

# Epigenetic biomarkers of promoter DNA methylation in the new era of cancer treatment

Keishi Yamashita<sup>1,2</sup>  | Kei Hosoda<sup>1</sup>  | Nobuyuki Nishizawa<sup>1</sup> | Hiroshi Katoh<sup>1</sup> | Masahiko Watanabe<sup>1</sup> 

<sup>1</sup>Surgery, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan

<sup>2</sup>Division of Advanced Surgical Oncology, Research and Development Center for New Medical Frontiers, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan

## Correspondence

Keishi Yamashita, Division of Advanced Surgical Oncology, Research and Development Center for New Medical Frontiers, Kitasato University School of Medicine, Sagamihara, Kanagawa, Japan.  
Email: keishi23@med.kitasato-u.ac.jp

Promoter DNA methylation, which occurs on cytosine nucleotides across CpG islands, results in gene silencing and represents a major epigenetic alteration in human cancer. Methylation-specific PCR can amplify these modifications as markers in cancer cells. In the present work, we rigorously review the published literatures describing DNA methylation in the promoters of critical tumor suppressor genes; detection of promoter DNA methylation in various body fluids permits early detection of cancer cells during perioperative courses of clinical treatment. The latest whole-genome comprehensive explorations identified excellent epigenetic biomarkers that could be detected at high frequency with high specificity; these biomarkers, which are designated highly relevant methylation genes (HRMG), permit the discrimination of tumor tissues from the corresponding normal tissues; these markers are also associated with unique cancer phenotypes, including dismal prognosis. In humans, HRMG include the *CDO1*, *GSHR*, *RASSF1* and *SFRP1* genes, with these markers permitting discrimination depending on the organs tested. The combination of several HRMG increased the early detection of cancer and exhibited reliable surveillance potential in human body fluids. Cancer clinics using such epigenetic biomarkers are entering a new era of enhanced decision-making with the potential for improved cancer prognosis.

## KEYWORDS

body fluid, cancer, *CDO1*, methylation, tumor suppressor gene

## 1 | INTRODUCTION

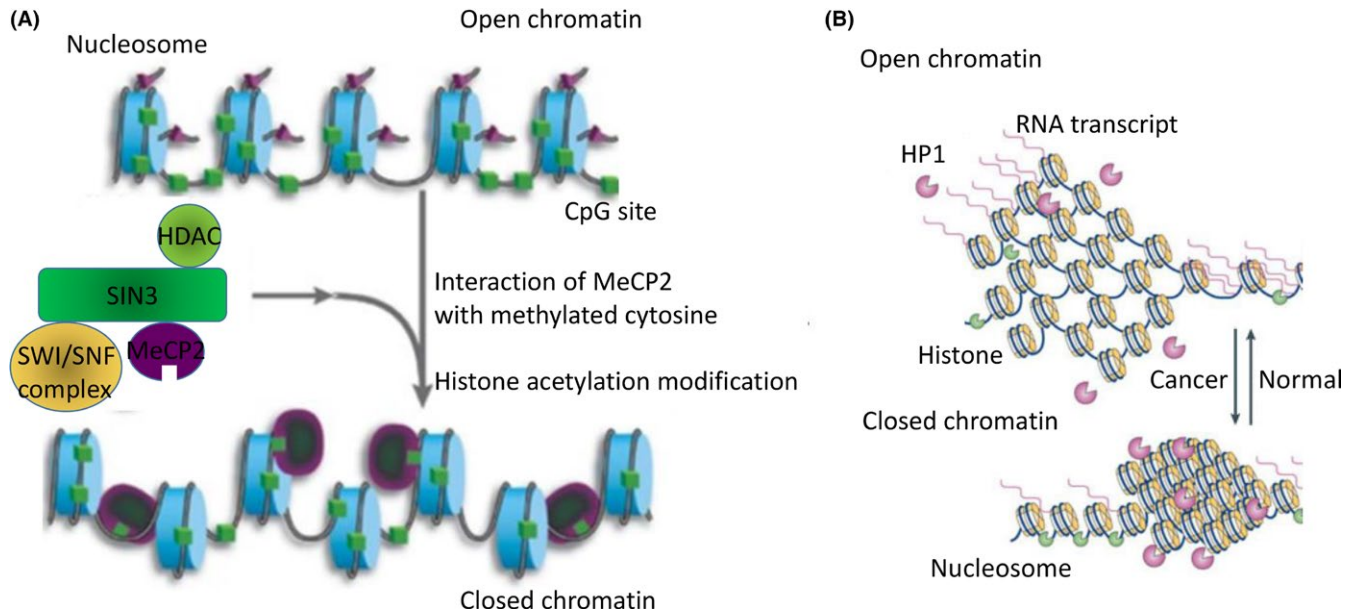
Epigenetic alterations are found in primary human cancers, and such aberrations are composed of DNA methylation and its linked histone modification.<sup>1</sup> DNA methylation occurs in cytosine residues among the CpG islands of the promoter regions of individual genes; methylated cytosine has been referred to as the “5th nucleotide,” in addition to the 4 canonical DNA bases (adenine, cytosine, thymine and guanine). Methylated cytosines are totally different from unmethylated cytosines from a phenotypic point of view. Methylated

cytosines can be bound by methyl-CpG-binding protein 2 (MeCP2), and the resulting protein-nucleotide can be incorporated into protein complexes that include histone modification enzymes (eg, histone deacetylase complex [HDAC])<sup>2</sup> leading to dynamic changes in chromatin structure (Figure 1A).<sup>3</sup> As a result, DNA methylation can result in gene silencing due to impaired access of transcription factors through condensed and closed chromatin (Figure 1B).

Promoter DNA methylation of tumor suppressor genes (TSG) is accompanied by gene silencing in human cancers, and combination treatments using demethylating agents (5-aza-2'-deoxycytidine)

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**FIGURE 1** A, Methylated cytosines (green boxes) can bind to MeCP2 protein, which is itself a complex composed of proteins with SWI/SNF and HDAC activities, leading, in turn, to alterations of chromatin remodeling and histone acetylation (respectively). These activities regulate the state (open or closed) of chromatin. B, Open chromatin facilitates access by the various transcription factors, permitting transcription to generate RNA. Heterochromatin protein 1 (HP1) functions include gene repression by heterochromatin formation

and HDAC inhibitors in cancer cell lines reactivate silenced TSG expression. Techniques such as pharmacological unmasking microarrays (PUM) have been used to identify novel, critical TSG candidates.<sup>4</sup> Nevertheless, frequent cancer-specific methylation is rare across the whole genome, and few methylated genes have been validated as sites of its frequent aberration with high specificity in human cancer.<sup>5-7</sup> Rigorous screening by PUM has repeatedly unveiled novel cancer-prone methylation genes associated with tumor suppressive functions, such as the encoding *homeobox only protein homeobox (HOPX)* gene has been identified in various cancers,<sup>8-12</sup> and *HOPX* expression has been independently revealed to be a biomarker representing differentiation or quiescent stem cell signatures in normal organ tissues.<sup>13-17</sup> Hence, epigenetic changes in differentiation markers may be essential for the initiation of cancer cell growth.

DNA methylation in primary cancer tissues does not necessarily represent cancer-specific methylation. For example, actual cancer specificity has been confirmed only in a very limited number of genes in primary gastric cancer.<sup>9</sup> In a screen that used direct sequencing to distinguish the “wheat” (genes with cancer-specific methylation) from the “chaff” (other methylated genes), *HOPX* was selected with the highest ranking (frequently methylated in 90% of primary tumors), followed by *Reprimo* (80%) and *NMDAR2B* (70%); high-throughput analysis using quantitative methylation-specific PCR (Q-MSP) validated these priorities.<sup>9,18</sup> Q-MSP can be used to screen for such cancer specificity because of its high-throughput nature; recent searches for the best performance showed high (exceeding .9) area under curve (AUC) values in human cancer for genes like that encoding *cysteine dioxygenase 1 (CDO1)*.<sup>19</sup> Among many other promising genes that have also been reported to be

methylated in primary tumor tissues, few have been validated for their cancer-prone nature using the “gold standard” method, Q-MSP.

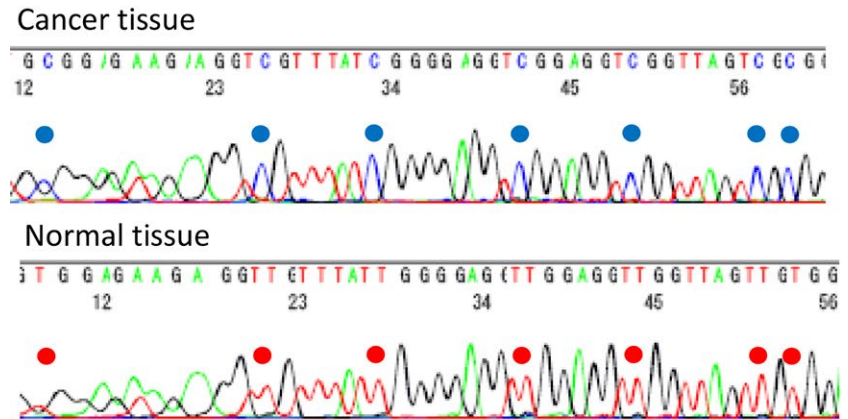
Such candidate biomarkers for methylated genes are widely scattered across the entire genome, amounting to a total of 200-300 genes.<sup>6,8,20</sup> After rigorous validation, superior biomarker candidates representing cancer-specific methylation are now considered ready for use in clinical decision-making in the development of therapeutic strategies, with possible use extending even to cases where cancer surgery is indicated.<sup>21</sup> In the present review article, the realistic potential of the epigenetic markers in cancer clinics is described.

## 2 | METHYLATION ASSAY

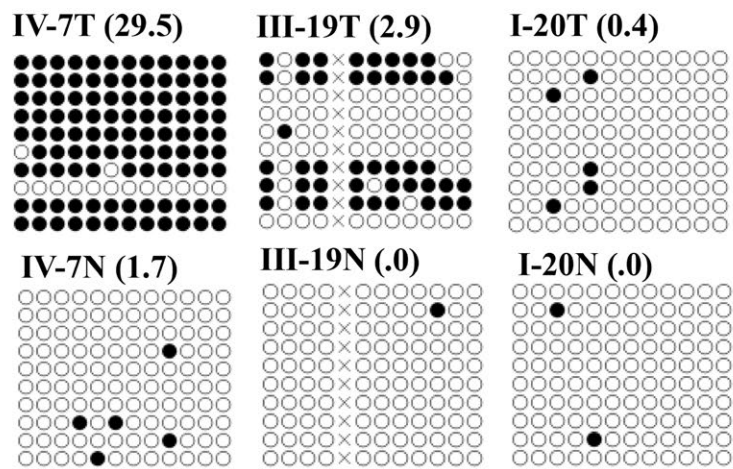
### 2.1 | Direct sequencing

Bisulfite treatment of DNA converts unmethylated cytosine to thymine without yielding a change to methylated cytosine. Following PCR amplification of the bisulfite-treated sequences, direct sequencing (ie, without first cloning the fragment) permits “direct” visualization of either the cytosine or thymine. This method is appealing for discovery screening of novel methylation events that represent novel TSG in primary tumors,<sup>4-7</sup> as well as the clear differentiation of primary tumors from the corresponding normal tissues (Figure 2A). In addition to direct sequencing, cloned sequencing (ie, sequencing of individual fragments after cloning of the PCR products) can elucidate the fine-scale mapping of methylation of the cytosines<sup>21,22</sup> (Figure 2B). However, cloned sequencing can be costly (in both time and money) and so is inappropriate for high-throughput analysis.

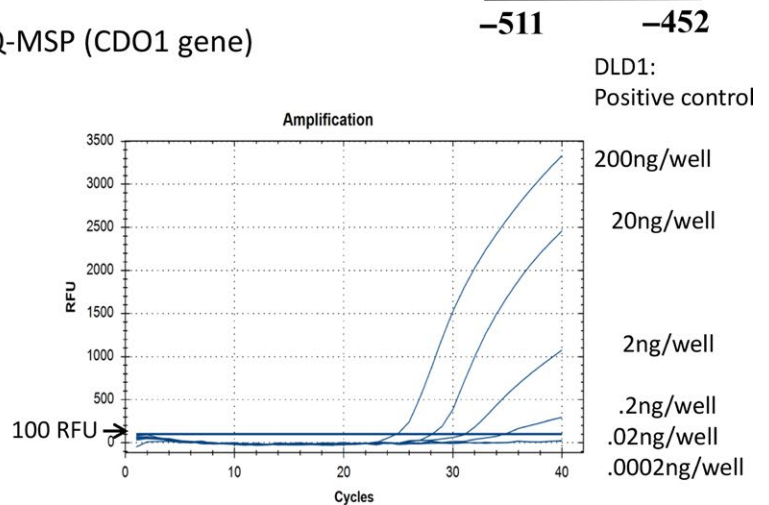
## (A) Direct sequencing (HOPX gene)



## (B) Cloned sequencing (HOPX gene)



## (C) Q-MSP (CDO1 gene)



**FIGURE 2** A, Direct sequencing revealed cytosine methylation (at the blue location) in primary tumor tissues (upper panel), while no cytosine methylation was detected (at the red locations) in the corresponding normal tissues. These cytosine methylation sites were consistent with CpG islands in the promoter DNA of the *HOPX* gene. B, Cloned sequencing after PCR amplification of products revealed finer-scale status of the methylated cytosines of the CpG portions of the *HOPX* gene promoter. Black circles represent methylated residues, while white circles indicate residues that lacked methylation (as assessed by cloned sequencing). TaqMeth values by Q-MSP ( $HOPX$  value/ $\beta$ -actin value  $\times$  100) are indicated within parentheses (right corners of each panel). C, Q-MSP for *CDO1* gene methylation was performed in DLD1 cells, revealing almost full methylation (93.3% of the CpG sites were methylated as judged by cloned sequencing) at various dilutions. PCR amplification detected signals at dilutions of 10-, 100- and 1000-fold, but not at 10 000- and 100 000-fold dilutions

## 2.2 | Real-time methylation-specific PCR

Methylation-specific PCR (MSP) is appropriate for high-throughput analysis of DNA methylation,<sup>23</sup> and quantitative MSP using a TaqMan probe (Q-MSP) permits the investigation of both the tumor tissues and the corresponding normal tissues

of cancer patients in a high-throughput manner; evaluation of the resulting receiver operating characteristic (ROC) curves permits discrimination of the tumor from the normal mucosa based on the most objective optimal criterion (optimal cut-off value).<sup>9,18</sup> The AUC of the ROC curve is a "gold standard" for identifying excellent epigenetic cancer biomarkers. However,

“the cut-off value or below” does not necessarily represent non-methylated status. It is more accurate to say that methylation values exceeding the cut-off value represent relative hypermethylation. For example, in primary gastric cancer, the most optimal cut-off value of the *HOPX* TaqMeth value (*HOPX* methylation value/*beta-actin* methylation value  $\times$  100%) was calculated as 3.6; this value permitted discrimination of the tumor from the normal tissues by Q-MSP.<sup>9</sup> When this cut-off value was used, *HOPX* hypermethylation was seen in 84% of the tissues defined as primary gastric tumors (based on classical histopathology), while *HOPX* hypermethylation was seen in 10% of the corresponding “normal” mucosa (again, as defined by classical histopathology). Intriguingly, this definition of hypermethylation by the Q-MSP technique was consistent with the results (presence or absence of methylation) of direct sequencing in gastric cancer cell lines.<sup>9</sup> The use of a Q-MSP cut-off value to discriminate cancer tissues from normal tissues, therefore, is highly consistent with the empirical results of direct sequencing. Representative methylation values of the *HOPX* gene based on the cloned sequencing are shown for primary cancer and normal mucosa tissues in Figure 2B.<sup>22</sup> These results demonstrate that hypomethylation is not synonymous with complete unmethylation.

At best, Q-MSP can detect methylation at dilution levels 1/1000 that of full methylation (Figure 2C), although Q-MSP is unable to detect a 1/10 000 to 1/100 000 dilution of methylation, a detection level that would be equivalent to that of digital PCR technique that is the most sensitive system available at present.<sup>21,24</sup> However, there are currently few papers on the use of digital PCR for methylation analysis in cancer clinics.

### 3 | THE BEST PERFORMANCE AS AN EPIGENETIC CANCER BIOMARKER: *CDO1*

Cancer-specific methylation should be designated only after comparing methylation in tumor tissues with that of the corresponding non-cancerous tissues. Nevertheless, many methylation genes have been so-called without such validation. CpG island methylator phenotype (CIMP) was defined based on the methylation profiles of multiple well-known tumor suppressor genes (e.g. *p16*, *MLH1* and *TIMP3*) in the same tissue samples; however, the individual genes showed methylation frequency of <50% in most primary tumors.<sup>25,26</sup> Such methylation frequencies are rather low compared to the >60% methylation seen in human cancer loci recently identified as highly relevant methylation genes (HRMG).<sup>27</sup> The relations of the CIMP phenotype to HRMG methylation status in human cancer have remained elusive, although several reports have suggested that HRMG are closely associated with CIMP. For example, methylation of *RIZ1*, an HRMG in gastric cancer (hypermethylated in 69% of primary tumors), was closely associated with the CIMP phenotype ( $P = .002$ ).<sup>28</sup> While HRMG differ in various organs (Table 1), and the *CDO1* gene is one of the

most common HRMG reported to data. The story thus begins at the *CDO1* gene followed by other HRMG.

Cysteine metabolism plays a pivotal role in cell stemness through regulation of reactive oxygen species (ROS),<sup>29</sup> and *CDO1* can actually augment ROS generation to induce apoptosis.<sup>19,30</sup> The *CDO1* protein can also bind peroxisome proliferation-activated receptor (PPAR) gamma to activate the critical onco-transcriptional factor CCAAT-enhancer-binding proteins (CEBP) alpha,<sup>31</sup> which can inhibit transduction of the Wnt signal.<sup>32</sup> Hence, *CDO1* may play a critical tumor suppressive role in tumorigenesis.

Since 2010, the frequent hypermethylation of the *CDO1* gene has been reported in primary breast,<sup>33,34</sup> lung,<sup>19</sup> biliary tract,<sup>35</sup> esophageal squamous cell carcinoma (SCC)<sup>36</sup> and adenocarcinoma,<sup>37</sup> gastric,<sup>19</sup> colorectal,<sup>19,38</sup> bladder,<sup>19</sup> penile (SCC),<sup>39</sup> kidney,<sup>40</sup> prostate,<sup>41</sup> endometrial,<sup>42</sup> pancreatic<sup>43</sup> and gallbladder cancer.<sup>44</sup> The frequencies of the aberrations in *CDO1* methylation in these cancers are high (Figure 3A). Intriguingly, however, there are several unique cancers in which the *CDO1* gene did not show an HRMG phenotype: for instance, renal cell clear carcinoma.<sup>28</sup> The frequencies were largely determined by generating an ROC curve to determine the most optimal cut-off value of methylation after comparing the tumor with the corresponding normal tissues in Q-MSP; an example of such analysis is shown in Figure 3B for pancreatic cancer.<sup>43</sup>

In contrast, the clinical relevance of *CDO1* gene in primary tumor tissues has also been demonstrated in several cancers. Specifically in esophageal cancer, *CDO1* methylation was significantly higher in advanced SCC tumors with cStage II/III than in the superficial tumors with cStage I,<sup>36</sup> and was significantly higher in larger-sized adenocarcinomas than in smaller-sized adenocarcinomas.<sup>37</sup> Aberrant methylation of the *CDO1* gene accumulated as the tumor progressed, as demonstrated in colorectal<sup>38</sup> and gallbladder tumorigenesis<sup>44</sup> (Figure 4A,B). These findings indicate that promoter DNA methylation of the *CDO1* gene commonly accumulates with progression in human cancers.

Cases with *CDO1* hypermethylation also showed poorer prognosis than those with hypomethylation in primary breast,<sup>34</sup> prostate,<sup>41</sup> colorectal,<sup>38</sup> gallbladder<sup>44</sup> and esophageal cancers.<sup>36,37</sup> Interestingly, in primary breast cancer, *CDO1* hypermethylation did not correlate significantly with markers of tumor progression such as TNM factors, while *CDO1* hypermethylation was the strongest independent prognostic factor.<sup>34</sup> Importantly, the prognostic relevance of *CDO1* methylation was confirmed even in a prospective nationwide cohort study in the Netherlands of patients with renal cell carcinoma.<sup>40</sup> These findings suggest that the methylation status of the *CDO1* gene could be used as a prognostic marker in various human cancers. However, the cut-off values of *CDO1* methylation as prognostic markers were always higher than those used to discriminate the tumors from the corresponding normal tissues, suggesting that cancer progression resulting in dismal prognosis is associated with stronger epigenetic changes than those observed at initial tumorigenesis.

**TABLE 1** Methylation frequencies of highly relevant methylation genes (HRMG) in human cancers

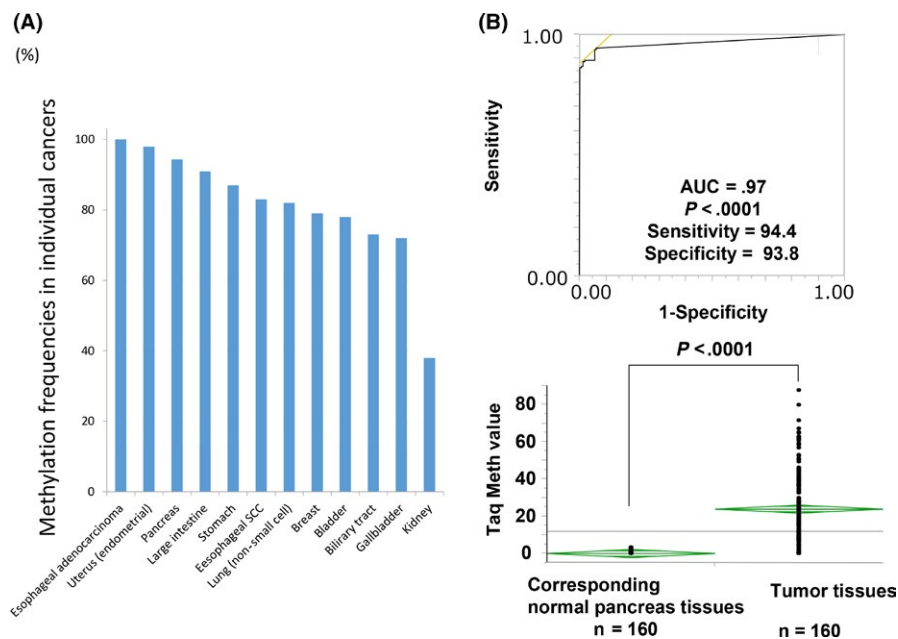
Organ	1	2	3	4	5
Head and neck SCC	<i>HOXA9</i> (.81, 60%)	<i>NID2</i> (.79, 71%)	<i>UCLH1</i> (.78, 66%)	<i>DCC</i> (.77, 75%)	<i>KIF1A</i> (.76, 72%)
Esophageal SCC	<i>ZNF582</i> (.95, 86%)	<i>NEFH</i> (.93, 86%)	<i>CDO1</i> (.91, 84%)	<i>NMDAR2B</i> (.91, 78%)	<i>PAX1</i> (.89, 100%)
Esophageal adenocarcinoma	<i>SFRP1</i> (96%)	<i>CDO1</i> (95%)	<i>APC</i> (92%)	<i>CDH1</i> (84%)	<i>TIMP3</i> (74%)
Lung	<i>GHSR</i> (1.0)	<i>CDO1</i> (.87, 92%)	<i>HOXA9</i> (96%)	<i>SHOX2</i> (94%)	<i>TAC1</i> (87%)
Stomach	<i>CDO1</i> (.95, 87%)	<i>DLEC1</i> (.87, 93%)	<i>HOPX</i> (.85, 84%)	<i>Reprimo</i> (.77, 69%)	<i>FLNC</i> (.72, 93%)
Large intestine	<i>CDO1</i> (.96, 91%)	<i>SFRP1</i> (.96, 85%)	<i>GFRA1</i> (.95, 89%)	<i>SEPT9</i> (.94, 100%)	<i>DCLK1</i> (.93, 82%)
Biliary tract	<i>SFRP1</i> (.95, 84%)	<i>OPCML</i> (.93, 89%)	<i>CDO1</i> (.91, 85%)	<i>ZSCAN18</i> (.77, 65%)	<i>DCLK1</i> (.75, 58%)
Gallbladder	<i>SEPT9</i> (.82, 77%)	<i>CDO1</i> (.74, 72%)	<i>14-3-3 sigma</i> (90%)	<i>3-OST-2</i> (72%)	<i>Maspin</i> (70%)
Pancreas	<i>GHSR</i> (1.00)	<i>CDO1</i> (.97, 94%)	<i>HOPX</i> (.85, 83%)	<i>NPTX2</i> (100%)	<i>UCLH1</i> (100%)
Breast	<i>GHSR</i> (.98, 92%)	<i>CDO1</i> (.84, 79%)	<i>MAL</i> (95%)	<i>14-3-3 sigma</i> (91%)	<i>VEGF</i> (89%)
Uterus (cervical)	<i>NKX6-1</i> (.97, 93%*)	<i>SOX9</i> (.96, 92%)	<i>SOX1</i> (.95, 88%*)	<i>ZNF516</i> (.92, 90%)	<i>LMX1A</i> (.9, 89%*)
Uterus (endometrial)	<i>GALR1</i> (.97, 100%)	<i>COL14A1</i> (.96, 92%)	<i>ZNF177</i> (.95, 92%)	<i>ZNF154</i> (.94, 82%)	<i>TMEFF2</i> (.90, 65%)
Bladder	<i>CDO1</i> (.87, 78%)	<i>APAF-1</i> (100%)	<i>Twist1</i> (98%)	<i>NID2</i> (96%)	<i>PCDH17</i> (92%)
Prostate	<i>RASSF1</i> (.99, 96%)	<i>MDR1</i> (.98, 88%)	<i>APC</i> (.97, 90)	<i>GHSR</i> (.97)	<i>GSTPI</i> (.96, 93%)

(Area under curve [AUC] of receiver operating characteristic [ROC] curve to differentiate the tumor from the normal counterpart, positive methylation frequency).

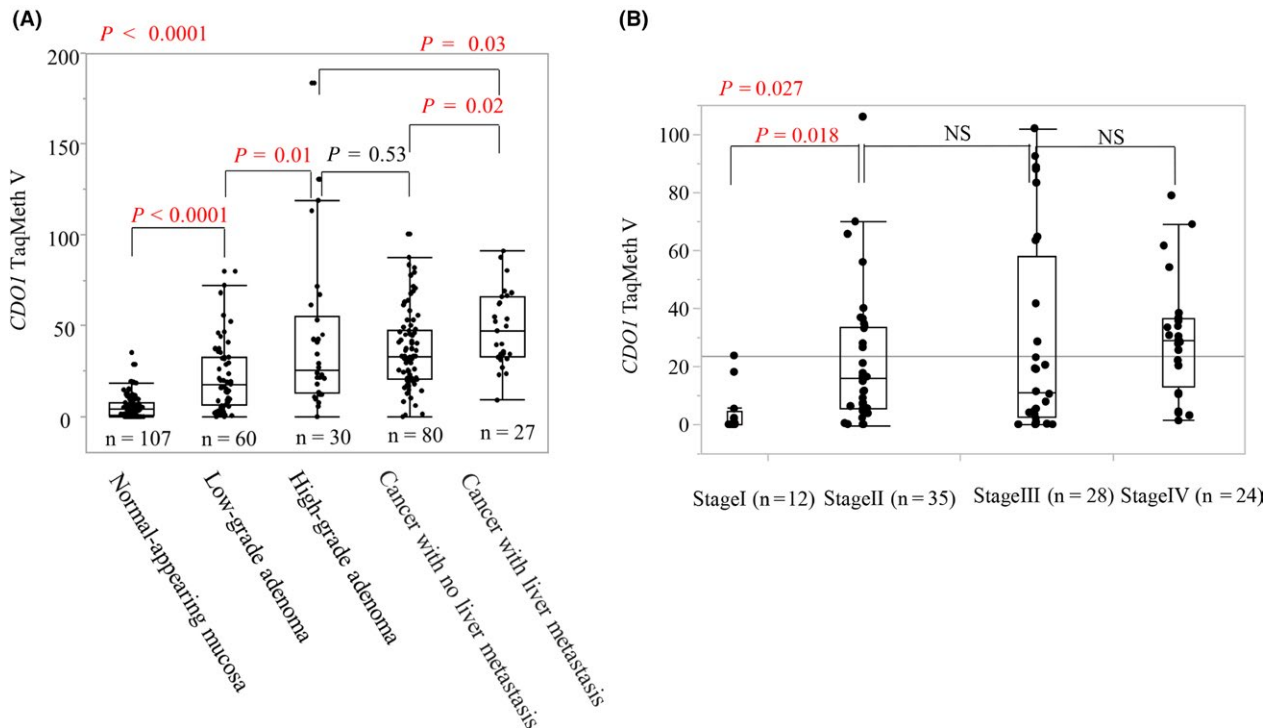
Asterisks were assessed by scrape samples.

Area under curve (AUC) could not always be included in this table due to lack of data.

SCC, squamous cell carcinoma.



**FIGURE 3** A, Methylation of the *CDO1* gene is shown in order of the frequencies in various cancers of esophageal adenocarcinoma (100%), endometrial adenocarcinoma (98%), pancreatic adenocarcinoma (94%), large intestine adenocarcinoma (CRC; 91%), stomach (gastric) adenocarcinoma (87%), esophageal squamous cell carcinoma (SCC) (83%), lung (non-small cell) cancer (82%), breast adenocarcinoma (72%), bladder cancer (78%), biliary tract cancer (73%), gallbladder cancer (72%) and kidney cancer (38%). B, Area under curve (AUC) of the receiver operating characteristic (ROC) curve to discriminate primary tumor tissues from the corresponding normal tissues is shown for pancreatic cancer. Quantitative methylation-specific PCR (Q-MSP) showed significant difference ( $P < .0001$ ) in *CDO1* methylation between the primary tumor tissues and the corresponding normal tissues



**FIGURE 4** Methylation value increased as the tumor progressed. A, In colorectal carcinogenesis, the *CDO1* methylation level increased significantly during the course of Vogelstein adenoma carcinoma sequence ( $P < .0001$ ). B, In gallbladder carcinogenesis, the *CDO1* methylation level increased significantly as the disease progressed from stage I to stage II

#### 4 | CANCER DETECTION MARKER OF *CDO1* GENE METHYLATION IN HUMAN BODY FLUIDS OR CONVENTIONAL BIOPSY/CYTOLOGY TEST

Highly relevant methylation genes could also be used as detection markers for minute cancer cells, because of the cancer-specific and prevalent nature of HRMG. Promoter DNA methylation of the *CDO1* gene is one of the most relevant changes across the whole genome, so methylation of this gene's promoter could be a highly promising epigenetic cancer biomarker candidate, even in human body fluids. Q-MSP can detect, at most, a 1/1000-dilution level of the fully methylated genes (Figure 2C); however, this detection level would not provide satisfactory sensitivity for detection of the marker in the plasma DNA of colorectal cancer (CRC) patients (in whom the *CDO1* promoter is seen in 40% of stage IV disease).<sup>45</sup> Digital PCR for *CDO1* methylation (which can detect a 1/100 000 dilution of fully methylated *CDO1*) might be sufficient to detect cancer cells in plasma, as shown for *VIMENTIN* (*VIM*) gene hypermethylation in patients with CRC.<sup>46</sup> However, *CDO1* gene methylation was reported to be readily detected in the plasma of patients with lung cancer,<sup>47,48</sup> where Q-MSP detection of *CDO1* methylation in plasma or serum showed 65% sensitivity with 74% specificity, and, when assessed in combination with the methylation of other HRMG, showed high sensitivity even in stage I disease. This extraordinarily high sensitivity of detection of cancer cells

in plasma has been for the first time experienced in cancer clinics. Plasma surveillance will play a pivotal role in evaluating therapeutic efficacy during cancer management including surgery.

*CDO1* hypermethylation could be useful for assisting preoperative disease diagnosis in critical body fluids other than plasma: for instance, through testing of endoscopic retrograde cholangiopancreatography (ERCP) brushing solution in biliary tract cancer<sup>49</sup> and cervical scrapings in endometrial cancer.<sup>42</sup> These body fluids are important for diagnosis, because inaccurate diagnosis would lead to unnecessary invasive surgery (false positive) or to missing the true disease (false negative). Cytology testing of ERCP brushing solution can be used to diagnose biliary tract cancer in approximately 60% of cases, while the *CDO1* methylation cytology test showed a high AUC of .93 in predicting biliary tract cancer.<sup>49</sup> Cervical scrapings were reported as a potential source of material for molecular testing in endometrial cancer, and *CDO1* hypermethylation amazingly showed an 82% sensitivity with 94% specificity for detecting endometrial cancer in cervical scrapings.<sup>42</sup>

DNA testing using *CDO1* hypermethylation may be useful for intraoperative diagnosis in gastric cancer surgery. The peritoneal DNA cytology test-positive gene (*CY1*) is a critical prognostic marker of gastric cancer, and *CY1*-positive cases represent stage IV disease. However, the conventional cytology test does not achieve sufficient sensitivity because peritoneal recurrences have been recognized in cytology-negative (*CY0*) cases. DNA cytology testing using *CDO1* methylation showed 2-fold higher detection rates of the minimal residual disease than did conventional *CY* tests in

the peritoneum, which was all positive for CY1 cases (Figure 5A), and can predict peritoneal recurrence accurately.<sup>50</sup> DNA CY1 diagnosis in the peritoneum would be beneficial for planning future multimodal therapy in gastric cancer after curative surgery. Cytology tests targeted at the peritoneum are critical for other types of cancer, such as colorectal<sup>51</sup> or ovarian cancer,<sup>52</sup> and DNA CY1 information may be useful for decision-making in the context of various abdominal cancers other than gastric cancer.

*CDO1* methylation could also be used to evaluate conventional biopsy samples evaluating tumor eradication after neoadjuvant therapy in esophageal cancer.<sup>36</sup> In esophageal SCC showing histological grade 2/3 that has been treated with neoadjuvant chemotherapy, the *CDO1* methylation level is significantly lower than in other cases (Figure 5B). These findings suggested that *CDO1* methylation can reflect the presence of remnant cancer cells in the biopsies after treatment by neoadjuvant chemotherapy. If confirmed by rigorous validation, such information could affect surgical indications in the near future.

## 5 | CLINICAL DECISION BY COMBINATION OF HIGHLY RELEVANT METHYLATION GENE EPIGENETIC BIOMARKERS

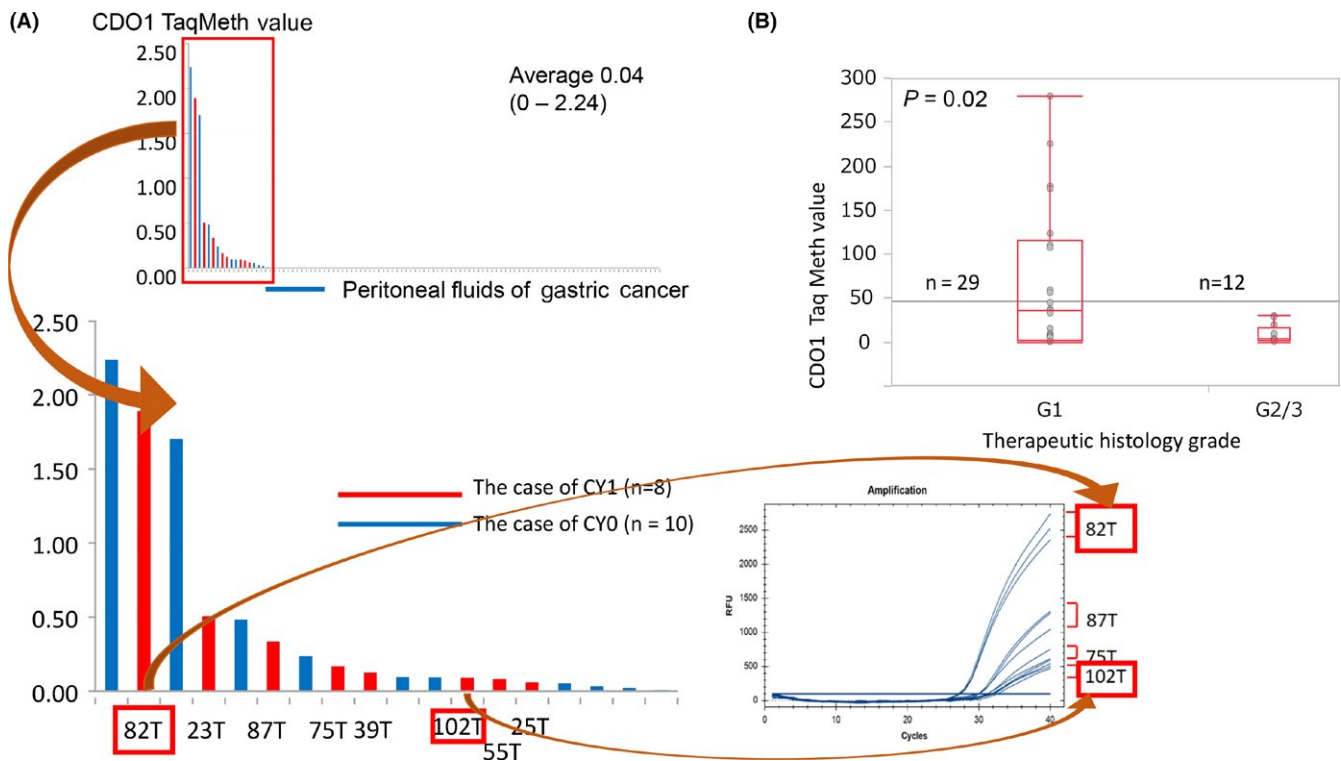
Epigenetic biomarkers using HRMG can affect the accuracy of: (i) preoperative diagnosis; (ii) intraoperative diagnosis; (iii) pathological

diagnosis; and (iv) follow-up surveillance on the therapeutic decision in cancer clinics.

### 5.1 | Preoperative diagnosis

In hepato-pancreato-biliary (HPB) cancer, pancreaticoduodenectomy, which has a high mortality rate, is needed for cure, and surgeons would prefer to definitively confirm cancer preoperatively. However, biopsy samples of HPB tumors cannot be obtained directly without invasive procedures because tumors of this type are located off the luminal mucosa. Cytological testing of the ERCP wash solution is currently the only method in a less invasive manner diagnose the tumors preoperatively.

In pancreatic cancer, 4 sequential case-control studies (discovery, technical validation, biological validation and clinical piloting) were conducted to demonstrate the diagnostic utility of HRMG analysis of *CD1D* (encoding a member of the CD1 family of transmembrane glycoproteins) methylation. Results of those studies showed that *CD1D* methylation in the pancreatic juice yielded an AUC value of .92 for patients with pancreatic cancer compared to patients with normal pancreas and chronic pancreatitis.<sup>53</sup> *CD1D* methylation in the pancreatic juice detected pancreatic cancer with 75% sensitivity and 95% specificity. That work also reported that *CD1D* methylation showed an AUC value of .94 in primary pancreatic cancers compared to the corresponding normal tissues.<sup>53</sup> Subsequent analyses



**FIGURE 5** A, In gastric cancer, DNA cytology test using *CDO1* methylation showed an approximately 2-fold higher detection rate of minimal residual disease in the peritoneum, among cases that were positive for CY1 ( $n = 104$ ). B, In esophageal squamous cell carcinoma (ESCC) with neoadjuvant chemotherapy showing histological grade 2/3, *CDO1* methylation level was significantly lower than in other cases ( $n = 41$ )

identified HRMG with higher performance in pancreatic cancer than *CD1D*, including the *GHSR*<sup>54</sup> and *CDO1*<sup>43</sup> genes (Table 1). Liquid biopsy of pancreatic juice using such novel HRMG (or combinations thereof) holds great promise.

In biliary tract cancer (BTC), bile solution was used to detect cancer using *CDO1/CNRIP1/SEPT9/VIM* methylation.<sup>49</sup> These genes were HRMG of BTC, and the combination detection in bile solution showed 85% sensitivity with 95% specificity. The AUC of the combination yielded an AUC of .94, but *CDO1*, the marker with the best performance, also exhibited an AUC of .93. Liquid biopsy using such HRMG showed superior diagnosis (73%-91%) and high specificity (95%-100%) compared to the conventional cytology test (58%-63%). Recently, additional HRMG with high performance have been identified, including *SFRP1*,<sup>55</sup> *OPCML*<sup>55</sup> and *SHOX2*<sup>56</sup> (Table 1), and additional optimization of the HRMG analyses may yield further improvements in performance.

Lung cancer also presents similar issues with regard to preoperative diagnosis. The conventional sputum cytology test shows low sensitivity; however, recent research using HRMG has shown excellent performance in lung cancer diagnosis. The *TAC1/HOXA7/SOX17* methylation combination showed 88% sensitivity with 71% specificity (AUC, .89).<sup>47</sup> These genes were HRMG in lung cancer and increased methylation was detected in the plasma of lung cancer, but the best discrete combination of the methylated genes (*CDO1/TAC1/SOX17*) exhibited the highest performance (91% sensitivity with 62% specificity).<sup>47</sup> Relatively low specificity is a concern in lung cancer. Ooki et al<sup>48</sup> selected a methylation panel of 6 genes (*CDO1/HOXA9, AJAP1/PTGDR/UNCX/MARCH11*) from the TOGA dataset. Even in serum samples from the stage IA subjects and population-matched control subjects, the gene panel yielded a sensitivity of 72.1% and a specificity of 71.4%.

In contrast, in gastrointestinal cancer, screening by endoscopy provides excellent diagnosis, and direct biopsy can guarantee the accuracy of preoperative diagnosis. Therefore, a cytological test is being developed for the sake of supplemental information. The identification of HRMG in oral rinse or gastric rinse specimens may be beneficial for the purpose of disease screening. On the other hand, a fecal DNA test providing quantitative molecular assays for *K-ras* mutations, aberrant *NDRG4* and *BMP3* methylation, with reference gene  $\beta$ -actin was shown to increase the diagnostic accuracy of colonic neoplasms by adding HRMG analysis to the conventional fecal immunochemical test (FIT).<sup>57</sup> Colonoscopy is an invasive procedure with a high risk of complications, so a fecal DNA test would be a promising tool for assessment prior to colonoscopy.

In oral cancer, a saliva test using HRMG such as *HOXA9/NID2* detected 50% of cancers with 90% specificity.<sup>58</sup> This sensitivity was not satisfactory as a liquid biopsy using HRMG, putatively due to inferior discriminating markers of oral SCC cancer (representing AUC of approximately .8 in primary tumors). However, saliva testing using another combination of HRMG (*EDNRB/DCC*) yielded improved diagnostic accuracy of oral neoplasms including precancerous lesions when combined with professional classification of clinical risk.<sup>59</sup>

In gynecological and urological cancer research, methylation markers were applied to the testing of cervical scrapings and urine specimens. Both tests are kinds of cytological assays, and various methylation markers showed superior clinical outcomes for diagnosis of the individual cancers as described below. This improved performance was especially notable for prostate cancer, where recurrence was defined as biochemical recurrence by serum PSA. These results demonstrate that biomarkers could have a significant role in disease surveillance, beyond the use of imaging modalities.

Screening by HRMG analysis of cervical scrapings using HRMG was applied to both cervical cancer and endometrial cancer. In cervical SCC, *SOX1* and *PAX1* methylation have been reported to have 74% sensitivity with 97% specificity in scrapings.<sup>60</sup> In combination with the Pap test, the sensitivity reached 89%.<sup>61</sup> Early detection of cancer would improve the clinical outcome of less invasive surgical treatments. However, *CADM1/MAL* combination analysis showed 92% sensitivity compared to CIN3 and 100% sensitivity for cancer.<sup>62</sup>

The HRMG profile of endometrial cancer is different from that of cervical SCC cancer; however, the combination of HRMG showed more successful performance in the former than in the latter. Specifically, the *BHLHE22/CDO1/CELF4* combinations of methylation markers showed 92% sensitivity with 95% specificity in scrapings tests.<sup>42</sup> Given that there have been no descriptions of methylation frequencies of primary tumors for assessing whether *CDO1* is an HRMG in this disease, that gene was not included as an HRMG for endometrial cancer in Table 1.

In urological cancers as well as in gynecological cancers, DNA diagnosis in urine sediments has been rigorously explored using HRMG analysis. In bladder cancer, urine sediments using *Twist1/NID2* methylation showed 90% sensitivity with 95% specificity, compared to 48% sensitivity in conventional urine cytology test.<sup>63</sup> In bladder cancer, there is little information regarding the AUC values assessed for both tumor and corresponding normal tissues by Q-MSP for genes such as *CDO1*.<sup>19</sup>

In prostate cancer and renal cell cancer, methylation biomarkers are likely to be promising for early detection of cancer cells. In prostate cancer, the combination of *GSTP1/p16/ARF/MGMT* methylation in urine sediments showed 87% sensitivity with 100% specificity.<sup>64</sup> In prostate cancer, many excellent HRMG have been reported,<sup>54,65</sup> and further optimization is anticipated. The *CDO1* methylation level is significantly higher in prostate cancer than in benign prostate hyperplasia or normal prostate tissues; however, neither AUC nor frequencies were reported in that study.<sup>41</sup>

In gynecological and urological cancer research, comparisons of tumor tissues with the corresponding normal tissues have seldom been investigated, but test samples such as urine sediments or scrapings have been examined directly. To date, it has not been proved whether methylation markers are actually HRMG, although in the present review, the HRMG was defined using AUC of scraping samples. Accurate diagnosis using such HRMG is expected to lead to appropriate treatment strategies including surgery.



## 5.2 | Intraoperative diagnosis

In the abdominal oncology field, the intraoperative peritoneal cytology test is an emerging modality that is used in the staging system. However, histological findings are not sufficient to detect minimal residual disease; molecular markers are anticipated to have great potential in this context. *Carcinoembryonic antigen (CEA)* mRNA was initially applied to this cytology test, where the technique was able to predict poor prognosis;<sup>66</sup> however, mRNA is unstable, and unlikely to be appropriate for the routine test. DNA markers are more stable than mRNA, but there has been (to date) no highly relevant marker.<sup>67</sup> Nevertheless, a combination of methylation markers was tested for use in cancer detection in the intraoperative peritoneal cytology test. Testing of methylation at *BNIP3/CHFR/CYP1B1/MINT25/SFRP2/RASSF2* showed that 7%-20% of cases had no peritoneal dissemination, and 75% of those results were consistent with those of the peritoneal cytology test. This multiplex analysis predicted peritoneal dissemination in 33% of the DNA cytology test-positive cases but in only 3% of the DNA cytology test-negative cases. This finding suggested that DNA cytology test-positive results represent viable cancer cell detection in the peritoneum. In short, the results of this analysis confirm the need to use multiplex assays (ie, using gene combinations), because the methylation frequency in the primary tumor tissues was not as high as that in individual genes, and there were cases that yielded conventional cytology test-positive results but were DNA cytology test-negative.

Using HRMG analysis, this point could be resolved using a peritoneal cytology test. *CDO1* methylation is one of the most frequent aberrations in gastric cancer tissues. A DNA cytology test using *CDO1* methylation detected cancer cells in all CY1 cases (100%), and, moreover, it detected cancer cells in 20% of all cases of gastric cancers, a rate that is twice that of the conventional cytology test.<sup>36</sup> This DNA cytology test can predict peritoneal recurrence in gastric cancer with type III and type IV gastric cancer. A prospective trial (UMIN000026191) is currently being conducted to confirm the utility of a DNA cytology test using *CDO1* methylation in 400 cases of gastric cancer. Table 2 provides a comparison between the clinical features of *CDO1* methylation and those of *CEA* mRNA (cDNA) and methylation combinations in the peritoneal fluid washing cytology test.

**TABLE 2** Clinical features of *CDO1* methylation compared with those of *CEA* and methylation combinations in peritoneal fluid washing cytology test

	mRNA	Non-HRMG	<i>CDO1</i> (HRMG)
Sample stability	△	○	○
Sensitivity	23%	25%	20%
% of CY0	10%	7%-20%	10%
% of CY1	69%	75%	100%
Marker	<i>CEA</i>	<i>BNIP3/CHFR/CYP1B1/MINT25/SFRP2/RASSF2</i>	<i>CDO1</i>
Reference	66	67	36

HRMG, highly relevant methylation gene.

## 5.3 | Pathological diagnosis

The most critical information representing cancer phenotypes must be obtained from the primary cancer tissues. Epigenetic information in the primary tumors should be considered in clinical decisions. Recent comprehensive exploration in large-scale DNA methylome analyses identified many HRMG representing cancer phenotypes as described above. HRMG has often been linked to crucial cancer phenotypes, because these markers may reflect functional aspects of tumor aggressiveness. HRMG have been demonstrated to be associated with aggressive tumor phenotypes such as lymph node metastasis or distant metastasis and frequently were predictive of dismal prognosis.<sup>34,36-38,41,44</sup> Promoter DNA methylation accumulates with disease progression, and the groups with the highest methylation values showed the poorest prognosis. Among such candidates, several genes would affect therapeutic decisions after surgery.

Separately from methylation of the primary tumors, prognostic utility has been reported as a prognostic factor. This result was obtained in research that focused on minimal residual diseases in lymph nodes pathologically diagnosed as "negative." Using HRMG for negative lymph nodes in stage I lung cancer, minimal residual disease in lymph nodes was detected, and such patients showed poorer prognosis than the other patients.<sup>68</sup> This result indicated that the detected HRMG methylation may represent a micrometastasis of cancer cells in lymph nodes, which were not capable of being discerned by the conventional pathological searches. In stage I lung cancer, postoperative adjuvant chemotherapy was not indicated; however, methylation-positive cases with the pathology-negative lymph nodes showed a 70% survival rate, and this patient selection method may be appropriate in candidates for adjuvant chemotherapy. Such a therapeutic strategy could be applied to gastrointestinal cancer in pathology-negative cases,<sup>69</sup> given that there have been no reports describing prognostic significance of such molecular micrometastasis signatures in pathology-negative lymph nodes.

Methylation genes harboring predictive value for anti-cancer drug efficacy provide attractive information for use in the development of therapeutic strategies. *MGMT* methylation in brain tumors showed success as such a marker.<sup>70</sup> *MGMT* methylation was able to predict the chemosensitivity

to alkylating agents of brain tumors<sup>71,72</sup> and other cancers.<sup>73,74</sup> Brain tumor cases with *MGMT* hypermethylation exhibited better postoperative outcomes than those with *MGMT* hypomethylation. Specifically, patients with *MGMT* hypermethylation showed greater responsiveness to radiation therapy.<sup>75</sup> In the brain tumor clinics, nanogram was useful including this epigenetic information.<sup>76</sup>

In esophageal cancer, *CDO1* methylation was associated with histological grade of neoadjuvant chemotherapy.<sup>36</sup> In tumors that were grade 2/3 after neoadjuvant chemotherapy, *CDO1* methylation was not detected. The *CDO1* gene is an HRMG in esophageal cancer, so the failure to detect *CDO1* methylation in grade 2/3 tumors may represent cancer eradication by neoadjuvant chemotherapy. Issues of concern included cases in which genes exhibited hypomethylation before neoadjuvant chemotherapy, and those in which gene methylation status differed when compared before and after chemotherapy; these disparities will need to be investigated to clarify the clinical utility of screening biopsy samples for gene methylation. In this context, it would be beneficial to exclude patients in whom neoadjuvant chemotherapy was not effective, which would eliminate cases in which disease progresses despite neoadjuvant chemotherapy.

## 6 | CONCLUSIONS

Recent research approaches have identified many HRMG in individual human cancers, and these novel emerging epigenetic biomarkers are ready for use in testing liquid biopsies in the clinic. Information about HRMG is expected to permit cancer surgeons to avoid unnecessary operations, to provide early detection of cancer occurrence and cancer recurrence, and to facilitate convenient surveillance with high accuracy in outpatients. These techniques are expected to permit the development of more sophisticated and optimized therapeutic strategies, yielding improved prognosis in cancer patients compared to current therapies. However, the optimization of screens employing known HRMG has not yet been achieved. The advantages of such markers are expected to depend on the specific clinical situations, and much research is ongoing to demonstrate the clinical significance of these approaches. Low levels of cancer-derived DNA in body fluids can be detected using HRMG analysis with Q-MSP; however, the use of digital PCR is expected to expand the clinical applicability of HRMG analysis to liquid biopsies. The surgical decisions that are expected to be most immediately affected by epigenetic markers are those relating to indications of adjuvant therapy for stage I lung cancer or glioblastoma, although cancer diagnosis and surveillance also will be improved by the epigenetic markers.

## CONFLICT OF INTEREST

There is no conflict of interest in this study.

## ORCID

Keishi Yamashita  <http://orcid.org/0000-0002-8492-0300>

Kei Hosoda  <http://orcid.org/0000-0002-9787-2889>

Masahiko Watanabe  <http://orcid.org/0000-0001-9038-0446>

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