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Extended Abstract: *Abrogation of Cell Cycle Checkpoint Control in Preneoplastic Cells*

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SUMMARY Genomic integrity is maintained by a network of cellular activities that assesses the status of the genome at a given point in time and that provides signals to proceed with or to halt cell cycle progression. Recent studies have identified cellular proteins that are the targets for the viral oncoproteins involved in these processes. We demonstrate that the expression of human papilloma virus type 16 E6 and E7 oncoproteins in normal mortal cells disrupts the integration of the network of signals that maintain genomic integrity. *Radiat Oncol Invest 1996;3:320–322.* **© 1996 Wiley-Liss, Inc.**

Key words: gene amplification, genomic instability, human papillomavirus, p53, pRb

INTRODUCTION

Genetic instability is a well- documented event in virally transformed cells. Multiple karyotypic rearrangements have been reported in both in vivo samples as well as in tissue culture samples after expression of viral genomes. Although the documentation of these changes is well known, the basis for their occurrence is not known.

GENE AMPLIFICATION IN NORMAL AND NEOPLASTIC CELLS

Several years ago, we postulated that the in-depth genetic and molecular analysis of one type of genome rearrangement might provide insights into the cellular processes that maintain genomic integrity or that promote genomic lability. For this purpose, we initiated studies that examined the regulation of gene amplification. We have used amplification ability as a marker of genomic instability and as a prototype of how the acquisition of genetic instability may be involved in neoplastic initiation and progression.

In early studies, we found a correlation between the ability of a cell population to amplify endogenous genes and its ability to form a tumor when injected in the appropriate animal [1]. In a panel of rat liver epithelial cells, we observed a striking parallel between the ability of these cell lines to become resistant to the drug N-phosphonacetyl-L-aspartate (PALA) and their ability to form tumors after injection into day-old syngeneic rats. Cells exposed to PALA became PALA-resistant by amplification of the CAD gene. Molecular analyses of independent PALA-resistant subclones confirmed that, in each case, this resistance was due to amplification of the CAD gene. The frequency of gene amplification ranged from 10^{-3} in highly tumorigenic cell lines to 10^{-6} in nontumorigenic but immortalized cell lines. The Luria-Delbrück fluctuation analysis allowed us to determine that amplification of the CAD gene in the rat liver epithelial cells arose in a spontaneous fashion in the population. Our data indicated that PALA was not selecting for a small subpopulation of preexisting resistant cells; instead, amplification of the CAD locus occurred spontaneously in all rat liver epithelial cell lines studied [2]. In stark contrast, we and others found that gene amplification was undetectable ($<10^{-9}$) in primary diploid cell populations [3,4] when using the clonogenic assay, demonstrating a dramatic difference between primary diploid cell

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populations and transformed populations in their ability to generate PALA-resistant colonies. These results indicate a significant alteration in genomic stability between these two cell types and provide an assay for the identification of its molecular basis.

The above observations allowed us to address the genetic control of amplification potential in normal and neoplastic cells. We used somatic cell hybrids between cells that have a high frequency of amplification and those that have no detectable frequency of amplification to determine whether the ability to amplify was a dominant or a recessive trait. Such hybrids were suppressed in their ability to amplify the CAD gene [5]. We analyzed parental and hybrid cells for their ability to amplify the endogenous CAD gene by measuring PALA resistance. PALA-resistant subclones arose by amplification of the CAD locus. The GM2291 fibroblast and the normal diploid GM0077 fibroblasts (NHF) showed no detectable gene amplification. In contrast, the highly tumorigenic fibrosarcoma (HT1080) and carcinoma (D98/AH-2) cell lines amplified the CAD gene at high frequencies. The hybrid cells (fusion of a normal fibroblast and a fibrosarcoma cell line) displayed an amplification frequency several orders of magnitude lower than that measured in the parental tumorigenic cell line. Similar results were seen with other hybrid cell lines [5]. The next goal was to identify the genes responsible for this suppression of amplification.

The p53 gene presented a compelling and intriguing candidate for one of these genes for several reasons. In our published study [6], we asked whether the mutation or loss of one or both p53 alleles was sufficient to allow gene amplification to occur. We measured CAD gene amplification frequency in two different systems: LFS fibroblasts and embryonal fibroblasts from mice carrying a germline disruption of p53. These data suggested that p53 is one determinant in a pathway that regulates the ability to amplify endogenous genes in mammalian cells. Further studies, however, demonstrated that some human tumorigenic cells showed detectable gene amplification despite the presence of only wild-type p53, suggesting that alternate pathways can bypass the role of p53 and that p53 is not the sole determinant for the regulation of gene amplification.

GENE AMPLIFICATION IN CELLS EXPRESSING VIRAL ONCOPROTEINS

In more recent studies, we have developed a human model system for analyzing carcinogenic progression. We chose to examine normal human fibroblasts that were infected with viral oncoproteins. Two different viral oncoproteins were chosen, i.e., the E6 and E7 proteins of human papillomavirus (HPV) type 16. Introduction of the viral oncoproteins into normal human fibroblasts had a dramatic effect on the cellular potential to amplify [7].

To analyze amplification potential in these cells, we incubated them in PALA under the standard conditions of the clonogenic assay. Uninfected NHF cells (described above) and those containing the neo control vector lacked a detectable frequency of CAD gene amplification. In contrast, cells containing the expressed E6/E7 viral oncoproteins generated PALA-resistant colonies at a frequency of 10^{-5}. Cells that expressed E6 alone or E7 alone also generated PALA-resistant colonies, with the E7-containing cells exhibiting a frequency that was tenfold lower than that observed for the E6- or E6/E7-containing cells [7]. These frequencies are consistent with those previously observed in preneoplastic and neoplastic cell lines (from 10^{-5} to 10^{-3}). Cell cycle analysis in the presence of PALA indicated that none of the HPV-infected populations were arrested in the cell cycle, whereas the normal NHF cells were. Fluorescent in situ hybridization analysis of the E6-expressing PALA-resistant subclones verified intrachromosomal CAD gene amplification as the mechanism of resistance to PALA, whereas hybridization analysis of the E7-expressing PALA-resistant subclones verified aneuploidy as the mechanism of resistance to PALA. All PALA-resistant subclones were mortal, with the HPV 16 E6/E7 PALA-resistant clones exhibiting an extended life span, similar to the parental population [7]. These data imply that inactivating p53 alone or the cellular proteins that bind E7 (members of the Rb family) is sufficient to permit the normal human cells to undergo rearrangements. However, it is well known that these viral oncoproteins bind other cellular proteins as well, some of which may be involved in the phenotypic change we observed.

In summary, the data generated have allowed us to identify the first genes that modulate the ability of a cell to amplify. Both E6 and E7 expression individually can alter a normal diploid human fibroblast and allow amplification to occur. Each gene also has been found to relax cell cycle checkpoint control: Cells no longer respond to a negative growth signal by arresting proliferation. In addition, in-depth studies with p53 in both human and rodent cells have demonstrated that the generation of aneuploidy is also affected and that there is a dosage effect. The effects that these gene products elicited in normal human fibroblasts precede crisis, demonstrating that immortalization is not obligatory for these types of genomic changes to occur.

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