Detecting Monoclonal Lesions in Archival Tissue Sections Using PCR: Experience in the Endometrium

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Abstract:

We hypothesize that precancers might be recognized by the presence of non-random X chromosome inactivation, a consistent feature of monoclonal cancers in women. X chromosome inactivation patterns in archival paraffin embedded tissues were determined by amplification of a hypervariable trinucleotide repeat in the X-linked androgen receptor gene following pre-digestion with *Hha*1, which destroys the unmethylated active X allele but not the methylated inactive allele. We have studied well differentiated endometrial cancers and their putative precursors, atypical adenomatous hyperplasias, using this approach.

All informative cases of well differentiated endometrial carcinoma, were shown to have non-random X inactivation by this method, confirming them as monoclonal. Clonality could not be ascertained in approximately half of the cases for several reasons including: lack of heterozygosity for the chosen marker, X inactivation skewing of matched control polyclonal tissues, and structural changes in the AR gene. X inactivation skewing in polyclonal tissues is due to the small number of endometrial stem cells at the time of embryonic X chromosome inactivation. With appropriate controls, this method is highly specific and moderately sensitive in detection of monoclonal proliferations.

A small group of potential endometrial precancers (atypical adenomatous hyperplasia), evaluated showed results similar to that of their putative sequelae, well differentiated endometrial carcinoma. All scorable cases were monoclonal, with the remaining being non-informative.

PCR assessment of X chromosome inactivation patterns is possible in archival paraffin-embedded materials, and may be used to identify monoclonal growth patterns, a common feature of cancers and precancers.

Tumor Clonality: Rationale

Clonal proliferation of a specific cell lineage occurs in limited circumstances in mammals, for example, in creation of B-cell clones capable of producing large quantities of particular antibodies. Otherwise, most somatic tissues are polyclonal unless there is a significant growth advantage within a subset of cells which proliferate at the expense of surrounding tissues. As such, clonal proliferation in most somatic tissues may be viewed as evidence of the creation of a cell or group of cells with a

growth advantage - a characteristic of neoplastic processes.

Laboratory Assessment of Clonality:

There are several tested methods to determine clonality. Karyotypic alterations common to all cells of a tumor confirm clonal origin, but requires fresh unfixed tissue, a condition that cannot be met with precancers. Molecular demonstration of a genetic lesion, as small as a point mutation, within all cells of a lesion is evidence that a common stem cell contributed that mutation. This approach applies only to that fraction of cases in which a "marker" mutation can be identified and specifically fails to identify clonal proliferation that might take place prior to creation of a specific genetic lesion. Previous attempts to evaluate clonality by PCR have concentrated on detection of gene rearrangements, primarily in lymphoid and hematopoietic cells[5].

X Inactivation and Clonal Analysis:

The randomness of X chromosome inactivation in 46,XX female tissues provides a marker system to distinguish monoclonal from polyclonal proliferations[8,31,32]. Many genes on the X chromosome are polymorphic, with detectable DNA and/or protein heteromorphisms that permit distinction between the maternally and paternally inherited X chromosomes. Inactivation of one copy of the X chromosome in each cell takes place at about the time of embryonic implantation and is faithfully maintained in all progeny cells of the adult except germ cells[15]. A polyclonal tissue, for example, contains a mixture of paternally or maternally derived inactive X chromosomes. If one cell in that tissue proliferates clonally and forms a tumor, the X chromosome inactivation pattern of the stem cell is maintained in all progeny cells, producing a mass of tissue with either the paternal or maternal copy of the X inactivated in every cell.

A chemical modification of DNA, methylation, has been associated with several polymorphic genes on the inactive X chromosome. Thus, it is possible with G6PD[28], HPRT[2], and androgen receptor[1] genes to directly identify the inactivated X chromosomal DNA by methylation-specific restriction enzyme digestion. Isoschizmers like *HpaII* and *Msp*1 cut DNA at the same recognition site with base composition CCGG, but *Hpa*II only cuts if the region is unmethylated. Differential digestion between the methylated inactive allele and unmethylated active allele with *Msp* 1 produces restriction fragment length differences demonstrable by Southern blot hybridization. Paraffin-embedded tissue is not suitable for such Southern blot studies routinely, because of the small quantity of DNA available and its frequent degradation.

A PCR assay for Clonality (Non-random X inactivation): AR-a/b PCR

An informative locus amenable to PCR-based detection of non-random X chromosome inactivation must fulfill the following criteria: 1) high frequency of

heterozygosity (polymorphism information content, PIC); 2) a site which is differentially methylated in the active and inactive copy of the X chromosome, and thus subject to differential digestion by methylation-sensitive restriction endonucleases; 3) the methylated and polymorphic regions must be in sufficient proximity for amplification by one PCR primer set, preferably within a few hundred bases of each other; and 4) the target must amplify efficiently and reliably. Several possible targets may be excluded based on known features of their genomic structure: DXS255 has a methylated site separated by 1kb from a hypervariable repeat that itself ranges up to 6kb in length[7,12] - too big for PCR; the fragile X gene, FRA1 and closely linked FRAXC2 amplify very inefficiently due to highly stable secondary structures of the single stranded melted DNA[21]. Others have used a restriction fragment length polymorphism of the PGK gene to develop a PCR assay for clonality[11,29]. This is complicated by reasonably low polymorphism information content (PIC=0.33) and requirement for post-amplification digestion for recognition of distinct alleles.

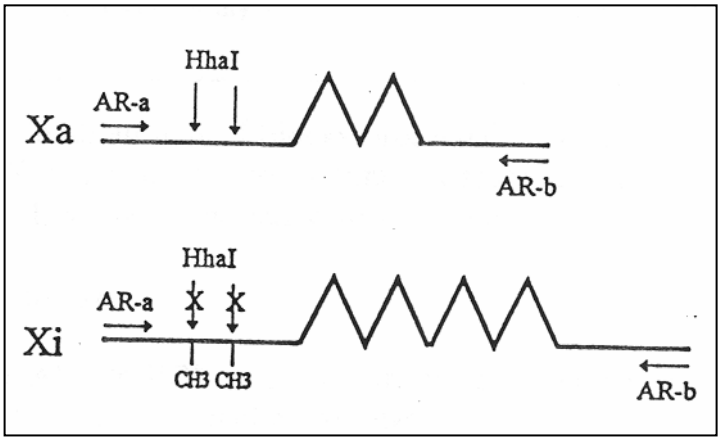
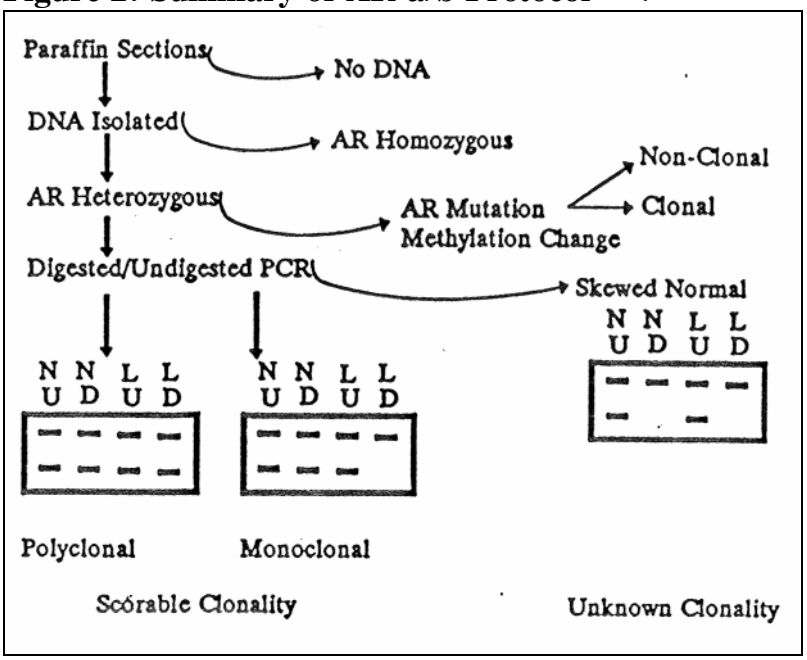


Figure 1: Diagram of PCR method to detect non-random X chromosome inactivation. AR-a and AR-b PCR primers target two X-chromosome linked androgen receptor alleles containing variable numbers of repeat elements (zig-zag line) flanked by *Hha*I sites methylated on the inactive X. Pre-digestion with *Hha*I cuts the active X allele, destroying this as a template for PCR, leaving only those from the inactive X for successful amplification. The X chromosome inactivation ratio is measured by comparison of PCR product generated with or without preceding digestion.

The androgen receptor gene has a hypervariable trinucleotide repeat[25] with PIC approximately 0.9 located less than 100bp from several *Hha*I sites known to be methylated on the inactive but not the active X chromosome[1](Figure 1). This genomic structure meets all of the stated requirements for a PCR clonality assay, and has led to its use by several groups for this purpose[1,17,18]. Genomic DNA is amplified with primers AR-a/b in the presence of trace amounts of ^{32}P-TTP, which produces a radiolabelled PCR product which can then be separated by molecular weight with high resolution on a non-denaturing polyacrylamide gel. Under these conditions 90% of women have a heterozygous genotype in 90%.

Figure 2: Summary of AR-a/b Protocol[18,19].



Starting with a paraffin section, and ending with an autoradiogram showing interpretation of androgen receptor alleles (Schematically shown in boxes), the protocol described is shown. Clonality of unsuccessful assays (curved arrows) will be unknown and occurs in about half of blocks processed. Code for autoradiogram schematics: N=normal polyclonal control tissue; L=Lesional tissue; U=undigested DNA; D=DNA digested with *Hha*1 prior to PCR.

Adaption to Archival Paraffin Sections:

Successful application of the AR-a/b PCR assay to paraffin embedded tissues is not trivial[18,19]. A brief discussion of critical parameters follows.

Efficiency of DNA recovery:We have found[20] that direct addition of the paraffin sections to lysis buffer warmed just above the melting temperature of the wax (60°C) yields 5-10 times more DNA than previously deparaffinized and re-hydrated tissue. Addition of glycogen or rRNA carrier directly to the lysis buffer enhances recovery during precipitation steps. Prolonged digestion in Proteinase K (48 hours), with replenishment of enzyme after 24 hours, also improves efficiency of extraction.

***Purity of DNA*:**Common DNA contaminants such as heavy metals, blood breakdown products, or unidentified materials may be difficult to remove and inhibit enzymatic modification either during restriction digestion or PCR. We routinely remove heavy metal residues with a chelating resin, Chelex[13] with improvement in PCR efficiencies. To improve upon the DNA quality further we borrowed a protocol originally used for pre-digestion cleanup of crude plasmid-containing cell lysates[27] and found to be effective in purifying dirty and damaged archaeological DNAs[6] for PCR. Following

digestion and phenol extraction, ethidium bromide is added under high salt conditions and removed by the subsequent phenol/chloroform extractions[6]. The method by which this works is unknown, but one possibility is that the intercalating ethidium displaces loosely associated contaminants which then partition into the organic extraction phase.

Controlled endonuclease digestion (HhaI):Digestion of paraffin isolated DNAs must be under very controlled conditions. We digest paraffin DNAs for 1-3 hours only and quench digestion by phenol extraction. This produces amplifiable template in more than 90% of digested samples.

PCR Conditions for Primers AR-a/b:The primers we have designed[18,19], designated AR-a and AR-b (5' CCG AGG AGC TTT CCA GAA TC 3' and 5' TAC GAT GGG CTT GGG GAG AA 3', respectively) use annealing temperatures of 55°C and Mg^{++} concentrations of 1.5mM, determined empirically. Product specificity was significantly improved by addition of a "Hot Start" step[4], whereby the PCR reaction is heated to 97°C for five minutes prior to addition of Taq polymerase at an 85°C holding temperature. The first three amplification cycles have prolonged (4 minutes instead of 30 second) 95°C denaturation steps to promote melting of this GC-rich target. Thermal cycling follows the sequence: 1) initial denaturation in the absence of Taq polymerase at 97°C for 5 minutes, 2) dwell at 85°C for 10 minutes, during which Taq polymerase is added; 3) 3 cycles at 95°C for 4 minutes, 55°C for 45 seconds, and 72°C for 90 seconds, 4) 27 cycles of 95°C for 30 seconds, 55°C for 45 seconds, and 72°C for 90 seconds, and 5) final extension at 72°C for 7 minutes.

Separation of Commingled Lesional and Normal Tissue:

Recently a novel method of selective DNA isolation based on selective ultraviolet irradiation has been described[24]. Briefly, desired areas of a tissue section are masked with opaque ink, and the DNA of unmasked areas is destroyed by short wave UV exposure. The entire tissue section is then scraped off of the slide, and only the shadowed areas can contribute intact PCR template. We have used this approach with great success after the following modifications: 1) Unstained wax sections are visualized under darkfield optics (using a high quality dissecting microscope, Wild M5A) to identify areas of interest - rehydration and staining of the sections diminishes DNA yields. 2) A highly opaque colloidal graphite in an isopropanol base is applied directly to the wax section. This formulation has excellent spreading qualities on hydrophobic wax surfaces, is non-reactive with the wax and DNA, and is essentially UV impervious. When applied with a disposable p200 pipet tip used as a quill, a spot 0.5mm in diameter, or swath of any desired size can be applied. 3) A high energy 254nm calibrated UV source is used for the irradiation (Stratalinker apparatus, Stratagene) - this minimizes time needed for exposure, and can be set to deliver a desired energy dose irrespective of changes in bulb intensity which occur with age.

Xi skewing in polyclonal tissues.

Given the small number of inner cell mass (embryo-destined) cells at the time of X chromosome inactivation, it is unreasonable to expect that every polyclonal somatic tissue in every adult will contain equal numbers of inactivated paternally and maternally derived X chromosomes. Even if the choice of X chromosome to be inactivated is random, the balance of maternal and paternal X's inactivated will follow a binomial distribution, the variation of which is determined by the number of progenitor cells at the moment of X inactivation[8,9]. For example, if there are only two stem cells for the future endometrium at the time of X inactivation, the chance that the same X would be inactivated in both cells is very high, 50%. The measured population (between individuals) variation in X chromosome inactivation can be used to calculate the primordial stem cell pool size according to the simple formula: variance observed = pq/N where p and q are probabilities of inactivating a particular X (both 0.5), and N is the stem cell pool size[9]. Actual X chromosome inactivation ratios can be measured by any quantitative method, including biochemical demonstration of X-linked isozymes[9,14] or Southern blot analysis of methylated sites adjacent to informative RFLP's[10].

We have determined the X inactivation ratio of a series of normal endometria, and preliminarily estimated that approximately 9 cells constitute the number of endometrial stem cells at the time of X chromosome inactivation. This estimate of 9 endometrial stem cells is in the range of that calculated for several other tissues: 10 for T-cells[22], 17.2 for liver[33], 10 for skeletal muscle[9], and 15 for skin fibroblasts[9].

Using the calculated number of 9 endometrial stem cells, the X inactivation ratios in the polyclonal endometrium of a larger population of women was predicted. Approximately 18% of women will have X inactivation ratios skewed more than two-fold: either less than 30% or more than 70% inactivation of one parental chromosome, easily seen by quick visual assessment. If skewing of tumor X inactivation is the sole criterion for determination of monoclonality, monoclonality can be falsely identified in the 18% of women in which polyclonal tissues demonstrate significant X inactivation skewing (82% specificity). Patients in whom normal tissues have skewed X inactivation patterns can, however, be identified by direct examination of those normal tissues, and the tumor results can therefore be discounted as non-informative. This disqualification of some tumor samples would increase the specificity to a very high level, certainly over 95%.

Specificity and Sensitivity of the PCR assay in identification of monoclonal proliferations:

Nonrandom X chromosome inactivation, defined as skewed inactivation of one X chromosome over the other, constitutes the basis for identification of monoclonal populations of cells. It is of great importance to predict and understand any

circumstances which might alter this relationship. To this end, the preceding data has been synthesized into overall estimates of specificity and sensitivity of detecting monoclonal endometrial proliferations by PCR using the androgen receptor locus.

Specificity: estimated to be 95-100% for well differentiated endometrial cancers, with proper controls

Imbalances of X chromosome inactivation may occur in polyclonal tissues of the female genital tract so this feature is not in itself specific to monoclonal tissues. Inactivation of individual cells in polyclonal tissues is random, but the small number of cells present at the time of X inactivation produces a broad variation in the net ratio of inactivated maternal vs. paternal X's. Thus if polyclonal control tissues which share a common embryologic lineage with the tumor source tissues are analyzed, those individuals with skewing of polyclonal tissues may be excluded from analysis as "non-informative", preventing ascertainment errors.

It is crucial to recognize that this assay assumes a close relationship between marker gene methylation status and X inactivation state. Any condition which demethylates methyl-cytosine residues on the inactive X, or methylates cytosine residues on the active X, will confound use of this methylation pattern to discern X inactivation status. This is of particular concern in malignancies, as destabilization of methyl-cytosine has been documented in some cancers[3,16,23,26,30]. In fact, in less differentiated endometrial cancers even monoclonal tumors may not have demonstrable skewing of methylation between the active and inactive X chromosomes. Fortunately in the case of endometrial cancer these constitute a subset of lesions which, unlike their well-differentiated counterparts, have never been associated with a precursor state, and thus of little relevance to investigation of the precursors themselves. It is necessary to document performance of this assay with the particular tumor of interest, before potential analytical errors in evaluation of their precursors may be assessed.

Karyotypic sex chromosome abnormalities, either numeric or structural, may confound the assay. This might occur by deletion or duplication of a marker locus, or a change in the number of inactive X's in each cell (tetraploid cells have two inactive X's, whereas XO cells have none). The extent to this will interfere with interpretation may be assessed by review of the karyotypic features of the tumor of interest.

Sensitivity: estimated to be 57%

Detection of a monoclonal proliferation by the PCR AR-a/b assay requires that 1) DNA capable of PCR amplification is isolated ($p=0.9$); 2) the androgen receptor is heterozygous ($p=0.9$); 3) normal control tissues do not demonstrate a widely skewed X inactivation pattern ($p=0.82$, as above); 4) digestion by *Hha*I is complete ($p=0.9$, as assessed by amplification of digested control template); and 5) methylation of the

inactive copy of the AR locus is maintained (probability unknown, p=0.95 used for this calculation). In aggregate, we can expect that 57% (sensitivity) of monoclonal lesions will be recognized as monoclonal. Since factors 1-4 will be reason to exclude any analysis from interpretation, whether monoclonal or polyclonal, it is not expected to introduce ascertainment bias.

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May 18, 1994

Dear Ms. Sullivan:

Enclosed please find an abstract and syllabus materials for my talk in "Recent Advances in Molecular Pathology."

Call me at (617) 732-6987, beeper 1191 if I can be of further assistance.

Sincerely,

George L. Mutter, MD
Asst Prof. of Pathology