

A Cytogenetic Footprint for Mammary Carcinomas Induced by PhIP in Rats

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ABSTRACT

PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine), a mutagen/carcinogen belonging to the class of heterocyclic amines (HCAs) found in cooked meats, is a mammary gland carcinogen in rats and has been implicated in the etiology of certain human cancers including breast cancer. To gain insight into the genomic alterations associated with PhIP-induced mammary gland carcinogenesis, we used comparative genomic hybridization (CGH) to examine chromosomal abnormalities in rat mammary carcinomas induced by PhIP, and for comparison, by DMBA (7, 12-dimethylbenz[*a*]anthracene), a potent experimental mammary carcinogen. There was a consistent and characteristic pattern of chromosome-region loss in PhIP-induced carcinomas that clearly distinguished them from carcinomas induced by DMBA.

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PhIP belongs to the class of heterocyclic amine (HCA) food mutagens/carcinogens derived from cooked meats, and is the most prevalent of the known carcinogenic HCAs in the human diet. Mammary carcinogenesis in the carcinogen-treated rat and in the human is similar in the site of origin of carcinomas from ductal elements, the susceptibility of the mammary gland to initiation during mammary gland development (i.e., period of adolescence), and the hormonal responsiveness of the tumors; thus mammary carcinogenesis in rats is recognized as a valuable model for the human disease [1, 2]. To determine the progression of PhIP-induced mammary carcinomas in rats, we have used a series of procedures to analyze the genomes of the cancerous cells, from gross chromosomal aberrations to single base mutations.

We examined by CGH carcinomas that were induced in female Sprague-Dawley rats by either PhIP or DMBA, a potent experimental mammary gland carcinogen belonging to the class of polycyclic aromatic hydrocarbons. PhIP carcinomas were obtained from a previous study in which tumors were induced over a 25-week period after administration of PhIP (75 mg/kg, p.o., once per day, for 10-days) or DMBA (10mg/kg, p.o., single dose) to 50-day old rats [3]. Vehicle control animals were run in parallel in both the PhIP and DMBA studies, and none of the control rats developed mammary carcinomas [4]. This technique produces cancers only in animals treated with the specific agent, avoiding spontaneous tumors that can complicate analyses [5, 6].

Although routinely applied to studies of the genomic alterations in mouse and human tumors, CGH has not been widely utilized in studies of the rat genome. It is used to detect genomic changes such as losses, gains and amplifications of chromosomes and chromosome regions, and can provide valuable information about solid tumors from which metaphase cells are difficult to obtain [7-9]. We examined 9 different tubulopapillary carcinomas, 6 induced by PhIP and 3 induced by DMBA. All 9 carcinomas contained genomic changes detectable by CGH as either losses or gains/amplifications of chromosomal regions. We found a consistent and characteristic pattern of chromosome-region loss in PhIP-induced carcinomas that clearly distinguished them from carcinomas induced by DMBA.

With the long-term goal of determining which genes in these common deleted regions are involved in PhIP-induced mammary carcinogenesis, we developed a method, referred to as gene recovery microdissection (GRM), to generate chromosome region-specific cDNA libraries to characterize and map the expressed genes in normal rat mammary gland. This approach is generally applicable for studies of species with incompletely characterized genomes, such as the rat, and will enable us to characterize the PhIP signature at the genetic level.

While the rat is a valuable experimental model for studies of carcinogenesis, rat genetics remains relatively understudied compared with mouse and human. Since the rat genome has not yet been mapped to the extent of humans or even mice, it is currently difficult to determine which genes in these deleted regions might be important for tumorigenesis. Thus, another means must be used to identify the known or novel genes in these regions that are expressed in normal rat mammary tissue and might therefore be important in carcinogenesis. We have developed a technique that combines HeadStart microdissection [10] with a process variously referred to as preparative in situ hybridization [11] or microdissection-mediated cDNA capture [12, 13]. GRM is a means for creating a chromosome region-specific cDNA library for any species and tissue from a single microdissected normal metaphase chromosome, eliminating the need to dissect multiple copies of the target. The process involves hybridizing a cDNA library specific for the species and tissue of interest onto degenerate oligonucleotide primed-PCR amplified metaphase chromosomes. This step also normalizes the library, increasing the ratio of less prevalent to more prevalent expressed genes [14]. PCR is used again to amplify the cDNA molecules in situ

following hybridization, and the desired chromosome regions are isolated by microdissection. The cDNA molecules hybridized to the dissected genomic DNA are then amplified by PCR in a tube by using primers specific for the linker arms on the cDNA. The cDNA molecules are then cloned and sequenced. In situ amplification followed by microdissection allows complex libraries to be made from single microdissected chromosomes and chromosome regions, possibly by increasing both the number of targets for in situ hybridization and probe accessibility to the target chromosomes. Once likely target genes have been selected, cDNA libraries from tumors can be made and the mRNA representing the expressed gene can be isolated and sequenced. This allows variation in the genetic sequence to be found. The GRM technique provides a valuable link between the cytogenetic and physical mapping techniques that will quickly and reliably determine which genes are expressed in specific chromosomal regions of a particular tissue.

Once mutations in a particular gene have been discovered, it is desirable to learn where in the tumor these changes occur, as the location of a particular mutation can give some indication of its role in tumor initiation and progression. To locate these point mutations, we have modified the procedure called rolling circle amplification (RCA) to function in fixed cells and tissue sections. The utility of RCA has been well documented [15-19]. RCA is a molecular cytogenetic technique used in conjunction with a 'padlock' oligonucleotide probe to locate and detect single base changes in DNA and RNA. Padlock probes are sequences comprised of ~100 nucleotides designed to hybridize to targets of approximately 30 bases, of which 15 are present in opposite orientation at each end such that the hybridized probe forms a circle with adjacent 3' and 5' termini [20, 21]. At 10 bases per helical turn, the hybridized probe is wrapped around its target 3 times, with the remaining 70 bases forming an unhybridized single-stranded loop. Post-hybridization DNA ligation is used to connect the two ends of the probe in the middle of the 30 base binding region. The 70 base loop that is not bound to the target not only facilitates circularization of the probe, but permits approximately 20 bases to serve as a primer recognition site enabling DNA polymerase to replicate the circle. RCA is an isothermal process in which the polymerase progresses continuously around the loop until the 100 bases have been replicated hundreds or thousands of times. Incorporating a labeled nucleotide during the RCA reaction produces sufficient signal to permit easy visualization of the target. To detect point mutations, the padlock probe is designed to place one of the two terminal bases directly over a pre-determined base of the target. To ascertain whether a particular base is mutated, two or more nearly identical vectors are constructed, one containing the complement to the normal base and the others complementary to the mutated base. If the terminal base in the probe is not complementary to the target, that base will not hybridize, preventing ligation and blocking the polymerase from progressing continuously around the loop with the result that no fluorescent signal will be generated. Consequently, the mutant and normal target sequences will only be detected by their respective probes. Designing a series of vectors to produce signals in different colors following the RCA allows, in effect, single nucleotide polymorphisms to be detected rapidly and efficiently.

When used in succession, CGH, GRM and RCA enable carcinomas to be analyzed from the gross cytogenetic level to the single base mutation. In this way, the genetic progression of the tumor can be studied.

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