than that of colorectal cancer; 518 genes were located on chromosome No. 2 (41.98%), 236 on No. 5 (19.12%), 357 on No. 8 (28.93%), and 123 on No. 10 (9.97%). According to clustering analysis, DNA differentially methylated sites were mainly on genes of cell adhesion molecules regulation, signaling pathways, energy transduction, cell cycle and apoptosis. The positive rate of RASSF1A and WIF-1 methylation in the tissues of LSTs patients were higher than that of PAs, and lower than that of colorectal cancer; differences were statistically significant ($p<0.05$). The relative expression levels of RASSF1A and WIF-1mRNA in the tissues of LSTs patients were lower than that of PAs, higher than that of colorectal cancer, and the difference was statistically significant ($p<0.05$).

CONCLUSIONS: The administration of high-density gene chip technology has a good application value to screen the differential expression of LSTs gene methylation phenotype. Results are consistent with the identification results.

Key Words:
PCR Colorectal laterally spreading tumor, Whole genome methylation phenotype, High-density gene chip, Methylation-specific PCR.

Introduction

About 80-90% of colorectal cancer cases occur through adenomatous polyps and colorectal laterally spreading development type tumor (LSTs), which are a special form of adenomatous polyps that grow laterally along the intestinal mucosal surface with less invasion to the deep layers, and higher malignancy potential than PAs[1,2]. Activations of proto-oncogene and inactivation of tumor suppressor genes are important mechanisms for the occurrence and development of tumors. The abnormal methylation of the promoter region is the main epigenetic alteration of the inactivation of tumor suppressor genes[3,4]. Studies have confirmed that the abnormal methylation of genes regulates cell cycle, tumor susceptibility, metabolic enzymes and cell adhesion molecule expression. These are early events in tumorigenesis along with tissue heterogeneity and occur earlier than cancerous tumors. These lead to metabolic and morphological abnormality and have an im-

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portant application value in early diagnosis, identification and evaluation of therapeutic effect and prognosis[5,6]. The sample required for detection of methylation is less than RNA and protein detection, and the sensitivity is higher[7]. The research methods include overall methylation, gene-specific methylation and methylation sites verification. 450K Infinium Methylation BeadChip Technology is a combination of DMH, MeDIP or MIPA technology which can effectively detect weak expression, low mutation frequency of methylation, and can also achieve a direct comparison of different degrees of methylation with a chip. The result is highly sensitive, highly specific and is highly repeatable[8,9]. Based on this, our study aims to screen and identify the differential expression of LSTs gene methylation phenotype.

Materials and Methods

Tissue Specimens

3 tissue samples of PAs, 3 tissue samples of LSTs and 3 tissue samples of colon cancer were taken in the screening of differentially expressed genes DNA methylation in the whole gene. 50 cases of patients with PAs, 50 patients with LSTs, and 50 cases of colorectal cancer were selected by identification of methylation from January 2014 to January 2016 at our hospital without previous surgery, radiotherapy or chemotherapy history, inflammatory bowel disease, familial adenomatous polyposis. Endoscopic or surgical resection was taken for tumor tissue specimens, and frozen in liquid nitrogen at -80°C. This study was approved by the Ethics Committee of our Hospital and obtained the informed consent rights of the patients and their families.

Research Methods

High-density Gene Chip Technology

QIAamp DNA Mini Kit (Qiagen, Dusseldorf, Germany) was used to extract tissue DNA. The NaDROP2000 nucleic acid quantitative instrument (Applied Biosystems Company, Foster City, CA, USA) was used for determination of DNA content and purity. An OD260/280 of 1.70-1.90 met the requirement. A 2% agarose gel electrophoresis was used for detection of DNA genomic integrity, and genomic DNA bands were clear without miscellaneous towing. EpiTect 96 Bisulfite Kits (Qiagen, Hilden, Germany) were used for transformation of heavy hydrogen sulfite. For chip hybridization, DNA samples were transferred to a special MSA4 plate denaturation and neutralization, with incubation for amplification overnight, enzyme digestion, and PM1 precipitation. The suspension was added into the micro bead chip, and incubated in the hybrid furnace for 22 h. After the cleaning, the nucleotide was added, which was extended to cross, and the colored primer was coated to protect the micro beads. Methylation site scanning and data analysis were conducted as follows, the chip was transferred to iScan Reader, a methylation module of GenomeStudio software (Illumina, CA, USA) was used for data analysis, and the impact of the system variation in the experimental process was eliminated.

The quality control analysis of the methylation chip includes the quality control of the chip. The

Table I. Primer sequences of each gene.

Gene	Sequence	Length (bp)
RASSF1A (U)	F:5′-GGGGGTTTTGTGAGAGTGTGTTT-3′ R:5′-CCCAATTAAACCCATACTTCACTAA-3′	204
RASSF1A (M)	F:5′-CGAGAGCGCGTTTAGTTTCGTT-3′ R:5′-CGATTAAACCCGTACTTCGCTAA-3′	192
WIF-1 (U)	F: 5’ -GGGTGTTTTATTGGGTGTATTGT-3’ R: 5’ -AAAAAAACTAACACAAACAAAATACAAAC-3’	154
WIF-1 (M)	F: 5’ -CGCTCCACTGGGCGCACCGC-3’ R: 5’ -TCGCACCTCGCTCGCGCCAGC-3’	145
RASSF1A	F:5′-CAGATTGCAAGTTCACCTGCCACTA-3′ R:5′-GATGAAGCCTGTGTAAGAACCGTCCT-3′	249
WIF-1	F:5’ -GTCTAAACGGGAACAGCCCT-3’ , R:5’ -GCTGGCATTCTCTGTTGTGC-3’	354
β-actin	F:5′-AAAGACCTGTACGCCAACAC-3′ R:5′-GTCATACTCCTGCTTGCTGAT-3′	219

Table II. Signal transduction pathway analysis differential expression of gene methylation mainly involved in.

Gene	Methylation level	Delta Data	Diff Score	Pathway
RASSF1A	high	0.35624	64.52312	Cell adhesion molecules (CAMs)
WIF-1	high	0.32157	55.32487	Wnt/β-catenin
PTPRO	high	0.26549	-72.63245	Caspase
FBX036	high	0.24321	-81.32649	mTOR
c-myc	high	0.36295	56.53427	Wnt/β-catenin
NCOR2	low	0.10205	64.53251	Calcium signaling pathway
RARβ	low	0.06354	66.63529	T cell receptor signaling pathway
COL4A2	low	-0.35646	-56.62345	ECM-receptor interaction
DAPK1	low	-0.46756	-57.62438	TGF-beta signaling

red and green signals represent the levels of DNA and biotin monitoring sensitivity and efficiency (value >5000). For the single base extension step quality control, the green signal represents the GC base, and the red signal represents the AT base (value >5000). For hybrid quality control, the detection of hybridization effects with biotin-labeled probes of three concentrations (green signal) (value >10000) and Residual base impurity quality control (value <5000).

Results were used to analyze whole genome methylation phenotype by cluster analysis and a significance test; the Data Delta was screened. Delta Data showed the methylation levels and the value of Beta 0-1. Positive values meant methylation increase, and the negative meant a decrease. Delta Data >0.2 meant increased methylation, and <-0.2 means decreased methylation. Diff Score significance test: positive values showed increased methylation while negative values represented decreased methylation. > 50 or <-50 indicated $p<0.00001$ and, therefore, significant statistical difference. The screening criteria for differential expression of gene methylation. Any group is an effective gene ($p<0.05$), and the Diff score value of the test group >50 or <-50, Delta Beta >0.2 or <-0.2.

Differential Gene Identification

Methylation-specific PCR (MSP) was performed using the SYBR Green I method, and primers were synthesized by Invitrogen Co. (Carlsbad, CA, USA). Primer sequences are shown in Table I. The reaction system included 2.5 μl 10×buffer, 1.5 μl Mg2+ (25 mol/L), 1.8 μl of dNTP (2.5 mol/L), 50 ng DNA, upstream and downstream primers (10 μmol/L), 0.25 μl Taq enzyme (5 U/μl), and water was added to the total volume of 25 μl. The reaction conditions were 95°C for 5 min, 95°C for 1 min, 56°C for 1 min, 75°C for 1 min for a total of 40 cycles, and finally, 78°C for 10 min to build the dissolution curve. The product was analyzed by 2% agarose gel electrophoresis and gel imaging analysis system (Media Cybernetics Company, Rockville, MD, USA). A methylation positive control was selected. The number of copies of DNA was calculated, and PCR amplification was diluted by 10 times of the gradient as standard. Specimens were taken as methylation positive if they had >500 copies /ml.

For the RT-PCR process, RNA extraction was performed by the conventional TRIzol method. The concentration and purity of RNA were determined by UV Spectrophotometry, and the synthesis of cDNA was conducted by a reverse transcription kit (Takara, Otsu, Shiga, Japan). The primers were designed and synthesized by Japanese Takara Company, and the primers were shown in Table I. The reaction system included 2.5 μl 5×buffer, 1.5 μl $MgCl_2$, 0.5 μl dNTP + 1 μl each of Upstream and downstream primers, 0.3 μl Taq enzyme, 2 μl cDNA templet, and water was added to make a total volume of 25 μl. The reaction conditions were 95°C for 5 min, 95°C for 30 s, 62°C for 30 s, 72°C for 30 s for a total of 35 cycles, and 72°C for 10 min. The dissolution curves were constructed, and the relative expression of mRNA was calculated by the $2^{-\Delta\Delta}$Ct method.

Statistical Analysis

The SPSS20.0 statistical software (Version X; IBM, Armonk, NY, USA) was used. Measurement data were expressed as a mean ± standard deviation. Single-factor ANOVA analysis was used to compare among groups. The pairwise comparison was tested using the LSD-t method. Count data was expressed by some cases or (%). The comparison among groups was tested by χ^2. $p<0.05$ indicated that the difference was statistically significant.

Table III. The degree of methylation of RASSF1A and WIF-1 tissues [cases (%)].

Group	The number of cases	RASSF1A	WIF-1
PAs	50	13 (26.0)	12 (24.0)
LSTs	50	33 (66.0)	27 (54.0)
Colorectal cancer	50	42 (84.0)	38 (76.0)
χ^2		36.345	27.273
p		0.000	0.000

Results

Differential Expression Analysis of GeNome methylation

The degree of LST methylation was higher than that of PAs, and 1234 genes had lower methylation levels than colorectal cancer samples. 764 genes had higher methylation levels than colorectal cancer, and 559 of them had lower levels than PAs. The average methylation level of LSTs was higher than that of PAs, and lower than that of colorectal cancer (Figure 1). Chromosomal location was taken on these 1234 genes, which were higher than that of PAs, and lower than that of colorectal cancer; 518 genes were located on the chromosome No. 2 (41.98%), 236 on No. 5 (19.12%), 357 on No. 8 (28.93%), and 123 on No. 10 (9.97%). Clustering analysis showed that the differentially methylated sites were mainly cell on genes of adhesion molecules regulation, signaling pathways, energy transduction, cell cycle and apoptosis (Table II).

The Degree of Methylation of RASSF1A and WIF-1 tissues

The positive rate of RASSF1A and WIF-1 methylation in the tissues of LSTs patients was higher than that of PAs, and lower than that of colorectal cancer; differences were statistically significant ($p<0.05$) (Table III).

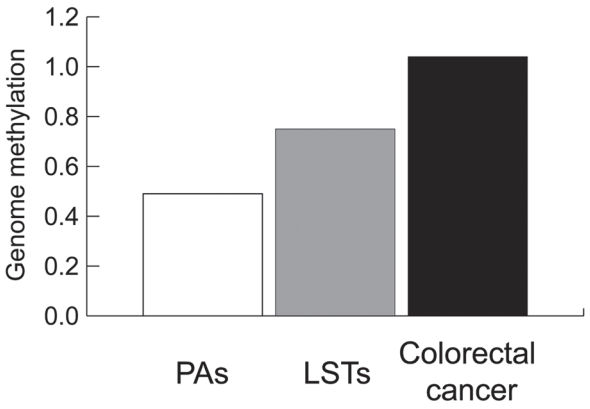

Figure 1. Differential expression analysis of genome methylation.

Analysis of Relative Expression Levels of WIF-1 mRNA and RASSF1A

The relative expression levels of RASSF1A and WIF-1 mRNA in the tissues of LSTs patients were lower than that of PAs, higher than that of colorectal cancer, and the differences were statistically significant ($p<0.05$) (Table IV).

Discussion

DNA methylation is the most common modification of eukaryotic DNA that is under the action of DNA methyl transferase (DNMT). S-adenosylmethionine is a methyl donor as it functions to transfer a methyl group onto a specific base. In the CpG cytosine dinucleotide, the 5-carbon atom is its target, and only affects the cytosine or guanine on the DNA chain. About 70-80% of normal DNA sequence CpG sites can have methylation, and CpG island with CpG dinucleotide is always unmethylated and often located near the gene transcriptional regulatory region. It has a close relationship with 50-60% of the human genome-coding gene[10].

A number of gene methylation studies for colorectal cancer have found that PCDH8, Tiam1, RASSF1A, RUNX3, hMLH1, KiSS-1 and methylation levels were closely related to the malignant

Table IV. Analysis of relative expression levels of WIF-1 mRNA and RASSF1A.

Group	RASSF1A	WIF-1
PAs	0.5324±0.1323	0.5213±0.1426
LSTs	0.3258±0.1132	0.3362±0.1033
Colorectal cancer	0.1234±0.0536	0.1025±0.0428
F	7.325	7.435
p	0.000	0.000

biological behavior such as tumor proliferation, differentiation, invasion and metastasis, which has an important value to guide chemosensitivity and predict clinical outcomes as the sensitivity index of early diagnosis of tumors[11,12]. Through the analysis of CpG island methylation phenotype (CIMP), the methylation status of CACNA1G, CDKN2A, CRABP1, MLH1 and NEUROG1 were obtained as the biological markers for the detection of CIMP[13]. Also, different from genetic change, epigenetic change is reversible and can restore the original function of the cell. LSTs are an important precancerous lesion of colorectal cancer, which has important clinical significance for its early diagnosis and effective intervention to improve the outcome of the tumor.

Through this research, we found that the degree of LST methylation was higher than that of PAs, and 1234 genes were found to have lower methylation levels than that of colorectal cancer. The average methylation level of LSTs was higher than that of Pas and lower than that of colorectal cancer. The genes were located on chromosome No. 2 (41.98%), on No. 5 (19.12%), on No. 8 (28.93%), and on No. 10 (9.97%). Clustering analysis indicated that DNA differentially methylated sites were mainly cell adhesion molecules regulation, signaling pathways, energy transduction, cell cycle and apoptosis. It is suggested that the application of high-density gene chip technology has a good application value in the screening of differentially expressed genes in the LST genome[14]. Further methylation levels of RASSF1A and WIF-1 genes were identified. The positive rate of RASSF1A and WIF-1 methylation levels in the tissues of LST patients was higher than that of PAs, and lower than that of colorectal cancer. The relative expression levels of RASSF1A and WIF-1 mRNA in the tissues of LST patients was lower than that of PAs, and higher than that of colorectal cancer; differences was statistically significant. It was suggested that the results of screening and identification were in good agreement. RASSF1A inhibits tumor formation by regulating cell cycle, apoptosis and genome stability; it is highly expressed in normal tissues, and loss of expression is closely related to the occurrence and development of tumor[15]. Wnt/β-catenin signaling pathway plays an important role in the occurrence, differentiation, proliferation, invasion and other biological behaviors of colorectal cancer[16]. C-myc and cyclinD1 are important downstream target genes, which regulate cell division cycle[17]. WIF-1 is a major tumor suppressor gene in the Wnt pathway, and its high methylation levels and low expression levels of mRNA have an important influence on the occurrence and development of colorectal cancer[18].

Conclusions

Therefore, the administration of high-density gene chip technology has a good application value to screen the differential expression of LSTs gene methylation phenotype. Results are consistent with the identification results.

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Conflict of Interest

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