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DNA Fingerprinting Techniques for the Analysis of Genetic and Epigenetic Alterations in Colorectal Cancer

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Abstract

Genetic somatic alterations are fundamental hallmarks of cancer. In addition to point and other small mutations targeting cancer genes, solid tumors often exhibit aneuploidy as well as multiple chromosomal rearrangements of large fragments of the genome. Whether somatic chromosomal alterations and aneuploidy are a driving force or a mere consequence of tumorigenesis remains controversial. Recently it became apparent that not only genetic but also epigenetic alterations play a major role in carcinogenesis. Epigenetic regulation mechanisms underlie the maintenance of cell identity crucial for development and differentiation. These epigenetic regulatory mechanisms have been found substantially altered during cancer development and progression. In this review, we discuss approaches designed to analyze genetic and epigenetic alterations in colorectal cancer, especially DNA fingerprinting approaches to detect changes in DNA copy number and methylation. DNA fingerprinting techniques, despite their modest throughput, played a pivotal role in significant discoveries in the molecular basis of colorectal cancer. The aim of this review is to revisit the fingerprinting technologies employed and the oncogenic processes that they unveiled.

1. Introduction. Genetic and epigenetic aberrations in human cancers

The isolation of the first human oncogenes and tumor suppressors [1–4] led to the prevailing hypothesis during the last decades postulating that the origin of cancer resides in the accumulation of somatic mutations in cancer genes, i.e. proto-oncogenes and tumor suppressors [5,6]. In addition to these oncogeneic mutations, the vast majority of solid tumors exhibit aneuploidy and chromosomal rearrangements. Whether somatic chromosomal alterations and aneuploidy are a driving force or a consequence of tumorigenesis remains controversial [7–9]. With the exception of the DNA mismatch repair mutator genes that underlie microsatellite instability, diagnostic of a strong mutator phenotype [10–12] the search for somatic mutations in genes involved in the preservation of genome integrity has been disappointing [13–17]. With the information at hand, it appears that somatic genetic alterations leading to such an active chromosomal instability do not have a great significance in cancer. The chromosomal alterations universally found in solid tumors may be in a greater extent due to clonal selection and evolution of errors of

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chromosomal segregation that, in some cases, can be originated by age-related epigenetic alterations.

A shift has recently occurred in cancer research with the realization that not only genetic but also epigenetic alterations play a major role in carcinogenesis. To date, three major types of epigenetic mechanisms have been identified in humans: DNA methylation, histone modifications, and, more recently, non-coding RNAs. These mechanisms, responsible for the cell identity and differentiation, have been found substantially altered during cancer development. Consequently, the study of cancer epigenetics has attracted considerable attention.

In vertebrates, DNA methylation occurs almost exclusively at the position 5 of cytosine residues within the dinucleotide CpG. These sites are found concentrated in some genomic regions denominated CpG islands (CGIs), generally associated to gene promoters. While most of the CpG sites outside CGIs are constitutively methylated, the CpG sites inside CGIs are frequently devoid of methylation. The methylation status of the CGIs located in promoters and other gene regulatory regions can exert a drastic effect on the transcriptional levels of downstream genes, providing an epigenetic mechanism to control gene expression. Up to 72% of the human gene promoters are specially rich in CpG sites [18], consistent with the notion that many genes are susceptible of epigenetic regulation through histone modifications and DNA-methylation.

Global DNA hypomethylation of cancer cells was discovered more than twenty-five years ago [19,20]. Soon after, the first example of somatic hypomethylation of cellular oncogenes in human cancer was reported [21]. Years later, it was found that some sporadic retinoblastoma tumors exhibited hypermethylation of the promoter of the tumor suppressor gene RB1, leading to transcriptional repression [22]. Since that seminal discovery, numerous genes have been found to undergo promoter hypermethylation in a large variety of cancers [23]. The role of gene promoter hypermethylation in carcinogenesis has been extensively studied, yielding cancer detection markers and chemotherapy predictors for cancer patients, as well as fostering the development of epigenetic drugs approved for the treatment of hematological malignancies [24].

Technologies to detect mutations, chromosomal copy number alterations and DNA methylation alterations have improved exponentially, fostered by the generalization of the microarray platforms in the mid nineties [25] and, more recently, the development of massively parallel sequencing platforms [26–29]. These technologies have reached an impressive throughput: today it is possible to analyze over one million genomic locations in just a single microarray chip and the massively parallel sequencing platforms are capable of delivering more than one hundred million sequences in a single experiment. Very recently, the analysis of colorectal tumors using a comprehensive high-throughput array-based relative methylation (CHARM) method [30], not biased for CpG island or promoter sequences, yielded a surprising discovery: the authors found that methylation alterations in colon cancer occur not only in promoters and CpG islands, but also in sequences up to 2 kb distant, which they termed 'CpG island shores'. CpG island shore methylation showed an inverse relationship with gene expression [31].

However, before these remarkable technological advances were achieved, a handful of ingenious techniques based on the generation of DNA fingerprints of matched normal and tumor tissues were successfully employed to detect genetic and epigenetic alterations and, despite their modest throughput, they were germane to significant discoveries that provided insights in the molecular basis of colorectal cancer. Some of these technologies still have a place in everyday lab research, as they usually require less complex and inexpensive

equipment. We will revisit in this review some of the fingerprint technologies employed in colorectal cancer research and their role in the discovery of fundamental oncogenic processes.

2. Fingerprinting techniques for the detection of chromosomal copy number changes

Few years after the development of the polymerase chain reaction (PCR) technique [32], two PCR-based fingerprinting techniques were almost simultaneously published, Arbitrarily Primed PCR (AP-PCR) [33] and Random Amplification of Polymorphic DNA (RAPD) [34]. RAPD fingerprints are obtained using a pair of very short primers, usually no longer than 10-mer, favoring unspecific annealing in the first several cycles of PCR amplification. The basis of the AP-PCR is the amplification of random genomic DNA sequences using a single primer of arbitrary sequence [33]. The first cycles of amplification are performed at a low annealing temperature, typically 40–50°C, favoring the primer annealing to many non-specific sequences in the genomic DNA. The annealing temperature is raised in subsequent cycles. A complex process of competition between the many initial amplification products results in only a subset of the initial amplicons to be further amplified in a very reproducible manner. Each arbitrary primer can amplify more than 100 DNA fragments ranging in size from less than 100bp to more than 2000bp, although the fragment size is typically less than 1kb [35]. AP-PCR amplicons are radioactively labeled, either adding $[\alpha\text{-}^{32}P]$-labeled dNTPs to the PCR reaction or labeling the 5' end of the arbitrary primer with $[\gamma\text{-}^{32}P]$-ATP prior to PCR amplification, and detected by autoradiography after electrophoretic separation in a denaturing polyacrylamide gel. In some protocols, ^{32}P is replaced with the lower-emission isotopes ^{33}P or ^{35}S, in order to obtain sharper autoradiography bands at the expense of longer exposure times [36,37]. Non-radioactive AP-PCR fingerprints have been also developed [38,39].

RAPD fingerprints have been employed in phylogenetic studies to distinguish between different cell strains or organisms, and have been also used to analyze chromosomal copy number changes in cancer cells [40–42]. Applications and achievements of RAPD technology have been recently reviewed by F.A. Atienzar and A.N. Jha [43]. Similarly, AP-PCR was originally designed to identify differences in the genomes of different prokaryotic and eukaryotic organisms and, consequently, it has been often used in genotyping and phylogenetic studies [43]. However, soon after its invention, the potential of this technique to detect DNA copy number alterations in tumorigenesis was established [44].

2.a. AP-PCR and the detection of chromosomal copy number changes

Comparison of AP-PCR fingerprints of genomic DNA from tumor cells and non-tumor tissue from the same individual allows the identification of both deletions and amplifications in randomly amplified *loci*. Losses are visualized as a reduction in the intensity of a band in AP-PCR fingerprint of the tumor genome compared to the normal, while an increase in the intensity reflect amplification in the tumor genome of the target sequence and hence their adjacent chromosomal sequences. A decrease in band intensity could, in theory, result from mutations in the primer annealing site preventing amplification [35], but this possibility is extremely infrequent. Theoretically, homozygous deletions could also be detected [45], but their frequency in cancer is very low. Thus, the vast majority of losses detected in the AP-PCR fingerprints represent heterozygous deletions often accompanying the loss of heterozygosity (LOH) unmasking tumor suppressor genes. Amplifications are observed as an increase in the intensity of the AP-PCR bands in the tumor genomic DNA fingerprint. The lower limit of detection is at the level of trisomies - tetrasomies [46].

AP-PCR offered several advantages over the previously available methods: the ability to analyze many arbitrary locations in a single experiment while being an inexpensive method that does not require complex equipment. AP-PCR amplicons exhibiting alterations can be easily eluted from the dried gel, re-amplified and subsequently sequenced to determine their genomic location. Once the AP-PCR fragments are identified, this technique can be used to generate a molecular karyotype, or amplotype, reflecting the chromosomal gains and losses in the tumor sample. Amplotyping confirmed many of the previously identified chromosomal alterations in colorectal cancer (ie. loss of 5q, and 17p), and discovered several alterations that were subsequently confirmed by other techniques, especially Comparative Genomic Hybridization (CGH). These include loss of chromosomes 4 and 14 in colorectal cancer and of 2q and 6p in breast cancers [47] as well as the very frequent gains of chromosomes 8q, 13q and 20 and the gains of chromosome 6 associated to metastatic colon cancers [44,46]. Of particular interest is the observation that gains of the region at 8q24 encompassing c-Myc were detected in around and above 50% of all of the tumors analyzed, including colorectal, gastric, liver, pancreas, lung, and breast cancers that made up over 50% of all human cancer worldwide, thus implicating c-Myc as perhaps the most common altered gene in human cancer ([48,46,47,49] and unpublished observations).

In addition, the fraction of bands exhibiting alterations in the AP-PCR fingerprints of tumors – or Genomic Damage Fraction (GDF) - provides an estimation of chromosomal copy number alterations. The distribution of the tumor specimens according to the extent of their genomic damage was found to be gradual but remarkably GDF provided an independent prognosis indicator in both gastric and colon cancers. Patients with low-GDF tumors had significantly better prognosis than patients with high-GDF tumors, independently of the cut-off point to stratify the tumors in either the low-GDF or high-GDF groups [36,37,49,50].

2.b. AP-PCR and the discovery of microsatellite instability and the mutator phenotype

In addition to copy number changes, AP-PCR unexpectedly detected small insertions and deletions in the template DNA, visualized as mobility shifts of one or a few fingerprint bands. This observation led to the discovery in 1993 of microsatellite instability (MSI) or the microsatellite mutator phenotype (MMP), a novel pathway for carcinogenesis [11]. About 12% of unselected colorectal cancers studied by AP-PCR exhibited mobility shifts of several of the AP-PCR fingerprint bands. When these bands were isolated and sequenced, it was found that all of them contained repeated Alu sequences, which had undergone somatic deletions of a few nucleotides in their poly(A) tails. Further experiments confirmed that microdeletions also occurred in other mononucleotide repeats and insertions and deletions in dinucleotide and trinucleotide microsatellite repeats.

The unbiased nature of the AP-PCR fingerprints allowed to extrapolate that these mutations originated by errors of replication due to slippage by strand misalignment surpassed the hundreds of thousands in the subset of tumors with MSI. Among these many irrelevant non-coding microsatellite sequence mutations some mutations will also hit cancer genes (oncogenes and tumor suppressors), ultimately leading to cancer [51,52]. Soon after the initial observation it was found that mutations in these microsatellite sequences accumulated because of mutations occurring in the DNA mismatch repair machinery genes [53,54]. MMR deficiency underlies the genome-wide microsatellite instability (MSI) phenotype in most of the hereditary non-polyposis colorectal cancers (HNPCC) and some sporadic gastrointestinal tumors [52,55].

The sequence of events in the MMP pathway can be summarized as follows: inactivation of MMR (mutator) genes causes a mutator phenotype, which causes oncogenic mutations, which cause cancer [56]. Therefore, cancer driven by mutator genes represents a "remote control" mechanism for carcinogenesis, as mutator gene inactivation does not immediately

lead to altered cell growth or survival [57,52,58]. MSI represents a distinctive pathway to cancer, with a particular spectrum of mutated cancer genes in MSI positive tumors [59–62]. MSI tumors display a low incidence in the APC and P53 tumor suppressor genes and K-ras oncogene, prototypical of colon cancer [11,63,57,64–66]. Instead, MSI positive colon tumors carry a plethora of different mutated genes, such as TGFβRII and Bax, which are rarely found in microsatellite stable (MSS) tumors [59,60,67,68]. This is because in a MMR deficiency background, mutations occur preferentially in genes with simple repeats in their coding or regulatory sequences [69,70].

2.c. DNA microarray platforms for detection of copy number alterations

Simultaneous hybridization of AP-PCR products (SHARP) was developed to determine the chromosomal location of the amplified products, without having to isolate, clone and sequence the individual AP-PCR bands [71]. In this technique, AP-PCR fingerprints are performed on genomic DNA from human/rodent monochromosome cell hybrids. Only the bands of human origin are visualized after hybridization, allowing the immediate determination of the chromosomal location of the different bands.

To increase the number of *loci* that could be analyzed in a single experiment, AP-PCR was transferred to a microarray format. The resulting technology was termed Chromosomal Hybridization of AP-PCR Amplicons (CHAPA) [72]. CHAPA employed a modified AP-PCR primer favoring the amplification of CA repeats, which are very common in the human genome, to maximize the number of different amplified PCR fragments. After AP-PCR amplification, the products were cloned into *E. coli* and the inserts of thousands of clones were individually identified by sequencing and printed onto microarray slides. DNA from normal and tumor samples was subsequently amplified using the same primer, labeled with different fluorescent dyes and hybridized onto the microarray. Differences in the ratio of the dyes accurately reflected copy number differences in the samples. Albeit CHAPA was proven to be suitable to detect copy number changes, its further development was hampered by the contemporary commercialization of CGH platforms facilitated by the information available form the Human Genome Project [73]. The higher resolution of the microarray platforms confirmed and extended the previous results obtained by allelotyping and AP-PCR amplotyping. Figure 2 shows the concordance in interpretation of results obtained with the original allelotype approach [74] that only detected losses (loss of heterozygosity or LOH) but not gains using polymorphic minisatellite sequences, AP-PCR amplotyping [46] and BAC CGH arrays [75].

In addition to AP-PCR, several technologies were developed in the early nineties to detect copy number alterations. One was Comparative Genomic Hybridization (CGH) [76–78]. Another approach, Representation Differential Analysis (RDA) [79,80] utilized PCR-derived genomic representations instead of the total genomic DNA to reduce complexity and increase hybridization signal intensity [81]. More recently, these technologies have been transformed to DNA microarray format. Pollack *et al.* utilized arrays of cDNA fragments for CGH analysis [82]. Pinkel *et al.* used arrays of BAC clones to increase the resolution up to 1 Mbp [83–85] and later tiling arrays were generated [86]. The next advance was the development of high density genomic arrays using oligoprobes derived from in silico analysis [87]. These platforms offer a coverage of the human genome without requiring cloning and sequencing individual probes before printing them onto microarray slides.

The analysis of colon cancer with Agilent 44K CGH arrays for copy number changes further validated the AP-PCR and CHAPA results. The larger number of *loci* analyzed by the CGH platform facilitated the detection of small alterations that might be missed by the AP-PCR amplotyping analysis. This CGH study revealed an interesting novel type of small genomic alteration: within large deleted regions there are smaller sequences that do not undergo the

deletion. They appear to have *escaped* from the loss. In some cases, the size of these alterations was just at the limit of the 44K arrays resolution, with just 3 or 4 probes within the *escaped* region, preventing a conclusive identification. Re-analysis of some of these alterations on the highest resolution arrays currently available from Agilent, the SurePrint G3 Human CGH Microarray Kit 1x1M, which have more than 974,000 coding and non-coding human sequences represented, confirmed the existence of some genes that *escape* from a deletion that affects the surrounding region (Figure 3). The implication is that these genes are under strong selection pressure against their loss during colorectal cancer development or progression. Some of the *escaped* genes participate in essential cellular functions, such as RNPOLII. Other genes, however, might be involved in the carcinogenesis process in a more direct way, like SMAD7, which has been found to be a factor in the risk to develop colorectal cancer [88].

3. Fingerprinting technologies for the analysis of epigenetic alterations

Most current DNA methylation analysis technologies rely on the DNA treatment with sodium bisulfite after chemical denaturation. This treatment deaminates all the unmethylated cytosine residues, converting them to uracil. Methylated cytosines, however, are resistant to deamination and remain unaffected. The bisulfite treated DNA is subsequently analyzed by PCR amplification, where the converted uracile residues in the sequence are substituted by thymine in the amplification product and therefore easily identified. Several techniques have been designed to study the methylation of regions of interest. Among them, the most popular technologies are bisulfite genomic sequencing [89], methylation-specific PCR (MSP) [90], MethyLight [91], combined bisulfite and restriction analysis (COBRA) [92], and more recently methylation microarrays [93]. Most of these techniques have been thoroughly reviewed [94,95] (see also Jorda and Peinado in this issue). However, before the widespread use of bisulfite-based techniques, several fingerprint approaches were developed based on methylation-sensitive restriction enzymes, whose activity is blocked by methylation of their target sequences.

3.a. Restriction Landmark Genomic Scanning for Methylation (RLGS-M)

RLGS is a high-resolution two-dimensional gel electrophoresis method based on the use of rare-cutter restriction enzymes to produce a discrete number of fragments [96]. The original method, based on a methylation-insensitive rare-cutter *Pac*I, was capable of detecting polymorphisms and copy number changes but not methylation differences. RLGS-M [97] is a variation of RLGS in which methylation-sensitive rare-cutters are employed, like *Not*I (G**CGG**CC**GC**) and *Asc*I (GG**CGCGC**C) that only cut when both internal CpG sites (in bold type) are unmethylated. RLGS-M is suitable to assess copy number changes as well as differences in methylation. After digestion, the 5'-protruding ends are labeled by filling the overhanging strand with radioactive nucleotides. The DNA is then subjected to a second digestion to produce smaller DNA fragments, most commonly with the restriction enzyme *Eco*RV, and separated in a first dimension agarose gel. After this electrophoresis, the sample is subjected to an *in situ* digestion with a third restriction enzyme, usually *Hinf*I, allowing adequate separation of the resultant smaller fragments in a second-dimension polyacrylamide gel electrophoresis. After autoradiography, the end result is a reproducible RLGS profile that displays hundreds of spots reflecting the combination of copy number and methylation status of individual *loci* [98]. RLGS-M profiles from different tissues can be compared to assess for tissue-specific methylation, or from tumor and adjacent normal tissue to investigate cancer-associated methylation aberrations. Deletions or hypermethylation are detected by the loss or reduction of signal intensity of a RGLS-M spot, while amplification or hypomethylation result in increased signal intensity or new spots. Using the surrounding single copy spots as internal controls, the loss or gain of intensity of a locus can be visually

estimated or quantified by densitometry. To facilitate the identification task, it is now possible to conduct automated RLGS fragment prediction and to download corresponding sequences for mouse [99] and human studies [100].

RLGS-M fingerprinting is one of the first methods capable of identifying many landmark fragments in a single run and the simultaneous identification of both gene copy number and methylation status [101]. However, this method has some drawbacks: it is rather labor-intensive, and only one sample per gel can be analyzed. Also, it requires at least a few micrograms of high-molecular-weight DNA to ensure the integrity of large pieces of DNA and to prevent nonspecific labeling of degraded fragments [98], so sample availability might be a limitation. Finally, the cloning step is critical and difficult as no PCR amplification is possible. [102]. Despite these limitations, RLGS-M has been successfully employed to analyze aberrant DNA methylation in breast, ovarian, colon, gastric, lung and hepatocellular cancers [103–110]. One RLGS-M study on colorectal cancer found that methylation of some CpG islands located in non-promoter regions of genes was associated with gene expression upregulation, indicating that alterations in the methylation status within CpG islands in colon tumors may have complex consequences on gene expression and tumorigenesis. [110].

3.b. Methylation-Sensitive Arbitrarily Primed PCR (MS-AP-PCR) and Restriction Fingerprinting (MSRF)

In 1997, two similar methods to generate methylation-sensitive fingerprints were published almost simultaneously, Methylation-Sensitive Arbitrarily Primed PCR method (MS-AP-PCR) [111] and Methylation-sensitive Restriction Fingerprinting method (MSRF) [112] (Figure 4). Both methods rely on the treatment of the genomic DNA with a methylation-insensitive frequent cutter yielding a methylation-independent DNA fragments library and, in a separate aliquot, with the same frequent-cutter and a methylation-sensitive restriction enzyme yielding a methylation-dependent DNA fragments library. In MSRF, the methylation-independent library is generated with the frequent cutter *Mse*I (TTAA) and the methylation-dependent library with a mixture of *Mse*I and the methylation-sensitive *Bst*UI (CGCG), which cuts only if both CpG sites within its recognition sequence are unmethylated. In MS-AP-PCR, two different methylation independent libraries are generated: one with *Rsa*I (GATC), and another one with a mixture of *Rsa*I and *Msp*I, which digests in C**C**GG sequences regardless the methylation status of the internal CG dinucleotide. The methylation-dependent library is generated with a mixture *Rsa*I and the methylation-sensitive *Hpa*II, which recognizes the same sequence than *Msp*I (CCGG) but only digests if the internal CG dinucleotide is demethylated. After enzymatic restriction, these DNA fragments libraries serve as template for PCR amplification in AP-PCR or RAPD conditions.

To maximize the likelihood of amplifying regions susceptible of being differentially methylated, primers containing at least one CpG dinucleotide in their 3' end are recommended. The rationale in both methods is that the methylated sequences, which are protected from restriction, will amplify in both libraries, whereas sequences containing unmethylated *Hpa*II (in MS-AP-PCR method) or *Bst*UI (in MSFR) sites are cleaved and will not yield any product in the methylation-dependent library. MS-AP-PCR includes an additional methylation-independent library generated with a mix of *Rsa*I and *Msp*I to discern whether the fragments amplified in both the *Rsa*I methylation-independent and the *Rsa*I-*Hpa*II methylation-dependent libraries contain internal *Hpa*II sites. The radioactive MS-AP-PCR and MSFR PCR products are resolved on high-resolution polyacrylamide gels under denaturing (MS-AP-PCR) or non-denaturing (MSRF) conditions. Comparison of methylation-dependent fingerprints from two samples, for instance a tumor and the surrounding normal tissue, reveals methylation differences. In both methods, hypermethylation is detected by increased intensity, whereas hypomethylation result in

decreased intensity of the fingerprint bands. In principle, changes in intensity of the fingerprints bands from the methylation-dependent libraries can arise also from copy number alterations. The nature of the alteration can be ascertained by comparing the fingerprints from the methylation-independent libraries. Since these libraries are generated with methylation-insensitive restriction enzymes, changes in band intensity in their fingerprints exclusively result from copy number alterations.

MSFR and MS-AP-PCR offered several advantages over previously published methods. Since the original sample is amplified by PCR, only a small amount of genomic DNA is required (100ng-1μg). Also, they provide a direct way to determine whether the change in intensity of a particular band reflects copy number and/or methylation changes. However, the number of *loci* that can be interrogated with these technologies is limited, especially with MSFR, and even when 4 to 8 sets of arbitrary primers are run on each gel, only a small number of candidates (30–40) can be identified in one autoradiography [102]. MS-AP-PCR, and to a lesser extent MSFR, have been widely used to identify aberrantly methylated genes in human cancers [111–127]. Specifically, since its implementation, MSFR has been instrumental in the detection of hypermethylation in the 5' CpG island of genes, such as EDNRB [117], PMP24 [119], ZHX2 [122], as well as hypomethylation of a repetitive element SATR-1 [120] in several human cancers.

Peter A Jones and colleagues developed MS-AP-PCR by screening genomic DNA from colon primary tumors, cell lines and normal tissues for methylation alterations [111]. They detected hypermethylation of a CpG island in the 5' end of the gene TPEF (transmembrane protein containing epidermal growth factor and follistatin domains) in both colon tumors and cell lines, which was shown to be associated with a reduction in expression [114]. In another study, a CpG-rich region in exon 5 of PAX6, which is a highly conserved transcription regulatory factor involved in embryogenesis, was found to be hypermethylated in colon and bladder cancer [115].

3.c. Amplification of Inter Methylated Sites (AIMS)

In 2002, Frigola and colleagues published an epigenetic fingerprinting method that they named Amplification of Inter Methylated Sites (AIMS, Figure 5) [128]. AIMS is a modified version of the methylated CpG island amplification (MCA) method [129], based on the differential cleavage of isoschizomers with distinct methylation sensitivity *Sma*I and *Xma*I. Both enzymes recognize the CC**C**GG octamer, but whereas *Sma*I activity is blocked by methylation of the central CpG site (in bold type), *Xma*I is methylation-insensitive. These isoschizomers also differ in the type of DNA ends generated after digestion. While *Sma*I generates blunt ends, *Xma*I generates 4-nt 5' protruding ends. In AIMS, the genomic DNA is first treated with *Sma*I, which cleaves all the unmethylated CpG sites leaving blunt ended DNA molecules. Then, the DNA is treated with *Xma*I, which cuts all the remaining methylated sites producing cohesive ends. Specific adaptors are ligated to the cohesive ends of the digested genomic DNA. The ligated sequences amplified by PCR using adaptor-specific primers extended at the 3' end with two to four arbitrarily chosen nucleotidic residues to reduce the complexity of the product. This method amplifies DNA sequences that have two close methylated *Sma*I sites and show homology to the nucleotides extended at the 3' end of the primer. Lack of methylation at either site will allow the digestion by *Sma*I, leaving blunt ends not compatible to the adaptor and therefore preventing amplification. Different fingerprints can be generated from the same sample by modifying the arbitrarily chosen 3' end of the amplification primer. After PCR, the amplicons are resolved in denaturing polyacrylamide-sequencing gels generating fingerprints that consist of multiple anonymous bands, ranging in size from about 200 to 1200 bp, representing DNA sequences flanked by two methylated sites. Individual bands can be excised from the gel,

reamplified using the same fingerprinting primer and characterized by sequencing [128,130,131].

AIMS is suitable to compare large numbers of samples and the simultaneous identification of hypomethylation and hypermethylation events. Hypomethylation is visualized as the loss of the fingerprint band in the tumor sample compared to its normal counterpart and hypermethylation is detected as the appearance of a new band in the tumor specimen. The method is especially powerful in detecting hypermethylation. Dilution experiments indicate that the technique is able to detect hypermethylated sequences even if they are present in <1% of the cells [128]. It detects hypomethylation as well, however the contamination of normal cells in the tumor samples limits its sensitivity.

Applications of AIMS to investigate epigenetic changes in cancer include the identification of recurrent hypermethylation associated with gene silencing [132,133], screening for both hypomethylation and hypermethylation in cancer cell lines with altered DNA methylation function [130,131]; and the genome-wide estimation of abnormal DNA methylation in cancers [132,133]. It has also been applied to identify epigenetic differences arisen during the lifetime of monozygotic twins [134]. In 2005, Frigola et al. employed AIMS to compare the levels of hypermethylation and hypomethylation alterations in colorectal carcinomas and adenomas. They found that the premalignant lesions already attained high levels of hypomethylation, but not hypermethylation, which suggested that this factor might play a key role in conferring the malignant potential since early stages. Also, they found that hypomethylation, but not hypermethylation, associated to poor prognosis [132]. Later, and based on their findings with AIMS, they described a novel mechanism in carcinogenesis involving the common repression of the entire 4-Mb band of chromosome 2q.14.2, by a coordinate long-range epigenetic gene silencing involving DNA hypermethylation and global methylation of histone H3K9 [133]. Remarkably, they found the transcriptional silencing of not only the genes undergoing promoter hypermethylation, but also the unmethylated neighboring genes, which also exhibited H3K9 methylation. Their work changed the view about DNA hypermethylation in cancer, which was previously envisaged as a local event silencing discrete genes, demonstrating that epigenetic silencing can span large chromosomal regions, affecting DNA-methylated and neighboring unmethylated genes that result coordinately suppressed by global changes in their histone methylation profiles.

3.d. Methylation Sensitive-Amplified Fragment Length Polymorphism (MS-AFLP)

The *Not*I-*Mse*I methylation sensitive-amplified fragment length polymorphism method (MS-AFLP) is also a DNA fingerprinting technology that allows for the simultaneous genome-wide detection of DNA hypermethylation and hypomethylation in tumor samples compared with normal tissue samples [135]. MS-AFLP is based on a previously published method, AFLP [136], but the restriction enzyme *EcoR*I was replaced with the methylation-sensitive restriction endonuclease *Not*I. This enzyme recognizes the **GCGGCCGC** sequence, which is frequent inside or in the proximity of CpG islands. The DNA methylation statuses of the two CpG sites in the octamer sequence are analyzed at hundreds of *Not*I sites in the genome by using this gel-electrophoresis based DNA fingerprinting technique. Epigenetic alterations, both hypo- and hypermethylation, in tumor tissue DNA are detected by comparing band intensities between normal and tumor tissue DNA fingerprints (Figure 6).

In short, genomic DNA is digested with the methylation-sensitive rare-cutter *Not*I and the methylation-insensitive frequent-cutter *Mse*I. All the *Mse*I sites are cleaved, and the *Mse*I ends are ligated to specific *Mse*I adaptors. The *Not*I sites are cleaved only when both of the cytosines of the two CpG dinucleotides in the octamer *NotI* recognition sequence are unmethylated. The *Not*I ends are ligated to specific *Not*I adaptors. When either one or both of the two cytosines are methylated, the *Not*I site is protected from cleavage and, therefore,

no adaptors are ligated. The fragment library is subsequently amplified by PCR with a ^{32}P-labeled primer complementary to the *Not*I adaptors, and a non-labeled primer complementary to the *Mse*I adaptors. Different combinations of primers differing in their 3' ends can be employed to increase the number of *Not*I sites analyzed. The PCR products are then resolved in standard sequencing gels and autoradiographied. Three different types of fragments are amplified: *Mse*I-*Mse*I, *Mse*I-*Not*I and *Not*I-*Not*I. The *Mse*I-*Mse*I fragments do not provide any information about DNA methylation, but they are not detected in the autoradiography since only the *Not*I-primer is labeled. The amplified products are DNA fragments in the range of 50–1,000bp, representing random anonymous genomic sequences.

MS-AFLP is highly reproducible and a relatively large number of bands (~100) are amplified with a high ratio of band signal/background noise [56]. Additionally, MS-AFLP requires little amount of template DNA. As little as five nanograms are sufficient for one MS-AFLP experiment with one pair of primers. In contrast with MSFR and MS-AP-PCR, bands in MS-AFLP fingerprints originate from the unmethylated (cleaved) *Not*I sites and therefore hypermethylation results in decreased intensity while hypomethylations results in increased intensity of the bands. DNA fragments exhibiting alterations can be directly cloned from fingerprint bands by amplification of gel-eluted DNA with the same pair of primers used for the fingerprint [135]. Several fingerprints generated with different primer combinations can be analyzed in parallel to maximize de number of *loci* per gel. As some MS-AFLP bands are common to several primer combinations, this provides an internal control for the alterations. Identification of consistent changes in a particular type of cancer can be facilitated by the analysis of multiple samples in the same gels.

Fluorescent-MS-AFLP (FL-MS-AFLP) is a non-radioactive adaptation of this technique in which fluorescently labeled *Not*I primers are employed. In FL-MS-AFLP the differences in methylation levels between normal and tumor samples are visualized by the difference in signal intensities in the electropherogram. MS-AFLP products were labeled with a fluorescent primer, TAMRA, and analyzed the changes in DNA methylation in cancer using an ABI Prism 377 automatic DNA Sequencer [135]. In a recent paper Kageyama and colleagues further developed the FL-MS-AFLP method to analyze the methylation levels of blood DNA from gastric cancer patients, as well as methylation differences between gastric tumor samples and adjacent normal tissue samples [137]. They labeled the *Not*I primer with the fluorescent dye FITC, and measured fluorescence intensity using the DSQ-2000 automatic DNA sequencer. They quantitatively evaluated the methylation status of more than 350 *Not*I sites in the human genome per run by FL-MS-AFLP. The use of fluorescence for MS-AFLP has not only increased the number of bands that can be analyzed, but also made the analysis of longer sequences (up to 1000 bp) possible. FL-MS-AFLP is safer than MS-AFLP because it does not require the use of radioactivity, which may be an important factor in clinical settings. In addition, multiple dyes with different absorption and emission range can be utilized [135]. Furthermore, as opposed to the short half-life of [32]P (14.3 days), fluorescently labeled primers have a longer shelf life if they are kept in the dark. However, using fluorescent tags for *Not*I-*Mse*I MS-AFLP has also disadvantages. The critical one is that the method does not allow direct cloning of DNA fragments after the identification of alterations.

MS-AFLP has also been applied to a DNA microarray hybridization format (DNA Microarray MS-AFLP) [138]. In short, in the microarray hybridization method PCR products are labeled with CY5 and CY3 (using the Klenow fragment and CY5-dCTP or CY3-dCTP), the two probes are mixed, and unincorporated dNTPs are removed. The probes are then used to hybridize DNA microarrays on glass slides in the CGH manner [77,83,139]. Fluorescent signal associated with each target spot is measured using a fluorescence scanner.

The fluorescence intensity correlates with the number of unmethylated *Not*I sites cleaved by the enzyme.

In our study describing the method, we performed a total of 30 (15x2 reciprocal labeling) DNA Microarray MS-AFLP hybridization experiments using genomic DNA from two breast and three prostate cancer cell lines in all the pairwise combinations. We also performed hundreds of Southern hybridization experiments to calculate the rates of false positives and negatives, and obtained the values of 2.6% and 3.6%, respectively. We also determined the sensitivity (78.5%) and specificity (97.1%) of the DNA Microarray MS-AFLP method. In the original gel electrophoresis-based MS-AFLP it is often difficult to quantify the signal due to noise from the bands in the background. An advantage with the DNA Microarray MS-AFLP method is that it is more quantitative, and even differences in copy number could also be detected, in addition to DNA methylation alterations. Furthermore, as in the FL-MS-AFLP, no radioisotopes are used. On the other hand, in the microarray format only two samples can be compared at a time. Another drawback is its limited use as a gene discovery tool because only the sequences on the microarrays can be analyzed. Nevertheless, gene identification is straightforward as each dot in the microarray is represented by a single sequence. This feature makes DNA Microarray MS-AFLP a valid method for DNA methylation analysis of larger number of *Not*I sites in a larger scale of analysis [138].

3.e. MS-AFLP and the absence of CpG island methylator phenotype in colorectal cancer

As previously mentioned, *Not*I sites are uncommon in the genome. Just 9853 *Not*I sites are found in the latest release (GRCh37) of the human genome sequence from the Genome Reference Consortium. Most of them, however, are located within CpG islands [140] and frequently associated with the 5' region of genes, but not in the highly repetitive elements Alu and LINEs. Hence, analysis of the methylation of *Not*I sites by MS-AFLP fingerprinting provides an unbiased estimation of the methylation status of CpG islands throughout the genome. This unbiased approach was key to demonstrate that CpG island hypermethylation in gastrointestinal cancers follows a gradual distribution [56,50], challenging the hypothesis of the existence of a cancer-specific CpG island methylator phenotype (CIMP). This hypothesis postulates the existence of two distinctive types of tumors: with and without a methylator phenotype that causes the concordant hypermethylation of some CpG islands. Which CpG islands and how many of them should be found methylated to unambiguously classify a particular tumor into CIMP+ or CIMP- have been a moving target in constant redefinition since the inception of the concept [141,129].

A recent study aimed to characterize *loci* markers for CIMP demonstrated that the original "classic" panel indeed exhibited a unimodal distribution and a completely redefined panel of CIMP markers was proposed [142]. It is still unexplained, however, what distinguishes these CpG islands that are targets and markers for CIMP from the many hundreds and thousands of CpG islands found also methylated in each one of the CIMP-negative tumors. Unquestionably, some CpG islands are more frequently methylated than others in tumors. It remains to be established whether the preferential hypermethylation of a subgroup of CGIs reflects an underlying mechanism targeting a particular subset of CGIs or is a mere consequence of the selective pressure over a stochastic widespread hypermethylation phenomenon, if the concomitant silencing of the affected genes favors the clonal expansion of the malignant cells. It has been over a decade now since the proposal of the CIMP hypothesis, and despite numerous CIMP publications, including editorials reassuringly confirmatory of the hypothesis reiterated every couple of years [143,144], we are still waiting to know what the methylator genes are [145]. This is in contrast with the identification and isolation of the first MMR mutator genes soon after the initial discovery of MSI (See Figure 7). The CIMP concept has been a remarkable example of the chase of

the wild geese and a red herring that for over a decade has confused and continues to confuse an important aspect of cancer epigenetics.

In conclusion, we are still essentially in the dark as the cause of the aberrant hypermethylation common in cancer. Recent growing evidence suggests a role of the Trithorax (Trx) and Polycomb repressor complexes (PRCs), the two main cell memory systems involved in the maintenance of a stem cell state [146–148]. It has been found that PRC-target genes are up to 12-fold more likely to have cancer-specific promoter DNA hypermethylation than non-targets [148] and that methylation on Lys27 of histone H3, a modification mediated by the Polycomb repressor complex 2 (PRC2), pre-marks genes for *de novo* methylation in cancer [147]. These findings support a stem cell origin of cancer in which reversible gene repression mediated by PRCs is replaced by permanent silencing (DNA methylation), locking the cell into a perpetual state of self-renewal and thereby predisposing to subsequent malignant transformation [148]. According to this hypothesis, the abnormal crosstalk between the Polycomb proteins and the DNA methylation machinery would be mechanistically underlying the aberrant promoter methylation of some genes, although only a minority of all the genes frequently hypermethylated in cancers seem to be PRCs targets. A notable exception is hMLH1, which is hypermethylated in the majority of sporadic MSI cases but has not been categorized as a PRCs target [149–151].

3.f. MS-AFLP and the association between DNA hypomethylation, aging and genomic damage

The results obtained by MS-AFLP on gastrointestinal cancers showed that not only hyper-but also hypomethylation accumulates gradually and in an age-associated fashion [50]. In addition, DNA hypomethylation detected by MS-AFLP strongly associated with the degree of aneuploidy estimated by AP-PCR. These findings led to the proposal of a novel "wear and tear" model linking aging and cancer through DNA hypomethylation. This pathway would explain the accumulation of genetic alterations in a certain fraction of gastrointestinal cancers not caused by an underlying microsatellite mismatch repair defect and subsequent mutator phenotype, or "spontaneous" mutations in cancer genes such as APC and p53. In this model, genome-wide DNA demethylation of CpG sites occurs as a result of a gradual and accumulative age-associated failure to preserve methylation replication fidelity, without the involvement of any particular genetic or epigenetic defect. When certain regions of the genome are hit, such as pericentromeric repetitive sequences, normally heavily methylated at birth, the probability of mitotic errors increases, leading to the accumulation of genomic damage and eventually to cancer [50].

4. Concluding remarks

In this review we have presented several fingerprinting techniques that have been applied to the study of genetic and epigenetic alterations in human colorectal cancers, and some of the discoveries that they made possible (Figure 7). These methods have now been surpassed in sensitivity and throughput by modern technologies, especially after the widespread commercialization of microarrays for both genetic and epigenetic alterations and, more recently, the development of massively parallel sequencing platforms [26–28]. As a discovery tool, the use of most of the fingerprinting methods presented in this review will be soon relegated by their more powerful successors. Some of them, however, can have a place in the day-to-day laboratory since they still offer certain advantages over the more sophisticated but expensive new methods. Its simplicity and affordability make them a reasonable option for the initial screenings of a large set of samples that, in a second phase, can be classified and analyzed in more detail using more powerful technologies.

AP-PCR was developed contemporarily with CGH and RDA. All of these approaches, designed to detect and estimate moderate changes in genome copy number, underwent a process of convergent evolution that after taking advantage of the information obtained from the human genome project, ended in a winner array CGH format with long synthetic oligos that are commonly used not only for the analysis of somatic alterations associated to the development and progression of sporadic cancer, but also for the study of germline copy number variations (CNV) and their impact in genome-wide association studies (GWAS) for cancer susceptibility.

The analysis of DNA methylation alterations by MS-AFLP in the *Not*I restriction site yields information of the methylation alterations occurring not only in CpG islands but also in CpG islands shores, which have been proven to be an important target of DNA methylation alterations [31]. This unforeseen lucky feature allowed to detect the association between aging and both hypermethylation and hypomethylation in gastrointestinal cancer and revitalized the interest on DNA hypomethylation as a relevant driving force in oncogenesis [50], which was overshadowed by the predominant focus on gene promoter hypermethylation.

It is worthy to reflect on how the use of relatively simple DNA fingerprinting techniques permitted original discoveries because of the panoramic views that they provided of the somatic alterations occurring in the colon cancer cell genome. For instance, due to the ability to study many samples in a few experiments, AP-PCR made possible a first insightful estimation of the high prevalence of altered 8q24 region pointing to a major role of c-Myc in human cancer. Also AP-PCR, due to its unbiased nature, allowed the extrapolation from the mobility shifts of a few fingerprint bands to the huge number of somatic clonal microsatellite mutations diagnostic of a genetic defect leading to a profound genomic instability. On the other hand, MS-AFLP fingerprinting also provided a panoramic view of the gradual distribution of hypermethyaltion alterations in colon cancers thus making impossible the distinction of tumors with and without a methylator phenotype. Thus, two DNA fingerprinting approaches, AP-PCR and MS-AFLP, directed to the analysis of somatic genetic and epigenetic alterations respectively, were instrumental for the discovery of the existence of a mutator phenotype and the non-existence of a methylator phenotype.

List of abbreviations

AIMS	Amplification of Intermethylated Sites
AP-PCR	Arbitrarily Primed PCR
CGH	Comparative Genomic Hybridization
CGI	CpG Island
CHAPA	Chromosomal Hybridization of AP-PCR Amplicons
CHARM	Comprehensive High-throughput Array-based Relative Methylation
CIMP	CpG Island Methylator Phenotype
CNV	Copy Number Variation
COBRA	Combined Bisulfite and Restriction Analysis
FL-MS-AFLP	Fluorescence MS-AFLP
GDF	Genomic Damage Fraction
GWAS	Genome-Wide Association Study

HNPCC	Human Nonpolyposis Colorectal Cancer
LOH	Loss of Heterozygosity
MCA	Methylated CpG-Island Amplification
MMP	Microsatellite Mutator Phenotype
MS-AFLP	Methylation Sensitive Amplified Fragment Length Polymorphism
MS-AP-PCR	Methylation-Sensitive AP-PCR
MSI	Microsatellite Instability/Instable
MSP	Methylation-Sensitive PCR
MSRF	Methylation-Sensitive Restriction Fiongerprinting
MSS	Microsatellite Stability/Stable
PCR	Polymerase Chain Reaction
PRC	Polycomb Repressor Complex
RAPD	Random Amplification of Polymorphic DNA
RDA	Representation Differential Analysis
RLGS	Restriction Landmark Genomic Scanning
RLGS-M	Restriction Landmark Genomic Scanning for Methylation
SHARP	Simultaneous Hybridization of AP-PCR Products

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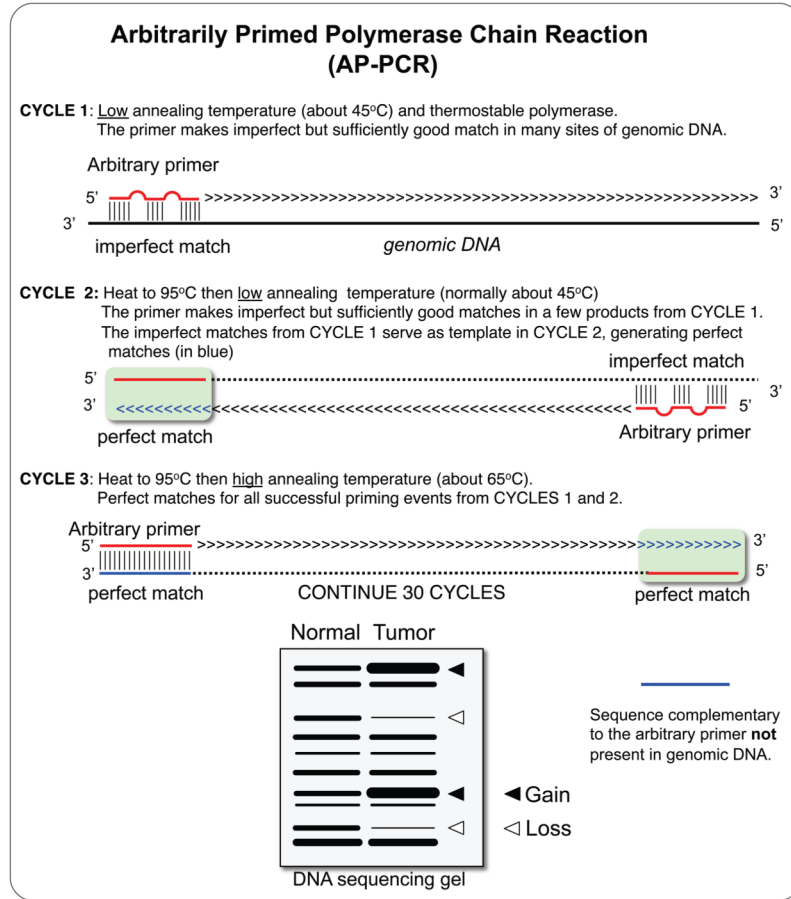
Figure 1.
The first cycles of amplification at low temperature allow the annealing of an arbitrary primer (in red) to multiple sites along the genome, and the amplification of sequences within two close annealing sites. The originally imperfect-match sequences will be transformed to perfect matches in the second round of amplification (in blue), where the DNA synthesized in the first round serves as template. DNA regions flanked by two primer-annealing sites are further amplified in the subsequent high-annealing temperature cycles, generating a reproducible fingerprinting pattern. Copy number alterations, both gains and losses, can be detected by comparing AP-PCR fingerprints from normal and tumor samples.
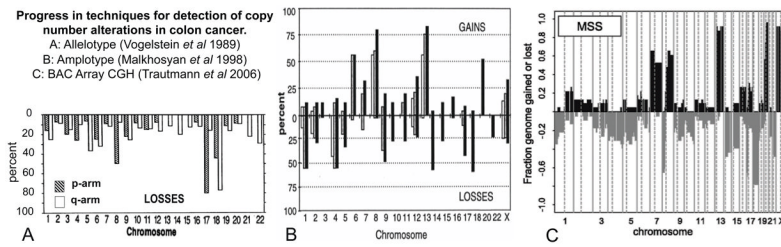
Figure 2.
Comparison of the copy number alterations in colorectal cancers detected by: (A) Allelotyping, (B) AP-PCR Amplotyping and (C) Array CGH. Allelotyping detects loss of heterozygosis by individually analyzing polymorphic minisatellite sequences in every chromosome arm. The use of AP-PCR made possible the detection of gains and losses in multiple genomic regions in a single PCR reaction. The results obtained with these two techniques were later confirmed by the higher resolution CGH arrays.
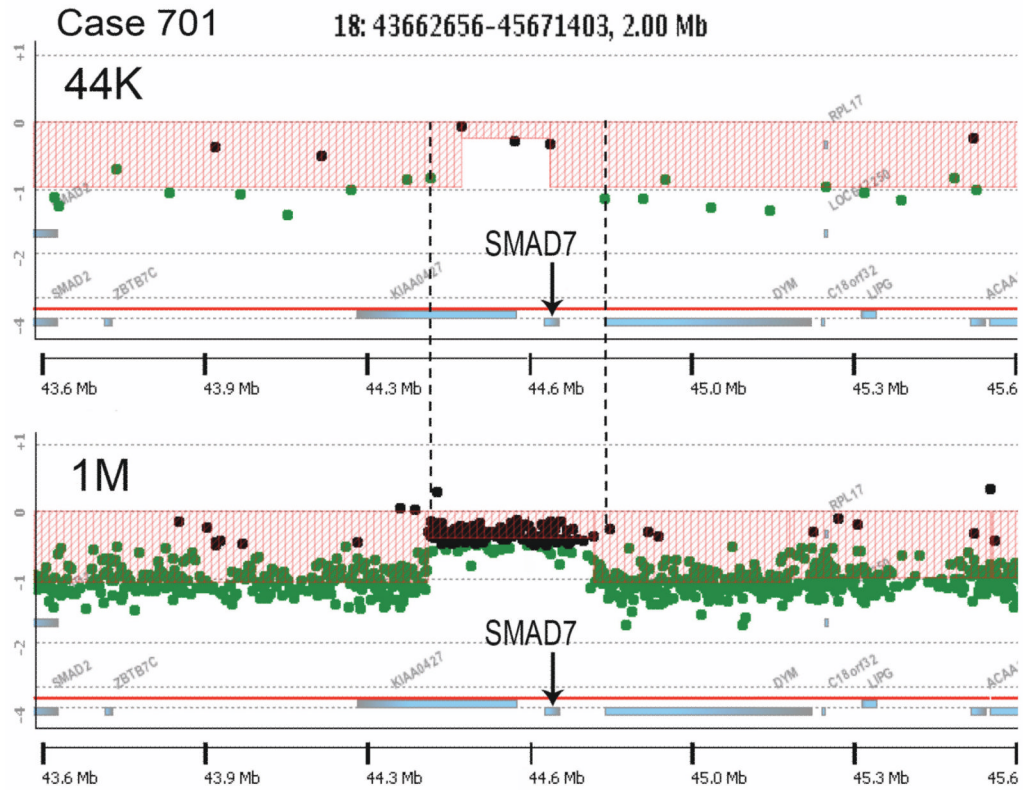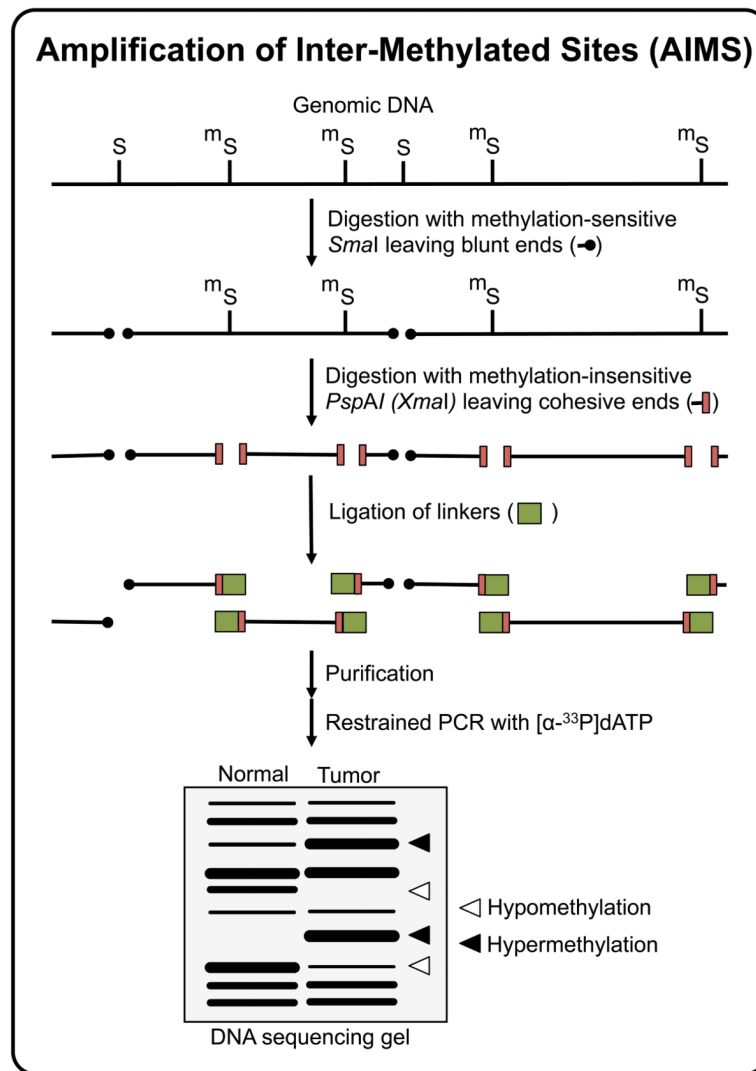
Figure 3.
Array CGH analysis with the Agilent 44K (top) and 1M (bottom) arrays of a 2Mb region of chromosome 18q21 from a colon cancer. Filled circles represent probes with no changes (black) and probes lost (green). 44K array analysis revealed a region close to SMAD7 that 'escaped' from loss (indent in the red area). The vertical dashed lines indicate the estimated maximum size of the region (~337Kb), determined by the closest neighbor probes that are lost (in green). The 1M array analysis confirmed that this ~300Kb region, containing the complete gene SMAD7, had escaped from the loss that affected the surrounding area.

Figure 4.
Methylation-Sensitive Arbitrarily-Primed PCR (left) and Methylation-Sensitive Restriction Fingerprinting (right) rely on the amplification of DNA libraries generated by methylation-sensitive and methylation-insensitive restriction enzymes. MS-AP-PCR libraries are generated by digestion with *Rsa*I (GTAC) alone or in combination with *Hpa*II (CCGG, methylation-sensitive) or *Msp*I (CCGG, methylation-insensitive). After digestion, PCR amplification of the DNA fragments libraries is performed with 20-mer primers following the principle of AP-PCR. MSRF libraries are generated by treatment of the genomic DNA with MseI (TTAA, methylation-insensitive) alone or in combination with BstUI (CGCG, methylation-sensitive), and the amplification is performed with a pair of shorter primers, following the RAPD protocol. In both techniques, changes in the intensity of the fingerprinting bands reflect changes in methylation of the methylation-sensitive enzyme recognition sites.

Figure 5.
Amplification of Intermethylated Sites (AIMS) technique. Non-methylated *Sma*I recognition sites (CCCGGG) are first digested using the methylation-sensitive *Sma*I restriction endonuclease, leaving blunt ends. A second digestion is then performed using the methylation-insensitive isoschizomer *Xma*I, leaving 5' protruding ends to which compatible adaptors are ligated. The DNA fragments in the range of ~200 to ~2000 bp and flanked by two ligated adaptors are subsequently amplified using primers that anneal to the adaptor sequence plus the restriction sequence and, to limit the number of amplified fragments, 1 or more arbitrarily chosen additional nucleotides. The increase of intensity of a particular AIMS fingerprint band reflects the hypermethylation of the flanking *Sma*I sites in the original sequence, while a decrease of intensity indicates hypomethylation.
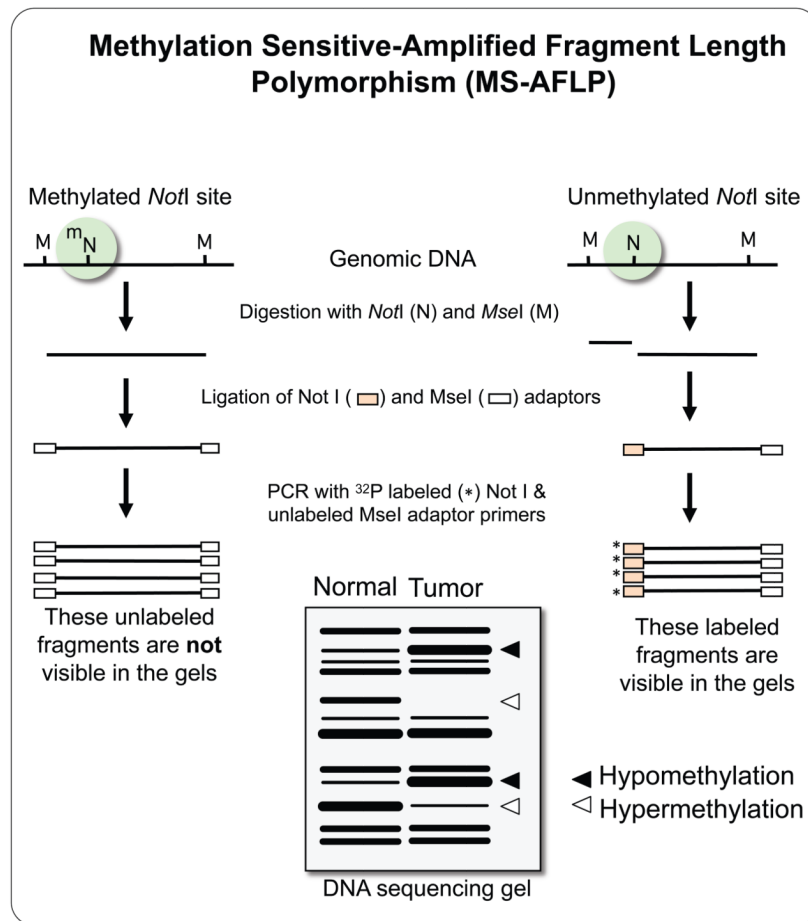
Figure 6.
Methylation-Sensitive Amplification Fragment Length Polymorphism (MS-AFLP).
Genomic DNA is treated with a mix of *Mse*I (TTAA, methylation-insensitive) and *Not*I
(GCGGCCGC, methylation-sensitive) restriction endonucleases. *Mse*I cuts all TTAA sites
leaving 5' protruding ends. *Not*I digests the unmethylated but not the methylated
GCGGCCGC, leaving 5' protruding ends. Compatible adaptors are ligated to the *Not*I and
*Mse*I cohesive ends, and the resulting DNA fragment library is amplified using primers that
anneal on these adaptors. To limit the number of fragments amplified during the PCR, one
or two arbitrarily chosen nucleotides are added to the sequence of the *Mse*I primer. Three
different types of fragments are amplified, methylation-independent *Mse*I-*Mse*I fragments,
and methylation-dependent *Mse*I-*Not*I and *Not*I-*Not*I fragments. The primer that anneals on
the *Not*I adapter is labeled, so only the methylation-dependent fragments are visualized after
autoradiography of the fingerprinting gels. Differences in MS-AFLP fingerprints band
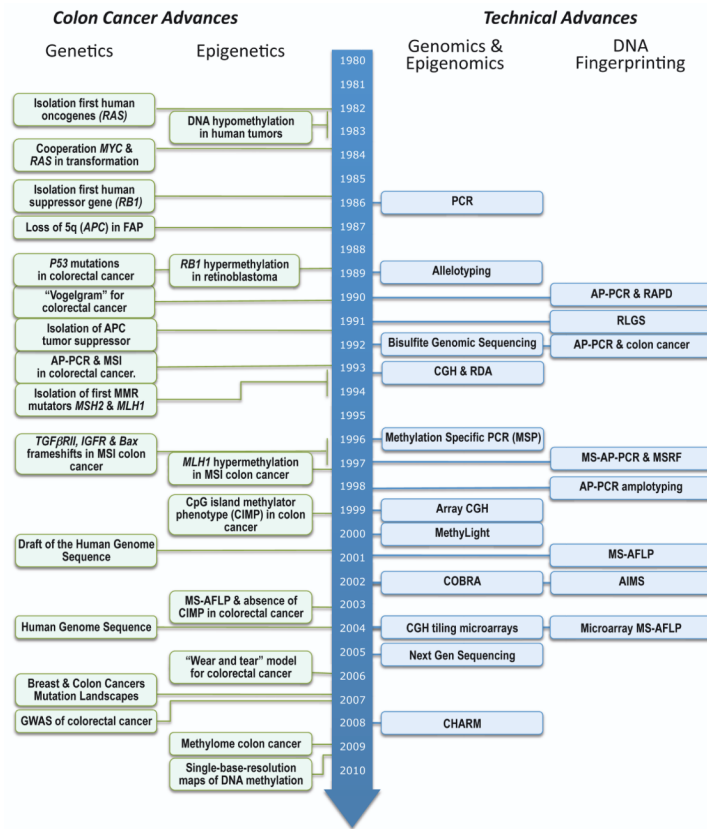intensity between normal tissue and tumor reflect changes in DNA methylation.

Figure 7.
Chronological diagram of some of the relevant advances in colorectal cancer research and the technical advances that made some of them possible.

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Table 1

Comparative features of DNA fingerprinting technologies

Technique (year)	Type[a]	Basis[b]	Resolution[c]	Detection[d]	Amount[e]	Throughput[f]	Cost[g]
RAPD (1990)	CNA	Arbitrary primer PCR amplification	Native electrophoresis	Ethidium bromide staining	>10ng	L: ~20–50 S: ~20	$1–$2
AP-PCR (1990)	CNA	Arbitrary primer PCR amplification	Denaturing electrophoresis	Autoradiography, silver staining or fluorescence	>10ng	L: ~50–100 S: ~40	$2–$3
CHAPA (2001)	CNA	Arbitrary primer PCR amplification	Microarray slides	Fluorescence	>100ng	L: >1,000 S: 2	~$100–$200
RLGS-M (1993)	MET	Digestion with *NotI* or *AscI*	Bidimensional electrophoresis	Autoradiography	>1µg	L: ~1,000 S: 1	~$20
MS-AP-PCR (1997)	MET	Arbitrary primer PCR amplification after digestion with *HpaII* and *RsaI*	Denaturing electrophoresis	Autoradiography	>100ng*	L: ~50 S: ~40	$3–$4
MSRF (1997)	MET	Arbitrary primer PCR amplification after digestion with *BstUI* and *MseI*	Native electrophoresis	Autoradiography	>100ng*	L: ~50 S: ~40	$3–$4
AIMS (2002)	MET	Adaptor-mediated PCR amplification after digestion with *SmaI* and *XmaI*	Denaturing electrophoresis	Autoradiography	>100ng*	L: ~50–100 S: ~20	$3–$5
MS-AFLP (2001)	MET	Adaptor-mediated PCR amplification after digestion with *NotI* and *MseI*	Denaturing electrophoresis	Autoradiography or fluorescence	>100ng*	L: ~50–100 S: ~20	$3–$5
Array-MS-AFLP (2004)	MET CNA	Adaptor-mediated PCR amplification after digestion with *NotI* and *MseI*	Microarray slides	Fluorescence	>1µg	L: ~10,000[h] S: 2	~$100–$200
OTHER NON-DNA FINGERPRINTING TECHNIQUES[i]							
Agilent CGH arrays	CNA	Hybridization of labeled genomic DNA fragments	Microarray slides	Fluorescence	>500ng	L: 10^4–10^6 S: 1–8	~$200–$500
Illumina Goldengate	CNA MET	Hybridization or single nucleotide extension of labeled DNA fragments	Microarray slides	Fluorescence	200ng–1µg	L: 10^4–10^6 S: 1–12	~$200–$500
Affimetrix arrays	CNA	Hybridization of labeled genomic DNA fragments	Microarray slides	Fluorescence	>500ng	L: 10^4–10^6 S: 1–96	~$200–$500
CHARM	MET	*McrBC*-fractionated DNA libraries	Microarray slides	Fluorescence	>3µg	L: 44,000 S: 2	~$200

[a] CNA: copy number alterations. MET: DNA methylation alterations.

[b] Key feature that underlies the method is listed.

[c] Method for resolving DNA sequences. Except RAPD that may use agarose gel electrophoresis, the rest of the fingerprinting techniques use polyacrylamide or derivatives (i.e., sequagel) gel electrophoresis.

[d] Method of detection of the DNA sequences.

[e]
Values indicate amount of genomic DNA for the initial digestions. Amounts of sample loaded in the electrophesis for the fingerprinting techniques (*) are less (5–10ng).

[f]
L: loci per sample; S: number of samples per gel. The values given for the fingerprinting techniques

[g]
The cost in US dollars per DNA sample is given as an approximation in a wide range (i.e., depending on whether the analysis is by autoradiography or fluorescence). Costs for fingerprinting techniques include the costs of electrophoresis but not the costs of sample DNA preparation. Costs can be calculated per Mb of genome covered by the techniques, by increasing the number of experiments with different primers. For instance, a map by AP-PCR of random genome sites at a 10Mb resolution for copy number alterations that could provide panoramic views of the degree of disruption of the cancer genome would cost around 6 (primers) X $2–3 = ~$15 per sample, and a map by MS-AFLP of *NoI* sites at ~10Mb resolution for DNA methylation alterations would cost around 6 (primers) X $3–$5 = ~$25. To reach 1Mb resolution would cost 10 times more, without counting the labor. More than 1Mb resolution would be impractical for DNA fingerprinting technologies.

[h]
The current limitation is imposed by the number of *NoI* sites in the genome, not by the technique itself.

[i]
The list is not exhaustive, and is only intended to contrast some differences in throughput and cost for comparative purposes. Massive sequencing approaches (i.e., Illumina Solexa Technology, Roche 454 or Applied Biosystems SOLiD) are not included because it is difficult to calculate the cost per sample depending on the extent of the analysis.