

Deregulated expression of HDAC3 in colorectal cancer and its clinical significance

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Conflict of interest

None declared

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Abstract

Background. To date, 4 classes of histone deacetylases (HDACs) have been identified in humans. Class I HDACs are zinc-dependent and NAD⁺-independent enzymes, and include 4 isoforms closely related to yeast RPD3: HDAC1, 2, 3, and 8.

Objectives. The aims of the study were to quantitatively evaluate the expression of HDAC3 in colorectal cancer (CRC) and to correlate its expression levels with clinicopathological parameters.

Material and methods. We characterized expression patterns of HDAC3 as class I HDAC isoforms in a cohort of 48 CRC patients by quantitative (real-time) reverse transcription polymerase chain reaction (RT-PCR). In addition, the potential relationship between HDAC3 expression levels and clinicopathological parameters in patients suffering from CRC was explored.

Results. We found that HDAC3 was highly expressed in colorectal tumors compared to normal colorectal tissues ($p < 0.05$). Furthermore, we found significant correlations between HDAC3 expression levels and tumor differentiation grades ($p < 0.05$).

Conclusions. In this prospective study we identified a pronounced HDAC3 expression pattern in CRC. Our findings support an important role of HDAC3 as a complementary molecular marker for existing histopathological diagnostic elements; it might also have applications in prognostic and targeted therapy. Furthermore, HDAC3 can be used as a biomarker to differentiate between tumor borders and margins, and it may also be useful for characterizing field cancerization in CRC.

Key words: HDAC3, deregulation, prognosis, colorectal cancer

Introduction

Every year in the world over 1.36 million new cases of colorectal cancer (CRC) are diagnosed and more than 600,000 patients die of the disease, making it the 3rd most common cancer and the 4th most common cause of cancer death in both men and women.^{1,2} The incidence of CRC varies considerably from country to country, but the rates are the highest in economically developed countries.³ Despite low rates of CRC incidence in south Asia, recent studies in Iran have indicated a significant increase in the rate of the disease in the past 3 decades.⁴ Approximately 3,641 new cases of CRC per year are diagnosed in Iran, out of which 2,262 die annually, accounting for roughly 6.3% of all cancer deaths in the country.⁵ The increased prevalence of obesity and decreasing physical activity in many parts of Iran, resulting from westernization, will probably continue to contribute to the growing CRC incidence and mortality, and make it a major public health burden.⁶ The progression of CRC is a multistep process that often develops over more than 10 years, which means there are opportunities for early diagnosis and even prevention.⁷ It begins as small adenomatous polyps and develops into an advanced large adenoma with high-grade dysplasia, and then progresses to invasive and metastatic carcinoma.⁸ The adenoma-to-carcinoma sequence requires multiple cumulative genetic changes and was first described by Fearon and Vogelstein.⁹

Genetic and genomic lesions, such as chromosomal translocations, point mutations, deletions, insertions, and amplification, have long been considered major causes of cancer. The activation of oncogenes and the inactivation of tumor-suppressor genes are end points of these changes. However, cancer formation and progression is not limited to these changes. Along with gene mutations, epigenetic alterations such as aberrant DNA methylation and aberrant posttranslational histone modifications, including acetylation, methylation, phosphorylation, etc., may also play a pivotal role in tumor initiation and progression. These changes have as their end point deregulated expression of oncogenes and/or tumor suppressor genes.^{10,11}

Up until now, the most widely studied epigenetic modification in human cancers has been cytosine methylation of DNA within the dinucleotide CpG, particularly the inactivation of tumor-suppressor genes by promoter hypermethylation.¹² Apart from cytosine methylations, there has been an increase in our knowledge about the involvement of aberrant patterns of posttranscriptional histone modifications in cancer development, including CRC. In CRC, acetylations and methylations of histone and their reversions are the best studied phenomena. In particular, acetylation of lysine residues of histone 3 and histone 4 has become one of the best studied modifications of this type.¹³ Acetylation of core histones by histone acetyltransferases (HATs) results in chromatin opening and the activation of gene transcription; in contrast, histone deacetylases (HDACs) remove the acetyl group from histones, allowing

compacted chromatin to reform, with transcriptional gene inactivation as the outcome.¹⁴ Dynamic levels of reversible acetylation are the result of the balance of the opposing activities of HATs and HDACs, which plays an important regulatory role in the transcription of many genes.¹⁵ Based on this balance, both positive and negative effects of HDACs on oncogenesis and inhibition of oncogenes can be expected. Disturbances in this balance might have dramatic outcomes on the cell phenotype. Studies on the pathogenesis of leukemias have provided the most informative evidence on how this balance is shifted in cancer cells.¹⁶ Indeed, acute promyelocytic leukemia was the first malignancy in which the involvement of HDACs was shown.¹⁷ HDACs are known to play a regulatory role in a wide variety of physiological cellular processes, including cell differentiation, cell cycle progression, DNA replication, transcription, gene silencing, and the response to genotoxic stress; however, these regulatory enzymes are also increasingly being found to be involved in cancer.¹⁸

To date, 4 classes of HDACs have been identified in humans. Class I HDACs are zinc-dependent and NAD⁺-independent enzymes, and include 4 isoforms – HDAC1, 2, 3, and 8 – that are closely related to yeast RPD3.^{19–21} Our study was designed to identify the *HDAC3* gene expression pattern in CRC. Furthermore, we aimed to investigate the potential applications of *HDAC3* expression analysis in accurately determining tumor margins during surgery and possible field cancerization. A tertiary objective in this study was to correlate *HDAC3* expression levels and clinicopathological variables in patients suffering from CRC. Finally, we used the receiver operating characteristic (ROC) curves and the areas under the ROC curves (AUC) to evaluate the feasibility of using *HDAC3* as a diagnostic biomarker for the detection of CRC.

Material and methods

Subjects

A cohort of 48 patients (22 males and 25 females) with colorectal tumors, all of which had matched tumor-adjacent normal (TAN) samples, was selected for gene expression analysis using quantitative (real-time) reverse transcription polymerase chain reaction (qRT-PCR). Tissue samples were gathered from consenting patients at the time of diagnostic procedures or during primary curative surgical resections at Imam Reza Hospital of Tabriz University of Medical Sciences (TUMS), Iran. Clinicopathological data were collected on all the patients in order to investigate correlations with HDAC3 expression levels. All the specimens were subjected to immediate snap-freezing in liquid nitrogen and archived at –80°C until the histopathological examination and review. Surgical pathologic staging was determined according to the TNM staging system of the American Joint Committee on Cancer and

World Health Organization (WHO) classifications. Ethical approval for this study was granted by the Ethics Committee of TUMS. The molecular studies for this work were done in the Tabriz Genetic Analysis Center of TUMS.

RNA extraction and qRT-PCR

For the HDAC expression analysis, 48 paired snap-frozen CRC and TAN samples were incubated overnight at 4°C in an RNA stabilization reagent (RNAlater, Qiagen, Hilden, Germany) and were subjected to total RNA extraction using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. After assessing the RNA concentration by spectrophotometer, the RNA was incubated with DNase to remove contaminating genomic DNA. Briefly, 3 µL of RNA was treated with 1 µL of DNase I, 1 µL of buffer and 5 µL of water, and incubated at 37°C for 30 min. In order to stop DNase, 1 µL of EDTA was added and incubated at 65°C for 10 min. Then, 2 µg total RNA was reverse transcribed to first strand cDNA using a random hexamer primer and reverse transcriptase. The reactions were incubated at 37°C for 10 min, followed by 50°C for 1 h and final denaturation at 70°C for 15 min. Negative control samples were included in each set of reactions. Then, cDNAs were diluted 5-fold and 2 µL was used in each PCR. The primers for *HDAC3* and glyceraldehyde phosphate dehydrogenase (*GAPDH*) were purchased from the Takapouzist Company (Tehran, Iran). The cDNA was amplified using qRT-PCR (the Rotor-Gene 3000, Corbett Life Science, Mortlake, Australia) with SYBR Green. For each gene, qRT-PCR was performed in triplicate in 25 µL of reaction volume consisting of 6 µL SYBR Green master mix (TaKaRa Bio Inc. Kusatsu, Japan), 0.5 µL of each gene-specific forward and reverse primer, 2 µL of cDNA from each sample, and RNase free water to bring the reaction mixture up to the final volume. Thermal

cycling parameters of 40 cycles were carried out as follows: 95°C for 5 min for 1 cycle, 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and 72°C for 5 min as the final extension. The PCR primer sequences of *HDAC3* and *GAPDH*, used as internal controls, are shown in Table 1.

Relative quantification

The relative expression of HDAC3 in tumor and TAN tissues was calculated using the comparative cycle thresholds (C_t) that were determined with the amplification plots within the logarithmic phase for each sample. C_t is defined as the number of PCR cycles at which the fluorescence signal is detected from the amplification of the target gene within a sample that increases to a threshold value of 10 times the standard deviation of the background emission. The starting amount of the target cDNA is inversely proportionate to C_t . ΔC_t values were calculated by subtracting *GAPDH* C_t from the test gene C_t . Relative mRNA levels were determined by subtracting normal control ΔC_t values from CRC ΔC_t values to give a $\Delta\Delta C_t$ value and conversion through $2^{-\Delta\Delta C_t}$.

Data analysis

The Spearman’s rank correlation coefficient was used for nonparametric data that were not normally distributed in our study. In addition, the Kruskal and Mann-Whitney tests were used for statistical analysis in each of the diagnostic groups. P-values <0.05 were considered statistically significant. The ROC curve and the AUC were used to assess the feasibility of using *HDAC3* as a diagnostic biomarker for the detection of CRC.

Results

HDAC expression levels in colorectal cancer

We used the qRT-PCR method to analyze the expression of the *HDAC3* gene in CRC. We used *GAPDH* for normalization of the gene expression data. In this cohort study, the expression of *HDAC3* was significantly higher in CRC compared to TAN tissues ($p < 0.03$) (Fig. 1).

Table 1. Primers used for PCR

Gene	Primer
<i>HDAC3</i>	F: TAGGGATGAGATACAGACAAGG
	R: GAAGCAGGGAAGAAATAAGG
<i>GAPDH</i>	F: CATGGCCTCCAAGGAGTAAG
	R: GCTTGAGCACAGGGTACTTTA

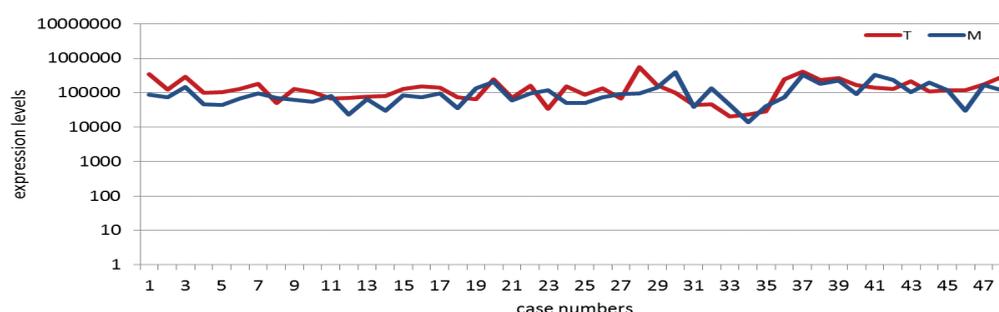


Fig. 1. HDAC3 is overexpressed in colorectal cancer (CRC). Analyses of the HDAC3 expression levels were performed in CRC tissues (n = 48) and tumor-adjacent normal (TAN) tissues. The HDAC3 expression level was significantly higher in CRC samples than in TAN tissues

T – tumor; M – marginal samples.

Correlation of HDAC3 expression with clinicopathological parameters

We further evaluated the relationship between the expression levels of HDAC3 in the patients' CRC tissues and clinicopathological characteristics of the patients using non-parametric tests. Regarding the clinicopathological variables, the patients were grouped according to their overall HDAC3 expression pattern, and we found that the HDAC3 expression significantly increased with tumor differentiation grade. High HDAC3 expression levels were associated with poor tumor differentiation, indicated by a high (G3) tumor grade ($p < 0.04$) (Table 2).

ROC curve assay for HDAC3 capability as a CRC marker

To shed light on the sensitivity and specificity of the HDAC3 expression levels as a tumor marker in CRC, an ROC curve was constructed and the area under the curve was calculated. The value of the AUC was 0.72 out of 1 for the HDAC3 assay (Fig. 2). This indicated that HDAC3 may be used as a potential diagnostic biomarker for CRC, and probably as a predictive and prognostic biomarker for CRC as well.

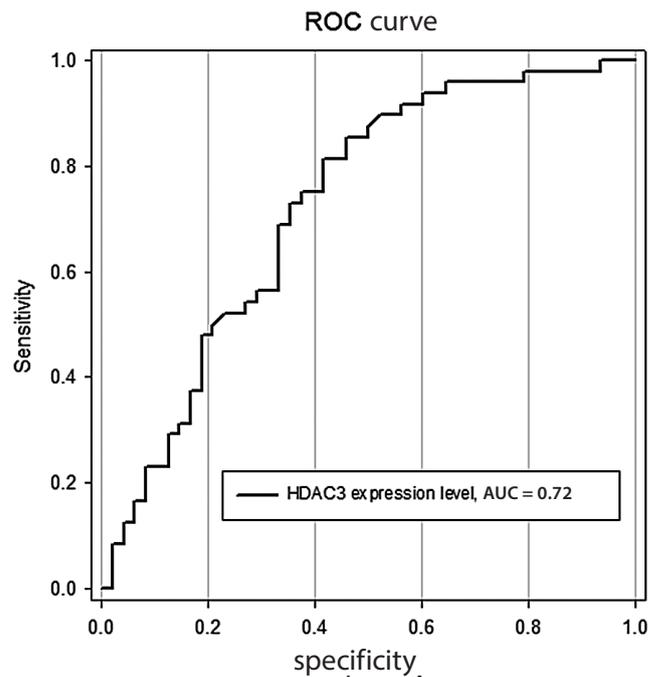


Fig. 2. The diagnostic performance of the HDAC3 expression data indicates that the AUC-ROC is 0.72 out of 1. ROC – receiver operating characteristics; AUC – the area under the ROC curve.

Table 2. Selected clinicopathological features of participants with CRC, and relationships between the HDAC3 expression levels in cancer tissue samples (n = 48)

Clinicopathological variables	Median	Min	Max	p-value HDAC3
Age				
<50	1.3405284855	0.25604870	3.94196477	0.22
>50	1.7360728985	0.43010254	3.86531151	
Gender				
male	1.4910755260	0.25604870	3.94196477	0.58
female	1.4761881470	0.28638478	3.04533229	
Tumor size				
<3	1.1112380320	0.54979096	1.19964893	0.17
≥3	1.6676598620	0.25604870	3.94196477	
Tumor differentiation				
grade 1: well	1.2094489460	0.25604870	3.86531151	0.04*
grade 2: moderate	1.3992817600	0.28638478	3.94196477	
grade 3: poor	2.4119166165	1.96567964	3.04533229	
Depth				
T ₂ ,T ₃	1.2456120785	0.41808787	2.45317053	0.77
T ₄	1.7376342220	0.25604870	3.94196477	
Lymph node metastasis				
absent	1.2247150570	0.25604870	3.14867080	0.20
present	1.8470684360	0.33843934	3.94196477	
Venous invasion				
absent	1.2399811680	0.28638478	3.86531151	0.45
present	1.6707825090	0.25604870	3.94196477	
AJCC stage classification				
I, II	1.656210461	1.184410345	2.630721395	0.21
III, IV	1.88508279	0.4017753	3.49364853	
Liver metastasis				
absent	1.3992817600	0.25604870	3.94196477	0.31
present	2.3796243590	0.46511126	3.04533229	

* the p-value for tumor differentiation is <0.05, indicating statistical significance; AJCC – American Joint Committee on Cancer.

Discussion

There are obvious logical reasons to study CRC. Based on new cases diagnosed worldwide, it is one of the most common causes of cancer, with at least 5-year survival rate. The prognosis of CRC patients is largely dependent on the stage at diagnosis. Early diagnosis is therefore critical for increasing survival time in CRC. Recently, non-invasive means of surveillance – in particular molecular markers – have facilitated early diagnosis of the disease.²² Notwithstanding the growing improvements in early diagnosis and treatment of CRC as a result of using a multidisciplinary approach, this disease remains seriously life-threatening for millions of people around the world, and the search for novel diagnostic and prognostic biomarkers is indispensable to prevent CRC-related mortalities.^{23,24} Thus, developing new strategies for CRC screening that will lead to higher rates of early CRC diagnosis is one way to reduce the socio-economic burden of CRC until the advent of more effective therapeutic strategies.²⁵

Investigating whether HDAC3 expression has clinical implications in CRC, sheds light on the role of HDAC3 in CRC. Our decision to undertake this study is supported by recent evidence that HDAC3 is an important member of the HDACs. HDAC3 is a well-studied epigenetic factor that is required for a wide repertoire of cellular processes due to its ability to regulate gene expression and function.^{26,27} To our knowledge, this is the first study to investigate HDAC3 expression in CRC using qRT-PCR. We used this technique for HDAC3 transcripts on 48 tumors and matched TAN tissues. We found HDAC3 to be highly expressed in the tumors of most patients with CRC ($p < 0.03$). Our findings are consistent with previous studies reporting noted changes of HDAC3 in a number of different human cancers. For example, deregulation of HDAC3 is frequently seen in ovarian carcinoma, breast carcinoma, prostate carcinoma, liver carcinoma, astrocytic glial tumors, pancreatic carcinoma, Hodgkin's lymphoma, acute lymphoblastic leukemia, and CRC.^{28–38} Most of the reports show the upregulation of HDAC3.

In line with our findings, Wilson et al. observed the deregulation of HDAC3 and other Class I HDACs in human colon cancer for the first time. Using Western blot analysis, they reported increased expression of HDAC3 protein in the tumor samples compared with adjacent normal tissue. In addition, they observed higher expression of HDAC3 in a panel of 10 established colon cancer cell lines when compared with a normal small intestinal cell line. Based on the results of cell culture studies done by this group, it has been suggested that in as much as the expression of Class I HDACs, including HDAC3, is restricted to the proliferative compartment of normal small intestinal and colonic epithelium cells, one physiological role of Class I HDACs may be to maintain cell proliferation. Consistent with this function, pharmacological suppression of HDACs in colon cancer cells leads to cell cycle arrest and stimulates the *p21*

promoter, consequently increasing the expression of *p21*, a Cdk inhibitor that is an important regulator of the cell cycle. This finding indicated that HDAC3 is involved in the inhibition of *p21*.³⁶ Indeed, inhibitors of HDACs have recently been noted for their potential to induce differentiation, apoptosis and transformed cell growth arrest in a wide spectrum of cancers.^{37,39–43} It should be noted that *HDAC3* is located on human chromosome 5q31.3 and at least 50 non-histone proteins (including RUNX3, GATA1, GATA2, E2F, c-Myc, p53, SHP, YY1, NF- κ B, STAT3, MEF2D, etc.) have been identified as its substrates.^{44,45} Although it has been proposed that HDAC3 is upregulated in colorectal carcinoma, it is not amplified at the DNA level. Furthermore, HDAC3 is expressed at higher levels in the proliferating cells of the colonic crypts, which might show that its levels are higher in colorectal carcinoma, because the cells are cycling.⁴⁶

In our study, HDAC3 showed an increased expression in association with one of the advanced disease clinicopathological parameters, namely poor differentiation. Thus, it can be used as a prognostic biomarker indicating a poor outcome of the disease. This is compatible with the first report of HDACs as adverse prognostic factors in colorectal tumors, which was demonstrated by Weicher et al. using immunohistochemical analysis.³⁷ Regulation of genes involved in the differentiation process in various tissues is one key role of HDACs, and their inhibitors induce terminal differentiation. Disturbance of the balance between proliferation and differentiation is one of the hallmarks of cancer. Cancer cells exhibit shifted or no differentiation, and display infinite proliferation that results in an undifferentiated, immature state. Interestingly, HDAC inhibitors restore the balance, stimulating tumor cells to differentiate and decreasing their proliferative ability.⁴⁷ No other correlations of HDAC3 with clinicopathological parameters were found.

The main finding of this study is the occurrence of overexpressed transcripts in tumor-adjacent histologically normal human colorectal tissues, as shown by the qRT-PCR expression analysis. As Fig. 3 shows, 13 of 48 tumor-adjacent histologically normal tissues had overlapped expression of HDAC3 with tumor tissues. Interestingly, most of the matched tumors of these patients were well-differentiated tumors. The most likely explanation for this result may be field cancerization, which is a description for the occurrence of genetic changes in histologically normal tissues adjacent to tumors.⁴⁸ However, the use of HDAC3 as a marker for characterizing field cancerization in CRC needs further evaluation and requires analyses of the deacetylation profiles of its several downstream targets. On the other hand, HDAC3 can be used as a biomarker to discriminate between tumor borders and margins. The use of the *HDAC3* gene expression data during surgery can help surgeons improve the rigor of their work, reducing surgical error in tumor removal. Similarly, Hashemzadeh et al. reported the same result for the *STC2*

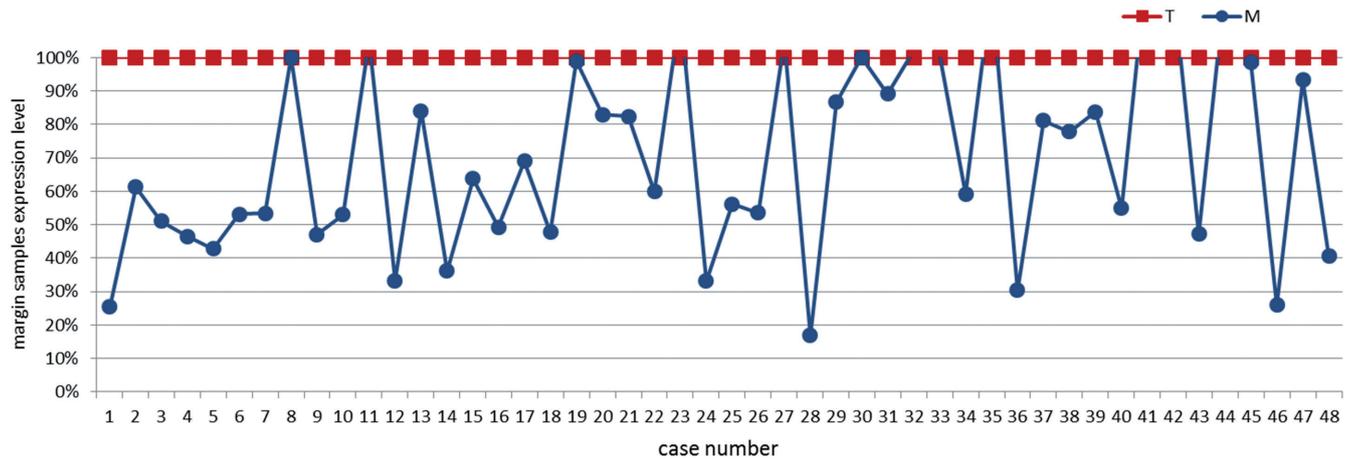


Fig. 3. Expression level of HDAC3 in marginal samples

T – tumor; M – marginal samples.

gene in CRC.⁴⁹ Taken together, our data may support the rare literature that shows field cancerization in CRC.⁵⁰ Furthermore, combining multiple gene expression data and correlating the data to distances from tumor margins might be effective for delineating the tumor margin and molecular alterations that are characteristic of field of cancerization in CRC. Finally, increasing our understanding of early events in the pathogenesis of CRC is crucial for identifying new targets for the prevention and treatment of this malignancy.

In conclusion, this study demonstrates that high levels of HDAC3 expression in qRT-PCR data are associated with poor prognosis in CRC. In addition, we propose that this data may be applicable to delineating the tumor margin. Finally, although some other biomarkers have been explored providing prognostic data in extensive CRC studies over the past few decades, our findings suggest that HDAC3 is a prognostic biomarker for CRC and can serve as a potential therapeutic target for this malignancy. One problem may arise from using these genes as biological markers for determining cancer status: the variability among different patients, even with the same type of cancer. This makes it impossible to use only one marker as a dependable method. For this reason, the integration of multiple expression data sets might be effective for diagnostic, prognostic and targeted therapy purposes.

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