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# Clonal Analysis of Solitary Intraductal Papilloma of the Breast by Means of Polymerase Chain Reaction

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***Clonality of solitary intraductal papillomas of the breast was analyzed using a method based on restriction fragment length polymorphism of the X-chromosome-linked phosphoglycerokinase (PGK) gene and on random inactivation of the gene by methylation. The application of polymerase chain reaction to this method enabled clonal analysis of such a small intraductal lesion as papilloma. Clonal analysis of DNA samples obtained from the nine solitary intraductal papillomas and adjacent normal breast tissues showed that all of the papillomas were monoclonal and all the normal breast tissues were polyclonal in origin. When DNA samples were obtained from four widely separated sites in the papillomas, clonal analysis showed that all were monoclonal and, in addition, the same allele of PGK gene was inactivated in each case. These results demonstrate that solitary intraductal papilloma arises as a single monoclonal tumor and extends along the ducts rather than occurring as multicentric monoclonal tumors and merging together subsequently. Immunohistochemical staining of smooth muscle α-actin, a marker protein of myoepithelial cells, revealed that solitary intraductal papilloma was composed of approximately equal mixtures of luminal epithelial and myoepithelial cells. Since solitary intraductal papillomas were shown to be monoclonal in origin, it was suggested that this disease originates from a common precursor that could differentiate into both luminal epithelial and myoepithelial cells. (Am J Pathol 1994, 144: 1320–1325)***

Solitary intraductal papilloma originates from the epithelium of the terminal ducts beneath the areola of the breast. Its size is usually 2 to 3 mm in diameter, and it attaches to the duct wall by a pedicle. Solitary intraductal papilloma may extend along the duct in which it originates and grow into its branches. Histologically, it is composed of anastomosing connective tissue stalks and an epithelium that clothes it. The epithelium of solitary intraductal papilloma, like that of the normal duct, consists of two types of cells, ie, an outer layer of luminal epithelial cells and an underlying layer of myoepithelial cells. Solitary intraductal papilloma is not thought to have premalignant significance, since it does not evolve into carcinoma.[1] It remains to be established whether this lesion is a true neoplasm.

Clonal analysis can provide valuable information on the origin of neoplasms. It is generally accepted that a rarely occurring set of somatic mutations are responsible for the development of cancer.[2] This somatic mutational theory predicts that the resulting tumors will be monoclonal in origin, since it is unlikely that an identical sequence of rare mutations will occur independently in two neighboring cells. Consistent with this theory, various types of benign and malignant human neoplasms have been shown to be monoclonal in origin.[3] On the other hand, in hyperplasia where multiple original cells respond to an exogenous or endogenous stimulus, a polyclonal expansion is to be expected. In this context, clonal analysis has been successfully applied for discriminating adenoma from hyperplasia in the parathyroid glands.[4]

The clonality of solitary intraductal papilloma is of interest because it may assist in the understanding of the histogenesis of this lesion and may also provide definitive information as to whether it is neoplasia or hyperplasia. In the present paper, clonal analysis was done through a newly established method based on restriction fragment length polymorphism of the X-chromosome-linked phosphoglycerokinase (PGK)

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gene and on random inactivation of the gene by methylation.[5] Through introduction of polymerase chain reaction (PCR), this new method has become applicable to clonal analysis of small lesions such as intraductal papillomas.[6,7] Taking advantage of this new method, we have demonstrated that solitary intraductal papilloma of the breast is monoclonal in origin and that it originates from a common precursor which retains the capability to differentiate into both luminal epithelial and myoepithelial cells.

## Materials and Methods

### Tissue Source

Twenty-two solitary intraductal papillomas of the breast, resected during the period from January 1991 to February 1993 at our institute, were utilized in this study. Of these, nine solitary intraductal papillomas that were heterozygous for the *Bst*XI polymorphism of PGK gene were subjected to clonal analysis. Bloody nipple discharge was an initial symptom in these nine patients. They underwent selective excision of a sector containing the affected ducts, which was preoperatively stained by an intraductal injection of 0.5% methylene blue. Surgical specimens were obtained from tumor tissues in the ducts and from adjacent normal breast tissues. In two papillomas extending several centimeters along the duct, surgical specimens were obtained from four widely separated sites. These specimens were embedded in OCT compound (Miles Inc., Elkhart, IN) and snap-frozen in liquid nitrogen, being kept at −80 C until DNA extraction. The remainder of the surgical specimens were subjected to routine histological examination, whereby a histological diagnosis of benign solitary intraductal papilloma was obtained in all cases.

### DNA Extraction

DNA extraction from frozen sections embedded in OCT compound was performed according to the method previously described.[7] In brief, two 5-μ sections and ten 10-μ sections were cut serially with a cryostat. The first 5-μ section was stained with hematoxylin and eosin for histological confirmation of sampling accuracy of tumor tissue. The second 5-μ section was subjected to immunohistochemical study with anti-smooth muscle α-actin antibody described below. DNA was extracted from the remaining ten 10-μ sections with pronase digestion followed by phenol/chloroform extraction.

### Primers

A restriction map of PGK gene and DNA sequence of the primers are shown in Figure 1. To amplify the region of PGK gene which contains *Bst*XI polymorphic site and the differentially methylated *Hpa*II site,

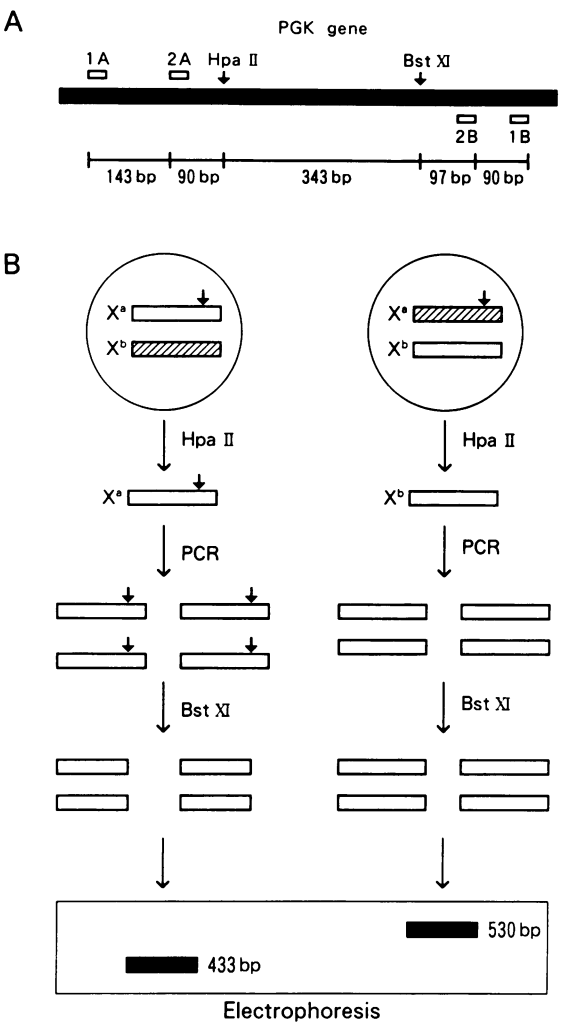


Figure 1. A: *Restriction map of PGK gene containing differentially methylated* Hpa*II site and polymorphic* Bst*XI site. DNA sequences of primers used for the amplification by PCR were:*
*1A:5′-CTGTTCCTGCCCGCGCGGTGTTCCGCATTC-3′,*
*1B:5′-ACGCCTGTTACGTAAGCTCTGCAGGCCTCC-3′,*
*2A:5′-AGCTGGACGTTAAAGGGAAGCGGGTCGTTA-3′,*
*2B:5′-TACTCCTGAAGTTAAATCAACATCCTCTTG-3′.*
B: *Schematic drawing of the strategy for the PCR-based method of clonal analysis.* $X^a$ *represents an X chromosome that contains a PGK gene with a* Bst*XI restriction site* (arrow); $X^b$ *represents an X chromosome that contains a PGK gene without a* Bst*XI restriction site. Somatic cells of females heterozygous for the* Bst*XI polymorphism of the PGK gene are composed of two types of cells, ie, those with inactive* $X^a$ *and those with inactive* $X^b$ *due to random inactivation of one of two X chromosomes by methylation. Active X chromosomes* (hatched bar) *are cleaved after methylation-sensitive* Hpa*II digestion. Methylated, inactive PGK allele is preserved after the* Hpa*II digestion and amplified by PCR. The PCR-amplified product is then digested with* Bst*XI and analyzed by 2% agarose gel electrophoresis.*

nested oligonucleotide primer pairs flanking this region were synthesized.

## PCR

To 20 μl of DNA solution prepared from the cryostat sections, 16 μl water and 4 μl of 100 mM Tris, 100 mM $MgCl_2$, 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 *Hpa*II (10 U/tube, Takara Shuzo Co., Ltd., Kyoto, Japan) for 12 hours at 37 C and then heated to 95 C for 3 minutes.

Twenty μl of *Hpa*II-digested or undigested DNA samples were subsequently used for PCR amplification of PGK locus by adding each sample to 80 μl of a solution containing 10 mM Tris, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% gelatin, pH 8.3, dNTPs (200 μM each), 20 pmol primer 1A (Figure 1), 20 pmol primer 1B (Figure 1), and 0.5 U Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). Samples were amplified in a Perkin-Elmer Cetus thermal cycler for 40 cycles (1 minute, 94 C; 2 minutes, 58 C; 3 minutes, 72 C). One-tenth volume of this PCR mixture was seeded into another reaction mixture. Primers 2A and 2B (20 pmol each) were added and the amplification was done as described above. After amplification, the mixture was extracted with 1 volume of phenol/chloroform and subsequently with 1 volume of chloroform and precipitated by adding one-third volume of 10 M ammonium acetate and 2.5 volumes of cold ethanol. After centrifugation at 12,000 × *g* for 30 minutes, the pellet was resuspended in 20 μl of 50 mM Tris, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, pH 7.5 containing 10 U of *Bst*XI (Takara Shuzo, Co., Ltd., Kyoto, Japan) and incubated for 12 hours at 45 C. Samples were stained with ethidium bromide and analyzed by 2% agarose gel electrophoresis.

## Immunohistochemistry

Cryostat sections were fixed in 10% buffered formalin and incubated with anti-smooth muscle α-actin monoclonal antibody (Sigma Chemical Company, St. Louis, MO) at 1:400 dilution for 2 hours at room temperature. It was then incubated with the streptavidin-biotinylated peroxidase complex at a dilution of 1:100 for 1 hour at room temperature. The reaction was developed by the diaminobenzidine-$H_2O_2$ procedure. The sections were counterstained with hematoxylin.

## Strategy of Clonal Analysis by Means of PCR

We conducted clonal analysis according to the method of Gilliland et al.[6] In females heterozygous for the *Bst*XI polymorphism of PGK gene on the X chromosome, somatic cells are composed of two types of cells, ie, those containing inactive PGK gene with *Bst*XI restriction site ($X^a$ in Figure 1) and those containing inactive PGK gene without this restriction site ($X^b$ in Figure 1), due to random inactivation of one of two X chromosomes by methylation. This random inactivation occurs at some time early in embryogenesis. While the initial choice of which X chromosome will be inactive in a given cell is made at random, once it is made, it is stable to cell division and therefore is fixed not only for that cell, but for all of its descendants.

In the method of clonal analysis by PCR, at first, DNA samples were digested with a methylation-sensitive restriction enzyme, *Hpa*II, to cleave the unmethylated active PGK gene (Figure 1, hatched bar). The methylated inactive PGK gene is preserved after *Hpa*II digestion. If tumors are polyclonal, tumor cells should be composed of two types of cells, ie, those with inactive $X^a$ chromosome and those with inactive $X^b$ chromosome. Thus, both $X^a$ chromosome and $X^b$ chromosome were preserved after *Hpa*II digestion. Amplification by PCR of PGK gene resulted in an amplification of PGK gene both with and without *Bst*XI site, giving rise to two bands, 530 and 433 bp, in the agarose gel electrophoresis. If tumors are monoclonal, tumor cells should be composed of one type of cells, ie, those with inactive $X^a$ chromosome or those with inactive $X^b$ chromosome. Thus, either $X^a$ chromosome or $X^b$ chromosome was preserved after *Hpa*II digestion. Amplification by PCR of PGK gene resulted in an amplification of either PGK gene with or without *Bst*XI site, giving rise to a 530 or 433-bp band in the agarose gel electrophoresis.

When DNA samples were amplified without *Hpa*II digestion, both PGK alleles were amplified. Thus, electrophoretic analysis of these amplified samples after *Bst*XI digestion gave rise to two bands 530 and 433 bp, regardless of the clonality of tumors.

# Results

## Analysis of Clonality of Solitary Intraductal Papillomas

DNA samples were prepared from the frozen sections of nine solitary intraductal papillomas and adjacent

normal breast tissues. Agarose gel electrophoresis of the PCR-amplified and *Bst*XI-digested products from normal breast tissue gave rise to two DNA bands, 530 and 433 bp, in every case, regardless of the presence or absence of a *Hpa*II precut, indicating that normal breast tissue is polyclonal (Figure 2). The ratio of intensity of the 530-bp band to the 433-bp band was 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 *Bst*XI site on each strand of a homoduplex would be digested.

On the other hand, clonal analysis of solitary intraductal papillomas showed a monoclonal pattern that either of the 433- or 530-bp bands completely or almost completely disappeared in the presence of a *Hpa*II precut in every case (Figure 2). When DNA samples prepared from four widely separated sites were analyzed, it was found that all of these samples were monoclonal in origin and, in addition, the same allele of PGK gene (Figure 3, 530-bp band in case 1 and 433-bp band in case 6) was preserved in the presence of a *Hpa*II precut regardless of the sampling sites in each case.

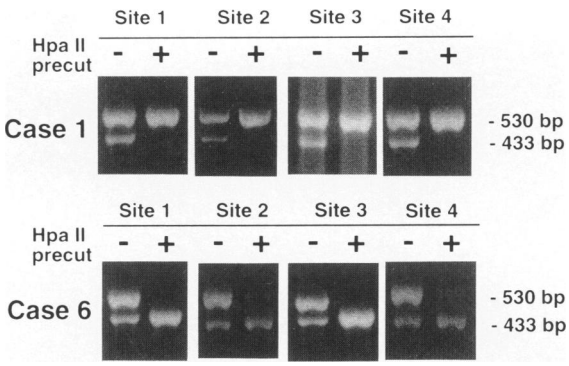


Figure 3. *Clonal analysis of DNA samples from four different sites in solitary intraductal papillomas. DNA samples were prepared from four widely separated sites in two solitary intraductal papillomas (Figure 2, cases 1 and 6). Clonal analysis of these samples was done as described in Materials and Methods. Results of agarose gel electrophoresis are shown.*

## *Proportion of Luminal Epithelial Cells, Myoepithelial Cells, and Stromal Cells in Solitary Intraductal Papilloma and Its Relation to Clonality*

Since epithelial cells are composed of luminal epithelial and myoepithelial cells, it is of interest to study whether these two types of cells originate from a common or different precursor. In order to separately count numbers of the luminal epithelial and myoepithelial cells, frozen sections were immunohistochemically stained for smooth muscle α-actin, which is a marker protein of the myoepithelial cells (Figure 4). Proportion of the luminal epithelial cells, myoepithelial cells, and stromal cells is shown in Table 1. Since stromal cells accounted for only less than 10% in every case, the results of clonal analysis shown in Figure 2 were considered to represent the clonality of epithelial cells. These results suggest that epithelial cells of solitary intraductal papillomas are monoclonal in origin.

The immunohistochemical study showed that solitary intraductal papillomas were composed of approximately 50% luminal epithelial cells and 40% myoepithelial cells in cases 1 to 7 in Table 1. Clonal analysis revealed that epithelial cells, which included

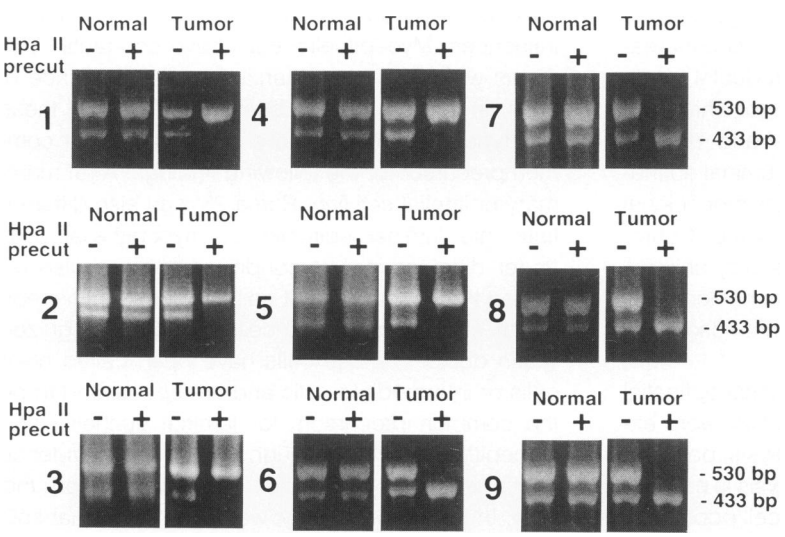


Figure 2. *Clonal analysis of solitary intraductal papillomas and adjacent normal breast tissues. DNA samples were prepared from cryostat sections of nine solitary intraductal papillomas (tumor) and adjacent normal breast tissues (normal) (cases 1–9). Clonal analysis of these samples were done as described in Materials and Methods. Results of agarose gel electrophoresis are shown.*

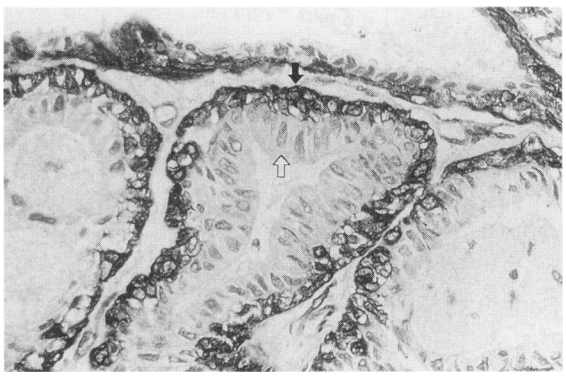

**Figure 4.** *Immunohistochemical staining of smooth muscle α-actin. Frozen sections of solitary intraductal papillomas were immunohistochemically stained with anti-smooth muscle α-actin antibody as described in Materials and Methods. Stained epithelial cells* (solid arrow) *correspond to myoepithelial cells, and unstained cells* (open arrow) *to luminal epithelial cells. A representative section is shown* (×300).

**Table 1.** *Proportion of Luminal Epithelial Cells, Myoepithelial Cells, and Stromal Cells in Solitary Intraductal Papillomas*[a]

| Section | Epithelial Cells | | Stromal Cells |
|---|---|---|---|
| | Luminal | Myoepithelial | |
| 1 | 53[b] | 43 | 4 |
| 2 | 51 | 45 | 4 |
| 3 | 51 | 44 | 5 |
| 4 | 50 | 43 | 7 |
| 5 | 58 | 39 | 3 |
| 6 | 50 | 43 | 7 |
| 7 | 52 | 39 | 9 |
| 8 | 78 | 14 | 8 |
| 9 | 73 | 18 | 9 |

[a] Frozen sections were cut from solitary intraductal papillomas and immunohistochemically stained with anti-smooth muscle α-actin antibody to discriminate myoepithelial cells from luminal epithelial cells.

[b] Numbers are percentages, taking the number of total cells included in a section as 100%.

both types of cells, were monoclonal in origin, ie, they were derived from a single cell. Thus, it seems reasonable to speculate that solitary intraductal papilloma originates from a common precursor which can differentiate into both luminal epithelial and myoepithelial cells. Another possibility is that luminal epithelial and myoepithelial cells have separate monoclonal origins and happen to inactivate the same X chromosome by chance. This, however, is very unlikely, since the probability that two different monoclonal cell populations inactivate the same X chromosome in seven cases is quite low, $2^{-7}$.

Cases 8 and 9 in Table 1, where luminal epithelial cells account for 70 to 80% of total cells, were excluded from the above-mentioned analysis, because the PCR-based method for clonal analysis is not sensitive enough to detect a monoclonal cell population which accounts for less than 20% of a total cell population.[7] Thus, results of clonal analysis in these cases would better be regarded to represent clonality of luminal epithelial cells but not myoepithelial cells.

## Discussion

In the present study, we have shown that solitary intraductal papilloma is monoclonal in origin. Our results, that the same allele of the PGK gene was inactivated in all the samples prepared from four different sites in each case, seem to support the thesis that this disease occurs as a single monoclonal tumor and extends along the ducts, rather than occurring as multicentric monoclonal tumors which subsequently merge. These results also support the concept that solitary intraductal papilloma is not a hyperplastic lesion but a true neoplasm.

In contrast with solitary intraductal papilloma, multiple intraductal papilloma is generally considered to be a precancerous lesion, since this disease occasionally progresses to papillary carcinoma or recurs as papillary carcinoma after local excision.[8] Multiple intraductal papilloma is a more extensive disease and affects many ducts; multiple papillomas grow independently in the ducts of a peripheral sector of the breast gland. Since multiple intraductal papilloma is histologically quite similar to solitary intraductal papilloma, it is speculated that each papilloma lesion within a multiple intraductal papilloma is also monoclonal in origin. With the PCR-based method described in this paper, it would be possible to analyze the clonality of each papilloma independently.

The ducts of normal breast gland are composed of two types of cells, luminal epithelial and myoepithelial cells. Both types of cells are derived from the embryonic ectoderm. Luminal epithelial cells are capable of producing milk under certain hormonal influences. Myoepithelial cells have contractile elements which are characterized by the presence of actin and myosin. It has been proposed that these two types of epithelial cells originate from a common precursor for the following findings. A rat mammary epithelial cell line, Rama 25, can also differentiate into luminal epithelial or myoepithelial cells under different *in vitro* conditions.[9] It was also reported that other types of cells besides luminal epithelial and myoepithelial cells can be recognized within ducts.[10] These cells have been called clear cells or intermediate cells and are speculated to be the common precursors for luminal epithelial and myoepithelial cells, although no definitive evidence has been made available as yet to support this view. In the present study, we have shown that solitary intraductal papilloma is composed of approxi-

mately equal mixtures of luminal epithelial and myoepithelial cells, and, in addition, that these two types of cells have a common single cell origin. This origin is considered to be a precursor which is capable of differentiating into both luminal epithelial and myoepithelial cells. These results seem to provide a supporting evidence that there are common precursors for these two types of cells within ducts of normal breast gland.

In conclusion, we have provided evidence that solitary intraductal papilloma is monoclonal in origin, and that luminal epithelial and myoepithelial cells originate from a common precursor. The PCR-based method of clonal analysis, which is applicable to small lesions, seems to be a powerful tool to investigate the histogenesis and progression of neoplasms.

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