

## Genomic determinants of prognosis in colorectal cancer

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### Abstract

Colorectal cancer progression is characterized by the sequential acquisition of multiple genetic aberrations. Insights into the biology of cancer cell and the development of novel methodologies has open a new frontier in the search of independent molecular factors to better predict outcome. Besides the generation of a large list of candidate markers, their applicability in routine clinical settings has been hindered by the heterogeneity of the disease. The analysis of cumulated genetic damage offers a more comprehensive measure of the cancer cell's genomic disruption and appears as a gauge of malignant potential. The prognostic application of different determinants of genomic damage is reviewed.

**Keywords** tumor progression, genomic instability, genetic alterations, prognostic marker

### 1. Accumulation of alterations in colorectal cancer. Genetic instability

Colorectal cancer is one of the best studied systems of multistage human carcinogenesis. Multiple genetic aberrations are required for tumor initiation and progression [1]. While alterations in certain genes appear to occur in a preferred order, the accumulation of genetic aberrations, rather than the sequence, appears to drive the malignant transformation [1]. In addition to the mutation in certain genes, chromosomal aberrations in the form of aneuploidy and structural rearrangements are early markers in colorectal carcinogenesis [2, 3]. Contrary to hematological malignancies, in which specific chromosome alterations frequently characterize a pathology, epithelial cancers are characterized by a large number of chromosome alterations, none of which appears to be specific [4]. Recurrent chromosomal

abnormalities often clustered in association patterns are also observed in most colorectal cancers [4]. Alternatively, a subset of colorectal tumors with few or none chromosomal alterations are characterized by ubiquitous somatic mutations at repeated sequences [5-7]. These tumors represent a distinctive pathway of tumor progression in which defects in the DNA mismatch repair machinery underlie the genetic instability expressed as an exacerbated microsatellite instability (MSI) [8]. Tumors exhibiting MSI constitute the first incontestable example of a mutator phenotype as the engine of tumor progression [9, 10].

Nowell, Foulds and Loeb studies pointed to a dynamic tumor model in which genetic instability would play a principal role in the generation of heterogeneity: genetic instability would increase the mutation rate and would facilitate tumor evolution from the early stages of tumor progression (reviewed in [11]). By analogy to microsatellite instability, Lengauer and coworkers have postulated the presence of chromosomal instability (CIN) in most sporadic colon cancers [12, 13]. Initially, their definition of chromosomal instability was limited to numerical abnormalities in colon cancer cell lines, although other studies have also stressed the prevalence of gross structural alterations in both cell lines and primary tumors [4, 14-20]. While chromosomal instability as the engine of tumor progression appears as an attractive hypothesis sustained by experimental and empirical data [13], the mechanisms underlying this type of instability in sporadic tumors are unknown. It has also been suggested that genetic instability is not necessary for the evolution of cancer cells as tumors can accumulate multiple genetic alterations in the absence of a mechanism that increases the frequency of mutations and only under the pressure of selection [21-23]. This issue is still a matter of debate and part of the problem in resolving it lies on the difficulty of analyzing and interpreting genomic damage in tumors.

## **2. Rationale to investigate genetic determinants as prognostic markers**

The extent of tumor bowel wall infiltration and lymph node metastases, both included in Dukes' stage and TNM classification systems, are the most important prognostic factors in colorectal cancer [24, 25]. Nevertheless, traditional morphologic criteria based upon pathologist's evaluation are accurate for predicting recurrence only in 50-75% of the patients with non metastatic invasive colon carcinoma. Therefore there is a need for additional, less subjective, independent factors to better predict outcome.

The advent of molecular biology techniques and the knowledge of molecular processes involved in tumorigenesis have raised expectations on the use of genetic markers in evaluating prognosis and diagnosis. Besides the large number of candidates of which an illustrative example is summarized in table 1, widespread applicability of molecular markers in routine clinical settings is still little bit more than a promise [26]. Multiple factors account for the poor impact that molecular biology has had in the clinical management of colorectal cancer patients, these include the wide heterogeneity in the spectrum of genetic alterations even in tumors with similar features and behavior, the heterogeneity in the manifestation of some alterations precluding the use of standardized strategies, the complexity of the pathways involved in tumorigenesis that prevents the obtention of a comprehensive picture of the deregulated processes [27, 28], and finally, intra-tumor heterogeneity [29], which results in a limited characterization of the molecular portrait.

**Table 1. Molecular markers associated to prognosis in colorectal cancer<sup>1</sup>**

Type of alteration	Molecular marker	Methods
Allelic imbalance / Chromosomal loss	17p, 18q, 3p, 4p, 8p	Analysis of LOH at specific polymorphic loci, CGH
Insertion/deletion in microsatellites	Microsatellite instability	Analysis of mobility shifts in microsatellite sequences
Point Mutation	p53, K-ras	DNA sequencing, SSCP
DNA methylation	p16	Methylation sensitive PCR
Gene amplification	MYC, SKI	Quantitative PCR, Southern blot
Overexpression	MYC, BCL2, Cyclin A, p27, PCNA, CD44	Quantitative RT-PCR, Immunohistochemistry
Underexpression	E-cadherin, beta-catenin	Quantitative RT-PCR, Immunohistochemistry

<sup>1</sup>This table is not an exhaustive list of markers with potential prognostic application. It only exemplifies different types of markers and some of the methods that are commonly used for the detection of each alteration. Abbreviations: CGH, comparative genomic hybridization; LOH, loss of heterozygosity; RT-PCR, reverse transcriptase PCR; SSCP, single strand conformation polymorphism.

### 3. Cumulated genomic damage as a prognostic indicator

The evidence that multiple chromosomal alterations accumulate through progression has prompted researchers to investigate the association between increased chromosomal disruption and malignant behavior as an alternative to the study of specific genetic markers. The heterogeneous nature of genetic alterations in cancer cells precludes a simple approach to their detection and characterization. Although a wide range of methodologies have been used to analyze genomic damage in tumors, little emphasis has been put on the comparison of results obtained from different approaches. Moreover, in an effort to find a functional meaning to the raw evidence of an alteration (for instance a loss of heterozygosity revealed by analysis of polymorphic markers), many authors have applied "flexible" interpretations to indirect observations. Therefore the nature and the role of genomic damage in the evolution of tumors remains largely unclear.

Genetic alterations are usually classified according to their nature [30], although the causes and functional consequences of similar alterations may be quite different. The type of cumulated damage analyzed depends on the methodology applied and direct comparison of cumulated genomic damage data obtained with different approaches is not feasible (reviewed in [31]). Because chromosomal aberrations are the most prevalent genetic change in colorectal cancer and a wide spectrum of techniques are available for the analysis of numerical and structural chromosomal alterations, most studies dealing with the impact of cumulated genomic damage refer to mutations detected at the chromosome level. A summary of different

techniques used to measure genomic damage and the types of genetic alterations they detect is shown in table 2.

**Table 2. Cumulated genetic alterations as prognostic markers in colorectal cancer**

Method	Measurement	Type of chromosomal alterations	References
Flow cytometry	Aneuploidy	Numerical alterations	[38, 40, 41, 84-90]
G banding cytogenetics	Cumulated chromosomal alterations	Numerical and gross (balanced and unbalanced) structural alterations	[43-45, 91]
CGH	Cumulated chromosomal alterations	Numerical and unbalanced gross structural alterations	[92]
Allelotyping	Fractional Allelic Loss (FAL)	Numerical and unbalanced structural alterations	[65-67]
DNA fingerprinting	Genomic Damage Fraction (GDF)	Numerical and unbalanced structural alterations	[42, 81]

### 3.1. Abnormal DNA content

The altered DNA content of tumor cells is due to numeric chromosomal alterations, either in the form of aneuploidy (a copy missing or three or more copies of one or several chromosomes) or polyploidy (more than two copies of the whole set of chromosomes). However, in general, the term ‘aneuploidy’ is used to describe an abnormal content of DNA. The DNA content may be easily determined by Flow Cytometry (reviewed in ref. [32]). The estimated limit of detection of the technique is usually 10% of variation in the cell’s DNA content; that is, aneuploidies of two or three chromosomes. A vast number of studies have investigated its utility as a prognostic factor in colorectal cancer (Table 2), although no consented conclusions have been reached to date [33-35]. The discrepancies may be attributed to different factors including the lack of standard methodology and criteria and variability in the type (frozen or paraffin-embedded) and quality of tissue analyzed among others. The main parameter considered is the DNA content of the most aneuploid population (DNA index, DI) as the index of aneuploidy of the tumor (reviewed in ref. [33]). Nevertheless, different studies have revealed a high variability in the extent of the aneuploid population within the tumor, suggesting that the genetic heterogeneity may indicate, and perhaps confer, an increased evolutive and malignant potential [2, 36-39]. In this sense it has been suggested the use of alternative measurements of DNA content that take into account the heterogeneity within the tumor [40, 41]. Because colorectal cancers appear to progress through different pathways, more recently, the assessment of the utility of some markers is being reevaluated in each group of samples. The rationale for this analysis assumes that specific genetic alterations may have different biological implications depending on the general genetic context. Therefore their impact on prognosis may be substantial in one group but not in another [42, 43].

### 3.2. Karyotypic abnormalities

G banding cytogenetics provides a powerful tool to analyze the whole of chromosomal alterations in tumor cells. Nevertheless the difficulty in obtaining good quality metaphases in solid tumors has precluded a wide use of this technique in colorectal cancer. Different studies have been able to karyotype relatively large series of colorectal tumors demonstrating that patients with tumors of complex karyotype show a lower survival rate than the rest of patients [43-45]. On the other hand, the analysis and comparison of metaphases of individual colorectal tumors has allowed the reconstruction of the chromosomal evolution of each tumor and the postulation of three different pathways of progression [4, 46]. Furthermore these groups of tumors are associated with clinical and molecular parameters, reinforcing the biological significance of this classification. Other classifications based on G-bands karyotyping [43, 47] or molecular techniques [10, 42, 48-58] have generated alternative classifications that are in part comparable to that of Dutrillaux and coworkers.

A recent paper by Bardi et al [43] probably constitutes the only study performed by conventional cytogenetics in as large series of colorectal cancers and specifically aimed to the prognostic assessment of karyotypic markers. While multiple significant data arise from this exhaustive work, including a karyotypic classification of the tumors, perhaps the foremost result is that not only the number of chromosomal alterations correlates with poor prognosis, but also that structural chromosomal aberrations are indicative of more aggressive tumors as compared to numerical aberrations.

Comparative genomic hybridization is the technique most frequently used as an alternative to classic cytogenetics allowing the investigation of specific chromosomal alterations together with global patterns of chromosomal disruption and their potential use as prognostic markers. Most recurrent chromosomal alterations previously detected by G-banding and allelotyping analyses, have been confirmed by CGH. Different studies have found that the number of chromosomal aberrations associates with advanced stages or worst survival [52, 57, 59-61], although opposite results have been also reported [54]. In any case, the number of cases included in each one of these studies is usually well below 50, and therefore these results need validation in prospective investigations performed in larger series. CGH studies have also revealed multiple single chromosomal alterations that are potential prognostic markers [60, 61], and have contributed to clarify some of the chromosomal imbalances previously detected in allelotyping analyses together with regions of gene amplification. CGH coupled with the power of microarray technologies [62, 63] appears as a promising alternative to overcome part of the limitations of chromosomal CGH. Nevertheless the lack of a standardized platform precludes a straightforward application in the near future.

### 3.3. Subchromosomal alterations

The discovery and mapping of a large number of short tandem repeat markers in the late 80s and early 90s allowed the analysis of allelic imbalances (losses of heterozygosity, LOH) in multiple tumors including colorectal cancer. Allelotyping studies contributed to identify tumor suppressor genes [64] and to measure generalized genomic damage in colorectal carcinomas [65-67]. The Fractional Allelic Loss (FAL) index determined by genome-wide allelotyping or microallelotyping provides an estimation of the fraction of the genome affected by allelic imbalances and has been found to associate with survival in colorectal cancer [68-71]. While numerical chromosomal alterations are also identified by allelotyping, the main outcome of a large genome-wide screening results in an appraisal of intra-

chromosomal damage. Very often LOHs are interspersed with regions of retention of heterozygosity [15, 17, 72, 73], indicating the structural nature of such alterations.

The Inter-Simple Sequence Repeat (InterSSR-) PCR is a DNA fingerprinting technique and consists of the amplification of DNA sequences between  $(CA)_n$  dinucleotide repeats using primers homologous to the repeats themselves but anchored at the 3' end by two nucleotides to prevent internal priming [74]. The Inter-SSR PCR has been applied to the analysis of overall genomic damage in colorectal cancer, although no correlations were found between the degree of damage and clinico-pathological variables [75]. It has also allowed the detection of genomic damage in premalignant colorectal polyps, suggesting that genomic destabilization is an early step in colorectal tumor progression [76].

Another DNA fingerprinting technique that has been applied to the quantification of genomic damage and that shares the same properties of rapidity and simplicity that InterSSR-PCR is the Arbitrarily Primed Polymerase Chain Reaction (AP-PCR). The AP-PCR is characterized by the use of primers of arbitrarily chosen sequence and low stringency of the annealing step in initial cycles of the reaction. AP-PCR generates complex reproducible fingerprints representing multiple loci distributed through out the genome [77]. As other allelotyping techniques, AP-PCR has been used to detect recurrent allelic losses and gains in colorectal cancer [78-80]. Because AP-PCR can simultaneously detect and differentiate between different types of genomic damage, it's application was instrumental in the discovery of the microsatellite mutator phenotype [5], and it has been also applied to the assessment of cumulated genome-wide damage in colorectal [81], lung [82] and gastric cancer [83]. In all cases high levels of genomic damage correlated with poor outcome. This correlation appeared to be independent in multivariate analysis. Genetic profiles obtained by AP-PCR fingerprinting have also contributed to distinguish pathways of tumor progression in colorectal cancer [19] and to establish a classification of colorectal carcinomas based on comprehensive genetic profiles [42].

The analysis of intra-chromosomal damage offers interesting perspectives because structural chromosomal instability is likely to play a major role in tumor progression [17, 20, 42, 43]. In spite of this, the routine application of the techniques currently available to determine intra-chromosomal damage has been impaired by the difficulty in the standardization of the technique among different laboratories.

#### **4. Conclusions**

Colorectal cancer is a complex and heterogeneous disease. Different molecular mechanisms are likely to underlie colorectal tumor progression. Alternative pathways of tumor progression would result in tumors displaying characteristic genetic profiles and differentiated biological behavior. Therefore individual genetic alterations are likely to show specific correlates in subsets of tumors representing the postulated pathways of progression. In consequence the potential clinical application of many of these factors is only conceivable after appropriate classification of cancers. Different factors are likely to confuse the classification (i.e. shared features, overlapping between pathways, convergent stepwise progression, etc.), specific signs or marks should also be preserved allowing the identification of the most probable pathway of progression. Estimates of global genomic disruption in its different forms are likely to reflect the marks (alterations at chromosomal, subchromosomal and sequence level) of the agent driving progression (i.e. genomic instability). Measurements of cumulated genomic damage constitute not only simplified pictures of the vestiges left during tumor history but also a

*magnetized needle* that may contribute to sight the malignant potential and to predict clinical outcome. Further methodological developments together with a better understanding of the biological processes implicated in tumorigenesis should aid in the obtention of truly useful prognostic markers.

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