Deficiency of Retinoblastoma Gene in Mouse Embryonic Stem Cells Leads to Genetic Instability

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Abstract

Genetic instability has been recognized as a hallmark of human cancers. Retinoblastoma (Rb) tumor suppressor protein has an essential role in modulating cell cycle progression. However, there is no direct evidence supporting its role in maintaining genetic stability. Here, we developed a sensitive method to examine the level of chromosome instability by using retrovirus carrying both positive and negative selectable markers that integrated randomly into individual chromosomes, and the frequency of loss of this selectable chromosomal marker (LOM) in normal mammalian cells was measured. Our results showed that normal mouse embryonic stem (ES) cells had a very low frequency of LOMs, which was less than $10^{-9}$/cell/generation. In Rb−/− mouse ES cells, the frequency was increased to approximately $10^{-7}$/cell/generation, whereas in Rb+/− ES cells, the frequency was approximately $10^{-7}$/cell/generation. LOMs was mediated mainly through chromosomal mechanisms and not through point mutations. These results, therefore, revealed that Rb, with a haplo-insufficiency, plays a critical role in the maintenance of chromosome stability. The mystery of why Rb heterozygous carriers have early-onset tumor formation with high penetrance can be, at least, partially explained by this novel activity.

Introduction

Genetic instability is one of the most important hallmarks of cancer (1). Associations of tumor suppressors with the process of chromosome behavior provide possible links between carcinogenesis and genetic instability. Aneuploidy, chromosome structural rearrangements, centrosome amplification, and gene amplification have been observed in p53-deficient cells (2) and, more recently, in APC-deficient cells (3), which supports a potential role of tumor suppressors in the maintenance of genetic stability.

The roles of Rb in cell cycle regulation and differentiation are well established and can explain how Rb suppresses tumor growth, but do not completely explain why cancer susceptibility results from loss of Rb function (4). In particular, the mystery as to why the inactivation of Rb leads to multiple genetic alterations that predispose cells to the process of tumorigenesis remains unsolved. In addition to its role in G1 progression, several lines of evidence suggest that Rb also plays a significant role at G2-M phases. First, the hypophosphorylated form of Rb, the functional form, is present in these cell cycle stages. It appears that Rb becomes dephosphorylated as Rb interacts with protein phosphatase 1 specifically during G2-M phases (5). The yeast homologue of protein phosphatase 1α has been shown to be essential for kineto-
prepared feeder layer, and amplified. To determine the genotype of the ES cells, genomic DNA was extracted, and PCR was performed as described previously (17). The selected three genotypes of the ES cells were karyotyped and had apparently normal chromosome pattern.

Selection of Individual ES Clones Infected with HygTK Retrovirus. The HygTK fusion gene (18) was inserted between the two LTRs derived from Moloney murine leukemia virus in a previously described vector (19). The resultant vector pHLL1-HygTK was transfected into the retroviral packaging cell lines and the HygTK virus was harvested. Approximately 2 × 10^5 ES cells, isolated as above, described at the 12th passage with the genotype of Rb^+/+, Rb^-/-, and Rb^-/-, respectively, were grown on a hygromycin-resistant feeder layer derived from embryonic fibroblasts of transgenic mice expressing the hygromycin phosphotransferase gene (The Jackson Laboratory). Cells were infected by the HygTK virus for 24 h in the presence of Polybrene and subsequently treated with 250 µg/ml hygromycin after another 24 h. Hygromycin-resistant clones were picked after 9 days of selection and afterward maintained in the ES cell culture medium containing 200 µg/ml hygromycin. Clones were individually amplified, and PCR analysis indicated that all of the hygromycin-resistant colonies carried the integration of the HygTK fusion gene (data not shown).

Results

Establishing a Method for Measuring the Globally Genetic Instability in Mammalian Cell. To follow the dynamic changes of individual chromosomes, a selectable marker was permanently integrated on the chromosome by retrovirus-mediated gene transfer, which would be expected to harbor a single copy of this marker randomly on individual chromosomes (19). By tracing this marker in multiple virally infected clones, one would have a global view of genetic alterations on chromosomes in general rather than at one specific locus. For the feasibility of detecting the presence or absence of the marker, we chose a fusion gene that combines the hygromycin phosphotransferase (Hyg) gene and herpes simplex virus type 1 thymidine kinase gene (TK) in frame. Translation of this fusion gene into a single bifunctional enzyme protein (designated hereafter as HygTK) confers both resistance to hygromycin and sensitivity to ganciclovir (18), which provides both positive and negative selectivity.

The HygTK fusion gene was inserted between two LTRs derived from Moloney murine leukemia virus (19); and thereby, its expression was under the control of the LTR promoter (Fig. 1A). In an attempt to explore the overall frequencies of LOMs regardless of the viral integration loci, more than 50 individual hygromycin-resistant colonies with approximately equal number of cells were mixed together. The mixture of cells was maintained in the hygromycin-containing medium, and then transferred to a selection-free medium in which the cells were to be propagated for about 10 generations. Before this propagation step, 1 × 10^7 cells were removed from the hygromycin-containing medium and immediately seeded in the ganciclovir-containing medium, and the ganciclovir-resistant colonies were counted to obtain Eo (Fig. 1B). After the propagation (in the selection-free medium), the cell number was counted; the number of generations through which the cells had been propagated was calculated (usually between 9 and 10 generations); 1 × 10^7 cells were seeded in the ganciclovir-containing medium; and, after 12 days of ganciclovir-selection, the ganciclovir-resistant colonies were counted to obtain Ep (Fig. 1B).

The ganciclovir-resistant colonies that arose were from those cells that had lost the functional TK gene and, thus, were no longer sensitive to ganciclovir. Maintaining the cells in the hygromycin-containing medium would have prevented the loss of the entire HygTK gene; therefore, ganciclovir-resistant cells were not detected or were detected at a frequency less than 10^-6. The appearance of ganciclovir-resistant cells before the cells were propagated in nonselective medium would be expected, because the accumulation of mutations during this long-term culture could have inactivated the TK gene in some hygromycin-resistant cells. This frequency, however, was subtracted from that of ganciclovir-resistant cells analyzed after the propagation, and the resultant subtraction represented the frequency of cells that had the TK gene inactivated during the propagation. Finally, this subtraction was sequentially divided by the total cell number and by the generation numbers to deduce the frequency of LOMs at the HygTK-integrated loci in one cell per division generation (Fig. 1C).

Loss of Rb Gene Increases Frequency of LOMs. The above method is ideal for cells with high colony forming efficiency. Normal human fibroblasts and mouse embryonic fibroblasts have poor colony-forming efficiency, at about 10^-3 to 10^-4. Therefore, it requires a substantial amount of cells for measuring LOMs by this method. Any immortalized cell lines, including cancer cell lines, are prone to genetic changes. The selection process in this procedure may create a bias toward certain changes and generate artifacts for this measuring. To circumvent these difficulties, we used ES cells to perform this experiment because of their normalcy and high colony-forming efficiency. Three mouse ES cell lines with normal karyotype from the same litter were used; one with the wild-type Rb, the other with one allele of Rb mutated at exon 20, and another one with both alleles mutated at exon 20 (15). Our results indicated that the LOM frequency of Rb^+/+ cells was lower than 10^-6, the frequency of Rb^+/− cells was between 10^-7 and 10^-6, and the frequency of Rb^-/- cells was higher than 10^-3 (Fig. 2). These results, therefore, suggest that the frequency of LOMs is increased in cells homozygous for the null mutation of Rb and moderately increased in cells heterozygous for Rb.

To determine whether the experiments with mixed viral-infected colonies would reveal the average frequencies of LOMs regardless of the viral integration loci, we examined the LOM frequencies at individual integration loci. Viral-infected cells were selected by hygromycin and hygromycin-resistant clones were randomly picked and individually cultured. PCR analysis indicated that all of the selected clones carried the integration of the HygTK fusion gene (as shown

Fig. 1. A method for measuring the frequency of LOMs using the retroviral-integrated HygTK fusion gene as reporter. A, schematic structure of the retrovirus carried the HygTK fusion gene. B, experimental steps outlined for measuring the frequency of loss of heterozygosity. C, formula for calculating the frequency of loss of heterozygosity. \( \text{Feq}_{LOM} \) is the frequency of loss of the marker per cell per generation. \( \text{Ep} \) and \( \text{Eo} \) are designated as in B. N, the number of generations.
RB PLAYS A CRITICAL ROLE IN GENETIC STABILITY

Loss of the Marker Involves Larger Deletion of Chromosomal Event Instead of Point Mutation. To explore the mechanisms underlying the loss of functional TK gene, we examined the status of the H
gTK fusion gene in the ganciclovir-resistant cells by PCR. As shown in Fig. 4, A and B, PCR was not able to amplify any fragments of the H
gTK fusion from genomic DNA of the ganciclovir-resistant clones that were randomly picked. This result indicated that ~90% of ganciclovir-resistant clones had lost the entire H
gTK fusion gene physically. It also suggested that point mutations or small deletions that could have inactivated the TK gene occurred at a low frequency (~10%). As suggested previously, potential mechanisms attributed to the loss of a whole gene include chromosomal loss, mitotic recombination, and interchromosomal rearrangement, which are all chromosome mechanisms (20). Therefore, increased frequency of LOMs observed in Rb+/- cells suggests that Rb-deficiency would lead to chromosome instability.

Interestingly, retaining one wild-type Rb allele does not appear to be sufficient for the maintenance of chromosome stability, because Rb+/- cells also have a moderately increased frequency of LOMs compared with the wild-type cells. It would, however, be possible that chromosome instability is a consequence of the loss of the remaining wild-type Rb allele. To examine this possibility, these ganciclovir-resistant clones derived from Rb+/- cells were genotyped for the wild-type Rb allele by PCR. The result indicated the clones picked randomly all retained one wild-type Rb allele (Fig. 4C), which suggested the haploinsufficiency of Rb in maintaining chromosome stability.

Discussion

Genetic instability in Rb-deficient cells could be responsible for further genetic alterations involved in cancer development. Germ-line

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Total 31 25 22

Fig. 3. Frequencies of LOMs in individual infected clones. A, number of clones of each genotype of ES cells is listed in the table (A) with frequencies of LOMs falling in each indicated logarithm range. B, histogram shows the percentage of clones falling in each indicated logarithm range deduced from A.
RB PLAYS A CRITICAL ROLE IN GENETIC STABILITY

Fig. 4. Genotyping PCR of the HygTK fusion gene and the Rb allele. A, schematic diagram of the genomic structure of the HygTK-integrated loci. PCR primers for amplifying various regions of the HygTK gene are indicated at their relative positions. B, genotyping PCR of the HygTK gene. Lanes 1–5, representatives of the hygromycin-resistant clones that carry the HygTK fusion gene; Lanes 6–15, representatives of ganciclovir-resistant clones. The approximate size of PCR products and corresponding primers used are indicated. Bottom panel, two pairs of primers as indicated were used in the same reaction. Primers amplifying a region of the Breal genome were used as control. C, genotyping PCR showed that the heterozygosity of the Rb allele was retained. Lanes 1–8, representatives of hygromycin-resistant clones of Rb+/− ES cells that carry the HygTK fusion gene; Lanes 9–27, representative of ganciclovir resistant clones of Rb+/− ES cells that have lost the HygTK fusion gene. 236 bp, PCR products derived from the target allele; 151 bp, products derived from the wild-type Rb allele.

mutations in one Rb allele lead to the development of retinoblastoma in human and pituitary tumors in mice at very early ages and with nearly complete penetrance (15, 21). The remaining wild-type allele is lost as a somatic event and, as suggested, mainly through chromosome mechanisms (20, 22). It has been estimated previously, based on the mean number of tumors occurring in carriers of Rb, that the mutation rates in both events are nearly equal (21).

In this study, by directly accessing the mutation rate, one view suggested that the mutation rate increases when the first Rb allele is inactivated. Chromosome instability in Rb-heterozygous cells could explain the high penetrance of tumor development with loss of the remaining wild-type allele. Nonetheless, the loss of the second Rb allele appears to be the threshold event in tumor development, given that it results in much more severe genetic instability, which might account for all of the following genetic alterations essential for tumorigenesis.

The possibility cannot be excluded that Rb could also suppress the frequency of point mutations or small deletions, considering that the HygTK gene may not serve as an appropriate reporter for small nucleotide changes. However, our observations implicating a low frequency of point mutations or small deletions in Rb-deficient cells is consistent with previous reports indicating that the loss of the remaining Rb allele in the majority of Rbs is mediated by chromo-

some mechanisms (20, 22). In addition, chromosome mechanisms appear to be the major cause of loss of heterozygosity during tumorigenesis based on studies with different tumor suppressor genes (23).

In previous studies, reporters such as the adenine phosphoribosyltransferase (APRT) gene at a specific chromosome locus have been used to evaluate the frequency of LOMs (23, 24). The new method used in this study, by using retroviral infection to integrate the reporter randomly on different chromosomes, allows us to access comprehensively all of the chromosome behaviors in a cell. In addition, the loss of reporter is prevented by positive selectivity conferred by the same reporter gene; thus, the new method is able to evaluate precisely the level of chromosomal instability.

Chromosome abnormality in Rb-deficient fibroblasts has not yet been reported. The average frequency of LOMs observed in Rb−/− cells is approximately 10−7. Classical methods such as multiplex fluorescent in situ hybridization and spectral karyotyping (25) would not be able to catch any aberrance if vast numbers of cells were not subjected to such analysis. By contrast, the new method appears to be more sensitive in evaluating the chromosome instability. However, unlike fluorescent in situ hybridization or spectral karyotyping, this new method does not reveal the types of chromosome aberrance directly. Our method has to be followed by traditional polymorphism marker analysis on the viral-integrated chromosome if one is interested in what types of chromosome mechanisms are involved.

Although nondisjunction is apparently a result of improper chromosome segregation, how other types of chromosome aberrance occur remains to be clarified. Chromosome mechanisms underlying LOMs in Rb-deficient cells are most likely comprised of multiple types of chromosome aberrance because Rb appears to be capable of modulating chromosome metabolisms from different, but intimately related, aspects including chromosome replication, segregation, and structural maintenance (4). Given that Rb is regarded as the prototype for tumor suppressors, it will be even more interesting to examine the role of other tumor suppressors in maintaining chromosome stability by this new method. It is conceivable that the other tumor suppressors have a common role in the maintenance of chromosome stability, and such a role may be pivotal for their functions in tumor suppression.

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References


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