X-inactivation patch size in human female tissue confounds the assessment of tumor clonality

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Most models of tumorigenesis assume that tumors are monoclonal in origin. This conclusion is based largely on studies using X chromosome-linked markers in females. One important factor, often ignored in such studies, is the distribution of X-inactivated cells in tissues. Because lyonization occurs early in development, many of the progeny of a single embryonic stem cell are grouped together in the adult, forming patches. As polyclonality can be demonstrated only at the borders of X-inactivation patches, the patch size is crucial in determining the chance of demonstrating polyclonality and hence the number of tumors that need to be examined to exclude polyclonality. Previously studies using X-linked genes such as glucose-6-phosphate dehydrogenase have been handicapped by the need to destroy the tissues to study the haplotypes of glucose-6-phosphate dehydrogenase [Fialkow, P.-J. (1976) *Biochim. Biophys. Acta* 458, 283–321] or to determine the restriction fragment length polymorphisms of X chromosome-linked genes [Vogelstein, B., Fearon, E. R., Hamilton, S. R. & Feinberg, A. P. (1985) *Science* 227, 642–645]. Here we visualize X-inactivation patches in human females directly. Results show that the patch size is relatively large in both the human colon and breast, confounding assessment of tumor clonality with traditional X-inactivation studies.

To examine the clonal architecture of normal tissue it is necessary to have a cellular marker that can be used to identify a subset of germ-line cells. Experimentally this can be achieved by using chimeric or mosaic animals. Because of the process of X inactivation, females heterozygous for X-linked polymorphisms are functionally mosaic at the mRNA and protein level. Previous studies have used X-linked genes such as glucose-6-phosphate dehydrogenase (G6PD) (1) or restriction fragment length polymorphisms (2) without reference to patch size. In female mammals the process of X inactivation occurs early during embryogenesis (day 16 in the human female). This process involves random inactivation of most of the genes on one or the other of the two X chromosomes by methylation of CpG islands (3, 4). The pattern of methylation is stable and inheritable so that it is passed on to all cellular progeny. The pattern of X inactivation is also widely believed to be stable during tumorigenesis (5). As X inactivation occurs at a relatively early stage of embryogenesis, although there is inevitably some mixing of cells during further development, in the adult mammal many of the progeny of a single X-inactivated embryonic cell are arranged together. In epithelia these groups of cells sharing a common X-inactivation pattern are termed patches. A single patch may be formed of the progeny of one cell or several cells all showing the same X-inactivation pattern. Thus cells in a single patch are monophenotypic but may be clonal or polyclonal in derivation. If adjacent cells in the middle of a patch are examined they will always be monophenotypic. There has been little investigation of the patch size of X inactivation in normal human female tissues. Limited data are available from PCR-based microdissection studies, which show that, in some tissues, the patch size can be relatively large, some 4 mm in diameter in the human aorta (6).

Materials and Methods

Tissue was collected from surgical resection specimens of Sardinian females heterozygous for the G6PD Mediterranean mutation (563 C → T). All patients had been previously shown to have reduced G6PD enzyme activity, and heterozygosity for the G6PD Mediterranean mutation was confirmed by PCR analysis of genomic DNA followed by MBOII restriction endonuclease digestion. Immediately after surgical resection samples of tissue were taken from unfixed surgical specimens, snap-frozen in liquid nitrogen, and stored at −40°C. Normal tissues were obtained from breast, colonic, small intestinal, and thyroid resection specimens. Frozen sections (7 μm) of the tissues were mounted on glass slides, air-dried at room temperature for 20 min, and stored at −40°C. Frozen sections were preheated at 53°C for 5 min to inactivate the Mediterranean mutant G6PD (7) followed by G6PD enzyme histochemistry to demonstrate G6PD activity (8).

Results

Epithelial cells showed a biphasic staining pattern being either strongly positive or completely negative for G6PD activity. Colonic and small intestinal crypts showed a monophenotypic staining pattern with no evidence of mixed crypts. The epithelium covering small intestinal villi showed a mixed pattern of staining, similar to that demonstrated in both mouse chimeras and humans (9), consistent with the fact that the epithelium covering each villus is derived from multiple crypts (Fig. 1).

Nine samples of normal colonic mucosa were examined from a single colectomy specimen resected from an 80-year-old female with colorectal carcinoma. Colonic mucosa was sampled away from the tumor. There was no history or histological evidence of inflammatory bowel disease or other colonic pathology. Colonic crypts stained either entirely positive or entirely negative for G6PD. A total of 10,538 crypts cut in cross section was examined. Of those, 50.2% of crypts stained positively (blue) and 49.8% negatively (white) for G6PD activity. None of 2,260 crypts examined at patch borders showed a mixed phenotype, confirming that human colonic crypts are clonal populations. This finding is consistent with results from previous direct (9) and indirect studies (10). Crypts were arranged in hexagonal arrays in large patches, with irregular patch borders, containing up to

Abbreviation: G6PD, glucose-6-phosphate dehydrogenase.

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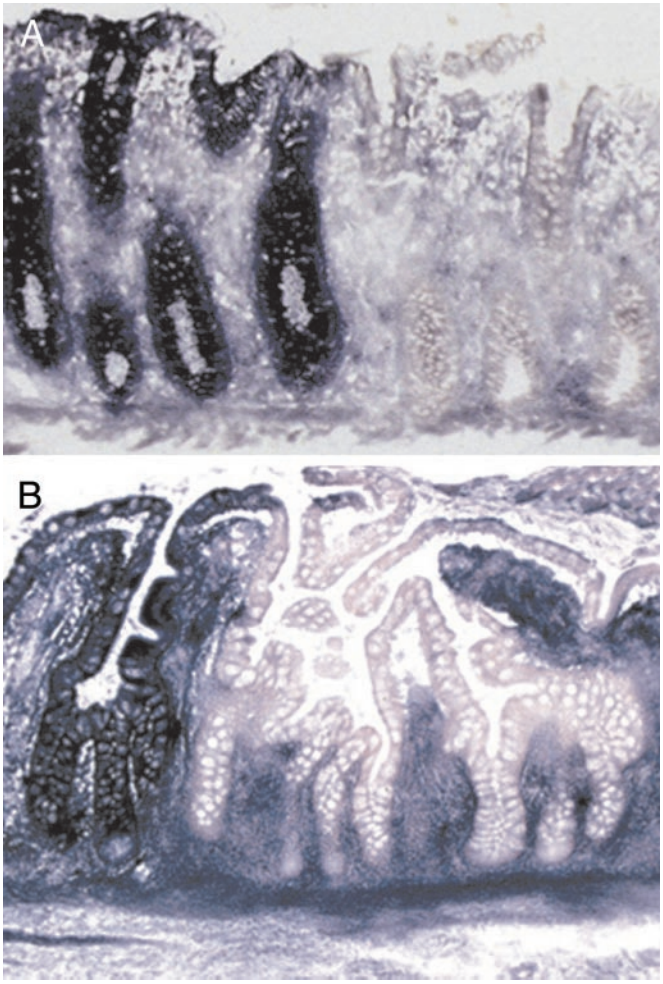

Fig. 1. G6PD staining pattern in intestinal crypts. (*A*) Colonic mucosa stained for G6PD activity showing longitudinal sections of colonic crypts. In individual crypts all epithelial cells show a similar staining pattern. (*B*) Small intestinal mucosa stained for G6PD activity showing longitudinal sections of crypts and villi. Epithelial cells in individual crypts show a similar staining pattern but villous epithelium shows areas of positive and negative staining. (Magnifications: ×90.)

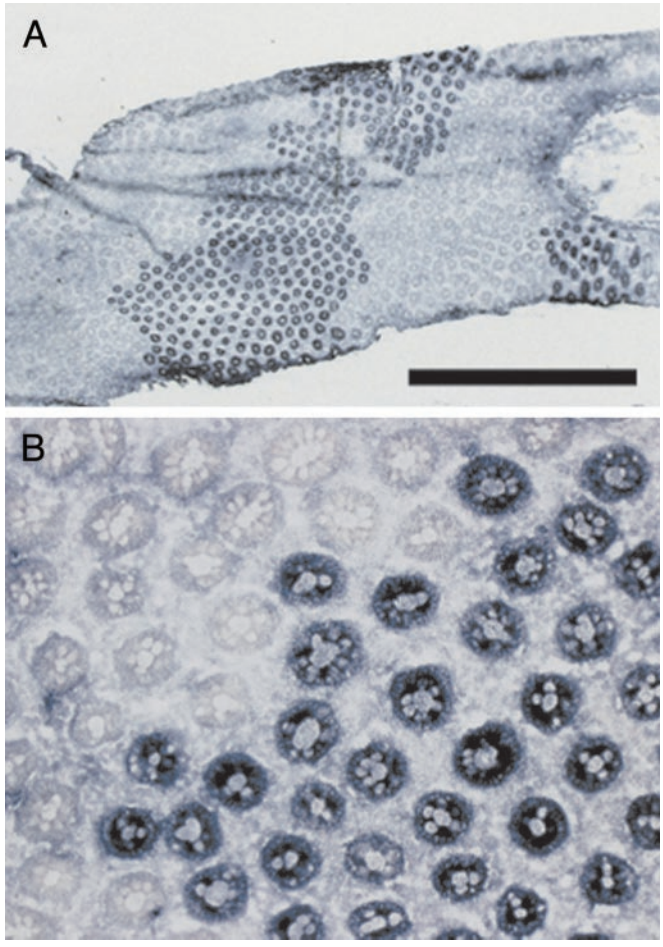

Fig. 2. G6PD staining pattern in colonic patches. Low-power (*A*) and high-power (*B*) views of normal colonic type mucosa stained for G6PD activity with crypts cut in cross section. Large patches of crypts with irregular patch borders are seen. (Scale bar: 2 mm.)

450 individual crypts (Fig. 2). Each crypt was examined in turn, and the staining pattern of all directly adjacent surrounding crypts was assessed. This crypt pair analysis showed that 8.2% of crypt pairs demonstrated a discordant staining pattern, a crypt pair phenotype index of 8.2% (11).

Multiple samples of breast tissue were examined from lumpectomy specimens from three adult females (aged 55, 82, and 87 years). G6PD staining showed that all 111 small ducts and 57 lobules examined demonstrated a monophenotypic staining pattern (Fig. 3). Both myoepithelial and luminal ductal cells always stained in the same fashion. A single large duct showed large patches of G6PD positivity and negativity. Thyroid tissue was examined from four patients (age range 41–67 years). Thyroid parenchyma showed a patchy distribution of staining with the epithelium of individual follicles showing areas of G6PD positivity and negativity (Fig. 4). This finding of polyclonal thyroid follicles is consistent with results from previous animal studies (12).

Discussion

Evidence supporting the clonal derivation of tumors comes both directly from clonality studies and indirectly from mutation analysis. In direct studies of human tumor clonality, early work focused on tissue derived from black females heterozygous for the G6PD A variant. These studies examined differences in electrophoretic patterns of G6PD A and B proteins extracted from tissue samples (1). Given the admixture of cells constituting a tumor (tumor cells, vessels, stromal elements, and inflammatory cells), it was not surprising that such tumors were commonly demonstrated to be polyclonal. More recent studies have used microdissection and RT-PCR analysis of the X-inactivation patterns of a variety of genes to examine tumor clonality. PCR studies typically involve the sampling of multiple small areas of tumor. Such sampling may miss polyclonality, particularly in large tumors where expansion of a dominant clone may have occurred. Furthermore very few of these studies take patch size into account, which is not surprising given the dearth of information available on patch size.

Previous studies of tumor clonality in the human colon have produced conflicting results: isoenzyme studies of G6PD in black females showed that a single sporadic colonic carcinoma (13) and seven colonic adenomas from three patients with Gardner syndrome were polyclonal (14). Analysis of X-linked restriction fragment length polymorphisms in microdissected tissue samples showed 50 human colorectal adenomas of both familial and sporadic type to be monoclonal (15). In the patient reported by Novelli *et al.* (9), who had familial adenomatous polyposis and was an XO/XY sex chimera, 76% of adenomas

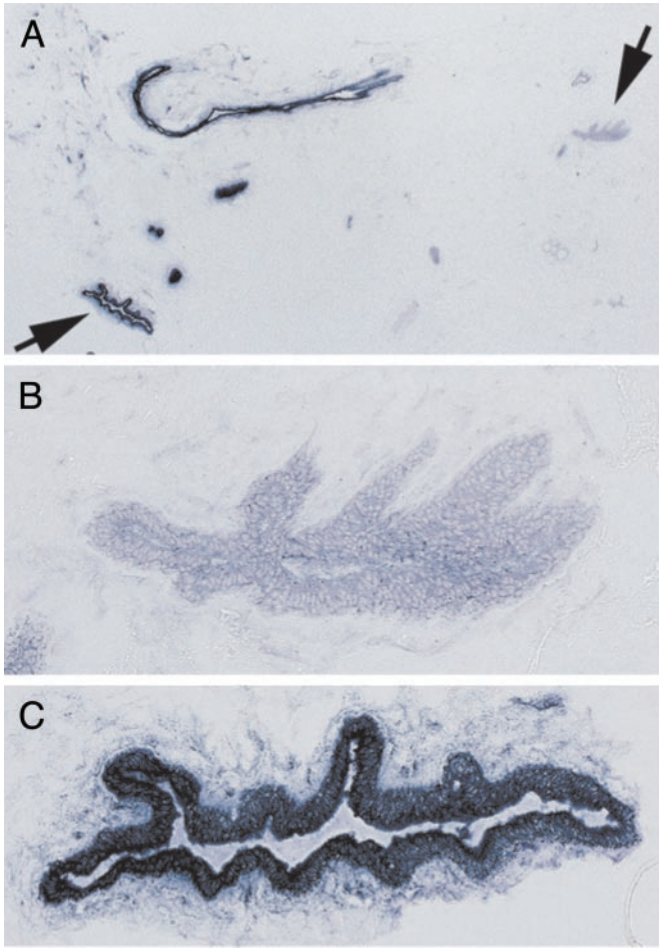

Fig. 3. G6PD staining pattern in human breast tissue. (*A*) A low-power view of benign breast tissue stained for G6PD activity. Arrowheads highlight positive and negative staining ducts shown at higher magnification in *B* and *C*. (*B*) A high-power view of a positive-stained duct. Both luminal and myoepithelial cells stain in a similar fashion. (*C*) A high-power view of a G6PD-negative staining duct. (Magnifications: *A*, ×14; *B* and *C*, ×94.)

(above monocryptal size) were polyclonal in origin. However, in this patient, the mean XO patch width was very small (1.24 crypts) and thus the chances of having discordant crypt pairs, and therefore of finding polyclonal tumors, was high.

Our direct observations here suggest that because of the large patch size in the colon, X-inactivation studies are heavily biased toward showing that tumors are monoclonal. With a crypt pair phenotype index of 8.2%, to exclude the possibility that all adenomas are polyclonal in origin every crypt in at least 43 adenomas would need to be shown to be monophenotypic (95% confidence interval). To exclude the possibility that 10% of adenomas are polyclonal 430 adenomas would need to be examined.

For some time it has been suspected that breast myoepithelial and epithelial cells share a common clonal origin, based largely

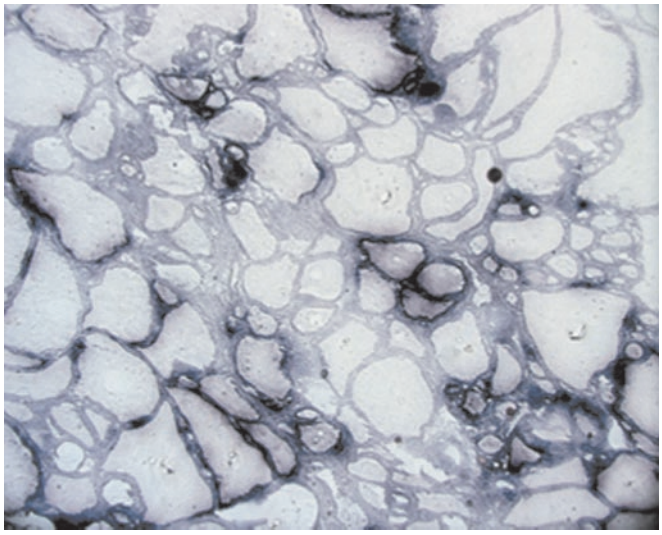

Fig. 4. G6PD staining pattern in human thyroid tissue. A low-power view of normal thyroid tissue showing patches of positive and negative G6PD staining. Many thyroid follicles show a mixed staining pattern. (Magnification: ×40.)

on studies in tissue culture (16, 17). Our studies show, again directly, that myoepithelial cells and epithelial cells have a common origin in humans. Lobules in the human breast measure from 1 to 8 mm in diameter and are drained by a terminal duct, which leads into the main duct system, the so-called terminal lobulo-ductular unit. Microarchitectural studies suggest that tumors arise from the terminal ductular/lobular unit (18, 19). In the human breast, molecular methods have given conflicting results where X-linked patch size is concerned, with some studies showing that normal breast tissue was polyclonal in origin, with a random distribution of X-chromosome inactivation (20) and others that epithelial patch size is quite large, and that the larger terminal ductal-lobular units represent the progeny of a single precursor or stem cell (21). Our studies show directly that this terminal lobulo-ductular unit often lies all within one patch/clone so that in studies using X inactivation it may be very difficult ever to demonstrate a polyclonal origin of breast tumors. Thus, not surprisingly, most studies using such markers report monoclonality in the early lesions from which breast cancers are considered to arise, such as intraductal carcinoma and atypical ductal hyperplasia (22, 23), with only a few studies suggesting a polyclonal origin (24).

In conclusion, we have demonstrated, directly, the size and shape of X-linked patch sizes in human tissues. We suggest that studies in human epithelial neoplasms involving X inactivation that fail to consider patch size cannot readily answer questions about tumor clonality. Ideally such studies should involve direct visualization of tumors and their relationship to patch boundaries.

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