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Molecular Cytogenetic Analysis of Telomere Rearrangements

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Abstract

Genomic imbalances involving the telomeric regions of human chromosomes, which contain the highest gene concentration in the genome, are proposed to have severe phenotypic consequences. For this reason, it is important to identify telomere rearrangements and assess their contribution to human pathology. This unit describes the structure and function of human telomeres and outlines several FISH-based methodologies that can be employed to study these unique regions of human chromosomes. It is a revision of the original version of the unit published in 2000, now including an introductory section describing advances in the discipline that have taken place since the original publication.

Keywords

telomere; copy number variants; chromosomal microarray

Introduction: Advances in the Field of Molecular Cytogenetic Analysis of Telomere Rearrangements Since 2000

Much has changed in the world of clinical cytogenetic testing in the 14 short years since this unit was originally written. We have rapidly moved from interrogating targeted regions of the genome, like telomeres, to whole-genome analysis for detecting deletions and duplications. In addition, the development and evolution of new technologies, such as oligonucleotide and single-nucleotide polymorphism (SNP) arrays, and more recently whole exome and genome sequencing, are allowing smaller and smaller regions of genomic imbalance to be detected.

In 2000, at the time our original unit was written, assays for genome-wide telomere screening were just becoming available, and were being used after normal results were obtained from G-banded chromosome analysis. In fact, only two studies were published, which estimated the frequency of telomere abnormalities in idiopathic intellectual disability (ID). These studies estimated a frequency of telomere abnormalities in 5% to 6% (Flint et al., 1995) and 7.4% (Knight et al., 1999) of individuals with ID, respectively.

Since that time, screening for telomere abnormalities on a clinical basis has escalated with the availability of multiplex commercial assays using Fluorescence In Situ Hybridization (FISH; see UNIT 8.10; Kashork et al., 2010), and other DNA-based approaches, such as Multiplex Ligation-dependent Probe Amplification (MLPA), based on identification of a set of human telomere clones representing the most distal unique DNA region for every

chromosome (Knight et al., 2000). Biesecker (2002) reviewed the early literature on telomere screening (14 studies including close to 2000 individuals) and estimated a rate of telomere abnormalities in ~6% of individuals with idiopathic ID and a normal karyotype, consistent with the initial studies. Hundreds of studies using targeted telomere FISH analysis have since been published. The largest study to date reported close to 12,000 cases referred for clinical telomere testing and found telomere imbalances in 2.5% of cases, demonstrating that telomere imbalances contribute significantly to ID and other developmental and congenital disorders for which clinical testing is routinely ordered (Ravnan et al., 2006).

Although telomere FISH allowed the initial detection of submicroscopic telomere imbalances, there were limitations to this methodology in that it could only ascertain copy number of a single genomic clone (e.g., BAC, PAC) per telomere region, with the results thus lacking more detailed information related to the size and gene content of the deleted or duplicated genomic region. In our original unit (see below), we recognized the potential that Comparative Genomic Hybridization (CGH) arrays using genomic clones had in being used for telomere and ultimately whole-genome screening. Although initially developed for use in cancer applications, we predicted that CGH arrays could revolutionize constitutional diagnostic cytogenetic testing. That prediction was realized in a step-wise manner that started with CGH arrays that contained “targeted” coverage of known clinically relevant regions of the genome, including the telomeres, and rapidly evolved to include whole-genome coverage.

Telomere coverage represented on genomic clone arrays moved from containing a single clone for each human telomere region to including an increased clone density covering ~5 Mb to measure the size of each imbalance (Ballif et al., 2007; Martin et al., 2007). This “molecular ruler” approach allowed some capability for sizing the extent of telomere imbalances; however, it was quickly realized that many imbalances extended beyond this targeted coverage, demonstrating the need for complete genome-wide copy number detection methods (Ballif et al., 2007). The movement from arrays using genomic clones to arrays that contain oligonucleotide or SNP probes allowed development and implementation of whole-genome copy number arrays that matched the power of a karyotype for genome-wide analysis, but far surpassed it in the level of resolution that could be obtained [Aradhya et al., 2007; Baldwin et al., 2008; also see *UNITS 8.12 & 8.13* (Miller et al., 2012, and Delaney et al., 2008, respectively)]. The adaption of this “chromosomal microarray” (CMA) for clinical testing quickly demonstrated the significant number of copy number imbalances that contribute to human disease but are below the resolution needed for detection by routine G-banding analysis (Miller et al., 2010). The increased diagnostic yield of CMA compared to karyotyping ultimately resulted in CMA being deemed the first-tier test for clinical cytogenetic testing, replacing the G-banded karyotype (Miller et al., 2010; Manning et al., 2010; South et al., 2013).

So, what have we learned about imbalances involving the telomere regions compared to other regions of the genome from these genome-wide copy number analyses? First, several studies of idiopathic developmental disorders and congenital malformations have now confirmed that imbalances involving telomere regions are over-represented compared to other chromosomal regions of the genome (Ballif et al., 2007; Baldwin et al., 2008; Shao et

al., 2008). Obvious contributing factors to this increased frequency is that terminal deletions only require one chromosomal breakpoint compared to two simultaneous breaks for interstitial deletions, and that most unbalanced translocations include terminal imbalances. Further, most telomere imbalances are significantly larger than originally suspected. One study showed that approximately 40% of telomere imbalances are greater than 5 Mb in size, indicating that the analytic sensitivity obtained from G-banded karyotype analysis is much lower than previously estimated (Ballif et al., 2007).

Another finding is that certain telomeres are involved in chromosome rearrangements more than others. Telomere regions most frequently involved in pathogenic imbalances include 1p, 10q, 4p, 22q, and 9q, listed in order from highest frequency to lowest (Ledbetter and Martin, 2007). Finally, similar to other regions of the genome, telomeric copy number changes can be interpreted as either pathogenic or benign copy number variants, depending on the genomic region involved. Common benign variants, such as deletions of the 2q telomere (Macina et al., 1994) and deletions and duplications of the 10q telomere region (Ravnan et al., 2006), have now been well documented, as have clinical phenotypes that correspond to certain telomere imbalances, such as 1p deletions (1p36 syndrome; Heilstedt et al., 2003) and 22q deletions (Phelan-McDermid syndrome; Phelan et al., 1992). The availability of publically accessible databases, such as ClinVar (<http://www.ncbi.nlm.nih.gov/clinvar>) and DECIPHER (see *UNIT 8.14*; Corpas et al., 2012), which catalog genotype and phenotype data from large-scale data-sharing efforts, will continue to help in defining genotype/phenotype correlations for the telomere and other regions of the human genome.

It has long been postulated that submicroscopic deletions/duplications in the genome may be responsible for a significant percentage of unexplained mental retardation. Since whole-genome scanning methods at resolutions higher than cytogenetics are not yet readily available, focusing on genomic regions that may be disproportionately represented in unbalanced chromosome rearrangements is an attractive approach to identifying subtle, very small genomic imbalances. Emerging evidence indicates that submicroscopic imbalances involving human telomere regions may account for as much as 5% to 10% of idiopathic mental retardation (Flint et al., 1995; Knight et al., 1999). In addition, emerging information on the structure of human telomeres suggests specific mechanisms that may promote interchromosomal pairing and exchange, leading to telomeric imbalances and resultant clinical consequences.

The analysis of telomeric chromosome bands is a significant challenge using conventional cytogenetics methods (G-banding analysis at the 450 to 550 band level; see *UNIT 4.2*; Schreck and Distèche, 1994), since most terminal bands are similarly G-negative in appearance, and subtle rearrangements (<3 Mb) are difficult to visualize. Patients reported to have normal karyotypes by conventional cytogenetics methods may actually have cryptic telomere rearrangements as the cause of their abnormal phenotype. The first example of such a cryptic translocation was reported by Lamb et al. (1989), during their investigation of a child who presented with α -thalassemia, dysmorphic features, and mental retardation. Although this boy's high-resolution karyotype was normal, he was later found to have an

unbalanced telomeric translocation through a combination of DNA and in situ hybridization analyses, which were prompted due to his specific clinical diagnosis.

Similar observations became more widespread with the advent of fluorescence in situ hybridization (FISH; see *UNITs* 4.3 & 8.10; Knoll and Lichter, 2005, and Kashork et al., 2010, respectively) in the study of other telomeric microdeletion syndromes. For example, in the authors' investigations of the Miller-Dieker lissencephaly syndrome (MDS), submicroscopic deletions (<3 Mb) in 17p13 were initially detected by Southern blot analysis (*UNIT* 2.7; Jarcho, 2000) using VNTR polymorphisms. After development of a FISH assay using cosmid clones from the critical deletion interval, cryptic translocations were unexpectedly found to be present in phenotypically normal parents in several families (Kuwano et al., 1991). This discovery highlighted the importance of distinguishing de novo deletion events from inherited unbalanced translocations in probands, as the latter are associated with a significantly increased recurrence risk.

This experience, along with reports of similar cryptic translocations occurring in families with Wolf-Hirschhorn syndrome (4p-; Altherr et al., 1991) and Cri-du-Chat syndrome (5p-; Overhauser et al., 1989), made it seem likely that cryptic telomeric rearrangements could be a major source of human pathology (Ledbetter, 1992). Therefore, the development of diagnostic tools for the efficient assessment of telomere integrity would be extremely valuable for genetic diagnosis. This unit discusses the development of DNA probes for all human telomeres and their use in single and multiplex assays for identifying cryptic telomere rearrangements.

Telomere Structure and Function

Telomeres are specialized DNA-protein complexes that cap the ends of eukaryotic chromosomes; they are necessary for chromosomal end replication and provide chromosomal stability by protecting chromosome ends from degradation (Blackburn, 1990). As shown in Figure 8.11.1, human telomeric DNA consists of between 3 and 20 kb of tandemly repeated (T₂AG₃)_n sequences, which have been shown to be evolutionarily conserved among vertebrate species (Moyzis et al., 1988; Meyne et al., 1989). Immediately proximal to the tandemly repeated (T₂AG₃)_n repeat in humans are subtelomeric repeats, or telomere associated repeats (TAR), that have a polymorphic chromosomal distribution among individuals (Brown et al., 1990a). Unique sequence DNA for each telomere is located proximal to the subtelomeric repeats, on average ~100 to 300 kb from the end of the chromosome (National Institutes of Health and Institute of Molecular Medicine Collaboration, 1996).

Many studies now document the fact that the unique sequence regions adjacent to human telomeres have the highest concentration of genes of any chromosomal regions in the genome (Saccone et al., 1992; Flint et al., 1997b). These data suggest that submicroscopic deletions or duplications at telomeres may have disproportionately greater clinical consequences than similar-sized imbalances elsewhere. Consistent with this notion is the characterization of a submicroscopic deletion of the 22q telomere shown to be less than 130

kb in size, yet associated with severe mental retardation and multiple physical anomalies (Wong et al., 1997).

Another feature of telomere biology that could affect the potential role telomeres play in mediating chromosomal rearrangements is the high genetic recombination rate at telomeres. Genetic recombination rates increase in the telomeric regions for both sexes in humans, but dramatically so in males. Although female recombination rates are generally higher than those of males for most regions of the genome, this pattern is reversed at telomeres, where male recombination is significantly higher than female (Donis-Keller et al., 1987; Rouyer et al., 1990; Blouin et al., 1995). The only known exception to this rule is human chromosome 14q, in which recombination rates for both sexes are increased, but female recombination remains higher than male (Wintle et al., 1997). This high rate of recombination at telomeres could play a role in occasional unequal exchange and gene-dosage imbalance, as has been observed in both pseudoautosomal regions (PAR) of the X and Y chromosomes. Unequal recombination in the PAR1 region (Xp;Yp) accounts for 80% of XX males (1/20,000 male births), due to translocation of the *SRY* gene to Xp (Rouyer et al., 1987; Weil et al., 1994). The reciprocal unequal product is observed in some XY females who are deleted for *SRY*. More recently, similar unequal exchange events affecting the PAR2 region (Xq;Yq) have been observed, leading to a severe mental retardation syndrome in males with a Yq karyotype and duplication of Xq (Lahn et al., 1994).

Telomeres also play a critical role in chromosome pairing in meiosis; they are the first regions of the chromosome to pair and synapse, thereby mediating homologous chromosome pairing. Although the precise mechanisms underlying the process of homology search and chromosome pairing remain relatively obscure, recent FISH studies on a variety of species have demonstrated highly conserved features of telomere behavior in meiotic prophase. In studies of humans, mouse, and maize, telomeres move to the nuclear envelope and cluster into a “bouquet” (Speed, 1988; Scherthan et al., 1996; Bass et al., 1997). This early event coincides with the first evidence of chromosome pairing, consistent with the notion that homology searches and pairing may initiate at the telomeres.

The occurrence of telomeric rearrangements could be propagated by promiscuous exchanges between nonhomologous telomeres that occur as the telomeres cluster in meiosis. Several telomere associated repeat (TAR) sequences have been identified and shown to be shared between different subsets of human telomeres (Brown et al., 1990b; Cross et al., 1990; Youngman et al., 1992; Flint et al., 1997a,b). In addition, recent sequencing data from a number of telomeric regions indicate the presence of two subdomains in human telomeres: the regions closest to the terminal repeat contain a mosaic of very short sequences shared by many chromosomes, while the more proximal subdomain contains longer sequences shared by fewer chromosomes (Flint et al., 1996). It is easy to imagine that these regions of shared homology between nonhomologous chromosomes could transiently pair, thus providing an opportunity for telomeric exchanges that might occasionally lead to chromosomal rearrangements.

Development of Unique Human Telomere Probes

A first-generation set of unique telomere probes for human chromosomes has previously been reported (National Institutes of Health and Institute of Molecular Medicine Collaboration, 1996). Multiple strategies were employed to isolate the chromosome-specific telomere probes. First, cosmid clones were derived from half-YAC clones, i.e., YACs that only have one yeast telomere and require for propagation a human insert that contains its own telomere sequences (Cross et al., 1989; Riethman et al., 1989). However, since the majority of cosmid clones internal to the half-YACs still contain subtelomeric repeats showing cross-hybridization to other chromosomes by FISH, an alternative strategy was to utilize the vector-insert junction of the half-YAC as the starting point for screening genomic libraries. Since most of the half-YAC clones are 150 to 300 kb in size, the resulting genomic clones, which extended proximal from this point, were more likely to only contain chromosome-specific sequences (Riethman, 1997). Other cloning strategies, such as chromosome walking from subtelomeric repeat sequences, were also employed to complete the telomere set (Ning et al., 1996).

Although humans have 24 different chromosomes, the total number of clones targeted for development in the first set of telomere clones was 41, rather than 48, because: (1) the X and Y chromosomes share homologous sequences at both telomeric pseudoautosomal regions, and (2) no efforts were made to obtain the telomeres of the short arms of the five acrocentric chromosomes, since loss of the short arms of these chromosomes does not cause phenotypic abnormalities. The initial set of probes consisted of 33 unique telomere probes, most of which were cosmid clones, that were within 300 kb of the end of the chromosome arm. For the eight chromosome arms that did not yet have true telomeric clones identified (1p, 5p, 6p, 9p, 12p, 15q, 16p, 20q), FISH probes were developed for the most distal marker on the integrated genetic/physical map for that chromosome arm (http://www.genome.wi.mit.edu/cgi-bin/contig/phys_map).

Subsequent to the report of the first-generation set of telomere probes, several groups have focused their efforts on optimizing and completing this valuable resource. Since the first set of clones consisted mostly of cosmid (35 to 40 kb) clones, which are suitable as single FISH probes but more difficult to use in multiprobe formats, one goal has been to expand the size of each telomere clone to a target size of 80 to 150 kb in a single P1, PAC, or BAC clone.

Another ongoing goal is to identify true telomere clones for the remaining chromosome arms previously represented by clones corresponding to distal genetic markers. Three new telomere clones (9p, 12p, and 15q) have been reported since the initial set of clones (Lese et al., 1999). These clones were identified using vector-insert junction sequence from newly identified half-YAC clones (9p and 12p) or distal markers from the genetic/physical maps (15q). Included in the characterization of these three new telomere clones was the determination of the genomic distance between each new telomere clone and its corresponding distal marker clone (which was reported in the first-generation telomere set). Interphase FISH analysis was employed to measure the genomic distances, and revealed distance estimates ranging from less than 100 kb up to 1 Mb. Figure 8.11.2 shows representative examples of interphase FISH results. These findings underscore the

importance of using telomere clones that are a known distance from the end of the chromosome, rather than uncharacterized distal marker clones that could be as much as a megabase from the telomere, thus missing smaller telomeric rearrangements. Although not yet formally published, new genomic clones suitable for FISH assays have been identified for the remaining telomeres (1p, 5p, 6p, 12p, 16p, 20q); these clones have been characterized through continuing efforts of the laboratories involved in the initial telomere set report (J. Flint, pers. comm.; C.M. Lese, unpub. observ.).

Methods for Identifying Telomere Rearrangements

Targeted Telomere Analysis

The availability of unique telomere probes for all human chromosomes has allowed cytogenetic analysis to reach a new molecular plateau. There is growing evidence that subtle deletions and duplications of human telomeric regions either occur more frequently than expected or have failed to be ascertained by routine cytogenetic analysis. The latter is likely, as small alterations of G-negative (pale-staining) chromosome regions, such as those located at most telomeres, are difficult to visualize with accuracy.

An increasing number of reported cases are emerging where FISH with chromosome-specific telomere probes has been used to complement and further elucidate results of routine cytogenetics. Suspected telomeric aberrations identified by G-banding analysis can now be verified or ruled out with FISH, using a telomere probe for the chromosome arm in question. As shown in Figure 8.11.3, FISH with a telomere probe for 12p was used to verify a deletion of chromosome 12 suspected by routine chromosome analysis (C.M. Lese and K. Kaiser-Rodgers, unpub. observ.). In addition, telomere FISH on patients with previously identified terminal deletions (by G-banding analysis) has unveiled cryptic unbalanced translocations or interstitial deletions. Brkanac et al. (1998) reported the identification of interstitial deletions in 5/35 patients previously reported to have 18q terminal deletions. Finally, as telomere microdeletion syndromes are being delineated, telomere probes are becoming more useful in targeted analyses to provide patients with specific genetic diagnoses.

Submicroscopic deletions at telomeres of a few hundred kilobases may harbor a large number of genes and lead to physical and developmental abnormalities. The increasing number of targeted FISH analyses of telomere rearrangements is beginning to reveal genotype/phenotype correlations pertaining to individual telomere aberrations. One well documented example is deletion of the 22q telomere, which has been associated with hypotonia, developmental delay, and absence of speech (reviewed in Precht et al., 1998). Figure 8.11.4 shows FISH results using a 22q telomere probe on a metaphase cell from a patient with a 22q deletion. The 22q telomere deletion phenotype shares some phenotypic overlap with Angelman syndrome and should, therefore, be considered part of the differential diagnosis for Angelman syndrome in individuals in whom genetic testing for Angelman syndrome fails to find any abnormality.

Genotype/phenotype correlations are gradually being defined for other telomere regions as well. Deletions of 1p have been shown to be associated with growth and mental retardation,

seizures, large anterior fontanelle, and facial dysmorphism (Shapira et al., 1997; Riegel et al., 1999; Wu et al., 1999). Although the phenotypic features reported for 1p deletions are not highly specific, they can be suggestive enough to pursue testing.

Deletions of the telomeric region of chromosome 2q have a variable phenotype which in some patients resembles Albright Hereditary Osteodystrophy, with short stature, obesity, round face, short metacarpals, brachydactyly, and mental retardation (Phelan et al., 1995; Wilson et al., 1995). The phenotype observed in 2q telomere deletion patients shares some characteristics with Prader-Willi syndrome.

Another telomeric deletion, which has been associated with consistent phenotypic features, is deletion of 11q. This deletion causes Jacobsen syndrome, characterized by psychomotor retardation, trigonocephaly, facial dysmorphisms, cardiac defects, and thrombocytopenia (Penny et al., 1995).

On the other hand, there are telomeric regions that do not appear to have phenotypic effects when deleted. This phenomenon has been observed for two telomeric regions, 10q and 17p, where a deletion of a few hundred kilobases of unique DNA results in a normal phenotype (C.M. Lese, unpub. observ.). As the characterization of human telomere rearrangements continues to improve, it will become possible to make phenotypic predictions based on such empirical data. Defined genotype/phenotype correlations will provide the ability to differentiate between telomeric aberrations involving critical genes, thus contributing to the pathology associated with their dosage imbalance, versus those rearrangements that will not have a phenotypic effect.

Multiplex Telomere Analysis

Cryptic translocations may be suspected in some individuals or families with idiopathic mental retardation, recurrent miscarriages, and/or a family history suggestive of a chromosomal rearrangement. For these cases, where there is not enough information for targeted telomere analysis, a multiplex assay for telomere screening, using FISH with multiple probes simultaneously, is an attractive diagnostic approach for assessing telomere integrity. A "telomere integrity assay" has been previously proposed, envisioned as a multicolor FISH assay using unique genomic clones as close to the ends of the chromosomes as possible to examine such cases (Ledbetter, 1992).

The determination of the frequency and pattern of cryptic deletions and translocations for all human telomeres in selected clinical populations is an important step in defining the mechanisms of telomere rearrangements. Such studies will allow multiple hypotheses to be tested, including: whether certain chromosomes are preferentially involved in telomere rearrangements; whether telomeric translocations more often involve chromosomes that share subtelomeric repeat sequences or other sequence homology; and whether chromosomes with length and/or sequence polymorphisms (e.g., 2q and 16p) are disproportionately represented in telomere rearrangements (Wilkie et al., 1991; Macina et al., 1994).

To date, two studies have been published that estimate the frequency of telomere abnormalities in idiopathic mental retardation. The first study, by Flint et al. (1995), examined 99 children and their parents using VNTR polymorphisms; three cases of cryptic deletions were identified. Since these authors tested only half of all telomeres, they estimated that as much as 5% to 6% of unexplained mental retardation may be caused by cryptic telomeric rearrangements. A subsequent study by this group used a FISH-based genome-wide telomere screen (41 telomere probes) to study children with unexplained mental retardation (Knight et al., 1999). They reported subtle telomeric abnormalities in 7.4% (21/284) of children with moderate to severe mental retardation and 0.5% (1/182) of children with mild retardation. This report concluded that telomeric rearrangements are the most common cause of idiopathic moderate to severe mental retardation (excluding recognizable syndromes). Additional data is needed to further confirm these studies and determine the incidence of telomere abnormalities for other clinical populations. There are multiple methods that are quickly becoming available for all-telomere screening, some of which will be addressed below in this unit.

Multicolor FISH assay—Standard multicolor FISH technologies, using single and/or combinatorial labeling strategies, are well suited for the development of genome-wide telomere-integrity assays. Unlike VNTR analysis, which requires the availability of parental samples for interpretation, FISH analysis can be performed and interpreted with the proband's sample only. In addition, FISH allows identification of balanced and unbalanced rearrangements, unlike all current molecular methodologies, which can only detect the unbalanced form.

Multicolor FISH includes, but is not limited to, FISH assays producing from 2 to 24 colors, depending on fluorophore availability and imaging capabilities. For accurate discrimination of each fluorochrome in a multicolor combinatorial hybridization, a high signal-to-background ratio for every fluorochrome is necessary and all hybridization signals should be balanced in intensity. Although thresholding software is able to compensate for minor differences in signal intensity, it is still necessary to optimize probe mixtures for best results. Variations in signal intensities between probes containing variable insert sizes (e.g., PACs and cosmids) can be problematic. This variability can be decreased by using a panel of PAC and BAC probes that are approximately the same size (average insert size of 100 kb). Some additional factors that must be considered when designing a FISH-based telomere screen include the use of directly or indirectly labeled probes, the number of hybridization areas to be used, and the number of metaphases required. Each of these factors will either affect the time required for the actual FISH procedure or the analysis time.

Over the past several years, more fluorochromes have become available, emitting in both the visible and the far-red region of the spectrum (Table 8.11.1). For example, Speicher et al. (1996) used five individual fluorochromes (FITC, Cy3, Cy3.5, Cy5, and Cy7), together with combinations of fluorochromes, to hybridize a different color along the length of every human chromosome (i.e., chromosome “painting”; see *UNIT 4.9*). A defined set of single-narrow-band excitation and emission filters was developed for this assay to discriminate each fluorochrome, as well as the DAPI counterstain. In order to image more than four different fluorochromes (including a DAPI counterstain), either a set of single-band-pass

filters matched to each fluorochrome or a combination of quad and single filter blocks is required. An automated microscope with automated filter block rotation can be used for image capturing (Eils et al., 1998). Alternatively, the filters can be moved manually using registration-compensation software for correct spatial alignment of signals (J. Piper, pers. comm.).

As shown in Table 8.11.1, an alternative set of fluorochromes in the visible region of the spectrum has been developed for direct labeling of DNA (Vysis). The use of a direct labeling scheme can significantly reduce the time for the FISH procedure by eliminating the need for detection steps.

The authors' laboratory has utilized up to six colors for the acquisition, enhancement, display, and analysis of multicolor images using a commercially available software package (ViewPoint from Vysis). This software, together with a Chroma 84000 filter set, enables performance of 6-color FISH using probe combinations labeled with the fluorescent nucleotides Spectrum Orange and Spectrum Green, as well as biotin-avidin Cy5. By using three different fluorescent labels separately and in combinations to label DNA, it is possible to generate a maximum of seven color combinations ($2^n - 1$ colors, where n equals the number of fluorochromes). With six different colors, both p and q telomeres of three chromosomes can be screened in one hybridization. The authors prefer to do telomere analysis using probes for both telomeres from a single chromosome in the same hybridization; the probes serve as internal controls and also aid in chromosome identification. Figure 8.11.5 shows 6-color FISH where 2q, 12p, and 12q were labeled with Spectrum Orange, Spectrum Green, and Cy5, respectively, and the other three telomeres were labeled with 1:1 mixtures of two fluorochromes. Once the signal from each fluorochrome has been captured, the software detects the presence or absence of each fluorochrome at every pixel, and assigns a different color to each pure fluorochrome and to each fluorochrome combination. The use of such multicolor technology will allow the telomere probe corresponding to each chromosome arm to be labeled a different color, enabling the detection of any telomeric alterations, including translocations or inversions. By using six colors, one could examine the integrity of all 41 telomere probes in seven hybridization areas.

An alternative approach to multicolor FISH is the interferometer-based spectral imaging system (Applied Spectral Imaging). This technology, termed spectral karyotyping, or SKY (also see *UNIT 4.9*; Lee et al., 2000), has been developed and successfully used for 24-color FISH with whole-chromosome painting probes (Schröck et al., 1996). This system has not yet been formally tested for genome-wide screening with unique DNA clones.

Both M-FISH and spectral karyotyping have the potential to be used as multicolor FISH assays for genome-wide telomere screening. Both methods would allow analysis of 24 different telomeres in a single hybridization using combinations of 5 fluorochromes, or all 41 telomeres could be hybridized simultaneously using combinations of 6 fluorochromes. For these types of analyses, multiple filters corresponding to each fluorophore would be needed for M-FISH; the different fluorophore combinations would be separated by their emission spectra using spectral karyotyping. For both methodologies, an extremely well

balanced probe mixture, containing all telomere probes, would be necessary to obtain satisfactory results.

Multiprobe hybridization format—A commercial multiprobe device, the Multiprobe-T system (Cytocell), uses a single slide for screening all telomeres. This two-color assay has 23 hybridization areas on a single slide, each containing the p (green)– and q (red)–arm telomere probes for a single chromosome. In this setup, the X and Y probes are contained in a single hybridization area, since both the p- and q-arm probes are located within the pseudoautosomal regions and there are no probes for the short arms of the acrocentric chromosomes. Telomere probes for the p and q arms for each chromosome are provided applied to 23 raised panels of a multiprobe coverslip. The assay is completed following the procedure outlined by the manufacturer. Briefly, hybridization solution is pipetted onto each of the 23 panels and the coverslip is brought into contact with the target slide containing metaphase spreads for codenaturation and hybridization. The target slide is prepared by pipetting individual drops of cell suspension into each of the 23 gridded areas (0.25 × 0.25–in. each). Following an overnight hybridization, post-hybridization washes and detection are completed.

Knight et al. (1997) tested the feasibility of this assay format by testing two normal cases and two cases with known telomere abnormalities; they were able to confirm the reliability of the technique by identifying the respective cases as either normal or abnormal. Since then, multiple studies have been published demonstrating the use of this device in identifying cryptic telomere rearrangements (Horsley et al., 1998; Brackley et al., 1999).

The advantage of this technique is that only a single patient slide is needed for a complete analysis. However, if one of the hybridization areas does not produce analyzable signals, or if there are not enough metaphases present in a grid (the authors' laboratory scores at least five metaphases per chromosome), additional hybridizations are necessary. Currently, the Multiprobe-T system uses probes that are indirectly labeled. This feature adds additional detection steps that lengthen the time needed for the FISH procedure; it can also produce a high background-to-signal ratio. This initial version could be converted to one using directly labeled probes for a more efficient assay.

Comparative Genomic Hybridization Arrays

Comparative genomic hybridization (CGH; *UNIT 4.6*; DeVries et al., 1995) is a powerful molecular cytogenetic technology that allows genome-wide analysis of DNA copy number in one assay, similar to the genome-wide scanning capability of conventional cytogenetic banding analysis (Kallioniemi et al., 1992; du Manoir et al., 1993). In this method, total genomic DNA from a patient or cell line is labeled with one fluorochrome while normal control DNA is labeled with a different fluorochrome. The two DNAs are mixed in equal ratios and simultaneously hybridized to normal metaphase chromosome preparations. This latter point is critical, as the major advantage of CGH to chromosome banding analysis is that only genomic DNA is required for analysis, not high-quality metaphase chromosome preparations, which are difficult to obtain in the case of some tumors and from archived materials. The ratio of fluorescence detected on the metaphase chromosomes is proportional

to the copy number of the test and control DNAs. Both decreases (deletions, monosomies) and increases (duplications, trisomies, gene amplifications) in patient/tumor copy numbers can be detected with accuracy. However, the limitation of this technique when applied to metaphase chromosomes is that the resolution is ~10 to 20 Mb, less than that of conventional cytogenetics.

Significant progress has now been reported by several groups in modifying the basic CGH method by using arrays of cloned DNA sequences such as cosmids, P1s, PACs, and BACs as the target (Solinas-Toldo et al., 1997; Pinkel et al., 1998). The resolution for deletion/duplication detection by this strategy is equivalent to the size of the target probe (e.g., 35 kb for cosmids up to 150 kb for BACs). In addition, a study by Pollack et al. (1999) described the use of cDNA clones as array targets to identify copy number changes corresponding to alteration in gene-expression levels. This methodology, termed CGH arrays, has the potential to revolutionize diagnostic telomere screening in probands where telomeric imbalances are suspected due to an abnormal phenotype. Obviously, this screening methodology would not be useful in normal individuals suspected of carrying a balanced cryptic rearrangement (e.g., ascertained for recurrent miscarriages) since it can only identify imbalances in the genome.

Although the initial reports describing CGH arrays focused on their use in examining copy number changes in cancer, the potential usefulness of this methodology in identifying constitutional DNA alterations was clearly recognized. As shown in cartoon form in Figure 8.11.6, for the purposes of telomere screening, clones corresponding to all telomeres could be placed on an array so that any telomeric imbalances could be identified in a single experiment. All apparent copy number increases or decreases identified by CGH-array analysis should then be tested by single-telomere FISH analysis for confirmation. For a more comprehensive screening test, clones corresponding to other genomic regions, such as common microdeletions, could also be included on the array.

Clinical Applications/Future Research

Telomere FISH analysis is beginning to be utilized more frequently for clinical applications. Currently, targeted telomere FISH analyses (described above) are used as an adjunct to routine cytogenetics to complement and further elucidate G-banding results. Although not yet used as widely as individual telomere probe analysis, all-telomere screening with the Multiprobe-T device is beginning to be offered on a clinical basis to rule out cryptic constitutional telomere rearrangements. As described below, additional studies assessing telomere integrity in well defined patient populations are clearly needed to determine the frequency of telomere abnormalities and estimate cost effectiveness.

To assess the incidence of telomere aberrations in individuals with mental retardation, several clinical populations can be defined that may have different frequencies of chromosomal etiology. The authors are currently dividing these clinical populations into three levels of risk for a chromosomal etiology: (1) 2 individuals in a family with mental retardation or one affected proband plus a significant history of miscarriage; (2) mental retardation with dysmorphic features; (3) mental retardation without dysmorphic features.

The first category, representing a pedigree suggestive of a balanced translocation segregating in the family, should yield the highest frequency of chromosomal translocations. Category 3, isolated developmental delay/mental retardation, is likely to yield the fewest abnormalities, but is the largest and a very important clinical population.

To date, two studies have been published that estimate the frequency of telomere rearrangements in idiopathic mental retardation (Flint et al., 1995; Knight et al., 1999). More studies are necessary to fully document the incidence of cryptic telomere rearrangements in selected clinical populations. Since the third category listed above is the largest, with potentially the lowest hit rate, it would be more economical to initially focus on the first two categories until more efficient and less expensive screening strategies are available. In this way, the frequency of telomere rearrangements in selected populations can be determined, and educated/informed decisions can be made whether this type of testing represents a valuable addition to clinical testing.

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Figure 8.11.1.

Schematic diagram showing the organization of DNA sequences in the telomeric regions of human chromosomes.

Figure 8.11.2.

Interphase FISH distance measurements on interphase nuclei from G0 fibroblast cells to determine the distance between either the 9p or 12p telomere probe and a corresponding probe for the most distal marker. Telomere probes are shown labeled in digoxigenin and detected with anti-digoxigenin rhodamine (red); probes for the most distal markers are shown labeled in biotin and detected with avidin-FITC (green). The arrowheads show hybridization signals for each probe set from a single nucleus, and the line in each figure represents 5 μ m. **(A)** Hybridization of clone RG41L13 (9p telomere; red) and clone GS56N11 (AFM274XE1; green); the genomic distance is estimated to be 250 kb between these two probes. Notice the close proximity of the green and red signals for the 9p clones. **(B)** Hybridization of clone GS8M16 (12p telomere; red) and clone GS93E1 (AFM303XD9; green); the genomic distance is estimated to be 600 to 800 kb between these two probes. Notice the greater signal separation between the 12p clones as compared to the 9p clones. Reprinted from Lese et al. (1999), with permission from Cold Spring Harbor Press.

Figure 8.11.3.

Hybridization of telomere probes for 12p, directly labeled with Spectrum Green (green), and 12q, directly-labeled with Spectrum Orange (red), to a metaphase chromosome preparation from a patient with a suspected deletion of 12p. Two hybridization signals are present for the 12q probe. Only one hybridization signal, on the normal chromosome 12 (closed arrowhead), is present for the 12p probe; the other chromosome 12 homolog shows no hybridization signal (open arrowhead), thus confirming a terminal deletion.

Figure 8.11.4.

Hybridization of a cosmid probe for the 22q telomere, labeled in digoxigenin and detected with anti-digoxigenin rhodamine (red), and an alpha-satellite probe for chromosomes 14 and 22, labeled in biotin and detected with avidin-FITC (green), to a metaphase cell from a patient with a 22q telomere deletion. Hybridization signal is visible on the normal chromosome 22 (closed arrowhead); the deleted homolog shows no hybridization signal (open arrowhead).

Figure 8.11.5.

Multicolor FISH with six probes for the telomeric regions of 2p, 2q, 11p, 11q, 12p, and 12q. Hybridization signals for each telomere are labeled with the corresponding probe name. Probes for 2q, 12p, and 12q were labeled with Spectrum Orange, Spectrum Green, and Cy5, respectively. The other three telomeres, 2p, 11p, and 11q were labeled with combinations of two of the above fluorochromes. Simultaneous hybridization of both the p and q telomere probes for a given chromosome aids in the identification of the chromosome.

Figure 8.11.6. Cartoon depiction of idealized results from a “telomere chip” analysis by CGH of a patient with Jacobsen syndrome due to an unbalanced 3p;11q translocation. For telomere clones present in normal dosage in the patient, equal ratios of control (green) and patient (red) DNA are depicted by a yellow hybridization result. For a 50% reduced copy number in the patient, a green hybridization signal for 11q indicates deletion. For the partial trisomy present on 3p, a relative excess of patient DNA is depicted by a red hybridization signal.

Table 8.11.1
Excitation and Emission Wavelengths of Fluorochromes for Multicolor FISH

Fluorochrome	Excitation wavelength (nm)	Emission wavelength (nm)
FITC	490	520
Cy3	554	568
Cy3.5	581	588
Cy5	652	672
Cy7	755	778
Spectrum Blue	400	450
Spectrum Aqua	433	480
Spectrum Green	497	524
Spectrum Yellow	530	555
Spectrum Orange	559	588
Spectrum Red	587	612
DAPI	367	452