Epigenetic Biomarkers for the Early Detection of Gastrointestinal Cancer

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Abstract
Background: Gastric cancer and colorectal cancer, the two most frequent cancers within the gastrointestinal tract, account for a large proportion of human malignancies worldwide. The initiation and progression of gastrointestinal cancer (GIC) is controlled by both genetic and epigenetic events. Epigenetic alterations, including changes in DNA methylation, specific histone modifications, chromatin remodeling and noncoding RNA-mediated gene silencing, are potentially reversible and heritable. Summary: In this article, we summarize the current advances in epigenetic biomarkers as potential substrates for GIC detection. The combined screening of a panel of methylated genes, hyperacetylated histones, microRNAs or other non-coding RNAs is currently under evaluation to improve sensitivity. Key Message: Current studies concentrated on the development of cost-effective epigenetic diagnostic biomarkers for GIC based on noninvasive blood or stool samples. The combined blood or stool test with a relatively high sensitivity could be a cost-effective screening tool for the detection of patients with asymptomatic cancers who could therefore choose whether or not to go for further examinations, such as endoscopy or colonoscopy. Practical Implications: A better understanding of epigenetic mechanisms has not only offered new insights into a deeper understanding of the underlying mechanisms of carcinogenesis, but has also allowed identification of clinically relevant putative biomarkers for the early detection, disease monitoring, prognosis and risk assessment of GIC. In particular, noninvasive biomarkers in serum or fecal samples for the detection of GIC could have potential for better compliance and can be incorporated into routine clinical practice in the foreseeable future, pending their validation in large-scale prospective trials.
Introduction

Gastric cancer (GC) and colorectal cancer (CRC) account for a large proportion of human malignancies, resulting in a high cancer-related mortality worldwide [1]. The accumulation of both genetic and epigenetic changes triggers the initiation and progression of cancer. Epigenetic aberrances play a fundamental role in the progress of gastrointestinal cancer (GIC), including changes in DNA methylation, specific histone modifications, chromatin remodeling and noncoding RNAs. In contrast to classic genetic alterations, epigenetic modifications affect gene expression without changing the DNA sequence and are potentially reversible [2]. A better understanding of epigenetic mechanisms will offer new insights into the discovery of biomarkers for the early diagnosis of GIC.

DNA Methylation

DNA methylation is mediated by enzymes called DNA methyltransferases and typically occurs in CpG islands (CGIs). CGIs are genomic regions that are unusually enriched with CpG dinucleotides and are commonly found in the promoter regions of almost 60% of human genes. The methylation of CGIs associated with the promoter region of genes is commonly associated with gene silencing, while methylation that occurs in CpG sites outside of promoter regions is associated with transcriptional activation [3]. In the process of tumor development, demethylation of the entire genome and hypermethylation of gene promoters mostly occur simultaneously [3]. Moreover, the methylation of promoter-associated CGIs may induce chromatin conformational modifications that inhibit the transcriptional process, thus altering gene expression levels [4].

Among all of the epigenetic mechanisms, DNA methylation has gained particular interest since it was discovered that promoter hypermethylation associated with gene transcriptional repression is an early event in carcinogenesis [2]. Furthermore, DNA methylation profiles represent a more stable source of biological information than RNA or the expression profiles of most proteins, making this marker particularly interesting for the early detection of GIC [5, 6].

DNA Methylation in CRC

Hundreds of genes show altered levels of DNA methylation across the average CRC genome, including genes within the Wnt signal transduction pathway such as APC, AXIN2, DKK1, SFRP1, SFRP2 and WNT5A, the DNA repair genes MGMT, MLH1 and MLH2, cell cycle-related genes such as CDKN2A, and RAS signaling genes such as RASSF1A and RASSF1B [7, 8]. DNA methylation mapping also shows that the highest CGI hypermethylation frequency takes place in GC [9, 10]. For instance, HOP homeobox methylation has shown potential as a biomarker with 84% of hypermethylated samples versus 10% of matched adjacent normal tissues [11]. Due to promoter hypermethylation, the expression of ADAMTS9 (A disintegrin and metalloproteinase with thrombospondin motifs 9), which belongs to the ADAMTS family, was silenced in 75% of GC cell lines and inhibited the expression of AKT/mTOR pathway genes [12].

Among all of the differentially methylated genes detected in various body fluids, searching for the specific ones with clinical relevance has attracted much attention [13]. Such genes can serve as useful, easily available and noninvasive biomarkers for GIC. Of mention is one landmark study that developed a new methodology called methyl-BEAMing for the absolute quantification of methylated molecules in DNA from plasma or fecal samples [14].
The current study concentrates on the development of cost-effective epigenetic diagnostic biomarkers for GIC based on noninvasive blood or stool samples. One of the first commercialized blood-based PCR tests to detect the presence of the methylated septin 9 gene in CRC patients has a sensitivity and a specificity of nearly 90% [15]. A blood-based CRC screening test using the methylated SEPT9 biomarker (septin 9), which encodes a GTPase involved in dysfunctional cytoskeletal organization, specifically detects the majority of CRCs at all stages and colorectal locations. The test has an overall sensitivity of 90% and a specificity of 88% [16]. Other potential blood-based methylation biomarkers, such as the genes that encode syndecan-2, thrombomodulin, secreted frizzled-related protein 2, runt-related transcription factor 3 and neurogenin-1, are currently under investigation [17]. The novel functional tumor suppressor gene, T-box transcription factor 5 (TBX5), a member of a phylogenetically conserved family of genes, is inactivated by promoter hypermethylation in 68% (71/105) of primary colon tumors. The detection of methylated TBX5 may serve as a potential biomarker for CRC [18]. Serum p16 methylation could be detected more frequently in CRC patients with lymph node metastases. Quantitative methylation-specific profiling has shown a high degree of sensitivity and could be used as a screening method for cancer [19]. One study has identified NPY, PENK and WIF1 as novel epigenetic markers and assessed their combined use as a panel of epigenetic markers for diagnostic accuracy in serum samples. The test performs the CRC detection of NPY, PENK and WIF1 with sensitivity/specificity values of 87/80% (higher sensitivity), 78/90% and 59/95% (higher specificity), respectively. This combined blood test could be a cost-effective screening tool for the detection of patients with asymptomatic cancers who could therefore choose whether or not to go for further examinations, such as colonoscopy [20]. The detected hypermethylated genes in GC are also of potential diagnostic significance, and perhaps will eventually override the value of classical serum tumor markers, such as carcinoembryonic antigen. For example, the aberrant hypermethylation of the SLC19A3 promoter in plasma may be a novel diagnostic biomarker for GC with 90% positive predictive values and a negative predictive value of 85% [21]. RNF180 (ring finger protein 180) has also been shown to be a novel preferentially methylated gene in the plasma of patients with GC, with a sensitivity of 63% and a specificity of 91% [22].

A commercially available stool-based test for the methylation of the gene coding for vimentin, combined with colonoscopy, has a degree of sensitivity for CRC that ranges from 40 to 80% [16]. Several other stool-based tests have been developed for the diagnosis of CRC to detect hypermethylated genes that encode for fibrillin-1, APC, CDKN2A, MLH1, MGMT, SFRP1, SFRP2 and NDRG4, amongst others; these have levels of sensitivity that range from 60 to 80% [23, 24]. TFPI2 is expressed in almost all colorectal adenomas (97%, n = 56) and stage I to IV CRCs (99%, n = 115). Furthermore, DNA-based stool assays have been used in patients with stage I–III CRC with a sensitivity of 76–89% and a specificity of 79–93%, which indicates the potential of investigating TFPI2 methylation levels in stool DNA as a noninvasive biomarker for the early screening of CRC [25]. Additionally, rather than detecting a single methylated gene, Imperiale et al. [26] found that the sensitivity of stool DNA testing combined with a panel of different biomarkers for the detection of CRCs was 92.3%. The combined screening of a panel of methylated genes is currently under evaluation to improve sensitivity [26].

**Histone Modifications**

Another critical epigenetic mechanism refers to post-translational covalent modifications at histone tails, such as acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, deamination and ribosylation [27]. However, compared to methylation-associated alterations, the understanding of the impact of histone modifications in human cancers
is limited. The consensus is that the post-translational modifications of histones establish a potentially reversible ‘histone code’ that permits active transcription with a euchromatin configuration, but inactivates transcription in a heterochromatin state to regulate gene expression [28]. Biomarker studies for the diagnosis of GIC have mainly focused on the expression of global histone-modifying enzymes.

It is agreed that hypoacetylated loci normally inhibit gene expression by histone deacetylases (HDACs), while hyperacetylated histones are involved in the activation of gene expression by histone acetyltransferases (HATs) [29]. Global H3 and H4 lysine hypoacetylation has been detected in GIC, and this is often associated with the downregulation of tumor suppressor genes [30]. The upregulation of several HDACs, such as HDAC1–3, HDAC5 and HDAC7, has been observed in CRC and linked to the downregulation of gene expression in the Wnt signaling pathway [31, 32]. Besides, HDAC2 upregulation has been found in the early stages of colonic carcinogenesis [32]. For one of the HATs, p300/CBP-associated factor (PCAF), expression is downregulated in GC tissues and correlates with gastric wall invasion, tumor size, node metastasis stage and poor overall survival rates [33]. Hyperacetylation of H3 in ZNF312b (FEZ family zinc finger 1) has also been found to promote the progression of GC [34].

The methylation of histone tails has been largely reported in GIC and cell lines, including the loss of trimethylation of H3K20, and the di- and tri-methylation of H3K4 (H3K4me2/me3), H3K9 (H3K9me2/me3) and H3K27 (H3K27me2/me3) [30]. The deregulation of HDAC activity has been strongly implicated in aberrant gene silencing and tumorigenesis. Overexpression of nuclear HDAC2 was observed in 81.9% of CRCs, 62.1% of colorectal adenomas and 53.1% of normal tissues [35]. A recent study showed that several of the above-mentioned marks correlate with the pathological features of GIC, such as tumor size, histological type and an increased tendency to metastasize. For example, overexpression of HDAC2 was found to be accompanied by hypoacetylation at H4K12 and H3K18 histones during the progression from adenoma to CRC, which suggests that increased levels of HDAC2 expression and the subsequent loss of acetylation are significantly associated with CRC progression [35]. One of the most studied histone-associated proteins, EZH2 (enhancer of zeste homolog 2), which encodes a H3 methyltransferase to induce polycomb-mediated repression of target genes, has shown poor prognostic effects and can promote CRC metastasis [36]. The expression levels of H3K9me2 were higher in CRC than in normal tissue and might correlate with poorer survival rates [37, 38].

**Noncoding RNAs**

MicroRNAs (miRNAs) are a class of 18–25-nucleotide noncoding RNA molecules that regulate the translation of genes. Owing to their smaller size, high stability in human tissues and crucial regulatory role for translation, miRNAs have a strong potential as better biomarkers than mRNA and proteins in most tumor types, including GIC [39]. In addition, tumor-derived miRNAs can be present in serum and feces at detectable levels and appear to be stably protected from endogenous ribonuclease activity in the circulation, thus providing an opportunity to develop these RNAs as potential candidates for diagnostic biomarkers for GIC [39]. Although previous studies regarding miRNA expression have been performed on tissue specimens, interest in using miRNA markers in blood and fecal specimens for the screening of GIC or precancerous lesions has been raised only recently.

In 2009, Ng et al. [40] demonstrated that miR-92 was significantly elevated in plasma, with a sensitivity of 89% and a specificity of 70% in discriminating CRC patients from controls, which suggests that it has potential as a noninvasive molecular marker for CRC screening. The
oncogenic miRNAs, miR-21 and miR-31, negatively regulate tumor suppressor genes such as PTEN and TPM1 and have been shown to be overexpressed in various human tumors [41]. The serum miR-21 levels can robustly distinguish CRC patients from controls (81.1% sensitivity and 76.7% specificity). Moreover, high levels of miR-21 expression in serum and tissue samples were significantly associated with tumor size, distant metastasis and poor survival rates. These observations show that serum miR-21 is a promising biomarker with the highest levels of sensitivity and specificity for the early detection of CRC [42]. Wang et al. [43] detected miR-194 in patients with advanced colorectal adenoma after polypectomy. The sensitivity/specificity of miR-194 as a predictor in their retrospective and prospective cohort studies were 71.0/78.0% and 76.1/77.2%, respectively, which demonstrated that miR-194 could be an independent predictor for adenoma recurrence in patients after polypectomy.

Many miRNAs have also been reported to be deregulated in GC. These miRNAs can be used as early diagnostic biomarkers in clinical practice. MiR-129-2 is silenced in GC and the restoration of its expression could induce apoptosis in GC cells [44]. The downregulation of miR-218 in GC inhibits its target, Robol, which activates the Slit/Robol signaling pathway, thus inducing the invasion and metastasis of GC. The detection of miR-218 may be a biomarker for the diagnosis of GC metastasis [45]. High levels of miR-17 and miR-106a in cancer cells have been confirmed in a study in which the value of the area under the ROC curve for a combined miR-17/miR-106a assay was 0.741 [46]. Wan et al. [47] further found that miR-9 could inhibit GC cell growth by targeting NF-κB, suggesting that miR-9 suppresses the pathogenesis of GC. Liu et al. [48] found from a genome-wide miRNA profile analysis that miR-378 showed a higher level in serum samples from GC patients, with a sensitivity of 87.5% and a specificity of 70.7%. Downregulated serum levels of miR-378 could be used to detect the early stages of GC. Interestingly, the positive detection rate of miR-31 in serum is significantly higher than that of serum carcinoembryonic antigen (68.29 vs. 21.95%), which indicates that it could potentially be a better diagnostic marker for GC [49].

Recent studies have demonstrated that stool-based tumor-derived miRNAs are present in a remarkably stable form with highly reproducible levels of detection. In 2010, Link et al. [50] demonstrated the feasibility of stool-based miRNA detection in patients with CRC. Stool-based miRNA can be used alone or be integrated into currently existing marker panels to give higher levels of sensitivity. Recently, Koga et al. [51] showed reasonable levels of sensitivity and specificity for the miR-17-92 cluster and miR-135 isolated from 206 CRC patients and 134 controls. The overall sensitivity and specificity according to a panel of miRNAs was 74.1% (146/197; 95% CI 67.4–80.1) and 79.0% (94/119; 95% CI 70.6–85.9), respectively. In the study by Wu et al. [52], the feasibility of two well-established oncogenic miRNAs, miR-21 and miR-92a, in stool was investigated as a screening tool for CRC and premalignant polyps. The expression of stool miR-21 and miR-92a was significantly higher in CRC compared to control stool samples (p < 0.0001). A recent meta-analysis revealed that the overall sensitivity and specificity of the most frequently studied miRNAs, including miR-92a, miR-21, miR-29a and their combinations with other miRNAs, were 81 and 79%, respectively, which suggests that miRNAs may be potential novel biomarkers for the detection of CRC [53].

In addition to miRNAs, emerging evidence has highlighted the importance of other noncoding RNAs in gastrointestinal carcinogenesis. For example, high levels of the long intergenic noncoding (Inc) RNA HOTAIR were detected in serum and tumor tissue samples of CRC patients and associated with poor prognosis [54]. LncRNA-p21, which is aberrantly expressed in CRC, regulates the Wnt/β-catenin signaling pathway [55]. Downregulation of the expression of ncrAN (lncRNA expressed in aggressive neuroblastoma) was detected in CRC samples from patients with liver metastases and predicted poor overall survival rates [56]. LncRNAs downregulated the targeted gene expression through chromatin remodeling and one lncRNA, called CCAT1-L, is transcribed specifically in human CRCs and regulates the chromatin state...
However, the noncoding RNA test may be better when not used alone as a screening tool, but in combination with other conventional tests, to improve the diagnostic sensitivity for GIC [53].

Conclusions

Most GICs follow the adenoma-carcinoma sequence, and the progression from an asymptomatic adenoma to carcinoma usually takes years. There is a need for a preventive strategy to stratify patients into appropriate surveillance programs. Several CRC screening tests, including fecal occult blood testing and colonoscopy, have been utilized in recent decades and have helped to reduce the mortality rate from GIC [58]. However, fecal occult blood testing has a low level of sensitivity and might miss a substantial proportion of malignant lesions. Furthermore, compliance with endoscopic or colonoscopic screening programs has been far from satisfactory. Recent studies of epigenetic changes have greatly extended our understanding of the pathogenesis and pathophysiology of GIC and have provided novel epigenetic biomarkers for the diagnosis of tumors. In particular, noninvasive biomarkers in serum or fecal samples for the detection of GIC could have potential for better compliance and can be incorporated into routine clinical practice in the foreseeable future, pending their validation in large-scale prospective trials.

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