Clonal Analysis of Fibroadenoma and Phyllodes Tumor of the Breast

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ABSTRACT

Clonality of fibroadenoma and phyllodes tumor of the breast was analyzed by means of the polymerase chain reaction using small DNA samples prepared from cryostat sections. The method of clonal analysis was based on restriction fragment length polymorphism of X-chromosome-linked phosphoglycerokinase gene and on differential methylation of the gene.

Specimens from 10 fibroadenomas and 5 phyllodes tumors heterozygous for the BstXI polymorphism of PGK gene were subjected to clonal analysis. It was found that fibroadenoma was polyclonal, but phyllodes tumor was made up of both monoclonal and polyclonal cell components. Since these tumors contained both epithelial and stromal components, clonality of each component was analyzed separately. Analysis of clonality of each cell component showed that both the epithelial and stromal cells were polyclonal in fibroadenoma and that the epithelial cells were polyclonal, but the stromal cells were monoclonal in phyllodes tumor. When DNA samples were prepared from widely separated sites of phyllodes tumors, every sample was found to contain a monoclonal stromal cell component. These results demonstrate that fibroadenoma is a hyperplastic lesion rather than a neoplasm, and that phyllodes tumor is a neoplasm of the stromal cells.

INTRODUCTION

Fibroadenoma is the third most frequent tumor of the breast, being exceeded only by gross cystic disease and carcinoma (1). The average age of patients with this disease is reported to be 20 to 30 years (1, 2). It presents as a well-demarcated and mobile tumor that is histologically constituted of both epithelial and stromal components. Natural history of fibroadenoma bears a benign nature. It grows slowly, and the growth often stops after the fibroadenoma reaches 2-3 cm in diameter (1). In a considerable proportion of patients, fibroadenoma becomes smaller in size or resolves completely (3). Thus, it is proposed that, as an alternative to surgical excision, cytologically diagnosed fibroadenomas can be clinically monitored because they have no intrinsic premalignant potential and tend to regress with time (2).

Phyllodes tumor is a relatively rare disease of the breast that accounts for 0.3-0.9% of all breast tumors (4). The average age of patients with phyllodes tumor is around 40 years, and 10 to 20 years older than those with fibroadenoma (1). Phyllodes tumor, like fibroadenoma, presents as a well-demarcated and mobile breast tumor and is indistinguishable from fibroadenoma by mammographic and ultrasonographic examinations. Phyllodes tumor also shares the same histological characteristics with fibroadenoma, in that the tumor has both epithelial and stromal components and their histological arrangement is somewhat similar. However, in phyllodes tumor, the stroma is more cellular and is made up of cells that vary in size and shape, and it sometimes undergoes a sarcoma-like change. Furthermore, there is a distinct difference in natural history between fibroadenoma and phyllodes tumor. Phyllodes tumor grows to be a large tumor usually measuring more than 10 cm in diameter, and, occasionally, it recurs locally or metastasizes to the lung. Thus, treatment of choice for phyllodes tumor is wide local excision or mastectomy.

Since clonal analysis provides valuable information on histogenesis and progression of a tumor, it is of interest to analyze the clonality of fibroadenoma and phyllodes tumor to provide new insights into their histogenesis and to elucidate the reason why their natural history is so different. Until now, no report has been available on the clonality of these tumors. In 1985, Vogelstein et al. (5) reported a method of determining clonality of human tumors based on restriction fragment length polymorphism on X-chromosome-linked genes (PGK and hypoxanthine phosphoribosyl-transferase) and on random inactivation of 1 of 2 X-chromosomes by methylation. This method was later modified and developed by introduction of the PCR and became applicable to small DNA samples (6). Recently, we have applied this method to clonal analysis of small DNA samples obtained from cryostat sections of breast cancer and have found that breast cancer is monoclonal in origin (7). In the present study, we have used the same method for clonal analysis of fibroadenoma and phyllodes tumor, and have found that fibroadenoma is composed of polyclonal epithelial and stromal cells, whereas phyllodes tumor is composed of polyclonal epithelial cells and monoclonal stromal cells.

MATERIALS AND METHODS

Tissue Source. Twenty-five fibroadenomas and 12 phyllodes tumors, resected during the period from April 1986 to September 1992 at our institute, were utilized in this study. Of these tumors, 10 fibroadenomas and 5 phyllodes tumors that were heterozygous for the BstXI polymorphism of PGK gene were subjected to clonal analysis. The median age of patients with fibroadenomas was 28 years (range, 21-38 years), and that of those with phyllodes tumors was 42 years (range, 16-52 years). The median sizes of fibroadenomas and phyllodes tumors were 2.5 (range, 2.0-3.5 cm) and 15.0 (range, 6.0-20.0 cm), respectively. All 5 phyllodes tumors were histologically diagnosed as benign. Surgical specimens of fibroadenomas and phyllodes tumors were embedded in OCT compound (Miles, Inc., Elkhart, IN) and were snap-frozen in liquid nitrogen. The samples were kept at -80°C.

DNA Extraction. DNA extraction from frozen sections embedded in OCT compound was performed according to the method described previously (7). In brief, 6 serial 10-µm sections (approximately 5 x 5 mm) were cut with a cryostat. The first section was stained with hematoxylin and eosin for a histological confirmation of accurate sampling of tumor tissue. The remaining 5 sections were subjected to DNA extraction with a Pronase digestion followed by phenol/chloroform extraction (7). Finally, extracted DNA was suspended in 20 µl of water.

To separately prepare DNA from the epithelial cell and stromal cell component, 10 serial 10-µm frozen sections were cut on glass slides. Each component was dissected using a 27-gauge needle with the assistance of an inverted microscope and reference to a section stained with hematoxylin and eosin. DNA extraction from the epithelial and stromal component was carried out as described above. Finally, extracted DNA was suspended in 20 µl of water.

Primers. A restriction map of PGK gene and DNA sequence of the primers are shown in Fig. 1. To amplify the region of PGK gene that contains BstXI polymorphic site and the differentially methylated HpaII site, nested oligonucleotide primer pairs flanking this region were synthesized.

PCR. To 20 µl of DNA solution prepared from the cryostat sections and from the epithelial and stromal cells, 16 µl water and 4 µl of 100 µM Tris, 100 mM MgCl2, 10 mM dithiothreitol, pH 7.5, were added and split into halves. These samples of DNA solution were incubated in the presence or absence of

Received 1/25/93; accepted 6/23/93.

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2 The abbreviations used are: PGK, phosphoglycerokinase; PCR, polymerase chain reaction; PT, phyllodes tumor.
**Clonality of Fibroadenoma and Phyllodes Tumor**

**Strategy of Clonal Analysis by Means of PCR.** We conducted clonal analysis of fibroadenoma and phyllodes tumor according to the method of Gilliland et al. (6). In females heterozygous for the BstXI polymorphism of PGK gene, somatic cells are composed of 2 types of cells, i.e., those with inactive PGK gene with BstXI restriction site and those with inactive PGK gene without BstXI restriction site. PGK gene inactivation of 1 of 2 X-chromosomes by methylation. In the method of clonal analysis by PCR, at first, DNA samples were digested with a methylation-sensitive restriction enzyme, HpaII, to cleave the unmethylated, active PGK gene (Fig. 1, hatched bar). The methylated, inactive PGK gene is preserved after a HpaII digestion. If tumors are polyclonal, tumor cells should be composed of 2 types of cells, i.e., those with inactive X′-chromosome and those with inactive X″-chromosome. Thus, both X′-chromosome and X″-chromosome were preserved after a HpaII digestion. Amplification by PCR of PGK gene resulted in an amplification of PGK gene both with and without BstXI digestion. Tumors are monoclonal, tumor cells should be composed of one type of cell, i.e., those with inactive X′-chromosome or those with inactive X″-chromosome. Thus, either X′-chromosome or X″-chromosome was preserved after a HpaII digestion. Amplification by PCR of PGK gene resulted in an amplification of either PGK gene with or without BstXI site, giving rise to 2 DNA base pairs, in the agarose gel electrophoresis. If tumors are monoclonal, tumor cells should be composed of one type of cell, i.e., those with inactive X′-chromosome or those with inactive X″-chromosome. Thus, either X′-chromosome or X″-chromosome was preserved after a HpaII digestion. Amplification by PCR of PGK gene resulted in an amplification of either PGK gene with or without BstXI site, giving rise to a 530-base pair band or a 433-base pair band in the agarose gel electrophoresis.

When DNA samples were amplified without a HpaII digestion, both PGK alleles were amplified. Thus, electrophoretic analysis of these amplified samples after a BstXI digestion gave rise to 2 bands, 530 and 433 base pairs, regardless of the clonality of tumors.

**RESULTS**

Clonality of Fibroadenoma and Phyllodes Tumor. Clonal analysis was done in 10 fibroadenomas and 5 phyllodes tumors using DNA samples prepared from cryostat sections. Representative results are shown in Fig. 2. Agarose gel electrophoresis of the PCR-amplified and BstXI-digested products from fibroadenomas gave rise to 2 DNA bands, 530 and 433 base pairs, regardless of the presence or absence of HpaII digestion before the PCR amplification, indicating that fibroadenoma is polyclonal. All 10 fibroadenomas analyzed were found to be polyclonal in origin. The ratio of intensity of the 530-base pair band to the 433-base pair band were approximately 3:1 when the samples were polyclonal. As suggested by Gilliland et al. (6), this phenomenon can be explained by the fact that 50% of the amplified strands will reanneal as heteroduplexes. Only the predicted 25% that have the BstXI site on each strand of a homoduplex would be digested.

When DNA samples were not precut with HpaII, electrophoresis of the PCR-amplified and Bst XI-digested products from phyllodes tu-

**Table 2. Clonal analysis of fibroadenoma and phyllodes tumor.** DNA samples were prepared from cryostat sections of fibroadenomas and phyllodes tumors. Clonal analyses of these samples were done as described in "Materials and Methods." Results of agarose gel electrophoresis of 2 representative fibroadenomas and phyllodes tumors are shown. bp, base pairs. Intensity ratios of the 530- to 433-base pair band quantified by a densitometer were 3.2, 2.8, 3.0, 2.8, 2.5, 6.8, 3.0, and 0.9 from left to right lanes, respectively.
tumors gave rise to 2 bands, the intensity ratio of which was approximately 3:1 (Fig. 2). However, when DNA samples were precut with $Hpa$ II, it gave rise to 2 bands, the intensity ratio of which was apparently different from 3:1 (Fig. 2). In PT-1, the intensity ratio of the 530-base pair band to the 433-base pair band was 6.8:1 and, in PT-2, it was 0.9:1. These results suggest that phyllodes tumor consists of both monoclonal and polyclonal cell components. Essentially, the same results were obtained in the other 3 phyllodes tumors (results of PT-3 and PT-4 are shown in Fig. 4).

Clonal Analysis of Epithelial Cells and Stromal Cells Prepared from Fibroadenoma and Phyllodes Tumor. Clonal analysis of epithelial and stromal cells of fibroadenomas (Fig. 3, FA-1 and FA-2) showed that both cellular components were since 2 bands (intensity ratio, approximately 3:1) appeared regardless of the presence or absence of a $Hpa$ II precut. Clonal analysis of epithelial cells of phyllodes tumors (Fig. 3, PT-1 and PT-2) also showed a polyclonal pattern. In the analysis of the stromal cells of PT-1 tumor, however, $Hpa$ II-precut samples gave rise to an almost single 530-base pair band and, in that of PT-2 tumor, the intensity of the 433-base pair band was far stronger than that of the 530-base pair band. These results indicate that the stromal cells of phyllodes tumors are predominantly composed of monoclonal cell component, although a small proportion of polyclonal component also exists.

In 2 phyllodes tumors, DNA samples were prepared from 3 widely separated sites to study the intersite variation of clonality (Fig. 4). In both tumors, every sample showed an electrophoretic pattern that a band was far stronger than that of the 530-base pair band. These results suggest that phyllodes tumor consists of polyclonal epithelial and monoclonal stromal cells. Thus, this tumor was preserved after $Hpa$ II digestion.

**DISCUSSION**

It is generally accepted that a rarely occurring set of somatic mutations, regardless of the number of steps required to induce a malignant state, are responsible for the development of cancer (8). This somatic mutational theory predicts that the resulting tumors will be monoclonal in origin, since it is unlikely that an identical sequence of mutations will occur independently in 2 neighboring cells. Consistent with this theory, various types of benign and malignant human neoplasms have been shown to be monoclonal in origin (9). On the other hand, in hyperplasia where multiple original cells respond to an exogenous or endogenous stimulus, a polyclonal expansion is to be expected. Thus, clonal analysis appears to be a powerful method of distinguishing neoplasia from hyperplasia. In parathyroid tumors, clonal analysis has been successfully applied for the differential diagnosis between hyperplasia and adenoma (10).

Although fibroadenoma is usually classified as a benign neoplasm of the breast in most texts, it seems to be consistent with a hyperplastic state or an abnormal development of a lobule rather than a neoplasm (3). Facts in support of this are (11): (a) use of elastic stains shows that each fibroadenoma develops from a single lobule; (b) fibroadenomas closely resemble hyperplastic lobules histologically that are common in normal breasts; (c) growth of most fibroadenomas stops after they reach 2 to 3 cm in diameter or a significant percentage of them spontaneously regress; and (d) fibroadenomas show the same hormonal dependency as the normal breast, i.e., some fibroadenomas grow rapidly during pregnancy and they lactate. Our observation that fibroadenoma consists of polyclonal epithelial and stromal cells is additional and definitive evidence in support of the thesis that fibroadenoma is not a neoplasm but hyperplasia of a lobule.

Clonal analysis has shown that phyllodes tumor consists of polyclonal epithelial cells and monoclonal stromal cells. Thus, this tumor can be regarded as a neoplasm of stromal cells. This finding is consistent with histological characteristics of this tumor: that cellularity of the stromal cells is high and that the epithelial component decreases as the size of tumor increases. In addition, monoclonal growth of stromal cells is compatible with the fact that the metastatic lesions of phyllodes tumor in the lungs are composed of only stromal cells.

Since phyllodes tumor is fairly often preceded or accompanied by fibroadenoma and both tumors have some histological similarity, it is suggested that histogenesis of these tumors is related (1). Our results on clonality also imply a histogenetic relationship of these tumors. Our hypothesis on the histogenesis of phyllodes tumor is as follows: The neoplastic component of phyllodes tumor is stromal cells, but monoclonal growth of the stromal cells is unlikely to occur from the onset. If it does occur from the onset, the resulting tumors should be made up of only stromal cells such as fibroma or fibrosarcoma. Phyllodes tumor, however, consists of both epithelial and stromal cells. Thus, it is speculated that phyllodes tumor begins as fibroadenoma (hyperplasia of a lobule) and, subsequently, a single stromal cell suffers somatic mutation and develops to form a phyllodes tumor composed mainly of monoclonal stromal cells and partially of polyclonal epithelial cells.

In conclusion, we have shown that fibroadenoma is composed of polyclonal epithelial and stromal cells and, therefore, it is better considered as a hyperplasia of a lobule rather than a neoplasm.
Phyllodes tumor has been shown to be a neoplasm of stromal cells but not of epithelial cells. These results may be clinically applicable to distinguish fibroadenoma and phyllodes tumor, since the PCR-based method for clonal analysis can be done on small DNA samples obtained by fine needle aspiration cytology. A prospective study is ongoing to evaluate the usefulness of clonal analysis on fine needle aspirates in the differential diagnosis of these tumors.

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