Original Article

TFAP2E and MLH1 Genes Methylation Pattern and Microsatellite Instability as Predictors of Rectal Cancer Response to Neoadjuvant Chemoradiotherapy

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ABSTRACT

Background: Neoadjuvant chemoradiotherapy (nCRT) prior to surgery in rectal cancer has several adverse effects. Predictive biomarkers for response to nCRT are needed to save patients unnecessary toxicities and to take a timely tailored treatment decision. Epigenetic modifications like DNA methylation patterns have been suspected to be potential predictive biomarkers.

Aim: To determine the role of TFAP2E and MLH1 genes' methylation status and microsatellite instability (MSI) in predicting response to 5-fluorouracil – based nCRT in rectal cancer.

Methods: DNA was extracted from 80 patients with newly diagnosed stage II / III rectal cancer. The methylation status of TFAP2E and MLH1 genes was determined by pyrosequencing and MSI was determined using 5 micro-satellite loci by conventional polymerase chain reaction and capillary electrophoresis.

Results: The cut-off values for TFAP2E & MLH1 genes 'methylation level were 40% and 15% by receiver operating characteristic curve analysis. Hypermethylated TFAP2E and MLH1 gene promotors and MSI were predominant among non-responders (p <0.001, <0.001 and =0.022; respectively). Other factors associated with significantly higher pathological response to nCRT were well/moderately differentiated adenocarcinoma, pretreatment carcinoembryonic antigen level ≤5 ng/ml and rectal tumor ≤5 cm from the anal verge.

Conclusion: Hypermethylated TFAP2E and MLH1 gene promotors and MSI in rectal cancer tissue were associated with poor response to 5-fluorouracil – based nCRT. They might be of value in predicting the response of rectal cancer to nCRT and in tailoring its treatment.

Keywords: Epigenetics, MLH1 gene, Microsatellite instability, Neoadjuvant chemoradiotherapy, Rectal cancer, TFAP2E gene

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Introduction

Colorectal cancer (CRC) is a disease with a major worldwide burden. It is the third most common cancer diagnosed in both males and females. It has heterogeneous clinical presentations, prognosis and individual treatment response [1].

Rectal cancer has a striking complication, which is the high recurrence rate; 4–33% [2]. Aiming at reducing the recurrence rate and downstaging the tumor prior to surgery, neoadjuvant chemoradiotherapy (nCRT) is recommended for a group of rectal cancer patients by the National Comprehensive Cancer Network (NCCN) and others [3,4].

The major benefits of pre-operative nCRT are to reduce tumor mass, decrease the thickness of invasion of tumor into the intestinal wall and

achieve clinical down-staging of tumor [5]. However, its outcome varies from a favorable 'pathological complete response', which occurs in 10–20 % of patients, to 'no benefit from treatment' and delayed opportunity of surgery with tumor growth during this period and even the development of distant metastasis [6, 7].

Considering the severe secondary effects of nCRT observed, reliable predictive biomarkers are strongly needed to predict the response of patients to the treatment and to identify non-responders and thus avoiding costly, long, and ineffective therapy with its adverse effects and getting benefit from other personalized treatment [8].

The recent introduction of epigenetic studies revealed characteristic DNA methylation pattern of cytosine guanine islands (CGIs) in rectal cancer. With these advances, a major potential lies in the use of DNA methylation patterns as a reliable and effective diagnostic, prognostic and predictive biomarkers to the response to 5-flurouracil (5-FU)-based nCRT in rectal cancer [9].

One of these predictive biomarkers is the transcription factor activating protein 2 epsilon (TFAP2E) gene which is located on chromosome 1p34 and has multiple CGIs with the potential for regulation of its expression by means of its CGIs' hypermethylation. It was found that hypermethylation of this gene is associated with its decreased expression, that is associated with clinical non-responsiveness to 5FU-based nCRT. Moreover, induction of TFAP2E expression was triggered by using de-methylating drugs [10, 11].

Another one is the mut L homolog 1 (MLH1) gene, which is located on chromosome 3p22.2. It is a member of a group of genes known as DNA mismatch repair (MMR) genes that are responsible for recognition and repairing of DNA errors that occur during DNA replication [12].

It was found that MLH1 gene hypermethylation with subsequent silencing of this gene correlates with loss of the MLH1 protein and DNA repairing mechanisms and provokes a characteristic molecular phenotype called microsatellite instability (MSI), displaying a form of genetic instability characterized by the accumulation of numerous mutations within repetitive sequences of DNA in non-encoding microsatellite regions, which has a predictive role in response to nCRT [13, 14].

It was reported that patients with MLH1 gene hypermethylation with MMR-deficient tumors and MSI did not respond to 5-FU-based nCRT, whereas those with MMR-proficient tumors and microsatellite stability (MSS) had a significant response to this treatment [15]. Moreover, it was reported that treatment of rectal cancer by de-methylating agents (which de -methylate genes like MLH-1) results in enhanced chemo-radiation sensitivity [16].

So, epigenetic methylation markers are suspected to be predictive markers to treatment response in rectal cancer. This information could be critical in the choice of chemotherapeutic treatment, especially considering recently developed new inhibitors of DNA methylation [17].

Insufficient attention has been paid to this important clinical issue. Moreover, to the best of our knowledge, published studies did not separate rectal cancer and studied rectal and colon cancer together [18].

In this study, we aimed at determining the predictive role of methylation markers (TFAP2E and MLH1 genes) and MSI status in response to nCRT among Egyptian patients with rectal cancer.

Methods

Patients

This study was conducted on 80 Egyptian patients with newly diagnosed rectal cancer stages II and III by magnetic resonance imaging (MRI) and biopsy, admitted to the Colorectal Surgery Department at Alexandria Main University Hospital, Alexandria, Egypt. All were scheduled to receive nCRT before surgery.

Full history was taken from all patients including; personal history (name, age, diet habits, smoking, alcohol intake and physical exercise), family history (CRC, gastro-intestinal, breast and genital cancers, age at diagnosis and outcome), past history (of exposure to previous chemotherapy, radiotherapy, X-ray, CT or MRI on colon and colorectal biopsy) and present history (regarding the main complaints as tenesmus, mucorrhae, anal bleeding, discharge, pain, abnormal bowel habits, weight loss and their duration). Digital rectal examination was done to assess the distance of the rectal mass from the anal verge.

Carcinoembryonic antigen (CEA)

Carcinoembryonic antigen was measured before starting nCRT, using ADVIA Centaur XP (Siemens Healthcare diagnostics, USA) [19].

Genomic study of endoscopic samples

1. Tissue sampling: Eighty endoscopic formalin fixed paraffin embedded (FFPE) rectal cancer tissue specimens were collected from 80 patients before starting nCRT. Five μm thick sections were dissected from each specimen, stained with haematoxylin and eosin (H&E) to determine locations of tumor tissue and adjacent normal tissue. Eight to ten cut sections, 10 μm thick, were dissected from each endoscopic FFPE biopsy from tumor tissue and from the adjacent normal tissue and were put into two separate Eppendorfs.

2. DNA extraction: DNA was extracted from tumor and adjacent normal tissues by QIAamp® DNA FFPE tissue kit for purification of genomic DNA from FFPE tissues according to the manufacturer's instructions (catalog # 56404, Qiagen, Germany) [20]. The DNA samples were stored at -20°C until use.

3. Assessment of TFAP2E and MLH1 genes' methylation level in rectal tumor tissue samples:

   3.1. DNA Bisulphite conversion [21]: DNA Methylation-Gold kit (Catalog # D5005, ZYMO RESEARCH, USA) was used to treat DNA with bisulfite, which converts unmethylated cytosines into uracils and methylated cytosines remain unchanged during the treatment.

   3.2. Polymerase chain reaction (PCR) amplification: The Bisulphite converted DNA was amplified according to manufacturer's instructions of Pyro Mark PCR Kit (200 runs, cat # 978703, Qiagen, Germany). The PCR reaction mix was set up as follows: 12.5 μl PyroMark PCR master mix (2x), 2.5 μl CoralLoad Concentrate (10x), 2.5 μl PCR primers (10x), 5.5 μl RNase-free water, 2 μl Bisulfite converted DNA, to bring a total volume of 25ul / reaction. Primers were included in the PyroMark CpG assay kit (cat # 978746, Qiagen, Germany). One set for MLH1 gene (PM00104832) and the other for TFAP2E gene (PM00001260) amplification (one primer in each set that was biotin 3' labelled). Thermal cycler (SimpliAmp, applied biosystems, Thermo Scientific, USA) was programmed according to the manufacturer's instructions. After amplification, the PCR product was

checked prior to pyrosequencing analysis by agarose gel analysis.

   3.3. Detection of promotor methylation status of TFAP2E and MLHI genes by Pyrosequencing Technique [22]: The detection of promotor methylation level of TFAP2E and MLHI genes in the amplified DNA was done according to manufacturer's instructions of Pyro Mark Gold Q24 reagents kit (24x5 runs, cat # 971802, Qiagen, Germany) on PyroMark Q24 sequencer as well as PyroMark Q24 software for analysis. Preparation of the reaction mix for pyrosequencing was done by adding 1 μl Sepharose beads (5 ml, lot no.10048166, GE Healthcare Bio-science, USA), 40 μl binding buffer, 29 μl high purity water and 10μl amplified biotinylated DNA sample bringing the total volume to 80μl in each Eppendorf.

   Preparation of Pyromark Q24 plates was done by adding 22.5μl of annealing buffer to 2.5 μl of sequencing primer which was included in the PyroMark CpG assay kit (cat # 978746, Qiagen, Germany). One set for TFAP2E gene (PM00001260) and the other for MLH1 gene (PM00104832). The pyrosequencing assays were designed to analyze the following sequences:

   - For TFAP2E gene promotor (with 6 CpG sites) (Figure 1): CTGG<u>C</u>GGCAA<u>C</u>GC<u>C</u>GAATCC<u>C</u>GCAGTG AC<u>C</u>G<u>C</u>GGA.

   - For MLH1 gene promotor (with 3 CpG sites): <u>C</u>GTAAGCTACAGCTRAAGGAAGAA<u>C</u>GT GAGCA<u>C</u>GA.

   Methylation percentage of each CpG site was displayed in a pyrogram, and was calculated as the cytosine: thymine ratio, methylated: unmethylated cytosine. Average % methylation of the whole sequence was calculated as the mean of the six CpG sites' methylation % in the region of TFAP2E gene and three CpG sites methylation % in the region of MLH1 gene.

4. Assessment of MSI status in the rectal tumor tissue and the adjacent normal tissue by conventional PCR:

   4.1. PCR amplification: The extracted DNA from both tumor tissue and adjacent

normal tissue from each patient was amplified using lyophilized primers (Invitrogen, Thermo Fisher Scientific, UK) for five loci (BAT-25, BAT-26, D5S346, D17S250 and D2S123), recommended by Bethesda protocol, and Taq Red Mix, master mix with loading dye (cat # 25044, Bioline, UK). The primer sequences of the five microsatellite loci were as follows:
BAT-25-F:5' CTCGCCTCCAAGAATGTAAGT-3'
BAT-25-R:5' CTATGGCTCTAAAATGCTCTGTTC-3'
BAT-26-F:5' TGACTACTTTTGACTTCAGCC-3'
BAT-26-R:5' AACCATTCAACATTTTTAACCC-3'
D5S346-F:5' ACTCACTCTAGTGATAAATCG-3'
D5S346-R:5' AGCAGATAAGACAGTATTACTAGTT-3'
D17S250-F:5' GGAAGAATCAAATAGACAA-3'
D17S250-R:5' GCTGGCCATATATATATTTAAACC-3'
D2S123-F: 5' AATGGACAAAAACAGGATGC-3'
D2S123-R:5' CCCTTTCTGACTTGGATACC-3'
They were dissolved in high purity water. 1 µl of forward primer, 1 µl of reverse primer, 12.5 µl of master mix, 200 ng of DNA and high purity water were dispensed into each 0.2 ml PCR tube with a total volume of 25 µl for amplification. The thermal cycler (SimpliAmp, applied biosystems, Thermo Scientific, USA) was programmed as follows: initial denaturation 94°C for 2 minutes then 35 cycles of three steps; denaturation 94°C for 15 sec, annealing 56°C (52°C for D17S250 only) for 30 sec, extension 72°C for 60 sec.

Detection and differentiation of PCR fragments were performed on the QIAxcel Advanced capillary electrophoresis system.

4.2. Detection of MSI status on QIAxcel Advanced Capillary Electrophoresis System [23]: Tumor and normal amplicons of each locus for each patient were tested side by side in the sample strip and their electropherograms were superimposed on each other to detect the absence or presence of instability in each locus [24] (Figure2).

4.3. Definition of MSI: Three categories of MSI have been defined by the National Cancer Institute; MSI-high (MSI-H) indicating instability in two or more loci, MSI-low (MSI-L) indicating instability in one locus only and microsatellite stability (MSS) indicating absence of instable loci. [24,25] In our study, cases of MSS and MSI-L were considered as MSS, and cases of MSI-H were considered as MSI, as previously grouped by Sargent *et al.* [14]

Neoadjuvant chemoradiotherapy and determination of tumor's response

Patients were subjected to long-course of nCRT. The radiation dose delivered was 50.4Gy (1.8Gy in 28 daily fractions) with infusional 5-FU (1000 mg/m^2 daily for five days during the 1st and 5th weeks of
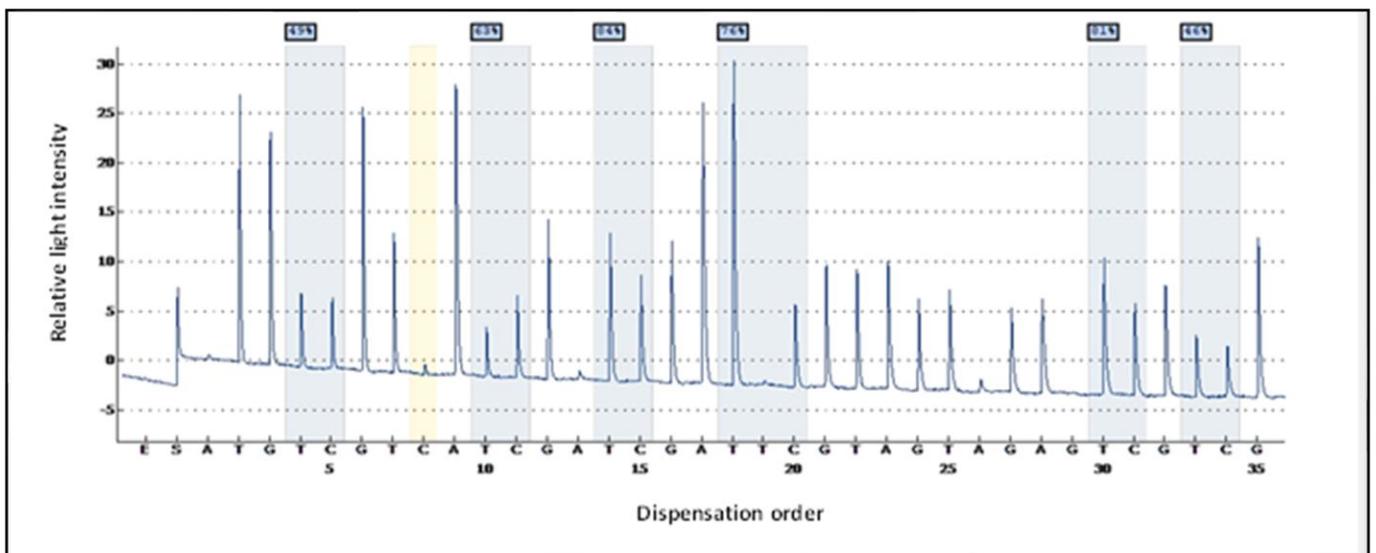
Figure 1: Pyrogram of methylation level of the six CpG sites in the TFAP2E gene promotor (The 6 blue-gray shaded areas of the pyrogram correspond to the 6 CpG sites interrogated in TFAP2E gene promotor; X axis: Dispensation order, Y-axis: Relative light intensity)
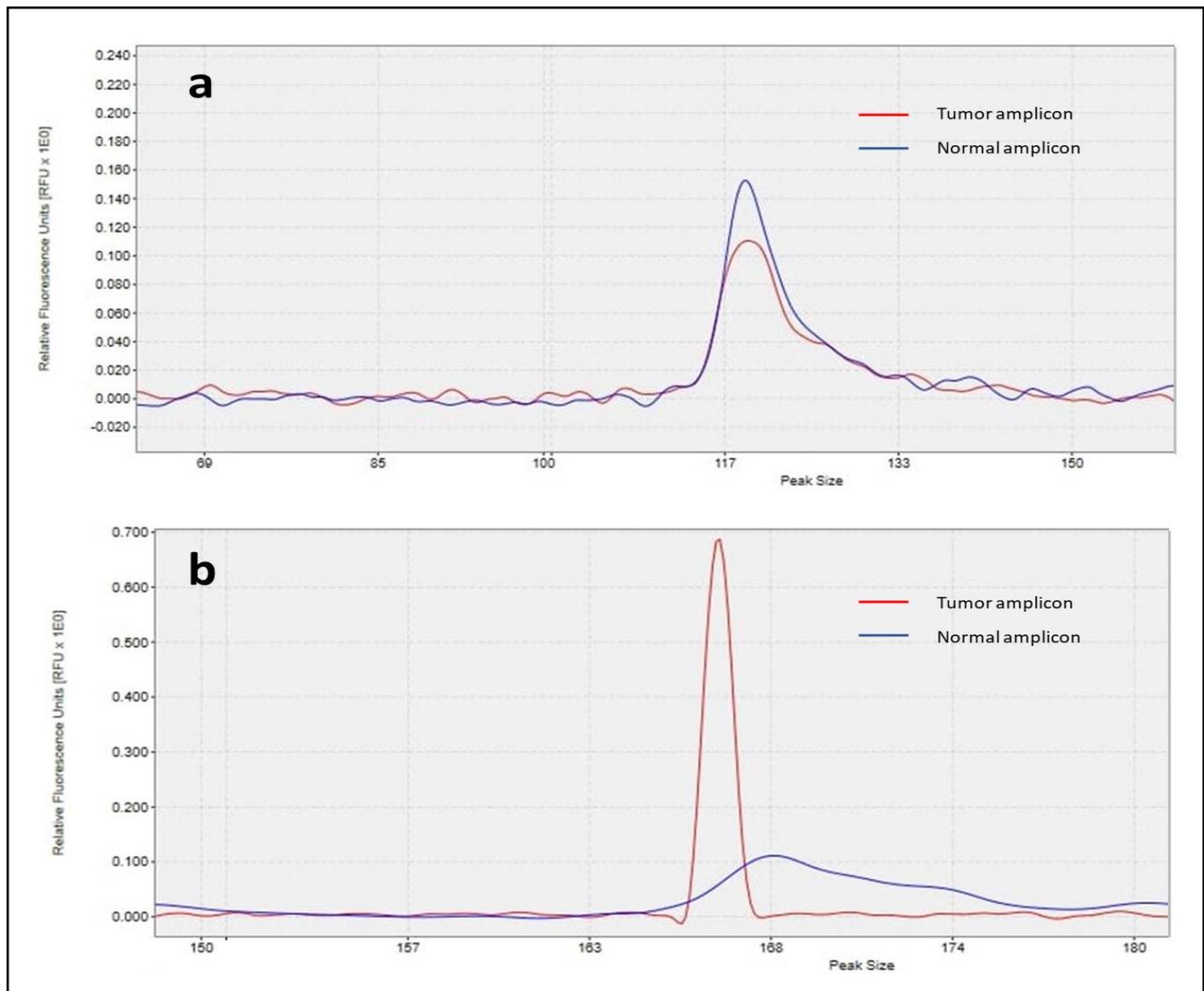
Figure 2: Electropherograms showing a) Stability of BAT-26 locus, b) Instability of D2S123 locus
(Tumor and normal amplicons of each locus were superimposed on each other in the electropherogram determining either stability, if they coincide together, as in a) for BAT 26 locus**,** or instability, if they do not coincide, as in b) for D2S123 locus.; X-axis: Peak size, Y-axis: Relative fluorescence units)

radiotherapy) or oral 5-FU all through the radiotherapy course at the Clinical Oncology Department - Alexandria Main University Hospital. Determination of the tumor's response to nCRT was done by pre and post neoadjuvant MRI according to Response Evaluation Criteria in Solid Tumors (RECIST) criteria [26] (patients with complete response [CR] or partial response [PR] were classified as responders and patients with progressive disease [PD] or stationary disease [SD] were classified as non-responders), and by post-surgical pathological examination according to Mandard tumor regression grade (TRG) classification [27] (patients were divided into responders [grades 1 to 3] and non-responders [grades 4 and 5]).

Statistical analyses

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). Qualitative data e.g. gender of patients was described using number and percent. The Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data e.g. age of patients was described using median and range (minimum and maximum) or mean and standard deviation. Significance of the obtained results was judged at the 5% level. The used tests were; Chi-square test to compare between different groups with categorical variables, Fisher's Exact or Monte Carlo correction for chi-square when more than 20% of the cells have expected count less than 5, Kappa test for agreement testing. Receiver

operating characteristic (ROC) curve was generated by plotting sensitivity (true positive) on Y axis versus 1-specificity (false positive) on X axis at different cutoff values. The area under the ROC curve (AUC) denotes the diagnostic performance of the test. Area more than 50% gives acceptable performance and area about 100% is the best performance for the test. The ROC curve allows comparison of performance between two tests.

Ethical considerations

The study was approved by the Institutional Human Research Ethics Committee of the Faculty of Medicine – Alexandria University (Approval #: 020870) and a written informed consent was obtained from all participants before enrolment in the study. Patients were all coded and detailed information was stored confidentially.

Results

Among the 80 included patients, 34 (42.5 %) were males and 46 (57.5%) females and their age ranged from 41 to 70 years with a mean of 57.1 years.

Forty-six (57.5%) patients had stage II rectal cancer, while 34 (42.5%) had stage III. Pathologically, 18 (22.5%) had well differentiated adenocarcinoma, 48 (60%) had moderately differentiated adenocarcinoma, while 14 (17.5%) had mucinous carcinoma. The distance between rectal cancer and anal verge was ≤ 5 cm in 26 (32.5%) patients and > 5 cm in 54 (67.5%) patients. The pretreatment level of CEA was ≤ 5 ng/ml in 36 (45%) patients and > 5 ng/ml in 44 (55%) patients.

Fifty (62.5 %) patients had hypomethylated while 30 (37.5 %) had hypermethylated TFAP2E gene promotor. Thirty-six (45 %) patients had hypomethylated while 44 (55 %) had hypermethylated MLH1 gene promotor. The cutoff values for TFAP2E & MLH1 genes 'methylation level (to distinguish between hypermethylated and hypomethylated genes) were 40% and 15% by ROC curve (AUC= 0.749 and 0.799 respectively, Figure 3).

Fifty-eight (72.5%) patients were MSS and 22 (27.5%) patients were MSI.

Assessment of response to nCRT by pathological TRG and radiological RECIST criteria were in good agreement with each other by kappa test (0.751 with p<0.001). Pathological response is more sensitive to changes at cellular level. The relation between clinico-pathological data and pathological

response to nCRT are summarized in Table 1. Factors associated with significantly higher pathological response to nCRT were well / moderately differentiated adenocarcinomas, pretreatment level of CEA ≤5 ng/ml, rectal tumors ≤5cm from the anal verge, microsatellite stability, hypomethylated TFAP2E gene and hypomethylated MLH1 gene.
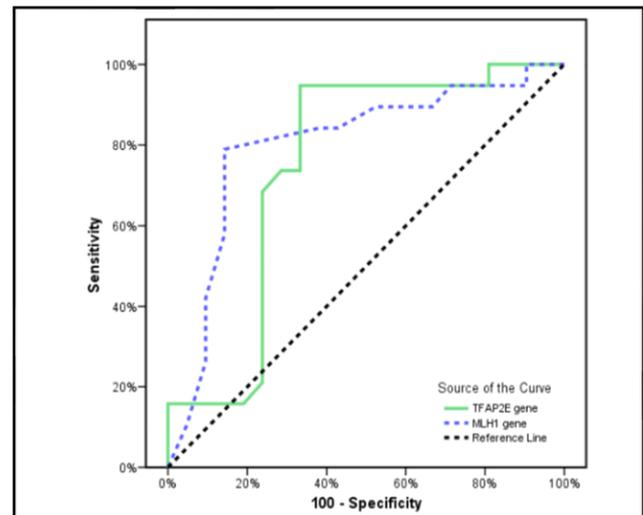
Figure 3: ROC curve analysis of methylation % values for TFAP2E and MLH1 genes

There was no statistically significant association between methylation level of the TFAP2E gene promotor and age, gender, TNM stage and histological type (Table 2). Similarly, there was no statistically significant association between these variables and the methylation level of the MLH1 gene promotor (Table 2).

Hypomethylated MLH1 gene (≤15 %) was significantly associated with MSS of rectal tumors (p=0.011). There were significant associations between gender of patients, histological type and microsatellite status of rectal tumors with predominance of MSI among females and those having mucinous carcinoma (Table 2).

Discussion

Hypermethylated TFAP2E and MLH1 gene promotors were found to be predominant among rectal cancer patients who did not respond to nCRT, while hypomethylated genes were predominant among responders. We did not find statistically significant associations between methylation levels of both TFAP2E and MLH1 genes and clinico-

pathological characteristics of patients (age, gender, histological tumor type and TNM stage).

In agreement with our study, Ebert et al [10] stated that the probability of response to 5- FU-based nCRT was six times higher among rectal cancer with TFAP2E gene hypomethylation (≤30% methylation level) than among rectal cancer with TFAP2E gene hypermethylation (>30% methylation level). Also, they reported that there was insignificant association between methylation level of TFAP2E gene and gender, histological type and TNM stage of rectal cancer. Murcia et al [28] conducted their study on 783 patients with stages II–IV CRC who received

5-FU-based CRT; 532 patients from a population-based multicenter cohort and 251 patients from a clinic-based trial. They aimed at evaluating the effectiveness of TFAP2E methylation and expression as a predictor of response of CRC patients to 5-FU-based CRT. The methylation status of TFAP2E gene was assessed by quantitative bisulfite pyrosequencing technique using PSQ HS 96A pyrosequencing system (QIAGEN). Optimal methylation cutoff was calculated by ROC curve analysis and was 40% with an AUC of 0.73. In contrast to our results, they reported that TFAP2E gene methylation level, either hypermethylated

Table 1: Relation between clinico-pathological data and pathological response of rectal cancer to neoadjuvant chemoradiotherapy

	Responders (*n* = 38)	Non-responders (*n* = 42)	*P*-value
	n (%)	*n* (%)	
Age (years)			
≤50	8 (21.1)	14 (33.3)	0.385*
>50	30 (78.9)	28 (66.7)	
Gender			
Male	18 (47.4)	16 (38.1)	0.554*
Female	20 (52.6)	26 (61.9)	
Stage			
II	18 (47.4)	28 (66.7)	0.218*
III	20 (52.6)	14 (33.3)	
Histological type			
Well differentiated adenocarcinoma	12 (31.6)	6 (14.3)	0.015**
Moderately differentiated adenocarcinoma	26 (68.4)	22 (52.4)	
Mucinous carcinoma	0	14 (33.3)	
Pre-treatment CEA level (ng/ml)			
≤5	26 (68.4)	10 (23.8)	0.005*
>5	12 (31.6)	32 (76.2)	
Distance from anal verge (cm)			
≤5	20 (52.6)	6 (14.3)	0.010*
>5	18 (47.4)	36 (85.7)	
TFAP2E gene methylation (%)			
≤40 (hypomethylation)	36 (94.7)	14 (33.3)	<0.001*
>40 (hypermethylation)	2 (5.3)	28 (66.7)	
MLH1 gene methylation (%)			
≤15 (hypomethylation)	30 (78.9)	6 (14.3)	<0.001*
>15 (hypermethylation)	8 (21.1)	36 (85.7)	
Microsatellite status			
Stable (MSS and MSI-L)	34 (89.5)	24 (57.1)	0.022*
Instable (MSI-H)	4 (10.5)	18 (42.9)	

CEA: Carcinoembryonic antigen; **MSS**: Microsatellite stability, **MSI-L**: Microsatellite instability- low, **MSI-H**: Microsatellite instability- high; *Pearson's chi-squared test, ** Monte Carlo test

Table 2: Relation between clinico-pathological data and promotor methylation of TFAP2E and MLH1 genes and Microsatellite Status

	TFAP2E gene promotor methylation level			MLH1 gene promotor methylation level			Microsatellite Status		
	≤40 (n = 50)	>40 (n = 30)	P - value	≤15% (n = 36)	>15% (n = 44)	P – value	Stable (n = 58)	Instable (n = 22)	P – value
	n (%)	n (%)		n (%)	n (%)		n (%)	n (%)	
Age (years)									
≤50	14 (28)	8 (26.7)	1*	6 (16.7)	16 (36.4)	0.286*	16 (27.6)	6 (27.3)	1*
>50	36 (72)	22 (73.3)		30 (83.3)	28 (63.6)		42 (72.4)	16 (72.7)	
Gender									
Male	22 (44)	12 (40)	0.804*	18 (50)	16 (36.4)	0.385*	32 (55.2)	2 (9.1)	0.012*
Female	28 (56)	18 (60)		18 (50)	28 (63.6)		26 (44.8)	20 (90.9)	
Stage									
II	24 (48)	22 (73.3)	0.117	18 (50)	28 (63.6)	0.385*	30 (51.7)	16 (72.7)	0.297*
III	26 (52)	8 (26.7)		18 (50)	16 (36.4)		28 (48.3)	6 (27.3)	
Histological type									
Well differentiated adenocarcinoma	16 (32)	2 (6.7)	0.061**	10 (27.8)	8 (18.2)	0.222**	16 (27.6)	2 (9.1)	0.018**
Moderately differentiated adenocarcinoma	30 (60)	18 (60)		24 (66.7)	24 (54.5)		38 (65.5)	10 (45.5)	
Mucinous carcinoma	4 (8)	10 (33.3)		2 (5.6)	12 (27.3)		4 (6.9)	10 (45.5)	
MLH1 gene promotor methylation level									
≤15	---	---	---	---	---	---	34 (58.6)	2 (9.1)	0.011*
>15	---	---		---	---		24 (41.4)	20 (90.9)	

*Fisher's Exact test, **Monte Carlo test

(>40%) or hypomethylated (≤40%), did not significantly play a major role in predicting response to 5FU-based CRT in rectal cancer patients (p=0.2). This dissimilarity in results may be due to differences in ethnic groups, larger sample size (783 *vs* 80) and selection criteria of patients (stages II-IV *vs* stages II and III CRC). However, in agreement with our study, they reported that none of the clinico-pathological characteristics of patients enrolled in their study was significantly associated with TFAP2E gene methylation level.

We found that hypermethylated MLH1 gene was significantly associated with MSI of rectal tumors. However, none of the clinico-pathological data such as age, gender, histological type and TNM stage of the tumor was significantly related to MLH1 gene methylation level.

Arnold et al [29] also reported that hyper methylation of MLH1 gene is significantly associated with the MSI phenotype in CRC. Also, Moreira et al [30] reported similar results. They found

that MLH1 gene hypermethylation (>15%) was prevalent in MSI tumors and MLH1 gene hypomethylation (<15%) was present in MSS tumors. They also reported that some of the clinico-pathological data, including age, gender and histological type of the tumor were not significantly related to MLH1 gene methylation status.

In our study, we found that MSS rectal tumors had a good response to 5-FU-based nCRT, while MSI tumors had lower response. MSI rectal tumors were predominant among female patients and patients with mucinous carcinoma with no statistically significant relation to either patients' age or rectal tumor stage.

Yang et al [15] conducted their study on patients with stage II rectal adenocarcinoma who received adjuvant therapy. In agreement with our study, they reported that 5-FU-based CRT had more benefits in MSS rectal cancer, but not with MSI. These results were supported by those of Sargent et al [14] and Ribic et al [31]. Ribic et al [31] reported that

some of the clinico-pathological variables including age and tumor stage were not significantly related to microsatellite status of the tumor, in contrast with the poorly differentiated histological type of the tumor that was reported to be significantly related to its MSI.

In contrast to our study, Thomas et al [32] reported that the benefit from 5-FU-based CRT in patients with rectal cancer was not significantly related to microsatellite status of the tumor, whether stable or not, and that the beneficial effect of 5-FU-based CRT was found in both MSI and MSS tumors. This dissimilarity in results may be due to ethnicity-related factors, difference in sample size (814 *vs* 80) and selection criteria of patients (stage C CRC, according to Australian Clinico-pathological Staging System, who received 5-FU-based chemotherapy *vs* stages II and III rectal cancer receiving nCRT). However, in agreement with our results, they reported that some of the clinico-pathological data enrolled in their study including female gender and poorly differentiated histological type of the tumor were significantly related to MSI of the tumor.

Among the strengths of the current study are the multiple clinical and laboratory factors studied to predict the response of rectal cancer patients to nCRT and the strict exclusion and inclusion criteria adopted for the selection of cases to avoid confounding factors that could affect the results as much as possible. Another strength is that the response to nCRT was assessed both pathologically and radiologically. The main limitation was the relatively small sample size due to limited budget.

To the best of our knowledge, this is the first study conducted on Egyptian patients with rectal cancer aiming at determining the role of some clinical and laboratory markers; TFAP2E and MLH1 genes and MSI in predicting their response to 5-FU-based nCRT.

Conclusion

Hypermethylation of TFAP2E and MLH1 gene promotors and MSI of rectal cancer are associated with a lower response rate to 5-FU-based CRT, indicating that they are potential predictors of treatment response in patients with rectal cancer who might benefit in the future from de-methylating agents.

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