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Title: Aberrant Methylation Profile and Microsatellit Instability In Turkish Sporadic Colorectal Carcinoma.

Başlık: Sporadik Türk Kolorektal Karsinomalı Hastalarda Metilasyon Profili ve Mikrosatellit İnstabilitesi

Kısa Başlık: Kolorektal Karsinomada Metilasyon Profili

Authors: Cumhur Gökhan EKMEKCİ[1] Mine GÜLLÜOĞLU[2] Yersu KAPRAN[3] Ferhunde DİZDAROĞLU[2] Uğur ÖZBEK[4]

Institutions: [1]Acıbadem Üniversitesi, LabGen, istanbul, Türkiye

[2]İstanbul Üniversitesi, İstanbul Tıp Fakültültesi, Patoloji, İstanbul, Türkiye

[3]Koç Üniversitesi, Tıp Fakültesi, Patoloji, İstanbul, Türkiye

[4]Acıbadem Üniversitesi, Tıbbi Genetik, İstanbul, Türkiye

Address for Correspondence: Cumhur Gökhan EKMEKCİ cgekmekci2@yahoo.com

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Sporadik Türk Kolorektal Karsinomalı Hastalarda Metilasyon Profili ve Mikrosatellit İnstabilitesi

Özet

Amaç: Bu çalışmanın amacı kolorektal kanserli olguların parafin bloklarından elde edilen genomik DNA da 6 farklı gen bölgesinin promoter metilasyonu ve mikrosatellite instabilitesi araştırılarak kolorektal kanser gelişimindeki, önemini değerlendirmektir.

Yöntemler: 76 sporadik kolorektal kanserli olguya ait parafin dokusundan tümörlü olduğu belirlenen bölgelerden kesit alınarak DNA izolasyonu gerçekleştirilmiştir. Bu DNA örneklerinden Metilasyon Spesifik PCZ (MS-PZR) yöntemi ile APC, hMLH1, p16INK4A, p15, p73 ve DAPK1 genlerine ait promoter bölgesi metilasyonu araştırılmıştır, aynı zamanda Mikrosatellite İnstabilitesi (MSI) varlığı 3 farklı STR bölgesi incelenerek belirlenmiştir.

Bulgular: Hasta örneklerimize ait genlerin metilasyon oranlarını hMLH1 için %24, APC için %31.5, DAPK1 için %19.6, p16 için %42.8, p15 için %30, p73 için %17 bulundu. Verilerin daha iyi anlaşılması için Metilasyon İndeksi hesaplandı. Bir örnek için Metile olan gen sayısının ile analiz edilen gen sayısına oranı şeklinde hesapladığımız MI değeri 0-0.83 arasında bulundu. Ortalama MI 0.271 idi(1.6 gen / numuneye karşılık gelir) ve medyan değeri 0.225 idi. Herhangi bir lokusta metilasyona uğramayan 15 örnek vardı. MSI'yi C-kit (21%), hMSH2 (18%) ve APC (15%) mikrosatellit bölgelerinde analiz ettik.

Sonuç: Literatüre göre Türk populasyonunda APC metilasyonunun diğer popülasyondan daha sık olduğunu belirledik. P16 promoter metilasyonun 6 gen arasında en fazla metilasyona uğrayan lokus olduğu ve kadın hastalarda daha sık metillendiği görülmüştür. P73 metilasyonu ise sıklıkla sol kolorektal kanserlerde belirlenmekte ve ek olarak da MSI ile ilişkili bulunmuştur.

Anahtar Kelimeler: Epigenetik, Promotör Metilasyonu, Kolorektal Kanser, Mikrosatellit instabilitesi, Metilasyonspesifik PZR.

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Aberrant Methylation Profile and Microsatellit Instability In Turkish Sporadic Colorectal Carcinoma.

Abstract

Objective: Genomic DNA obtained from paraffin blocks of the intended colorectal cancer cases was evaluated for promoting colorectal cancer by investigating the promoter methylation of 6 different gene promoter regions and microsatellite instability.

Methods: DNA was isolated from the paraffin tissue of 76 sporadic colorectal cancer patients by cross sections from the areas determined to be tumorous. The methylation specific PCR (MS-PCR) method was used for these DNA samples fo rmethlylation studies in promoter region of six different APC, hMLH1, p16INK4A, p15, p73 and DAPK1 genes. In the same samples, the presence of microsatellite instability (MSI) .

Results: The fequencey of methylation was 24% for hMLH1, 31.5% for APC, 19.6% for DAPK1, 42.8% for p16, 30% for p15, 17% for p73. We calculated a methylation index (MI=ratio between the number of genes methylated and the number of genes analyzed). MI was ranged from 0-0.83, with an average of 0.271 corresponding to 1.6 genes /sample and median was 0.225 and there were 15 samples which doesn't methylated in any loci. We analysed MSI in C-kit (21%), hMSH2 (18%) and APC (15%), microsatellite region.

Conclusion: We observed APC methylation was common then the other population for Turkish patient. P16 was the most commenest methylated loci among the 6 of gene and it seems storongly related with female patients. P73 was releated with left colorectal cancer and additionally it was related with the MSI.

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Key Words: Epigenetic, Promoter Methylation, Colorectal cancer, Microsatellite instability, Methylation Specific-PCR.

Introduction

Colorectal cancers (CRC) the third most common cancer type cause of death world wide and it is estimated that more than 1.3 million new diagnosed individuals are present on world wide and CRC may cause more than 700 000 deaths a year (1). Although the incidence varieties all over the world, then it was reported that India has the lowest incidence. On the other hand highest incidence are present for developed countries (2-3).

Immigrant data were shown that there are also international differences. Incidence of CRC has changed very quickly in Italy, Japan, poor regions of China, and Polynesianmales in Hawaii. Furthermore, it was highly sensitive to environment, suggesting that colon cancer is, in part, highly susceptible to environmental changes (2-3). The incidence of migrants and their descendants were quickly reached in rates of countries (4-5). The internationally stated rate of 20 times more frequent observation can be explained by diet habits and environmental differences in large section (2). However, It has been known since long time that colorectal cancer is common in some families (6-8). As a result, colorectal cancer is considered as a disease associated with genetic and environmental factors.

Despite the rapid and major advances in human cancer biology, no significant increase has been observed in the overall life span and survival rates of these patients over the past three decades. Despite the fact that many genetic alterations leading to cancer development have been identified and technical developments have been added, and molecular profiling has been established. The formation of a molecular profile is in its broadest sense and the identification of genetic abnormalities are occured within the cell. Moreover occurrence of events are in proteins or protein networks. These developments have been promising to increase the life span and rates of the cases with diagnostic and prognostic approaches (9).

Colorectal cancers (CRC) develop by accumulation of genetic and epigenetic changes. The development of CRC is assumed that primarily start with alteration of Wnt and TGF-Beta signal pathways and than activation of KRAS followed by the inactivation of APC and TP53 (10-12). Large group of CRC (80-90%) occur in this order. The remaining 10-20% of CRC mismatch repair system does not fulfill its mission adequately end result of this mostly characterized by mutations and microsatellite instability (MSI) (10-12). Another genetic classification model of CRC describes two subtypes: a chrosomal instability (CIN) type (which microsatellite are stable (MSS)) and as a 2nd type microsatellite instable (MSI) (13). First group was characterized with multiple chrosomal alterations together with P53 mutations and MSI, CpG island methylator phenotype (CIMP) on a genome wide level and BRAF mutations was observed in second group. (13-14). For CRC development DNA methylation accepted as third underlying mechanism after CIN and MSI. Changing gene expression under the influence of DNA methylation may contribute CRC initiation and development (15-17). It is very well known that genome wide epigenetic alteration are play important role not only for CRC and also for the most of the cancer development. Recently genome wide transcriptomic studies revealed five distinct Molecular classification are present for CRC end result of changes in genetic expression pattern (18-20).

Latest discoveries are also revealed that tumor location may also important CRC development (left side vs right side CRC). Underlying molecular mechanism of right side colon cancer development seems more relevant with MSI, CIMP and frequently BRAF mutations, and TP 53 mutations are relatively rare seen right side of CRC. And for left sided CRC we observe frequently copy number alteration, TP53 mutations and morerelevant with MSS phenotype and CIMP ar eobserved low status on this side of CRC (21-22).

Methylation profile of CRC are widely studied subject to investigate and identify any early detection molecular marker, any prognosis related marker, for diseases classification, any therapeutic target, and also for risk assesment (23-26). Early genome -wide DNA hypomethylation has been observed in CRC and LINE-1 (long interspersed nucleotide element 1) hypomethylation has been found connected with increased cancer related mortality and overall mortality in CRC patients (27-29).

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To understand methylation profile and MSI in Turkish colorectal cancer samples, we have used Methylation specific –PCR technique to examine six differentially methylated tumor-suppressor genes and we analysed three Microsatellite loci for MSI. Methylation of the APC, hMLH1, p16INK4A, p15, p73 and DAPK1 promoters was studied using methylation-specific PCR in 76 sporadic CRCs We perform these analyses on sporadic colorectal cancer samples from their paraffin embedded tissue blocks. We also performed MSI analysis of same samples and together with methylation profile we performed statistical analysis based on avaliable proporties of patient.

Methods

Patients and Specimens

The cases were surgical resection specimens of consecutive patients with CRC at XXX hospital in Turkey. **This study was a retrospective study that is why we couldn't take informed consent from the patients. P**araffin block samples that is stored along side associated information such as age, tumor type and tumor stage was used. Inclusion criteria were resection of CRC and availability of pathology tissue block of the primary tumor. Exclussion criteria were history of or pathologic evidence for familial adenomatous polyposis or idiopathic inflammatory bowel disease, or family history of hereditary non-polyposis colorectal cancer syndrome. Thus Pathology block of 76 patients were selected from pathology department of XXX in 2001 through 2004. While we were chosen patients from avaliable pathologic report data, we aimed to select equal number of disturibution according to sex, age, histological characterization, tumor localization and stage.

From all patient samples we collect both tumor tissue and non-neoplastic control tissue from each formalin-fixed paraffin embedded (FFPE) resection specimens were microdissected and DNA extracted as reported previously (30).

In this study, the anonymized paraffin block samples that is stored along side associated information such as age, tumor type and tumor stage was used. This study was conducted in accordance with the ethical principle of Helsinki Declaration.

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Bu çalışmada, yaş, tümör tipi ve tümör evresi gibi bilgileri depolanan isimsiz parafin blok örnekleri kullanılmıştır. Bu çalışma Helsinki Deklarasyonunun etik ilkelerine uygun olarak yürütülmüştür.

Microdissection from FFPE samples and DNA isolation

10-15 tissue sections of a 10-micrometer-thick paraffin block taken from tumorogeneous and none tumorogeneous distal part of the same patient were used.

Firstly, paraffin was removed using xylene from the samples and 100% ethyl alcohol was added and the samples were precipitated. Then tissue digestion buffer solution which includes proteinase K per tube to lyse cells. Phenol / chloroform mixture (1/1 ratio) was added and centrifuged at 14,000 rpm for 3 minutes. The supernatant was taken and this process was repeated once more. The supernatant was taken up in new tubes. 50 µl of 5M ammonium acetate was added to each of the tubes. After adding the chilled 100% ethyl alcohol, it was left at -20 ° C overnight. The next day samples were centrifuged at 14,000 rpm for 30 minutes, then the supernatant was discarded and drying. Samples were allowed to stand for 10-15 minutes at 55° C, then 50 µL of sterile H2O was added and they were allowed to stand at room temperature for at least 1 hour before measuring optical densities. Obtained DNA samples were maintained at -20 ° C until analysis.

Sodium bisulfite treatment of DNA

DNA treated with sodium bisulfite as previously described (30). Briefly, 5µg of DNA were denaturated at 42°C for 30 min with 0.4M NaOH, incubated in 10mM hydroquinone, 3M (Sodium Bisulfite, Sigma, St Louis, USA) at 55°C for 16 hrs and purified with the (GeneClean III kit, Bio 101, Vista, USA). Prior to ethanol precipitation, DNA was desulfonated in 0.4M NaOH for 15 min at 37°C. DNA was resuspended in water and stored at –80°C.

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Preparation of positive controls used in methylation (In Vitro Methylation).

(Sss I enzyme, New England BioLabs, Ipswich, USA), which methylates cytosines in the presence of CpG dinucleotide all trough whole genome, was used to generate positive controls. This process also uses S-Adenyl Methionine (SAM, New England BioLabs, Ipswich, USA), as the methyl donor group.

Methylation-specific PCR

Bisulfite-treated DNA was used in methylation-specific PCR (MSP) reactions for 6 genes. Table 1 shows the sequences of the primers used, specific for the methylated (M) and unmethylated (U) forms. And table 2 present the gene regions examined for methylation and the lengths of the regions amplified in the PCR. The amplifications reactions contained 25 pmoles of each primer, 200µM dNTP, 1U (Hotstart Taq polymerase and 1x Q buffer, Qiagen, Valencia, USA) with variable amounts of MgCl2 and cycling conditions as shown in Figure 1 All MS-PCR products were run in a 4% agarose gel.

Assay for Microsatellite Instability (MSI)

MSI was determined by fluorescently labeled PCR amplification kit for this purpose ("HNPCC Microsatellite Instability Test", Roche, Berlin, Germany). Normally it consist of 5 markers from panel described by American Cancer Association, International HNPCC colobaration group and German Cancer Research group. Altough with this kit 5 markers were provided we could manage to analyses only 3 locus BAT25 (c-kit), D5S346 (APC) and D17S250 (hMSH2). Primer sequences are given in Table 3.

Statiscal analysis:

To test methylation and MSI of one loci is cooparating with the other loci methylation statues and also for the rest of the avaliable outcome of patients we used Fisher Ki-squre test and within this group of analyses if the sample size smaller than 2, then we used Pearson test and its results. Comparison of methylation status and nonparametric outcome were tested by the nonparametric Mann-Whitney U Test. All test were done by (SPSS software 10.0, Chicago, IL, USA) for windows and

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P < 0.05 was considered statistically significant with only 2- sided results of the tests. This study was conducted in accordance with the ethical principle of Helsinki Declaration.

Results

We analyzed the methylation status of 6 genes in 76 sporadic CRC (27 – 86 years old, median age 58.8). We analysed MSI in neoplastic tissue and we compare it with normal peripheral mucosa within same patient group. Since we were particularly interested in the correlations of methylation of tumor suppressor genes previously confirmed to be methylated in CRC, we prioritized our selection of genes to include APC and hMLH1. Since our CRC patients were derived from a distinct geographical and ethnic back ground, we also wanted to revisit the pattern of methylation and included 4 genes (DAP-Kinase, p15, p16 and p73).

Amongs 76 samples tumor localization distrubition were; 26 (34.2%) rectum cancer, 18 (23.7%) sigmoid cancer, 13 (17.1%) right colon, 8 (10.7%) left flexura and left colon, 7 (9.2%) recto sigmoid, 3 (3.9%) transvers colon and in 1 (1.3%) tumor was observed in all colon. Histologic and morphologic classification was done accordingto WHO's TNM classification. Most of our sample group in stage II and III and only 7 samples were in stage IV. Table4 represent the number of patients and their stage according to TNM classification.

There was musinoz area in 22 samples but only 8 (10.5%) of them accepted mucinous adenocarcinoma. In 14 (18.4%) samples tumors were developed from tubulo villous adenoma. In 19 (25%) samples cripriform area was present. Pathologic demonstration was also representing that in all the samples out of 28 there was necrotic area, out of 17 samples there was desmoplastic area. Additionally in 13 samples there was blood vessels invasion, there was lenfatic invasion in 34 samples and there was perinoural invasion in 35 samples. there wasn't any patological tumor evidence in either proximal or distal surgical border in all samples.

Methylation specific PCR (MSP) was used to determine methylation of p15, p16, p73, APC, DAPK1 and hMLH1. MSP reactions for each gene were first standardized using in-vitro methylated DNA (IVM), which yielded a positive product with primers for the methylated form. Under our experimental MSP conditions a consistent absence of an amplification product for themethylated

form wasobserved in 15-20 different DNA samples obtained from peripheral blood mononuclear cells from healthy individuals. This indicated that methylation was tumor specific. Methylation was very common in CRC. Except, 15 samples (19.8%) there wasn't any detectable methylation among those six loci. The frequency of methylation of each gene varied notably. The frequency of methylation was shown in table5.

The frequencey of methylation was 24% for hMLH1, 31.5% for APC, 19.6% for DAP-Kinase, 42.8% for p16, 30% for p15, 17% for p73. To quantify the extent of methylation in our series of CRC, we calculated a methylation index (MI=ratio between the number of genes methylated and the number of genes analyzed) based on the 6 genes that demonstrated some degree of methylation. MI was ranged from 0-0.83, with an average of 0.271 corresponding to 1.6 genes /sample and median was 0.225 and when we removed 15 samples which doesn't methylated in any loci the average of MI was 0,337 (represent 2.13 genes/sample) and median was 0.33.

For analyzing whether the coexistence of interrelationship between two different loci, we performed that two-sided Fisher's exact test to understand whether one locus was methylated or unmethylated is affected by another locus methylation status. Statistical result was shown in table 6

We also performed MSI analysis for 3 loci by (ABI Prisim 310 sequencher, Applied Biosystem, Foster City, CA, USA) within these group of samples. We compared tumor tissue samples with normal mucosal specimen for the same patients samples. We analysed MSI in C-kit, hMSH2 and APC micro satellite region. Amongs these 3 region for c-kit loci that we got positive MSI in 14 patient out of 67 analysed sample, for hMSH2 loci; 12 positive results from 66 analysed samples and for APC loci; 10 positive MSI was present out of 67 analysed samples. In an overall samples MSI data there were one loci MSI in 18 patients, in 10 patients samples there was 2 loci MSI and in 4 patients there were 3 loci MSI.

All the analysed 3 loci of MSI and methylation of 6 gene are compared statistically with avaliable data (age, sex, tumor localization, tumor cell differentiation, histologic charecterization of tumor cell) of patientsby SPSS 10.0 for Windows software. We observed p73 methylation is significantly related with left colon cancer (p=0.003, 2-sided), p16 methylation was seen in female more than male (p=0.029, 2-sided), p15 methylation is seen more related with the well differentiated tumor cell

(p=0.028, 2-sided). Also APC methylation was correlated with stage I tumor (p=0.05, 2 sided), stage I tumor was related with rectum cancer (p=0.017). Age and Methylation Index were compared with the other parameters by nonparametric Mann Withney U Test (SPSS for Windows software). According to test results, MSI in APC loci was positively corelated with methylation index (p=0.029, MSI in APC additionally related with age (p=0.032) and one of the other age related MSI was c-kit loci (p= 0.011) for both loci instability was incrising with age. Amongs those 6 loci which we performed methylation analysis the only p73 methylation was related with age (p=0.017) and inverse relation was present in age and left colorectal cancer (p=0.015). MI was observed higher with stage I (p=0.006) and stage III (p=0.03).

In the same patient group, MSI studies were performed using DNA samples isolated from paraffin blocks of tumor and peripheral mucosa. DNA was used from same patient group but additionaly we used distal healty region paraffin blocks also used for MSI comparison.

The MSI kit was used for this aim and it was used to study microsatellite instability of three regions. These regions were microsatellites present in the C-kit, hMSH2 and APC regions. Microsatellite Unstable Tumors; In the unstable locus of fragment analysis, a new allele was observed, which was not observed in healthy tissue samples, or a decrease in the number of alleles were analysed when the normal - tumor tissues are compared.

Instability was determined in 14 of the 67 samples for the C-Kit microsatellite locus from 3 regions examined for MSI, 12 of 66 samples for the MSH2 microsatellite locus, and 10 for the APC microsatellite locus. 18 of the samples had MSI in at least one locus. MSI was observed in 2 loci in 10 specimens, 3 and 1 loci in 4 specimens.

Discussion

We investigated the methylation status of 6 gene regions using the methylation-specific PCR method from paraffin block samples of 76 sporadic colorectal cancer cases. The most frequent methylation was observed in the p16 gene region (42.8%), followed by the methylation ratios in APC, p15, hMLH1, DAPKinase and p73 regions, respectively.

In similar studies, p16 methylation was found to be 10% (10/65, 39% of sporadic) (31), 28% (27/97) for Americans, 27% (13/48) for Jordanian society and 10% (7/68), methylation rate was determined . We observed p16 gene methylation more frequent in females than in males in our study population ($p = 0.023$). Although p16 gene methylation was more associated with mucinous carcinomas than other gene regions, but there was no statistical significance ($p = 0.058$).

Similar studies have been published Pehlivan et al. from Turkey. They had studied on metastatic colon cancer samples and they found in MSI-H group. KRAS mutations is common and found 42.1 % in their samples group. They have observed P16 methylation in only one sample out of 17 total sample and it was in MSI-H group (32).

The methylation rate for p15 with the same chromosomal location was 69%; 39% (44/64) for sporadic and 30% (20/64) for our study group (31). On the other hand studies from United States has not identified any methylation on this region (33). In our study group, p15 gene methylation was associated with well-differentiated tumor cell ($p = 0.009$).

There are three members of the p53 gene family (p53, p63, p73) and transcriptional activation specific to the sequences of gene products occur (34-36). Functionally, overexpression of the p73 gene has been reported to activate the targets of p53, such as cell cycle inhibitor p21 (34-35). However, there are also significant differences between the genes that p53 and p73 activate. P53 and p73 proteins are involved in the development of the organism in response to DNA damage. It is known that p73 is involved in p53-like response to cell stress. Overexpression of P73 triggers apoptosis (34-37). These and similar observations suggest that p73 is a tumor suppressor gene that affect p73 expression in some tumor types. Different studies have shown that p73 broad methylation was present in different proportions and in different populations. Xu et al was found that p73 methylation in 63% (41/65) (31) of the patients, whereas in our group this rate was observed the lowest methylation pattern with 17%. Very strong correlation was found between left colon cancer and p73 gene methylation ($p = 0.003$), and we observed that MSH2 related with MSI. So it was related with P73 methylation ($p = 0.017$). In addition, we were found that the age was related with P 73 methylation ($p = 0.017$, Mann Witney U test) .

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Apopitosis-associated protein kinase (Dap-kinase1) is involved in different apoptosis systems as a calcium/calmodilin-dependent serine/thironine kinase. However, the mechanism of triggering apoptosis is not completely understood (38). We determined in our study group that the methylation rate of 19.1% in the DAPK1 region. Xu et al. did not identify 39 sporadic cases of methylation (31). Satoh et al. was investigated DAPK1 methylation in a series of 9 colorectal cancer in a tumor cell line by 3 and 17 in the series of stomach cancer. Pehlivan et al. was worked on Turkish CRC samples and researched for 5 genes methylation and 2 of them was the same with our studies DAPK1 and p16. It was found DAPK1 and 53% of methylation CRC samples and 29% of methylation from same samples healty part of CRC. They concluded DAPK1 was not directly related with cancer formation but it was considered an important factor for sensitivity (32). Mutation and methylation were found in MSI -H group. In our study group, there was no association between DAPK1 methylation, any features of the disease and methylation of other gene regions.

APC, a tumor suppressor protein the loss of function in the APC gene that it is known as the initiator and rate limiting mutation of the development of multi-step cancer in the large intestine (39). APC protein is directly related to β-catenin signaling proteins (40). APC proteins are located in the microvessel network in vivo, whereas in vitro they cause polymerizations of tubules into microtubules (41). Early-occurring mutant proteins lose the ability to suppress the β-catenin / Tcf signaling pathway in regions close to the carboxy end of the APC gene (42).

We observed APC methylation in our case group with 31.5%. There are reports in the literature that the rates of APC methylation are relatively low (8%) (31). Esteller et al. was determined the methylation of the APC region in 18% of 108 colorectal cancer cases examined (43). In another study was determined the methylation of the APC promoter to be 28% of 137 colorectal cancer cases. In the same specimens indicate that loss of heterozygosity (LOH) and immunohistochemical staining APC methylation is important in loss of gene expression and in the formation of second hit in the development of colorectal cancer (44).

Homozygous deletions of p16 and p14 genes are frequently observed in lung and breast cancers, which are not encountered in colorectal cancers (45). In contrast, methylation of p16 and p14 is

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more common in colorectal cancers (46). APC promoter methylation is a similar condition, although methylation in gastric cancers is a more common condition

Statistical analysis of the patient group showed that APC methylation was significantly associated only with the stage 1 patient group. This result was evaluated in the same direction as the rate limiting effect of the APC gene in the literature.

It is known that MSI as a genetic finding is an alternative pathway in the development of cancer after the detection from HNPCC and sporadic colorectal cancers (47). In our samples, there was a significant correlation between microsatellite instability in the APC region and hMLH1 methylation ($p = 0.032$) in the three regions where we performed the MSI study. HMLH1 methylation is not statistically related to MSI in the c-kit region ($p = 0.054$, 2-sided), and there was no significant correlation with MSI in the MSH2 region. We hypothesized that hMSH2 gene methylation may be significantly related to MSI in this region. It is reported that MLH1 and MSH2 mutations are not associated with low levels of MSI (48). It has been suggested that the methylation state in DNA repair genes not involved in the MMR mechanism may be the cause of the MSI (49). Interestingly, we found that only methylation in the p73 gene region was significantly associated with MSI in the MSH2 region ($p = 0.017$, 2-sided), while other methylation regions were not significantly associated with MSI screening

Furukawa and colleagues examined hMLH1 methylation in three regions and found to be associated with MSI-H, proximal colon cancer involvement with a type of methylation, termed type 1, which contained more than 80% of the CpG island. The methylation of the PTEN promoter region was highly related to the MSI-elevation and was claimed to be a second hit in the HMLH1 methylation (50). In our study group was identified with 24% HMLH1 methylation and it was found to be compatible with the literature (18%). In our samples were consisted sporadic patient group, there was no clinical relationship except HMLH1 methylation and MSI regions.

Co-methylation of some important tumor suppressor genes have been identified in some studies and it was defined as methylator phenotype of the relevant cancer. In this study, a similar relationship for colocal-rectal cancer was not demonstrated for the 6 regions in which we studied. The most striking aspect of our results, could be summiresed as, the association of p73 methylation in that in left colon

involvement and correlation with MSI, and also it was increased with age. The more frequent observation of P16 methylation in female gender, the fact that p15 methylation is associated with well differentiated cancers and it was the original result of our study for Turkish society.

Conclusion: We observed APC methylation was common then the other population for Turkish patient. P16 was the most commenest methylated loci among the 6 of gene and it seems storongly related with female patients. P73 was releated with left colorectal cancer and additionally it was related with the MSI.

We have no conflict of interest to declare.

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Table 1. Methylation and Unmethylation specific primers for each loci

DAP-Kinase;

DAPK1-M-F:	5’ GGA TAG TCG GAT CGA GTT AAC GTC 3’
DAPK1-M-R:	5’ CCC TCC CAA ACG CCG A 3’
DAPK1-U-F:	5’ GGA GGA TAG TTG GAT TGA GTT AAT GTT 3’
DAPK1-U-R:	5’ CAA ATC CCT CCC AAA CAC CAA 3’

p73;

p73 M-F	5’ GGA CGT AGC GAA ATC GGG GTT C 3’
p73 M-R	5’ ACC CCG AAC ATC GAC GTC CG 3’
p73 U-F	5’ AGG GAT GTA GTG AAA TTG GGG TTT3’
p73 U-R	5’ ATC ACA ACC CCA AAC ATC AAC ATC CA3’

APC;

APC M-F	5’ T C G A G A A C G C G A G C G A T T C G 3’
APC M-R	5’ G A C C A A T C C A A C C G A AA C G A 3’
APC U-F	5’ T T G A G A A T G T G A G T G A T TT G A 3’
APC U-R	5’ A A C C A A T C C A A C C A AAA C A A 3’

P15;

p15 M-F	5’ G T T C C A G A A C C G G C G C T A C A A G T G 3’
p15 M-R	5’ G C G T G C CC G A G C T C A G T C CC A G T T 3’
p15 U-F	5’ G T T C C A G A A C C G G C G C T A C A A G T G 3’
p15 U-R	5’ G C G T G C CC G A G C T C A G T C CC A G T T 3’

P16;

p16 M-F	5’ T T A T T A G A G GG T G GGG C G G A T C G C 3’
p16 M-R	5’ C C A C C T A AA T C G A C C T C C G A C C G 3’
p16 U-F	5’ T T A T T A G A G GG T G GGG T G G A T T G T 3’
p16 U-R	5’ C C A C C T A AA T C A A C C T C C A A C C A 3’

hMLH1;

hMLH1M-F	5’ TTC GCGTG TAT TTT TAG GTC GGT C 3’
hMLH1M-R	5’ CGA CAC AAC TCC TAC AAC GAC CG 3’
hMLH1U-F	5’ TTA TGA GTA TTT GTG TGT ATT TTT AGG TTG GTT 3’
hMLH1U-R	5’ CAC TAA CAA CAC AAC TCC TAC AAC AAC CA 3’

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Table 2. The gene regions examined for methylation and the lengths of the regions amplified in the PCR.

Gen	GenBank No.	Location of primers according to transcription start point	PCR product length (bp)
APC	U02509	-17 147 / -17 050	108
		-17 153 / -17 046	97
DAPK	NM_004938	-332 / -229	103
		-332 / -234	98
HMLH1	AB017806	-526 / -661	125
		-531 / -655	124
$p15^{INK4B}$	NM_004936	-318 /-188	130
		-312 / -187	125
$p16^{INK4A}$	NM_000077	-80 / +46	126
		-80 / +44	124
P73	AB031234	-1585 / -1505	80
		-1588 / - 1510	78

PCR: Polymerase Chain Reaction

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Table 3: Primers used in Determination of Microsatellite Instability

Name of Loci	Chromosomal localization	Primer sequences	PCR product size and label
BAT25 (c-kit),	4q12-4q12	F. 5' TCGCCTCCAAGAATGTAAGT 3' R. 5' TCTGCATTTTAACTATGGCTC 3'	110-130bp florasan 6-FAM
D5S346 (APC)	5q21- q22	F. 5'ACTCACTCTAGTGATAAATCGGG 3' R.5'AGCAGATAAGACAGTATTACTAG TT3'	100-130bp florasan HEX
D17S250 (hMSH2)	17q11.2- q12	F. 5' CAGGAAGAATCAAATAGACAAT 3' R.5'GTGCTGGCCATATATATATTTAAA CC3'	200- 230bp florasan 6-FAM

PCR: Polymerase Chain Reaction

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Table 4: Number of samples stage and their percentage distribution according to WHO based on TNM classification

Stages		
Stage	Number of sample	Percentage (%)
Stage I	8	10.5
Stage II	29	38.2
Stage III	32	42.1
Stage IV	7	9.2

Table 5: Represent the each locis methylation ratio and its percentage

	hMLH1 (%)	APC (%)	P16 (%)	P15 (%)	P73 (%)	Dap-Kinase (%)
Number of methylated samples / number of analysed samples	18 / 73 (%24)	23 / 73 (%31.5)	30 / 70 (%42.8)	20 / 64 (%30)	12 / 71 (%17)	10 / 51 (%19.6)

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Table 6. Relation of a locus methylation to other locus methylation

	hMLH1	APC	p16	p15	p73
DAP-kin	p=0.816	p=1,00	p=0,17	p=0,128	p=0,519
p73	p=0.431	p=0.317	p=0,392	p=0,97	
p15	p=0.523	p=0.099	p=0.117		
p16	p=0.558	p=0.62			
APC	p=0,168				

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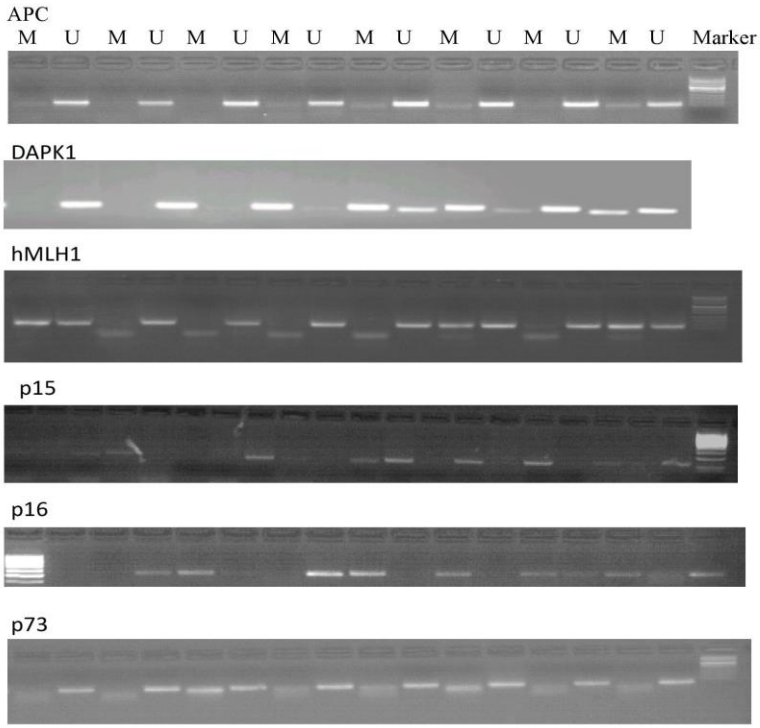

Figure 1. Agarose gel electrophoresis of Polymerase Chain Reaction product.

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