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Hypermethylated promoter profiles for tumour supressor *APC*, p53, *MSH6* and *MGMT* genes in CRC tumours

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Abstract

Background and Aim: Colorectal cancer (CRC) results from a multiple steps of genetic and epigenetic parameters that transform a normal cell into cancerous epithelium. Aberrant DNA methylation is a common epigenomic alteration in carcinogenesis. In the present study was aimed to investigate the possible DNA hypomethylation in three candidate tumour supressor (TS) genes in CRC tumours. Method: Twenty CRC solid tumours were analyzed in the current project by epigenetical profilling of target TS genes. Promoter methylation status was evaluated by MS-MLPA technique in current twenty CRC tumours for target p53, MLH6 and MGMT genes. Results: Results showed alterated methylation profiles for p53, MLH6 and MGMT genes in the current CRC tumours. Increased hypermethylation (HM) at different levels were detected all TS genes that examined in the current results. The HM percentages were; APC (20/100%), p53 (12/60%), MSH6 (12/60%) and MGMT (2/10%) respectivelly. No hypermethlation was detected for both BRCA 1-2 (control) genes in all tumoural samples that examined in the current results. Conclusions: Preliminary results from the current project suggest that altered DNA methylation status takes crucial role in CRC initiation and/or progression. Results need to confirm by large-scale of tumoural samples.

Keywords: CRC tumours, promoter hypermethlation, p53, *MSH6*, *MGMT*, MS-MLPA technique, epigenetic alteration.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common types of cancer with instabil phenotype and its incidence varies in different populations. CRC characterized by sequential accumulation of genetic and epigenetic alterations (Van Engeland, 2011). The interactions between some events such as; histone modifications, DNA methylation, microsatellite instability, chromosomal remodeling and variable non-coding RNAs may cause to epigenetic dysregulation in CRC (Migliore *et al.,* 2011; Farkas *et al.,* 2014; Juo *et al.,* 2014). The structural point mutations, cytogenetics alterations such as; translocations, deletions and epigenetic marks as DNA methylation, histone tail modifications, ubiquitinisation and variable non-coding RNAs expressions are the well known major tirggers in CRC. The investigations on epigenome profiles of solid tumours take crucial role in the potential therapeutic applications for CRC pathogenesis as claimed by Coppedè F. Sloane *et al.,* have claimed that the tumor suppressor gene USP44 is epigenetically inactivated in colorectal adenomas, but this alone is not sufficient to cause aneuploidy in colorectal neoplasia (Sloane *et al.,* 2014). Variable methylated

CpG-islands that are located in or near the promoter region of tumour supressor (TS) genes has been shown to be associated with transcriptional inactivation in a wide spectrum of human cancers (Kuhmann *et al.,* 2014; Tahara *et al.,* 2014; Carmona *et al.,* 2013). Juo *et al.,* have reported the prognostic value of CpG island methylator phenotype (CIMP) and CRC prognosis (Juo *et al.,* 2014).

DNA methylation pattern analysis is a biomarker for non-invasive diagnosis of CRC (Wu *et al.,* 2015). Decreasing the *MTHFR* enzyme function may cause to the global DNA hypomethylation due to lack of the intracellular methyl sources and initiates carcinogenesis process in approximately 25% of the patients have a family history of the disease (Van Engeland *et al.,* 2011; Ozdemir *et al.,* 2012; Rai, 2015). Recent literature findings show that combined effect of genetic and epigenetic alterations transforms the functional cells to carcinomas. Aberant DNA methylation is a common epigenomic alteration in carcinogenesis. The present study aimed to investigate the epigenetic changes (DNA methylation) in four candidate genes in CRC tumours. In the current study, it was aimed to identify the methylation status of tumour suppressor *APC*, p53, *MSH6* and *MGMT* genes in CRC solid tumours. The methylation status measured by MS-MLPA technique in current twenty CRC tumours.

MATERIALS AND METHODS

Patients, Clinical Diagnosis and Laboratory Assessment

In a total of 20 CRC patients; 16 colonal (80%) and 4 rectal (20%); 18 male (90%), 2 female (10%) and the mean age-min-max; 60.72±12.20(48-81) were included in the current report. The tumoral tissue samples were used for tissue specific tumour supressor gene profiling and peripheral blood-EDTA samples from each patients were used for germ-line *MTHFR* gene profiling (data not shown). Tumoural and peripheral blood-DNA samples were obtained during routine diagnosis from CRC patients in Cumhuriyet University Training and Research Hospital by the collaboration of department of Medical Genetics and General Surgery between June 2007 and January 2010. Blood samples were used for genotyping for point mutations of C677T and A1298C markers for *MTHFR* gene and solid tumoural samples were used for epigenetics profiling MS-MLPA method. Informed consent was obtained from all patients of the current cohort.

Methylation Pattern Analysis for Target TS Genes

Biological samples (Peripheric blood containing EDTA and tumoral tissues) from patients were used for high molecular weight genomic DNA isolation. Genomic tumoural DNA were isolated from same type of tissue and purified by the same method in the current study. The total genomic DNA was extracted by the MagnaPure Compact (Roche, Germany) and Invitek kit extraction techniques (Invitek®; Invisorb spin blood, Berlin, Germany) from solid tumours and blood-EDTA samples. SALSA MLPA probemix ME002-C1 Tumour Suppressor - 1 and 2 kits were used for methyl specific epigenetic analysis for target TS genes (SALSA, Lot C1- 0412, MRC-Holland). This ME002-C1 MS-MLPA probemix contains 27 MS-MLPA probes detecting the methylation status of promoter regions of 25 different tumour suppressor (TS) genes. The restriction enzyme *HhaI* was used for the identify the methylated and/or unmethylated samples in the current study.

DNA methylation patterns can be detected by using several methods, including sodium bisulfite sequencing and restriction digestion using methylation-sensitive endonucleases (methyl marker enzymes) such as R.*Msp*1, *Hpa*II and *Hha*I. Target *APC* gene digestions and some suspicious samples were corrected by methyl modifiying enzyme R.*Msp1* digestion. The methyl sensitive enzyme R.*Msp*1 (enzyme could not digest the DNA in the presence of 5-methylcytosine – 5mC instead of cytosine in CpG dinucleotides) was used for some suspicious samples in the current study. In this technique, tumoural genomic DNA samples were amplified by promoter regions specific primers for target TS genes after digestion with a methylation-sensitive restriction enzyme of R.*Msp*1 and compared to the blood samples from healthy individuals.

RESULTS

Presented results show alterated methylation profiles for *APC*, p53, *MLH6* and *MGMT* genes in the current CRC tumours. Tumoural tissue samples from CRC patients were examined in the current study. In a total of 20 CRC patients; 16 colonal (80%) and 4 rectal(20%), 18 male (90%), 2 female (10%) and the mean age-min-max; 60.72±12.20(48-81) were analysed. Most of tumours were in grade 2(13/65%) in the current CRC cohort histopathologically. Results showed four TS genes with the highest average hypermethylated (HM) percentages in the tumoural CRC tissues that examined in the current results (Figures 1 and 4). Various tumour suppressor genes are frequently silenced by promoter hypermethylation in different tumoural tissues, but the same genes are unmethylated in normal solid and blood samples that derived DNA of healthy individuals.

It is not possible to gain a PCR signal in MS-MLPA probe targets a single specific *HhaI* site in a CpG island; if methylation is absent for a particular CpG-site for target TS genes. The *HhaI* digested and/or undigested samples

Figure 1. The MS-MLPA signal generating mechanism. **A:** Hypermethylated inactive gene and generates PCR product due to uncut *HhaI* digestion **B:** Some hypo and/or hypethylated active/inactive genes without PCR product due to active *HhaI* digestion (Modified from ME002-C1 probemix kit, MRC-Holland).

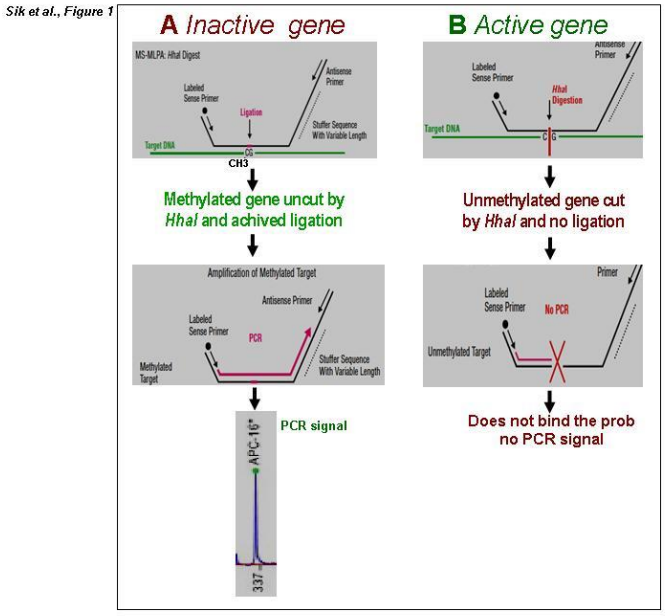


Figure 2. Shows the signals for tumoural MS-MLPA PCR products of target functional TS genes that provided by ME002-C1 probemix kit (MRC-Holland) without *HhaI* digestion (A). All stuided probes were generated PCR signals. Generated signals show the hypermethylated target genes (not all) from tumoural tissue(B). Shows the heterozygous inactive p53 and homozygous inactive TS APC in a solid tumour from one of the current male CRC patient after MS-MLPA analysis (B). Normal apperance of TS p53 and other target genes that undigested by *HhaI* is seen in diagram A.

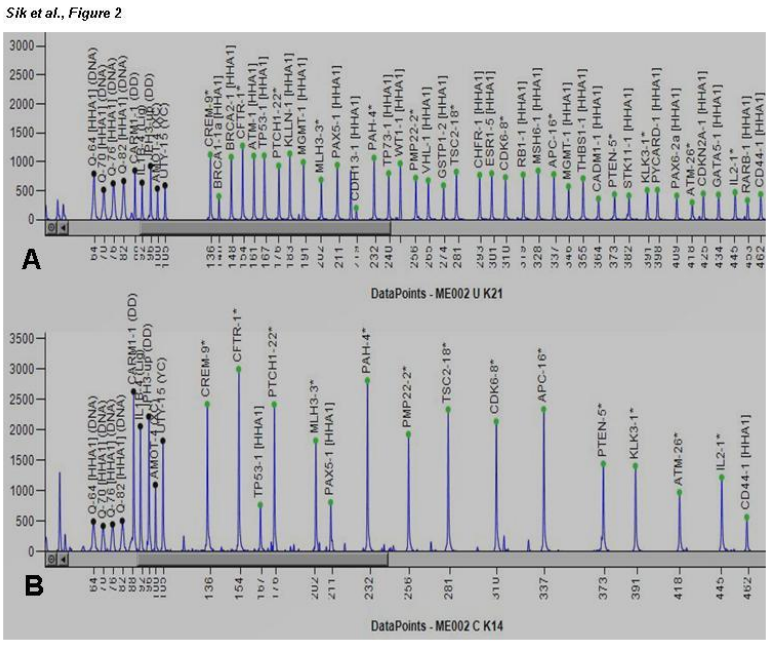
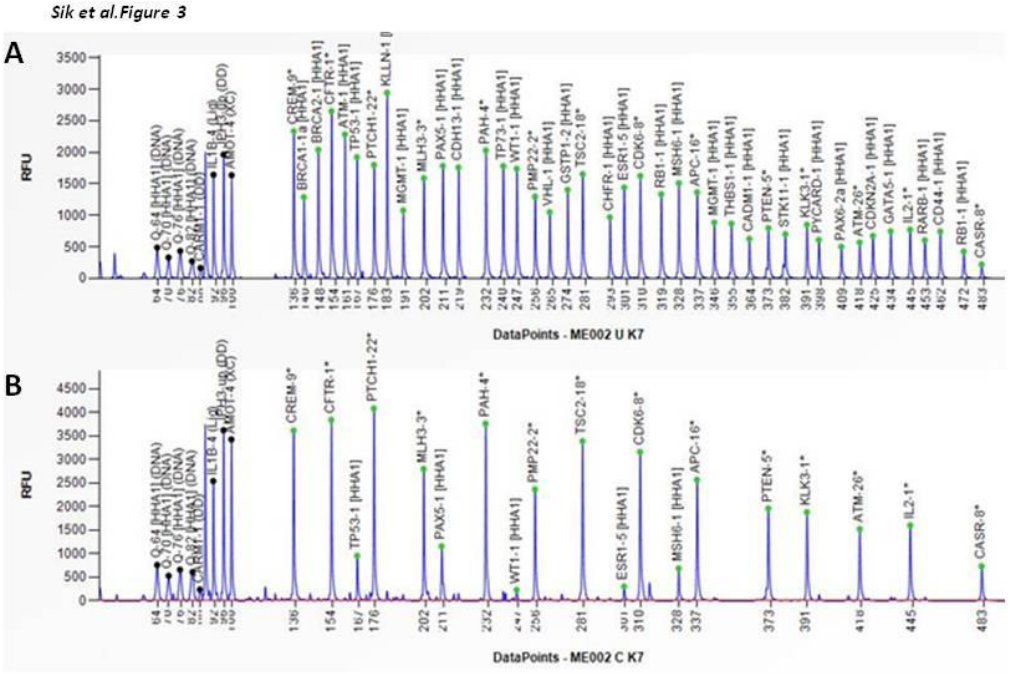


Figure 3. Various target gene inactivation profiles from a case of CRC. Shows the signals for tumoural MS-MLPA PCR products of target functional TS genes that provided by ME002-C1 probemix kit (MRC-Holland) without *HhaI* digestion (A).Normal signal appearances were generated for all TS and other control genes (A). Shows the heterozygous for p53, MSH6 and homozygous inactivation of TS APC in a solid tumour that digested by *HhaI* and *R.Msp1* and analyed after MS-MLPA (B).

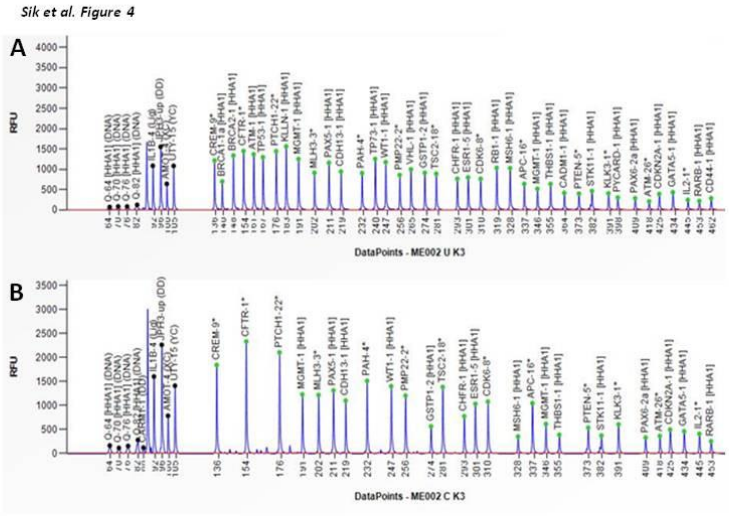


were evaluated due to signal generating system(Figure 1). The HM percentages were; APC (20/100%), p53(12/60%), *MSH6* (12/60%) and *MGMT*(2/10%) for the current studied TS genes respectivelly (Table 1). Heterozygous hypermethylated profiles were detected in five tumoural samples (5/25%) and seven were homozygous (7/35%) for p53 gene in the current results (Table 1). Homozygous hypermethylation profiles in both allelles were detected in a *APC* TS gene (20/100%) in all tumour that examined in the current results. Heterozygous hypermethylated profiles were detected in two tumoural samples (2/10%), two were homozygous (2/10%) for TS *MGMT* and twelve samples were homozygous hypermethylated for TS *MSH6* (12/60%) gene in the current results (Table 1). No hypermethlation was detected for both *BRCA* 1, 2 (control) genes in all tumoural samples that examined in the current results (Table 1).

DISCUSSION

CRC is a consequence of the accumulation of genetic and epigenetic alterations that result in the transformation of normal colonic epithelial cells to carcinomas. Abberant DNA methylation profiles were reported by some researchers in hereditary CRC patients (Sahnane *et al.,* 2015). Hypermethylated promoter profiles in various degree were reported for some tumour supressor *APC*, p53, *MSH6* and *MGMT* genes in the current CRC patients. Recent literature findings showed TS genes inactivation in certain proportions in diffrenet patient cohorts (Sahnane *et al.,* 2015; Khamas *et al.,* 2012; Garajová *et al.,* 2015; XL *et al.,* 2004). Demethylating agent 5-aza-2'-Deoxycitidine were used in CRC treatment for the TS genes reactivation and identified potentially affected genes by genome-wide screening technique (Khamas *et al.,* 2012).

Figure 4. Non-digested, normal appearance (A) and methyl specific RE enzymes digested variable appearance(B) signals in tumoural tissue from a current CRC case. Shows fully active p53, heterozygous for MSH6 and fully inactive MGMT, APC TS genes.



Sik et al. Table 1

Epigenetic Status		Tumour Supressor Gene Types that examined in the current CRC tumours (n:20)					
		APC	**p53**	***MSH6***	***MGMT***	***BRCA1***	***BRCA2***
Active n/%		0/0	8/40	8/40	16/80	20/100	20/100
Inactive n/%	Heterozygous	0/0	5/25	-	2/10	-	-
	Homozygous	20/**100***	7/**35***	12/**60***	2/10	-	-

: Significant

Table 1. The tumour suppressor gene types and functual status due to their promoter methylation degrees in the current CRC tumours

In the current results the solid CRC tissues were identified by MS-MLPA technique for the target TS genes. The ME002-C1 probe mix detecting kit (MRC-Holland) was used for hypermethylated promoter regions of target tumour suppressor genes in the current solid tumours-derived DNA samples. That kit allows that upon digestion, the peak signal obtained in unmethylated samples will be very small or absent. In contrast, when tested on in vitro methylated human DNA, these probes do generate a signal (Figure 1). Results showed alterated methylation status for *APC*, p53, *MLH6* and *MGMT* TS genes in the current CRC patients. Target TS genes were silenced by promoter hypermethylation in the current tumoural tissues with different tumour grades.

The HM percentages were; (20/100%) for *APC*, (12/60%) for p53, (12/60%) for *MSH6* and (2/10%) for the *MGMT* respectivelly. Heterozygous hypermethylated profiles were detected in five tumoural samples (5/25%) and seven were homozygous (7/35%) for p53 gene in the current results. Homozygous hypermethylation profiles in

both allelles were detected in a *APC* TS gene (20/100%) in all tumours that examined in the current results. Heterozygous hypermethylated profiles were detected in two tumoural samples (2/10%), two were homozygous (2/10%) for TS *MGMT* and twelve samples were homozygous hypermethylated for TS *MSH6* (12/60%) gene in the current results. The *BRCA* 1, 2 (control) genes were fully active and hypomethylated profiles in all tumoural samples that examined in the current results. These results summarize that the knowledge of hypermethylation biomarkers for TS genes in CRC allows the epigenomic diagnosis, progression and treatment outcome.

Most of the CRC cases are sporadic but approximately 25% of patients have a familial history. The systemic functional gene *MTHFR* is a one of the trigger molecule in human complex diseases including CRC (Khamas *et al.,* 2012). Gene has pluripotent affect on folate metabolism, cells methyl sources, gene regulation, DNA methylation, maintain the integrity and stability of DNA. In several studies, the 677TT genotype was associated with an increased risk of CRC (Kaneda and Yagi, 2015; Coppedè, 2014; Yan, 2015; Bae *et al.,* 2016). The current results with some of previous literature findings have pointed out the importance of the functional gene mediated CRC progression. As claimed by Perez-Carbonell *et al.,* the epigenetic dysregulation due to silencing TS genes by hypermethylated promoter regions are an important mechanism of colorectal carcinogenesis and allow the early detection of CRC patients (Perez-Carbonell *et al.,* 2015).

We validated that the CpG sites were hypermethylated for various TS genes (*APC*, p53, *MGMT*, *BRCA*1-2 and *MSH*6) in the current results. Results showed that epigenetic influences of TS genes play an important role in the initiation and/or progression of cancer and epigenetic instability may lead to effective prevention strategies for CRC patients. Results need to confirmed by large-scale of tumoural samples.

CONCLUSION

We validated that the CpG sites were hypermethylated for various TS genes (p53, *MSH6* and *MGMT*) in CRC tumours in the current results. Results also show epigenetic influences of TS genes may play an important role in the initiation and/or progression of cancer and that instability lead to effective prevention strategies in CRC theraphy. Results need to confirmed by large-scale of tumoural samples.

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COMPETING INTERESTS

All authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

All authors read and approved the final manuscript. M.S., F.S., and H.K.K.; acquisition of data, performed the bioinformatical work, analyzed the clinical data and designed the clinical experiments, O.O., and E.S; designed the experiments, performed PCR, analyzed the sequencing data, O.O., M.U; interpretation of data, performed the statistical analysis, O.O.; drafted and review the MN supervised the project.

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