Techniques for clonal analysis using X-chromosome inactivation patterns (XCIP) and their interpretation.

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Introduction

Most human malignant cells are derived from a single, common, abnormal or transformed ancestral cell and are thus clonal in origin. Where disease specific abnormalities such as cytogenetic aberrations are unknown, the assessment of X-chromosome inactivation patterns (XCIPs) is a useful means of performing clonal analysis. In mammalian female somatic cells only one of the two X-chromosomes remains active to prevent a lethal double dose of genes from the X-chromosome. Once X-chromosome inactivation is established at an early stage of embryogenesis it remains stable and is stably inherited, such that all daughter cells maintain the same inactive X throughout all future rounds of mitosis. Due to the random nature of X-chromosome inactivation examination of the XCIP for a particular tissue or cell type then reveals that the results are distributed in a Gaussian pattern about a mean of 50%.

Practical techniques

In order to perform clonal analysis using XCIPs there are two basic requirements, namely the ability to distinguish between the two X-chromosomes, usually achieved using an X-linked polymorphic marker, and secondly the ability to discriminate between the active and inactive X-chromosomes. Both DNA and RNA may be used for these assays. The different assays as summarised below and technical details provided in the references.

DNA A variety of relatively common polymorphisms have been identified in a number of X-linked genes that have been used to determine XCIPs at the DNA level. Here, active

and inactive chromosomes are distinguished by differential methylation patterns, detected by use of methylation sensitive restriction enzymes, such as *Hha* I and *Hpa* II, which will not digest methylated DNA. Southern blotting was initially used but is now superseded by polymerase chain reaction (PCR) techniques. Examples of genes that have been studied include phospho-glyerate kinase (PGK), monoamine oxidase A, which contain polymorphisms and, the human androgen receptor gene (HUMARA) and the fragile X gene -both of which contain a polymorphic VNTR. The HUMARA locus contains a trinucleotide (CAG) repeat or VNTR sequence within the first exon, and there are four methylation sensitive restriction enzyme recognition sites, two each of *Hha* I and *Hpa* II, within 100 base pairs of the 5' end of the VNTR. The HUMARA locus is highly polymorphic with each VNTR having 11-31 CAG repeat units; the two X-chromosomes can be discriminated using this assay in approximately 90% of females For this method of XCIP analysis the DNA must first be digested with a methylation sensitive enzyme, as PCR does not maintain methylation patterns, thus only the methylated (undigested) gene is amplified in the subsequent PCR step, which can be quantified using either a radiolabelled or fluorescent primer. This technique is summarised in figure 1.

RNA Recently polymorphisms have been described within the transcribed regions of X-linked housekeeping genes enabling direct analysis of the relative expression of the alleles corresponding to the two X-chromosomes. Thus RNA based clonal assays circumvent disadvantages of DNA analysis as they can do not rely upon methylation which may be affected by nongenetic factors, carcinogenesis, vary from gene to gene and RNA methods can be applied to enucleate cells. Single base substitutions have been reported in a number of genes so far including G6PD (a different polymorphism from the A/A- African variant), iduronate-2-sulphatase (IDS), the palmitoylated membrane protein p55, BTK, and FHL1. Several different methods of determining an XCIP have been described; including PCR followed by a restriction enzyme digestion or a ligase detection reaction, allele specific PCR which has more recently been combined with SSCP.

Technical pitfalls

There are however potential technical problems which may confound interpretation of results obtained using such PCR techniques, these include variability of methylation sensitive enzyme digestion, *Taq* polymerase slipping within the repeat unit and preferential amplification of shorter alleles of the VNTR at increasing PCR cycle number. Finally, when primers and/or nucleotides becoming limiting in the PCR reaction, heteroduplex formation may occur which can interfere with subsequent restriction enzyme digestion, as the heteroduplexes are undigested and falsely elevate the proportion of uncut alleles. It has also been suggested that methylation of inactive genes may be inconsistent between genes, affected by carcinogenesis and other nongenetic factors.

When transcriptional assays are used sample purity becomes more critical than for DNA assays as different tissues may contain highly variable transcipt levels, with for example a single T-lymphocyte being equivalent to 6 neutrophils or 800 platelets for the IDS mRNA.
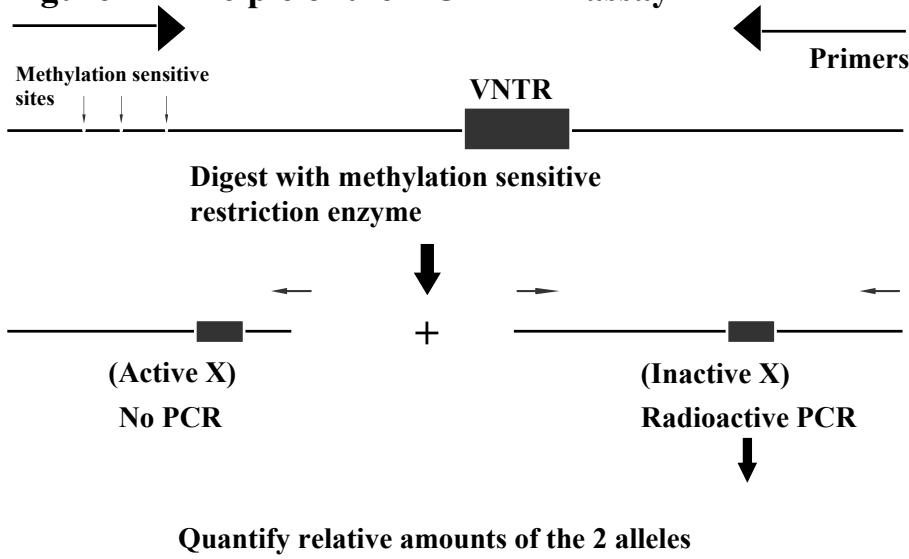
Interpretation of clonal analysis

The results of clonal analysis performed using XCIPs must be interpreted with appropriate reference to both the individuals' own constitutive XCIP and their age. An

arbitrary definition of a clonal population is a 75% expression of one allele and 25% of the other. This may also be expressed as an allelic ratio.
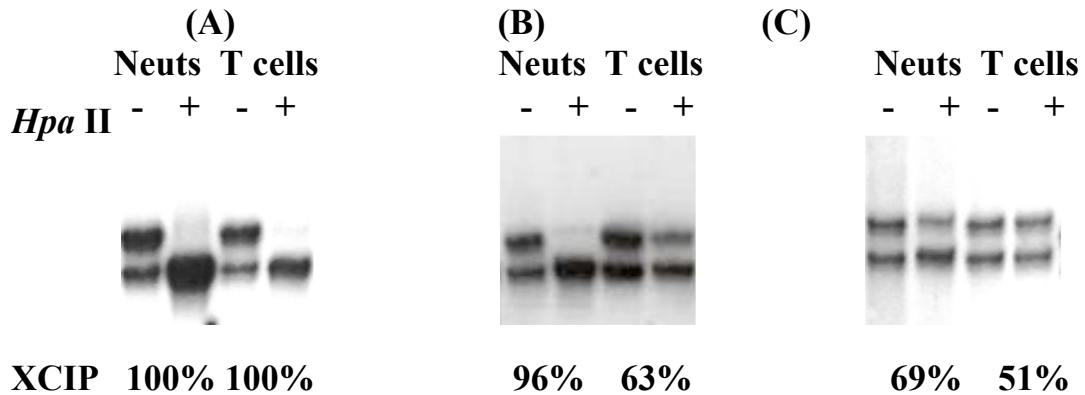
20-25% of haematologically normal females have a constitutively imbalanced or skewed XCIP with greater than 75% expression of one allele. This is likely to reflect the small number of haemopoietic stem cells at the time of Lyonisation. As a consequence of constitutive skewing, an imbalanced or skewed XCIP therefore can only be interpreted as clonal if control tissue (T lymphocytes for myeloid cells) from the same individual shows a balanced or significantly different pattern. Thus a control cell population must be tested in parallel to test population. The definition of a significantly different result between test and control results varies between laboratories but must be greater than interassay variation.

Skewed XCIP in myeloid cells of the elderly which may not be reflected in T lymphocyte XCIPs is known as age-related skewing. Possible explanations include some small selective advantage or disadvantage occurs to a particular X-chromosome, i.e. somatic cell selection, genetic predisposition and changes in stem cell usage (stochastic stem cell loss), This has important implications both for the interpretation of XCIPs in the elderly, and the usefulness of these techniques in the diagnosis of haematological disorders in older females. An imbalanced myeloid XCIP in the presence of a balanced T cell XCIP cannot be interpreted in elderly females (arbitrarily defined as older than 65 years of age) as evidence of clonal haemopoiesis. Conversely balanced myeloid XCIPs can be used to indicate the presence of polyclonal haemopoiesis.

Figure 1 Principle of the HUMARA assay

Primers

Methylation sensitive sites

VNTR

Digest with methylation sensitive restriction enzyme

+

(Active X)

No PCR

(Inactive X)

Radioactive PCR

Quantify relative amounts of the 2 alleles

Figure 2 Representative results from HUMARA analysis of T cells and neutrophils from ET patients

	(A)		**(B)**		**(C)**	
	Neuts	**T cells**	**Neuts**	**T cells**	**Neuts**	**T cells**
Hpa II	- +	- +	- +	- +	- +	- +
XCIP	100%	100%	96%	63%	69%	51%

Representative analyses of ET patients from each of the results groups:
(A) XCIPs skewed in both samples
(B) neutrophil XCIP skewed, T cell XCIP balanced
(C) both XCIPs balanced.

Additional reading

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