Multi-stage analysis of FOXM1, PYROXD1, hTERT, PPARA, PIM3, BMI1 and MCTP1 expression patterns in colorectal cancer

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ABSTRACT

Aim: To explore biomarkers with a tumor stage-dependent expression pattern in patients with colorectal cancer (CRC).

Background: The fourth most common cancer in the world is colorectal cancer (CRC). A variation in the gene expression rate is a common change in cancers initiation and the accumulation of these variation changes the behavior of normal cells and turns them into cancer cells.

Methods: Real-time RT-PCR was used to investigate the expression patterns of the FOXM1, PYROXD1, hTERT, BMI, PPARA, PIM3 and MCTP1 genes in 54 patients with stage I to IV CRC and their relation with clinicopathological features of CRC were analyzed. Results: FOXM1, hTERT and MCTP1 genes are overexpressed in CRC tumor tissues when compared to normal adjacent tissues in all the stages.

Results FOXM1, PYROXD1, hTERT, PIM3, BMI, PPARA and MCTP1 had-stage dependent expression. Investigation of the association between clinicopathological features and expression pattern of the studied genes revealed: a) a significant relationship between FOXM1 gene expression level and tumor stage, tumor size and lymph node involvement, b) a considerable association between alterations in PPARA and PIM3 expression and lymph node involvement, c) a notable correlation between hTERT expression level and the tumor stage and d) a strong correlation between MCTP1 expression and patient's age only.

Conclusion: Our study indicates that expression profiles of these genes either individually or together can be applied as potential biomarkers for prognosis of CRC.

Keywords: Colorectal cancer, Expression pattern, Real-time RT-PCR.

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Introduction

The fourth most common cancer in the world is colorectal cancer (CRC) and the worldwide burden of this disease is estimated to rise even further by 2030 (1). The prevalence and percentage mortality of CRC have been found to fluctuate by up to 10-fold globally (2). In Iran the prevalence of this cancer is on the rise and surprisingly, its occurrence in men under 50 years old is remarkable (3, 4). The most important diagnostic factor for this cancer is its stage at the time of diagnosis (5). The traditional method for cancer staging is the tumor-node-metastasis (TNM) classification system (5, 6). Although this classification system provides useful information to physicians, it cannot discriminate between the biological behaviors of different tumors. Many studies consider CRC progress as a stepwise
procedure with the accumulation of various genetic alterations (7, 8). Cancer tissue gene expression profiling is anticipated to bring new insights into the underlying causes and understanding of cancer biology as well as improving new methods of prognosis, diagnosis, prediction and therapy. Furthermore, a variation in the gene expression rate is a common change in cancers initiation and the accumulation of these variation changes the behavior of normal cells and turns them into cancer cells. Cancer initiation and growth is tightly controlled by the interaction between genetic and epigenetic factors which leads to differential gene expression. Normal cell growth is blocked with different ways which are critically coordinated alterations in gene expression during carcinogenesis (9, 10).

In the recent research at first CRC patients with different tumor stages were included to study. Then we went through the expression data in literature to elucidate new reported biomarkers that had driven the development of CRC. Previously, a panel of genes by Agenda which is a classifier of robust gene expression (ColoPrint) was identified to significantly improve the prognostic accuracy of pathologic factors in CRC patients with stage II and III.(11). Therefore, to investigate the relationship between the expression of certain genes and different clinicopathological factors, we selected: a) 4 genes from this panel randomly b) 3 other genes which were not in this panel but were cited a lot in literature. Consequently, we inspected the expression of this panel of genes in Iranian CRC patients, hoping to introduce possible diagnostic or prognostic biomarkers. According to the above description, seven genes (as mentioned in Table 1) were selected.

### Methods

#### Tumor Samples

This project was permitted by the National Institute for Genetic Engineering and Biotechnology (NIGEB). Colorectal cancer patients were accepted to Rasool e Akram Hospital (a referral governmental hospital) in Tehran (between the years 2010 to 2017). Written consent forms were taken from every case. Fifty four tumor and 48 adjacent normal tissues were prospectively obtained during surgery. The tissue specimens were then stored at -70°C prior to RNA extraction. All patient pathologic information was obtained from the Department of pathology. Colorectal cancer (CRC) tissue staging was carried out in accordance with the TNM classification system (12).

#### RNA purification and cDNA synthesis

The TriPure Isolation Reagent and RevertAid First Strand cDNA Synthesis Kits were used for RNA purification (Roche Applied Sciences, Germany) and cDNA synthesis (Thermo Fisher Scientific, Germany), respectively.

#### Real-time RT-PCR

Real-Time RT-PCR using the SYBR-Green master mix was carried out by Bosch's real-time PCR thermal cycler (Roche Applied Sciences, Germany). The amplification process was carried out in a 10 μL reaction volume using 0.1 μM vials, containing 0.5 μM of each primer, 1 μL of cDNA (as template), 5 μL of SYBR-Green master mix, 3 μL of water. The

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| Name/Gene ID | Accession number | Location | Description | Biological activity |
|--------------|------------------|----------|-------------|---------------------|
| FOXM1/2305   | NM_202002.2      | 12p13    | Forkhead box protein M1 | Transcription regulation |
| PPARA/5465   | NM_001001928.2   | 22q13.31 | Peroxisome proliferator-activated receptor alpha | Transcription regulation |
| PIM3/415116  | NM_001001852.3   | 22q13    | Serine/threonine-protein kinase pim-3 | serine/threonine kinase activity |
| MCTP1/79772  | NM_001297777.1   | 5q15     | Multiple C2 domains, transmembrane 1 | calcium-mediated signaling |
| PYROXD1/79912 | NM_024854.3      | 12p12.1  | pyridine nucleotide-disulphide oxidoreductase domain 1 | oxidoreductase |
| hTERT/7015   | NM_198253.2      | 5p15.33  | Telomerase reverse transcriptase | Ribonucleoprotein |
| BMI1/648     | NM_005180.8      | 10p12.2  | BMI1 proto-oncogene, polycomb ring finger | Transcription, Transcription regulation |
Multi-stage analysis of gene expression patterns in colorectal cancer

Table 2. Primer sequence details for PCR and Real RT PCR

| Gene name | Primer sequence (5’---3’) | Amplicon size (bp) |
|-----------|---------------------------|-------------------|
| FOXM1     | For: AGTGTGTACGTGGTCGAG   | 123               |
|           | Rev: GGGGATGAACGGGAGTCT   |                   |
| PPARA     | GCAGGAGGGGCGCAAAGGGT      | 164               |
|           | TGGGTCGAGTGATGGCATGG      |                   |
| MCTP1     | TAGACTTTATCTCAAGTAAAAAGC  | 172               |
|           | TAGAAGCTTTTATGCAAACATTTCA |                   |
| PIM3      | AAGAAGCTTCAACCTTGTGGGG    | 170               |
|           | GGTCATACCTTGTCGGCCCTG     |                   |
| PYROXD1   | TAGACAGAGGGGTGGTATGC      | 132               |
| hTERT     | ATCTTTCTCGTGATGCTGCCA     | 228               |
|           | TCATTGTCGTGGGACGATGT      |                   |
| BMI1      | ATCTTTCTCGTGATGCTGCCA     | 200               |
|           | TCGATGCCGATGATGGCATGG     |                   |
| GAPDH     | GCAGGAGGGGCGCAAAGGGT      | 219               |
|           | TGGGTCGAGTGATGGCATGG      |                   |

The thermal cycle program was as follows: 95°C for 5 min for the initial denaturation step, and an amplification program (95°C for 20, 60°C for 15 and 72°C for 20 seconds, respectively) repeated for 40 cycles. Primers were designed with the oligo7 software (Table 2). The specificity of the primers was theoretically tested by the BLAST database. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was selected as the housekeeping gene.

Statistical data analysis

The real-time RT-PCR raw data for each gene was evaluated with the Linreg software. Subsequently, the expression ratio results (sample group difference relative to the control group) for significance and statistical analysis were analyzed with the REST software and SPSS software V22.0 (SPSS, Inc., Chicago, IL). The normality assumption was checked by the Kolmogorov-Smirnov test, and variances between groups were analyzed by the one-way analysis of variance (ANOVA) and independent sample T tests.

Results

Patient pathologic analysis

In total, 54 CRC tissues and 48 adjacent normal tissues registered at the Rasool-e-Akrum Hospital were analyzed in this study (pathological information of 3 patients were absent). The median age of patients at the time of diagnosis was 50.5 years (ranging from 22-79 years). Cases were composed of 26 females and 25 males. The TNM staging information of patients was as follows: 8, 14, 18, and 8 patients were at stage I-IV. Among these samples, 26 were localized to the colon and 19 to the rectum, while for 5 cases the information was not available. Twenty five (%48) patients were lymph node positive (82% N1 & 18% N2) and 29 (%52) were lymph node negative.

H&E staining

For pathological purposes, frozen tissues were stained with hematoxylin and eosin (H&E). This was followed by evaluation of tumor content and verification of their histology (Figure 1).

Figure 1. A colorectal tumor cells and B normal colorectal cells characteristics. Hematoxylin –Eosin (H&E) staining verified by pathologist. Magnification (×400)
Expression profiles of FOXM1, hTERT, PPARA, PIM3, PYROXD1, BMI1 and MCTP1 in CRC patients from early stage I to advance stage IV.

FOXM1

Relative expression analysis showed that the FOXM1 gene expression rate was considerably different between tumor and normal tissues in a way that FOXM1 was upregulated in tumor tissues $P \ (H) = 0.03$ (Figure 2). Investigation of the association between clinicopathological features and the FOXM1 expression level demonstrated a significant association between the grade (stage), tumor size (T), lymph node involvement (N) and the FOXM1 expression level in our patients ($P \leq 0.05$), ($P \leq 0.045$) ($P \leq 0.05$). No association was detected between the FOXM1 expression pattern and age, tumor position, tumor stage, gender and differentiation. FOXM1 expression analysis at different stages (S1–S4) revealed that FOXM1 expression increased significantly at early stage I ($P \leq 0.007$), and no significant expression was observed at the stages S2, S3, and S4 (Figure 2).

![Figure 2](image)

**Figure 2.** A: Relative expression analysis of FOXM1 ($P \leq 0.05$). B: Stage-dependent expression of FOXM1 in CRC patients (S1–S4 stands for cancer stages from stage I to progressive stage IV).

hTERT

As expected, hTERT gene expression was not detectable in normal tissues while a significant overexpression of hTERT was detected in CRC patients. Expression of hTERT was considerably related to the grade of the tumor ($P \leq 0.03$), and its expression level was significantly amplified in all the grades. No other association was detected between hTERT RNA pattern and other clinical characteristics, such as lymph node involvement, age and tumor size.

PPARA

The PPARA expression pattern between tumor and normal adjacent tissues was not considerably different $P \ (H) = 0.7$. Interestingly, there was a considerable association between PPARA expression level and lymph node involvement in CRC patients ($P \leq 0.038$). The analysis of PPARA expression at different stages (S1–S4) indicated that PPARA expression decreased significantly in cancer versus control samples in S2, S3 and S4. Furthermore PPARA expression at Stage I was not considerably different (Figure 3).

![Figure 3](image)

**Figure 3.** Expression profile of PPARA at different stages (S1–S4).

PIM3

The rate of PIM3 expression did not increase in tumor tissues in contrast to relative to the adjacent normal tissues $P \ (H) = 0.335$. Moreover, a significant association was observed between PIM3 expression alteration in tumor tissues and lymph node involvement ($P \leq 0.044$). The analysis of PIM3 expression at different stages (S1–S4) showed that PIM3 expression increased significantly at early stage I ($P \leq 0.001$) and no
significant expression was found in the S2, S3, and S4 stages (Figure 4).

Figure 4. Expression profiles of PIM3 at different stages (S1-S4).

PYROXDI
Relative expression result illustrated that PYROXDI is up-regulated in sample group (in comparison to control group) by a mean factor of 6.780 ($P\leq0.006$). However, no significant association was observed between PYROXDI over expression and clinicopathological features. Additionally, PYROXDI expression level was significantly increased in all the tumor stages S1-S4 (Figure 5).

Figure 5. A: Relative expression analysis of PYROXDI ($P \leq 0.05$). A P value less than 0.05 were considered statistically significant.

BMI1
The expression rate of BMI1 did not increase in tumor samples when compared to normal samples ($p$-value>0.708). No other association was detected between BMI1 and CRC clinicopathological features. Furthermore, BMI1 upregulation was identified in both early stage and control samples (Figure 6).

Figure 6. Upregulation of BMI1 in early stages of CRC tumor tissues.

MCTP1
The expression rate of MCTP1 did not increase in tumor samples when compared to normal samples ($p$-value>0.708). No other association was detected between BMI1 and CRC clinicopathological features. Furthermore, BMI1 upregulation was identified in both early stage and control samples (Figure 6).

Figure 7. A. Relative expression of MCTP1 ($P \leq 0.001$). B. MCTP1 expression at different stages (S1-S4) of CRC tumor tissue.
The real-time RT-PCR data were evaluated to deduce the RNA pattern of the MCTP1 gene. There was considerable variation between tumor groups when compared to the control group and MCTP1 was upregulated in tumor groups by a mean factor of 2.844 (P ≤ .0.010). Also, variation in expression level of the MCTP1 gene in tumor tissues was strongly correlated with patient’s age (P ≤ 0.018).

Furthermore, MCTP1 upregulation was identified amongst advanced-stage and control samples (Figure 7). All the above results are summarized in Table 3 (Table 3).

**Discussion**

The fourth most common cancer in the world is CRC, and in Iran, it is much greater than the global average, with a prevalence of 160 out of every 100,000 people (13, 14). The most important diagnostic factor for this cancer is cancer stage at the time of diagnosis. The traditional method for cancer staging is based on the tumor-node-metastasis (TNM) system. Although this method provides effective clinical information of tumor's stage or grade, but, unfortunately, it is unable to give a precise biological classification, and more importantly cannot discriminate between the biological behavior of various tumors (15). Accordingly, it is essential to find an accurate and reliable method that can improve individual treatment. Numerous studies have compared CRC gene expression pattern in normal and cancer tissues at different stages of the disease (16, 17). Herein, we investigated the expression pattern of the selected genes in CRC patients to identify biomarkers which discriminate among colorectal cancers with altered stages. The purpose of this

| Clinico-pathological features | N (%) | FOXM1 | PPARA | PYROXD1 | hTERT | BMI1 | PIM3 | MCTP1 |
|------------------------------|-------|------|------|---------|-------|------|------|-------|
| Relative expression          |       | 0.03** | 0.7 | 0.006* | 0.05** | 0.708 | 0.335 | 0.096 |
| Age ≤50                      | 19 (36)| 0.028**| 0.746| 0.565   | 0.45   | 0.71  | 0.958 | 0.018**|
| Age >50                      | 34 (64)|       |      |         |        |      |      |       |
| Gender                       |       | 0.209 | 0.770| 0.318   | 0.323  | 0.09  | 0.659 | 0.285 |
| Female                       | 26(49) |       |      |         |        |      |      |       |
| Male                         | 27(51) |       |      |         |        |      |      |       |
| TNM stage                    |       | 0.636 | 0.747| 0.559   | 0.03** | 0.34  | 0.081 | 0.567 |
| I                            | 8 (18) |       |      |         |        |      |      |       |
| II                           | 11(23) |       |      |         |        |      |      |       |
| III                          | 20(42) |       |      |         |        |      |      |       |
| IV                           | 9 (18) |       |      |         |        |      |      |       |
| Tumor Size                   |       | 0.045**| 0.984| 0.075   | 0.88   | 0.3   | 0.297 | 0.567 |
| 5 cm<                        | 19(47) |       |      |         |        |      |      |       |
| 5-<8                         | 18(44) |       |      |         |        |      |      |       |
| 8-10                         | 4 (9)  |       |      |         |        |      |      |       |
| Lymph node Involvement       |       | 0.05** | 0.038**| 0.668 | 0.34   | 0.099 | 0.04**| 0.129 |
| N0                           | 20(42) |       |      |         |        |      |      |       |
| N1                           | 21(44) |       |      |         |        |      |      |       |
| N2                           | 7(14)  |       |      |         |        |      |      |       |
| Localization                 |       | 0.165 | 0.316| 0.194   | 0.3    | 0.66  | 0.444 | 0.865 |
| Colon                        | 24(54) |       |      |         |        |      |      |       |
| Rectum                       | 21(46) |       |      |         |        |      |      |       |

**MCTP1**

The real-time RT-PCR data were evaluated to deduce the RNA pattern of the MCTP1 gene. There was considerable variation between tumor groups when compared to the control group and MCTP1 was upregulated in tumor groups by a mean factor of 2.844 (P ≤ 0.010). Also, variation in expression level of the MCTP1 gene in tumor tissues was strongly correlated with patient’s age (P ≤ 0.018). Furthermore, MCTP1 upregulation was identified amongst advanced-stage and control samples (Figure 7). All the above results are summarized in Table 3 (Table 3).
research was to identify biomarkers with a tumor stage-dependent expression pattern so as to develop cancer staging procedures and explore the regulatory mechanisms of CRC (18).

The Forkhead box protein M1 transcription factor (FOXM1) has been shown to have a crucial function in cell cycle progress, and in the S and G2/M phases it has exhibited extreme expression (19, 20). Recently, a growing number of studies have described FOXM1 as a key oncogenic transcription factor as it can promote tumor progression (21). Emerging data has shown that FOXM1 regulates gene expression essential to proliferation, apoptosis, and cell-cycle progression, thereby signifying its overall function in tumor growth (22). Several researches have confirmed that FOXM1 is overexpressed in different cancers and this elevated expression has a vital role in cancer development (23, 24). Furthermore, it was previously been reported that FOXM1 overexpression is related to the presence of the progressive TNM stage and metastasis lymph node, suggesting that FOXM1 is possibly involved in cancer metastasis and invasion (24-27). In our research, FOXM1 overexpression was also significantly detected in tumor specimens (P ≤ 0.03). In the current study, it was revealed that the FOXM1 expression pattern was clearly related to grade, lymph node involvement and tumor size in CRC (P ≤0.05, P ≤ 0.045, P ≤0.05, respectively). Analysis of FOXM1 expression at different stages (S1-S4) revealed that its expression increased significantly at early stage I (P≤0.007), with no significant expression being observed at the S2, S3, and S4 stages. One possible interpretation is that since FOXM1 is a transcription factor, its high level of expression in the initial phases of tumor development can alter cell proliferation and cell-cycle progression, thereby aiding tumor formation.

The Nuclear receptor subfamily 1 group C member 1 protein (NR1C1) also well-known as the peroxisome proliferator-activated receptor alpha (PPARα) is a nuclear protein (28), which belongs to the subfamily of peroxisome proliferator-activated receptors. The fatty acid products and their derivatives are mediated by these receptors at the transcriptional level. Regarding the regulatory role of PPARs in lipid metabolism, these receptors control cell survival, proliferation and differentiation through these pathways, thus monitoring tumorigenesis in different tissues (29, 30). A large number of studies have shown that PPARα targets more than a hundred genes (18, 31, 32). To date, PPARα pattern in colorectal malignancy has not been studied. Accordingly, the RNA pattern of PPARα in CRC, and the association of PPARα expression and the patients’ clinicopathological features was investigated in CRC. Our result showed that the RNA pattern of PPARα was not considerably altered in tumor and normal parallel tissues (P ≤0.05). Interestingly, there was a considerable association between the PPARα expression level and lymph node involvement in CRC patients (P ≤0.038). These data suggest that alteration in PPARα expression level may be involved in colorectal tumor invasion. The analysis of PPARα 'expression at different stages (S1-S4) indicated that its expression decreased significantly in cancer versus control samples at the S2, S3 and S4 stages. Furthermore, PPARα expression at Stage I was not considerably different. In this study, in contrast to the robust gene expression classifier (ColoPrint) (33), the PPARα expression level was not only considerably different between tumor and normal adjacent tissues, but its expression level was also found to decrease in certain stages of CRC.

Human telomerase reverse transcriptase (encoded by the hTERT gene) is crucial for the replication of chromosome ends (34). Furthermore, hTERT has various molecular functions and is involved in numerous essential biological processes (http://www.uniprot.org/uniprot/O14746). It has been found that hTERT expression increases in various human cancers (35). As expected, hTERT gene expression was not detectable in normal tissues while a significant overexpression of hTERT was detected in CRC patients. The hTERT RNA level was considerably related to cancer grade (P ≤0.03). Its level was noticeably amplified in all the grades. There was not any association between hTERT pattern and clinical features. One possible interpretation for this finding is that in the primary stages involving precancerous lesions, most tumors
go through constant telomere shortening, thereby activating the telomerase enzyme which leads to tumor progression.

Pyridine nucleotide disulphide oxidoreductase domain 1 (PYROXD1) gene belongs to flavoprotein family and catalyze the pyridine-nucleotide-dependent reduction of thiol residues in proteins. (36). One of the cause of chronic inflammation is oxidative stress and the activation of chronic inflammation pathways mediates most chronic diseases and cancers(37, 38). The role of PYROXD1 in cancer biology and other disease has not yet understood. For the first time we studied PYROXD1 expression level in colorectal cancer and we showed that PYROXD1 is up regulated in this cancer. However, no significant association was observed between PYROXD1 over expression and clinicopathological features. Additionally, PYROXD1 expression level was significantly increased in all the tumor stages S1-S4. The consistent expression of PYROXD1 across all the cancer stages may indicate that PYROXD1 gene contribute in many major biological pathways involved in cancer formation and progression.

The PIM3 gene codes for the provirus integrating site moloney murine leukemia virus (Pim) family of proteins which have serine /threonine kinase activity (39, 40). Literature review introduces PIM3 as a proto-oncogene which can prevent apoptosis and help tumorigenesis by delivering survival signaling and inducing the release of anti-apoptotic proteins (41-43). In this study, PIM3 was not overexpressed in cancerous tissues of CRC patients. Moreover, a significant association was observed between alteration in PIM3 expression in tumor tissues and lymph node involvement. The analysis of PIM3 expression at different stages (S1-S4) showed that PIM3 expression increased significantly in early stage I (P≤0.001) and no significant expression was found at stages S2, S3, and S4. In this study, although PIM3 expression was generally not considerably different between tumor and normal adjacent tissues, but it was found that PIM3 was significantly expressed at early stage I. Regarding the aberrant expression of PIM3 and its function as a proto-oncogene in various cancers, it seems that in CRC, PIM3 has stage-dependent expression and in stage I of CRC, PIM3 acts as a proto-oncogene, helping tumor formation. However, PIM3 expression was found to be not significant in the other stages of cancer.

The BMI1 gene encodes a ring finger protein that belongs to the Polycomb group (PcG) (44), which has a critical function in maintaining proliferation, cell differentiation, regulating cellular memories and stem cell self-renewal (45, 46). The existing literature suggests a significant BMI1 function in malignancy and its upregulation in different cancers (47, 48). In this study, although BMI1 was not upregulated in tumor tissues when compared to adjacent normal tissues, however, its upregulation was observed at stages I and II of CRC relative to the control samples. These results suggest that BMI1 also has stage-dependent expression, and thus, these data support the biological role of BMI1 in CRC, especially at stages I and II.

The multiple C2 domain and transmembrane protein 1 (encoded by the MCTP1 gene) is composed of multiple C2 domains and binds to calcium in the absence of phospholipids via the C2 domains (49). It is involved in calcium signaling, with calcium acting as a secondary messenger. Calcium signaling has important roles in a wide range of physiological processes including cell growth and proliferation, enzyme activity, permeability of ion channels and other processes (50, 51). Real-time PCR revealed a considerable alteration in sample cases relative to the control cases. Variation in the expression level of the MCTP1 gene in tumor tissues was strongly correlated with patient’s age. Furthermore, MCTP1 upregulation was identified in advanced-stage tissues when compared to the control samples.

While only the FOXM1, PYROXD1, hTERT and MCTP1 genes were overexpressed in CRC tumor tissues relative to the normal adjacent tissues at all the stages, but FOXM1, PYROXD1, hTERT, PIM3, BMI1, PPARA and MCTP1 were found to have stage-dependent expression. The FOXM1, hTERT, MCTP1, BMI1 genes were involved in cell growth, PIM3 was involved in cell death, and PYROXD1 was involved in oxidative stress. We hope such
efforts of using molecular staging signatures may develop cancer staging procedures by introducing potential biomarkers and significantly benefit the development of personalized medicine in CRC.

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Conflict of interests

The authors declare that they have no conflict of interest.

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Multi-stage analysis of gene expression patterns in colorectal cancer

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