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Review

Long interspersed nucleotide element-1 (LINE-1) methylation in colorectal cancer

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ABSTRACT

Colorectal cancer (CRC) represents a group of molecularly heterogeneous diseases characterized by genetic and epigenetic alterations. Long interspersed nuclear elements (LINEs) are a form of retrotransposable element found in many eukaryotic genomes. These LINEs, when active, can mobilize in the cell and steadily cause genomic rearrangement. Active LINE reorganization is a source of endogenous mutagenesis and polymorphism in the cell that brings about individual genomic variation. In normal somatic cells, these elements are heavily methylated and thus mostly suppressed, in turn, preventing their potential for bringing about genomic instability. When LINEs are inadequately controlled, they can play a role in the pathogenesis of several genetic diseases, such as cancer. In tumor cells, LINE hypomethylation can reactivate the mobilization of these elements and is associated with both an advanced stage and a poor prognosis. In this article, we summarize the current knowledge surrounding LINE methylation, its correlation to CRC and its application as a diagnostic, prognostic and predictive biomarker in colon cancer.

1. Introduction

Colorectal cancer (CRC) constitutes a significant global health burden, having led to over 700,000 deaths globally in 2012. It currently stands as the third most common cancer in men and the second most common in women [3], with a yearly incidence of over one million cases worldwide [1,2]. In the United States and Europe, it is considered the third and second leading cause of cancer mortality, respectively [1]. The primary cause of death is due to liver metastasis [4] and the median survival rate with metastases is approaching 30 months [5,6]. Approximately 50% of patients with CRC develop tumor recurrences [7–9]. Fortunately, due to the implementation and growth of wide spread cancer screening assays, such as colonoscopy and image-based detection, as well as increasingly effective therapies, the mortality of CRC is subsiding in many countries [1].

CRC represents a group of molecularly heterogeneous diseases characterized by genetic and epigenetic alterations along a tumorigenesis sequence [10,11]. In addition to genetic mutations, it is known that epigenetic changes such as DNA methylation, the loss of genome imprinting, and histone modification are important factors for tumor initiation, development and progression [12–14].

DNA methylation is a fundamental epigenetic process that, in most cases, modulates genetic expression levels [15]. In tumor cells, abnormal DNA methylation may be classified into two categories: site-specific CpG island promoter hypermethylation and global DNA hypomethylation [16]. Several studies in cancer cells have reported site-specific CpG island promoter hypermethylation in tumor suppressor genes and global DNA hypomethylation in repetitive sequences [17–20]. This is contrasted in normal somatic cells, where long interspersed nuclear elements (LINEs) are heavily methylated, limiting the activities of retrotransposal elements and thus preventing genomic instability [21,22].

There exists a group of LINEs named, LINE-1 retrotransposon, which constitute a notable portion (approximately 17%–18%) of the human genome. Accordingly, the methylation status of LINE-1 retrotransposons could reflect the global DNA methylation level of the genome [16,23–28]. A low degree of methylation correlates to chromosomal instability and CRC dysplasia progression [10,29,30]. The

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structure, retrotransposition process and correlation to CRC of LINE-1s are discussed in detail in this literature review.

2. The structure of LINE-1

Repetitive sequences compose half of the human genome and are subdivided into two main types. The first type is referred to as satellite repeats, also called tandem repeats. These refer to a repeating sequence of one or more nucleotide located adjacent to one another. The second type consists of interspersed repeats, or repeated sequences, that, unlike satellite repeats, are scattered throughout the genome [16]. The human genome has numerous interspersed repetitive sequences originating from DNA transposons and retrotransposons [31]. The former of these two elements moves through a "cut-and-paste mechanism" mediated by an element-encoded protein called transposase [16]. Three categories of retrotransposons are known: LINE-1, SINE or Alu, and SVA elements. The first of these categories are autonomous element, capable of self-propagation through RNA intermediates, and the latter two are non-autonomous, which depend on LINE-1 for activation. LINE-1retrotrasposons are present on both homologous chromosomes [16]. Nearly 500,000 of these LINE-1 s are truncated [32] such that only 3000 to 5000 can be considered full length LINE-1 sequences throughout the human genome [25]. In general, the majority of LINE-1 s have lost their retrotransposition competency due to 5′ truncations, inverted re-arrangements, point mutations occurring during reverse transcription or subsequent chromosomal replication of the inserted element.

The average human genome contains approximately 50–120 active LINE-1 s, with highly active groups (about 5–10% of active elements) [33]. These active LINE-1 s utilize a "copy-and-paste" mechanism to insert themselves throughout the genome, with potentially disruptive effects on neighboring genes or regulatory sequences. A full-length LINE-1 has an internal promoter in its 5- untranslated region (UTR), which ranges from +1 to 909 base pairs (bp) [34]. The initial 460 bp region of the 5′- UTR includes 29 CpG sites, whose methylation status has been extensively investigated and shown to be heavily methylated in normal somatic cells [35–37]. Most methylated CpGs are located in the 5′ region and can behave as internal promoters [22]. A full-length LINE-1 element contains two promoters within its 5′-UTR region that are aligned in opposing directions. One of these promoters acts as a sense-promoter and regulates the transcription of retrotransposon-related genes such as an RNA-binding protein, an endonuclease, and a reverse transcriptase. The second promoter which is aligned in the opposite orientation, functions as an antisense promoter, and generates an alternative transcription start site that results in induction of transcription of downstream genes [26,38]. In addition, LINE-1 retrotransposons consist of two open reading frames (ORF1 and ORF2) flanked by a 5′- and 3′- UTR. These two ORFs encode an RNA-binding protein, and proteins with endonuclease and reverse-transcriptase activities, respectively [39].

3. LINE-1 mobilization

3.1. Activity in normal cells

LINE-1 hypomethylation is normally active in the germline and during embryogenesis, while being epigenetically suppressed in somatic cells. For the most part, LINE-1 s are highly methylated and inactivated in these somatic cells, however some continue to retain their capacity to retrotranspose into new genomic locations [40] (Fig. 1). In tumor cells, LINE-1 s can be reactivated through hypomethylation and participate in the pathological processes of cancer initiation and progression [25,31,40,41]. LINE-1 activity differs in cellular function, varies within and among cancer types and fluctuates during cancer evolution [42–45]. The LINE-1 methylation levels in normal tissues are strongly based on the tissue type; the range of LINE-1 methylation levels is narrow in some tissues (such as the liver, kidneys, breast, lungs and stomach) and wide in other tissues (such as the thyroid and esophagus) [46]. The LINE-1 methylation status has been also proposed to vary at individual genomic locations. One study of human cancer cell lines demonstrated that the methylation levels differ at nine LINE-1 loci [47]. Another study that evaluated the methylation patterns of 17 LINE-1 loci in several cell types has shown that the methylation levels at these loci are influenced differentially, depending on the location of the particular sequences in the genome [48]. In can be said that the changing methylation status observed in different sets of LINE-1 loci may lead to different cellular phenotypes [16].

3.2. Activity in tumor cells

LINE-1 mobilization can, over time, rearrange the genome, and thus affect genetic expression in many ways. Active LINE-1 s can continually reorganize the human genome, becoming a source of in-house mutagenesis and polymorphism, which results in individual genome variation *via* recombination and rearrangement. These events can participate in the pathogenesis of many genetic diseases, such as cancer [16,49–53]. For instance, in cancer cells, LINE-1 retrotransposons can inactivate surrounding gene function through insertional mutagenesis, aberrant splicing or DNA breaks, leading to genomic instability [41,54] (Fig. 2). It should be noted that somatic LINE-1 insertions occur more often in intergenic or heterochromatic regions [45], in cancer-specific hypomethylation regions [43], and in genes commonly mutated in cancer, suggesting an oncogenic effect of LINE-1 insertions [38,55–57]. As a protective response to the LINE-1-induced mutagenesis effect in tumorogenesis, eukaryotic cells have developed several mechanisms to neutralize LINE-1 mobilization including siRNAs or piRNA-mediated mechanisms. The bidirectional promoters within the LINE-1 5′-UTR can give rise to sense and antisense RNAs, which, when bound to each other, form double-stranded RNAs (dsRNAs). These dsRNAs are subsequently sliced by the enzyme Dicer into endogenous (*endo*)-siRNAs. The resultant *endo*-siRNAs can then degrade LINE-1 mRNAs and silence LINE-1 retrotransposition by triggering the RNA interference mechanism, thereby creating a negative regulatory loop. Like siRNA, piRNA can also exert a negative regulation effect on LINE-1 s. In addition to siRNA and piRNA, other defensive strategies against LINE-1 mobilization include the RNA helicase MOV10 (which degrades LINE-1 mRNAs and suppresses their translation) and APOBEC3 family members (which inhibits LINE-1 retrotransposition by varying mechanisms) [31].

4. LINE-1 methylation levels and molecular alterations in CRC

Some molecules such as folate play a crucial role in cellular methylation, and their deficiency can contribute to DNA hypomethylation [2]. Figueiredo et al. reported that folic acid supplementation does not influence LINE-1 methylation levels in the normal colonic mucosa [58]. In contrast, Liu et al. elucidated an association between the relative distribution of folate species and global DNA hypomethylation in the normal human colorectal mucosa [59].

The relationship between LINE-1 methylation levels and endogenous molecular alterations in CRC is relevant to our general understanding of the disease. The hypomethylation of LINE-1 was shown to inversely correlate with microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP) in CRC according to a study performed by Hur et al. [26]. On the contrary, Kuan et al. were not able to elucidate an association between tumor LINE-1 hypomethylation and the molecular alterations including specific gene mutations, MSI and elevated microsatellite alterations at selected tetranucleotides [60]. Similarly to the Hur study, Iacopetta and colleagues showed that the LINE-1 methylation levels were inversely associated with the CpG island methylation of specific genes including *MLH1*, *P16* (*INK4A*), *APC*, *TIMP3*, *ER*, *MYOD* in normal colonic mucosa [61]. Ogino et al. indicated that LINE-1 methylation level was higher in MSI-positive

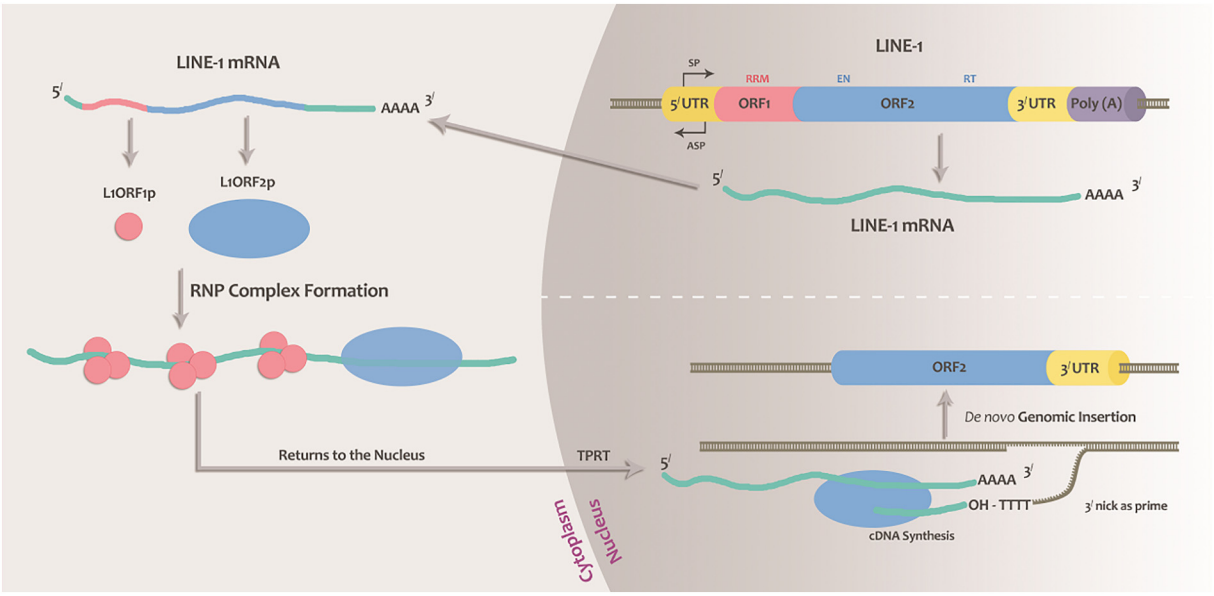

Fig. 1. Mobilization of a LINE-1 element. LINE-1 is a retrotransposon, meaning that its replication cycle involves an RNA intermediate. It is also an autonomous element, which means that it encodes its own protein machinery for reverse transcription and integration of its sequence into the genome. Full-length LINE1 elements comprise a 5′ UTR sequence that contains an internal bidirectional promoter (sense promotor, SP and antisense promotor, ASP), followed by open reading frame −1 (ORF1) and − 2 ORF2, a 3′ UTR and a poly (A) tail. ORF1 encodes an RNA-binding protein (L1ORF1p) and the protein encoded by ORF2 (L1ORF2p) has an endonuclease (EN) domain and reverse transcriptase (RT) domain. It is assumed that nuclear import is required for ORF2p to access the genome. ORF2p has endonuclease activity that nicks the DNA at an insertion site and leaves a 3′OH group. ORF1p is a major component of the LINE-1 RNP complexes formation. The order of events during the mobilization of a LINE-1 element, which results in a 5′ truncated insertion is: Step 1: LINE1 transcription in the nucleus; Step 2: LINE-1 mRNA exportation to the cytoplasm; Step 3: ORF1, ORF2 translation and ribonucleoprotein (RNP) complex formation; Step 4: reverse transcription of the RNA by ORF2p-mediated target primed reverse transcription (TPRT); and Step 5: *de novo* double-stranded insertion.

tumors than in MSI-negative tumors, as well as in *BRAF* mutation tumors than in *BRAF* wild-type tumors in primary CRCs [62]. Baba et al. manifested no significant relationship between *KRAS* and *PIK3CA* mutations and LINE-1 methylation level in primary CRCs [63]. In a study by Murata et al., it was demonstrated that in metastatic CRC tumors, the LINE-1 methylation level was not associated with MSI status or *KRAS*, *BRAF* and *PIK3CA* mutations. However, their study was limited by a rather small sample size [24]. A few other studies have shown the association of LINE-1 hypomethylation in CRCs with inferior survival, but, once again, their relatively small sample sizes might also affect their results [64,65]. Moreover, the association of LINE-1 hypomethylation with inferior survival appeared to be stronger in MSI-high cases with a family history of CRC rather than in MSI-high cases without a similar family history [10]. This conclusion may be overinterpreted since the MSI-high/*BRAF* wild-type subtype and the MSI-high subtype with a CRC family history include Lynch syndrome cases [66].

In CRC, LINE-1 hypomethylation may also significantly correlate with various clinical and pathological variables. The relationship between LINE-1 hypomethylation and early-onset CRC has recently been the focus of much scientific attention. Early-onset CRC presents a clinically distinct CRC phenotype and is often associated with an unfavorable prognosis. Baba et al. showed that severe LINE-1 hypomethylation (methylation < 40%) appear significantly more in younger patients than in older individuals [63]. Antelo et al. identified a similar relationship between early-onset CRC (≤50 years of age) and LINE-1 hypomethylation [67]. Thus, LINE-1 hypomethylation may be a potentially important marker for early-onset CRC [16]. Furthermore, a recent study has shown that LINE-1 hypomethylation is a prognostic biomarker in early-stage rectal cancer, although the study did not examine the entire lower bowel for possible cancer [68]. An additional

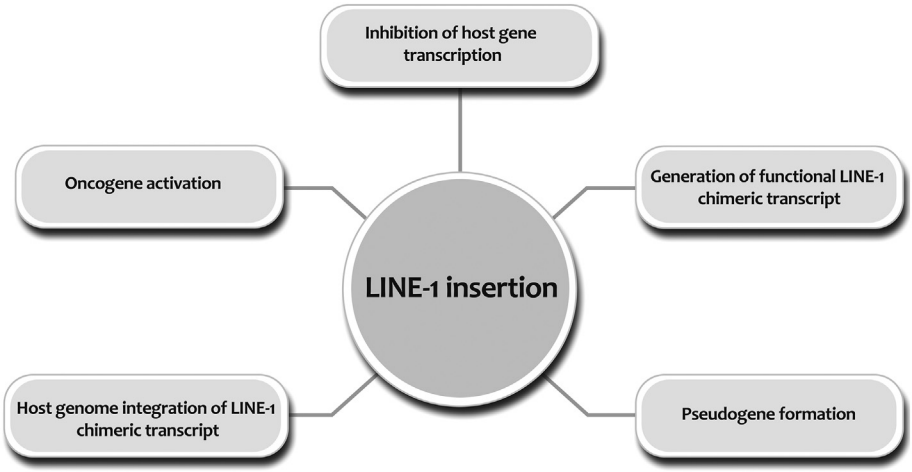

Fig. 2. The potential pathogenic functions of LINE-1 in cancer.

study by Rhee et al. showed that LINE-1 hypomethylation is associated with adverse prognoses in MSI-high CRCs [69]. The association of LINE-1 hypomethylation with lower bowel cancer survival rates was found to be stronger in the MSI-high/BRAF wild-type subtype, which is linked to the most favorable prognosis among subtypes [70]. The potential mechanisms of interaction between LINE-1 methylation levels and MSI status have been reported in several studies. Compared to microsatellite stable (MSS) tumors, MSI-high variants are characterized by numerous somatic mutations [71] that may be more readily influenced by LINE-1 hypomethylation leading to chromosomal instability. Other possible mechanisms may involve inflammatory mediators, variation in locus specific methylation patterns, and non-coding RNAs describing the interaction of LINE-1 methylation level and MSI status [10].

5. Role of LINE-1 hypomethylation in CRC

In 1992, cancer-associated with LINE-1 mutagenesis was reported when a somatic LINE-1 insertion into the *APC* gene was found to have caused a gene disruption in a colon cancer sample [50]. In a study by Lee et al., somatic LINE-1 insertions were more frequently found in CRC than other cancers such as prostate and ovarian cancers, and interestingly, no somatic LINE-1 insertions were detected in glioblastoma and multiple myeloma [43]. In cancer tissues obtained from CRC patients, the numbers of somatic LINE-1 insertions ranged from 2 to 106, indicating the possibility of the LINE-1 retrotransposition profile as a signature for different subtypes of cancer [31]. In 2014, Barchitta et al. reported a meta-analysis explaining the role of LINE-1 hypomethylation in human cancer. They demonstrated that LINE-1 methylation levels were significantly lower in cancer patients than in control samples, especially in certain cancer types. The results were confined to tissue samples, both fresh/frozen and FFPE specimens [72]. Another meta-analysis supported the correlation between LINE hypomethylation and poor prognosis in cancer patients [73]. More specifically, LINE-1 hypomethylated in CRC had been associated with advanced stage and poor prognosis [10,74].

5.1. LINE-1 as a diagnostic biomarker in CRC

Several studies have investigated the associations between LINE-1 methylation levels and cancer risk, progression, and prognosis, with a majority of these studies supporting the correlation between tissue LINE-1 hypomethylation and increased cancer risk [42,44,75]. For instance, in patients with a family history of CRC, colonic LINE-1 hypomethylation was linked to a higher CRC risk indicating an association between genetic predisposition and somatic epigenetic changes [75]. LINE-1 methylation levels in normal colon tissues have also been explored in multiple studies. Kamiyama et al. evaluated the LINE-1 methylation levels in cancer tissues obtained from CRC patients and matched them with non-cancerous colonic mucosa, The study found elevated LINE-1 relative demethylation levels in the non-cancerous colonic mucosa compared to tumor lines [76]. In African Americans with CRC, LINE-1 was found to be progressively hypomethylated in the normal adenoma cancer sequence [42]. Bariol et al. also demonstrated that the global DNA hypomethylation level was greater in CRC neoplastic lesions (including hyperplastic polyps and adenoma) than in the normal colonic mucosa [77]. Similarly, Sunami et al. showed a linear correlation between LINE-1 demethylation progression and TNM stage progression, suggesting that the onset of LINE-1 demethylation occurs early during the normal colorectal mucosa's progression to adenoma. Moreover, genomic methylation levels have been shown to continuously decrease during CRC development and progression, which suggests that aberrant DNA methylation analysis allows for CRC subtype identification [74]. This phenomenon strengthens the potential utility of methylation tests for detection of CRC and suggests that LINE-1 hypomethylation may be a useful diagnostic marker in both sporadic and inherited CRCs [78]. The LINE-1 methylation level in normal colonic mucosa have been shown to be independent of age, body mass index, sex, smoking status, alcohol consumption and race [58]. In addition, LINE-1 methylation has minimal variability throughout the colon in contrast to other loci [79], which is an important characteristic for a diagnostic biomarker. Hur et al. reported that in CRCs with liver metastasis, LINE-1 methylation levels were lower in the metastasis *versus* the primary CRC tissue [26]. These examples indicate that LINE methylation can change with tumor development and progression but not all studies have confirmed this finding. In one particular study of 869 population-based CRC tumors, Ogino et al. demonstrated that LINE-1 methylation levels were not associated with tumor stage [62]. Murata et al. also found identical LINE-1 methylation levels between primary tumors and liver metastases. In addition, they reported the absence of heterogeneity of the LINE-1 methylation level in superficial regions with that of the invasive front regions in primary tumors. It was assumed that cancer cells acquired the tumoral characteristic of global DNA hypomethylation during the early stage of tumor development and the cells maintained this characteristic throughout the process of metastasis [24]. These studies were in accord with an additional study by Matsunoki et al. [80], which reported that LINE methylation was relatively stable during CRC progression. More studies with larger sample sizes are needed to explain the current discrepancies.

5.2. LINE-1 as a prognostic biomarker in CRC

Most research on the LINE-1 methylation levels in gastrointestinal cancers has originated from CRC tumors, in which the levels of LINE-1 methylation are variably diminished. In *in vitro* studies, the levels of LINE-1 methylation in colon cancer cell lines (COLO205, HCC-2998, HCT116, HCT15, HT29, RKO, SW48, KM12, LOVO, SW620) are highly variable, ranging from 30 to 70% [81]. In a study of 643 CRCs in two independent prospective cohorts, Ogino et al. demonstrated that the extent of LINE-1 hypomethylation is associated linearly with tumor invasiveness. They observed an approximately five-fold increase in cancer-specific mortality as the LINE-1 methylation levels of the tumor ranged from high to low [64]. Ahn et al. showed that a lower LINE-1 methylation level in CRC is more commonly associated with unfavorable prognoses in patients with resected stage III proximal, but not distal cancers [65]. If a potential informative high-risk biomarker exists in adenomas associated with concomitant or synchronous CRCs, a potential biomarker with the ability to predict the risk of metachronous neoplasia and provide revealing information on potentially missed synchronous lesions could very well be considered [11]. In a meta-analysis performed by Ye et al., LINE-1 methylation was significantly associated with the survival of CRC patients, which could be a predictive factor for CRC prognosis [82]. Hypomethylation in LINE-1 and MSI-high in CRC have been associated with inferior and superior survival, respectively; however, it remains uncertain whether the prognostic association of LINE-1 hypomethylation differs by MSI status. Inamura et al. elucidated that the association of LINE-1 hypomethylation with inferior survival is stronger in MSI-high CRCs than in MSS CRCs. Hence, the low LINE-1 methylation level in CRC could also be recognized as a prognostic biomarker. It could be a useful prognostic biomarker to identify aggressive carcinomas among MSI-high CRCs [10] and LINE-1 methylation assay may also be a useful prognostic marker in both sporadic and hereditary CRCs for the early detection of cancer [9,70].

5.3. LINE-1 as a preventive biomarker in CRC

LINE-1 hypomethylated CRC has been associated with a young age at onset [71] and CRC familial clustering [83]. Tumor LINE-1 methylation levels are an increasingly attractive potential biomarker for predicting survival benefits from adjuvant chemotherapy. Surgery supplemented with adjuvant chemotherapy using oral fluoropyrimidines extends the lifespan of patients with low LINE-1 methylation

levels, while apparently conferring no survival benefits in patients with high LINE-1 methylation levels [84]. LINE-1 hypomethylation, a surrogate biomarker for global DNA methylation, is associated with worse progressive-free survival and overall survival following FOLFOX (5-florouracil, folinic acid and oxaliplatin)-based chemotherapy. In other words, LINE-1 hypomethylationis have resistance to FOLFOX treatment [85].

6. Conclusion and future directions

As mentioned above, not all studies have confirmed that LINE-1 methylation levels vary with tumor development and progression. These discrepancies may be due to differences in molecular methods used to assess the LINE-1 methylation or simply due to chance variation between independent studies. Factors such as the side of tissue samples could influence the experimental results since LINE-1 methylation levels in normal mucosa obtained from the right side of the colon are noticeably lower than those obtained from the left side of the colon when observed [58]. In addition, an important problem in the assessment of LINE-1 methylation status in CRC is that this disease is highly heterogeneous and contains different infiltrating cells including stromal cells, lymphocytes, and endothelial cells. This heterogeneity may confound molecular analysis of LINE methylation, since LINE-1 retrotransposon are highly methylated in normal cells. It is necessary to precisely isolate tumor cells from normal tissue specimens, in order to obtain accurate interpretations. In this regard, Sunami et al. used integrated lasers to capture microdissection in their LINE-1 methylation analyses and showed that a mean methylation level of 80% was found in normal subjects, indicating a potential threshold for pathologic activity [74]. These issues should be further examined with both increasingly standard and precise techniques and in independent studies with greater sample sizes.

Determining the histopathology associated with the onset of LINE-1 hypomethylation and studying its trend along its progression sequence can illuminate the role of LINE-1 hypomethylation in CRC. In contrast to irreversible genetic changes, epigenetic changes of LINE hypomethylation may provide potentially reversible molecular targets for both cancer cell therapies and chemoprevention. Further investigations in this field would provide new insights into the pathogenesis of CRC and could help to develop new therapeutic strategies.

Disclosure

The authors have declared no conflict of interest.

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