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Review

Assessment of cumulated genetic alterations in colorectal cancer

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Summary. Widespread genetic alterations are a common feature of most colorectal cancers. While specific recurrent alterations may reveal the involvement of a gene or set of genes in the biology of the disease, the cumulated genomic damage is likely to reflect the biological history of the neoplastic cells. Furthermore, the functional implications behind many of these genetic changes may show the evolutionary potential of the neoplastic cells. Different approaches, ranging from the gross determination of total nuclear DNA content to cytogenetic and molecular approaches, reveal different types of chromosomal and subchromosomal alterations and have been applied to measure generalized genomic damage in colorectal carcinomas. High levels of genomic damage usually appear associated with increased aggressiveness in colorectal cancer, and the use of different assessments of genomic damage as independent prognostic factors has been proposed. Therefore, appropriate definition of the extent of cumulated alterations and their functional consequences may be of interest in the understanding and management of cancer. The different methodologies and clues to the interpretation and integration of the results obtained with each technique are discussed in this review.

Key words: Tumor progression, Genomic instability, Genetic alterations, Methods

A role for cumulated genomic damage in tumorigenesis

The idea that the genetic alterations present in cancer cells could be responsible for the growth and progression of the tumors was first proposed by Theodor Boveri almost ninety years ago (Boveri, 1914). A subset of the genetic alterations identified in tumor cells are clearly associated with the ethiopathology of the transformation. These include specific translocations, gene amplification and mutations in a series of genes coding for proteins with oncogenic-related functions. The study of particular genetic alterations with known or suspected direct involvement in the tumorigenic process (those affecting oncogenes and tumor suppressor genes) is not the target of this review, but rather the analysis of cumulated genomic damage from a broad point of view. The extent of genetic alterations (referred to herein as genomic damage) in most cancer cells involves far more than changes in a few genes. Multiple and heterogeneous genetic aberrations ranging from sequence changes to chromosome number variations are detectable in a typical neoplasm (Jackson and Loeb, 1998; Lengauer et al., 1998). Different sorts of studies in colorectal and other types of tumors appear to indicate that the greater the genomic damage, the worse the prognosis (see below), suggesting that the assessment of global genomic damage could have clinical utility. Although substantial advances have been made in the molecular characterization of the genetic events underlying malignant transformation, we are still far from completely understanding what the role of cumulated genetic alterations in the evolution of the tumors is and what its causes and consequences are (Schar, 2001). One of the most accepted theories postulates that the cumulated genomic damage is the consequence of genetic instability. Genetic instability would increase the mutation rate and would facilitate tumor evolution from the early stages of tumor progression (Nowell, 1986; Loeb and Loeb, 1999). Nevertheless, 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 (Tomlinson and Bodmer, 1999; Tomlinson et al., 2002b). 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.

The heterogeneous nature of genetic alterations in cancer cells precludes a simple approach to their

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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.

In summary, the study of cumulated genomic damage is likely to reveal clues to the understanding of the factors governing the molecular evolution of tumors. Furthermore its assessment may be useful in the clinical management of human cancer. This review intends to shed some light on the evaluation, interpretation and integration of genomic damage data. Since colorectal cancer is the paradigm in the study of tumor progression, we will focus on the results obtained for this type of tumor.

Types of genetic alterations

With an aim to characterizing cumulated genomic damage in cancer cells, alterations are usually classified according to their nature and extent and independently of their functional consequences (Table 1). Smaller alterations occurring in the DNA sequence include substitutions, deletions or insertions of one or a few nucleotides (Lengauer et al., 1998). Alterations affecting larger stretches of DNA (usually referred to as chromosomal aberrations) can be subdivided into numerical (aneuploidies and polyploidies) and structural alterations (amplifications, duplications, deletions, inversions and translocations of fragments of chromosomes). Numerical alterations are the consequence of losses and gains of whole chromosomes. The length of the fragments involved in structural chromosomal alterations is very variable (affecting from a few kb to whole chromosome arms). The term subchromosomal alteration is used to refer to deletions or amplifications that affect small chromosomal regions. Obviously, the different nature and extent of the alterations precludes single approaches for their combined analysis. Furthermore, the complete characterization of every alteration is not always feasible in single-shot screenings, which are unlikely to distinguish between different types of alterations (Table 1). This is especially relevant when investigating chromosomal alterations and implies that estimation of genomic damage does not always reflect the actual rate of genetic alterations, but rather represents an arbitrary index that arises from the detection of uncharacterized alterations. In an attempt to gain insights into the nature of the results arising from each technique, the rest of the manuscript has been organized following the rationale of the different methodological approaches that are most often used to assess genomic damage in cancer cells.

Approaches for the assessment of global genomic damage

The survey of cumulated genomic damage may be obtained by the computation of data obtained in specific genetic analyses of multiple loci or by using approaches based on the global screening of genetic alterations. Epigenetic alterations also play an important role in the disruption of the genome of cancer cells (Jones and Baylin, 2002), but due to the different nature and specificity of the techniques used in their analysis this issue will not be discussed here.

Genetic instability at the DNA sequence level

The assessment of cumulated genetic alterations affecting subtle sequence changes has only been intensively studied in tumors displaying microsatellite instability (MSI) (Perucho et al., 1994). The genomic damage related to this type of instability consists of

Table 1. Types of genomic damage in colorectal cancer.

CUMULATED GENOMIC DAMAGE	UNDERLYING ALTERATINS	DETECTION OF THE DAMAGE	TYPE OF GENOMIC INSTABILITY
Subtle nucleotide variations	Point mutation	DNA sequencing, SSCP and others	None detected
	Deletions/Insertions in short tandem repeats	Analysis of microsatellites (PCR/electrophoresis)	Microsatellite instability (defective DNA mismatch repair system)
Aneuploidy	Loss and gain of chromosome copies	Flow cytometry, G-banding cytogenetics, CGH, LOH, FISH, DNA fingerprinting (AP-PCR)	Numerical chromosomal instability (defects in chromosomal segregation)
Structural chromosomal alterations	Chromosome rearrangements	G-banding cytogenetics, M-FISH, SKY, CGH[b], LOH[b], DNA fingerprinting (AP-PCR)[b]	Structural chromosomal instability

[a]Abbreviations: SSCP: Single strand conformation polymorphism, CGH: Comparative genomic hybridization, LOH: Loss of heterozygosity, FISH: Fluorescence in situ hybridization, M-FISH: Multiplex-FISH, SKY: Spectral Karyotyping, AP-PCR: Arbitrarily primed PCR. [b]These techniques allow the detection of unbalanced but not balanced rearrangements.

small deletions or insertions within short tandem repeats (microsatellite sequences) in tumor DNA compared with the corresponding DNA from normal tissue (Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993) (Fig. 1). Microsatellite instability is the symptom of defects in the DNA mismatch repair system (Boland, 1997). As well as in sporadic colorectal cancer a common form of familial cancer, the Hereditary Non-Polyposis Colon Cancer (HNPCC), is caused by inactivating mutations in different genes involved in mismatch repair (Peltomaki, 2001). Since the DNA polymerase is prone to produce errors in the replication of repetitive sequences, the lack of repair is especially manifested in microsatellite sequences. In the human genome most microsatellite sequences lie in non-coding regions and therefore mutations are unlikely to play a significant role in tumorigenesis. Nevertheless, multiple genes contain short mononucleotide tracts within the coding region and therefore are also a preferential target for instability (Duval et al., 2001). It is of note that colorectal tumors displaying microsatellite instability constitute a distinctive group with well-defined molecular and clinicopathological features that are related to the accumulation of damage in short tandem repeats (Janin, 2000; Atkin, 2001; Fishel, 2001; Peltomaki, 2001). Accordingly, a number of studies indicate that assessment of microsatellite instability may have implications for the clinical management of colorectal cancer (Aaltonen et al., 1998; Gonzalez-Garcia et al., 2000; Gryfe et al., 2000). Although tumors with impairments in the DNA mismatch repair system accumulate mutations in the order of hundreds of thousands (Perucho et al., 1994), diagnostic criteria usually rely on the analysis of a few microsatellite sequences. It is widely accepted that two or more microsatellites mutated from a panel of five analyzed is indicative of microsatellite instability (Boland et al., 1998). Due to the intrinsic instability of microsatellite sequences, spurious mutations are observed even in tumors with an intact mismatch repair system. This observation prompted some investigators to propose the existence of a group of colon cancers with low microsatellite instability (Boland et al., 1998; Jass et al., 2001; Kambara et al., 2001). Nevertheless, recent studies dismiss the biological and clinical relevance of such low instability (Gonzalez-Garcia et al., 2000; Halford et al., 2002; Laiho et al., 2002; Tomlinson et al., 2002a). In summary, microsatellite instability is the consequence of an unpaired DNA repair system that results in extensive genomic damage in tumor cells. Moreover, the cumulated genomic damage is the propelling force behind the evolution of these tumors in a distinctive pathway of progression.

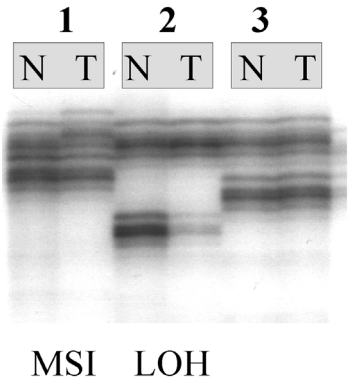

Fig. 1. Detection of genetic alterations in microsatellite DNA sequences. This example illustrates the analysis of a CA microsatellite (D4S405) from three paired normal mucosa (N) and colon carcinomas (T). All three cases are heterozygous for this marker. Tumor from patient 1 displays band mobility shifts with respect to its paired normal tissue. This alteration is indicative of microsatellite instability (MSI). The tumor from patient 2 displays loss of one of the alleles. This alteration is indicative of loss of heterozygosity in the chromosomal region where this sequence maps (4p15). The tumor from patient 3 retains both alleles and is stable.

No clear evidence exists for other types of genetic instability resulting in subtle variations of the DNA sequence in colorectal tumors. Cumulated dispersed mutations have been described occurring in a colon cancer cell line, although at low rates (Harwood and Meuth, 1991). More recently, the screening of 3.2 Mb of coding DNA in a short series of colorectal cancers failed to find evidence of a mutator phenotype at the nucleotide level (Wang et al., 2002). It is of note that an increased mutation rate resulting in a high transversion mutation frequency has been observed in mammary tumorigenesis (Liu et al., 2002). Future investigations should increase our knowledge on this issue.

Multiple approaches are available for the analysis of subtle sequence variations. For most of them the accuracy of the result relies on the specificity of the screening due to the qualitative nature of the changes detected and the experimental outcome. Hence, discrepancies between studies are likely to reflect sensitivity differences and/or populational variations rather than a bias generated by the method or in the interpretation of the raw data. In consequence, controversy is usually limited to the definition of degrees of instability (as discussed above for the microsatellite mutator phenotype) and therefore we will not treat the different methodologies available here, these may be obtained from the references cited above and specific reviews (Perucho, 1994; Rossiter and Caskey, 1994).

Chromosomal alterations

Chromosomal alterations are the most frequent form of genomic damage observed in colorectal and other tumor cells. In 1997, Lengauer and coworkers hypothesized the existence of chromosomal instability (by analogy to the microsatellite mutator phenotype) as the driving force of cumulated chromosomal aberrations in most colorectal cancers (Lengauer et al., 1997). However, the nature of this instability and the underlying mechanisms are unclear and its existence is still a matter of debate. There is also a hypothesis which is the other

way round: that chromosomal alterations (in the form of aneuploidy) cause genetic instability (Duesberg et al., 1999; Li et al., 2000). Most of the entanglement in the analysis of chromosomal damage arises from the heterogeneity of methodologies used for their analysis and the flexible interpretation of alterations revealed by diverse techniques that are often considered as equivalent. Here we will address the applicability, strengths and pitfalls for those used most frequently in the analysis of cumulated genomic damage.

Flow cytometry

This technique allows the quantification of the alteration of the DNA content of tumor cells by comparison with the DNA content of normal diploid cells (Macey, 1994). 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 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.

Because aneuploidy is one of the most frequent characteristics of colorectal tumors (Dutrillaux, 1995) and because its routine analysis in clinical specimens is feasible, multiple studies have determined its utility as a prognostic factor. However, contradictory results have been obtained and no definitive conclusions have been drawn to date (Bauer et al., 1993; Compton et al., 2000; Bast et al., 2001). Different factors appear to put the applicability of DNA content measurement by flow cytometry in the prognostic assessment of colorectal cancer patients in doubt. To cite a few: the lack of standard methodology; tissue type variability (frozen or paraffin-embedded); possible contamination of the sample with non-tumor tissue; small samples; scarce use of multivariate analysis; arbitrary establishment of cut-off points; and the use of confusing nomenclature (diploid, non-diploid, near diploid, aneuploid, tetraploid, near tetraploid, low aneuploid, high aneuploid, etc.) among others. Another aspect that may play a significant role in the interpretation of the results is the biased use of the DNA content of the most aneuploid population (DNA index, DI) as the index of aneuploidy of the tumor. 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 (Remvikos et al., 1988; Giaretti, 1994; Tollenaar et al., 1997; Flyger et al., 1999; Buglioni et al., 2001; Risques et al., 2001). In a recent study it has been shown that an index reflecting both the degree and the extent of aneuploidy might be used as an independent predictor of survival (Risques et al., 2001). As a whole, ploidy determination in colorectal cancer may be considered as a simple approach to obtain a gross view of genomic disruption in tumor cells and its assessment may be useful in understanding the progression of tumors. This may eventually have applications in the clinical management of patients.

Classic cytogenetics

The term 'classic' or 'conventional' cytogenetics refers to the different banding techniques developed during the 70s which allow the identification of each chromosome by specific banding patterns and hence the determination of chromosome alterations, either numerical or structural. The main disadvantage of this technique is the need for good quality metaphases, which are very difficult to obtain in the case of solid tumors. In spite of this inconvenience, different studies have been able to karyotype a considerable number of colorectal tumors (in total, around 250) and in some of the series it has been demonstrated that patients with tumors of complex karyotype show lower survival rate than the rest of patients (Heim and Mitelman, 1995; Mitelman, 2000). 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 associated with three specific types of colorectal tumors: firstly, monosomic type (70% of tumors), characterized by loss of chromosomes 17p and 18, the presence of monosomies, deletions and structural rearrangements and a tendency to endoreduplication; secondly, trisomic type (20-25% of tumors), characterized by the presence of trisomies and the absence of polyploidies and structural rearrangements; and thirdly, normal type (5-7% of tumors), with stable karyotype (Muleris et al., 1988, 1990a; Dutrillaux, 1995). Furthermore these groups of tumors are associated with clinical and molecular parameters, reinforcing the biological significance of this classification. Trisomic and normal type tumors are preferentially located in the proximal colon, have low incidence of p53 mutations and present microsatellite instability; meanwhile, monosomic tumors show the opposite characteristics (Dutrillaux, 1995). Besides the power of classic cytogenetics to characterize numerical and structural chromosomal aberrations, the difficulties in its application to dynamic settings and to the study of clinical samples has precluded its wider use in cancer genetics.

Molecular cytogenetics

The advent in recent years of different molecular cytogenetic techniques has solved some of the problems inherent to classic cytogenetics such as the need for good metaphases. On the other hand, they have filled the gap of resolution existing between classic cytogenetic techniques (megabase pairs) and molecular biology techniques (base pairs) (Ried et al., 1997). The most

important of these techniques are Comparative Genomic Hybridization (CGH), Fluorescence In Situ Hybridization (FISH), and the techniques of multicolor karyotyping: Spectral Karyotyping (SKY) and multiplex FISH (M-FISH). Furthermore the combination of these techniques wih each other or with classic cytogenetics has allowed the complete karyotyping of cell lines (Chen and Nierman, 1994; Masramon et al., 2000; Tsushimi et al., 2001).

Comparative Genomic Hybridization (CGH) consists of a competitive in situ hybridization between normal and tumor DNA, each one labeled with a fluorochrome and hybridized to a normal metaphase (Kallioniemi et al., 1993). The differences in color along the chromosomes allow the detection of regions with gains or losses in the tumor DNA. CGH is especially suitable for the analysis of solid tumors because it provides a global screening of alterations using genomic DNA. Therefore, CGH has contributed not only to confirming some of the previous findings of classic cytogenetics (Muleris et al., 1990b; Bardi et al., 1995) but also to increasing our knowledge of the chromosome alterations in solid tumors in general and colorectal cancers in particular. The most novel results have come from the analysis of clinical specimens, where the consistent identification of non-random and recurrent chromosomal alterations has been allowed, including losses, gains and gene amplifications (Ried et al., 1996; Meijer et al., 1998; Nakao et al., 1998; De Angelis et al., 1999; Georgiades et al., 1999). Although survival analysis has not been performed in most of the studies, it has been shown that the number of chromosomal alterations increases with disease progression, either when comparing adenomas vs. carcinomas (Ried et al., 1996; Meijer et al., 1998), or different Dukes' stages in carcinoma (De Angelis et al., 1999) or carcinomas vs. metastases (Al-Mulla et al., 1999). Moreover, aneuploid tumors show more chromosomal alterations than diploid tumors (De Angelis et al., 1999) and the number of alterations are positively correlated with the DNA index of the tumors (Meijer et al., 1998). Finally, recent studies that combine the analysis of genomic damage by CGH and by flow cytometry together with the determination of microsatellite instability have found that there is a subset of near-diploid tumors that show neither microsatellite instability nor major chromosome alterations, suggesting that these tumors follow a pathway of progression characterized without the apparent need for genetic instability (Georgiades et al., 1999; Chan et al., 2001).

The FISH consists of the hybridization of a fluorescently-labeled DNA probe to either chromosome

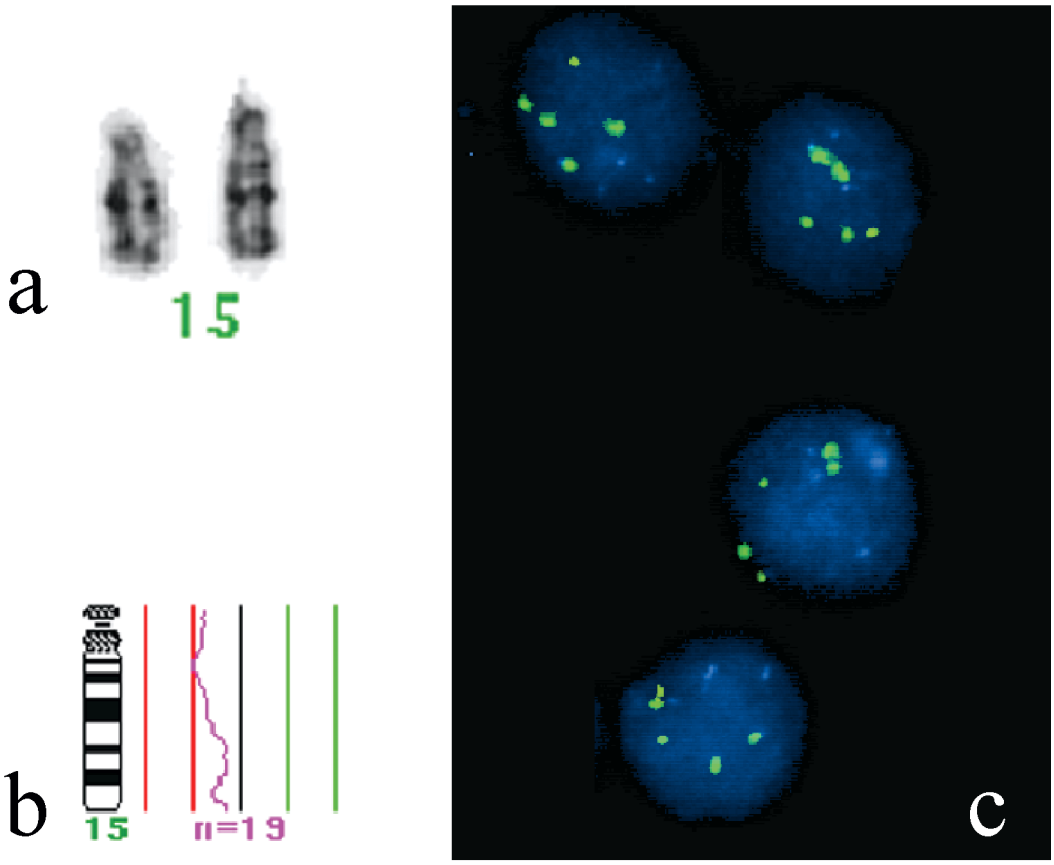

Fig. 2. Analysis of alterations in chromosome 15 in SW480 colon cancer cell lines by three complementary approaches. **a.** G-banding cytogenetics shows a normal pair of chromosome 15. b. Comparative Genomic Hybridization shows a slight decrease along the chromosome due to the high modal chromosome number or SW480 cells (n=57). **c.** Fluorescence In Situ Hybridization using a centromeric probe for chromosome 15 reveals the presence of 5 centromeres. The remaining three centromeres are probably part of rearranged chromosome markers.

metaphases or to interphase nuclei (Gozzetti and Le Beau, 2000). There are three types of FISH probes: gene-specific probes, repetitive-sequence probes (centromeric or telomeric); and chromosome-painting probes. In the three cases only a few target sequences can be analyzed simultaneously, making FISH a technique useful for detecting specific alterations, but not for searching for new ones. As an example of FISH gene-specific assays, MYC amplification can be detected in interphase nuclei of colon cancers (Obara et al., 2001). Centromeric FISH probes have been used to investigate the clonality and heterogeneity of tumors and cell lines (Nanashima et al., 1996; Lengauer et al., 1997; Di Vinci et al., 1999; Bomme et al., 2001; Roschke et al., 2002). In these studies, an abnormal number of fluorescent signals per nucleus is interpreted as aneuploidy. Nevertheless, structural aberrations, which are very common in a high proportion of colorectal cancers, may result in chromosome markers with a centromere which does not represent the actual chromosome. For instance, FISH analysis using a centromeric probe for chromosome 15 revealed five signals per cell in SW480 cells, while G-banding and CGH analysis showed only two copies of this chromosome per cell (Fig. 2). Since cross-hybridization of the FISH probe was discarded because other cell lines with a normal number of chromosome 15 displayed the expected two signals, it suggests that the remaining three centromeres are a part of chromosome markers. Therefore, centromeric FISH results should be interpreted with caution.

FISH-derived techniques of multicolor karyotyping (SKY and M-FISH) allow the simultaneous analysis of the complete chromosome complement and, in consequence, screening for new tumor-specific chromosomal aberrations (Schrock and Padilla-Nash, 2000). These techniques are based on the hybridization of 24 differentially-labeled chromosome-painting probes on tumor metaphases, so after the image analysis all chromosomes are visualized simultaneously and each pair 'painted' with a different fluorescent color. These techniques have been applied in several studies with colorectal cell lines and have contributed to a better characterization of chromosomal aberrations in some of them (Melcher et al., 2000) as well as providing some clues about chromosome instability (Ghadimi et al., 2000; Abdel-Rahman et al., 2001; Tsushimi et al., 2001; Roschke et al., 2002). However, as with classic cytogenetics, multicolor karyotyping requires good metaphases, giving it a limited application in solid tumors.

Analysis of polymorphic markers

The analysis of the highly polymorphic variable number of tandem repeats (VNTRs) (Jeffreys et al., 1985) has been applied to the detection of polymorphisms during malignant transformation (Thein et al., 1987; de Jong et al., 1988) and to studies of clonality of tumors, both primary and metastatic (Fey et al., 1988). The use of probes for specific loci has also allowed the study of profiles of chromosome loss (loss of heterozygosity, LOH) in colorectal cancers (Fearon et al., 1987; Solomon et al., 1987; Law et al., 1988; Monpezat et al., 1988; Delattre et al., 1989; Sasaki et al., 1989; Vogelstein et al., 1989). The recurrent observation of LOH in the same chromosomal region in a given tumor type is considered to be an indication of the presence of a tumor suppressor gene, whose loss promotes neoplastic transformation (Skotheim et al., 2001). For this reason the screening for LOH in tumors has been widely used and has facilitated the localization of several tumor suppressor genes, such as p53 and DCC (Fearon and Vogelstein, 1990; Weinberg, 1991). The advent of the Polymerase Chain Reaction (PCR) and the identification and mapping of multiple microsatellite sequences (which have the advantages of being highly polymorphic and abundant along the genome) has displaced the use of VNTRs in the assessment of LOH. Nowadays, the most common procedure for detection of LOH consists of the comparison of alleles in tumor and normal tissue after PCR amplification of microsatellite loci followed by gel electrophoresis and detection by radioactivity or silver staining (Fig. 1). This process has been improved with the introduction of fluorescently-labeled primers, which permit the detection of the PCR products by automatic gel sequencers or capillary electrophoresis, simplifying the technique and increasing the quality of the results (Canzian et al., 1996; Skotheim et al., 2001).

In classical studies, Vogelstein and coworkers (Vogelstein et al., 1989) applied intensive LOH screening to estimate global genomic damage in colorectal cancers. The frequency of allelic deletions present in the tumor (called Fractional Allelic Loss, FAL) was found to be associated with an increased probability of recurrence of the disease and death (Kern et al., 1989; Vogelstein et al., 1989). In a second study with the same samples, it was also shown that aneuploid tumors (as determined by flow cytometry) displayed higher FAL levels (Offerhaus et al., 1992), suggesting the relationship between the two measures of genomic damage. The availability of a large number of highly polymorphic microsatellite markers distributed genome-wide, together with the high sensitivity, low requirement of starting material and method simplicity, has allowed thousands of cancer researchers to screen all sorts of neoplastic specimens in search of loci with a high rate of LOH. This kind of technique is useful to reveal specific alterations or to unmask gross aberrations, but very often the extent and the nature of the alteration cannot be inferred directly from the determination of LOH. For instance, allelic losses and gains are often indistinguishable and actual LOHs may be produced by heterogeneous non-related alterations (i.e. mitotic recombination, structural unbalanced chromosomal alterations, numerical alterations) (Tischfield, 1997). Therefore, few studies have pursued the determination of

cumulated genomic damage by allelotyping analysis in colorectal cancer. These investigations (Thorstensen et al., 1996; Massa et al., 1999; Weber et al., 1999) have basically confirmed previous findings.

Arbitrarily-Primed PCR (AP-PCR) and Inter-Simple Sequence Repeat PCR (Inter-SSR PCR)

The AP-PCR is a DNA fingerprinting technique based on the amplification by PCR of genomic DNA using primers of arbitrarily chosen sequence and initial cycles of low stringency. Because the primer anneals to multiple sites, many PCR products are generated and result in a reproducible fingerprint when analyzed by electrophoresis (Welsh and McClelland, 1990). The Inter-SSR PCR is very similar to the AP-PCR and shares the same properties of rapidity and simplicity. It 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. Due to the abundance of these repeats and their location scattered randomly throughout the human genome, the PCR yields a multitude of products that, like the AP-PCR, produce a fingerprint of bands after electrophoresis.

In colorectal cancer, the AP-PCR has been applied to the study of allelic losses and gains (Peinado et al., 1992) and because it can simultaneously detect and differentiate between different types of genomic damage, it has also been instrumental in the discovery of the microsatellite mutator phenotype (Ionov et al., 1993). AP-PCR has been used to easily generate allelotypes of primary and metastatic colorectal tumor cells (Malkhosyan et al., 1998; Arribas et al., 1999). Furthermore, due to its capacity to simultaneously screen for multiple alterations genome-wide in an unbiased manner, it has also been applied to the assessment of cumulated genomic damage (Fig. 3) (Arribas et al., 1997). This study showed that high levels of genomic damage correlated with poor outcome in a series of colorectal carcinomas. This correlation appeared to be independent in multivariate analysis. The Inter-SSR PCR has also 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 (Basik et al., 1997). 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 (Stoler et al., 1999). Besides the simplicity, low requirement of material, and expected power of results generated by these DNA fingerprinting techniques, its routine application has been precluded by difficult standardization of the technique and the lack of shared objective criteria for the interpretation of the fingerprints.

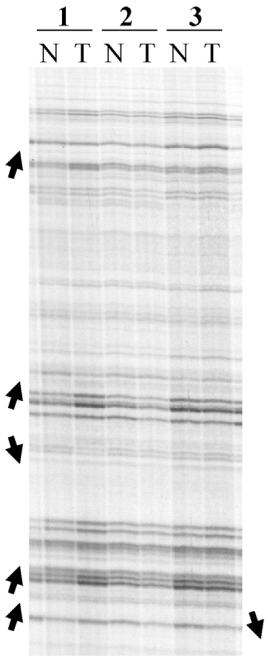

Fig. 3. AP-PCR analysis of three paired normal (N) and tumor (T) DNAs. Arrows next to cases 1 and 3 indicate gains (up-arrowhead) and a losses (down-arrowhead) in the tumor with regard to its paired normal tissue. Assay conditions and primer sequences may be obtained from the authors.

Concluding remarks

In this review we have tried to summarize the different approaches that have been used to assess the cumulated genomic damage of colorectal tumors. The complexity of the situation is evident, since probably there are different pathways of tumor progression, and the causes, nature and consequences of the genomic damage in each pathway are unlikely to be the same. While the rationale of using a measure of global genomic damage to improve the prognostic assessment and treatment of colorectal cancer patients may have a solid base, no general application is feasible. That is to say, specific analyses are probably needed for the different subsets of tumors. Future studies should address the analysis of a large series of samples with a wide array of techniques in order to provide enough information to establish associations between the different types of genomic damage, the clinico-pathological parameters of the tumors and the survival of the patients. This is becoming more feasible with the development of high throughput technologies (for instance CGH on microarrays) and the knowledge of the sequence of the human genome. Furthermore, abnormal patterns of DNA methylation and genetic instability may also be be intrinsically linked. Therefore, comprehensive approaches using a combination of techniques should be used to elucidate the different pathways of tumor progression as well as to better understand the underlying mechanisms of malignancy. We can imagine tumor progression as a picture hidden by a piece of paper. Each technique is a pattern of holes which is made in the piece of paper, each hole disclosing just a small area of the picture. There are probably several ways to identify the picture but certainly, the

overlapping of different patterns seems to be one of the best strategies.

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