European Journal of Human Genetics (2003) 11, 879–883

www.nature.com/ejhg

SHORT REPORT

Karyotyping of human synaptonemal complexes by cenM-FISH

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The purpose of this work was to adapt the recently described centromere-specific multicolour (cenM-) FISH technique to human meiotic cells, and evaluate the usefulness of this multiplex fluorescence method for karyotyping human synaptonemal complex (SC), previously analysed by immunocytogenetic approaches. The results obtained demonstrate that cenM-FISH is a reliable one-single-step method, which allows for the identification of all SC present in pachytene spreads. Moreover, when cenM-FISH is applied after immunocytogenetic analysis, the number and distribution of MLH1 foci per chromosome can be established and recombination analysis for each chromosome can be performed easily.
European Journal of Human Genetics (2003) **11,** 879–883. doi:10.1038/sj.ejhg.5201067

Keywords: cenM-FISH on synaptonemal complexes; immunocytogenetics; meiotic recombination

Introduction

The synaptonemal complex (SC) is a protein-rich structure that is formed during the first stages of prophase I, and which reaches the completed stage at pachytene.[1] The use of silver or phosphotunstic acid-staining at early stages of meiosis (prophase I) allows for the visualisation of SC by light and electron microscopy. Studies using this method have been performed both in males – control[2] and carriers of structural chromosome aberrations[3–5] – and females[6] and have provided information about the pairing process of homologous chromosomes during meiosis I. A new and wide field in the study of SC and recombination processes has been opened with the combined use of immunocytogenetic and FISH techniques.[7] The application of both analyses in parallel allows not only for the identification of specific pairs of bivalents (up to four[8]), but also to study meiotic exchange rates of the target chromosomes.[8,9] Previously, this has only been possible by chiasma analysis at the diakinesis–metaphase I stage, following triple staining for the identification of individual bivalents.[10,11]

The introduction of multiplex fluorescent FISH methods (M-FISH, SKY, Rx-FISH, MCB, chromosome bar code) has allowed the simultaneous detection and/or high-resolution analysis of each chromosome in mitotic metaphases,[12] and have been successfully used to detect numerical aberrations as well as many structural chromosome rearrangements (for overview see: http://mti-n.mti.uni-jena.de/~huwww/MOL_ZYTO/mFISHlit.htm). However, none of these multicolour FISH techniques was completely accurate in the identification of secondary heterochromatic blocks and centromeric regions present in a significant number of small supernumerary marker chromosomes (SMCs). Those SMCs carrying an α-satellite sequence in the great majority of the cases can be identified using the recently described centromere-specific multicolour (cenM-) FISH technique.[13] This M-FISH technique combines the centromeric probes of almost all human centromeric regions, except for chromosome 1, where a probe specific for the heterochro-

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Received 24 March 2003; revised 28 May 2003; accepted 10 June 2003

matic subcentromeric region in 1q12 was employed, in order to identify all human chromosomes, in a single assay. This approach allows for the identification of constitutional and acquired SMC,[14,13,15] and also for the identification of other aneuploidies.[16]

In this work, we have adapted the cenM-FISH method to SC spreads, previously analysed by immunocytogenetic techniques in order to detect MLH1 foci (putative recombination sites). We have achieved for the first time the individual identification of all SCs, in a single assay. Then, recombination foci can be pinpointed for each bivalent. Our results show that cenM-FISH is a very useful tool for karyotyping SCs and for further investigations into mechanisms of the meiotic process.

Material and methods

Testicular biopsies were obtained from infertile patients under a general anaesthetic. One of them was a carrier of a reciprocal translocation (t(10;14)(q24;q32)), and was ascertained because of repeated reproductive failures; the other was an infertile man with apparently normal mitotic karyotype. Written consent was obtained from all patients, and the study was approved by the Institutional Ethics Committee. The testicular tissue was macerated in an isotonic solution (NaCl 0.9%) until a cell suspension was obtained, and then placed in a centrifuge tube in order to separate the seminiferous tubules from the testicular cells. The supernatant was recovered and placed in another tube, centrifuged for 5 min at 600 *g*, and finally resuspended in a few drops of NaCl 0.9% until achieving the desired concentration. Fixation and spreading of the cells were performed using a method described elsewhere,[7] with some modifications: after a brief hypothonic treatment (less than 1 min), 15 μl of cell suspension were mixed with 15 μl of 0.003 Lipsol solution on a clean microscope slide. After 10 min, 90 μl of 2% formaldehyde containing 0.02% SDS pH 8.4 were added to the mix and allowed to stand for 10 min more. Finally, the slides were rinsed with distilled water and allowed to dry at room temperature.

Immunocytology of spermatocytes was performed following Barlow and Hultén.[17] The primary antibodies used in this work were rabbit anti-SCP3 antibody (a gift from Dr Christa Heyting; University of Wageningen, The Netherlands) and mouse anti-MLH1 antibody (Pharmingen; San Diego, CA, USA), which were applied at 1:1000 and 1:500, respectively, in PBT (PBS, 0.15% fetal calf serum, 0.1% Tween-20) overnight at room temperature. The fluorescence secondary antibodies applied were TRITC-conjugated goat anti-rabbit IgG antibody and FITC-conjugated goat anti-mouse IgG antibody (both from Sigma; Madrid, Spain), at 1:500 in PBT for 4 h at room temperature. After three 5 min washes in PBT and a brief rinse in distilled water, slides were allowed to air-dry, and were counterstained with antifade (Vector lab Inc.; Burlingame, CA, USA), containing DAPI at a concentration of 0.032 ng/ml (Sigma; Madrid, Spain). Each slide was evaluated using a fluorescent photomicroscope (Olympus Bx60) and all observed pachytene nuclei with anti-MLH1 antibody foci were captured and processed using a Power Macintosh G3 with Smartcapture software (Digital Scientific; Cambridge, UK). After this, slides were stored at −20°C until hybridisation.

SC spreads were hybridised with cenM-FISH probes, which were prepared using the procedure described by Nietzel *et al*.[13] Briefly, DOP-PCR (50 μl volume) was used to amplify the cloned centromere or subcentromere sequences of all chromosomes. None of the probes were commercially supplied. A secondary DOP-PCR (20 μl volume) was used to label the probes, with d-UTPs carrying biotin, diethylaminocoumarine, Spectrum Red, Spectrum Orange or FITC as ligands. The labelling scheme (see Figure 1) has been changed slightly compared to the initial paper.[13] Probe solution was denatured for 5 min at 75°C and prehybridised for 10 min at 37°C.

Prior to postfixation, DAPI was removed from SC spreads using 4 × SSC/0.05% Tween-20 solution (5 min), followed by an ethanol series (70, 85 and 100%). Then, slides were dipped in PBS for 5 min, postfixed with 3% formaldehyde

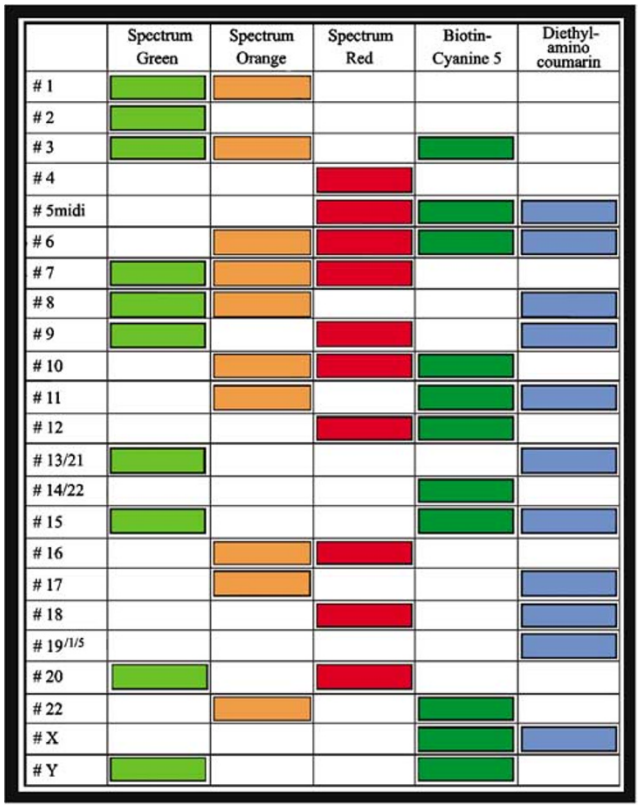

Figure 1 Label scheme showing the fluorochrome combination used for each human chromosome.

in 50 mM $MgCl_2$/PBS (10 min), dipped again in PBS, briefly rinsed with purified water and finally dehydrated in 70, 85 and 100% ethanol (2 min each) at room temperature and air-dried. Slides were placed on a hotplate warmed to 68°C; then, 100 μl of denaturing solution (70% formamide/ $2 \times$ SSC) were dropped onto each slide and covered with a $24 \times 60\,mm^2$ coverslip. After 2 min, slides were removed from the slide warmer, passed through an ethanol series at room temperature, and allowed to air-dry. A measure of 10 μl of probe mix was added to SC slides beneath $24 \times 24\,mm^2$ coverslips. Coincubation was performed overnight at 37°C in a humidified chamber. Then, slides were washed in $0.4 \times$ SSC at 70°C for 2 min, followed by 2 min in $4 \times$ SSC/0.05% Tween-20. After the washes, biotin was detected using a Cy5-avidin/biotynilated anti-avidin system. Finally, slides were counterstained with antifade containing DAPI. Visualisation and capture were made using a Zeiss Axioplan microscope (Zeiss; Jena, Germany) equipped with a CCD camera. Analysis was performed using the ISIS digital FISH-imaging system (MetaSystems; Altlussheim, Germany).

Results and discussion

CenM-FISH has been successfully applied on SC spreads from two different male patients. All of the SC spreads had been previously analysed by immunocytogenetic techniques. The immunocytogenetic method allows for the detection of the DNA mismatch repair protein MLH1, which localises in the late recombination nodules that can be seen during pachytene. MLH1 shows a correlation with crossover recombination events.[17] When pachytene nuclei are analysed using an antibody against the MLH1 protein combined with an antibody against the SCP3 protein, which localises in the lateral element of the SC,[18] a labelling pattern consisting of crossing-over foci appears as bright spots along the SC. Several authors have reported immunocytogenetic analysis combined with FISH on human spermatocytes, using whole chromosome painting (WCP), centromeric (CEP), telomeric (Tel) and also unique DNA probes in order to determine the number and position of recombinant foci for some specific chromosomes.[7] To date, the simultaneous detection of four different chromosomes has been possible.[8] Recently, identification of all bivalents on SCs of mouse spermatocytes has been reported by using M-FISH techniques, in two consecutive hybridisation rounds.[19]

By using cenM-FISH, all chromosomal bivalents, both autosomal and sex chromosomes, have been detected in the analysed SC spreads, in a single step and with similar fluorescence intensities for all centromeres (Figure 2a). CenM-FISH analysis has allowed us to identify unequivocally all chromosomes in 92% of the studied cells ($n = 60$). As stated by Barlow and Hultén,[7] the application of FISH on antibody-labelled human spermatocytes leads to a partial loss of fluorescence intensity of the antibody signals. However, the SC still can be observed under fluorescent microscopy, so their characterisation can be performed. The specific position of X and Y chromosomes within the sex vesicle could be determined in all SC spreads, independently of the condensation stage of the sex bivalent. The labelled cen-DNA appears as a diffuse signal of variable size situated either on only one or on both sides of the SC, and with its origin located on the corresponding SC. This diffuse appearance of the FISH signals is probably due to the morphology and chromatin

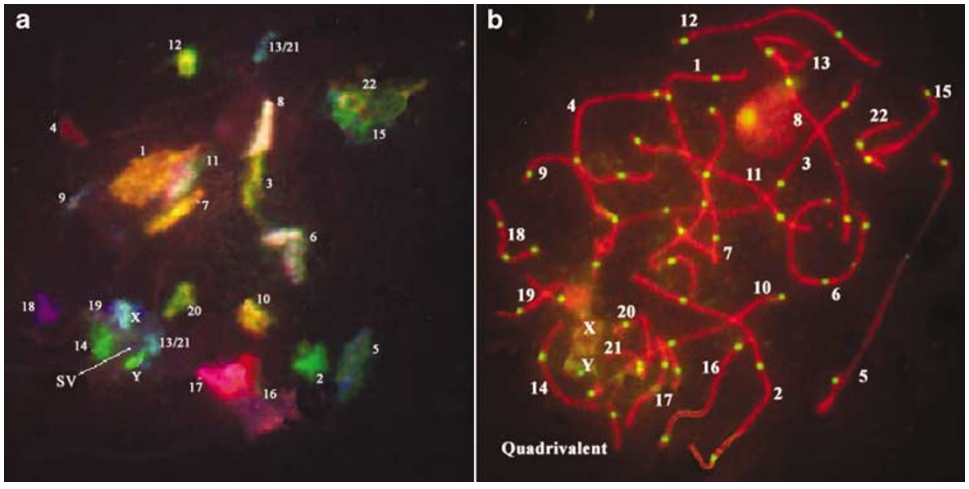

Figure 2 (**a**) CenM-FISH image of a SC spread from a translocation carrier t(10;14)(q24;q32). Image was captured and processed using the ISIS digital FISH-imaging system (MetaSystems; Altlussheim, Germany). (**b**) Immunocytogenetic image of the same SC spread, after identification of all bivalents. Image was captured and processed using a Power Macintosh G3 with Smartcapture software (Digital Scientific; Cambridge, UK).

condensation adopted by the bivalents after the formaldehyde-fixation treatment. Each centromere probe hybridise with the two homologous centromeres of each bivalent and its size would depend on the size of the target DNA region (smaller in centromeric DNA or larger in heterochromatic subcentromeric DNA). One of the most interesting cenM-FISH advantages is that its use provides the identification of all SCs in a single round of hybridisation. Use of multi-FISH techniques based on WCP, such as M-FISH or SKY, on human SC spreads is not adequate, mainly because their application on the formaldehyde-fixed chromatin would result in a halo of overlapped signals, making the correct identification of each bivalent difficult. CenM-FISH is an alternative, which avoids the problem caused by the high degree of chromatin superposition, and its application on SC spreads will give an important push to meiotic recombination analysis. Owing to the high homology between the α-satellite DNA of chromosomes 13 and 21 (99.7%),[20] these two chromosomes share the same colour code combination, and they cannot be differentiated from each other. However, this does not represent a problem when meiotic chromosomes are analysed, because distinction between chromosomes 13 and 21 can be made on the basis of their size.

Once all SCs are identified (Figure 2b), the quick elaboration of a SC karyotype could be possible. Years ago, karyotypes of microspread pachytene spermatocytes according to the SC physical length and the centromeric index were reported.[1] Using the cenM-FISH technique, we have observed that some SCs displayed a physical length longer than expected. For instance, chromosome 19 has been frequently found to be longer than chromosome 18. In fact, this phenomenon has been previously noticed by other authors in human and other organisms.[8,10,21] Recently, a covariation of SC length has been described.[9] By comparing two different sets of chromosomes, one with chromosomes displaying different physical size but similar genetic length (chromosomes 16 and 19) and the other with chromosomes of similar physical size but different genetic length (chromosomes 21 and 21), it has been found that SC length reflects genetic length instead of physical length of a concrete chromosome.

When applied to the analysis of reciprocal translocations, cenM-FISH also defines the position inside the quadrivalent of the chromosomes involved in the reorganisation. This can be very helpful for the recombination analysis in translocations carriers, as it has been shown that the chiasma frequency and distribution in translocation carriers may vary for the same chromosome when compared to the normal situation.[22]

In conclusion, our results indicate that cenM-FISH is a good tool for the analysis of SCs and meiotic chromosomes. The combined use of this technique with immunocytogenetic methods on SCs and also the application of cenM-FISH on meiotic chromosomes, would help us to better understand some of the mechanisms involved in the meiotic process, including those which lead to the generation of chromosome aberrations.

Acknowledgements

We thank Dr Christa Heyting for the gift of the rabbit anti-SCP3 antibody. We also thank Àngels Niubó for technical assistance. We acknowledge the financial support given by FIS, (PI020258), CIRIT (2001 SGR-00201), the Herbert Quandt Stiftung der Varta AG and the EU (ICA2-CT-2000-10012). M Oliver-Bonet was the recipient of a grant (2001BEAI200094) from the Generalitat de Catalunya.

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