Full Length Research Paper

Hypermethylated promoter profiles for tumour suppressor 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 suppressor (TS) genes in CRC tumours. Method: Twenty CRC solid tumours were analyzed in the current project by epigenetical profiling 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 altered 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%) respectively. 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 hypermethylation, 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 triggers 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 suppressor (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. Ablant 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 suppressor 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 (Peripheral blood containing EDTA and tumoral tissues) from patients were used for high molecular weight genomic DNA isolation. Genomic tumoral 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 Hhal 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 bisulphite sequencing and restriction digestion using methylation-sensitive endonucleases (methyl marker enzymes) such as R.Msp1, HpaII and HhaI. Target APC gene digestions and some suspicious samples were corrected by methyl modifying enzyme R.Msp1 digestion. The methyl sensitive enzyme R.Msp1 (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 primer regions specific primers for target TS genes after digestion with a methylation-sensitive restriction enzyme of R.Msp1 and compared to the blood samples from healthy individuals.

RESULTS

Presented results show altered 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 Hhal site in a CpG island; if methylation is absent for a particular CpG-site for target TS genes. The Hhal 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 studied 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 appearance 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 $H_{ha}\ell$ 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 $H_{ha}\ell$ and $R_{Msp1}$ and analyzed after MS-MLPA (B).

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 alleles were detected in an 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 hypermethylation 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 suppressor APC, p53, MSH6 and MGMT genes in the current CRC patients. Recent literature findings showed TS genes inactivation in certain proportions in different patient cohorts (Sahnane et al., 2015; Khamas et al., 2012; Garajová et al., 2015; XL et al., 2004). Demethylating agent 5-aza-2'-Deoxycytidine 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.

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

<table>
<thead>
<tr>
<th>Epigenetic Status</th>
<th>Tumour Suppressor Gene Types that examined in the current CRC tumours (n=20)</th>
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<tbody>
<tr>
<td></td>
<td><strong>APC</strong></td>
</tr>
<tr>
<td>Active n/0%</td>
<td>0/0</td>
</tr>
<tr>
<td>Inactive n/0%</td>
<td>Heterozygous</td>
</tr>
<tr>
<td></td>
<td>Homozygous</td>
</tr>
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</table>

*: Significant

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 altered 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** respectively. 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 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, BRCA1-2 and MSH6) 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 therapy. Results need to confirmed by large-scale of tumoural samples.

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