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An expression based clonality assay at the human androgen receptor locus (HUMARA) on chromosome X

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X-chromosome inactivation based clonality assays, first described by Fialkow and co-workers (1), have the important advantage of not relying on any specific tumor markers, and can thus potentially determine clonal derivation of cells in any informative female. These assays rely on the ability to distinguish between (i) the *maternal* vs. *paternal* X chromosome using polymorphisms and (ii) the *active* vs. the *inactive* X chromosome using either differential expression of genes or differential methylation of the active vs. inactive X. Fialkow's original approach was based on a rare coding polymorphism at the glucose-6-phosphate dehydrogenase (G6PD) locus on chromosome X, and is limited by low informativeness. An alternative strategy developed by Vogelstein *et al.* takes advantage of changes in methylation patterns that accompany inactivation of the X chromosome, which can be delineated with methylation sensitive restriction endonucleases such as *Hpa*II. Informative restriction fragment length polymorphisms (RFLP) associated with the phosphoglycerate kinase (PGK) and hypoxanthine phosphoribosyl transferase (HPRT) loci, and the highly informative VNTR at the DXS255 locus have expanded informativeness to include most females. However, there has been concern that differential methylation at these loci is not a reliable marker for the state of activation of the X chromosome, or that variable methylation may occur in association with malignancy. This is well exemplified at the DXS255 locus analyzed with the M27β probe which is hypermethylated in a significant proportion of acute myelogenous leukemia blast populations (3). Until recently, no locus has had a coding polymorphism coupled with differential methylation sites which would allow comparison of both the expression and methylation based assays at the same locus. Such a combination would allow validation of differential methylation based assays in comparison with expression analysis at the same locus.

The human androgen receptor assay (HUMARA) is a newly described clonality assay that takes advantage of a highly polymorphic trinucleotide repeat in the coding region of the first exon of the human androgen receptor gene (4). The CAG repeat is closely linked to four methylation sites that have served as the basis for studying X-inactivation patterns in female carrier of X-linked hemopathies (4). The methylation sites are unmethylated on the active X chromosome and methylated on the inactive X chromosome. We have devised an expression assay at this locus using a reverse transcriptase (RT) PCR strategy that allows clonality determination without relying on differential methylation of X chromosomes. We have compared the expression assay with the differential methylation based assay in informative female patients with hematologic malignancies (4) and brain tumor (1) and found concordant results in each case. The expression clonality assay described in this report has the potential of being informative in more than 90% of females, which is a significant advance for the field. Furthermore, this expression assay allows for a unique validation of the differential methylation based assay, confirming the reliability of the methylation patterns at the human androgen receptor in this small series of patients. However, usefulness of the HUMARA expression assay may be limited by the level of expression of the androgen receptor in different tumors or tissues.

Human androgen receptor expression assay: reverse transcription

The RT-PCR assay was based on a nested primer strategy described by Baier *et al.* (5). Briefly, RNA was isolated from peripheral blood mononuclear cells (4 patients) and from tumor specimen (1 patient) using the RNAzol™ (Tel-Test, Inc.) method according to manufacturer recommendations and from a brain tumor specimen (1 patient) by the method of Chirgwin (6). Two μg of total RNA was added to a tube containing 18 μl of a mix of dNTP (1 mM of each); RT buffer (5×: 250 mM Tris–HCl, 40 mM $MgCl_2$, 50 mM KCl 1, 5 mM dithiothreitol; pH 8.5); nested primer: 5′-GGCTCCAGGCTCTGGGACGC-AACCTCT-3′ (20 pmol); AMV reverse transcriptase (40 units, Boehringer Mannheim). Reverse transcription was performed at 42°C for 60 min and enzyme was inactivated at 90°C for 10 min. A mock reverse transcription lacking reverse transcriptase was performed as a control for DNA contamination. PCR *amplification of the HUMARA locus*: 2 μl of reverse transcription products were added to 23 μl of a PCR mix containing buffer (10×: 500 mM NaCl, 100 mM Tris–HCl, pH 8.2; 15 mM $MgCl_2$, 0.1% gelatin); dNTPs (200 μM each); primer HUMARA I: 5′-GCTGTGAAGGTTGCTGTTCCTCAT-3′, and primer HUMARA II: 5′-TCCAGAATCTGTTCCAGAGCGT-

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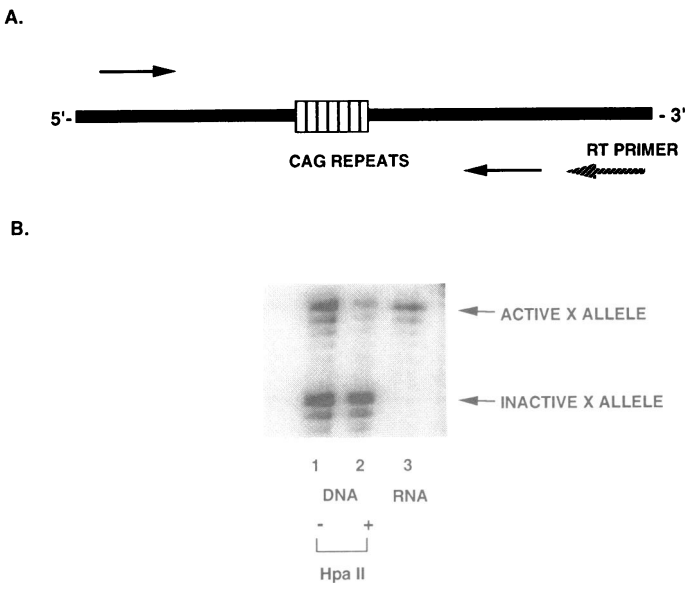

Figure 1. HUMARA EXPRESSION ASSAY: **A.** Human androgen receptor locus: RNA is reverse transcribed using a nested primer strategy. cDNA is amplified with primers that flank the informative CAG repeat. **B.** Clonality analysis of DNA and RNA specimen from a female patient with acute myelogenous leukemia (AML). 1) Non precut DNA shows the two X-linked alleles corresponding to the paternal and maternal copies of the androgen receptor locus. Shadow bands below the major band are characteristic of amplified repeat sequences. 2) DNA precut with *Hpa*II gives a single dominant allele, reflecting clonal derivation of >90% of the cells in sample. The amplified allele corresponds to the methylated *inactive X allele*. 3) Reverse transcription of RNA isolated from blood, followed by amplification of the HUMARA locus. Since only genes on the active X are transcribed only the *active X allele* is amplified; demonstrating that cells were derived from a single progenitor. Similar data were obtained in patients with chronic myelogenous leukemia (CML, 3 patients) and brain tumor (1 patient), confirming reliability of the methylation-based HUMARA DNA assay.

GC-3′ (12.5 pmol each); DMSO (0.75 μl, Sigma); γ-^{32}P end labeled HUMARA I primer (1.25 pmol), [*kinasing protocol*: 2 μl (5 pmol/μl) of primer HUMARA I is added to 10× kinase buffer (1 μl, Boehringer Mannheim), γ^{32}P dATP (6 μl, 3000 μCi/mmol), polynucleotide kinase (0.6 μl, Boehringer Mannheim) and H_2O (0.8 μl), followed by incubation at 37°C for 30 min and inactivation of PK at 90°C for 2 min]; Taq polymerase (0.5 units, Cetus); H_2O to final volume of 23 μl. Samples were amplified on a programmable thermal cycler (MJ Research, Inc.) initial DNA denaturation at 94°C for 3 min then 28 cycles starting with 94°C for 45 sec, 60°C for 30 sec and 72°C for 30 sec. At the end of amplification 12.5 μl of formamide loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to each sample, and samples were denatured at 95°C for 3 min and chilled rapidly. Amplified PCR products (8−10 μl) were electrophoresed on a 4% acrylamide-urea-formamide denaturing gel at 80 watts for 3.5 hours. The gel was dried and exposed to a phosphor screen for 24 hours, scanned on a Phosphorimager (Molecular Dynamics) and allelic ratios were quantitated with ImageQuant software.

Differential methylation based clonality assay

The HUMARA assay was carried out as previously described (4) with minor modifications. Briefly, genomic DNA was precut by mixing sample DNA (100 ng−1 μg in 2 μl) with *Hpa*II (1 μl, high concentration, 40 U/μl), *Rsa*I (0−5 μl, high concentration, 40 U/μl), L buffer (2 μl, Boehringer Mannheim) and H_2O (14.5 μl). An auto-control was precut in the same way except that *Hpa*II was omitted from the mix. Samples were incubated at 37°C overnight, and heat inactivated at 95°C for 10 min prior to amplification. The HUMARA locus was amplified as described above.

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