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METHYLATION PROFILES OF EXOCRINE AND NEUROENDOCRINE COLORECTAL CARCINOMAS USING METHYLATION-SPECIFIC MULTIPLE LIGATION-DEPENDENT PROBE AMPLIFICATION

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INTRODUCTION

Epigenetics in cancer

In human cancers, aberrant epigenomics are known to contribute to various phases of neoplastic development including initiation, promotion, invasion and chemotherapy resistance. Epigenetics refers to the study of heritable changes in gene expression that occur without alterations in DNA sequence. Histone modifications and DNA methylation are the key processes responsible for epigenetic regulation. Post-translational modifications to histone tails influence chromatin structure which plays an important role in the control of the transcriptional status of genes in a particular locus [1,2]. DNA methylation exerts critical effects on gene-expression regulation in normal cells, and alterations on the normal methylation patterns characterize, and likely contribute, to cancer development and progression. Reversible methylation of cytosines takes place within CG dinucleotides clustered at the promoter region of around 60% of all human genes and in repetitive sequences interspersed along the genome. It constitutes an essential mechanism that is present in normal "healthy" cells. As a regulatory system, this process contributes to the maintenance of genome integrity by keeping endoparasitic sequences inactive through heavy methylation; it maintains tissue-specific expression patterns and defines genomic imprinting.

In terms of DNA methylation, cancer cells show genome-wide hypomethylation and site-specific CpG island promoter hypermethylation [3,4]. Loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality to be identified in cancer cells more than 25 years ago [5]. It was recognized that a widespread DNA hypometilation may convey diverse effects upon epithelial cells, including an increase in genome instability due to the altered chromatin structure and the activation of transposable elements like LINEs and SINEs [6]. In addition, gene-specific hypomethylation has been identified as a way to

activate proto-oncogenes, such as *MAGE* in melanomas and *CAGE* in stomach and liver cancers among others [7,8]. Furthermore, hypomethylation at specific promoters can activate the normally methylated parental allele inducing loss of imprinting (LOI). For example, LOI affecting *IGF2* is reported as a common event in cancers of colon and of other sites [9,10].

Aberrant methylation of gene promoters can mimic genetic mutation or deletion by abolishing the expression of genes involved in all cellular pathways, and it is of particular significance in the context of inactivation of tumour suppressor genes, thus allowing malignant progression [11,12]. Methylation process takes place at the 5' position of cytosine (m5C, 5-methylcytosine) and is catalyzed by the enzymes of DNA methyltransferase family (DNMTs). DNA methylation can inhibit gene expression directly, preventing the binding of transcription factors, and indirectly, by recruiting methyl-CpG-binding domain proteins (MBDs). These proteins in turn recruit histone-modifying and chromatin-remodeling complexes at methylated sites [13] (figure 1).

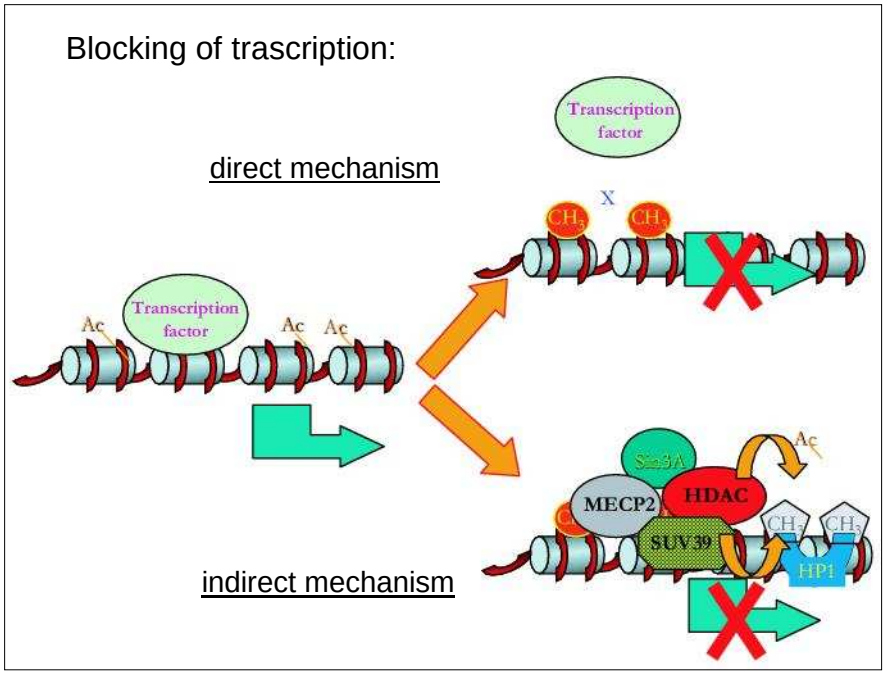

Figure 1. Silencing of genes by methylation. DNA methylation can silence genes by either direct or indirect mechanisms. An active gene, shown to the left, is unmethylated. The direct mechanism

is shown in the top right panel. Methylation of a CG residing in the recognition site for a transcription factor inhibits the binding of a critical transcription factor to the gene, resulting in blocking of transcription. In the second mechanism, shown at the bottom right, methylated CGs attract DNA binding factors which in turn recruit co-repressors, resulting in a change in chromatin structure to an inactive configuration. (Modified from Szyf et al. [14]).

Epigenetics and genetics interplay has recently become to emerge [15]. Mutations and deletions or even polymorphic variations occurring in just mentioned proteins belonging to the epigenetic machinery has been documented in several studies (reviewed in [16]). On the other hand, deregulated epigenetic mechanisms may initiate genetic instability resulting in the acquisition of inactivating mutations in tumour suppressor genes (TSGs) and activating mutations in oncogenes. Furthermore, m5C is highly mutable by deamination, resulting in transitional mutations (i.e. C to T).

Epigenetics in colorectal cancers

Colon cancer has become a paradigm in epigenetic research. In the last 20 years, many works have reported a great number of genes which are targets of aberrant methylation process in colon disease.

DNA hypermethylation-induced gene silencing is a common event that affect TSGs promoter and serves as an alternative mechanism to genetic mutation for the loss of tumor suppressor functions [11,12]. As reported in table 1, these genes are involved in many cellular processes, such as DNA repair, cell cycle control, Ras signalling, apoptosis, metastasis, vitamine response and p53 network among others.

Although the mechanisms that underlay aberrant DNA methylation in cancer cells remain to be elucidated, current evidence suggests that it may be an early and possibly even an initiating event in the development of colorectal cancer (CRC).

Table 1. **Frequent targets of aberrant methylation processes in tumorigenesis**

Gene	Role in colorectal tumorigenesis	Histological grade
CDH13 H-Caderin	It is a regulator of the dynamic cellular adhesion–deadhesion processes, and its inactivation contributes to the dissemination of cancer cells	Aberrant crypt foci and adenomas
CRBP1 Retinol binding protein1	Encodes a carrier protein involved in the transport of retinol (vitamin A alcohol). Methylation may contribute to the loss of retinoic acid responsiveness	Adenomas
DAPK1 Death-associated protein kinase	Its silencing contributes to the inactivation of gamma-interferon induced programmed cell death	Hypermethylation was already observed in colon mucosa adjacent to carcinoma
IGF2 Insulin-like growth factor	Loss of imprinting at this locus is a risk factor for CRC predisposition. It affects cell proliferation	Colonic normal mucosa

Gene	Role in colorectal tumorigenesis	Histological grade
MGMT O6-methylguanine DNA methyltransferase	Hypermethylation contributes to colorectal field cancerization.	Histologically normal-appearing mucosa of cancer patients
WIF1 Wnt inhibitory factor-1	It contributes to the aberrant activation of Wnt signaling	Proposed as a valuable biomarker in plasma for early detection of CRC
RASGRF2 Ras guanine nucleotide-releasing factor 2	Implicated in Ras-mediated signaling and development	Aberrant crypt foci and adenomas
APC Adenomatous polyposis coli	Antagonist of the Wnt signaling pathway, it's involved in aberrant cell migration, adhesion, transcriptional activation and apoptosis	Adenomas
COX2 Prostaglandinendoperoxide Synthase 2	Involved in inflammation and mitogenesis, tumour angiogenesis acting through VEGF and metastasis	Adenomas
GATA4 GATA binding protein 4	Suppresses colony formation, proliferation, migration, invasion, and anchorage-independent growth of colorectal cancer cells	Aberrant crypt foci and adenomas
GATA5 GATA binding protein 5	Suppresses colony formation, proliferation, migration, invasion, and anchorage-independent growth of colorectal cancer cells	Aberrant crypt foci and adenomas
HIC Hypermethylated in cancer	Modulates the transcriptional stimulation of genes regulated by Wnt/beta-catenin signaling. Inactivation in cancer leads to abnormal cell proliferation at early stages	Adenomas
hMLH1 mutL homolog 1	Its inactivation causes microsatellite-unstable tumours	Adenomas
p14ARF Cyclin-dependent kinase inhibitor 2A	Tumour suppressor gene, stabilizer of the tumour suppressor protein p53 and involved in cell cycle control	Adenomas
p16INK4a Cyclin-dependent kinase inhibitor 2A	Tumour suppressor gene, negative regulator of cell growth and proliferation in the G1 phase of the cell cycle, and is an important regulator of the angiogenic switch	Adenomas
RARb Retinoic acid receptor	It mediates the growth-inhibitory action of retinoic acid	Adenomas
RASSF1a Ras association domain family member 1	It inhibits the accumulation of cyclin D1, and thus induces cell cycle arrest. Methylation of this gene is an early event in CRC	Adenomas

Modified from Carmona et al.[17]

In fact, several studies identified aberrant promoter methylation in early precursor lesions, such as aberrant crypt foci (ACF) [18], adenomas [19] and even in tumour-adjacent normal-appearing mucosa [20]. Many reports now are being published about the changes in the methylation status of normal background mucosa during the development of colorectal neoplasia. The "field effect" model suggests that precancerous cells proximal to the cancer cells harbour some of the genetic alterations that are present in fully developed cancer. For example, Shen et al. [21] detected methylation of the promoter region of *MGMT* in the normal mucosa adjacent to colon tumor tissue in 22 (50%) out of 44 patients whose tumors exhibited *MGMT* promoter methylation but in only three (6%) out of 51 patients without it. These results indicate that some CRC may arise from a field effect defined by epigenetic alteration of *MGMT*.

Many attempts have been made to identify the methylation patterns appearing at the various stages of colorectal cancer progression, although the different techniques employed, the uneven criteria for selecting samples and the biased validation methods have yielded little consensus about the number of loci defining each step of cancer development. In 1999, Toyota and co-workers identified a series of methylation markers whose status defined the CpG island methylator phenotype (CIMP) [22]. This proposal emerged as a new pathway for colorectal tumourigenesis, in addition to the classic mutator or chromosomal instable (CIN) and microsatellite instable (MSI) categories, standing for a subset of sporadic colorectal tumours bearing excessive cancer-specific promoter hypermethylation. This molecular subgroup of tumours claimed to group up to 75% of sporadic CRC with MSI, and was initially characterized for exhibiting concordant tumour-specific promoter hypermethylation in a series of markers (*CDKN2A, MLH1, THBS1, MINT1, MINT2, MINT31*). Later, Weisenberger et al. established another panel to support the CIMP as a distinct molecular trait of CRC, identifying a new diagnostic panel

of markers (*CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1*) [23]. Subsequently, in two large prospective cohort studies, Ogino et al. validated the usefulness of eight methylation markers including markers established by Weisenberger et al. (*CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1, CDKN2A/p16, CRABP1,* and *MLH1*) in CIMP classification [24].

However, some authors did not observe the bimodal distribution of methylation frequencies. This controversy might be due to the inclusion of MSS and/or MSI-H in these studies or due to tumor heterogeneity [25]. In fact not all CpG islands are methylated in a similar manner in CRC and thus the choice of markers can affect the features of CIMP [26,27].

However, later, considerable research activity has been performed regarding markers able to accurately identify CIMP tumors. Among others, a comprehensive analysis of CIMP has been performed in CRC using a large population-based sample (904 tumors) [28]. Utilizing an expanded set of methylation markers (16 CpG islands), this study revealed that CIMP+ tumors are independently associated with poor differentiation, MSI-H and BRAF mutation. This group of tumors is also inversely correlated with LINE-1 hypomethylation and β- catenin (*CTNNB1*) activation. In a later study [29], the CIMP+ tumors were further subdivided into two molecularly distinct subclasses by genetic characteristics. By an integrated genetic and epigenetic analysis with 27 promoter-associated CpG islands, Shen et al. [29] found that CIMP+ cases fitted into two subgroups (CIMP1 and CIMP2) and that they corresponded to very distinct genetic profiles. CIMP1 are characterized by a high frequency of MSI and *BRAF* mutations (more than 50%) with few *KRAS* and *TP53* mutations. CIMP2 is associated with a high frequency of *KRAS* mutations (92%), but MSI and *BRAF* mutations occurred rarely with a low rate of *TP53*

mutation. Conversely, CIMP− cases that showed low or less methylation for all genes examined have a high rate of *TP53* mutations (71%) with lower rates of MSI (12%) or

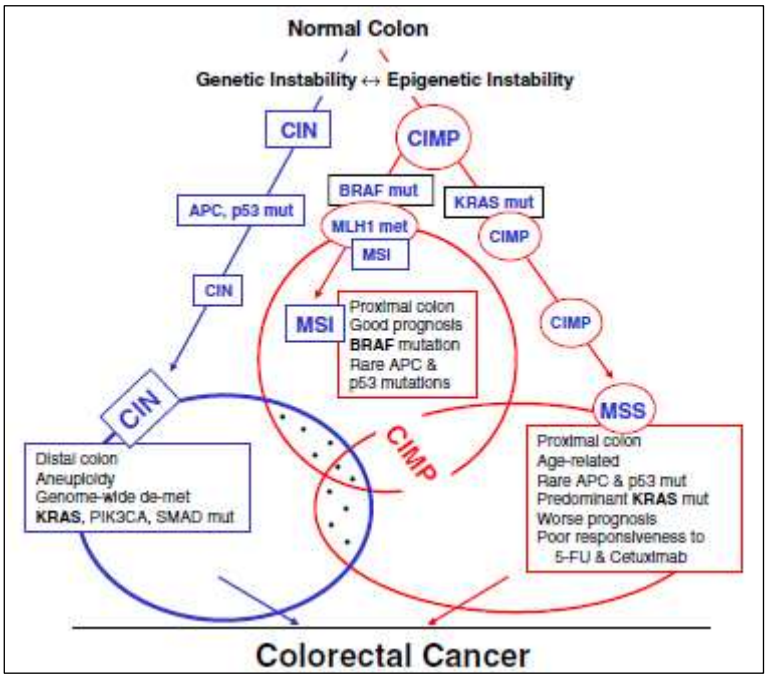

Figure 2. Multiple pathways to progression of colorectal tumors. Three distinct parallel pathways (CIN, CIMP+/MSI, and CIMP+/MSS) for the progression of CRC from normal colon mucosa have been recently proposed by Issa. Modified from Kim et al.[30]

mutations of *BRAF* (2%) or *KRAS* (33%).

Even though the CIMP approach to the classification of CRC has accelerated understanding of CIMP in colon carcinogenesis, the absence of a consensus panel defining the CIMP groups makes the comparison of the literature difficult. In addition, inclusion of genes showing age-related methylation or genes whose methylation is associated with better prognosis might lead to an underestimation of true frequency or prognostic value of CIMP [31]. For example, using different marker panels, CIMP+ was found to correlate with a worse prognosis in a report [32,33] but not in another study [34].

The concordant methylation of multiple genes has been observed in other types of cancer, suggesting that CIMP is a common occurrence in human neoplasms. However, it is still not clear whether the CIMP molecular phenotype represents a biologically distinct group of cancer, whether it is only observed in selected groups showing different degrees of methylation at particular loci, or whether it is derived from a group of tumors that are part of a normal distribution with regard to aberrant DNA methylation. It is also controversial whether this "epigenetic instability" is integral to tumor initiation and progression.

Finally, CpG island methylation is an early and widespread phenomenon in colorectal carcinogenesis and a lot of studies have shown evidence for a CIMP-phenotype also in stable (MSS) CRCs. Moreover, some authors [35-37] found a percentage of CIMP+ phenotype also among poorly differentiated neuroendocrine carcinomas (NECs) of colon and rectum, which are rare and highly malignant cancers that typically harbour high chromosomal instability [38,39]. However, to date there is little information about the incidence of the CIMP phenotype and the specific epigenetic profiles in NECs, mainly due to the smallness of the series analyzed.

Methods and strategies to investigate promoter methylation

Many different strategies are available for assessing the epigenetic patterns of normal and cancer cells. So far, most studies addressing aberrant methylation in colorectal and other cancers have focused on genes already known to be involved in cancer pathogenesis, especially those for which no genomic mutation had been identified [40]. Using the candidate-gene approach, a high number of genes inactivated by promoter hypermethylation in a given tumour has been recognized [17,40]. On the other hand, some authors opted for a genome-wide manner to consider DNA methylation events (i.e., by studying a limited number of genomic sites that are representative of the genome). The first reports relied on restriction enzyme-based methods suitable for comparing large series of samples and the simultaneous identification of hypomethylation and hypermethylation events (reviewed in Carmona et al.[17]). Techniques such as restriction landmark genomic scanning (RLGS), which was the first large-scale method for investigating methylation and differential methylation hybridisation (DMH), which was the first array method to be optimized for identifying novel methylated loci in cancer, are among the first examples of large-scale epigenomic techniques [41]. Nevertheless, these ingenious approaches suffer many technical limitations, including first of all laborious and time-consuming protocols and outputs difficult to elaborate. However, despite some pitfalls, studies based on these techniques succeeded in identifying a surfeit of abnormally methylated loci in cancer, and provided a picture of particular CpG methylation patterns shared by many tumour types and of markers exhibiting distinct tumour-type specificity.

Later, genetic unmasking strategies opened up a new line of investigation in the search for hypermethylated loci. Specifically, the disruption of the two major DNA methyltransferases (DNMT1 and DNMT3b) in the colorectal cancer cell line HCT-116 [42]

provided a useful tool for identifying differentially methylated loci on a global genomic scale, by comparing the methylation profile of the double knock-out (DKO) with the unmodified HCT-116 cell lines. Other methods have exploited the plasticity of epigenetic modifications by using pharmacological agents such as 5-aza-2-deoxycytidine (5-aza), which is widely employed as a DNA methylation inhibitor to induce gene expression by covalent trapping of DNMT, or histone deacetylase inhibitors, such as trichostatin A (TSA). Nevertheless, it is important to note that due to the pleiotropic effects produced by these epigenetic drugs, many genes not directly affected by aberrant methylation – or not even epigenetically regulated – were transcriptionally over-activated. This leads to quite a high frequency of false-positive results.

The advent of the bisulphite treatment of DNA, which converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected, was a fundamental contribution to cancer epigenetics research [43,44]. The implementation of this technique in conjunction with PCR amplification, for example methylation-specific PCR (MSP) [45], allows the sensitive examination of DNA methylation of any sequence, and requires only very small amounts of material. Also, the promoter CpG island hypermethylation in different human cancers was comprehensively analyzed using bisulphite sequencing [46], which, to date, has been considered the gold standard technique for directly studying DNA methylation and for validating results obtained using other approaches. Moreover, pyrosequencing method offers, in addition, a quantitative approach to detect different levels of methylation [26].

These techniques are employed also at a clinical level. MSP, for example, and Methylight, developed as the quantitative version of the MSP technique [47], are being implemented to conduct routine diagnostic tests, such as the detection of *MGMT* methylated promoter for patient stratification in the therapy of glioblastoma multiforme [48]. Moreover, traditional PCR-based techniques are now complemented by several new PCR-based methods for

detecting DNA methylation that display increased analytical sensitivity and provide quantitative information. For instance, methylation sensitive melting-curve analysis (MS-MCA) takes advantage of the differential resistance of DNA to melting, depending on the relative GC content. A melting analysis performed straight-on after the amplification differentiates the products based on their sequence as two different temperature peaks should be noticed [49]. An improvement of this technique, also based on melting analysis of PCR amplicons, is the high-resolution melting-curve analysis (MS-HRM) which is a more sensitive approach [50].

All these methods mentioned above are currently used in a candidate-gene strategy, performing a single-gene analysis at time and taking advantage from bisulfite-DNA treatment, known to be a time-consuming process. Furthermore, some of them (for example Methylight and Pyrosequencing) are quite expensive and need an accurate optimization before use. Methylation-Specific Multiple Ligation-dependent Probe Amplification (MS-MLPA) is a fast new method that detects methylation status of cytosine exploiting methylation-sensitive enzyme thus avoiding bisulfite conversion. It allows the screening of multiple genes at time by a simultaneous amplification of 20-30 sequences of interest in a single PCR reaction.

Methylation-Specific Multiple Ligation-dependent Probe Amplification (MS-MLPA)

Over the years, the interpretation of studies investigating CIMP in CRC has been complicated by the use of several different methods, gene panels, and criteria for defining this phenotype [11,17]. Most current methods involve single-gene analysis performed with labour intensive approaches. By contrast, high throughput methylation approaches are not suitable in clinical practice because they are expensive and generally not applicable to DNA from FFPE tissues [17,30]. Methylation-Specific Multiple Ligation-dependent Probe Amplification (MS-MLPA) is a fast new inexpensive method that detects methylation in a large set of genes simultaneously using only small amounts of DNA from FFPE tissues. This technique uses multiple probe sets each consisting of two oligonucleotides, both containing a sequence-specific region used for hybridization to the genomic DNA, tagged with common tails complementary to a universal primer set (Figure 3). One of the two oligonucleotides additionally contains a stuffer sequence of a characteristic length, allowing separation of the individual loci analyzed. In practice, after over-night hybridisation, the sample is divided into two aliquots. In one part of the sample, adjacently hybridized probes are joint through ligation, whereas for the other half of the sample, ligation is combined with a methylation sensitive restriction enzyme HhaI (recognition site GCGC) that digests the unmethylated fragments. Both ligation and ligation-digestion probe sets are then amplified by polymerase chain reaction (PCR) and analyzed by capillary electrophoresis. By comparison of the electropherogram of ligated sample with the one of ligation-digestion sample, the amount of methylation can be calculated as a ratio value for each probe.

To date, the number of studies that employed this technique is reported in literature to be about sixty overall. Since this method has been published for the first time in 2005 [51]

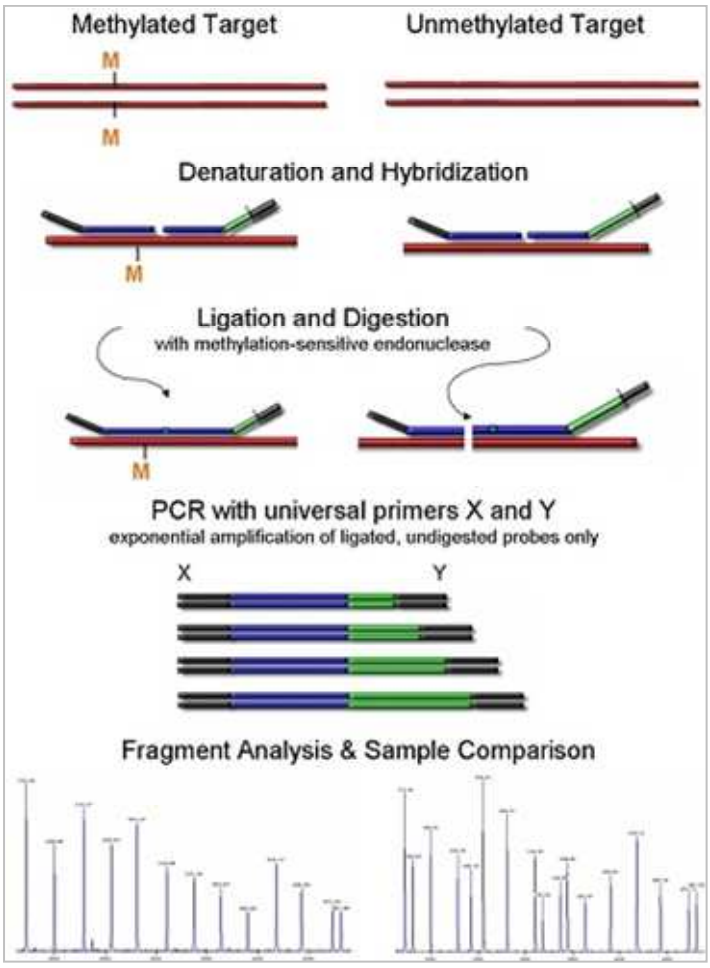

Figure 3. Diagram of the steps of MS-MLPA procedure. The MS-MLPA procedure can be divided in five steps: 1. DNA denaturation and hybridisation of MLPA probes; 2. ligation and digestion; 3. PCR; 4. separation of amplification products by capillary electrophoresis and 5. data analysis.

many works have been described especially in the research field of imprinting disorders or hematologic diseases. In recent years the clinical utility of this method is strongly emerging as a promising tool to rapidly screen the routinely standard-processed tumors or the archival tissues in retrospective studies [52-54]. However, the translational application of MS-MLPA and its potential utility for analysing CRCs has not been fully investigated yet.

AIM

The aims of our work were to investigate the methylation status of 34 tumor suppressor genes in a series of 104 formalin-fixed and paraffin-embedded specimens, including 83 exocrine adenocarcinomas and 21 neuroendocrine cancers.

First we evaluated the feasibility and the effectiveness of the employment of MS-MLPA to rapidly screen an archival series of FFPE tissues. We analyzed the accuracy of this method by assessing sensitivity and specificity through ROC analysis.

Clinico-pathological and molecular characteristics were collected for all cases and used to correlate methylation profiles in subsets of clinically and biologically different tumours. Furthermore, we studied samples of colorectal mucosa from 13 CRC patients and from 12 patients with negative history for colorectal cancer. In particular we evaluated whether:

- ✓ gene methylation patterns, if present in normal-appearing mucosa of CRC patients, showed cancer-specific signature or were similar to those of colonic mucosa of non-CRC patients;
- ✓ methylator phenotype (CIMP) was a peculiarity of unstable tumours or if other subsets of cancer shared this phenotype;
- ✓ distinct patterns of methylated genes could characterize exocrine versus neuroendocrine tumours;
- ✓ CIMP phenotype or single gene status could serve as prognostic marker.

MATERIALS AND METHODS

Patients and samples

This study was performed on 104 tumor samples, including 83 sporadic CRCs and 21 NECs. The clinico-pathological and molecular profiles of all 83 exocrine cancers were previously examined and published [55]. The NECs consisted of a series of archived FFPE tissue samples from 21 patients who underwent curative surgical resection between 1989 and 2009 at the Ospedale di Circolo, Varese. The inclusion criterion for these cases was the availability of tumor tissue for molecular analysis. All samples were histologically reviewed by two independent pathologists according to the WHO classification of tumors of the digestive system [56,57] and the TNM staging system [58,59].

Outcome data were collected by consulting clinical records and/or the Tumor Registry of the Lombardy region (Italy) and were available for all but six patients (94.2% of patients). Thirty-three patients (33.7%) died of disease while fifty-eight patients (59.2%) were alive (median follow-up time of 16 months and 75 months, respectively). Seven patients (7.1%) were excluded from the survival analysis because they died within the first 30 days after tumor resection. *BRAF* and *KRAS* mutation tests, microsatellite instability (MSI-H) and loss of heterozygosity (LOH) studies on chromosomes 5q, 17p and 18q were previously performed [55]. Molecular and clinico-pathological informations of all 104 tumors are summarized in table 2.

Twenty-five samples of normal-looking colonic mucosa from 25 patients were also included in this study. The selection of these 25 normal samples was carried out in order to represent all colorectal segments; the median age of the patients was 70 years, without significant differences between the two groups. Thirteen of these samples were derived from normal tissue at the resection margins of 13 patients with CRCs included in this study. The remaining normal specimens were obtained from 12 individuals who had

Table 2: Clinico-pathological and molecular data of the 104 colorectal cancer patients

Variable	CRCs (N=83)		NECs (N=21)	
Gender				
Male	44	53%	13	62%
Female	39	47%	8	38%
Age				
Median (minimum-maximum)	68	(41-91)	69.5	(44-85)
Site				
Right	36	43%	10	48%
Left	47	57%	11	52%
Histotype				
Glandular	65	78%	-	
Mucinous	13	16%	-	
Diffuse	1	1%	-	
Signet-ring cell	3	4%	-	
Grade of differentiation				
G1-G2	66	80%	0	0%
G3	17	20%	21	100%
TNM stage				
Stage I-II	38	49%	4	20%
Stage III-IV	39	51%	16	80%
Microscopic growth				
Expansive	19	23%	0	0%
Infiltrative	64	77%	9	100%
Neuroinvasion				
Absent	60	72%	7	44%
Present	23	28%	9	56%
Angio-lymphoinvasion				
Absent	55	66%	4	24%
Present	28	34%	13	76%
Histological heterogeneity				
Absent	50	60 %	21	100 %
Present	33	40 %	0	0 %
Tumor infiltrating lymphocytes				
Absent	20	24%	11	92%
Present	63	76%	1	8%
Microsatellite instability status				
MSI-H	19	23%	2	10%
MSS	64	77%	19	90%
Loss of Heterozygosity (5q, 17p, 18q)				
Absent	28	36%	1	7%
Present	50	64%	13	93%
BRAF mutation				
Absent	72	87%	19	95%
Present	11	13%	1	5%
KRAS mutation				
Absent	65	81%	14	70%
Present	15	19%	6	30%

undergone surgery for ischemic colorectal disease or for diverticulitis without a personal history of CRC.

DNA extraction and evaluation of amplifiability

DNA samples were obtained from formalin fixed and paraffin embedded tissues using representative 8-µm sections. Three sections of every specimen were treated twice with xylene, and then washed twice with ethanol. DNA was extracted using a QIAamp® DNA FFPE Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Neoplastic areas were manually microdissected for DNA extraction and contained at least 70% of tumor cells, to minimize contamination by normal cell. Each DNA sample was evaluated for integrity and amplifiability by BIOMED-2 multiplex PCR [60] in order to correlate DNA fragmentation with MS-MLPA reproducibility. Briefly, as illustrated in figure 4, five pairs of control gene PCR primers were used to amplify products of exactly 100, 200, 300, 400 and 600 bp from the following four target genes: *TBXAS1* (exon 9), *RAG1* (exon 2), *PLZF* (exon 1), *AF4* (exon 11), *AF4* (exon 3) [60]. DNA fragmentation and MS-MLPA reproducibility were correlated.

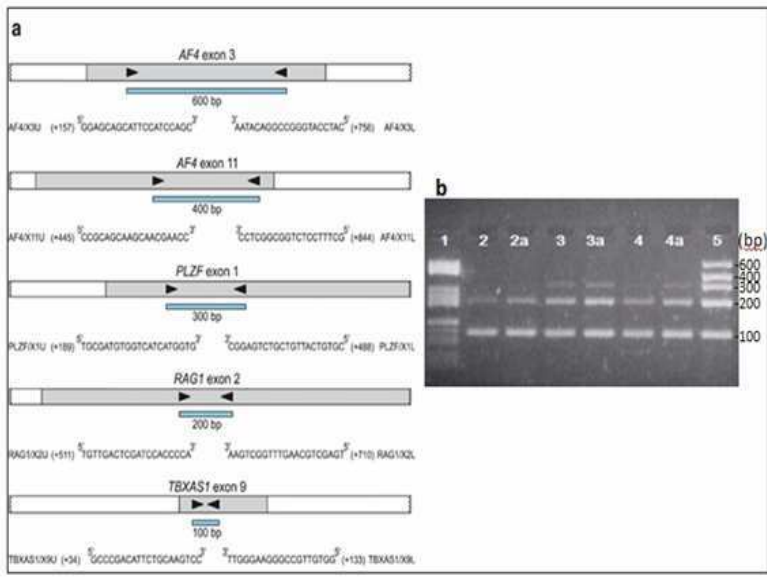

Figure 4. Control gene PCR for the assessment of amplifiability and integrity of DNA samples. a) Schematic representation of control genes exons and primer sets. b) PCR products separated in a 3% agarose gel. Modified from van Dongen et al.[60]

Methylation-specific multiplex ligation-dependent probe amplification

The SALSA MS-MLPA ME001 Tumor suppressor-1 Kit and SALSA MS-MLPA ME002 Tumor suppressor-2 Kit (MRC-Holland, Amsterdam, The Netherlands) were used to perform promoter methylation analysis on all 104 tumors and 25 normal samples previously mentioned.

Each kit used in our study contains 27 MS-MLPA probes that detect the methylation status of promoter regions of 24 tumor suppressor genes. All these genes are frequently silenced by methylation in tumors of different sites, but are unmethylated in blood-derived DNA of healthy individuals. Using both kits, we examined a total of 34 different tumor suppressor genes due to a partial overlapping between the two probe-mixes. Seventeen genes were addressed by two probes that recognized different HhaI restriction sites in their promoter regions. All probes, target genes, sequence accession number and amplicone size are reported in table 3 with the exception of two genes, CDKN2B and MSH6, that were excluded from further analyses after checking the specificity of the test. Additional information, such as probe sequences and chromosomal locations can be found at www.mlpa.com.

All MS-MLPA reactions were done according to the manufacturer's instructions with minor modifications, using 100-150 ng of DNA. The probe-mix is added to 5ul of denatured DNA and allowed to hybridize for 16 hours at 60°C. Subsequently, the sample is divided in two: one half is ligated by adding 10ul of ligase-mix, whereas in the other half ligation is combined with digestion by adding 10ul of ligation-digestion mix. These sample are incubated for 30 minutes at 54°C, then the HhaI enzyme is inactivated by denaturation at 95°C for 1 minute. Since the unmethylated sequences are cut by the

Table 3: Genes examined by MS-MLPA analysis

N°	gene	position	ID number	Amplicon size
1	*TP73*	1p36.3	NM_005427.2	400*; 238**
2	*CASP8*	2q33.2	NM_001228	265*
3	*VHL*	3p25.3	NM_000551	355*; 265**
4	*RARB*	3p24.2	NM_000965	193*; 454**
5	*MLH1*	3p22.3	NM_000249	166*; 463*
6	*RASSF1*	3p21.3	NM_007182	328*; 382*
7	*FHIT*	3p14.2	NM_002012	409*
8	*APC*	5q22	NM_000038	148*
9	*ESR1*	6q25.1	NM_000125	373*; 301**
10	*PAX5A*	9p13	NM_016734	211**
11	*CDKN2A* (p14)	9p21.3	NM_058195	160*
12	*CDKN2A* (p16)	9p21.3	NM_058195	427**
13	*DAPK1*	9q22	NM_004938	346*
14	*PTEN*	10q23.3	NM_000314	292*; 184**
15	*MGMT*	10q26.3	NM_002412	193**; 382**
16	*CD44*	11p12	NM_000610	319*; 463**
17	*GSTP1*	11q13	NM_000852	454*; 274**
18	*PAX6*	11p13	NM_001604	409**
19	*WT1*	11p13	NM_000378	247**
20	*ATM*	11q23	NM_000051	184*; 160**
21	*IGSF4*	11q23	NM_014333	427*; 355**
22	*CDKN1B*	12p13.2	NM_004064	274*
23	*CHFR*	12q24.3	NM_001161344	238*; 292**
24	*BRCA2*	13q13.1	NM_000059	301*; 148**
25	*RB1*	13q14.2	NM_000321	319**; 472**
26	*THBS1*	15q15	NM_003246	346**
27	*PYCARD*	16p11.2	NM_013258	400**
28	*CDH13*	16q23.3	NM_001257	436*; 220**
29	*TP53*	17p13.1	NM_000546	166
30	*HIC1*	17p13.3	NM_006497	220*
31	*BRCA1*	17q21.3	NM_007294	247*; 142**
32	*STK11*	19p13.3	NM_000455	373**
33	*GATA5*	20q13.3	NM_080473	436**
34	*TIMP3*	22q12.3	NM_000362	142*

Legend: * ME001-C1 MS-MLPA kit; ** ME002-A1 MS-MLPA kit (MRC-Holland, The Netherlands).

restriction enzyme, this process resultes in the ligation of the methylated sequences only. Eight microlitre of the two aliquots are then amplified in a 25ul PCR reaction using Veriti thermo cycler (Applied Biosystems, Foster City, USA) with this thermal protocol: 33 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 1 min with a final extension of 20 min at 72°C. In order to assess MS-MLPA reliability two replicates were performed for each sample and positive and negative controls using fully methylated DNA (CpGenome Universal Methylated DNA, Millipore) and unmethylated DNA (CpGenome Universal UnMethylated DNA, Millipore) were included in each MS-MLPA experiment. Aliquots of 1.5 ul of the PCR reaction were combined with 0.5 ul TAMRA internal size standard (Applied Biosystems, Foster City, USA) and 13.5ul of deionized formamide. After denaturation, fragments were separated and quantified by electrophoresis on an ABI 310 capillary sequencer and Genemapper analysis v.4.0 (Applied Biosystems). Values corresponding to peak size (base pairs) and peak height were used for further data processing by Coffalyser V7 software (MRC-Holland). Methylation dosage ratio (MR) was obtained by the following calculation: MR= $(P_x/P_{ctrl})_{Dig\ /}\ (P_x/P_{ctrl})_{Undig}$ where P_x is the peak height of a given probe, P_{ctrl} is the sum of the peak heights of all control probes, Dig stands for HhaI digested sample, and Undig stands for undigested sample.

A methylation ratio (MR) for a given gene may range from 0 (0% of alleles methylated) to 1.0 (100% of alleles methylated) and threshold values of 0.3 and 0.7 are suggested by the manufacturer to consider a locus as hemi-methylated or fully-methylated, respectively. However we determined in our laboratory sensitivity and specificity of each probe as well as the of accuracy MS-MLPA to provide semi-quantitative results by a titration experiment using mixtures of fully methylated DNA (CpGenome Universal Methylated DNA, Millipore) and unmethylated DNA (CpGenome Universal UnMethylated DNA,

Millipore). Serial dilutions of 0%, 10%, 30%, 50%, 80% and 100% of methylation were analysed three times resulting in three data points for each dilution. MRs obtained in the titration experiments were evaluated by Receiver Operating Characteristic (ROC) curve analysis [61,62].

Validation of MS-MLPA results by methylation-specific PCR and by bisulphite pyrosequencing

To validate the MS-MLPA results, we used methylation-specific PCR (MSP) [63] and bisulphite pyrosequencing [64] to analyse the same promoter regions interrogated by MS-MLPA probes. MS-PCR was applied to analyse *hMLH1* methylation status using previously published primers and protocol [65]. All MSP data were obtained by capillary electrophoresis on an ABI 310 Automatic DNA Sequencer (Applied Biosystems, Foster City, CA).

Bisulphite pyrosequencing was used to confirm methylation patterns of the *MGMT* gene. Bisulfite modification of genomic DNA (300 ng) was performed with an EpiTect Bisulfite Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Bisulfite-modified DNA was amplified and sequenced by using primers supplied in a PyroMark *MGMT* kit (Diatech, Jesi, Italy). The biotinylated strand of each PCR product was isolated from 20 µl of each PCR product using a Vacuum Prep Tool (Qiagen). Pyrosequencing was carried out with PyroGold reagents on a PyroMark Q96 ID system (Qiagen). Pyrogram outputs were analyzed by the Pyromark Q24 software using the Allele Quantification software (Qiagen) to determine the percentage of methylated alleles at each of the four CpG sites examined. Aberrant methylation was scored when the mean value of the percentage of methylated alleles was higher than 15%.

Statistical analysis

Univariate comparisons of continuous data were carried out using Student's t-test and discrete variables were compared with χ^2 test or Fisher's exact test. The association between discrete outcome and continuous predictor was evaluated with a logistic regression model. All comparisons were two-sided and a p-value <0.05 was considered to be significant.

The cut-off value of the methylation ratio (MR) of each gene with corresponding sensitivity, specificity and accuracy was estimated by Receiving Operator Characteristic curve analysis [61,62]. The best statistical cut-off was calculated to achieve the highest levels of accuracy to detect at least 10% of methylation in each gene.

Survival analysis was performed using Kaplan Meier curves, log-rank test and the Cox regression model. The data were statistically analysed using STATA software (version 10.0).

RESULTS

MS-MLPA quality assessment: evaluation of reproducibility and accuracy.

Reproducibility of the MS-MLPA assay was assessed by performing in duplicate the analysis of 142 FFPE DNA samples, after checking the integrity and amplifiability by BIOMED-2 multiplex PCR generating products of 100, 200, 300, 400 and 600 bp. MS-MLPA raw data were reproducible in all the 104 tumor samples (Table 2, materials and methods) and the 25 normal samples included in this study. All these DNA samples showed products of 200 bp or more in the multiplex PCR. By contrast, this analysis led to the exclusion of a total of 13 DNA samples (9% of cases). These cases, showing amplified products smaller than 200 bp, displayed discordant raw data between the two replicates. Representative MS-MLPA results with respect to DNA amplifiability are shown in figure 5.

Normal values for the 53 MS-MLPA probes included in the two kits were determined by testing normal controls from lymphocytes of healthy donors. Reproducible results with all the probes were found, with the exception of the probes 0607-L00591 (*CDKN2B* gene) and 01250-L00798 (*MSH6* gene) which provided false positive results because of incomplete HhaI digestions. For this reason these two probes were not taken into account in the subsequent analyses.

To further assess the robustness of the MS-MLPA assay, raw data derived from two different probes interrogating the same gene were compared for 17 of the 34 genes examined (Table 3). As illustrated in figure 6, high levels of concordance were observed by comparing the MR data obtained with the two probes for all 17 genes (average concordance: 91% ± 7.6). Only for MGMT probes we found a low level of concordance, equivalent to 55%. Thus, promoter methylation of *MGMT* gene was tested with a comparative analysis using pyrosequencing method (see "Validation of MS-MLPA results by MS-PCR and by pyrosequencing").

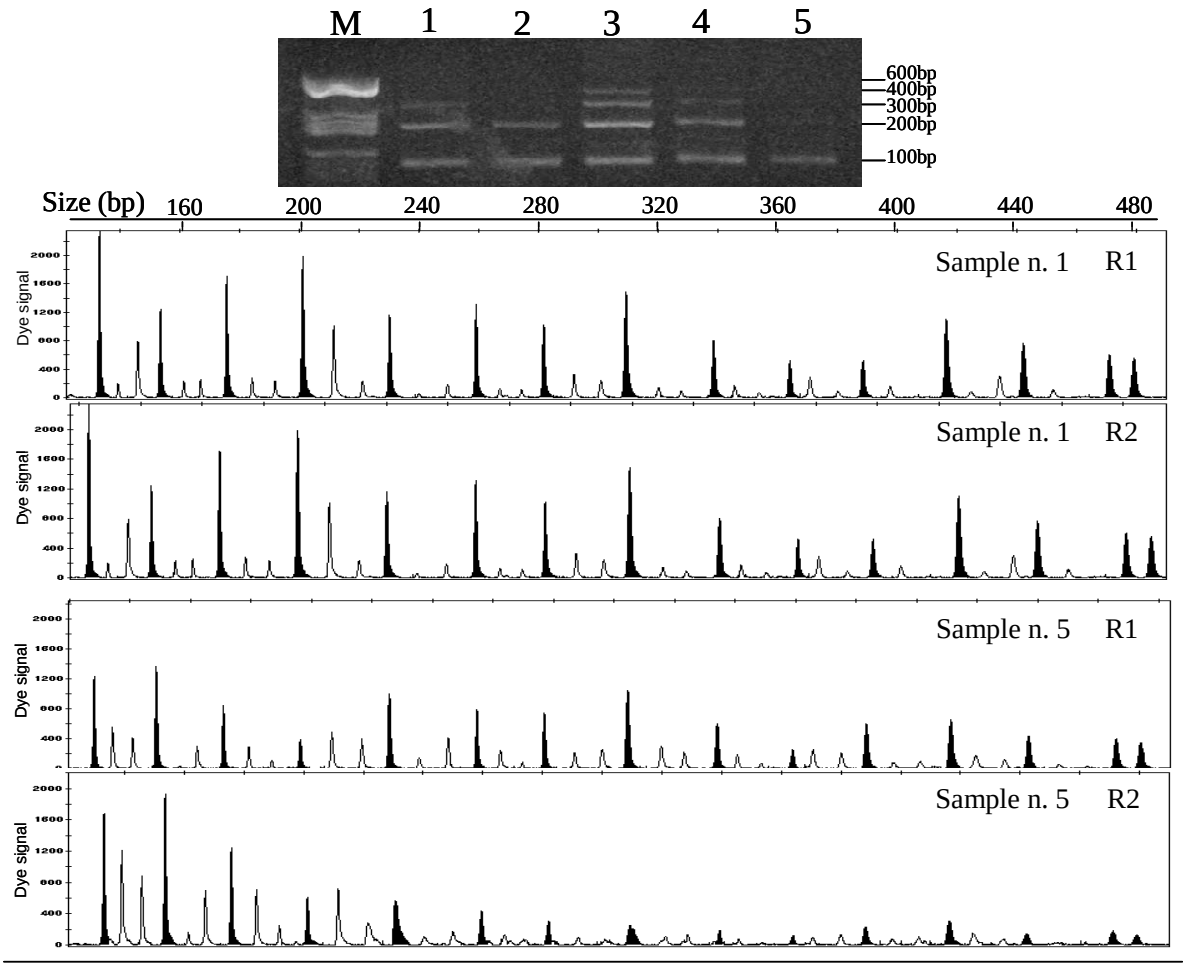

Figure 5. Examples of MS-MLPA results with respect to amplifiability of DNA samples.

A 3% agarose gel shows control genes PCR products of five DNA samples obtained from FFPE tissues. Electopherograms show replicates (R1 and R2) of MS-MLPA raw data from two representative DNA samples displaying an amplifiability of 300bp and 100bp (case 1 and case 5 respectively). As evident, sample 5 did not allow to obtain reproducible results.

Accuracy of the MS-MLPA assay was evaluated through the assessment of sensitivity and specificity of the method performing ROC analysis on titration experiment data. The methylation status of the 34 genes under study was tested on dilutions of increasing percentages of *in vitro* methylated DNA and unmethylated DNA (0%, 10%, 30%, 50%, 80% and 100%). As illustrated in Table 4, the MRs in the MS-MLPA analysis gradually increased with the degree of methylation.

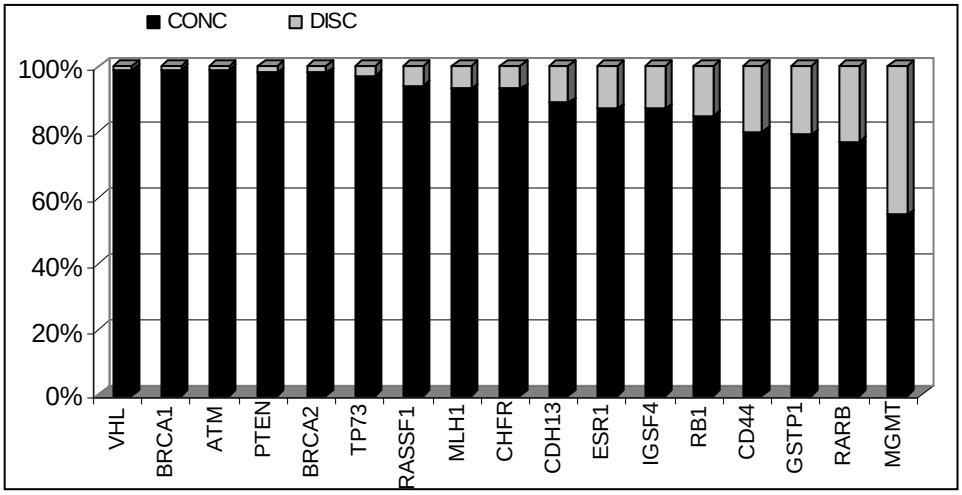

Figure 6. Concordance levels between two probes interrogating the same gene.

Percentage of concordance of results are displayed in y-axis. CONC, concordance; DISC, discordance.

Sensitivity was 100% for all probes because no false negatives were observed. By contrast, specificity differed depending on the gene probe, ranging from 83% to 50%. In general, the titration experiment demonstrated that MS-MLPA analysis can discriminate, almost for all the probes, samples containing 10%, 30% and 50% methylated sequences while no distinction can be made among samples with more than 50% of methylation. Moreover, some probes (see for example RASSF1) were not able to discriminate between 10% and higher levels of methylation. For all these motives, we scored aberrant methylation as a categorical variable. Furthermore as the application of an universal cut-off (0.3 suggested by the manufacturer) for all the 34 probes to score absence or presence of methylation was not applicable indiscriminately to all probes due to the peculiar specificity of each probe, a specific MR threshold was calculated for each gene. These values corresponded to the highest levels of accuracy to detect 10% of methylation (Table 4).

Table 4: Results of the titration assay to assess linearity, sensitivity, specificity and accuracy of MS-MLPA

Genes	MS-MLPA methylation-ratios in DNA mixing analysis						ROC analysis results			
	0%	10%	30%	50%	80%	100%	**MR threshold**	accuracy (%)	specificity (%)	sensitivity (%)
TP73	0.06	0.21	0.42	0.73	0.84	0.94	**0.32**	95.8	83	100
ESR1	0.06	0.27	0.56	0.92	1.00	0.88	**0.42**	95.8	83	100
CDKN2A p16	0.06	0.21	0.49	0.82	0.95	1.00	**0.24**	95.8	83	100
CDKN2A p14	0.06	0.16	0.46	0.74	0.95	1.00	**0.36**	95.8	83	100
DAPK1	0.00	0.21	0.39	0.67	0.83	1.00	**0.22**	95.8	83	100
ATM	0.00	0.16	0.38	0.65	0.85	0.92	**0.26**	95.8	83	100
IGSF4	0.05	0.23	0.47	0.82	0.92	0.89	**0.35**	95.8	83	100
CDKN1B	0.03	0.19	0.36	0.67	0.85	0.97	**0.25**	95.8	83	100
CHFR	0.00	0.20	0.46	0.78	0.99	0.99	**0.34**	95.8	83	100
BRCA2	0.02	0.22	0.51	0.85	1.00	1.00	**0.36**	95.8	83	100
THBS1	0.03	0.18	0.49	0.73	0.83	0.96	**0.25**	95.8	83	100
TP53	0.04	0.14	0.43	0.70	0.82	0.89	**0.21**	95.8	83	100
BRCA1	0.02	0.18	0.46	0.77	0.98	0.96	**0.31**	95.8	83	100
GATA5	0.05	0.18	0.45	0.72	0.97	0.99	**0.24**	95.8	83	100
TIMP3	0.00	0.19	0.38	0.67	0.91	0.95	**0.28**	95.8	83	100
VHL	0.01	0.27	0.68	1.00	1.00	0.93	**0.17**	91.7	67	100
MLH1	0.00	0.24	0.44	0.70	0.88	1.00	**0.2**	91.7	67	100
FHIT	0.01	0.26	0.44	0.86	0.92	0.86	**0.24**	91.7	67	100
APC	0.00	0.43	0.58	0.76	0.96	1.00	**0.44**	91.7	67	100
PTEN	0.05	0.25	0.53	0.85	1.00	1.00	**0.16**	91.7	67	100
MGMT	0.05	0.44	0.55	0.80	1.00	0.90	**0.38**	91.7	67	100
CD44	0.01	0.65	0.99	1.00	1.00	0.85	**0.66**	91.7	67	100
GSTP1	0.00	0.41	0.85	1.00	1.00	1.00	**0.28**	91.7	67	100
PAX6	0.00	0.24	0.49	0.75	0.93	0.91	**0.23**	91.7	67	100
WT1	0.10	0.33	0.53	0.83	0.94	0.89	**0.31**	91.7	67	100
RB	0.03	0.29	0.69	1.00	1.00	1.00	**0.2**	91.7	67	100
PYCARD	0.00	0.19	0.39	0.67	0.91	0.98	**0.21**	91.7	67	100
CDH13	0.11	0.56	0.75	1.00	1.00	0.94	**0.58**	91.7	67	100
HIC1	0.00	0.35	0.68	1.00	1.00	1.00	**0.22**	91.7	67	100
STK11	0.02	0.35	0.84	1.00	1.00	1.00	**0.23**	91.7	67	100
CASP8	0.01	0.56	0.68	0.82	0.88	0.99	**0.41**	87.5	50	100
RARB	0.00	0.31	0.52	0.81	0.97	1.00	**0.27**	87.5	50	100
RASSF1	0.00	0.81	0.79	0.94	0.98	1.00	**0.57**	87.5	50	100
PAX5	0.00	0.32	0.55	0.74	0.94	0.90	**0.3**	87.5	50	100

Legend: MR threshold: MS-MLPA ratio chosen to score aberrant methylation in each gene (>MR value). Based on ROC curve analyses these values correspond to the highest levels of accuracy to detect at least 10% of methylation.

Validation of MS-MLPA results by MS-PCR and by pyrosequencing

MS-MLPA was validated for methylation patterns in *hMLH1* and *MGMT* genes by MSP and by PCR-pyrosequencing. As is evident from both comparative analyses reported in table 5, there was a very high correlation between MS-MLPA and the other two methods ($p<10^{-14}$). In particular, MS-MLPA and pyrosequencing showed a concordance of results in 96% of tumor samples analyzed for *MGMT* methylation (92 out of 96 cases). Among the four discordant cases, 3 of the 96 samples (3%) were negative in the MS-MLPA analysis and showed a mean percentage of methylation in the five CpG dinucleotides of about 15-20% in the pyrosequencing analysis. On the contrary, one case was positive with the MS-MLPA method but negative with pyrosequencing. Comparing the MS-MLPA and MSP methods, there was agreement between the two approaches in 95% of the samples (97 of 102 cases). All five discordant samples showed methylation of *hMLH1* using MSP but were negative in the MS-MLPA analysis.

Table 5: Validation of MS-MLPA results by methylation-specific PCR (MSP) and by bisulphite pyrosequencing

Genes	MS-MLPA/PYROSEQUENCING				P
	+/+	**-/-**	**+/-**	**-/+**	
MGMT	25 (26%)	67 (70%)	3 (3%)	1 (1%)	10^{-17}
	MS-MLPA/MSP				P
	+/+	**-/-**	**+/-**	**-/+**	
MLH1	15 (15%)	82 (80%)	0 (0%)	5 (5%)	10^{-12}

Legend: MSP, Methylation-Specific PCR; +, methylated; -, unmethylated; +/+, positive for both; -/-, negative for both; +/-, positive MS-MLPA/negative pyrosequencing or MSP; -/+, negative MS-MLPA/ positive pyrosequencing or MSP.

Overall frequencies for promoter methylation in normal colorectal mucosa and in CRC samples

Methylation patterns of all 34 genes were examined by MS-MLPA in the 104 tumor samples, in adjacent mucosa of 13 CRC patients, as well as in colonic mucosa from 12 patients without evidence of malignancies. The selection of these 25 normal samples was carried out in order to represent all colorectal segments; the median age of the patients was 70 years, without significant differences between the two groups.

In order to exclude age related methylation, we correlated patient age (continuous variable) with methylation of each gene through the logistic regression model and we found a weak association between increasing age and presence of methylation of ESR1 and of THBS1 genes (OR values: 1.07, C.I.: 1.002-1.141, p=0.045 and 1.09, C.I.: 1.003-1.205, p=0.043). For all the other genes, no associations were present.

As reported in figure 7, a significant increase in the level of gene methylation is evident going from samples of normal mucosa of control cases to samples of normal mucosa of CRC patients to CRC specimens (p<0.0001).

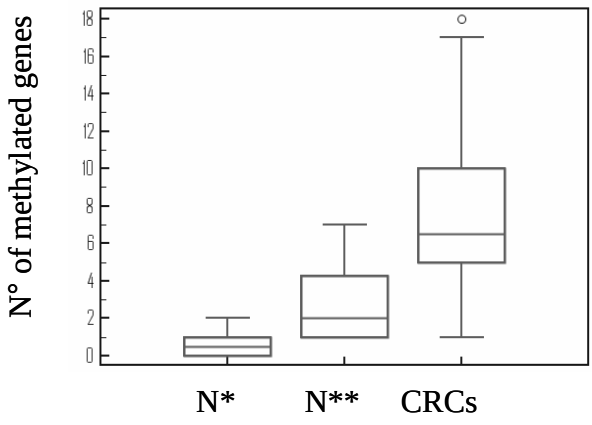

Figure 7. Number of methylated genes in normal and tumor samples. The box plots describe the different frequencies of methylated genes in colorectal mucosa of non neoplastic patients (N*), in the normal mucosa of patients with CRC (N**) and in CRCs.

In the first group we did not observe gene methylation, with the sole exception of *ESR1* and *STK11* genes that showed methylation in 3 and in 4 samples, respectively, as shown in detail in figure 8. Higher levels of methylation were detected in normal mucosa of CRC-patients and the following genes showed methylation: *GATA5* (77%), *RARB* (38%), *MGMT* (31%), *PYCARD* (23%), *WT1* (23%) *PTEN* (23%), *ESR1* (15%).

In the group of tumor samples *ESR1*, *WT1* and *GATA5* were methylated in more than 80% of cases. Intermediate levels of methylation between 10% and 45% were observed for the following genes: *CDH13*, *CDKN2A* (p16), *RARB*, *MGMT*, *CHFR*, *PAX6*, *PAX5A*, *TIMP3*, *DAPK1*, *STK11*, *APC*, *hMLH1*, *PTEN*, *HIC1*, *IGSF4*, and *RB1*. The remaining genes were rarely methylated, showing methylation in less than 10% of cases.

MS-MLPA profiles for clinico-pathological and biological stratification of CRCs

A subgroup of 27 colorectal cancers (26% of cases) showed extensive gene methylation involving more than 25% of the promoters examined. This finding suggested the presence of a CIMP+ phenotype in these samples (Figure 8). The methylation of the following 12 genes was significantly correlated with CIMP+: *RARB*, *hMLH1*, *CHFR*, *TIMP3*, *HIC1* ($p<0.0001$), *THBS1* ($p=0.0007$), *PAX5A* ($p=0.003$), *MGMT* ($p=0.004$), *PAX6* ($p=0.006$), *CDH13* ($p=0.007$), *APC* ($p=0.01$), and *CDKN2A* (p16) ($p=0.04$).

The presence of CIMP was observed with similar frequency in exocrine CRCs and in NECs (25% *versus* 29%, respectively). Among the exocrine CRCs, CIMP+ was significantly associated with the presence of MSI (58% of MSI tumors *versus* 16% of MSS tumors). Among NECs, we observed MSI in two cases that also showed CIMP. Overall the presence of MSI was the marker most strongly associated with widespread methylation ($p<0.0001$).

We first assessed the clinico-pathological and biological characteristics significantly associated with CIMP considering all 27 CIMP+ tumors regardless of the MSI status. This analysis highlighted that CIMP+ tumors were more frequently located in the proximal

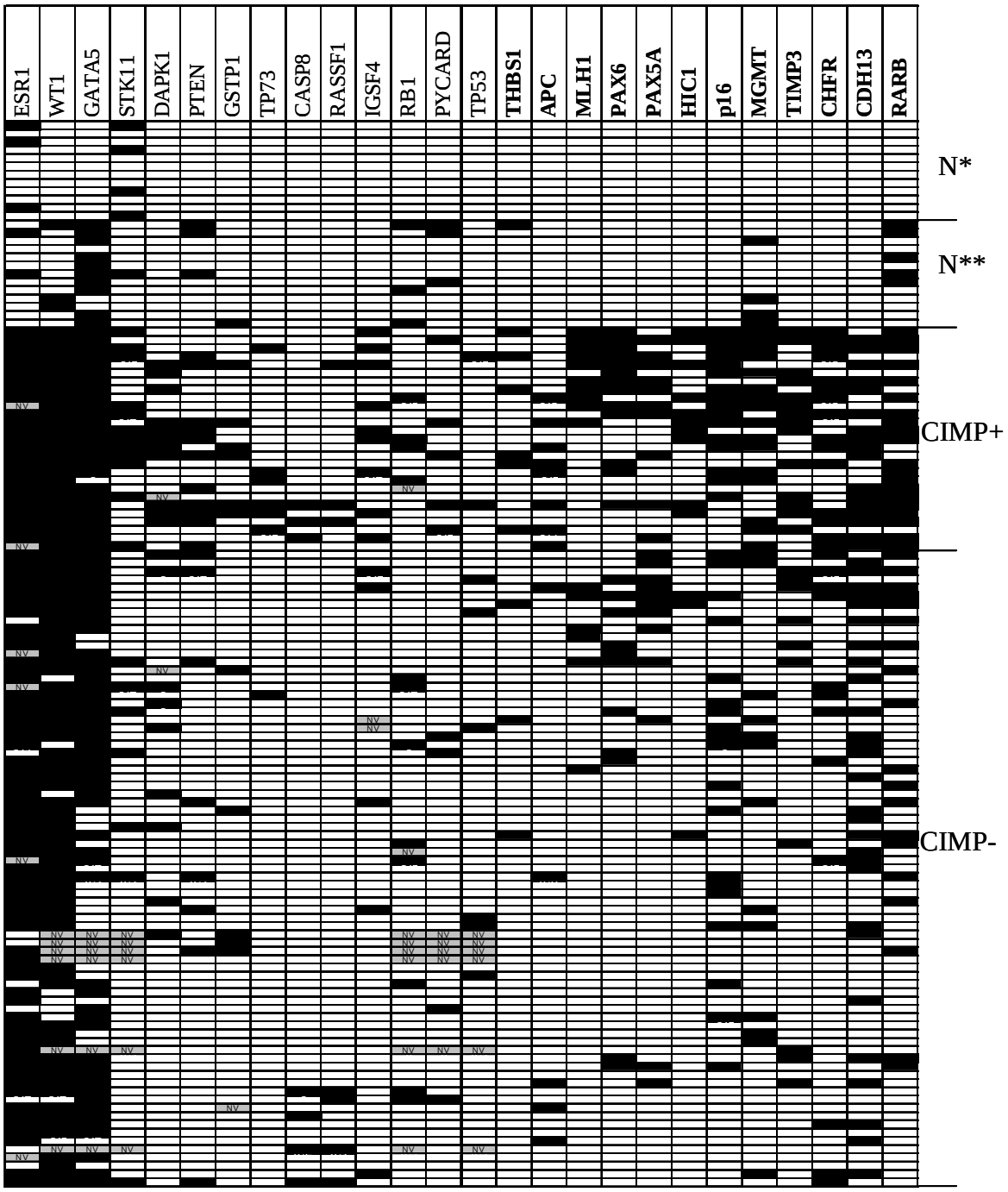

Figure 8. Methylation profiles of normal and tumor samples. Analytic description of presence of methylation (black cells) or absence of methylation (white cells) in each gene is illustrated for each sample. Genes whose methylation is associated with the CIMP+ phenotype are reported on the right side of the graph and are highlighted in bold. Gray cells are used for unavailable data.

colon (p=0.02), showed poor differentiation (p=0.05) and, among exocrine CRCs, mucinous histology (p=0.001) and histological heterogeneity (p=0.02). CIMP+ tumors more frequently exhibited *BRAF* mutation (p=0.005) and the absence of loss of heterozygosity at 5q, 17p and 18q (p=0.008).

However, figure 9 clearly emphasizes that all these features were typical of CIMP+ tumors with MSI and that the associations found between CIMP and specific clinico-pathological/molecular features were strongly influenced by MSI status. Indeed, when considering only the group of MSS tumors, the statistical significance of all associations reported above was lost.

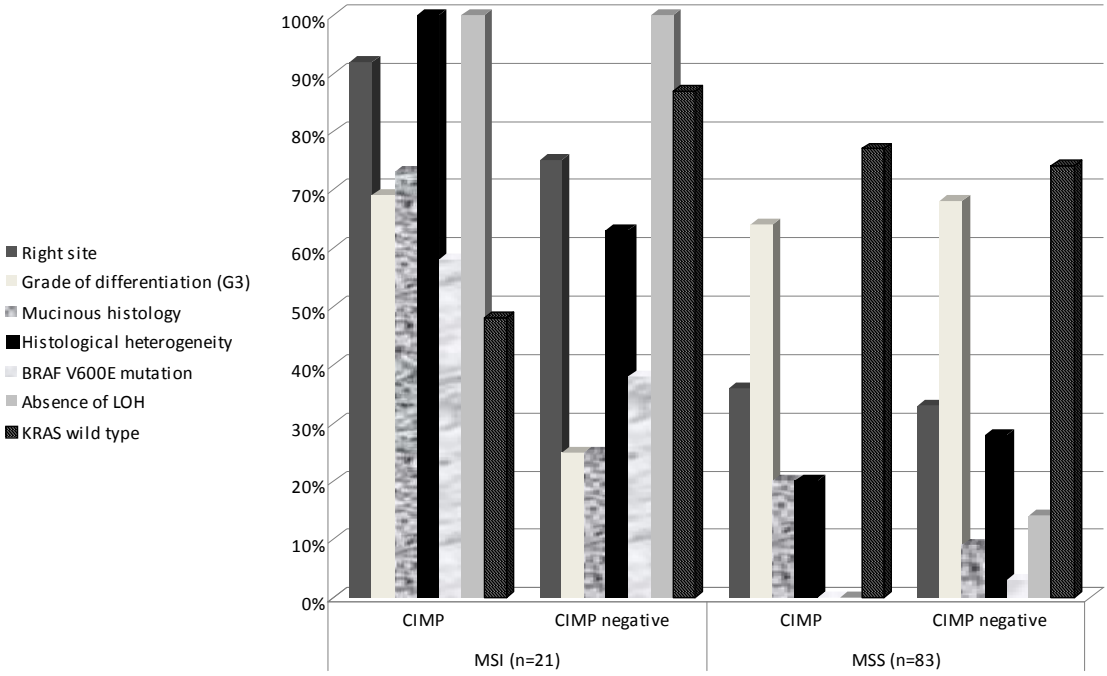

Figure 9. Distribution of clinico-pathological and molecular characteristics in MSI and MSS tumors with regard to CIMP+ and CIMP- phenotype.

When considering CIMP status and cancer-specific survival, we observed a trend towards a better prognosis in patients with CIMP+ tumors. However, this association was not

independent of MSI status because the presence of MSI was positively associated with an increased cancer-specific survival (p=0.04). Although not statistically significant because of the small number of cases, CIMP+ tumors with MSI were associated with a very good prognosis compared with all the remaining three classes of cancers (Figure 10).

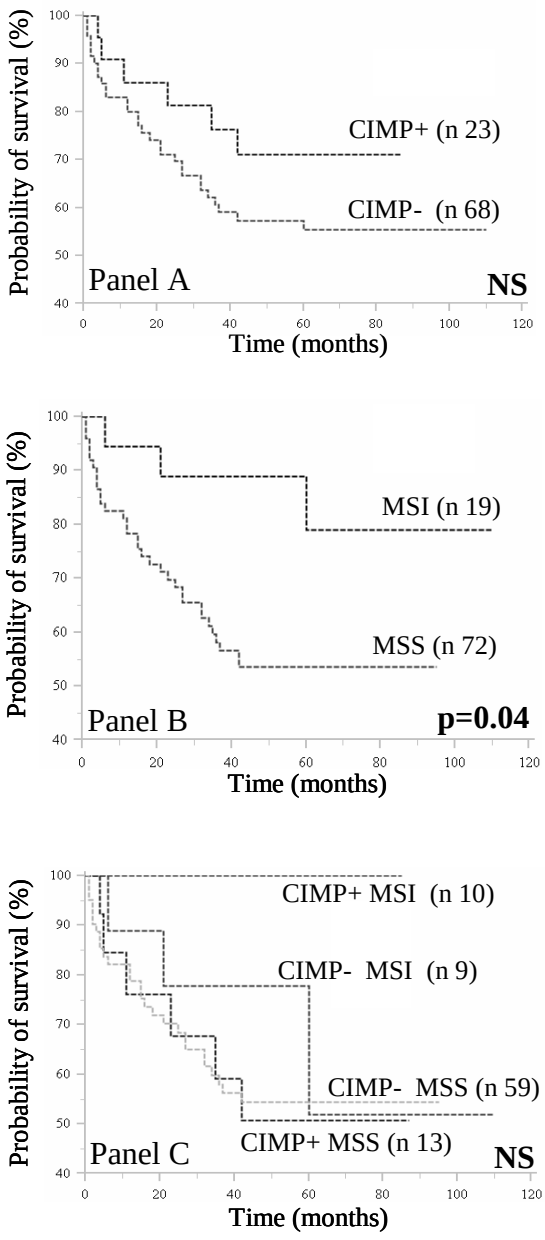

Figure 10. Kaplan Meier survival analysis by CIMP status (panel A), by MSI status (panel B) and by the combination of both CIMP and MSI status (C). Log rank test was used to calculate p values for differences between groups. NS = p value not statistically significant.

Intriguingly, this result was observed also among NECs because the only two long surviving patients with NECs (alive with no evidence of disease after 215 and 93 months, respectively) showed CIMP+ tumors with MSI.

Specific methylation patterns of individual genes were observed comparing exocrine CRCs with NECs. In exocrine CRCs the most frequently methylated genes were *CDKN2A* (p16) and *PAX6* ($p<0.0001$ and $p=0.04$, respectively). By contrast, methylation of *RASSF1*, *CASP8* and *APC* was typical of NECs ($p<0.0001$, $p<0.0001$ and $p=0.01$, respectively.

DISCUSSION

MS-MLPA is a new approach, described for the first time in 2005 [51], that has mainly been applied in the study of genomic disorders associated with the loss of imprinting. So far, MS-MLPA has rarely been exploited in the analysis of gene methylation in tumors and very few studies about methylation profiling of CRCs using this method have been published [26,66].

In this work we have optimized and successfully applied MS-MLPA analysis to an archival series of 104 CRCs and 25 normal colonic mucosa samples confirming that this method works very well with DNA from FFPE tissues providing highly reproducible methylation results. MS-MLPA is rapid and simple to perform, relatively inexpensive and allows screening for multiple predefined candidate genes simultaneously, using small quantities of input DNA. In addition, the assay can be performed in 96-well format, making it suitable for high throughput analysis and requiring only equipment widely available in molecular genetics laboratories. A particular strength of MS-MLPA is its ability to perform methylation analyses without labour intensive approaches based on sodium bisulphite treatment of the DNA. In this work we used two commercial MS-MLPA kits and we demonstrated that methylation ratios for all but two probes under study were consistent and reproducible.

A critical aspect of MS-MLPA is related to the use of a methylation-sensitive restriction enzyme to discriminate between methylated and unmethylated sequences. Incomplete digestion of unmethylated targets may lead to false-positive results and is a caveat inherent in most restriction enzyme-based methylation assays [67]. However, this problem can be easily overcome by using at least two probes for each target sequence and introducing in each experiment positive and negative controls. Moreover it is possible to improve the application of the short-probe MS-MLPA approach, as recently described [68].

The quality of DNA samples utilized in MS-MLPA reaction is another crucial question.

For all cases, we evaluated the reproducibility of replicates in the light of their amplifiability determined by control gene PCR, and we found a positive association between poor quality and low reproducibility, suggesting that DNA quality assessment is an important pre-requisite for MS-MLPA application.

In our experience, another limitation of the assay is that MS-MLPA is not a quantitative method to assess DNA methylation. Indeed, an important finding of our work is the demonstration that MS-MLPA shows a differential probe-to-probe capacity to discriminate among percentages of DNA methylation and that the highest values of accuracy are achieved defining aberrant methylation as a categorical variable by using specific cut-offs of MR for each gene. Moreover, the comparison of results obtained by two probes addressing the same promoter found concordance values higher than 77% for all probes, except for the one addressing *MGMT* promoter. However, subsequent validation tests of *MGMT* and *MLH1* promoter methylation using pyrosequencing and MSP methods, confimed MS-MLPA results with an agreement of 96% and 95%, respectively. Overall these observations confirm that MS-MLPA may be considered an effective and simple first line tool to study DNA methylation in wide series of archival clinical samples. Importantly, the clinical translational application of this technology may also be related to the intrinsic flexibility of the MS-MLPA platforms and to the opportunity to develop targeted tumor-specific customized assays as well as to optimize a panel of "universal methylation markers" that could be useful in the analysis of gene methylation in different sites.

Moreover, the employment of this technique, even using two commercial assays including predefined genes not specific to any tumour type, allowed us to reproduce findings that have been previously described by others using different methods [69-71] about

increased methylation in CRCs, the presence of CIMP in these tumours and its associations with specific clinico-pathological and molecular features.

In the present work, we identified a total of 27 CIMP+ tumors consisting of 14 MSI cancers (12 exocrine CRCs and 2 NECs) and 13 MSS cancers (9 exocrine CRCs and 4 NECs). Our result suggested that CIMP+ tumors with MSI represent a biologically and clinically distinct entity. In agreement with a large body of data in the literature [69-71], these tumors showed strong positive associations with proximal colon (92% of cases), poor differentiation (69% of cases), mucinous histology (73% of cases), histological heterogeneity (100% of cases), *BRAF* mutation (58% of cases) and absence of loss of heterozygosity at 5q, 17p and 18q (100%).

Although all these associations remained significant when CIMP+ MSS cancers were added to the analysis, it was evident that this second subset of tumors was much more heterogeneous, showing the above mentioned features less frequently or not at all. Furthermore, the methylator phenotype lost its prognostic meaning when we considered survival probability in stable tumors.

On the other hand, in unstable tumors CIMP phenotype seems to select a subset of cases with a very good prognosis. Intriguingly, this result was observed also among NECs because the only two long surviving patients (alive with no evidence of disease after 215 and 93 months) showed CIMP+ tumors with MSI. However, this hypotesis could not be definitively determined in our study because of the small number of cases and needs further investigations.

NEC tumors are rare and highly malignant cancers, most often located in the right colon and known to be associated with chromosomal instability and p53 alterations and, to date, very few works dealing with the role of aberrant methylation in these tumor entity have been published. In this work we demonstrated that DNA methylation as well as CIMP are

present in sporadic CRCs and in NECs with very similar frequencies. The observation that gene hypermethylation is a common feature of such different tumors supports the hypothesis that epigenetic abnormalities can characterize the earliest steps of tumors destined to progress differently. On the other hand, if the disruption of epigenetic machinery in the earliest phases of carcinogenesis seems to be a process common in the two tumor types, the presence of a gene-specific signature in these two entities suggests the involvement of aberrant methylation also in later steps, in association with sequence variations, thus determining the biologic fate of different tumour types.

Indeed, our study identified two different mehtylation pattern in exocrine and neuroendocrine tumors. Specifically, *RASSF1*, *CASP8* and *APC* were more frequently methylated in NECs than in exocrine CRCs. These findings are consistent with previous studies reporting high frequencies of *RASSF1* methylation in neuroendocrine tumors of the gastroentero-pancreatic system [36,72] and high frequencies of *APC* methylation in colorectal NECs [36]. Similarly to our data for the colorectal site, unique profiles for gene methylation have been generated that can distinguish between non small cell lung cancer and small cell lung cancer [73,74]. Intriguingly, small cell lung cancers that show clinico-pathological features indistinguishable from those of NECs of various sites exhibit significantly higher frequencies of *CASP8* and *RASSF1* methylation compared with non small cell lung cancers [75]. On the contrary, exocrine CRCs were characterized by high levels of methylation in CDKN2A/p16 and PAX6 promoters. Moreover, exocrine unstable tumours exhibited, as reported in many works [17], a specific profile of methylation including *hMLH1*, *CDKN2A*/p16, *RARB*, *HIC1*, *PAX5A*, *PAX6*, *TIMP3* and *CHFR* genes.

MS-MLPA analysis was performed also on 25 colorectal histologically normal mucosae. The comparison between methylated genes in normal colonic tissues of 12 non-CRC patients versus 13 patients with CRCs included in this study allowed us to identify a

"cancer-type methylation" in the last group of samples, as aberrant methylation was absent in the control group. Specifically, in histologically normal tissues of patients with CRC, methylation occurred in many genes, while only ESR1 and STK11 were methylated in mucosae of non neoplastic patients. We also checked whether the aberrant gene-specific methylation in tumor-adjacent normal tissues were found in matched tumours but we did not find the same pattern (data not shown). According to this observation, Belshaw and co-workers [76] recently demonstrated that variation in methylation levels existed between crypts from the same biopsy suggesting that human crypts are prone to random acquisition of aberrant gene methylation.

An important finding of our work was the observation that some genes, found hypermethylated with high frequencies in cancers, are also methylated in most of the colonic mucosae of CRC patients. Among these genes, *GATA5* showed extensive methylation in a considerable number of tumour samples and it has been widely observed in normal mucosa of patients bearing CRCs (77% of cases) and never in the control group. Indeed, recent studies have highlighted the potential diagnostic value of *GATA4/5* methylation for early detection and risk assessment of colon cancer [77,78]. Our analysis has also pointed out the potential usefulness of *WT1* gene as an early diagnostic marker because of the very high frequencies of methylation in the present series. To date, a widespread methylation of this gene has been reported in colorectal cancer, although very few studies have been published on this issue [79,80]. In our opinion, this finding deserves to be further explored, especially with respect to the disruption of methylation patterns described in CRCs to occur in 11p15 where *WT1* maps together with *IGF2*, a known target of LOI events [10,81,82].

Taken together, these data have important clinical and scientific implications suggesting the involvement of these genes in tumour initiation and proposing them as markers of

early neoplastic phases. Promoter methylation analysis of serum and stools DNA has recently been described as a potential tool to be used as a non-invasive test for the early diagnosis of CRC [78,83,84].

In conclusion, our experience indicates that the MS-MLPA assay is an easy and reliable system for epigenetic characterization of tumor tissues and presents innovative aspects that may have important scientific and clinical implications.

Aberrant gene methylation is a common abnormality in CRC and may be observed in tumors with very different genetic and clinico-pathological profiles. The use of DNA methylation alterations as a molecular marker system could potentially be a powerful approach to population-based screening for the early detection and for risk assessment of colorectal cancer.

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