Molecular diagnosis of sex chromosome aneuploidy using quantitative PCR

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ABSTRACT

Numeric sex chromosome imbalances, or aneuploidies, are present in several pathological conditions including tumors, abnormal gestations, and clinical syndromes. Here we report a method to identify karyotypic imbalances of the X and Y chromosomes using the polymerase chain reaction (PCR). The polymerase chain reaction was used to quantitatively coamplify the sex chromosome linked genes ZFX and ZFY. Quantitation was facilitated by 1) use of a single primer set which recognizes both templates, 2) incorporation of radiolabelled nucleotides during amplification, and 3) use of amplification conditions which minimize heteroduplex formation. High accuracy of the method was confirmed by concordance with values expected from titrated male and female DNAs and cells from patients with sex chromosome aneuploidy. This approach provides a rapid and reproducible method of evaluating relative abundance of allelic genes, and might be applied to detection of autosomal aneuploidy.

INTRODUCTION

Existing methods of detection of numeric chromosome abnormalities have a variety of limitations. Conventional karyotyping is labor intensive and susceptible to sampling error. Automated nuclear DNA cytometry (flow cytometry or robotic microspectrophotometry) permits larger populations of cells to be sampled, but at the expense of information about the identity of specific chromosomes. It is now possible to use DNA isolated from a large population of cells as the basis for quantitative allele-specific studies. In the case of the X and Y chromosomes, the molecular genetic approach is simplified by the recent cloning of sex chromosome linked (non-pseudoautosomal) genes, some with extensive inter-chromosomal homology(1–3). Of the molecular methods amenable to quantitative study the polymerase chain reaction has several advantages: it is less labor intensive and more sensitive than *in situ* hybridization and, unlike Southern blot analysis, does not require transfer to a membrane and hybridization for quantitation.

Molecular sex chromosome analysis has largely been qualitative to date, focused primarily on antenatal diagnosis of gender by detection of Y chromosome sequences (4,5). Sex chromosome aneuploidy is, however, a frequently encountered abnormality, accounting for approximately 40% of chromosome anomalies in livebirths (6). These include Turner syndrome (45,X and variants), Klinefelter syndrome (47,XYY and variants), and 47,XXX and 47,XYY genotypes, usually diagnosed by clinical evaluation and documentation of the attendant sex chromosome aneuploidy or mosaicism. Previous attempts to use molecular sex chromosome markers to study the genotype of these patients have been at best semi-quantitative (7,8). More extensive efforts have been made to quantitate sex chromosome abundance in populations of haploid male germ cells(9–13). Quantitative identification of sex chromosomes is also of use in diagnosis of hydatidiform mole (14,15), and qualitative identification has application in forensic diagnosis.

We have developed a method to identify sex chromosome aneuploidy by PCR which uses a single primer set directed against homologous but distinct genes on the X and Y chromosomes. This system provides a basis for defining accuracy and precision of quantitative PCR without the need to add exogenous marker template or use multiple primer sets. We have chosen the X and Y chromosome linked non-pseudoautosomal alleles of the zinc finger coding genes, ZFX and ZFY (16) as markers for the sex chromosomes. A pair of primers with 100% homology to both target alleles was used to generate a 406 base pair (bp) product with sex chromosome specific restriction fragment length polymorphisms.

MATERIALS AND METHODS

DNA Isolation

DNA for titration experiments was isolated from normal human male or female somatic tissues (liver or uterus) using a standard lysis solution (0.1 mg/ml Proteinase K, 100 mM NaCl, 10 mM Tris (pH 8), 25 mM EDTA, 0.5% SDS) (17). All samples were incubated overnight at 50°C prior to phenol extraction and ethanol precipitation the following day. DNA concentration was calculated from the OD₂₆₀ and diluted to

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Table I. Estimation of Y chromosome content in human DNAs amplified with zf a/c primers

Sample ^a	DNA ^b	n ^c	Mean X1/Y1 ^d	Expected %Y ^e	Calculated % Y ^f Mean (SD) ^g
100M(A)	100% Male	11	1.49	50.0	50.3 (2.4)
100M(B)	100% Male	13	1.53	50.0	49.5 (2.2)
75M	75% Male	4	2.87	37.5	34.5 (2.5)
50M	50% Male	4	5.48	25.0	21.7 (2.2)
25M	25% Male	4	11.83	12.5	11.3 (1.1)
CL1	47,XY	15	0.73	66.7	67.5 (3.0)
CL2	47,XY	5	0.90	66.7	62.9 (4.2)
CL3	48,XXY	5	1.69	50.0	47.2 (2.3)
CL4	47,XXY	5	3.74	33.3	28.9 (3.1)
CL5	47,XXY	15	2.61	33.3	37.0 (4.1)
CL6	49,XXXXY	5	6.05	20.0	20.2 (2.8)

^aTitrated male and female DNAs (upper, '% Male') and aneuploid cell line DNA (lower, 'CL').

^bTitrated DNA is a mixture of normal male and female DNA. Karyotypes are given for aneuploid cell lines.

^cNumber (n) of separate PCR reactions, each using 500ng template amplified 25 primary and 2 secondary cycles as described in Materials and Methods. Each DNA included tubes amplified on different days and analyzed on different gels.

^dAverage relative signal intensity of X1 to Y1 fragments generated by amplification with zf a/c primers (see Materials and Methods).

^eY chromosome percentage of all sex chromosomes, based upon measured titration or karyotype.

^fY chromosome percentage of all sex chromosomes, calculated from the observed X1/Y1 ratios (see Materials and Methods).

^gMean $\pm$ standard deviation (SD).

100 ng/ μ l. Male DNA was mixed with female DNA to yield the following titrations of male DNA: 75%, 50%, and 25%. Human cell lines established from patients with sex chromosome aneuploidy were obtained from the Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, Camden, NJ (Table I concordance with Coriell accession numbers: CL1,GM09326; CL2,GM01250; CL3,GM04375A; CL4,GM03102; CL5,GM00325; CL6,GM01202C) . Cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 20% fetal calf serum prior to harvesting and DNA isolation as described above. Cell line DNAs were assigned a reference code, analyzed blindly, and decoded at the time of data compilation.

PCR Amplification

500 ng of purified DNA was amplified in a 50 μ l polymerase chain reaction using the 20-mer oligonucleotide primers zf-a and zf-c representing nucleotides 256–275 and 642–661 respectively, of the ZFY containing plasmid pDP1007 as recorded in the GenBank database (16). Primer zf-a is (5'–3') ACCAC-CTGGAGAGCCACAAG; primer zf-c is (5'–3')TCACACT-TGAATGGCATCTC. Comparison with the homologous sequence of ZFX (pDP1065) (3) allowed prediction of expected allele-specific *Hae*III restriction fragments (Figure 1). Trace amounts (final 25–250 nM) of ³²P-TTP (800 Ci/mmol) were added to the reaction mixture (10 mM Tris pH 8.4, 50 mM KCl, 20 μ g/ml gelatin, 1.5 mM MgCl, oligonucleotide primers 0.2 μ M each, 0.2 mM each dNTP, 25U/ml Taq polymerase (Perkin Elmer Cetus, Norwalk, CT)) to label amplified products. Magnesium concentration and annealing temperature were empirically optimized at 1.5 mM and 50°C, respectively. In each experiment a negative control composed of PCR reaction mix without template was included, and the X1 (ZFX) band served

Fig. 1. Panel A: Schematic diagram of amplified ZF region on the human X and Y chromosomes. The region targeted by the 20-mer oligonucleotide primers zf-a and zf-c (3). Both primers anneal to completely homologous targets, flanking an intervening stretch in ZFX and ZFY with 95% homology. Within the amplified product ZFY contains a *Hae*III site not present in ZFX, yielding Y chromosome specific *Hae*III restriction fragments of 84 bp (Y2) and 280 bp (Y1), in addition to a 42 bp fragment (X/Y) common to X and Y chromosomes. *Hae*III digestion of the amplified product ZFX gives a 364 bp X chromosome specific (X1) restriction fragment in addition to the common 42 bp fragment (X/Y). *Hae* III sites are shown by arrows. Panel B: zf a/c primed PCR products from male and female DNA. Autoradiograph (72 hour exposure) of undigested and *Hae*III digested PCR products generated by amplification of male (lanes 1 & 3) or female (lanes 2 & 4) somatic DNA using primers zf-a and zf-c. Lane M contains Phi-X 174 molecular weight marker digested with *Hae*III. A 406 bp amplification product (Uncut) can be discriminated upon digestion with *Hae*III into ZFX (X1) or ZFY (Y1,Y2) specific fragments.

as a positive internal control in all DNAs examined. Thermal cycling followed the sequence: 1) initial denaturing at 93°C for 4 minutes; 2) repeated cycles at: 93°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 seconds; and 3) final extension at 72°C for 7 minutes. In cases where secondary PCR was performed, 1–5 μ l of the primary PCR product were added to a fresh 50 μ l reaction mixture and cycled as outlined above for 1–20 additional cycles. Products were digested with *Hae*III and electrophoresed at 400–600 V in an 8% polyacrylamide gel made in TBE (45 mM Tris-borate, 1 mM EDTA). Gels were dried and autoradiography performed with preflashed Kodak XAR film at –70°C with intensifying screens.

Densitometry

Autoradiogram optical density was measured with an EC model 910 optical densitometer and the resultant plot processed using the GS365W Electrophoresis Data System, v.2.0 (Hoeffer Scientific Instruments, San Francisco, CA). The baseline of plots was corrected by the best straight line fit, and peak area calculated. The relative area associated with fragments X1 and Y1 (Figure 1) was expressed as a ratio (X1/Y1). This ratio in normal male somatic DNA (50% of sex chromosomes are Y,

Fig. 2. Effects of Secondary PCR on X1/Y1 ratio. Male somatic DNA was amplified with zf-a and zf-c primers in the presence of ^{32}P -TTP for 20, 25, or 30 cycles of primary PCR followed by secondary PCR using 1 μl of the primary reaction products as input (Materials and Methods). Secondary PCR was performed for 0, 2, 5, 7, 10, 15 or 20 cycles. X1/Y1 ratios were determined by densitometry of autoradiograms. In the absence of heteroduplexes, the X1/Y1 ratio detected by ^{32}P -TTP incorporation in male DNA (50% Y) should be approximately 1.4. Densitometry shows X1 bias introduced into X1/Y1 ratios with increasing cycle number. This is largely reversed by 2–5 cycles of secondary PCR, and recurs with continued secondary amplification.

designated '50Y') was used to establish relative signal intensity generated from equimolar starting amounts of ZFX and ZFY templates. Relative ZFX and ZFY contents of each sample (ZFX/ZFY) were calculated relative to the 50% Y standard (50Y) as follows: $\text{ZFX/ZFY} = (\text{X1/Y1})/(\text{X1}_{50\text{Y}}/\text{Y1}_{50\text{Y}})$. This ratio was then translated into percentages for ZFX and ZFY using: $\%Y = 100 \times \text{ZFY}/(\text{ZFY} + \text{ZFX})$.

RESULTS

PCR Products

The 20-mer oligonucleotide primers zf-a and zf-c generated amplified products of expected molecular weight (Figure 1) which hybridized on Southern blot analysis to the ZFY probe pDP1007 (data not shown). The 364 bp ZFX-specific *Hae*III fragment X1 and the 280 bp ZFY specific fragment Y1 were used as markers for the X and Y chromosome, respectively, in subsequent experiments.

Control of Heteroduplex Formation

Depletion of PCR components or saturation of Taq polymerase activity, evident as a loss of amplification efficiency (plateau), might bias ZFX/ZFY quantitation towards ZFX by formation of ZFX-ZFY heteroduplexes with a ZFX-like restriction pattern. The logarithmic phase of amplification was identified by amplifying 500 ng of template DNA for a variable number of cycles and the quantity of product was measured by incorporation of radiolabelled TTP (Data not shown). Amplification was found to be logarithmic through approximately 24–25 cycles, beyond which the rate of product accumulation plateaus.

Relative autoradiographic intensity of radiolabelled sex chromosome specific fragments X1 and Y1 was measured in DNA following different numbers of PCR cycles (Figure 2), both in logarithmic and plateau phases. Normal male DNA, assumed

Fig. 3. Autoradiography of titrated male/female DNA PCR products. Autoradiogram (48 hour exposure) of *Hae*III fragments resulting from zf a/c primed amplification of titrated somatic DNAs. Samples include 100% male DNA (50% Y, 50% X), 75% male DNA (37.5% Y, 62.5% X), 50% male DNA (25% Y, 75% X), 25% male DNA (12.5% Y, 87.5% X), and female DNA (0% Y) (see Materials and Methods). Primary amplification for 25 cycles followed by 2 secondary cycles and electrophoresis are described in Materials and Methods.

to have equimolar concentrations of the ZFX and ZFY templates, was used as starting material. A prediction of the expected relative intensity of the X1 and Y1 bands labelled with ^{32}P -TTP incorporation during synthesis was generated by counting the number of A-T pairs synthesized in each fragment: 201 and 148 respectively. The expected X1/Y1 ratio of TTP incorporation is thus 1.4. The observed X1/Y1 ratios as measured by densitometry of autoradiograms (see Materials and Methods) slightly exceeded this predicted value during logarithmic amplification and increased dramatically in the plateau phase (Figure 2, primary PCR only, 0 cycles secondary PCR). With increasing cycle number beyond 20 cycles, extraneous, unpredicted bands appeared.

We reversed the effects of reagent depletion during (primary) PCR by adding an aliquot of amplification products to a fresh (secondary) reaction mixture. Product produced from either 20, 25, or 30 (primary) cycles of amplification was diluted into fresh PCR reaction mix with primers and reamplified for a variable number of (secondary) cycles. In this manner the X1/Y1 ratio was restored near the predicted level of 1.4 (Figure 2), and extraneous species disappeared. As secondary amplification proceeded reagent depletion recurred and X1/Y1 ratios increased.

Standardization of PCR Conditions

For all subsequent quantitative studies (Figures 3 and 4, Table I) we used 500 ng of template in a 25-cycle primary PCR reaction (see Materials and Methods), followed by transfer of 10% of the products to a fresh reaction mixture for two cycles of secondary PCR.

PCR Amplification: Titrated Male and Female DNAs

The relative ZFX and ZFY content of titrated male and female DNAs was determined (Figure 3, Table I). Repetitive analysis of male DNA samples, with equimolar starting amounts of ZFX and ZFY targets, consistently showed an X1/Y1 ratio of 1.5; this number was used in subsequent calculations to extrapolate non-equimolar ratios.

PCR Amplification: Patients with Sex Chromosome Aneuploidy

DNA extracted from cell lines of individuals with sex chromosome aneuploidy was quantitatively amplified (Table I) using zf-a and zf-c primers. The calculated Y chromosome

Fig. 4. Precision of *zf a/c* primed quantitative PCR. Dot plot of individual values obtained on repeat determinations showing extent of variation in calculated Y chromosome percentage, as a fraction of all sex chromosomes. Each dot represents one determination which was used to generate summaries in Table I. For methods and sample designations see Table I.

content was quite close to that anticipated from the karyotype. Two samples of 66.7% and 33.3% Y chromosome content (Samples CL1 and CL5, Table I and Figure 4) were tested repeatedly to determine precision at different Y chromosome abundances. The coefficient of variation was greater at lower Y chromosome abundances, measuring 4.4% and 11.1% for CL1 and CL5 respectively.

DISCUSSION

Evaluation of the method

Our technique of quantitative PCR provides an accurate and precise method to identify sex chromosome imbalances when both X and Y chromosomes are present. Close agreement between Y chromosome content calculated from quantitative PCR and that predicted by karyotype or DNA titration indicates a high degree of accuracy (Table I). Accuracy was lowest in titrated DNAs with low Y chromosome contents, perhaps due in part to errors in calculating DNA concentration and pipetting during actual mixing of samples. These sources of error were not present in aneuploid cell line samples. The precision of Y chromosome values calculated from repetitive sample determinations is also high (Figure 4), with coefficients of variation measuring 4.4%–13.8%. This compares closely to errors of 10% (18) reported by others for single primer set co-amplification of homologous targets, or 7–10% (19) for double primer set coamplification of non-homologous targets. It is likely that quantitation of samples with Y chromosome contributions lower than those measured in this study would be subject to greater error as the Y1 peak is obscured by background. Background was, however, very low and not a problem in the range tested.

Qualitative simultaneous detection of both alleles is enhanced by longer autoradiographic exposures, permitting identification of Y chromosome sequences representing less than 10% of those targeted.

Two similar PCR products of equal molecular weight were effectively identified by template-specific restriction digests, and then quantitated by autoradiography. Specific digestion can be complicated by formation of DNA heteroduplexes (18,20), a process avoided by termination of amplification during logarithmic phase, or reversed by rediluting PCR products into fresh reagents for a few cycles of secondary amplification (18). We chose to do a two step amplification because the consistency of results was relatively refractory to minor variations in the starting amount of template, and differing amplification efficiencies of individual DNA isolates. Autoradiography of radionucleotides incorporated during amplification avoids the necessity to transfer and hybridize the products without sacrificing sensitivity. When combined with the high resolution of polyacrylamide gel electrophoresis, densitometric scanning of autoradiograms yields clean peaks with flat baselines eminently suitable for quantitation. Ethidium staining is inadequate in most cases to clearly demonstrate the small amounts of products present during early and mid logarithmic amplification making it unsuitable for situations where heteroduplex formation is a concern. In our experience ethidium poststaining can also produce irregular backgrounds and diffused bands, complicating densitometry.

Quantitative PCR and Cytogenetics

Quantitative PCR of gene targets with known linkages can provide ploidy data, as can cytogenetic analysis. One limitation is that PCR cannot distinguish balanced aneuploidy such as 48,XXYY from 46,XY euploidy. The karyotype simultaneously surveys ploidy of all chromosomes, whereas analysis directed at specific alleles is necessarily constrained to the targeted regions. PCR could be applied to any polymorphic locus, permitting identification and quantitation of autosomal aneuploidies. By using several primer sets, simultaneously or in parallel, it is feasible to screen for the more common trisomies with a panel of PCR assays—a procedure amenable to automation.

If the sex chromosomes are structurally abnormal, PCR and the karyotype provide complementary information. Deletion or insertion of the sex-determining region of the short arm of the Y chromosome (Yp) in intersex patients frequently involves ZFY(8,16), presumably because it is tightly linked to SRY(21), a candidate gene for the testis determining factor(22). Extrapolation of karyotype from ZFX and ZFY analysis would be inaccurate in these cases. Similarly, rearranged or translocated regions of the Y chromosome unidentified by karyotype in the intersex patient may be specifically recognized by presence of Yp molecular markers(7,8).

Fluorescent *in situ* hybridization techniques may also be used to obtain sex chromosome ploidy data in interphase nuclei, but there are situations where quantitative PCR may be preferred. *In situ* hybridization is more reliable than PCR only if tissue is well preserved, a variable that sometimes cannot be controlled. This is especially true of archival material. For interphase sex chromosomes the best *in situ* probes are repetitive. In the case of the Y chromosome these are confined to the centromere (alpha satellite(23)), and long arm (classical satellite(24))—unfortunately leaving out the region of the short arm that is so critical for sex determination.

Other Applications

Perhaps the greatest diversity of sex chromosome quantitation strategies has been applied to sexing sperm, an area of tremendous interest in predicting or choosing the gender of offspring. Of the methods used previously, F-body scoring(25,26) is subject to tremendous error, and karyotype of decondensed sperm following rodent egg fertilization(9,10) is laborious. Sperm are an example of a cell where *in situ* hybridization studies using Y chromosome specific probes have been hindered by the technical problem of accessing condensed target chromosomes(12) combined with high background(27). Experiments to evaluate sex chromosome transmission distortion by quantitative PCR are currently underway in our laboratory.

In certain pathological conditions with a limited repertoire of stable karyotypes molecular ploidy analysis can provide valuable information about the karyotype. This is the case with hydatidiform moles. These abnormal conceptuses are generally diploid or triploid. Several reports indicate that it is the diploid moles that are more likely to progress, especially those that are heterozygous as documented by an 46,XY karyotype or presence of Y chromosome sequences (14,15). These must be distinguished from histologically similar but apparently benign triploid hydatidiform moles (69,XXY, 69,XYY, or 69,XXX).

This report has emphasized the quantitative aspects of zf a/c primer design, but there are obvious advantages for qualitative studies. This primer set should always yield a specific product in karyotypically normal cells with intact DNA, providing an internal positive control. This is a distinct advantage over other PCR based sexing strategies which by focusing solely on the Y chromosome have a finite false negative rate. This is especially important in archival or forensic specimens where template integrity is unknown.

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