Epigenetic alteration: new insights moving from tissue to plasma – the example of PCDH10 promoter methylation in colorectal cancer

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Background: Tumour-released DNA in blood represents a promising biomarker for cancer detection. Although epigenetic alterations such as aberrant promoter methylation represent an appealing perspective, the discordance existing between frequencies of alterations found in DNA extracted from tumour tissue and cell-free DNA (cfDNA) has challenged their practical clinical application. With the aim to explain this bias of agreement, we investigated whether protocadherin 10 (PCDH10) promoter methylation in tissue was associated with methylation pattern in matched cfDNA isolated from plasma of patients with colorectal cancer (CRC), and whether the strength of concordance may depend on levels of cfDNA, integrity index, as well as on different clinical–pathological features.

Methods: A quantitative methylation-specific PCR was used to analyse a selected CpG site in the PCDH10 promoter of 67 tumour tissues, paired normal mucosae, and matched plasma samples. The cfDNA integrity index and cfDNA concentration were assessed using a real-time PCR assay.

Results: The PCDH10 promoter methylation was detected in 63 out of 67 (94.0%) surgically resected colorectal tumours and in 42 out of 67 (62.7%) plasma samples. The median methylation rate in tumour tissues and plasma samples was 43.5% (6.3–97.8%) and 5.9% (0–80.9%), respectively. There was a significant correlation between PCDH10 methylation in cfDNA and tumour tissue in patients with early CRC (P < 0.0001). The ratio between plasma and tissue methylation rate increases with increasing cfDNA integrity index in early-stage cancers (P = 0.0299) and with absolute cfDNA concentration in advanced cancers (P = 0.0234).

Conclusion: Our findings provide new insight into biological aspects modulating the concordance between tissues and plasma methylation profiles.

Epigenetic inactivation of multiple tumour-suppressor genes (TSGs) is a key molecular event in the multistep genetic pathogenesis of cancer. Epigenetic silencing through aberrant methylation of CpG islands in TSG promoter regions occurs in virtually all tumour types as well as in premalignant lesions, and has been recently proposed as a new candidate cancer biomarker.

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silenced in multiple human cancer types (Ying et al, 2006) and promoter methylation has emerged as the leading mechanism for its downregulation in CRC (Zhong et al, 2013).

MATERIALS AND METHODS

Patients and samples. The study cohort included 67 consecutive patients undergoing surgery for CRC at the University Hospital of Verona (Italy) between January 2010 and December 2010. Blood specimens were collected before intervention. Paired tumour and adjacent normal mucosa were obtained during surgical procedure, immediately frozen in liquid nitrogen, and stored at −80 °C. Histological diagnosis was established on standard haematoxylin and eosin-stained sections according to the 2000 WHO classification system for tumours of the digestive system. The tumour stage was determined according to the AJCC staging system (Edge and Compton, 2010). Only patients with primary colorectal adenocarcinomas untreated with neo-adjuvant radiochemotherapy were included in the study. The CRC patients with a personal history of HIV or herpes virus B or C infection, previous history of cancer, or symptoms of severe acute or exacerbated chronic disease were excluded.

Ethical approval was obtained from the hospital and informed consent gained from all patients before sample collection. Clinical and pathological information were retrieved from medical records.

DNA isolation from plasma and tissue samples. Blood samples were collected in 7 ml EDTA tubes and processed within 1 h from collection. Plasma was separated by double centrifugation (800 g for 10 min, separation, and 1600 g for 10 min) and the aliquots were immediately frozen at −80 °C until DNA isolation. DNA was extracted from 1 ml of plasma by the QIAamp DNA Blood midi kit (Qiagen, Hilden, Germany) as previously described (Xue et al, 2009). Genomic DNA was also extracted from 10 μm fresh frozen tissue sections using the Gentra Purgene Kit (Qiagen) according to the manufacturer’s instructions.

Plasma DNA concentration and integrity index. The total amount of cell-free plasma DNA as well as DNA integrity index were assessed by two quantitative PCR (qPCR) assays targeting, respectively, a 115-bp amplicon and a 247-bp amplicon of a consensus sequence of human ALU interspersed repeats. The primer set for the 247-bp amplicon (ALU115) amplifies both shorter (truncated by apoptosis) and longer DNA fragments, whereas the primer set for the 247-bp amplicon (ALU247) amplifies only longer DNA fragments. Sequences of primers have been reported elsewhere (Umetani et al, 2006b). The shorter amplicon was used to quantify total DNA, whereas the ratio between the absolute concentration of the longer amplicon and the shorter one defined the integrity index 247/115, which was used to assess the fragmentation level of cfDNA.

Because the annealing sites of ALU115 are within the ALU247 annealing sites, an integrity index close to 1 indicates that template DNA is not truncated, whereas a value close to 0.0 means that cfDNA is truncated into fragments smaller than 247 bp. The absolute equivalent amount of DNA in each sample was determined by means of a calibration curve with serial dilutions (10 ng to 0.01 pg) of a genomic DNA obtained from peripheral blood leukocytes of a healthy volunteer. A negative control (without template) was run in each reaction plate. All qPCR assays were performed in a blinded manner, without knowledge of the specimen identity, and mean values were calculated from duplicate reactions. The PCR products were subjected to electrophoresis on 2% agarose gels to confirm product size and specificity of PCR.

The reaction mixture for each ALU-qPCR consisted of a template, 0.2 μM each of forward primer and reverse primers
Sodium bisulphite conversion. Purified genomic DNA was subjected to bisulphite modification treatment using the Epitect Bisulfite Kit (Qiagen). Briefly, all unmethylated cytosines in bisulphite reaction are deaminated and sulphonated, to be converted into uracil bases, whereas 5-methylcytosine remains unchanged. Thus, the sequence of treated DNA will differ depending on whether the DNA is originally methylated or unmethylated. In short, 1 μg of DNA was treated with conversion reagent and incubated at 50 °C for 10 min and 50 °C for 16 h. Samples were applied to columns, washed, desulphonated, washed again, and then eluted with 40 μl of elution buffer. Bisulphite-converted DNA samples were stored at −20 °C for up to 1 week.

Methylation-specific PCR (MSP). After sodium bisulphite treatment, enzymatic amplification of modified DNA was performed by Real-Time PCR using a Sybr green-based quantitative MSP. Primers for MSP were designed using the web-based software MethPrimer (http://www.orogene.org/methprimer) to specifically amplify either a bisulphite-sensitive, unmethylated strand or a bisulphite-resistant, methylated strand on the PCDH10 gene promoter region. In particular, we focussed on CpG island spanning the transcriptional start site on the exon 1 as we would expect it to be heavily methylated in cancer subjects. This assumption is derived by recent finding according to which the promoter activity of PCDH10 is located on this fragment and its methylation is responsible for gene silencing (Li et al., 2011). Detailed sequences of primer sets are as follows:

Methylated (M)-Fo: 5′-AGTTATAGGTTTATAGTAGCTG-3′ (−112 to −88);
M-Rev: 5′-ATATTTCTACTCTCTACCTAGCTA-3′ (+76 to +90);
unmethylated (U)-Fo: 5′-GAAAGTTATAGGTTTTTATGTAGTTG-3′ (−115 to −88);
U-Re: 5′-GAAAGTTATAGGTTTTTACGTAGTTG-3′ (+76 to +90).

In order to verify the efficiency of the newly designed primers, CpGenome Universal Methylated DNA (Chemicon, Millipore Billerica, MA, USA) was used as 100% methylated control DNA. DNA extracted from peripheral blood mononuclear cells of normal individuals was used as unmethylated control DNA. Defined mixtures of methylated and unmethylated DNA were used to assess if the methylated DNA was amplified in proportion.

The relative signals as exemplified by the cycle threshold (Ct) value specific for the methylated state (Ctm) and unmethylated state (Ctu) were used to calculate the methylation rate (methylation rate = 100/(1 + 2^(-Ctm-Ctu))). The proportion (%) of methylation rate detected in plasma with respect to methylation rate detected in tissues was expressed as plasma/tissue ratio (p/t ratio).

After detection of optimal conditions that allowed the same protocol to be performed for two sets of forward and reverse primers, 3 μl of bisulphite-modified DNA was used as template for amplification on 7500 Applied Biosystems real-time PCR system. The qPCR assay used a reaction mixture consisting of the following final concentration: 0.5 μM of forward and reverse primers, 250 μM of each dNTP (GE Healthcare, Little Chalfont, UK), 1 × HotStart Buffer (Qiagen), 1.5 mM MgCl₂, 1.5 units HotStart polymerase (Qiagen), 2 μM SYTO 9 (Invitrogen, Life Technologies, Carlsbad, CA, USA), and 1 × ROX reference dye (Invitrogen), in a total reaction volume of 20 μl with 3 mM MgCl₂. Real-time PCR amplification was performed with preincubation heat activation of DNA polymerase at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 64 °C for 30 s, and extension at 72 °C for 30 s in an 7500 Applied Biosystems real-time PCR system (Foster City, CA, USA).

RESULTS

Methylation profiles in tissues and plasma. Aberrant PCDH10 promoter methylation was detected in 94.0% (63 out of 67) of surgically resected colorectal tumours and in 10.4% (7 out of 67) of paired surrounding mucosae, respectively. The same test performed in plasma samples showed that 42 out of 67 (62.7%) samples were methylated. The rate of concordance between methylation status of tissues and plasma samples is shown in Table 1. The four patients who were negative for both tissue and plasma methylation were excluded from further analysis. The median calculated methylation rate in tumour tissues and plasma samples was 43.5% (63–97.8%) and 5.9% (0–80.9%), respectively. A statistically significant correlation was found between tissue and plasma methylation rate (r = 0.464, P < 0.001), and this association remained strongly significant for early-stage cancers (r = 0.677, P < 0.001), whereas it was lost for advanced cancers (r = 0.278, P = 0.178) after partitioning of patients according to cancer stage.

Table 2 summarises the PCDH10 methylation rate of the 63 colorectal tumours and corresponding plasma sample, and their association with various clinicopathological factors. In both tumour tissues and plasma samples, no significant associations were found for gender, tumour location, venous and lymphatic invasion, as well as tumour differentiation. In terms of pathological stage classification, the median methylation rate was significantly higher in advanced-stage (stage III/IV) cancer than in the early stage (stage I/II) (52.4%, 17.4–96.6% vs 36.7%, 6.3–97.8%). An opposite trend was found in plasma samples (7.3%, 0–55.0% vs 14.0%, 0–80.9%; Figure 1).

Association between the methylation profile, cfDNA concentration, and integrity index. The results of measurements revealed a wide spectrum of DNA concentrations in plasma of cancer patients, between 26.0 and 295.0 ng ml⁻¹, with a median value of 37.1 ng ml⁻¹. The cfDNA concentration and cfDNA integrity index positively correlated with tumour stage (r = 0.322, P = 0.01 and r = 0.316, P = 0.012, respectively). A positive correlation was found between p/t ratio and the integrity index (r = 0.353, 0.012, respectively). A positive correlation was found between p/t ratio and the integrity index (r = 0.353, 0.012, respectively). A positive correlation was found between p/t ratio and the integrity index (r = 0.353, 0.012, respectively). A positive correlation was found between p/t ratio and the integrity index (r = 0.353, 0.012, respectively). A positive correlation was found between p/t ratio and the integrity index (r = 0.353, 0.012, respectively). A positive correlation was found between p/t ratio and the integrity index (r = 0.353, 0.012, respectively). A positive correlation was found between p/t ratio and the integrity index (r = 0.353, 0.012, respectively). A positive correlation was found between p/t ratio and the integrity index (r = 0.353, 0.012, respectively). A positive correlation was found between p/t ratio and the integrity index (r = 0.353, 0.012, respectively). A positive correlation was found between p/t ratio and the integrity index (r = 0.353, 0.012, respectively). A positive correlation was found between p/t ratio and the integrity index (r = 0.353, 0.012, respectively).
The perspective to use molecular analysis of blood samples for cancer screening and surveillance is particularly attractive because of simplicity, moderate invasivity of venous blood collection, potential for assay automation, and large-scale population analysis. In particular, the assessment of epigenetic alteration in cfDNA has shown the potential to become a viable alternative to current screening strategy, especially for those tumours such as CRC where adherence rate to existing programs is still low because of poor compliance (Inadomi et al, 2012). Although several studies have attempted to identify abnormal methylation pattern in cfDNA of patients with CRC (Lee et al, 2009; Nishio et al, 2010; Tänzer et al, 2010; Hibi et al, 2011; Kim et al, 2011; Warren et al, 2011; Wu et al, 2011; Cassinotti et al, 2012; Pack et al, 2013; Summers et al, 2013), the results of many of these are however contradictory. Some investigations described high detection rates of cancers, whereas others reported opposite outcomes despite the use of rather similar techniques. The main source of this heterogeneity is indeed attributable to difference in terms of clinical performance of various epigenetic markers tested, whereas a significant bias may also arise from the nature of circulating DNA (Diehl et al, 2005).

To establish whether or not a direct connection exists between methylation profile detectable in tissues and cfDNA, we selected the same marker and an identical methodology in matched clinical samples, that is, PCDH10, which is one of the tumour-suppressor genes to be epigenetically silenced in CRC (Zhong et al, 2013). We also reported our findings in a qualitative approach, as either positive or negative for the presence of PCDH10 methylated sequences, as well as in a quantitative manner to assess the ratio of methylated and unmethylated DNA in both tissue and plasma samples.

Taken together, our results confirm that methylation of PCDH10 promoter region is a common epigenetic event in colorectal tumours, and that this cancer-specific aberration can be frequently found in the circulation. We could detect hypermethylation in nearly 67% of cfDNAs extracted from patients in whom somatic alterations were identified, a value that is in substantial agreement with those reported in previous scientific literature data (Lin et al, 2012). We also showed that the degree of cfDNA methylation is associated with some characteristics of cfDNA, such as its concentration and integrity, and that these correlations vary in strength and direction in parallel with tumour stage. The marker used in the present study has been selected because of its high pretest probability to be heavily methylated in CRC patients. Although our confirmatory findings might induce to speculate on possible use of this marker as a noninvasive CRC detection system or – more realistically – in CRC monitoring, the evaluation of clinical performance, with regard to its specificity and sensitivity, goes beyond the aim of the present study, and further case–control studies are warranted to address this issue.

The methylation profile of candidate promoters, the quantification of absolute cfDNA concentration, and the assessment of cfDNA integrity have been used by many authors as independent markers for noninvasive cancer detection so far. Moreover, an approach based on the simultaneous determination of these three biomarkers has recently improved the diagnostic performance in colorectal tumours, and that this cancer-specific aberration can be frequently found in the circulation. The innovative finding of this study is that the methylation rate of PCDH10 in stages I and II. The correlation between p/t ratio and cfDNA concentration was not statistically significant (r = 0.014, P = 0.931). The correlation between p/t ratio and integrity index became negative (r = -0.401, P = 0.047) in stages III and IV, and the correlation with cfDNA concentration also achieved statistical significance (r = 0.452, P = 0.023). No statistically significant correlations were found among the three markers in the entire study population.

### Characteristics of methylated and nonmethylated cfDNA

The rate of concordance between tissues and plasma samples was 66.6%, with 42 out of 63 patients showing aberrant methylation of PCDH10 promoter region in both tissue-extracted DNA and cfDNA. More specifically, in early CRC a concordance was found in 29 out of 38 (76.3%) samples, whereas a concordance for 14 out of 35 (56.0%) samples was observed in the group of advanced-stage patients (P = 0.105 for the difference between the groups). Among the 20 patients with nonmethylated cfDNA, 3 were at stage I, 6 at stage II, 8 at stage III, and 3 at stage IV.

The differences between cfDNA-positive and -negative samples after dividing patients in two groups according to cancer stage are shown in Table 3. In patients at early stage, the probability to detect aberrant methylation of PCDH10 promoter region in plasma increases with the extent of methylation in the primary tumour and with integrity index of cfDNA. On the contrary, the concentration of cfDNA itself is the factor that mainly affects the possibility to detect methylated cfDNA in advanced-stage cancer patients.

### DISCUSSION

The differences between cfDNA-positive and -negative samples after dividing patients in two groups according to cancer stage are shown in Table 3. In patients at early stage, the probability to detect aberrant methylation of PCDH10 promoter region in plasma increases with the extent of methylation in the primary tumour and with integrity index of cfDNA. On the contrary, the concentration of cfDNA itself is the factor that mainly affects the possibility to detect methylated cfDNA in advanced-stage cancer patients.

| Tissue Tissues | Methylated PCDH10 | Unmethylated PCDH10 | Total |
|----------------|-------------------|---------------------|-------|
| All patients (n = 67) |                   |                     |       |
| Methylated PCDH10 | 42                | 21                  | 63    |
| Unmethylated PCDH10 | 0                 | 4                   | 4     |
| Total               | 42                | 25                  | 67    |
| Agreement 46 out of 67 (68.8%), \( \kappa = 0.204 \pm 0.09, P = 0.0751 \) |

| Early-stage patients (n = 41) |                     |                     |       |
| Methylated PCDH10 | 29                | 9                   | 38    |
| Unmethylated PCDH10 | 0                 | 3                   | 3     |
| Total               | 29                | 12                  | 41    |
| Agreement 32 out of 41 (78.0%), \( \kappa = 0.320 \pm 0.15, P = 0.0052 \) |

| Advanced-stage patients (n = 26) |                     |                     |       |
| Methylated PCDH10 | 14                | 11                  | 25    |
| Unmethylated PCDH10 | 0                 | 1                   | 1     |
| Total               | 14                | 12                  | 26    |
| Agreement 15 out of 26 (57.7%), \( \kappa = 0.089 \pm 0.08, P = 0.2707 \) |

Abbreviations: cfDNA = cell-free DNA; PCDH10 = protocadherin 10.
Table 2. Associations between protocadherin 10 (PCDH10) methylation in the tumour and plasma of colorectal cancer patients and their clinicopathological parameters

| Variables | N (%) | PCDH10 methylation rate (median, range) | Tumour tissues | P-value | Plasma samples | P-value |
|-----------|-------|----------------------------------------|----------------|---------|----------------|---------|
| Gender (mean age ± s.d.) | | | | | | |
| Male (69.0 ± 12.4) | 41 (65.1) | | | 52.42 (7.80–96.85) | 0.1144 | | |
| Female (67.0 ± 12.7) | 22 (34.9) | | | 37.13 (6.30–97.76) | 0.5631 | | |
| Lymphatic invasion | | | | | | |
| Negative | 40 (63.5) | | | 46.44 (6.30–97.76) | 0.0563 | | |
| Positive | 23 (36.5) | | | 40.58 (9.87–96.65) | 0.2905 | | |
| Vessel invasion | | | | | | |
| Negative | 48 (76.2) | | | 44.13 (6.30–97.76) | 0.5932 | | |
| Positive | 15 (23.8) | | | 43.50 (20.22–96.65) | 0.0912 | | |
| Tumour differentiation | | | | | | |
| G1/G2 | 46 (73.0) | | | 48.53 (6.30–97.76) | 0.0178 | | |
| G3 | 17 (26.0) | | | 40.15 (12.47–96.65) | 0.0912 | | |
| Tumour location | | | | | | |
| Proximal | 34 (54.0) | | | 53.33 (6.30–96.85) | 0.1601 | | |
| Distal | 29 (46.0) | | | 33.17 (7.50–97.76) | 0.0782 | | |
| Pathological stage | | | | | | |
| I | 12 (19.0) | | | 15.90 (6.3–97.76) | 0.1601 | | |
| II | 26 (41.3) | | | 40.42 (7.8–96.85) | 0.0912 | | |
| III | 17 (27.0) | | | 52.42 (17.37–93.64) | 0.5932 | | |
| IV | 8 (12.7) | | | 52.37 (25.29–96.65) | 0.0782 | | |

*Stage II vs stage III.

Figure 1. Comparison of PCDH10 methylation rate in the tumours and plasma of patients at early and advanced CRC.

Table 3. Differences between unmethylated and methylated cell-free DNA (cfDNA) in cancer patients

| Stages I and II (n = 38) | N (%) | Tumour tissue methylation rate (median, range) | P-value | cfDNA concentration (median, range) | P-value | cfDNA integrity index (median, range) | P-value |
|--------------------------|-------|-----------------------------------------------|---------|------------------------------------|---------|-------------------------------------|---------|
| Unmethylated | 9 (23.7) | 20.0 (7.50–64.79) | 0.0178 | 33.0 (26.2–55.0) | 0.8100 | 0.35 (0.13–0.60) | 0.0122 |
| Methylated | 29 (76.3) | 50.51 (6.30–97.76) | 0.0178 | 32.0 (26.4–81.0) | 0.56 (0.06–1.0) | | |

| Stages III and IV (n = 25) | N (%) | Tumour tissue methylation rate (median, range) | P-value | cfDNA concentration (median, range) | P-value | cfDNA integrity index (median, range) | P-value |
|--------------------------|-------|-----------------------------------------------|---------|------------------------------------|---------|-------------------------------------|---------|
| Unmethylated | 11 (44) | 46.34 (32.41–93.64) | 0.3914 | 36.0 (26.0–90.3) | 0.0214 | 0.65 (0.49–1.0) | 0.2060 |
| Methylated | 14 (56) | 59.16 (17.37–96.65) | 0.0214 | 80.0 (30.2–295.0) | 0.59 (0.3–1.0) | | |

Bold characters indicate the significance (P<0.05).
detected in plasma increased with increasing methylation rate in tumour tissues only in early-stage cancers, whereas this correlation was apparently lost in advanced cancers. The probability to detect aberrant methylation of PCDH10 promoters in cfDNA also increased with enhanced integrity of cfDNA in early-stage cancers, and with total cfDNA concentration in advanced-stage cancers, respectively.

These findings are consistent with a suggestive model, wherein hypoxia induces necrosis of tumours, leading to the subsequent release of long DNA fragments into the circulation. The sensitivity of blood-based methylation assay increases with the rate of cfDNA integrity in early-stage cancers. In these patients, the leading factor that may limit the detection of methylated cfDNA is the presence of short (truncated by concurrent apoptosis) wild-type DNA fragments deriving from cell death in normal tissues. As tumours become more aggressive, the degree of necrosis increases so that the absolute amount of circulating methylated DNA and the integrity index correspondingly rise. In these advanced cancers, the necrosis entails the death of neoplastic cells and surrounding stromal and inflammatory cells within the tumour. Thus, the methylation rate of circulating DNA becomes independent from methylation rate of primary tumour because the DNA released from necrotic regions is likely to contain a greater proportion of wild-type fragments. In this setting, the higher is the total concentration of cfDNA, the greater is the possibility to detect tumour-specific aberrations.

The findings of this study, along with the model proposed for their interpretation, are in some parts consistent with those of Diehl et al. (2005), who reported that the ‘extra’ DNA in advanced-stage patients is not derived from the neoplastic cells themselves, because only a minor fraction of circulating APC fragments was found to be mutant. On the other hand, these authors showed that most of DNA fragments containing mutations were of relatively small size, whereas larger fragments tended to be wild type (Diehl et al., 2005). This finding is in contrast with our results on the association between the plasma methylation rate and the integrity index of cfDNA (which prompted us to conclude that necrosis instead of apoptosis is the main source of tumour-derived cfDNA), but may be interpreted as partially consistent with data obtained in advanced-stage patients. It is noteworthy, however, that different types of analyses are not directly comparable. Moreover, the conflicting conclusions emerged here and in the previous literature are mainly attributable to the fact that, although cfDNA was detected in peripheral blood 30 years ago (Leon et al., 1977), the exact mechanisms at the basis of the presence of cfDNA in blood under normal and pathological conditions are not fully understood. A reasonable explanation is that more than one mechanism may be involved (Alix-Panabieres et al., 2012). Moreover, the prevalence of one tumour cell mechanism over the others may depend on specific cancer identity (Ellinger et al., 2008) and, according to our findings, may vary during cancer development (Thierry et al., 2010). In such a puzzling scenario, more work is needed to assess the many variables influencing the relative contributions and the interactions between different mechanisms of release.

The major strengths of this study include the novelty and timeliness of this first critical comparison between methylation profiles in tissue and plasma specimens evaluated on well-characterised case patients with colorectal neoplasia. Nevertheless, the modest size of samples might negatively affect the power of our findings on one side, even if controlled and standardised sample preparation and careful characterisation of optimal conditions for all measurements are in support of reliable results.

In the era where robust, sensitive, specific, and reproducible analytical techniques have become available for detecting epigenetic alterations in cfDNA, the chance to implement these relatively simple blood-based assays appear as a new tool for noninvasive cancer diagnosis and monitoring. In this perspective, our findings provide new insights into the biological aspects modulating the concordance between tissues and plasma methylation profiles, thus adding novel and useful information to achieve the goal of translation of basic research into clinical practice.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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PCDH10 methylation in colorectal cancer: tissue vs plasma

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