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Mutation and expression analysis of the *IDH1*, *IDH2*, *DNMT3A*, and *MYD88* genes in colorectal cancer

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ABSTRACT

Colorectal cancer (CRC) is one of the leading causes of death around the world. Its genetic mechanism was intensively investigated in the past decades with findings of a number of canonical oncogenes and tumor-suppressor genes such as *APC*, *KRAS*, and *TP53*. Recent genome-wide association and sequencing studies have identified a series of promising oncogenes including *IDH1*, *IDH2*, *DNMT3A*, and *MYD88* in hematologic malignancies. However, whether these genes are involved in CRC remains unknown. In this study, we screened the hotspot mutations of these four genes in 305 CRC samples from Han Chinese by direct sequencing. mRNA expression levels of these genes were quantified by quantitative real-time PCR (RT-qPCR) in paired cancerous and paracancerous tissues. Association analyses between mRNA expression levels and different cancerous stages were performed. Except for one patient harboring *IDH1* mutation p.I99M, we identified no previously reported hotspot mutations in colorectal cancer tissues. mRNA expression levels of *IDH1*, *DNMT3A*, and *MYD88*, but not *IDH2*, were significantly decreased in the cancerous tissues comparing with the paired paracancerous normal tissues. Taken together, the hotspot mutations of *IDH1*, *IDH2*, *DNMT3A*, and *MYD88* gene were absent in CRC. Aberrant mRNA expression of *IDH1*, *DNMT3A*, and *MYD88* gene might be actively involved in the development of CRC.

1. Introduction

Colorectal cancer (CRC) is one of the most prevalent cancers throughout the world with a 5-year survival rate of 30–65% and it affects men and women almost equally (Haggar and Boushey, 2009; Savas and Younghusband, 2010). Although the incidence of CRC has stabilized and/or declined gradually in the last 30 years in parts of Europe and the United States, the incidence is increasing rapidly in some Asian countries such as China, Japan, and Singapore, among others (Haggar and Boushey, 2009; Jemal et al., 2008). Ulcerative colitis (UC), familial adenomatous polyposis (FAP), and hereditary nonpolyposis cancer (HNPCC) constituted the top three high-risk factors for the development of CRC (Kaluz and Van Meir, 2011). During the past decades, a series of genes, including *APC*, *TP53*, and *KRAS*, was identified to be altered in CRC (Kaluz and Van Meir, 2011). Among them, genes involved in the EGFR signal pathway (*KRAS*, *BRAF*, and *APC*) are the most frequently mutated in CRC patients. However, there is still a large portion of CRC cases that do not contain mutations in these canonical oncogenes and tumor-suppressor genes, and the molecular underpinnings of CRC have not been clearly elucidated (Aissi et al., 2013).

Recent genome-wide association studies and/or whole-exome sequencing analyses in different cancers have led to the identification of many non-canonical candidate oncogenes, i.e., *IDH1/2*, *MYD88*, *SF3B1*, and *MAP2K1/2* (Mardis et al., 2009; Ngo et al., 2011; Nikolaev et al., 2012; Parsons et al., 2008; Puente et al., 2011; Quesada et al., 2012).

Abbreviations: CRC, colorectal cancer; *APC*, adenomatous polyposis coli; *KRAS*, Kirsten rat sarcoma viral oncogene homolog; *TP53*, tumor protein p53; *IDH1*, isocitrate dehydrogenase 1 (NADP+), soluble; *IDH2*, isocitrate dehydrogenase 2 (NADP+), mitochondrial; *DNMT3A*, DNA (cytosine-5-)-methyltransferase 3 alpha; *MYD88*, myeloid differentiation primary response 88; UC, ulcerative colitis; FAP, familial adenomatous polyposis; HNPCC, hereditary nonpolyposis cancer; *EGFR*, epidermal growth factor receptor; *BRAF*, v-raf murine sarcoma viral oncogene homolog B; *SF3B1*, splicing factor 3b, subunit 1; *MAP2K1/2*, mitogen-activated protein kinase kinase 1/2; 2-HG, 2-hydroxyglutarate; AML, acute myeloid leukemia; TLR, Toll-like receptor; *IL-1R*, interleukin 1 receptor; NF-κB, nuclear factor-kappa B; RT-qPCR, real-time quantitative PCR; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; DSS, dextran sodium sulfate; α-KG, α-ketoglutarate; HepG2, human hepatocellular liver carcinoma cell line; *HIF-1α*, hypoxia-inducible factor 1-α; *GLUT1*, glucose transporter 1; *VEGF*, vascular endothelial growth factor; *HOXA2*, homeobox A2; *HOXA4*, homeobox A4; *HOXA6*, homeobox A4; WT, wild-type.

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Foremost among the list are isocitrate dehydrogenase 1 (*IDH1*) and isocitrate dehydrogenase 2 (*IDH2*), which are frequently mutated in glioblastoma and several kinds of myeloid malignancies (Gross et al., 2010; Ichimura et al., 2009; Mardis et al., 2009; Zou et al., 2010). The most important mutations in these two genes are *IDH1* p.R132, *IDH2* p.R140, and *IDH2* p.R172 that are located in the active site of the enzymes (Xu et al., 2004; Yan et al., 2009). Subsequent investigations discovered that these mutants promote the production of 2-hydroxyglutarate (2-HG) (Figueroa et al., 2010; Xu et al., 2011). The increased level of 2-HG could result in genome-wide histone and DNA methylation alterations, which have been proven to play active roles in the development of cancer (Figueroa et al., 2010; Jia and Guo, 2013; Kondo and Issa, 2004; Xu et al., 2011). Although the first *IDH1* mutation was identified in a CRC patient and the gain-of-function mutation was also validated in colorectal cell lines, there has been no other *IDH1* mutation reported in colorectal cancer thus far (Jin et al., 2013; Sjoblom et al., 2006; Yen et al., 2010). Another gene involved in DNA methylation, DNA methyltransferase 3A (*DNMT3A*), was found to be associated with colorectal cell proliferation, apoptosis, and senescence (Ng et al., 2009; Zhang et al., 2011). A series of functional mutations in the coding region of *DNMT3A* was identified in acute myeloid leukemia (AML), but the mutation status and expression level of this gene in CRC and its relevance to CRC pathogenesis is still unknown (Ley et al., 2010; Lin et al., 2011; Yan et al., 2011).

MYD88 is a key adaptor mediating the signal transduction from Toll-like receptor (TLR) and interleukin 1 receptor (IL-1R) family members (Han, 2006). Activation of MYD88 could give rise to multiple downstream signaling cascades including nuclear factor-kappa B (NF-κB) signal pathway, which plays a crucial role in pathogenesis of CRC through suppression of apoptosis, induction of epithelial growth, promotion of angiogenesis, and cancerous cell invasion (Wang et al., 2009). Recently, two groups independently identified that mutation p.L265P in the *MYD88* gene is a recurrent mutation in human lymphoma (29%) and chronic lymphocytic leukemia (2.9%) (Ngo et al., 2011; Puente et al., 2011). Several cancer-associated signaling pathways (including NF-κB) are activated in the presence of *MYD88* mutation (Ngo et al., 2011; Puente et al., 2011). However, whether the increased NF-κB activity in CRC could be attributed to the *MYD88* p.L265P mutation has not been analyzed.

To investigate whether the four genes *IDH1*, *IDH2*, *DNMT3A*, and *MYD88* are actively involved in the development of CRC, we analyzed the reported pathogenic mutations of these genes in 305 CRC patients and quantified the mRNA levels of these four genes in paired cancerous and paracancerous normal tissues from 65 patients in this study. No hotspot mutations were identified except for an *IDH1* mutation p.I99M. Decreased mRNA expression of *IDH1*, *DNMT3A*, and *MYD88* were identified in cancerous tissues. These results suggested that aberrant mRNA expression of *IDH1*, *DNMT3A*, and *MYD88* genes might be associated with the onset and/or development of CRC.

2. Materials and methods

2.1. Patients

305 patients were collected at the First Affiliated Hospital of Kunming Medical University from 2010 to 2011. All patients were histopathologically confirmed to have CRC by two pathologists independently. The colorectal cancer staging was determined according to the TNM (Tumor, Node, Metastasis) system. The TNM system assigns a number based on three categories. "T" denotes the degree of invasion of the intestinal wall, "N" denotes the degree of lymphatic node involvement, and "M" denotes the degree of metastasis. T1: Cancer has grown through the muscularis mucosa. T2: Cancer has grown through the submucosa. T3: Cancer has grown through the muscularis propria. T4: Cancer has grown through the wall of the colon or rectum and is attached to or invades into nearby tissues or organs. N0: No cancer in nearby lymph nodes. N1: Cancer cells are found in or near 1 to 3 nearby lymph nodes. N2: Cancer cells are found in 4 or more nearby lymph nodes. M0: No distant spread is seen. M1: Cancer has spread to distant organs or distant lymph nodes. Detailed characteristics of the patients were described in our previous report (Xiao et al., 2013). Paired colorectal cancerous tissues and paracancerous normal tissues were collected for each patient. This study was approved by the institutional review board of the Kunming Institute of Zoology. Written informed consents conforming to the tenets of the Declaration of Helsinki were obtained from each participant prior to the study.

2.2. Sequencing of the IDH1, IDH2, DNMT3A, and MYD88 genes

Genomic DNA was extracted from cancerous tissues or peripheral blood by using the standard phenol–chloroform method or AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen Scientific, CA, USA) according to the manufacturer's instruction. Fragments containing mutation R132 of the *IDH1* gene and mutations R140 and R172 of the *IDH2* gene were amplified using the primer pair of hIDH1f (Balss et al., 2008)/IDH1r (Zou et al., 2010) and IDH2-4F/IDH2-4R, respectively (Supplementary Table 1). Amplification of exon 5 harboring mutation p.L265P in *MYD88* and exon 23 covering mutation R882 of the *DNMT3A* gene were performed by using primer pairs MYD88-3U/MYD88-4195L and hDNMT3Af/hDNMT3Ar (Li et al., 2012), respectively (Supplementary Table 1). The PCR reaction was performed in a volume of 25 μL using the following conditions: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 52 °C (for *IDH1*)/56 °C (for *IDH2*)/62 °C (for *MYD88* and *DNMT3A*) for 30 s, and 72 °C for 30 s, and ended with a final extension at 72 °C for 7 min. All the purified PCR products were sequenced using the amplification primers and/or specific primers for each gene (Supplementary Table 1) and the Big Dye Terminator v.3.1 Cycle Sequencing Kit on an ABI Prism 3730 DNAsequencer (Applied Biosystems, CA, USA).

2.3. Cell culture, transfection, and quantification of mRNA expression

HepG2 cells were transfected with pcDNA3.1 empty vector, wild-type IDH1, IDH1 mutant p.I99M (c.A297G) and p.R132H (as a positive control). After transfection for 48 h, total RNA was extracted and the mRNA expression levels of α-KG-dependent enzymes and related downstream genes (*HIF-1α*, *GLUT1*, *VEGF*, and H3K79 dimethylation associated HOXA genes (*HOXA2*, *HOXA4*, and *HOXA6*)) were determined by quantitative real time qPCR (RT-qPCR). Detailed experimental information was reported in our recent study (Lu et al., 2014). As we performed the transfections for all vectors at the same time, we presented the result of p.I99M together with the reported results for pcDNA3.1 empty vector, wild-type IDH1, and IDH mutant p.R132H in our recent study (Lu et al., 2014).

2.4. Quantification of IDH1, IDH2, DNMT3A, and MYD88 mRNA levels

Total RNA was isolated from paired cancerous tissues and paracancerous normal tissues of 65 CRC patients (Supplementary Table 2) who received no radiotherapy and/or chemotherapy treatment before the surgery by using TRIZOL (Invitrogen, Carlsbad, CA). One microgram of total RNA was used to synthesize single-strand cDNA using an oligo (dT) 18-mer as primer and MMLV Reverse Transcriptase (Promega, Madison, WI) in a final reaction volume of 25 μL. Primers used for determining the mRNA expression levels of *IDH1*, *IDH2*, *DNMT3A* and *MYD88* were listed in Supplementary Table S1. The *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) gene was amplified for normalization (Supplementary Table S1). RT-qPCR was performed on the IQ2 Real-Time PCR system (Bio-Rad, Hercules, CA) with the SYBR Premix Ex Taq II (Tli RNaseH Plus; TaKaRa, Otsu, Shiga) and the following amplification condition was used: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 15 s, 53 °C (for *IDH1*)/59 °C

(for *IDH2*)/60 °C (for *MYD88* and *DNMT3A*)/55 °C (for *GAPDH*)/for 15 s, and 72 °C for 20 s, and a final extension cycle at 72 °C for 5 min. Each sample was performed in two duplicates.

2.5. Statistical analysis

The distribution of the mRNA expression levels of the four genes was normal. The difference of mRNA expression levels of the four genes between cancerous tissues and paracancerous normal tissues was determined by paired *t*-test (two-tailed) or one-way ANOVA (for those more than 2 groups) using the mean values. Comparisons were performed between cancerous tissues and paracancerous normal tissues of all TNM stages. mRNA expression changes of the four genes in cancerous tissues among different stages were also evaluated. *P* values less than 0.05 were considered to be statistically significant. All the statistics were performed by using Prism 5 software (GraphPad, San Diego, CA).

3. Results

3.1. Mutation status of IDH1–R132, IDH2–R140/R172, DNMT3A–R882, and MYD88–L265P in CRC samples

We analyzed the mutation status in exon 4 of *IDH1* (for R132) and *IDH2* (for R140 and R172), exon 23 of *DNMT3A* (for R882), and exon 5

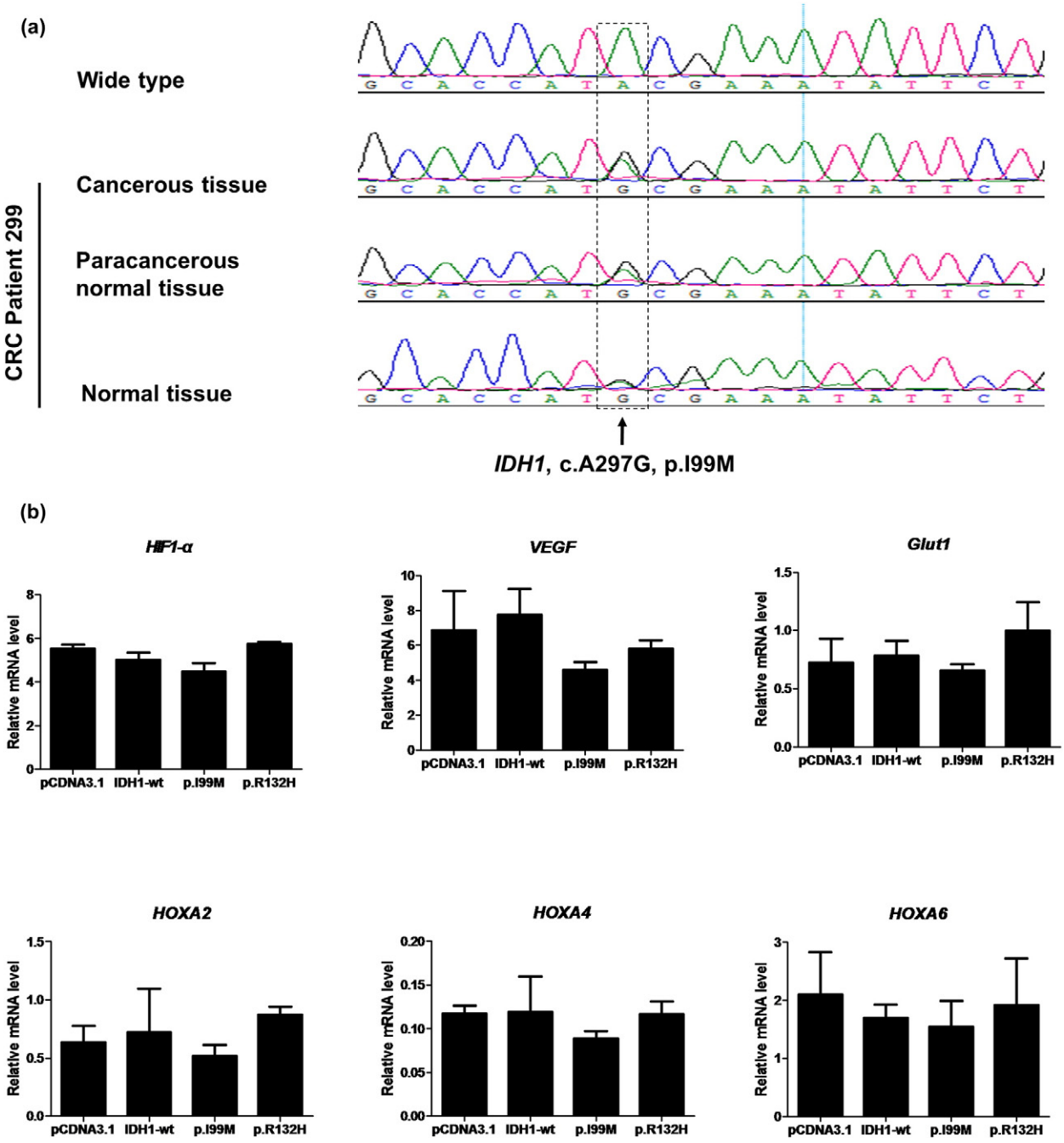

Fig. 1. Identification and functional characterization of IDH1 p.I99M mutation. (a) The mutation status in cancerous, paracancerous and normal tissues of the CRC patient was determined. Sequencing electropherogram of one wide-type patient was used as the reference. Arrow indicates the mutated position. (b) The HepG2 cells were transfected with expression vectors for IDH1 wild-type, p.I99M, p.R132H (as a positive control) and pcDNA3.1 empty vector. The relative mRNA expression levels of genes (*HIF-1α*, *Glut1*, and *VEGF*) involved in the HIF-1α pathway and genes (*HOXA2*, *HOXA4*, *HOXA6*) associated with H3K79 dimethylation were detected by RT-qPCR. Values are shown in mean ± SD. Results are representative of three different experiments. No significant effect was observed for p.I99M compared with wild-type IDH1 (IDH1-wt) and positive control (p.R132H).

of *MYD88* (for p.L265P) in 305 CRC patients by direct sequencing. A heterozygous p.I99M mutation in the *IDH1* gene was identified in one CRC patient. This mutation was observed in cancerous tissue, paracancerous tissue, and paracancerous normal tissue of the patient (Fig. 1a), suggesting that it is unlikely to be somatic. We did not find any of the reported hotspot mutations in the four genes in our samples.

3.2. Effect of IDH1 p.I99M on the mRNA expression level of α-KG-dependent enzymes and downstream target genes

To further explore whether the *IDH1* p.I99M mutation would have an effect in carcinogenesis, we assessed the mRNA expression level of a number of genes associated with activation of the HIF-1α signaling pathway and histone methylation (*HIF-1α*, *GLUT1*, *VEGF*, *HOXA2*, *HOXA4*, and *HOXA6*) in HepG2 cells overexpressing IDH1 p.I99M. We found that p.I99M had no significant influence on the mRNA expression levels of these genes compared with the wild-type or positive control (*IDH* p.R132H) (Fig. 1b). However, we cannot rule out the possibility that this negative result was caused by the use of an inappropriate cell line (liver cell but not colon cell) and the inability to measure 2-HG concentration.

3.3. mRNA expression levels of IDH1, IDH2, DNMT3A, and MYD88 in paired cancerous tissues and paracancerous normal tissues

In order to assess the potential role of the four genes in the pathogenesis of CRC, we evaluated the mRNA expression levels of these genes by RT-qPCR in paired cancerous tissues and paracancerous normal tissues from 65 CRC patients (Supplementary Table S2) who did not received treatment prior to surgery. The mRNA expression levels of *IDH1* ($P = 0.024$, Fig. 2a), *DNMT3A* ($P = 0.013$, Fig. 3a) and *MYD88* ($P = 0.009$, Fig. 4a) are significantly decreased in cancerous tissues compared with the corresponding normal tissues. When the samples were sub-grouped by tumor location, expression of *DNMT3A* ($P = 0.042$, Fig. 3b) and *MYD88* ($P = 0.029$, Fig. 4b) was significantly reduced in colon specimens. Similar mRNA expression levels of these genes were observed between colon and rectum (Figs. 2d, 3d, 4d).

To figure out whether the changes in mRNA expression are associated with the malignancy or stage of CRC, we sub-grouped the samples according to the TNM staging system (Figs. 2e–o, 3e–o, 4e–o). A decrease in *IDH1* mRNA level was seen in cancerous tissues compared with paracancerous normal tissues in T4 ($P = 0.034$, Fig. 2g), N0 ($P = 0.048$, Fig. 2i), and M1 ($P = 0.013$, Fig. 2n). *IDH1* mRNA expression level in cancerous tissues was similar among different TNM stages (Fig. 2h, l, o). Decreased

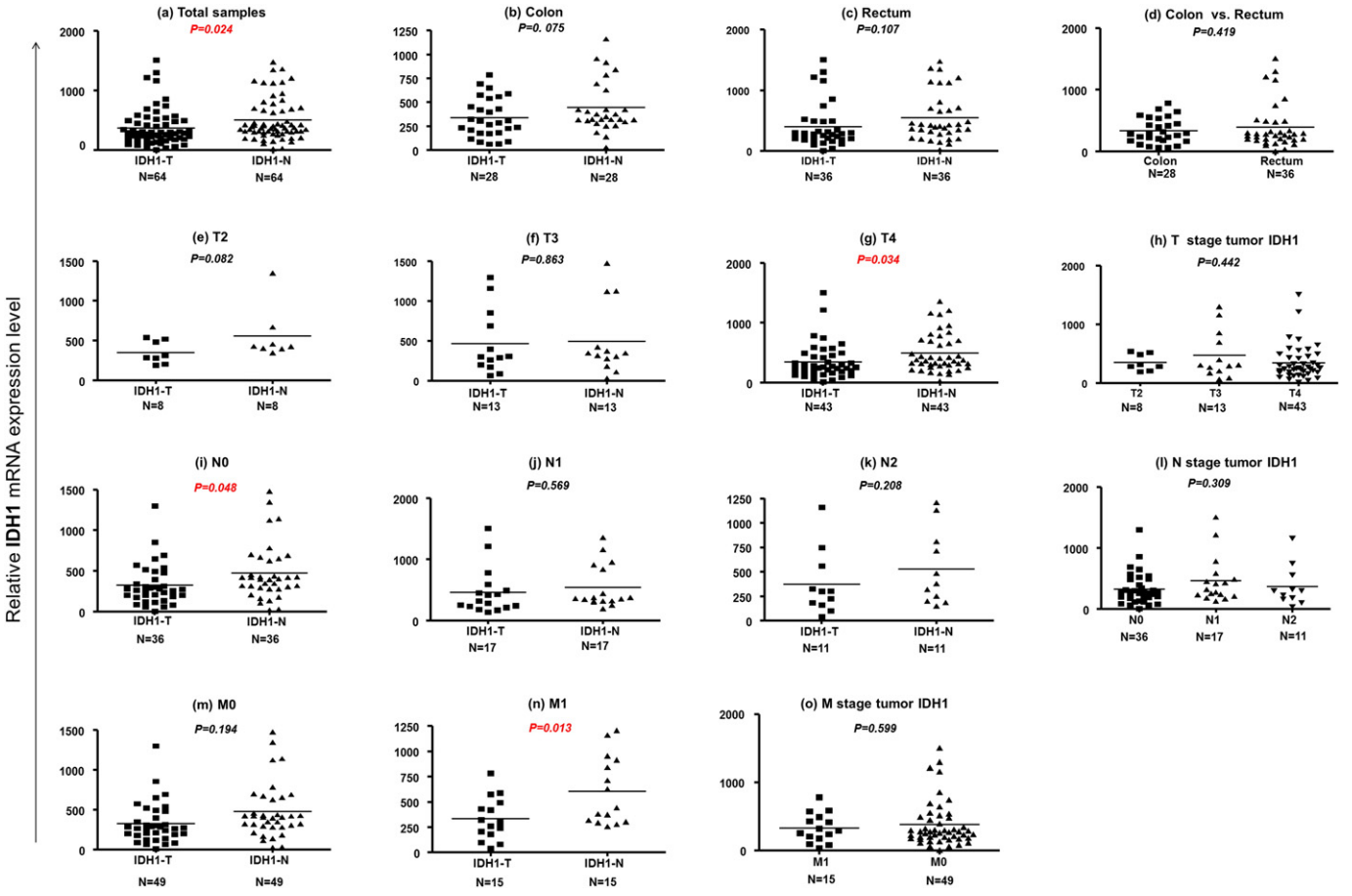

Fig. 2. Relative mRNA expression levels of *IDH1* gene in paired cancerous and paracancerous normal tissues from 65 patients with different pathological characteristics. A total of 65 patients were analyzed. One patient was excluded from the analysis because of RT-qPCR failure. In subgroup analysis for colon specimens, rectal specimens were excluded. Similarly, in subgroup analysis for rectal specimens, colon specimens were excluded. For the TNM subgroup analysis, mRNA expression levels were compared between cancerous tissues and paracancerous normal tissues from patients belonging to the same stage. Differences in cancerous tissues among the specimens belonging to different TNM stages were also compared. The *IDH1* mRNA expression level is significantly reduced in cancerous tissues compared with the corresponding paracancerous normal tissues in total samples (a). Significant decrease of IDH1 expression in cancerous tissues was observed in patients of T4 stage (g), N0 stage (i), and M1 stage (n). No significant expression change of IDH1 in cancerous tissues between the colon and rectal specimens (d) and among the specimens belonging to different TNM stages (h, l, o).

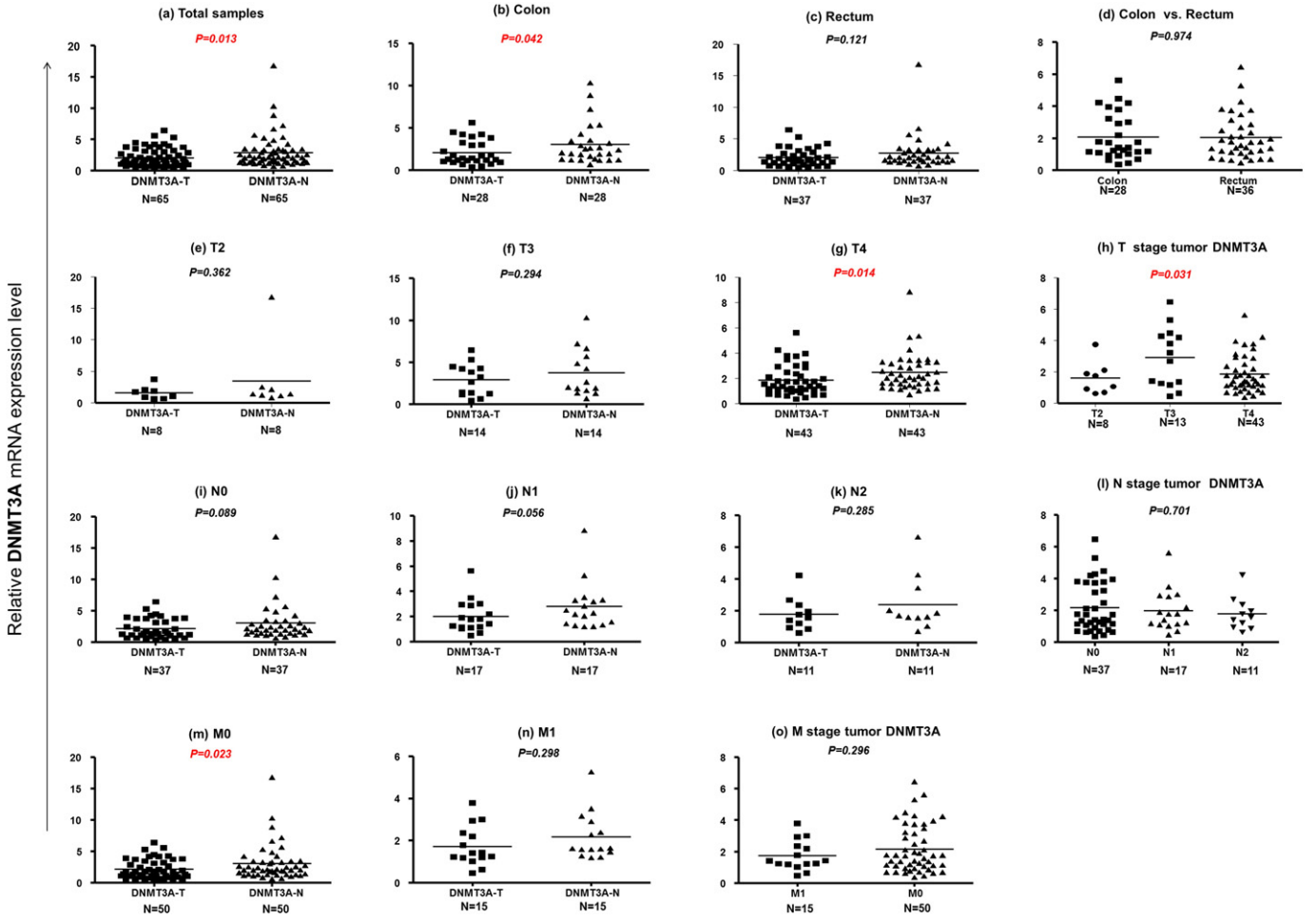

Fig. 3. Relative mRNA expression levels *DNMT3A* gene in paired cancerous and paracancerous normal tissues from 65 patients with different pathological characteristics. A total of 65 patients were analyzed. For information about grouping the specimens in the subgroup analysis, refer to legend of Fig. 2. The *DNMT3A* mRNA expression level in cancerous tissues was significantly lower than that in the corresponding paracancerous normal tissues (a). Patients of T4 stage (g) and M0 stage (m) have significantly reduced *DNMT3A* mRNA expression levels in cancerous tissues. Significant difference of mRNA expression level of *DNMT3A* in cancerous tissues was observed among specimens belonging to different T stages (h).

DNMT3A mRNA expression was observed in T4 ($P = 0.014$, Fig. 3g) and M0 ($P = 0.023$, Fig. 3m). Significant difference of *DNMT3A* ($P = 0.031$, Fig. 3h) mRNA expression level among T stage (Tumor invasion) cancerous tissues was identified. Significantly decreased *MYD88* mRNA expression level was observed in T4 ($P = 0.028$, Fig. 4g), N0 ($P = 0.048$, Fig. 4i) and M0 ($P = 0.016$, Fig. 4m). Significantly altered *MYD88* mRNA expression level ($P = 0.049$, Fig. 4i) among N stage (lymphatic node involvement) cancerous tissues was also identified. These results with TNM staging are consistent with the results sub-grouped by metastasis (Supplementary Fig. 1).

Moreover, we found that the mRNA expression level of *MYD88* increases as the N stage grows ($P = 0.049$, Fig. 4i). As to the T stage (Fig. 4h) and M stage (Fig. 4o), the trend remains, though the differences did not reach statistical significance. This observation showed a potential prognostic role of *MYD88* expression level for tumor malignancy during the process of CRC development.

For *IDH2*, the mRNA level was unchanged between paired cancerous tissues and paracancerous normal tissues either in total samples or in sub-grouped samples (Supplementary Fig. 2).

4. Discussion

The pathogenesis of CRC is an intricate and multi-step processes regulated by intrinsic and extrinsic factors, including genetics, age, diet, population and lifestyle (Chan and Giovannucci, 2010; Haggar and Boushey, 2009). Great achievements have been made in deciphering the genetic mechanisms of CRC onset, and a relatively limited number of the most prominent oncogenes and tumor-suppressor genes were identified over the past 30 years (Fearon, 2011). There has been insufficient study showing whether the recently identified cancer-causing genes *IDH1*, *IDH2*, *DNMT3A* and *MYD88* are also involved in CRC pathogenesis.

In this study, we screened 305 CRC patients for reported pathogenic mutations of three epigenetic related genes (*IDH1*, *IDH2* and *DNMT3A*), together with *MYD88* (which is of great importance in mediating inflammation response). We identified only one patient with a heterozygous *IDH1* p.I99M mutation (Fig. 1a), and no patients with the reported hotspot mutations. The low frequency of *IDH1* mutations in this study is consistent with that of previous studies with small sample sizes (Bleeker et al., 2009; Holdhoff et al., 2009; Kang et al., 2009). Mutation p.I99M was first observed in a Han Chinese patient with AML (Zou et al., 2010) and in another AML patient by Chotirat et al. (2012). This mutation is located in the substrate-binding pocket of isocitrate, which corresponds to the active region in IDH2 (Zou et al., 2010). In a previous study in multiple solid cancers, Bleeker et al. (2009) identified an *IDH1* p.G97D mutation, which is also located in the isocitrate binding active region, suggesting that mutations in this region may have a deleterious effect. The impaired isocitrate affinity of *IDH1/2* caused the production of 2-HG. The accumulation of this metabolite could aid in the development of neoplasm (Dang et al., 2009; Gross et al., 2010; Ward et al., 2010). Our functional characterization of the effect of *IDH1* p.I99M on the mRNA expression levels of HIF-1α genes, its target

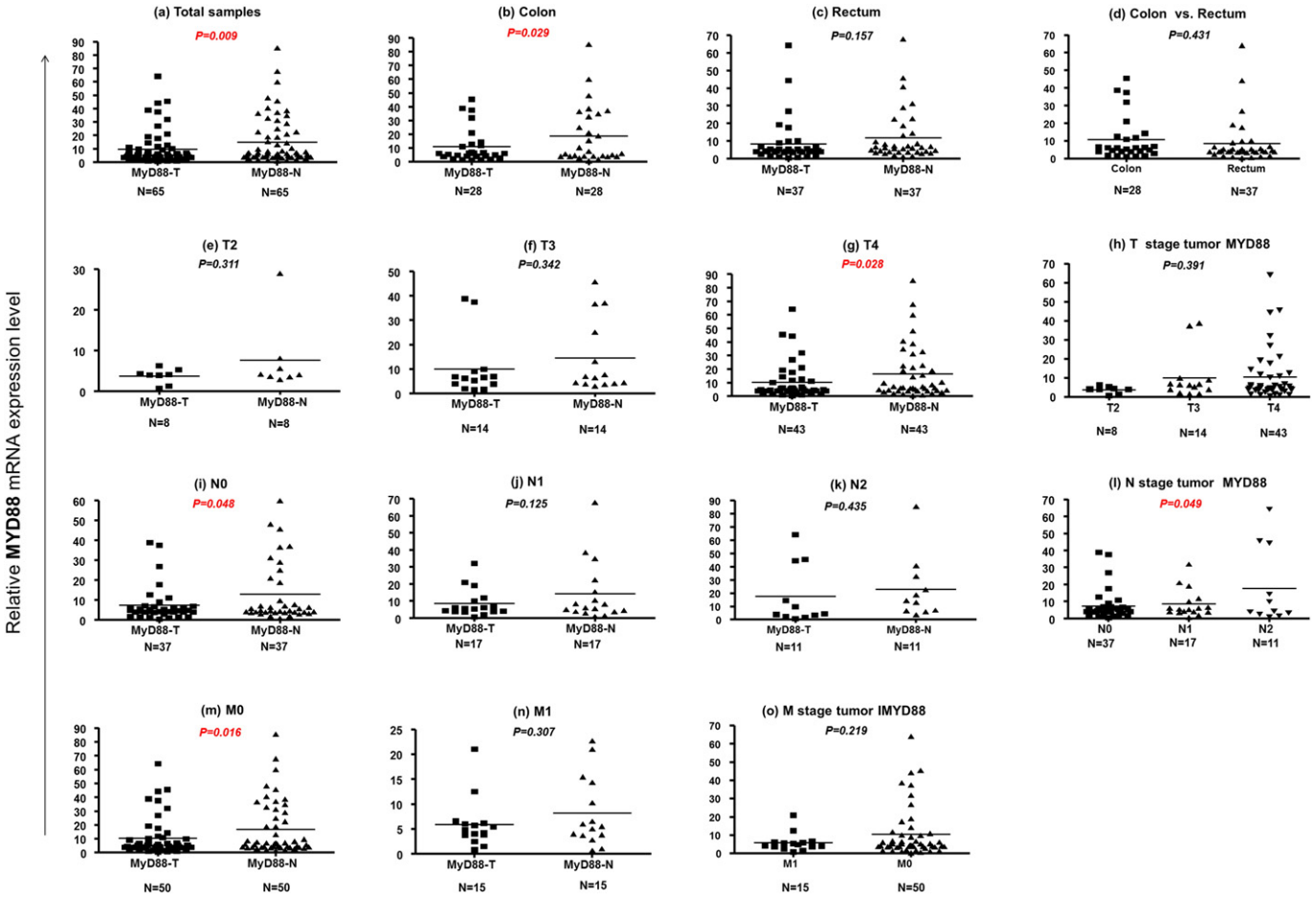

Fig. 4. Relative mRNA expression levels *MYD88* gene in paired cancerous and paracancerous normal tissues from 65 patients with different pathological characteristics. A total of 65 patients were analyzed. For information about grouping the specimens in the subgroup analysis, refer to legend of Fig. 2. The *MYD88* mRNA expression level in cancerous tissues was significantly lower than that in the corresponding paracancerous normal tissues (a). Patients of T4 stage (g), N0 (i), and M0 stage (m) have significantly reduced *MYD88* mRNA expression levels in cancerous tissues. Significant difference of mRNA expression level of *MYD88* in cancerous tissues was observed among specimens belonging to different N stages (l).

genes (*GLUT1* and *VEGF*), and H3K79 dimethylation associated genes (*HOXA2*, *HOXA 4* and *HOXA 6*) gave rise to negative results (Fig. 1b). It was consistent with two recent reports (Ward et al., 2012; Zhang et al., 2013) that showed that *IDH1* p.I99M behaved similarly to the wild-type. Note that we did not measure the 2-HG level and that the cell line used might not be appropriate to show the effect. The exact role of *IDH1* mutation p.I99M in CRC needs further study. The absence of reported hotspot mutations in the four genes that were frequently identified in gliomas and/or hematologic malignancies suggested that these cancer driving mutations might be tissue specific but not actively involved in the initiation and/or development of CRC.

To further characterize the potential role of the four genes in CRC, we determined the mRNA levels of these genes in 65 paired cancerous tissues and paracancerous normal tissues. Our data showed that mRNA levels of *IDH1*, *DNMT3A*, and *MYD88* were significantly decreased in cancerous tissues compared to the paired paracancerous normal tissues (Figs. 2a, 3a and 4a). Hu et al. (2010) found that lower level of IDH1 expression was associated with decreased expression of p53 (which is a tumor suppressor gene in many cancers) and shorter survival time in osteosarcoma. This reported result that *IDH1* expression was negatively correlated with tumor metastasis was consistent with our observation that *IDH1* expression decreased in tissues with distant metastasis (all types of metastasis) (Supplementary Fig. 1B). Robbins et al. (2012) also demonstrated that decreased *IDH1* expression might be correlated with tumor promotion. As cancer cells do not prefer to use the citric acid cycle for energy, this may be one of the reasons that IDH1 was down-regulated in CRC cells. The altered expression of *IDH1* in CRC and other cancers need to be validated in future studies.

DNMT3A plays an essential role in the maintenance of methylation patterns during embryogenesis (Raddatz et al., 2012). Aberrant DNA methylation in colorectal carcinoma cells was discovered, and reduced levels of DNA methylation have also been depicted as a major characteristic of human colon cancer methylomes (Berman et al., 2012). The loss of *DNMT3A* expression was found to be common in lung cancer (46.4%) and was associated with the allelic loss (Kim et al., 2013). In addition, Gao et al. (2011) reported that deletion of Dnmt3a promotes lung tumor progression. In this study, we observed a decreased mRNA level of *DNMT3A* in CRC cancerous tissues, which would suggest that abnormal expression of this gene is responsible for the low level of DNA methylation and may contribute to the development of CRC.

Signal pathways coupled with *MYD88* were engaged in cancer promotion in several mouse carcinogenesis models (Salcedo et al., 2010). Under chronic colitis conditions induced by DSS-induced mucosal damage, *MYD88* had a protective role in colon cancer (Salcedo et al., 2010). However, two previous studies for the *MYD88* expression in CRC offered controversial results, with one reporting a similar level of *MYD88* expression in both cancerous tissues and normal tissues (Je et al., 2012) and the other one reporting a significant increase of this gene in cancerous tissues (Wang et al., 2010). Je et al. (2012) also identified that the expression level of *MYD88* was increased in gastric cancer cells but decreased in colorectal cancer cells compared to normal cells. Our study showed that the *MYD88* mRNA expression level was decreased in

cancerous tissue compared to corresponding paracancerous normal tissues. Moreover, we found that mRNA expression level of *MYD88* increases as the tumor stage grows, suggesting a potential prognostic role of *MYD88* mRNA expression level for tumor malignancy during the process of CRC development. As Salcedo et al. (2013) reviewed, MYD88 has divergent effects in cancer. In CRC, MYD88 may protect against tumor formation through its involvement in tissue repair.

The expression level of *IDH2* was found to be reduced in CRC (Lv et al., 2012). In contrast, we detected no alteration of *IDH2* mRNA expression level between paired cancerous tissues and paracancerous normal tissues (Supplementary Fig. 2). The exact reason for this discrepancy remains unknown. Because of the relatively limited samples in both studies, further research with higher sample number is necessary to clarify this issue.

Our study has several limitations. First, we only screened the reported hotspot mutations instead of the entire region of the *IDH1*, *IDH2*, *DNMT3A* and *MYD88* genes. There is a possibility that other mutations in these genes may be involved in CRC. Second, we analyzed the mRNA expression of these genes in a limited number of specimens. Further validation of the mRNA and protein levels in CRC was needed. Third, our functional characterization of IDH1 p.I99M may not be optimized to show its effect.

In conclusion, we screened the hotspot mutations in *IDH1*, *IDH2*, *DNMT3A* and *MYD88* in 305 CRC patients and we did not find any mutations except for *IDH1* p.I99M in one patient. Our quantification of mRNA expression of these four genes revealed a decreased mRNA expression level of *IDH1*, *DNMT3A* and *MYD88* in cancerous tissues relative to the corresponding paracancerous normal tissues. Further studies are essential to unravel the molecular underpinnings of these genes in CRC.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2014.05.070.

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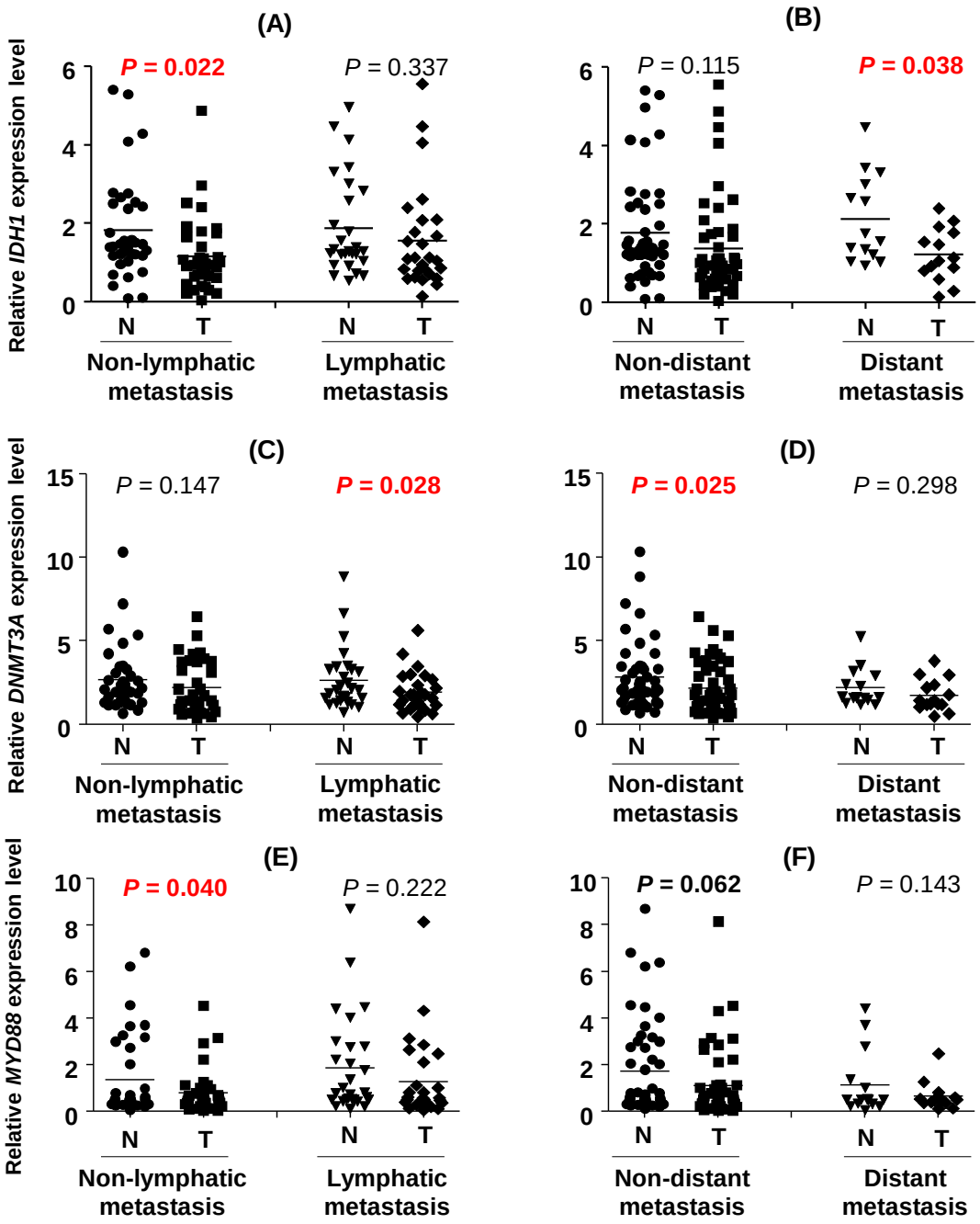
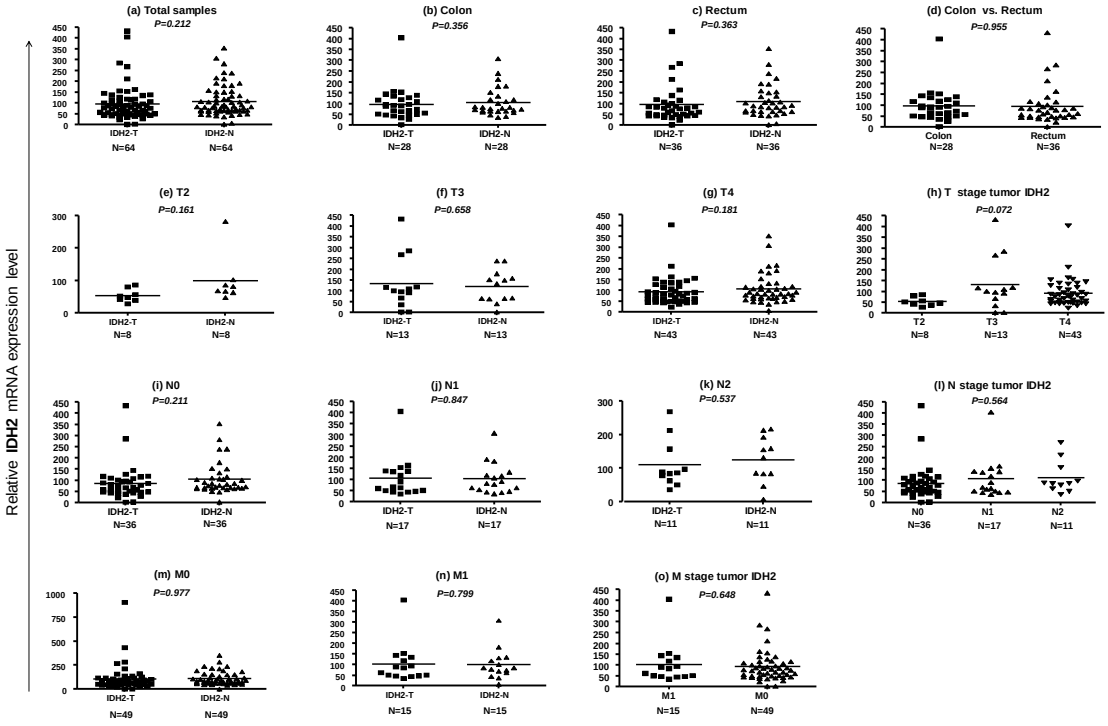


Supplementary Figure 1. Relative mRNA expression levels of *IDH1*, *DNMT3A* and *MYD88* genes in paired cancerous and paracancerous normal tissues with different metastasis status. All the samples were divided into two groups according to the features of lymphatic metastasis or distant metastasis. *IDH1* mRNA levels in non-lymphatic metastatic and distant metastatic cancerous tissues are significantly declined comparing with the related paracancerous normal tissues (A and B). *DNMT3A* mRNA levels in lymphatic metastatic and non-distant metastatic cancerous tissues are significantly declined comparing with the related paracancerous normal tissues (C and D). The decrease of *MYD88* mRNA expression in cancerous tissues was observed only in non-lymphatic metastatic group (E and F).



Supplementary Figure 2. Relative mRNA expression level of *IDH2* gene in different stages. A total of 65 patients were analyzed. One patient was excluded from the analysis because of failure to be detected. There is no significant difference of the mRNA expression change of *IDH2* between cancerous and paracancerous normal tissues either in total samples (a-d) or divided stages (e-g, i-k, m-n). No significant difference of *IDH2* mRNA expression was observed in cancerous tissues among the TNM stages (h, l, o).

Supplementary Table 1. Primers used for PCR amplification, sequencing and mRNA quantification

Gene	Primers	Primer sequences (5'→3')	Note	Reference
Mutation analysis				
IDH1 (R132)	hIDH1f	TGCTGCAGAAGCTATAAAGAAG	PCR; Sequencing	(Balss et al., 2008)
	IDH1r	GCAAAATCACATTATTGCCAAC	PCR	(Zou et al., 2010)
IDH2 (R140/172)	IDH2-4F	AGACTCTACTGCCTTCCTCAT	PCR	this study
	IDH2-4R	TCTGGTCTCAAGCAATCCTGC	PCR; Sequencing	this study
	IDH2rc	TGTGGCCTTGTACTGCAGAG	Sequencing	this study
MYD88 (L265P)	MYD88-3U	GGGCACTTTCTCTGAGGAG	PCR	this study
	MYD88-4195L	CCCCAGGAGACCCAGAGCTATG	PCR	this study
	MYD88-3R	GAGCACAGATTCCTCCTAC	Sequencing	this study
DNMT3A (R882)	hDNMT3Af	AGGAGTTGGTGGGTGTGAGT	PCR	(Li et al., 2012)
	hDNMT3Ar	TGCTCCTATCTGATCAGGCT	PCR	(Li et al., 2012)
Quantification for mRNA expression				
IDH1	IDH1-mRNA-U	TTGGCTGCTTGCATTAAAGGTT	qPCR	this study
	IDH1-mRNA-L	GTTTGGCCTGAGCTAGTTTGA	qPCR	this study
IDH2	IDH2-mRNA-U	GCTGGAGAA GGTGTGCGTG	qPCR	this study
	IDH2-mRNA-L	TGTTCAGGAAGTGCTCGTTCAG	qPCR	this study
MYD88	MYD88-RT-U	TGGTTCTGGACTCGCCTTG	qPCR	this study
	MYD88-RT-L	AGGAGGCAGGGCAGAAGTACAT	qPCR	this study
DNMT3A	DNMT3A-qPCR-F	CAGCTTCCACGTTGCCTTCT	qPCR	(Pattyn et al., 2003)
	DNMT3A-qPCR-R	CAGCTTCCACGTTGCCTTCT	qPCR	(Pattyn et al., 2003)
GAPDH	GAPDH-RT-F	CAACTACATGGTTTACATGTTC	qPCR	(Xiao et al., 2013)
	GAPDH-RT-R	GCCAGTGGACTCCACGAC	qPCR	(Xiao et al., 2013)

Supplementary Table 2. Histopathological characteristics of 65 Han Chinese patients with colorectal cancer selected for mRNA quantification

Sample No.	Patient ID	Gender	Age (Years)	Tumor location	Differentiation	Lymphatic metastasis	Distant metastasis	TNM [a]
1	1	F	69	Rectum	Moderate	No	No	T3N0M0
2	2	M	76	Sigmoid colon	Moderate	No	No	T3N0M0
3	3	M	47	Rectum	Moderate	Yes	Yes	T4N2M1
4	5	M	70	Rectum	Moderate	No	No	T2N0M0
5	6	F	52	Sigmoid colon	Moderate	No	No	T4N0M0
6	8	F	58	Sigmoid colon	Moderate	Yes	Yes	T4N1M1
7	9	M	77	Rectum	Moderate	No	No	T2N0M0
8	10	M	26	Rectum	Poor	Yes	No	T4N2M0
9	11	M	33	Sigmoid colon	Moderate	No	Yes	T4N0M1
10	13	M	64	Rectum	Moderate	No	No	T3N0M0
11	14	M	60	Rectum	Moderate	No	No	T4N0M0
12	16	F	74	Transverse colon	Moderate	No	No	T2N0M0
13	17	M	65	Transverse colon	Poor	No	No	T3N0M0
14	18	F	70	Rectum	Moderate	No	No	T4N0M0
15	19	F	56	Rectum	Moderate	No	No	T2N0M0
16	20	F	65	Right hemicolon	Good	Yes	No	T3N1M0
17	21	F	64	Rectum	Moderate	Yes	No	T2N1M0
18	22	M	72	Sigmoid colon	Moderate	Yes	Yes	T4N1M1
19	23	F	56	Rectum	Moderate	No	No	T4N0M0
20	24	F	53	Sigmoid colon	Moderate	No	No	T4N0M0
21	26	M	80	Right hemicolon	Moderate	Yes	Yes	T4N1M1
22	27	F	90	Sigmoid colon	Good	No	No	T4N0M0
23	28	F	64	Rectum	Moderate	Yes	No	T4N1M0
24	29	M	56	Descending colon	Moderate	No	No	T4N0M0
25	30	F	71	Rectum	Moderate	Yes	No	T4N1M0
26	31	M	61	Rectum	Moderate	No	No	T4N0M0
27	32	F	39	Sigmoid colon	Moderate	No	No	T3N0M0
28	33	M	41	Rectum	Moderate	No	No	T2N0M0
29	35	M	69	Rectum	Moderate	Yes	No	T4N1M0
30	36	M	71	Rectum	Moderate	Yes	Yes	T4N2M1
31	37	F	63	Ascending colon	Moderate	Yes	Yes	T4N1M1
32	38	M	40	Rectum	Moderate	Yes	No	T4N2M0
33	39	F	64	Sigmoid colon	Moderate	Yes	No	T4N2M0
34	40	M	49	Rectum	Moderate	No	No	T4N0M0
35	41	M	66	Cecum	Poor	Yes	No	T4N1M0
36	42	M	70	Rectum	Moderate	No	No	T3N0M0
37	43	F	68	Sigmoid colon	Moderate	Yes	No	T4N2M0
38	44	F	62	Descending colon	Moderate	No	No	T4N0M0
39	45	F	61	Descending colon	Moderate	No	No	T3N0M0

40	46	M	66	Sigmoid colon	Moderate	No	Yes	T4N0M1
41	47	M	57	Rectum	Moderate	No	No	T2N0M0
42	48	M	69	Rectum	Moderate	No	No	T3N0M0
43	49	F	70	Ascending colon	Moderate	Yes	Yes	T4N1M1
44	50	M	64	Sigmoid colon	Moderate	No	Yes	T4N0M1
45	51	M	52	Rectum	Good	No	Yes	T4N0M1
46	52	M	48	Rectum	Moderate	Yes	No	T4N2M0
47	53	F	80	Rectum	Moderate	No	No	T4N0M0
48	54	F	39	Rectum	Moderate	Yes	No	T4N1M0
49	55	F	39	Rectum	Moderate	Yes	No	T4N1M0
50	56	M	46	Ascending colon	Moderate	No	No	T4N0M0
51	57	F	61	Rectum	Moderate	Yes	Yes	T4N1M1
52	58	M	85	Rectum	Moderate	No	No	T4N0M0
53	59	F	29	Rectum	Moderate	Yes	Yes	T4N2M1
54	60	F	83	Rectum	Moderate	Yes	Yes	T4N1M1
55	62	F	70	Rectum	Moderate	No	No	T3N0M0
56	63	F	57	Rectum	Moderate	Yes	No	T4N2M0
57	64	F	68	Right hemicolon	Poor	No	No	T3N0M0
58	65	M	59	Sigmoid colon	Moderate	Yes	Yes	T4N1M1
59	66	F	58	Sigmoid colon	Moderate	Yes	No	T4N1M0
60	67	M	67	Sigmoid colon	Moderate	No	No	T3N0M0
61	68	F	72	Rectum	Moderate	Yes	No	T4N2M0
62	69	F	54	Rectum	Moderate	No	No	T4N0M0
63	70	F	57	Rectum	Moderate	No	No	T2N0M0
64	71	M	69	Rectum	Moderate	Yes	No	T3N2M0
65	72	F	66	Left hemicolon	Moderate	No	No	T3N0M0

[a] The stage of cancer was classified as the international standard following the 7th edition of AJCC Cancer Staging Handbook.

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