PCR Techniques for Clonality Assays

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Clonal overgrowths represent the hallmark of neoplastic proliferations, and their demonstration has been proved useful clinically for the diagnosis of malignant lymphomas based on the detection of specific and dominant immunoglobulin and/or T-cell receptor gene rearrangements. Nonrandom genetic alterations can also be used to test clonal expansions and the clonal evolution of neoplasms, especially analyzing hypervariable deoxyribonucleic acid (DNA) regions from patients heterozygous for a given marker. These tests rely basically on the demonstration of loss of heterozygosity (LOH) resulting from either hemizygosity (nonrandom interstitial DNA deletions) or homozygosity of mutant alleles observed in neoplasms. LOH analyses identify clonal expansions of a tumor cell population, and point to monoclonal proliferation when multiple and consistent LOH are demonstrated. Based on the methylation-related inactivation of one X chromosome in female subjects, X-linked markers (e.g., androgen receptor gene) will provide clonality information using LOH analyses after DNA digestion with methylation-sensitive restriction endonucleases. Therefore, both non-X-linked and X-linked analyses give complementary information, related and not related to the malignant transformation pathway respectively. Applied appropriately, these tools can establish the clonal evolution of tumor cell populations (tumor heterogeneity), identify early relapses, distinguish recurrent tumors from other metachronic neoplasms, and differentiate field transformation from metastatic tumor growths in synchronic and histologically identical neoplasms.

Key Words: Clonality—X chromosome inactivation—Microsatellites—Tumor suppressor genes—Tumor progression—Paraffin-embedded tissues.

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Conceptually, a clone is a group of genetically identical cells descended from one common ancestor. Currently, clonal derivation of cells is the hallmark of neoplasia and strongly implicates acquired somatic mutations giving survival advantage to a clonal cell population (22). Most somatic tissues are polyclonal unless a marked kinetic advantage within a subset of cells causes them to proliferate at the expense of surrounding tissues, resulting in monoclonal tissues.

There are several tests to determine clonality. The markers for clone detection must define a readable and reliable feature linked to a particular cell type and must include karyotypic alterations and single point mutations, among others. The presence of a common and nonrandom alteration in all cells of a tumor confirm clonal origin, but requires in many instances fresh, unfixed tissue—a condition that cannot be met with preneoplasia. Molecular demonstration of a genetic lesion, as small as a point mutation, within all cells is evidence that a common progenitor cell contributed to that mutation. This approach applies only to that fraction of cases in which a “marker” mutation can be identified and fails specifically to identify clonal proliferation that may have taken place before the creation of a specific genetic lesion (Fig. 1). In addition, those particular marker features must also be transmitted to descendant cells and must not induce apoptosis. Under the latter circumstance, any genetic alteration does not result in a dominant clone (15).

A given clone should show proliferative advantages over the remaining nonselected cell population to explain tumor growth. This proliferative advantage does not have to be related to a higher proliferation rate. Apoptotic indices lower than those required normally for regular turnover can increase the total cell number. The most important kinetic feature is the imbalance between cell proliferation and cell loss, ending in cellular overgrowth (15,20). Genetic alterations in a kinetically active tumor cell population lead to progressive clone selection and enhance genetic diversity in a given neoplasm as well. Therefore, the inherent genetic instability within a neoplasm results in heterogeneity in tissues derived from a single cell. As soon as the neoplastic transformation

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FIG. 1. X-linked and non-X-linked clonality assays provide complementary information. This polyclonal tissue by X-linked analysis would be termed *monoclonal* for a given marker (**black star**) after neoplastic-related clonal expansion takes place.

takes place and neoplastic growth ensues, genetically identical cells no longer exist. Nevertheless, certain genetic markers can be used to test clonal expansions within a tumor cell sample. These include gene rearrangement analyses of immunoglobulin genes for B cells and T-cell receptor genes for T cells (3,16).

We review the principles and requirements of the proper application of polymerase chain reaction (PCR) techniques in the detection of clonality using formalin-fixed and paraffin-embedded material. Two main groups of techniques are presented: those based on the analysis of microsatellites (linked or not to X chromosome) and those related to gene rearrangements. Their potential applications in tumor pathology and pitfalls are summarized briefly. Lastly, some methodological recommendations to obtain reliable results are given.

CLONALITY ASSAYS BASED ON MICROSATELLITE ANALYSIS

Currently, successful clonality assays using paraffin-embedded tissues are based mainly on analyses of widely dispersed, hypervariable regions composed of repetitive deoxyribonucleic acid (DNA) sequences (microsatellites) (36). Several of these microsatellites have been used extensively for DNA fingerprinting and are very useful in genetic linkage analyses, based on their high percentage of heterozygosity in the general population. The loss of heterozygosity (LOH) of a given genetic marker could be linked to loss of tumor suppressor genes (TSG) by DNA deletions (32–34), which would also contribute to the multistep carcinogenesis process selecting cells with growth advantages (44). This progression of genetic events has been found in most inherited cancer syndromes and plays an important role in sporadic cancer development.

Microsatellites have assumed an increasingly important role in this task because of their ubiquity, PCR “typability” (except for [dA]_n multimers, whose size polymorphisms are difficult to type by PCR), Mendelian codominant inheritance, and extreme polymorphism (2,4). Microsatellites belong to the family of highly polymorphic and repetitive noncoding DNA sequences. It must be noted that, although widely distributed in the human genome, microsatellites are not uniformly spaced. For example, they are underrepresented in subtelomeric regions of chromosomes. Although their origin and function are not clear (36), their polymorphism has been demonstrated to be very useful in delineating cell lineage (2,4). The different length of these tandemly arranged repeats in the paternally and maternally inherited alleles explains their high polymorphic information content and obvious applications in genome mapping and positional cloning, personal identification, population genetic analysis, and the construction of human evolutionary trees. For clonality purposes, both X-linked and non-X-linked clonality assays can be designed.

Tumor allelotyping (57,58)—the genotypic analysis of all human chromosome pairs for regions of interstitial deletion—is based conceptually on deletions of TSG alleles. The term *allelic imbalance* is associated closely with LOH and it is preferred when quantitation of minute DNA amounts is not reliable (6,7,18,48), like in paraffin-embedded tissues after microdissection. Both findings are evidence of clonal expansion in a tumor cell population, regardless of the DNA region deleted. Conversely, if the selected polymorphic region is related to known TSG, there is high probability of mutation or function dysregulation of the corresponding gene. Such a situation can explain the growth advantage of the tumor cells carrying that genetic alteration.

X-linked Clonality Analysis

X-linked clonality analysis can be used only for clonality analysis in informative female subjects, does not assess tumor heterogeneity, and does not provide any data on the precise genetic alteration responsible for clonal proliferation (see Fig. 1). All methods of clonality analysis based on X chromosome inactivation (XCI) include the ability to determine the paternally derived X chromosome (X_p) from the maternally derived one (X_m). Many genes on the X chromosome are polymorphic and permit the distinction between maternally and paternally inherited X chromosomes. Their informativeness is related directly to the frequency of their polymorphism in a population, from 29% heterozygosity for hypoxanthine phosphoribosyl transferase to more than 90% for the human androgen receptor gene (26).

According to Lyon’s hypothesis (41), all X chromosomes in a cell in excess of one are inactivated on a

random basis during early embryogenesis. Inactivation of most, but not all, genes on one of the X chromosomes is mediated by a gene called *Xist*, and represents a gross imprinting of many genes to achieve a mammalian dose compensation for X-linked genes, which renders maternal and paternal chromosomes nonequivalent functionally. The mechanisms leading to XCI have not been characterized fully, but DNA methylation may maintain the inactive state once it is established. Approximately 60% of all Cytosine-phosphate-Guanidine (CpG) dinucleotides in the DNA of vertebrates are methylated at the C5 position, but the frequency at particular sites varies between cell types (31). These methylation patterns are transmitted by clonal inheritance through the strong preference of mammalian DNA (cytosine-5)—methyltransferase for hemimethylated DNA. Methylation patterns are established during gametogenesis and early embryogenesis (primordial cell pool of 16–30 cells) (23) and involve, among other DNA regions, promoter regions of alleles on the inactive X chromosome (X^i), whereas alleles on the active X chromosome (X^a) are normally unmethylated (41). Given the small number of embryo-destined cells, it is reasonable to expect unequal numbers of inactivated X_p and X_m , although X chromosome is inactivated randomly in each cell. Thus the Lyonization ratio (X_p^a/X_m^a) in the population follows a binomial distribution. Allelic variation is determined by the number of progenitor cells at the moment of inactivation (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). The average Lyonization ratio is close to 50:50 in large cell populations, although individual variation has been found (23), resulting in skewing toward one allele. This is the reason for using as controls for unequal Lyonization the most closely related tissue thought not to be involved in the disease process (14).

The distinction of X^a from X^i can be made by gene expression analysis (messenger ribonucleic acid and protein derive only from genes on X^a) or can be delineated at the DNA level using methylation-sensitive restriction endonucleases (Fig. 2). The last method can be performed in paraffin-embedded tissues, although it has the absolute requirement of invariable and differential methylation at the polymorphic locus in X^a and X^i . A perfect X-linked clonality assay would unite highly informative polymorphism with an absolute differential methylation pattern between X^a and X^i . Therefore, an informative locus amenable to PCR-based detection of nonrandom XCI must fulfill the following four criteria:

1. High frequency of heterozygosity
2. A site that is methylated differentially in X^a and X^i , and thus is subject to differential digestion by methylation-sensitive restriction endonucleases
3. Polymorphic and methylated regions that are in suf-

FIG. 2. Dual approach for the analysis of X-chromosome inactivation in females. (**Left**) Gene expression analyses are based on messenger ribonucleic acid (mRNA) and protein polymorphism from alleles located in the active X chromosome (X^a). (**Right**) Conversely, deoxyribonucleic acid (DNA) analysis provides information through the amplification of alleles located on inactive (methylated) X chromosome (X^i) after DNA digestion with methylation-sensitive restriction endonucleases. PCR, polymerase chain reaction.

ficient proximity for amplification by one PCR primer set

4. A target that amplifies efficiently and reliably (Fig. 3). Noninvolved normal tissues related closely to the lesion must be tested simultaneously for unequal Lyonization in each particular patient.

Several possible targets have been tested, but currently the best option is the human androgen receptor gene (55,56). This gene has a hypervariable CAG trinucleotide repeat in the coding region of its first exon, located less than 100 base pairs (bp) from four methylation sites recognized by *HhaI*, and known to be methylated on X^i but not on X^a (see Fig. 3).

Non-X-linked Clonality Analysis

These tests rely basically on the demonstration of LOH, resulting from either hemizygosity (nonrandom interstitial DNA deletions) or homozygosity of mutant alleles observed in neoplasms, but only provide information when the genetic marker represents or is linked to a TSG involved in the malignant transformation. These tests represent the first option to study tumor heterogeneity if several samples are taken from a single neoplasm (17), but two main problems must be considered. First, there is no specific sequence of genetic alterations for a given tumor, and therefore several genetic markers must be tested to get information on clonality. Different sequences of genetic alterations have been proposed for several tumor types and locations based on statistical

FIG. 3. (A) Structure of the androgen receptor (AR) gene (exon 1). A highly polymorphic CAG repeat sequence location 100 base pairs (bp) downstream of a potentially methylated CG island allows a reliable yield of both components in a single polymerase chain reaction amplification (between 250 and 300 bp). Both alleles can be differentiated easily by size, and can be amplified only from the inactive X chromosome (X^i , digestion-resistant allele) in the HhaI-digested sample. (B) Allelic patterns of AR gene in monoclonal and polyclonal tissues. The presence of a single allele in HhaI-digested samples (D) in informative cases (two alleles in undigested samples [U]) defines monoclonal tissues (left, right), whereas the retention of both alleles characterizes polyclonal ones (center). Each cell shows the allelic structure after HhaI digestion to emphasize that the polymerase chain reaction amplification comes from the intact allele only (methylated X chromosome). HMW, high molecular weight; LMW, low molecular weight.

analyses of case series (5,30,57), but the frequency of each genetic alteration is variable. Second, the background level of LOH in normal tissues has been reported to be between 4% and 20%, regardless of the detection system used (8,13,51,59). A similar LOH frequency must be assumed as background in the evaluation of tumor tissues (51). Considering the worst scenario of all genetic lesions being equally important and frequent (21), the probability of randomly finding coexisting genetic alterations in normal tissues would be $0.2^2 = 4.0 \times 10^{-2}$ for two genetic loci, $0.2^3 = 8.0 \times 10^{-3}$ for three genetic loci, and so on. No single genetic alteration of TSG proves by itself that a given proliferation is monoclonal: The LOH

for that particular marker informs only on clonal expansion and cellular selection in genetically heterogeneous tumor cell populations. Only the accumulation of genetic lesions in TSG supports a monoclonal origin of tumors (15), especially if multiple samples from the same tumor show concordant genetic alteration (14,18) (see Methodological Aspects).

CLONALITY ANALYSIS BASED ON SPECIFIC GENE REARRANGEMENTS

This application will not be covered in detail because it is better known and applicable to malignant lymphomas only. It has been demonstrated to be useful in the diagnosis of malignant lymphomas, in which the presence of homogeneous-appearing lymphoid cell overgrowths are considered histologic evidence of clonal expansion and malignancy (3,10,16).

Committed lymphoid precursors undergo unique sequential assembly of the heavy and light chains of immunoglobulin (B-cell precursors) and T-cell receptor chains (T-cell precursors) during their maturation (16). Their diversity is based on somatic DNA deletions, template-independent nucleotide additions, and specific splicing (39). These processes involve, in the case of immunoglobulin heavy chain, three highly variable regions, complementarity-determining regions, which separate four framework regions (FR) (10). The locations of DNA breaks are determined by short DNA sequences (7 and 9 bp) recognized by the recombination-activating proteins. The presence of length-specific spacer sequences provides the right splicing and determines the DNA rearrangement order (e.g., D-J and V-DJ in the case of immunoglobulin). Template-independent nucleotide additions give the final DNA rearrangement of immunoglobulin or T-cell receptor chains. All gene rearrangements in lymphoid precursors take place only in cells expressing terminal deoxynucleotidyl transferase (16).

The PCR design for gene rearrangement detection has to consider the special situation of DNA sequence addition and deletion. It is particularly important for the primer binding regions, which must be located in less variable sequences (FR regions for immunoglobulin gene rearrangement) to avoid failed amplification resulting from their loss. This factor also helps to explain false-negative results in the detection of clonal rearrangement in well-differentiated lymphoid neoplasms (related to gene hypermutability). Complete rearrangements may be associated with the loss of inner FR regions (in B cells) and with δ -chain (in T cells), the most sensitive primer binding regions to detect early rearrangement. Therefore, a broad approach is recommended for detecting clonal rearrangement, including at least two different set of primers (FR III–FR IV and FR I–FR IV) in B-cell lymphoid lesions (16), and several primer sets

for the T-cell receptor γ -chain in T-cell lymphoid lesions (3).

All PCR-based analyses must be run with appropriate controls, including internal positive (provided by non-specific amplifications of locus-homologous sequences), positive (lymphoid proliferation with clonal rearrangement), negative (polyclonal lymphoid proliferation), and technique efficiency (mixture of monoclonally and polyclonally rearranged DNA tested previously) (16). The last control is needed essentially for the clinical application of these tests when sensitivity is an absolute requirement (16). Their interpretation must also consider the presence of false-positive and false-negative cases, and their causes (3,16). It should be emphasized that clonal gene rearrangement does not mean malignancy, because even some benign conditions can show it. Furthermore, well-defined monoclonal PCR bands can be observed when the lymphoid DNA template is present in trace amounts. Nonamplifiable DNA may result from the failure of PCR amplification for technical reasons (contamination, highly fragmented DNA, etc.) or when the presence of rearranged DNA is less than the sensitivity level (approximately 1% of the total cell population). Lastly, oligoclonal proliferation (especially in immunodeficiency-related lymphomas) can result in smear patterns from overlapped clonal bands.

GENERAL INTERPRETATION CRITERIA AND QUALITY CONTROLS OF CLONALITY MICROSATELLITE ANALYSES

Two complementary aspects are evaluated with X-linked and non-X-linked clonality analyses (see Fig. 1). XCI takes place early during embryologic development, usually before and unrelated to any genetic event involved in the malignant transformation. Therefore, it is really informing about clonality in tumors and precancerous conditions, although it is not able to inform on the specific molecular alterations. Different polymorphic regions have been related to TSG (recessive trait genes). Therefore, LOH analyses enable us to study the molecular pathways involved in the malignant transformation, and to test clonal expansion and tumor heterogeneity if various molecular markers are assessed in samples from different tumor areas. Moreover, the correlation of these molecular markers with other pathologic parameters (like tumor cell invasion, nuclear grade, proliferation indices, etc.) can help us to identify high-risk patients and to understand the process of multistep carcinogenesis. All nonrandom gene rearrangements, as markers of malignancy, fulfill the criteria mentioned for LOH analyses.

Any molecular analysis must be run with appropriate controls, including known positive (monoclonal proliferations, homozygous for the marker) and negative (polyclonal proliferations, heterozygous for the marker)

controls, and from embryologically related tissues for XCI analysis to exclude a skewed Lyonization ratio (18, 20,43). The PCR approach for microsatellite analysis (including both clonality assays) must amplify the right locus and accurately identify informative patients (two different alleles present in control tissues; Fig. 4). This issue becomes especially important for microsatellite analysis when the presence of extra bands is not exceptional, especially in cases of internal labeling. A sine qua non requirement for clonality analysis is the identification of a polymorphic locus in the normal control (see Fig. 4). In every case, the tumor sample must be compared with normal controls from the same patient to test patient heterozygosity for the marker—a feature that must be maintained in HhaI-digested samples in tests based on DNA methylation, such as XCI assays (Fig. 5). Patients with two identical alleles or showing skewed Lyonization in control tissues should be considered noninformative and should be excluded from clonality evaluation. Another cause of noninformative cases is the anomalous expansion or reduction of tandem repeats resulting from microsatellite instability that results in extra bands (see Fig. 4). True new bands, as true evidence of microsatellite instability, are located normally in the expected size range (usually approximately 100 bp), above or below the expected PCR product.

Therefore, LOH and allelic imbalances of gene loci can be interpreted as evidence of monoclonal proliferation (for X-linked assays of templates digested by methylation-sensitive restriction enzyme, such as HhaI) or clonal expansion (for non-X-linked assays, including gene rearrangements), if noninformative cases have been excluded previously (see Fig. 1 and 4). Positive allelic

FIG. 4. Polymerase chain reaction-based analysis of microsatellites in clonality assays. Allelic polymorphism (P) in any given control identifies informative (I) patients (left). The tumor (T)–control (C) comparison allows case classification as normal (retention of heterozygosity [ROH]) or abnormal (loss of heterozygosity [LOH] and microsatellite instability [MSI]). Cases showing MSI could be either monoclonal or polyclonal and should be excluded from clonality assays. NP, no polymorphism; NI, noninformative patient.

FIG. 5. Gel patterns of androgen receptor (AR) alleles from controls. Only polyclonal controls (two-band pattern on HhaI-digested samples [D]) from tissues with AR-allelic polymorphism (two-band pattern on HhaI-undigested samples [U]) are informative for X-linked clonality assays. Monoclonal controls (even from polymorphic tissues) and tissues with AR-allelic monomorphism lack informativeness for clonality purposes.

imbalances are determined case by case, in relation to the densitometric allelic ratio in the normal control (43), which requires a threshold of at least 4:1 in skewed data. The host cell contamination of tumor samples could give false heterozygous results that would require careful microdissection and microscopic control of the sample collection.

APPLICATIONS AND PITFALLS

Clonality assays can be useful in the analysis of different biologic processes. They have been used mainly in the study of malignant transformation and tumor progression. At this level, the complementary information provided by X-linked and non-X-linked markers contributes to the definition of the real nature of the lesion, as mentioned earlier. Monoclonal patterns would support the neoplastic nature, although they have been described in other proliferative processes such as aggressive fibromatosis (1) or focal nodular hyperplasia of the liver (25). Similarly, the polyclonal patterns reported in sacrococcygeal cystic teratomas suggest their hamartomatous nature, opposed to the monoclonal immature teratoma (54). The acquisition of additional genetic deletions in certain histologic areas favors a molecular progression as reported for the adenoma–carcinoma sequence in colon (52), or sporadic neuroendocrine tumors of the pancreas (45). The acquisition of genetic changes has been considered evidence of molecular progression that also results in tumor heterogeneity. Tests demonstrating those abnormalities only prove clonal expansions, but would support monoclonality if several markers from different tumor areas show concordant genetic alterations (15, 18).

The identity of synchronic or metachronic tumors can be tested at the molecular level using these clonality markers. Both extremes have been reported in tumors. Some coexistent tumors have revealed the same pattern of genetic markers (both X linked and non-X linked), suggesting that a common progenitor contributed to those lesions, and thus supporting a multifocal rather than a multicentric origin. This particular situation has been demonstrated in bladder tumors (18,53), human immunodeficiency virus-associated Kaposi's sarcoma (47), disseminated peritoneal leiomyomatosis (46), or multifocal C-cell hyperplasias and nodular adrenal medullary hyperplasias associated with multiple endocrine neoplasia 2A (20a). In the case of malignant neoplasms, this shared genetic alteration would support a metastatic origin for the tumors (11), or a common cellular origin for biphasic neoplasms (60). Opposite findings have been reported for prostatic tumor foci when the heterogeneous genetic composition suggests either an independent evolution of those foci from a common progenitor or a completely different origin (28,29).

Lastly, any well-characterized genetic alteration can be used for the early detection of recurrences, both local and systemic. These alterations pick clonal expansion from selected groups of cells, and they have been proposed as tools to study the resection margins in conservative surgery, as reported for head and neck squamous cell carcinomas (35). Likewise, the presence of minimal residual disease can be defined better at the molecular level by detecting circulating tumor cells with specific gene alterations, more frequently gene rearrangements, as reported for malignant lymphoma (16) or sarcomas of the Ewing family (12).

These molecular studies should be interpreted with caution. Some considerations should be made to set the proper value of these techniques. These considerations include tumor cell heterogeneity, sample size, tissue control, restriction enzyme digestion and abnormal methylation, and artifactual allelic dropout.

Tumor Cell Heterogeneity

Tumor cell heterogeneity is linked to genetic instability and biologic progression. This genetic heterogeneity is often reflected in phenotypic expression. Examples include the presence of a *Ki-ras* point mutation in carcinomatous areas from adenomatous polyposis coli (*APC*)-mutated sporadic colorectal adenomas (52). Similarly, mutations in cell cycle regulators (e.g., tumor protein 53, retinoblastoma, cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors) have been related to either proliferative advantages or apoptotic dysregulation (9). So, it can explain their association with tumor grade as far as nuclear atypia (pleomorphism, chromatin features, and size variability) is expression of both proliferation and apoptosis. Tumor heterogeneity must be

studied using several tumor samples of appropriate size from each tumor (see the following section).

Sample Size

Sample size is a limiting factor. To increase sample homogeneity and to avoid normal cell contamination, very small samples (even single cells) have been used in genetic analyses. However, the lower the number of cells, the higher the probability of false monoclonal patterns based on inadequate sampling. Although early XCI takes place at random and usually gives a chessboard pattern, small cell populations descended from a common stem cell may grow together like a clone (patch size concept or contiguous cellular regions of the same lineage). For this reason, monoclonal XCI patterns are reported in breast lobules when studied from single samples. Multiple samplings from different areas and sample sizes larger than 100 cells or 0.25 mm² can avoid this problem.

The sample size in tumor cell analysis is, therefore, an important parameter, particularly in light of tumor cell heterogeneity. Microdissection techniques allow us to pick up very small samples selectively, which can show false cellular homogeneity, based on LOH or allelic imbalance. If the tumor cell populations selected for molecular analysis are taken before they become a biologically prominent component (with proliferative or invasive advantages), the results obtained may be confusing and irrelevant clinically, and need to be evaluated in the proper biologic context. This would be the case with microheterogeneity in tumors that tend to give disparate results with meanings that remain essentially unknown. Except for intraepithelial proliferation, all cell samples with microdissection provide target cell-rich samples with a varying degree of host cell contamination (including stromal, inflammatory, and endothelial cells). Taking all these factors into consideration, multiple samples from the same case should always be studied, and assays should be performed in duplicate before accepting the results as relevant.

Tissue Control

Tissue control from the same patient is an absolute requirement to test patient heterozygosity for a particular TSG marker (as mentioned earlier; see Fig. 5 and Fig. 6). Ideally, embryologically related tissues are the best to show a reliable X-linked clonality pattern. Oncogene genetic changes such as RAS point mutations that are not found in the germline are excluded from this approach and do not require heterozygosity control.

Restriction Enzyme Digestion and Abnormal Methylation

Every single step for all molecular tests must be controlled for completion to avoid false results. Appropriate

FIG. 6. Methylation level and allelic patterns of androgen receptor (AR). **(A)** Monoclonal tissues would only change the clonality pattern if the amplified AR locus was hypermethylated. A pseudopolyclonal pattern would be revealed on gel electrophoresis (**right**). **(B)** Polyclonal tissues would only change the clonality pattern if the amplified AR locus was hypomethylated. A pseudomonoclonal pattern would be revealed on gel electrophoresis (**left**). PCR, polymerase chain reaction; U, HhaI-undigested sample; D, HhaI-digested sample; HMW, high molecular weight; LMW, low molecular weight.

controls must be run, especially for restriction enzyme digestions. Because XCI analysis is based on differential DNA methylation of one allele from X-chromosome genes (e.g., human androgen receptor gene) and relies on endonuclease digestion by methylation-sensitive restriction enzymes, suboptimal enzymatic digestion provides a changed clonality pattern in the case of monoclonal tissues. Likewise, abnormal methylation provides potentially the same false result in the case of hypermethylation, whereas hypomethylation could affect the clonality pattern in polyclonal tissues giving a pseudomonoclonal one (see Fig. 6). Additionally, methylation abnormalities occur during the course of malignant transformation (37). Hypomethylation has been described in relation to increased proliferation during early stages of neoplasms, whereas hypermethylation has been linked to

late stages associated with a higher mutation rate and tumor progression (42).

Artifactual Allelic Dropout

PCR bias against one allele (especially the larger one) can result in preferential amplification of the other allele (usually the smaller) (24,49). An appropriate extraction method providing DNA of enough quality (3,19), and PCR designs including both long denaturation and extension during the first three cycles, and 7-deaza-deoxyguanine triphosphate (dGTP) in the amplification mixture to improve the amplification of CG-rich DNA regions reasonably avoids that bias (18,20,49).

METHODOLOGIC ASPECTS

Formalin-fixed, paraffin-embedded tissues are analyzed more easily at the DNA level, resulting in part from the better preservation of this nucleic acid. Therefore, any PCR-based technique applied to this material should consider DNA extraction, DNA modification (such as restriction enzyme digestion), target amplification, and adequate gel resolution of the products.

DNA Extraction Process

Several chemical modifications are induced in tissues by fixation and processing, including cross-linking between basic amino acids of proteins and the amino groups on DNA bases. Nonspecific amplifications are caused mainly by primer-independent, but DNA polymerase- and cycling-dependent, incorporation of nucleotides into DNA, possibly related to DNA repair and/or internal priming (40). This is the reason for complete and intense protein digestion before DNA purification (19). Denaturing reagents can break the cross-linked strands but in turn provide short-length DNA strands, precluding their use in protocols that require DNA of 250 to 500 bp in length. The general DNA quality of the extracted DNA should be tested by gel electrophoresis of the protein-digested sample, universal DNA amplification using degenerated oligonucleotide primer-PCR, or amplifying β -globin gene with primer sets at least 100 bp lengthier than the final DNA target.

The easiest protocol giving the highest DNA quality is 55 to 60°C prolonged proteinase K digestion (5–7 days, with every-day enzyme replacement) (19). The standard phenol-chloroform purification protocol results in the best contaminant-free DNA for any PCR application. Negative amplification resulting from sample contamination (specific amplification lacking with no primer dimers) can be avoided by diluting the sample.

DNA Modifications

Certain applications need original DNA strands, such as those based on the genomic imprinting of XCI. The

presence of methylated cytosine can be tested by sample digestion by methylation-sensitive restriction endonucleases. In any case, appropriate internal control should be included to prove complete digestion. The samples already show smear patterns, and even they may be undetectable using gel electrophoresis (especially for microdissected samples). A logical way to accomplish this issue is to include DNA mimickers in every sample undergoing restriction enzyme digestion. These mimickers are normally viral DNA (such as phages) and should fulfill some requirements. They must be linear and double stranded (like human genomic DNA), and they must contain base sequences recognized by the tested enzyme with reliable pre- and postdigestion patterns. In addition, no sequence similarity able to give nonspecific amplification in further PCR should be present. Xho-I-linearized ϕ X174RII phage represents an ideal mimic for HhaI digestion used for XCI analysis.

XCI analysis tests the differential methylation level in a CpG island approximately 100 bp upstream of the CAG repeat (56). Different methylation-sensitive restriction enzymes have been used, especially HhaI and HpaII. The first provides more reliable results because of its activity with single-strand DNA (that activity has not been demonstrated for HpaII). We must keep in mind that the embedding process partly denatures DNA and, therefore, single-strand DNA is a normal component in archival material.

Target Amplification by PCR and Gel Resolution

All PCR methods should consider the appropriate conditions regarding Mg^{2+} and primer concentrations, nucleotide concentration, number of cycles in case of PCR-based quantitative analyses, PCR product labeling, and detection methods. The standards for all PCR technique need specific optimization for each set of primer according to Mg^{2+} (normally 1.5 mM) and nucleotide concentration (in the 50- μ M range for microsatellite analyses). PCR cycling conditions should always consider the number of cycles to avoid product saturation unacceptable for any quantitative PCR design; in general, between 25 and 30 cycles give adequate amplification in accordance with the initial DNA concentration. We optimized experimentally the conditions for PCR as follows: the reactions were run with 1.5 mM $MgCl_2$, using 0.3 μ M each primer, 200 μ M each dNTP (including 7-deaza-dGTP instead of dGTP) and 1 μ L template. A long denaturation (4 minutes) was used in the first three cycles, the annealing temperature was 55°C, and the number of cycles was optimized experimentally to 28. A "hot start" (addition of primers to mixtures kept at 85°C) should also be included to facilitate the complete denaturation of DNA strands with high CG content in the initial amplifications (18, 20, 43).

DNA samples from microdissected tissues are not ideal for reliable quantification and are generally run with unknown target DNA concentration. So, relative quantification of both allelic bands must be taken into consideration to determine whether LOH (or *allelic imbalance*) is present. This issue brings us to consider the labeling and detection method. ^{32}P - and ^{33}P -based radio-labeling represent the standard protocols, including both external (one primer is 5' labeled) and internal (labeled nucleotide in the PCR mixture) methods. Although the latter usually gives more background, it permits the highest sensitivity for microdissected paraffin-embedded samples. Allelic separation can be achieved by running the samples far enough into high-resolution denaturing polyacrylamide gels (variable concentrations of formamide and urea). Some other detection methods have been used, including fluorescent labeling (6,7) and silver staining of PCR products (38). The highest sensitivity is achieved by radioisotopic methods, which remains the standard for molecular detection of genetic alterations, especially in formalin-fixed, paraffin-embedded material. Additionally, the ratio between signal and initial DNA amount is highly variable for silver-stained gels, making the applications of this technique less reliable for quantification. Different technical approaches have been used to detect interstitial DNA deletion and single base changes (mutations/polymorphisms), including single-strand conformational polymorphism, denaturant gradient gel electrophoresis, mutant allele-specific amplification, ribonuclease (RNase) protection, etc. (27,50). Although the final proof for any mutation must be direct sequencing, one of the most sensitive methods for detecting single base changes is PCR/denaturant gradient gel electrophoresis, which is able to distinguish DNA strands differing in only one base (3,18).

Lastly, the linear ratio between radioactive emission and signal deposition can be maintained by film preflashing to get a 0.1 to 0.2-OD unit absorbance increase at 540 nm in the preflashed film. In addition, signal stabilization during autoradiogram development requires -70°C storage. The allelic ratio has to be quantitated in normalized samples to exclude any potential contamination with normal tissue (43). At that level, different computer software is available to aid in analysis. □

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