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# DNA methylation patterns as molecular biomarkers: an overview in colorectal cancer

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## SUMMARY

DNA methylation patterns may be used as innovative biomarkers for some pathologies including cancer. They show a great accessibility due to their stability and presence in body fluids. In addition, these epigenetic modifications may be used as prognosis markers or therapeutic targets. Concretely, in colorectal cancer (CRC), the third most common cancer in the world in both men and women, a continuous genetic and epigenetic alteration occurs during neoplastic transformation in colonic epithelial cells. This accumulation of alterations leads to the transformation of normal colonic epithelial cells to adenocarcinomas, and these genetic alterations are promoted by aberrant methylation of promoter regions and subsequent gene silencing. Many of these genes have been reported to be methylated in the tissue, plasma and stool of CRC patients, suggesting that they may have great potential to be used as biomarkers for the early detection of CRC. The aim of this study is to review changes in the methylation pattern of the genes that can be used as novel diagnostic and prognostic biomarkers of CRC.

**Key words:** Colorectal cancer – Biomarkers – DNA Methylation – Prognosis

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## INTRODUCTION

DNA methylation, which may be detected in most of the CpG sequences of the genome, induces the apparition of cancer through hyper- or hypomethylation of the promoters of genes implicated in essential cell signaling pathways in the mechanisms of cell cycle control (Verma and Kumar, 2017). DNA hypermethylation decreases gene expression while DNA hypomethylation influences chromosome stability (Reis et al., 2017). Abnormal methylation of promoters could result in silencing of tumor suppressor genes resulting in the proliferation of cancer (Lakshminarasimhan and Liang, 2016). The possibility to detect DNA methylation in body fluids is a promising approach for cancer diagnosis and prognosis (Lissa and Robles, 2016; Wang et al., 2017)

Aberrant methylation phenomena that alter gene expression without changing their DNA sequence contribute to the development of colorectal neoplasia (Galanopoulos et al., 2017). Classically, colorectal cancer (CRC) has been associated to age (Li et al., 2015a), alcohol (Arranz et al., 2012), obesity (Gribovskaja-Rupp et al., 2011) inherited genetic conditions (Rustgi, 2007) and type 2 diabetes (Bae et al., 2015). Early detection of colorectal polyps by CRC screening tests is effective in reducing CRC mortality (Hamzehzadeh et al., 2017). 38% of CRC patients have shown a mean of 5-year survival rate and 17% after 10 years (Navarro -Freire et al., 2015). Invasion of the tumor through the bowel wall and the presence of nodal metasta-

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ses are two prognostic factors studied in patients with CRC. Bowel obstruction occurs in 15-20% of patients with CRC, and is related to an increase of the postoperative morbidity, mortality and a decrease of the survival rate (Repici and de Paula Pessoa Ferreira, 2011). The alteration of the DNA mismatch repair (MMR), a system for repairing mis-incorporation of bases or their insertion or deletion during DNA replication and recombination (Perazzoli et al., 2017), has been shown to be relevant as a prognostic factor in cancer, including colon cancer (Zaanan et al., 2017). 15% of CRC confirm MMR deficiency, as a result of extensive microsatellite instability (MSI) (Poynter et al., 2008), specially caused by CpG island hypermethylation of cancer-related genes (Fang et al., 2012; Sangplod et al., 2014). MSI has been associated with a better prognosis regarding microsatellite-stable tumors (MSS) (Sinicrope and Sargent, 2012; Popat and Houlston, 2005). The continuous genetic and epigenetic alterations leads to the transformation of normal colonic epithelial cells into adenocarcinomas (Grady and Carethers, 2008), and these genetic alterations are promoted by aberrant methylation of promoter regions and subsequent gene silencing (Sangplod et al., 2014). Regarding the above methylated promoters and their close relationship with cancer prognosis and survival, new treatments have been assayed. For example, in vitro use of 5-aza-2'-deoxycytidine has been used to demethylate the MGMT promoter activity in glioblastoma cell lines (Perazzoli et al., 2015). Several genes such as CDKN2A/p16, MGMT, MLH1, GTAT5, SNCA, FBN1, SFRP1, WIF-1 and SEPT9 among others have been reported to be methylated in the tissue, plasma and stool of CRC patients, suggesting that they may have great potential to be used as biomarkers for the early detection of CRC (Frattini et al., 2008; Lee et al., 2009, Zhang et al., 2015; Li et al., 2015b; Salehi et al., 2012; Rawson and Bapat, 2012; Toth et al., 2014) (Figure 1). These epigenetic alterations in CRC may be used as reliable biomarkers for diagnostic but also for predictive purposes to improve therapeutic response (Puccini et al., 2017). The possibility to detect abnormal DNA methylation patterns in samples from blood, feces or tissue are the basis of these clinical ad-

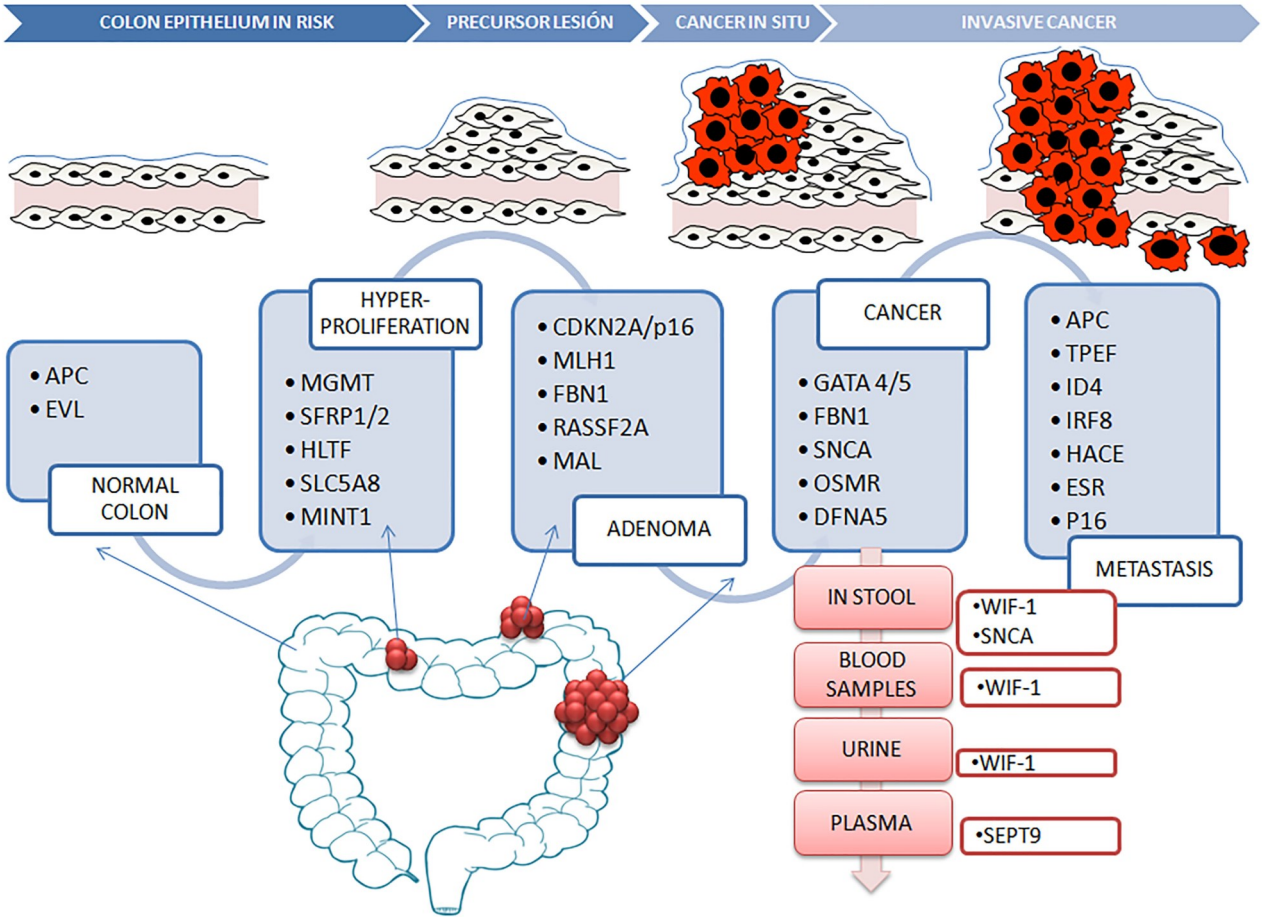


**Fig 1.** Potential DNA methylation biomarkers in colon cancer. Colon cancer can be diagnosed using multitude of DNA methylation biomarkers along the process of carcinogenesis. Tumor-specific methylated DNA can be situated in colorectal tissues in different stadiums of the disease, in the same way that in samples of blood, plasma or urine. A small amount of tumor-specific methylated DNA is in stool samples due to the easy transportation from the colon into the rectum. Free tumor DNA as well as cancer cells lacking the capacity to metastasize can also access the circulatory system. Potential DNA methylation markers are shown in preneoplastic lesions and metastasized CRC.

vances (Galanopoulos et al., 2017).

### *DNA METHYLATION MARKERS IN CRC*

Neoplastic transformation is induced by continuous genetic and epigenetic alterations in colonic epithelial cells (Kim et al., 2010). The methylation of some gene promoters may be critical to the development of some tumors. It has been demonstrated that aberrant methylation contributes to colon carcinogenesis inducing alteration in the expression of several genes which play a relevant function in specific oncogenic pathways (Dimberg et al., 2013; Furlan et al., 2013). DNA methylation is regulated by a family of DNA methyltransferase enzymes (DNMT1, DNMT3A, and DNMT3B) responsible for transferring the methyl groups from S -adenosylmethionine to the 5 position of cytosine bases in the CpG- island (Herman and Baylin, 2003). DNMT1 looks after the correct replication of genomic DNA methylation patterns during the S phase of the cell cycle (Miremadi et al., 2007; Ferguson-Smith and Greally, 2007), while DNMT3A and DNMT3B perform the new methylation of DNA and its maintenance (Okano et al., 1998). These 3 DNMTs are overly expressed in several tumor types, such as the colon, breast, ovarian, kidney and gliomas (Kar et al., 2014; Arai et al., 2006; Deplus et al., 2002; Rajendran et al., 2011; Subramaniam et al., 2014). The inhibition of DNMT1 expression decreases the cellular transformation and the cellular growth (Chik and Szyf, 2011). The overexpression of DNMT3B is linked to aberrant DNA methylation in cancer and promotes intestinal tumurigenesis in mouse models (Linhart et al., 2007), and its depletion decreases the genomic methylation in colon cancer cells (Rhee et al., 2002). Successive accumulation of genetic mutations that result to CRC may induce diverse tumor populations with differences in chromosomal instability (CIN), KRAS, BRAF, MSI and the CpG island methylator phenotype (CIMP) CRCs with high degrees of methylation have different epidemiology, histology and molecular features, by which these tumors may be classified in distinct groups (Hagland et al., 2013). In fact, it is possible to consider a subgroup of CRC with differentiated pathological, clinical, and molecular features and with different hypermethylated gene clusters. So, the CIMP is related to the emergence of CRC and may be used as a predictive biomarker (Shima et al., 2011).

### *CPG ISLAND METHYLATOR PHENOTYPE*

The CIMP is widely detected in CRCs and it is related with the silencing of determined genes by methylation in their promoter (Issa, 2004). This hypermethylation promoter performs an important role in colorectal tumurogenesis (Li et al., 2014; Hinoue et al., 2012; Kang et al., 2015). The CIMP testing is used for prognosis and detection of cancer and residual diseases, which has a great clinical applicability (Heichman and Warren, 2012). For example, CIMP detection allows discrimination between serrated adenomas (CIMP-positive) and tubular adenomas (CIMP-negative) (Langner et al., 2015). That suggests that CIMP CRCs may arise from serrated adenomas, so the early detection of CIMP in the early adenomas is of great clinical relevance (Yano et al., 2011; Dhir et al., 2011). CIMP-positive has been associated with serrated polyps and CIMP-negative with tubular ones. Thus, immediately when the biopsy of the polyps is carried out, if they are serrated, the patient will be diagnosed with CIMP-positive CRC; and if it is tubular, CIMP-negative. Only with the pathologist identifying these types can the oncologist prescribe a treatment for CIMP-positive or negative CRC (Kim et al., 2008; Langner et al., 2015). According to the level of CpG-island methylation (Dhir et al., 2011; Itzkowitz et al., 2008; Yano et al., 2011; Toyota and Issa, 1999), the CRC patients are classified as one group with rare methylation (CIMP-negative) or with aberrant methylation (CIMPhigh). Berg et al. (2014) studied the CIMP status of 64 biopsies by means of 2 different panels. The Ogino gene panel, composed of the genes CDKN2A, MLH1, SOCS1, RUNX3, NEUROG1, CRABP1, IGF2 and CACNA1G; and the Weisenberger, a panel composed of NEUROG1, CACNA1G, SOCS1, RUNX3 and IGF2 samples (Itzkowitz et al., 2008) showed CIMP status constant and independent from the panel used (34 as CIMP-negative, and 13 as CIMP-high). They tested a number of methylation sites for the genes: 3 probes each for RUNX3, CACNA1G and IGF2; 4 probes each for MLH1, CRABP1, SOCS1 and CDKN1A; and 6 probes for NEUROG1. A greater increase of levels of expression was revealed in CIMP-high rather than in CIMP-negative samples. Only four of these 31 probes did not show a difference in the CIMP-high samples. Other studies with a CIMP panel marker composed of MLH1, MGMT, CDKN2A/p16, APC, MINT1, MINT31, and RUNX3 genes, revealed a higher percentage of CIMP-low patients with respect to CIMP-high and CIMP-negative. Also, CIMP-high was significantly associated with worse prognosis compared to CIMP-negative and CIMP-low (Li et al., 2014). The relationship between CIMP and MSI status has been exhibited in a recent population-based cohort study. CIMP-high/MSI status was related to an important increase in colon cancer-specific mortality, while CIMP-high/MSS cancers had a poor outcome (Kang et al., 2015). Besides, BRAF and KRAS have also been associated with CIMP. Mutation of BRAF is more frequent in CIMP-high, while KRAS is more frequent in CIMP-low (Hinoue et al., 2012; Siraj et al., 2014). In the study of Siraj et al. (2014), 2.5% CRC showed BRAF gene mutations, of which 90% were BRAF V600E somatic mutation. These findings were associated with CIMP, MSI status, right-sided tumors and high expression of Ki67. Recently, CIMP status was associated with inferior outcome for CRCs, used to stratify the poor prognostic patients with MSS BRAF mutated tumors (Vedeld et al., 2017) and combined with MLH1 or p16 INK4a methylation to determine CRC patient prognosis (Saadallah-

Kallel et al., 2017). We will outline some of the most currently CRC methylation molecular biomarkers (Table 1), and we will also illustrate their diagnostic therapeutic and prognostic applications.

### *CDKN2A/p16*

Cyclin-dependent kinase inhibitor (CDKN2A/p16) is a tumor suppressor that binds to CDK4 and CDK6 in the G1-S transition. Several cancer types have shown CDKN2A promoter methylation and therefore silencing gene (Herman et al., 1995). A meta-analysis of 27 cohort studies of CRC patients (Caucasian and Asian populations) associated CDKN2A/p16 promoter methylation with pathological characteristics of CRC such as tumor, localization, size, Dukes stage, lymph node metastasis, nodes, histologic grade and metastasis stage.

Shima et al. (2011b) studied the methylation status of CDKN2A/p16 promoter in tumor specimens from patients with CRC. 30% of tumors showed methylation at the CDKN2A/p16 promoter and 25% of them loss of CDKN2A/p16 protein expression, corroborating similar findings of other studies (Malhotra et al., 2010; Lam et al., 2008).

CDKN2A/p16 is more frequently silenced by CpG promoter methylation in CRC (Shima et al., 2011b; Bihl et al., 2012; Furlan et al., 2013) and is often included in the panel of markers used to assess the CIMP phenotype, which is related to metastatic cancer (Ogino et al., 2009). However, the panel of markers used to evaluate the CIMP phenotype usually is different among studies, hindering direct comparison. For this reason more studies based on identical CIMP and cancers are needed (Xing et al., 2013).

Serum samples from patients with CRC with adenoma and healthy volunteers were used to determine methylation status of CDKN2A/p16 promoter by methylation-specific PCR using immunoprecipitation with anti-histone antibodies. 81% of CRC patients displayed CDKN2A/p16 promoter methylation, but no CDKN2A/p16 promoter methylation was found either in the serum of adenoma patients or volunteers. Also, this hypermethylation was linked to tumor invasion. 83% of CRC patients with CDKN2A/p16 methylated promoter displayed venous invasion and 60% were without lymphatic invasion (Sakamoto et al., 2010). Hence, the study of methylation states of CDKN2A/p16 promoter could be a powerful biomarker for CRC. On the other hand, the association between CDKN2A methylation with MSI, BRAF mutation and higher tumor grade is known, but not with KRAS mutation (Bihl et al., 2012). Nonetheless, Kohonen-Corish et al. (2014) discovered a relationship between CDKN2A/p16 methylation and KRAS mutations. In a cohort study of 381 rectal cancers, 39% and 20% of them showed KRRAS mutations and CDKN2A/p16 methylation, respectively. This relationship was correlated with worse overall and cancer-specific survival.

Frattini et al. (2008) studied the methylation status of CDKN2A/p16 promoter in patients with primary and recurrent CRC. Fluorescent-methylation specific PCR was used to determine the CDKN2A/p16 promoter methylation status. Plasma samples obtained at the time of surgery showed the same levels of CDKN2A/p16 promoter methylation as in the primary tumor. The analysis of relapse-free patients showed absence of CDKN2A/p16 promoter methylation in all cases. And finally, patients that appeared disease free showed absence of CDKN2A/p16 promoter methylation. However, DNA abnormalities were displayed in the plasma of patients with metastasis, recurrence or carcinomatosis. KRAS mutations were linked to CDKN2A/p16 promoter methylation. These findings suggested CDKN2A/p16 promoter methylation could be useful as a clinical biomarker to detect primary CRC along with metastases and/or recurrences. By contrast, Sugai et al. (2017) recently demonstrated that others genes showed high methylation and may be useful to predict cancer risk of surrounding normal mucosa (SFRP1 and SFRP2I methylated - crypts type I- and DKK2 and mir34b/c methylated -crypts type II-) while CDKN2A/p16 was not methylated (normal crypts or type III).

**Table 1.** Principal DNA methylation biomarkers in blood and stool samples of CRC patients

| Biomarkers | Sample | References |
|---|---|---|
| *CDKN2A/p16* | Blood | Sakamoto et al., 2010 |
| MGMT | Blood and stool | Lee et al., 2009 |
| *hMLH1* | Blood | Gradi net al., 2001 |
| *GATA4* | Stool | Hellebrekers et al., 2009 |
| *SNCA* | Stool | Li et al., 2015 |
| *FBN1* | Tissue | Lind et al., 2011 |
| SFRP1/2 | Blood and stool | Vatandoost et al., 2016 |
| *WIF* | Blood and stool | Amiot et al., 2014 |
| *SEPT9* | Blood | Warren et al., 2011 |
| *ALX4* | Blood | Ebert et al., 2006 |
| *NEUROG1* | Blood | Herbst et al., 2011 |
| *VIM* | Stool | Chen et al., 2005 |
| *NDRG4* | Stool | Melotte et al., 2009 |

**ALX4**: ALX homeobox 4; NEUROG1: Neurogenin 1; VIM: vimentin; NDRG4: N-myc downstream regulator gene 4. Others methylated genes are indicate in the text.

### *MGMT*

O6-methylguanine-DNA methyl-transferase (MGMT) is a DNA-repair enzyme, a regulator of DNA mismatch repair, that protects cells from alkylating agents by taking out 5 adducts from the O6 position of guanine (Gerson, 2004). Methylation of MGMT promoter, and secondary loss of expression of MGMT, is usual in human cancers, including CRC. Shima et al. (Shima et al., 2011a), detected 38% of MGMT promoter hypermethylation and 37% of loss of MGMT expression in 855

samples of CRC. Also, MGMT promoter methylation in normal colorectal mucosa adjacent to cancerous tissue has been correlated with the CRC (Kim et al., 2010). Recently, Sambuudash et al. (2017) confirmed the increase in the MGMT promoter methylation and other 14 genes (DSFRP1, MYOD1, CD8a, SPOCK2, ABHD9, etc) in CRC samples in comparison to adjacent normal mucosa samples. Farzanehfar et al. (2013), studied the levels of the MGMT promoter in normal colorectal mucosa adjacent to cancerous tissue with and without methylation of MGMT promoter. Greater levels of methylation of the MGMT promoter were observed in the tumors with methylated MGMT promoter than in the tumors without methylated MGMT promoter. Recently, fourteen studies were included in a meta-analysis after screening 120 articles. The overall survival of CRC patients was found not to be significantly associated with MGMT methylation, but the frequency of MGMT methylation was significantly higher and increased in CRC more so than in normal tissues (Li et al., 2015c).

Also, hypermethylation of the promoter region of MGMT is inversely related to Dukes' stage in CRC patients (Nagasaka et al., 2003). In an analysis performed by Nagasaka et al. (2008), the MGMT methylation status was significantly related to the stage and differentiation of CRC, suggesting that the MGMT methylation status was strongly related to tumor recurrence and hence had the potential to be a useful prognostic factor for CRC patients. However, compared to other CIMP specific, such as CDKN2A/p16 and MLH1, the sensitivity and specificity of MGMT methylation for CIMP-High is greater (Kawasaki et al., 2008). Presently, methylation status of MGMT promoter has been associated to inflammatory bowel disease. 60% of patients with this disease develop CRC displaying a 10-15% mortality rate (Goel et al., 2011; Herrinton et al., 2012). Mokarram et al. (2015) studied the MGMT promoter methylation status in tissue from inflammatory bowel disease patients and healthy patients. The findings showed 71% methylation of MGMT promoter in inflammatory bowel disease patients, and no methylation was found in normal tissues. Moreover, loss of MGMT expression is related to G to A mutations in KRAS gene (Esteller et al., 2000), BRAF-V600E mutation and MSI-high status (Weisenberger et al., 2006). A comparative study between KRAS wild type and KRAS mutated carcinomas demonstrated KRAS mutated carcinomas more frequently, being associated with MGMT methylation and different MSI status, but no difference in overall survival was observed (Rosty et al., 2013).

Detection of MGMT promoter methylation in plasma and stool samples from CRC patients has been correlated to MGMT promoter methylation in their tumor tissue (Taback et al., 2006; Kang et al., 2011; Azuara et al., 2010; Lee et al., 2009). MGMT promoter methylation was detected in stool sample from CRC (52.5%), colon adenomas (41.7%), hyperplastic polyps (15.8%) and was undetected in healthy controls (Kang et al., 2011), suggesting MGMT promoter methylation as a clinical biomarker for early detection of CRC.

In fact, a recent study using 50 patients demonstrated that that both blood and tumor tissue MGMT and ERCC1 methylation were associated with non-metastatic cancer rectum and proposed this biomarker for the diagnosis of malignant tumors of the rectum (Shalaby et al., 2017). Recently, PCR digital to determine MGMT promoter methylation in metastatic CRC patients has been used to enhance patient selection in relation to temozolamide treatment (Sartore-Bianchi et al., 2016).

***MLH1***

Somatic MutL homolog 1 (MLH1) is a regulator of DNA mismatch repair, with an significant role in the development of sporadic CRC and usually included in CIMP marker sets of promoter CpG island (Toyota et al., 1999). Aberrant methylation of CpG sites of MLH1 promoter is included in 60% of CIMP-high CRCs (Ogino et al., 2007) and is correlated with loss of MLH1 protein expression (Deng et al., 1999), advanced ages and the female gender (Thiel et al., 2013). Hokazono et al. (2014) detected MLH1 methylation in 9.6% of CRCs, of which 31.6% were classified as CIMP-high and 5.3% as CIMP-low, and no MLH1 methylation for CIMP-negative tumors. These findings suggest the link of CIMP- high with aberrant methylation of MLH1. Also, single-nucleotide polymorphism of MLH1 gene has been correlated to MLH1 promoter methylation. Studies concerning single- nucleotide polymorphism of MLH1-93G/A genotypes A/A, G/A and G/G) revealed that only MLH1-93G/A was significantly correlated to MSI-High, loss of MLH1 expression and MLH1 promoter methylation in CRC (Whiffin et al., 2011; Miyakura et al., 2014).

De Vogel et al. (2009) researched hypermethylation of MGMT and MLH1. Only 10% of patients showed hypermethylation of both, it being higher in women than in men for MLH1. The MLH1 promoter hypermethylation was more often in proximal colon tumor, and the patients with positive family history of CRC exhibited a greater MGMT and/or MLH1 methylation. Similar findings were found in the comparative study between germinal and somatic MLH1 promoter methylation. The prevalence of MLH1 promoter methylation was less in germinal than in somatic. Somatic MLH1 promoter methylation frequency was associated with proximal colon, female gender and MSI CRC. Neither germinal nor somatic MLH1 promoter methylation was associated with overall survival of CRC (Wang et al., 2014b). In fact, Fu et al. (2016) correlated the combination of CIMP and the methylation status of MLH1 with patients with CRC (stage II). Patients with CCIMP+/MLH1 unmethylated tumors showed the poorest clinical evolution. In addition, some authors included MLH1 in a group of genes (type III) not frequently methylated in normal crypts and with a low capacity to predict cancer risk (Sugai et al., 2017). Ma et al. (2017) have recently demonstrated that MLH1 methylation could

be associated to vascular invasiveness not only in CRC but also in lung cancer patients.

Within CRCs, MLH1 gene has been closely related to hereditary non polyposis colon cancer, or Lynch syndrome, an inherited syndrome which effects up to 5% of all CRCs (Hegde et al., 2014). Crucianelli et al. (2014) showed that Lynch syndrome patients (36%) displayed abnormal MLH1 promoter methylation. In addition, a 15% of the CRCs analyzed by Moreira et al. (2015), showed MLH1 promoter hypermethylation. BRAF mutant carcinomas are correlated with methylation of the MLH1 promoter and therefore with CIMP (Vasen et al., 2013). In fact, tumor BRAF V600E mutation and MLH1 promoter methylation may be predictors of negative MMR mutation status in CRCs (Parsons et al., 2012). In a study of 33 patients with CRC, 21.2% of them showed BRAF mutation/ MLH1 promoter methylation, suggesting sporadic cancers; and 51.5% of them showed BRAF no mutation/MLH1 promoter no methylation, indicating Lynch syndrome (Kim et al., 2014). In addition, Farchoukh et al. (2016) showed that MLH1-hypermethylated BRAF wild-type colorectal carcinomas can harbor KRAS mutations.

Grady et al. (2001) detected methylated hMLH1 promoter DNA in the serum of patients with microsatellite unstable colon cancer. Lee et al. (2009) determined the methylation status of MLH1 promoter in plasma of CRC patients by MSP, and these findings were similar to their tumor tissue. Also, MLH1 promoter has been detected in stool samples from CRC patients, being significantly higher in disease-free patients compared with adenoma patients (Elliott et al., 2013; Petko et al., 2005).

***GATA5***

GATA5, a member of the GATA family of proteins, is a zinc-finger transcription regulatory factor with an important role in cell lineage specification and cell differentiation during embryonic development (Molkentin, 2000; Morrisey et al., 1997). Also, GATA5 is a potential tumor suppressor whose methylation has been associated to several cancers (Peters et al., 2012; Wang et al., 2014a; Sacristan et al., 2014; Rankeillor et al., 2014), especially CRC. Methylation status of GATA5 and GATA4 promoters has been studied in colorectal mucosa tissue of CRC patients and patients without cancer. GATA5 and GATA4 promoter methylation were detected in 79% and 70% of CRC tissues, respectively. No correlation was detected between GATA4/5 promoter methylation and MSI, V600E BRAF mutation, tumor- node-metastasis stage (TNM), tumor location, gender, age at diagnosis, histologic type, or grade of differentiation (Hellebrekers et al., 2009). Nonetheless, Worthley et al. (2011) found a relation among GATA5 promoter methylation, age and rectal mucosal proliferation.

GATA5 promoter methylation (Zhang et al., 2015) has been observed in plasma of CRC patients (61.4%) and patients with adenoma (43.33%) by MSP. Only 21.28% was detected in plasma samples of control patients. Also, GATA5 promoter methylation was significantly correlated with increased tumor differentiation status, tumor node metastasis stage and lymph node metastasis. Hellebrekers et al. (2009) analyzed promoter methylation of GATA4/ in CRC patients using both tissue and fecal DNA. In addition, GATA4/5 was overexpressed in human CRC cell lines to determine its potential as tumor suppressor. Results showed a significant inhibition of colon cancer cell proliferation by GATA4/5 and that their methylation in fecal DNA may be of interest for CRC detection.

***SNCA***

Synuclein alpha (SNCA) is a presynaptic protein highly expressed in the vertebrate brain and strongly linked to Parkinsons's disease and Down syndrome (Pihlstrom et al., 2015; Ramakrishna et al., 2016). Synuclein alpha promoter hypermethylation and loss of expression has also been consequently associated to CRC, whereas normal mucosa has rarely been detected as being methylated (Li et al., 2015b; Lind et al., 2011). In a study of 74 CRC patients, SNCA promoter methylation was detected in 54 of them, but not in the healthy controls subjects (Lind et al., 2011). Similar findings were detected in tissue and in stool samples from CRC patients. 71% CRC tissue samples and 1.1% matched normal mucosa tissue samples showed SNCA promoter methylation, which was related to SNCA promoter methylation detected in stool samples (69.66% CRC and 0% in healthy ones). No correlation was found in overall methylation with age, gender, tumor location, pathological pattern, or Dukes' stage (Li et al., 2015b).

***FBN1***

Fibrillin-1 (FBN1) gene, a member of the fibrillin family, encodes the major component of extracellular microfibrils, and is highly linked to Marfan syndrome (Groth et al., 2016; Wang et al., 2015). At present, the methylation of its promoter has been related to cancers such as non-Hodgkin lymphomas, prostate, ovarian and mainly CRC (Wang et al., 2005; Bethge et al., 2014). FBN1 promoter methylation has been identified in 81% CRC, 68% in adenoma and 2% in normal mucosa, and has been related to loss of expression, tumor size, MSI and BRAF mutations (Lind et al., 2011). Also, FBN1 promoter methylation is found in CRC tissue but not in normal tissue. Li et al (Li et al., 2015b) found FBN1 promoter methylation in 78.7% of CRC tissue samples and 3.4% of normal mucosa, and compared these findings to methylation status of FBN1 promoter in CRC stool samples. 70.8% of CRC stool samples were methylated and only 6.7% of stool for normal individuals. However, no correlation was found in overall methylation with age, gender, tumor location, pathological pattern, or Dukes' stage. Similar results were found by Gou et al (Guo et al., 2013), advancing the detection of hypermethylation status in the promoter of FBN1

as a highly specific and sensitive noninvasive biomarker for CRC.

***SFRP1***

Secreted Frizzled-related protein 1 (SFRP1) gene, belonging to the SFRP family, encodes an antagonist protein of the Wnt signaling pathway, highly activated in CRC and with an important role in tumorigenesis (Suzuki et al., 2004). Its methylation status has been related to human cancers, appearing as a novel tumoral biomarker (Andresen et al., 2015; Li et al., 2015d). Meta-analysis of 8 cohort studies (942 CRC patients) revealed a significantly higher prevalence of SFRP1 promoter methylation in CRC tissues than those of normal, adjacent, and benign tissues (Li et al., 2015d). SFRP1 promoter methylation and both the consequence downregulation of SFRP1 as a tumor suppressor gene and the loss of SFRP1 expression may collaborate to the activation of the Wnt signaling pathway (Salehi et al., 2012; Rawson et al., 2011). Salehi et al. (2012) found lower SFRP1 expression in CRC tissue (62.8%) than in normal tissue (95%), which was related with deep invasion, left lesion site and advanced TNM stage. Similar results were found by Dallol et al. (2012), who also associated metastasis rectal cancer, male gender and MSI-high to SFRP1 promoter status. Recently, hypermethylated PPP1R16B, SFRP1 and SYNE1 were detected in CRC tissue in comparison to normal tissues although SFRP1 hypermethylation was also detected in adenomas (Galamb et al., 2016).

Detection of SFRP1 promoter methylation in serum (Lyu et al., 2014) and in stool of CRC patients is proposed as a non-invasive genetic screening method for CRC (Salehi et al., 2012; Zhang et al., 2007). Recent studies still propose the detection of SFRP2 promoter methylation in serum and stool of CRC patients as screen methods for early detection of CRC (Zhang et al., 2015; Vatandoost et al., 2016). Studies of Barták et al. (2017) corroborated that methylation of SFRP1, SFRP2, SDC2, and PRIMA1 promoter sequences appears in the plasma samples from patients with CRC and adenoma patients. When applied this panel of genes, both colorectal adenocarcinomas and adenomas could be distinguished from controls with a 91.5% and 89.2% of sensitivity, respectively.

***WIF-1***

Wnt inhibitory factor-1 (WIF-1) gene encodes a secreted glycoprotein antagonist of Wnt ligands and therefore inhibits the Wnt signaling pathway (Aguilera et al., 2006). WIF-1 is often silenced by promoter CpG island hypermethylation in several cancers (Yang et al., 2010), and frequently in CRC and especially in serrated adenoma as showed Fang et al., (2014). In this study a 34.5% of sessile serrated adenoma and a 55.2% of traditional serrated adenoma showed methylation of the WIF-1 promoter. This status of methylation and loss gene expression were correlated with distant metastasis and vascular invasion (Abdelmaksoud-Dammak et al., 2014), although several studies corroborate the relationship between WIF-1 promoter methylation and loss expression (Yang et al., 2010; Taniguchi et al., 2005). A study including CRC cell lines, carcinomas, nonpolypoid and polypoid adenomas showed an increase in the methylation of SFRP2, WIF-1, DKK3 and SOX17 (four Wnt-antagonists) in the majority of samples compared to controls (Voorham et al., 2013).

Recent studies have been able to detect WIF-1 promoter methylation in serum, urine and stools samples from CRC patients. Amiot et al. (2014) detected higher WIF-1 promoter methylation in stool (19.3%), in serum (26.7%) and in urine (32.6%) samples from CRC patients than that of the control individuals. Other studies corroborated the use of WIF-1 promoter methylation in stools and plasma samples as a novel and non-invasive biomarker of CRC (Lee et al., 2009; Zhang et al., 2014).

***SEPT9***

Septin 9 (SEPT9) gene belongs to SEPT family, which encodes a group of GTPbinding proteins with an important role in several cellular processes (Hall and Russell, 2004). SEPT9 promoter methylation is related to CRC (Mitchell et al., 2014) and is used as an important biomarker to detect CRC with high efficacy easily in plasma (Toth et al., 2014, Kang et al., 2014; Heichman, 2014; Potter et al., 2014; Gyparaki et al., 2013). Although it has been not been associated with neither tumour recurrence nor cancer-specific survival, it has been associated with cancer specificity (Tham et al., 2014). Ahmed et al. (2012) detected higher methylation in CRC (82%) and adenomas (88%) than in normal mucosa tissue (3%).

Similar findings were detected by Toth et al. (2014): adenomas (100%), CRC (97.1%) tissue and only 4.2% in healthy tissue. Septin 9 methylated DNA has been also detected in blood of patients with CRC (Warren et al., 2011). Semaan et al. (2016) showed that SEPT9/SHOX2 methylation may help to distinguish CRC and adenomas from advanced and non-advanced adenomas and inflammatory colonic tissue. Compared to plasma samples, 30.8% was detected from adenoma, 88.2% from CRC and 8.3% from healthy patients. He et al. (2010) still reported the parallel level SEPT9 promoter methylation in tissue (78%) and in plasma (75%) samples of CRC patients. Non-conclusive results were obtained from the most recent clinical trial carried out to evaluate SEPT9 as a biomarker for CRC cancer screening (NCT00855348). Recently, Rasmussen et al. (2017) proposed the use of seven hypermethylated promoter regions including SEPT9 (ALX4, BMP3, NPTX2, RARB, SDC2, SEPT9, and VIM) to detect CRC founding a 90.7% sensitivity for detection. The utility of the SEPT9 methylation testing in plasma as diagnostic tool has been corroborated in other cancers such as head and neck squamous carcinoma (Schrock et al., 2017). Recently, the SEPT9 assay has been capable to detect a 62.5%

of advanced adenoma with high-grade dysplasia (Song et al., 2017).

## CONCLUSIONS AND PERSPECTIVES

Several studies demonstrate that the methylation of some promoters is related to the emergence of CRC. The research of their methylation status may be a relevant approach as well as a new biomarker for the early detection of CRC. At present, clinical studies are ongoing but only a few have completed the clinical trials and have been made commercially available. An example is ColoVantage®, a DNA methylation assay commercial based on the detection of SEPT9 methylation in blood (Warren et al., 2011). The use of non-invasive genetic biomarkers screening method for CRC is a novel ability in a recent clinical practice that should continue to be researched. Probably, epigenetics study in CSC should be complementary to other biomarkers such as miRNA, with the aim to develop a cluster of biomarkers as a more effective diagnosis tool. On the other hand, analysis of large populations and the development of clinical trials are urgently necessary to determine the clinical significance of these biomarkers by comparing the sensitivity and specificity of classical methods for diagnosis. In this context, the recent NCT02928120 clinical trial analyses blood samples from 193 CRC patients to determine methylation biomarkers for CRC recurrence after surgery and the NCT03146520 clinical trial try to validate the Syndecan 2 methylation status as a marker for early diagnosis of CRC. In any case, despite the different techniques used to determine a new biomarker o cluster of biomarker applicable for screening purposes, future studies will be necessary to come up with a solution that solves the argument about the predictive and prognostic values of the proposed markers, including methylation patterns. In this context, a most deep study of the molecular background of colon cancer could drive to methods that be useful to obtain an effective CRC screening that could detect this malignancy. In addition, the methylation status could be a new molecular marker that permits a more individualized approach to the CRC therapy. The objective is to determine relevant biomarkers that will be cost effective, that improve the prognosis of the patients and that may be easily applied in a clinical setting.

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