[CANCER RESEARCH 54, 5059–5063, October 1, 1994]

Perspectives in Cancer Research

Microsatellite Instability: Marker of a Mutator Phenotype in Cancer[1]

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Introduction

There is increasing evidence that multiple mutations are present in many human tumors. Molecular techniques are progressively making it feasible to dissect the eukaryotic genome, from chromosomes, down to genes and nucleotide sequences, and eventually even to three-dimensional structures. With each deeper level of exploration, more and more mutations are being documented in cancer cells. The emerging concept is that genomes of cancer cells are unstable, and this instability results in a cascade of mutations some of which enable cancer cells to bypass the host regulatory processes.

Cancer as a Mutator Phenotype

Based on the high frequencies of chromosomal abnormalities and mutations in human cancers, I offered the hypothesis that cancer is manifested by a mutator phenotype (1). This hypothesis was based on the argument that the spontaneous mutation rate in normal cells is insufficient to account for the high frequency of mutations in human cancer cells (1, 2). Moreover, the frequency of mutations in cancer cells may be even higher than that which is detected. Current methods are biased towards detecting large rearrangements; deletions, frameshifts, base substitutions, and small rearrangements are likely to be missed. In fact, we lack methods to efficiently identify random single base substitutions in the nucleotide sequence of genes. The prediction is that more and more mutations will be found in cancer cells as we clone and sequence genes from tumors and then compare their nucleotide sequences with those in adjacent normal tissues. The multiple chromosomal changes currently detected in cancer cells may be the tip of the iceberg. There may be a much larger number of single nucleotide substitutions, frameshifts, small deletions, and insertions. A feature of cancer may be the continual accumulation of mutations within individual cells. In this Perspective, I will focus on point mutations and mutations occurring in repetitive nucleotide sequences.

Is the rate of spontaneous or background mutations in normal cells sufficient to account for the many mutations in human cancers? A compendium of studies of somatic mutation rates of human cells in culture using hypoxanthine-guanine phosphoribosyltransferase (3) and adenine phosphoribosyltransferase (4) suggests a background mutation rate of approximately 1.4×10^{-10} mutations/base pair/cell generation (1). This rate is similar to that deduced by Chu *et al.* (5), based on the presence of electrophoretically distinct protein variants at unselected loci in cultured human lymphoblastoid cells. Considering that cancers arise in one or a few cells, we have estimated that the background mutation rate in normal cells can account for only two or three mutations in each tumor and not the much larger number of mutations that are reported (8–11), or the even greater numbers that are likely to be found as methods for detection become more sensitive. At some time during the life of a tumor, the mutation rate must be greater than in normal cells; *i.e.*, cancer cells must exhibit or have exhibited a mutator phenotype (1, 8, 12).

The concept that oncogenesis involves a mutator phenotype has been considered by several investigators (1, 8, 12). An alternative hypothesis to account for the multiple mutations in tumors can be formulated based on mutation-driven clonal repopulation. Let us assume that the first mutation leads to a strong proliferative advantage. If the mutant cell expands to 10^{10} progeny within the tumor, then a second mutation occurring at a normal frequency of 10^{-10} would occur with a high probability in cells already harboring the first mutation. Among the early mutations would have to be ones that confer immortality or at least extend cellular life span. If this and each subsequent mutation results in a similar clonal proliferation, multiple mutations could accumulate in each tumor cell in the absence of an increase in the mutation rate. However, this hypothesis is less attractive than that of a mutator phenotype, because it requires that each mutation, even a single mutation in one of two recessive alleles, causes a profound growth advantage. Despite this limitation, the fact that tumors are predominantly clonal in origin indicates that many mutations result in cellular proliferation. Moreover, clonal evolution and a mutator phenotype are not exclusive; both may be required to account for the frequency of mutations in cancers.

Multiple Mutations in Cancers

Until recently, the hypothesis that cancer cells exhibit a mutator phenotype received little experimental support (1). Most comparisons between normal and cancer cells used rodent cells in which there is less DNA repair than in human cells, or between nontumorigenic and tumorigenic cell lines, both of which are immortal and already likely to contain multiple mutations. Experiments on the fidelity of DNA synthesis using the SV40 replication complex did not reveal differences between normal cells and malignant cells (13) and thus failed to support the hypothesis of a mutator phenotype. However, experiments on the fidelity of DNA synthesis are insensitive; they can only detect errors in DNA synthesis which occur more frequently than 10^{-6}, which is four orders of magnitude greater than the background mutation rates exhibited by somatic cells. In contrast to these negative studies, the recent demonstration of microsatellite instability in different human tumors has provided strong evidence for a mutator phenotype (14–16). Each microsatellite contains multiple repetitive nucleotide sequences that are relatively constant in normal cells but vary in length in certain tumors. Even though microsatellite variations might not affect the phenotype of the cell, they are by definition mutations. Microsatellite instability in some of these cancers (17) occurs coordinately with mutations in genes that are homologous to those involved in mismatch repair in bacteria (18) and yeast (19). The implication is that mutations in these mismatch repair genes decrease the capacity of that system to correct errors made during DNA replication, particularly errors in repetitive nucleotide sequences. Mutations in the mismatch repair genes could be an early event in the carcinogenic process, since these mutations have been demonstrated in diploid colon cancer cells (16). Microsatellite repeats are likely to be hot spots for mutagenesis (20), and mutations within these se-

Received 6/21/94; accepted 8/4/94.

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[1] This work was funded by Grant OIG CA 39903 from the National Cancer Institute.

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quences might be a marker of increased replicative errors throughout the genome of cancer cells.

Microsatellite Instability

The human genome is punctuated with repetitive nucleotide sequences or microsatellites. These repetitive di- tri-, and tetranucleotides are frequently, but not invariably, located between genes and have been classified as "junk" DNA. The number of tracts containing repetitive sequences in the human genome is enormous. For example, there are about 100,000 CA/GT repeats, each with a chain length greater than 24 (21). Early studies on DNA synthesis demonstrated that DNA polymerases can increase the length of oligonucleotides consisting of repetitive sequences by the slippage of one strand relative to the other (22). The predominant nucleotide sequence of the repeats in the product of the reaction is a copy of that in the template. Early investigation of microsatellite instability, *i.e.*, variations in the number of repetitive unit sequences in each microsatellite, reported changes in the fingerprint pattern of DNA in a variety of cancers, including colorectal carcinomas, but not in adjacent normal tissues from the same individuals (23). Findings of the expansion of specific repetitive sequences in certain inherited human diseases, including the fragile X syndrome (24), brought into focus the inherent instability of these repetitive sequences and raised the possibility that these microsatellites might provide a sensitive indicator for genetic instability in tumors.

Cancer and Mismatch Repair

The finding that the length of selected CA repeats is variable in HNPCC[3] provided the first definitive demonstration of a mutator phenotype in cancer cells (14, 16, 25, 26). The presence of a mutation in a gene in HNPCC that is homologous to *mut*S [16, 27; the gene responsible for mismatch recognition in methyl-directed mismatch repair in bacteria (28)] is found in HNPCC families. Another gene that is a homologue of the bacterial mismatch repair gene, *mut*L is mutated in other families with HNPCC (29, 30). Furthermore, it has been directly demonstrated that extracts from colorectal cancer cell lines are defective in mismatch repair (25, 31). Taken together, these findings tightly link mismatch repair and microsatellite instability and provide a logical mechanism for the generation of microsatellite instability in HNPCC. Variations in microsatellites are generated by errors in DNA replication. These errors create regions of non-complementarity that are normally corrected by the mismatch repair system. Mutations in the genes that comprise this system result in defective proteins that fail to correct replication errors, including those resulting from slippage during DNA replication. The nucleotide sequence homologies between the mutant genes in families with HNPCC and the mismatch repair genes in bacteria and yeast imply that these genes have similar functions (25). In bacteria, this repair pathway corrects a variety of errors resulting from DNA replication and other DNA synthetic processes (18). These include single base substitutions, deletions, additions, and frameshifts, as well as small nucleotide sequence rearrangements. Three of the genes that are required for mismatch repair in *Escherichia coli*, *mut*S, L, and H, are believed to encode proteins that function primarily if not exclusively in mismatch repair (32). Mutations in each of these genes result in a mutator phenotype in *E. coli*; it has been estimated that this repair pathway appears to confer a 10- to 400-fold enhancement in the accuracy of DNA replication (33). In yeast, mutations in any of three of the genes involved in mismatch repair (*PMS1*, *MLH1*, and *MSH2*) result in a 100- to 700-fold increase in additions and/or deletions within repetitive GT tracts (19). These findings clearly demonstrate that mutations in mismatch repair are correlated with variations in the length of repetitive sequences in eukaryotic cells. Experiments are necessary to go beyond this correlation and demonstrate that the expansion of microsatellite sequences which occurs during tumorigenesis is caused by mutations in mismatch repair genes.

Unsolved Questions

The exciting advances in our knowledge about chromosomal instability in tumors have brought into focus new questions and paradoxes: 1. It is now established that microsatellite instability is a frequent occurrence in HNPCC. This finding raises the question of whether microsatellite instability may be a common occurrence in a variety of other types of cancers; 2. What is the relationship between microsatellite instability in cancers and microsatellite expansion in certain inherited neurodegenerative diseases? 3. In bacteria, the mismatch repair system corrects a variety of errors in DNA synthesis. Will other kinds of errors be found in tumors amongst these repetitive sequences? Is there a generally enhanced frequency of mutations in the coding sequences of genes in tumors that contain mutations in mismatch repair? 4. Repetitive nucleotide sequences in DNA are synonymous with junk noncoding DNA. Does expansion of these noncoding sequences result in, or is it coordinate with, clonal expansion? 5. Are mismatch repair genes only a few of the many target genes that when mutated bring about the large number of mutations observed in tumor cells? Are mutations in other genes that normally provide chromosomal stability associated with other hereditary predispositions to cancer? Are these mutations found in other sporadic cancers?

1. Is Microsatellite Instability Limited to HNPCC? Recent evidence establishes that inherited mutations in h*MSH2* (17) and h*MLH1* (30) account for the majority of cases of HNPCC (Lynch syndrome). Mutations in h*MSH2* map to chromosome 2 and to the gene that encodes a human homologue of the *E. coli* mismatch repair protein, *mut*S, that in *E. coli* binds to the mismatch and initiates the repair process (32). In families with HNPCC, a mutation in one gene copy is inherited, and a somatic mutation in the second copy is associated with microsatellite instability in the tumor (16, 25). Mutations in h*MLH1*, a homologue of the bacterial repair gene *mut*L, map to chromosome 3 and are also found in families with HNPCC (29, 30). Families with HNPCC are delineated as having at least three relatives in two generations with colorectal cancer, with one diagnosed before age 50 (34). These families also have elevated incidences of cancers of the endometrium, stomach, gall bladder, pancreas, and urinary tract. In addition to HNPCC, these other tumors also display microsatellite instability. Muir-Torre syndrome, an inherited predisposition to sebaceous gland tumors as well as to colorectal carcinomas, is also associated with microsatellite instability (35). Thus, microsatellite instability is prevalent in many familial cancers.

In addition to these tumors in cancer-prone families, microsatellite instability also occurs in sporadic cancers of the colon (36), stomach (37), and endometrium (38). The presence of microsatellite instability in lung cancer is controversial. Peltomaki *et al.* (39) failed to demonstrate instability in analyzing 8 different microsatellites in 86 lung cancers. In contrast, Shridhar *et al.* (40) detected instability in 13 of 38 lung tumors and Merlo *et al.* (41) reported similar results. A lack of microsatellite instability has been reported in breast cancers and in male germinal cell cancers (42). In evaluating the overall situation, it should be noted that false positives can arise by the same types of replication errors postulated to occur *in vivo* and during *in vitro* synthesis in the polymerase chain reaction (43). Anomalous polymerase chain reaction products are often produced during the amplification of repetitive sequences in normal DNA, and upon gel electro-

[3] The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer.

phoresis, these products yield a ladder-like pattern. This artifact can be the result of slippage or recombination of truncated products (44). However, since most of the studies reported measured microsatellite variations by comparing DNA from tumor and normal cells from the same individual, it seems unlikely that *in vitro* errors constitute a major source of false positives. Presumably the same errors would be made using both normal and tumor DNA. Furthermore, the presence of length variations in different DNA satellites from the same tumor would render random *in vitro* errors an even less likely possibility. Thus, in well-documented studies, false positives appear to arise infrequently. In contrast, false negatives, *i.e.*, the lack of expansion, could simply reflect the limited number of microsatellite sequences examined. Thus, an overall assessment of the studies on sporadic cancers indicates that microsatellite instability is not limited to tumors associated with HNPCC. Instability might become evident in many cancers if a large number of repetitive sequences were analyzed.

2. Microsatellite Instability in Other Diseases. Expansion of tracts of repetitive sequences has been shown to be diagnostic of seven different neurodegenerative diseases. In each of these inherited diseases, a unique repetitive nucleotide sequence is expanded meiotically or during early embryogenesis. Each of these diseases is associated with the lengthening of a trinucleotide repeat within or adjacent to an actively transcribed gene. In the fragile X syndrome, the expanded sequence is a CGG repeat (45); in Huntington's disease, it is a CAG repeat (46); and in myotonic dystrophy, it is a CTG repeat (47). In carriers with mutations in the fragile X gene, lengthening of the CCG repeats is limited, whereas in affected individuals, the expansion can be extensive and involve hundreds of repeats (45). The relationship of this extensive expansion (from 30 to 2000 repeats in fragile X) to the addition of a few repetitive units in tumors remains to be established. Both clearly provide documentation of the instability of these repetitive sequences in the human genome. However, it is important to note that there is no evidence for increased incidence of malignancies in any of these neurodegenerative diseases, nor is there evidence for neurodegenerative lesions in patients with cancers.

The mechanism for expansion of repetitive sequences in the two situations, *i.e.*, cancer and neurodegenerative diseases, may be entirely different. It has been inferred that expansion of repetitive stretches both in neurodegenerative diseases and in cancers is the result of slippage in the copying of repetitive sequences during DNA replication. *In vitro*, it has been clearly established that slippage by DNA polymerases occurs during the copying of repetitive nucleotide sequences (20). The occurrence of increased slippage during DNA replication in either of these pathological states remains to be established. Thus, it may be premature to infer that these syndromes exhibit a replication error-prone phenotype (26). In addition, I am unaware of the expansion of CA repeats (the ones most frequently reported in cancers) in the fragile X syndrome, or conversely, of the expansion of CGG repeats in HNPCC. The responsible mutations in HNPCC have been shown to involve the mismatch repair system (25), presumably resulting in a deficit in the correction of errors in DNA replication that occur more frequently in repetitive nucleotide sequences. Even though the genetic alterations in the different hereditary neurodegenerative diseases are associated with microsatellite instability, there is no evidence that the responsible mutations are in the mismatch repair system. Thus, the possible connections between expansion of microsatellites in cancers and neurodegenerative diseases are still tenuous.

3. Mutations within Genes. The mismatch repair system corrects a variety of errors in DNA replication. Bacteria harboring mutations in mismatch repair exhibit a mutator phenotype; the mutations are predominantly single-base substitutions (33). Thus, it seems likely that defects in human mismatch repair will include single base substitutions within repetitive nucleotide sequences, as well as within other genes. In fact, Bhattacharyya *et al.* (48) reported that, in selected cell lines from patients with HNPCC, the mutation rates for 6-thioguanine resistance and ouabain resistance are increased 1000- and 500-fold, respectively, over those observed in normal diploid fibroblasts. These data provide the first evidence linking HNPCC with mutations other than in repetitive sequences.

4. Function of Microsatellite DNA. The CA repeats that are expanded in tumors occur in noncoding sequences. It is frequently assumed that this noncoding or junk DNA is a vestigial product of abortive evolution of genes and/or an accumulation of uncorrected errors in DNA replication. This assessment may be in part faulty. It can be argued that the maintenance of extensive repetitive nucleotide sequences is evolutionarily costly and would have a negative impact on selection. Junk DNA may have a function. One possibility arises from the fact that repetitive DNA does not contain only perfect repeats; instead, they are interrupted by base substitutions that may act as a barrier to homologous recombination (49). Frequent misincorporations during DNA replication could accumulate and punctuate repetitive DNA with random nucleotide substitutions. In cancer cells, expansion of these sequences could break down this barrier to recombination and permit chromosomal exchanges which are a common finding in most tumors.

A major question is does junk DNA render tumors clonal? In HNPCC microsatellites, variability of specific repetitive tracts occurs in the majority of cancer cells within a tumor. Can these changes in repetitive sequences result in clonal proliferation? If so, it implies that the initial repetitive sequences had acted to down-regulate growth. An alternative possibility is that the mutator phenotype is nonselective; *i.e.*, other mutations occur with high frequency throughout the genome and are the driving force for clonal proliferation. The important point is that instability of repetitive sequences may merely be a sensitive indicator of genomic hypermutation. The recent findings of increased mutation rates for hypoxanthine-guanine phosphoribosyltransferase and ouabain resistance in HNPCC cell lines support this possibility (48).

5. Candidate Mutator Genes. Mutations in h*MSH2* or *HML1*, genes homologous with either *mut*S or *mut*L, have been established as the major inherited defect in HNPCC and have been associated with variability in repetitive nucleotide sequences in DNA from these tumors (16). However, the possible converse correlation remains to be investigated. Is instability of repeats in tumors invariably associated with mutations in h*MSH2* or *HML1*, or can mutations in other genes account for microsatellite instability? It remains to be determined how frequently mismatch repair enzymes are mutated in sporadic cancers that exhibit microsatellite instability. Umar *et al.* (31) designed a frameshift assay based on the replication of SV40 DNA *in vitro* to measure expansion or contraction of repetitive sequences in DNA. Complementation assays indicated the presence of at least four different mutations that generate microsatellite instability in extracts from endometrial and colorectal cell lines. It is important to determine the biochemical basis for the complementarity, especially since these assays may provide a unique means to delineate components of the human mismatch repair complex that are mutated in different tumors.

It was initially proposed that mutations in a large number of genes that insure genetic stability, including those involved in mismatch repair, could generate a mutator phenotype in tumors (1). The underlying premise was that mutations in one or more of these stability genes is an early event in carcinogenesis. These initial mutations can be inherited, as in HNPCC, or arise from endogenous or exogenous DNA damage. It was suggested that these mutations in "stability" genes generate a series of secondary mutations throughout the genome, some of which would involve oncogenes that alter the regulatory mechanisms of the cell. The concept of a cascade of mutations that accumulate during tumor progression is illustrated in the model in

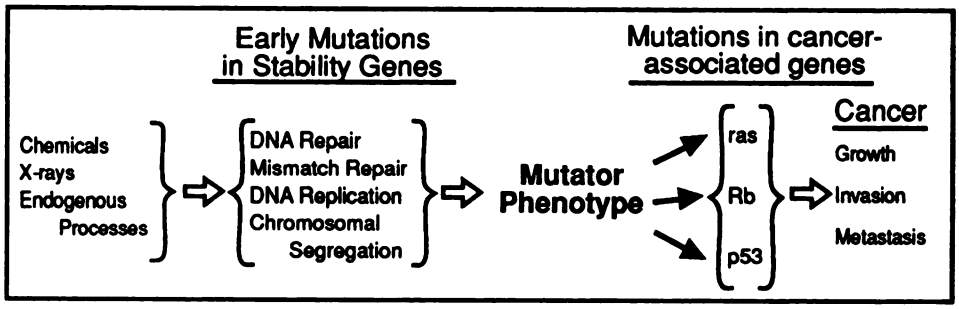

Fig. 1. Schematic representation of the sources for multiple mutations in cancer. The hypothesis proposes that mutations in stability genes may be an early event in carcinogenesis. Mutations in these genes would increase the overall level of mutations throughout the genome and initiate a cascade of further mutations, resulting in yet greater genetic instability and in heterogeneity among cancer cells. Mutations in cancer-associated genes, such as *ras*, *Rb*, and *p53* could in part account for the ability of the cancer cells to grow where they ought not, to invade, and to metastasize.

Fig. 1. Studies in *E. coli* have indicated that approximately 10 gene products are involved in mismatch repair, and mutations in more than 20 known genes can dramatically increase the rate of mutations throughout the genome. In principle, proteins that maintain genetic stability include those involved in DNA replication, DNA repair, deoxynucleotide metabolism, those that monitor the integrity of DNA, those functioning in chromosomal segregation, and others that determine the location of genes in chromosomes and control the copy number of genes (1, 2, 50). Mutations in any of these gene products could likely increase mutagenesis. However, many of these gene products are required for viability, and it is unlikely that inactivation of both alleles can be inherited. Even a single mutation in one of the two alleles may not be compatible with development. In the inherited case, the second mutation is likely to be somatic, occurring in the cancer-prone tissue, and introducing a mutator phenotype. Further mutations could occur randomly throughout the genome, some involving genes associated with the ability of cancer cells to proliferate, invade, and metastasize.

In summary, there are multiple genes that are required for the accurate transfer of genetic information from one cell to its progeny during each division cycle. Mutations in any of these genetic stability genes may be an early event in tumorigenesis, and the generator of the multiple mutations observed in tumors. Relaxed genomic stability could be initiated by primary alterations in genes involved in DNA replication, DNA repair, or chromosomal segregation. The recent demonstration of mutations in mismatch repair and the expansion of repetitive sequences in DNA may constitute the initial indication of more generally prevalent mutations that characterize the cancer phenotype.

Acknowledgments

I thank Drs. A. Blank, F. Christians, A. Skandalis, S. Sorof, and R. Prehn for critical comments, review, and counsel.

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Cancer Res 1994;54:5059-5063.