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REVIEW

FISHing Chromosomes in Endocrinology

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Fluorescence *in situ* hybridization (FISH) is currently recognized as a reliable, sensitive, and reproducible technique for identification of copy number and structure of chromosomes providing information on the single-cell level. The technique permits cytogenetic investigation of metaphase spreads and interphase nuclei. Several protocols have been used for preparations from fresh samples or archival material. Alphoid or telomeric DNA probes can detect specific chromosomes, and cosmid probes can localize single copies of the segment of interest. FISH is a powerful tool in understanding physiologic mechanisms, in generating high-resolution physical genetic maps, and in resolving problems of the pathogenesis of several diseases. FISH may shed light on the cytogenetic background and chromosomal alterations in the field of endocrinology, resulting in a better understanding of functional activities and various endocrine disorders.

Key Words: Chromosomes; cytogenetics; endocrine glands; fluorescence *in situ* hybridization.

Introduction

Analysis of chromosomes with standard cytogenetics has proven valuable for the study of genomic abnormalities. However, karyotyping requires tissue culture, resulting in selective cell growth and loss of overall genetic information from the examined material. In addition, it is often difficult to obtain metaphase spreads from cells with a low proliferation potential. Several other molecular techniques using PCR protocols enable identification of copy number and structure of chromosomes. Although sensitive, these methods provide no information at the single-cell level. Fluorescence *in situ* hybridization (FISH) is currently recognized as the most valuable alternative technique for cytogenetic investigation with multiple applications in several areas of clinical and basic research.

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Interphase cytogenetics was initially applied in 1986 by Cremer et al., who utilized a radioactive DNA probe to demonstrate chromosomal aberrations in nonmitotic human nuclei. The same year, Pinkel et al. (1986) introduced a nonisotopic, high sensitivity fluorescence technique for cytogenetic analysis. Since then, several protocols of FISH have been developed, using various types of DNA probes and labeling systems for chromosome visualization.

Technical Insights

Types of Probes and Utilities

Telomeres and centromeres represent structural and functional elementary components of all eukaryotic cells. In humans, telomeres contain the same highly conserved repetitive DNA sequence $(TTAGGG)_n$. The centromeric, and to a lesser degree, the telomeric regions are composed of one or more highly repeated satellite DNA families (Meyne and Moyzis, 1994). Alphoid (pericentromeric) DNA plays an important role in the centromeric function. Alphoid probes specifically recognize a pair of homologous chromosomes (Haef and Ward, 1994). α-Centromeric DNA probes specific for individual chromosomes are widely used to detect numerical chromosomal aberrations and, thus, to estimate aneuploidy. These probes are, however, not appropriate to detect structural abnormalities.

Small, unique DNA sequences of chromosomes by incision in a larger cosmid or plasmid vector vehicle to increase the target size can be used as probes. Cosmid DNA probes can detect single copies of the segment of interest on the target chromosome. By labeling of hundreds of unique sequence probes from the entire length of a single chromosome, whole chromosome painting probes can be obtained (Wolman, 1994). In addition, specific DNA probes can hybridize the entire genome (Kallioniemi et al., 1992). Since the introduction of FISH technique (Pinkel et al., 1986), chromosome-specific DNA libraries have been established (Pinkel et al., 1988; Kuto et al., 1991; Smit et al., 1991). In addition to the detection of numerical chromosomal abnormalities, FISH can be applied to augment G-banded interpretations of chromosomal aberrations

(Sullivan et al., 1993). Since marker chromosomes cannot be readily identified with standard high-resolution techniques, FISH represents a sensitive alternative method for characterization. In addition, chromosome painting DNA probes can identify other structural aberrations, such as chromosome duplications, balanced or unbalanced translocations, and complex rearrangements. FISH is the appropriate technique in generating high-resolution physical genetic maps for isolation of normal and defective genes (Kiechle-Swartz et al., 1991; Smit et al., 1991).

Synthetic oligomeric probes can rapidly be synthesized by PCR. They are small, single-stranded, usually of <50 nucleotides length, and thus, allow good penetration during hybridization, which is a great advantage especially for the interphase procedure. Oligomers are stable and do not self-anneal during hybridization permitting reduction of hybridization time (Meyne and Moyzis, 1994).

Detection Systems

Direct or indirect fluorescence systems, using biotin or digoxigenin label, are currently recognized as the most sensitive for the cytogenetic *in situ* hybridization technology. They are available for double or multicolor labeling and signal amplification. Fluorescein, Texas red, rodamin, and aminomethylcumarin acetic acid are some of the most popular fluorochromes. Multicolor labeling probes conjugated with different fluorochromes enable simultaneous detection of two or more different regions on the same chromosome or identification of two or more different chromosomes (Poddighe et al., 1992; Wolman, 1994).

Tissue and Fixation

FISH can be applied to metaphase spreads and to interphase nuclei. Blood lymphocytes and other biologic material, such as semen, or imprints and cytologic smears from solid fresh tissues obtained by fine-needle aspiration (FNA) biopsy or surgery can be used for FISH (Wolman, 1994). In addition, cytospin preparations of fresh or frozen tissues, and of formalin-fixed and paraffin-embedded archival material can provide sufficient sampling for interphase FISH after enzymatic digestion and cell dispersion (Hopman et al., 1991). It should also be mentioned that FISH can be directly applied on paraffin sections for cytogenetic evaluation (Thompson et al., 1994).

Several fixatives, such as 70% ethanol or formalin, can be used. We found fixation in chilled acetone to be an excellent procedure for fresh touch preparations (Kontogeorgos and Kapranos, 1996). Depending on the type of fixative, the duration of fixation and the period of storage, complementary digestion with proteolytic enzymes, such as proteinase K or pepsin, is required. Enzymatic pretreatment is necessary particularly for solid tumors to reverse the effect of fixation, to remove the cytoplasm, and to increase the penetration of DNA probe into the dense nuclear material (Poddighe et al., 1992).

Pitfalls

Smear or touch preparations containing intact nuclei provide a reliable estimation of the DNA content. In contrast, tissue sectioning leads to loss of nuclear material causing a decrement of hybridization signal. Nuclear overlapping, low intensity of positive signals, and minor binding sites are common artifacts that can affect the estimation of chromosome copy numbers. Crossreaction of some α-satellite probes, such as for chromosome 13 with other chromosomes or with minor hybridization sites, leads to overproduction of the number of signals. The amount of DNA content of alphoid repeats varies among different chromosomes, even between homologs (Poddighe et al., 1992; Meyne and Moyzis, 1994; Wolman, 1994).

Applications

During the last 10 years, studies utilizing FISH technique have been reported with increasing frequency. This article is focusing on findings related to endocrine glands.

Pituitary

A cDNA for human thyrotropin-releasing hormone (TRH) receptor isolated from a human pituitary cDNA library was used as a biotinylated probe. The gene encoding the TRH receptor was localized to chromosome 8q23 by FISH (Morrison et al., 1994). The human gonadotropin-releasing hormone (GnRH) receptor gene has been recently cloned from the human pituitary gland. The FISH technique was employed to determine the chromosomal localization of the human GnRH receptor gene, which was found to be localized to the chromosome band 4q13 (Kakar and Neill, 1995). Similar studies revealed by FISH that the oxytocin receptor gene mapped to chromosome 3p26.2, and the gene coding for the human pituitary-specific V3 vasopressin receptor is located on chromosome band 1q32 (Inoue et al., 1994; Rouseau et al., 1995). In a recent study, an α-satellite centromeric DNA probe was applied to demonstrate abnormalities of chromosome 11 in four somatotroph adenomas, using the direct FISH technique in interphase cells. All tumors exhibited numerical chromosomal abnormalities; 8–23% of the cell population contained one to three extra copies of chromosome 11. These abnormalities were found to be more prominent in sparsely granulated somatotroph adenomas compared with the densely granulated variant (Kontogeorgos and Kapranos, 1996).

In a single study of six suprasellar teratomas, four were investigated by dual FISH performed on nuclei from paraffin blocks using centromeric DNA probes specific for X and Y chromosomes. By static image analysis, three tumors were diploid. The substantial majority of cells in two of these tumors comprising one mature and one immature teratoma from male patients showed 2X and 1Y signals. The third, and immature teratoma from a male host showed 2X and 2Y signals. The fourth tumor, an immature teratoma

from a female patient, exhibited 4–6X signals, an excess of 70% of cells. No sufficient material from this tumor was available for ploidy studies (Yu et al., 1995).

Thyroid

Aberrations of chromosomes 7 and 12 frequently occur in follicular thyroid adenomas. To evaluate possible cytogenetic similarities between follicular adenomas and goiters, the single or double FISH was applied to isolated nuclei from frozen and paraffin-embedded material of thyroid follicular adenomas and goiters using α-satellite, centromere-specific DNA probes for chromosomes 7 and 12. Polysomies of chromosome 7 were detected in 35.7% of thyroid follicular adenomas and in 10.7% of goiters. Polysomies of chromosome 12 were detected in 29.6% of thyroid follicular adenomas and 6.7% of goiters. In addition, enzymatic *in situ* hybridization on frozen sections revealed polysomies only in lesions of predominantly microfollicular pattern. Trisomy was the most frequent aberration, whereas tetrasomy and monosomy were infrequently encountered. DNA aneuploidy determined in a subset of adenomas showed a close correlation with the numerical abnormalities of chromosomes 7 and 12 (Criado et al., 1995). In a similar study, double FISH using DNA probes specific for the pericentromeric regions of chromosomes 3, 7, 9, 11, 12, 18, and X detected numerical chromosomal changes in thyroid lesions, including two nodular hyperplasias, two adenomas, and seven papillary carcinomas. The changes were more prominent in the follicular lesions, showing the highest degree of trisomy for chromosome 12. Severe monosomy of chromosome 9 was observed in a single metastatic papillary carcinoma, whereas monosomies of a moderate degree for different chromosomes were noted in one follicular adenoma and two papillary carcinomas (Taruscio et al., 1994). Trisomy 7 detected by classic cytogenetics in a multifocal metastasizing papillary thyroid carcinoma was further investigated by FISH using a satellite probe D7Z1 on fresh metaphase harvests, and on touch preparations from frozen specimens of the tumor and the lymph node metastases. Trisomy 7 was found in 6–13.2% of cells from both primary and metastatic lesions (Herrmann and Lalley, 1992).

Parathyroids

DiGeorge syndrome represents a developmental field defect of the third and fourth pharyngeal pouches accompanied by hypo- or aplasia of the thymus and parathyroids, in addition to congenital heart defect and characteristic facial dysmorphism. Molecular analysis carried out in 16 cases employing FISH and DNA dosage analysis to detect haploinsufficiency of chromosome 22q11 revealed deletion of this region in all patients (Levy-Mozziconacci et al., 1994). The significant role of these molecular techniques in diagnosis, prognosis, and genetic counseling should be emphasized. In another study, 23 patients with DiGeorge syndrome were investigated by prometaphase chromosome analysis and/or by FISH using a set of six cosmid probes. Four patients displayed a cytogenetically visible interstitial deletion in band 22q11.2, whereas in 18 patients, the deletion was disclosed only by FISH. In 21 patients, the deletion encompassed the six loci tested, whereas in one patient, only the most telomeric of these loci was conserved. The remaining patient did not show any deletion (Demczuk et al., 1994).

Pancreas

Pancreatic regenerating (reg) gene is markedly increased in regenerating islets and decreased when insulin gene expression is suppressed, suggesting that reg gene may be involved in the regeneration and function of β-cells. Human metaphase chromosomes used for FISH confirmed that the reg gene is located on the short arm of chromosome 2 near the centromere at band 2p12 (Perfetti et al., 1994).

For identification of acquired genomic alterations of pancreatic adenocarcinoma, classic cytogenetics and FISH analysis were employed in 62 primary pancreatic adenocarcinomas obtained from surgical resections. Chromosomal gains were detected mostly in chromosomes 20 and 7, whereas more frequent chromosome losses were noted in chromosomes 13, 12, 17, and 6. Four tumors with known 6q deletions were analyzed to determine whether the apparent partial losses of 6q were real. FISH using a biotin-labeled microdissection probe from 6q24ter verified loss of one copy of this region in three tumors. In addition, double minute chromosomes were identified in eight cases (Griffin et al., 1995). Pancreatic adenocarcinomas analyzed by FISH showed frequent somatic mutations and deletion of the p16(MTS1) gene. This gene is implicated in cell cycle arrest during the G_1 phase, and it is presumed to function as a tumor suppressor gene. Allelic deletions of 9p21-p22 were found in 85% of tumors. Analysis of MTS1 in pancreatic adenocarcinoma xenografts and cell lines revealed homozygous deletions in 41% and sequence changes in 38%. Sequencing of MTS1 from the primary tumors confirmed the mutations (Caldas et al., 1994).

Adrenals

A cosmid marker was used for rapid molecular diagnosis in subjects with complex glycerol kinase deficiency, which is characterized by congenital adrenal hypoplasia. FISH analysis employing a cosmid 35 in combination with an Xq control probe provided accurate information on submicroscopic X-chromosomal deletions, particularly in female carriers (Worley et al., 1995). In another study, families that had males affected with X-linked adrenal hypoplasia congenital and hypogonadism were evaluated for mutations involving the DAX1 gene, which represents a new member of the nuclear hormone receptor gene superfamily. Two independents exhibited an identical frameshift mutation owing to a single base pair deletion, whereas

in the third pedigree, a deletion of the entire DAX1 locus was noted. One of these families was further evaluated for prenatal diagnosis by FISH using a cosmid probe containing the DAX1 gene in order to detect larger deletions. The results showed a deletion of the DAX1 locus and the contiguous glycerol kinase region. Specific diagnosis of the genetic alterations of this disease is fundamental for the affected child and members of the family (Guo et al., 1995). In another study, concerning a young male with congenital adrenal hypoplasia associated with hyperpigmentation, marked elevation of blood ACTH, and absent response to IV ACTH challenge, a *de novo* duplication of 5p (p13.3 and p15.1) was confirmed by FISH technique (Chen et al., 1995). In yet another study of an individual with adrenal hypoplasia, three markers (C7, B24, and L1) that map distal to Duchenne muscular dystrophy, glycerol kinase deficiency, and adrenal hypoplasia loci on chromosome Xp21.3 were analyzed. Only C7 appeared to be deleted when all three markers were hybridized to interphase cell nuclei of Nijmegen1 (Trask et al., 1992). Direct preparations of uncultured interphase cells from a human adrenal carcinoma investigated by FISH exhibited high aneuploidy. The high frequencies of trisomy for the studied chromosomes indicated that most of the tumor cells were in the triploid range. In contrast, cultured cells from the tumor showed normal karyotype (Rosenberg et al., 1995).

Gonads

Difficulties in detecting sex chromosome in patients with mosaic karyotypes cause phenotypic-genotypic discrepancies. FISH permits the genomic identification of these patients in metaphase spreads or interphase nuclei by using an X chromosome-specific DNA probe. In a patient with Turner's syndrome displaying 46,XX/45,X0 mosaicism, previously established by standard cytogenetics, the genomic constitution of the ovary was evaluated by FISH using centromeric-specific alphoid DNA probe for the human X chromosome. Although ovarian sections and blood samples showed the presence of both 46,XX and 45,X0 cell lines, the genomic constitution of the germ cells/oocytes in ovarian primordial follicles was shown to be normal (Novak et al., 1995). An unusual case of a female infant with 45,X/46,XY/47,XYY/48,XYYY mosaicism with a phenotype including ambiguous genitalia, bilateral Fallopian tubes, and a left infantile testis with epididymis was investigated by FISH to identify the presence of 46,XY cells (Fox et al., 1995). In another case of a 2-yr-old male with 45,X/47,XYY mosaicism, FISH was applied to disclose tissue-specific distribution of the two cell lines in biopsies of the streak gonad and dysgenetic testis. The prepubertal germ cells showed mostly a 47,XYY karyotype, Sertoli cells exhibited both karyotypes, and the remaining tissue was composed mainly of 45,X cells (Ragg et al., 1995). In another report, a patient with peripheral blood karyotype 45,X associated with ambiguous genitalia was studied in order to resolve the phenotype/genotype discrepancy. Gonadectomy at age 7 mo revealed a left prepubertal testis and a streak gonad on the right side. The karyotype obtained from the left gonad (45,X/46,XXq) was different from that of the right gonad (45,X). Utilizing three different techniques, including PCR amplification, FISH, and chromosome painting for X and Y chromosomes, the presence of Y chromosome sequences was confirmed. A high percentage of Y chromosome-positive cells were found in the left gonad, whereas no Y chromosomal material could be detected in the right gonad, indicating that the Xq contained Y chromosomal material (Kocova et al., 1995). In another study, gonadal tissue obtained from two patients with 45,X karyotype was analyzed by Southern blot and FISH techniques. FISH employing a mixture of probes hybridizing both the centromere and long arm of Y chromosome was employed. No signals were detected with the other Y chromosome-specific DNA probe in the first patient. In the second patient, very few cells were hybridized with the mixture of probes (Kocova et al., 1996). A girl with DiGeorge syndrome associated with ovarian hypoplasia and combined manifestations of 46,XX,i(18q) karyotype with monosomy 18p and trisomy 18q was further investigated by FISH. Chromosome painting with a panel of chromosome 8-specific DNA probes confirmed the cytogenetic findings of a isochrome 18q (van Essen et al., 1993).

Human semen was recently studied by FISH to detect aneuploid populations. Chromosome analysis of spermatozoa from three normal individuals investigated by dual-color FISH procedures, using probes specific for the X and Y chromosomes and chromosomes 1 and 12, revealed a low numbers of hyperhaploid populations. The frequency of hyperhaploid sperm was 0.8, 1.03, and 2.27/thousand for XX, YY, and XY respectively, whereas 1.67/thousand sperms were diploid. Comparing these results with estimates of sex chromosome aneuploidy in humans, it is conceivable that a selective disadvantage may exist for sperms carrying two sex chromosomes (Goldman et al., 1993). The three-color FISH procedure allows the identification of hyperhaploid spermatozoa and diploid spermatozoa (with 24 and 46 chromosomes, respectively) and their meiotic origin (meiosis I or II). Analysis of spermatozoa from a male with 46,XY/47,XXY karyotype using α-satellite DNA probes specific for chromosomes X and Y revealed proportions of X- and Y-bearing sperm to be 52.78 and 43.88%, respectively. The frequencies of disomy and diploidy were low, whereas the frequency of hyperhaploid 24,XY spermatozoa was significantly higher compared with controls. These results suggest that a few 47,XXY germ cells may complete meiosis and produce mature spermatozoa (Chevret et al., 1996).

Numerical aberrations were demonstrated by interphase analysis of nuclei isolated from fresh seminoma tissue, utilizing a set of 12 centromeric DNA probes. Using these probes, aneuploid numbers of fluorescent spots were found

in all cases, compared with flow cytometric analysis (van Dekken et al., 1990). The significance of trisomy 12, a characteristic nonrandom numerical chromosome aberration commonly found in ovarian neoplasms, particularly sex cord stromal tumors, remains to be evaluated. In a study of 10 ovarian sex cord stromal tumors, FISH was employed on frozen sections to analyze chromosome 12 abnormalities using a centromeric DNA probe. The range of trisomy was 12–32% in granulosa cell tumors and 8–22% in fibromas, whereas in a single thecoma, trisomy was present in 8% and in one Sertoli-Leydig cell tumor in 4% of the nuclei examined. These results showed that trisomy 12 represents a common aberration in ovarian sex cord stromal tumors (Taruscio et al., 1993). The presence of trisomy 12 was also investigated by FISH in a series of 75 primary ovarian tumors, including 20 fibromas, 24 granulosa cell tumors, 13 serous cystadenomas, 7 mucinous cystadenomas, and 11 borderline epithelial tumors, on sections from formalin-fixed and paraffin-embedded tissues. Trisomy 12 was detected in 27% of the serous borderline tumors and in 40% of fibromas, but was not observed in any other benign epithelial tumors. These results, in conjunction with those of previous cytogenetic studies, suggest that trisomy 12, although rare in benign epithelial ovarian tumors, is often the sole anomaly in borderline epithelial neoplasms (Persons et al., 1994). A series of 16 ovarian sex cord stromal tumors, obtained from patients with long-term follow-up, was analyzed by interphase FISH on paraffin-embedded sections for trisomy 12. The findings showed that trisomy 12 occurs in the great majority of clinically benign sex cord stromal tumors, indicating its limited prognostic significance for sex cord stromal tumors (Shashi et al., 1994). The artifact introduced by nuclear slicing in the screening for trisomy 12 was evaluated in 19 ovarian juvenile granulosa cell tumors assessed by FISH. Intact tissue sections and cytospin preparations of nuclei from the same tissue blocks were analyzed. The mean percentages of signal gain for chromosome 12 probe were 19% in tissue sections and 33% in cytospin preparations; thus, lack of demonstration of trisomy 12 in intact tissue sections may be partly owing to slicing artifact (Halperin et al., 1995).

Abnormalities of other chromosomes have been found in ovarian carcinomas and human carcinoma cell lines. Deletion of 6q is a frequent finding in ovarian carcinoma. Chromosome 6q abnormalities were analyzed by G-banding and FISH in six ovarian carcinoma cell lines using a chromosome 6 paint probe, a chromosome 6 centromere-specific DNA probe, and cosmids that map to q24-27 bands. Abnormalities of 6q were found in three cell lines, indicating that gene or genes localized in bands 6q26-27, mostly in the region proximal to 6q24, play a critical role in the development or progression of ovarian carcinoma (Lastowska et al., 1994). In a recent report, seven regions from different cases of ovarian cancer were studied for identification of genomic composition and chromosomal origin using combined techniques of chromosome microdissection and FISH. Twelve specific chromosome band regions were identified as amplified, including 11q, 12p, 16p, 19p, and 19q (Guan et al., 1995). In another study, the presence of chromosome 12 abnormalities was investigated by bicolor double FISH in diverse gonadal and extragonadal human germ cell tumors (GCT) and GCT-derived cell lines. Three different probes were used for specific identification of the entire chromosome 12, its short arm, and its pericentromeric region. One or more copies of a genuine i(12p) chromosome were demonstrated in three testicular, one ovarian, one dysgenetic, and one extragonadal intracranial GCT (Suijkerbuijk et al., 1992).

Conclusions

FISH represents a fascinating, sophisticated approach to cytogenetics. It is a powerful tool that combines molecular genetics with classical cytogenetic information and brings them together to a single framework for morphologic evaluation. It can offer a large body of new information on physiological mechanisms and resolve problems in the pathogenesis of many diseases. Several applications of FISH are currently used for mapping genes encoding hormones, hormone receptors, enzymes, and other substances related to endocrine function. In addition, FISH technology is useful for diagnosis, prognosis, and genetic counseling, as well prenatal screening of families with members affected with genetic endocrine disorders.

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