

Neocentromere formation in a stable ring 1p32-p36.1 chromosome

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Abstract

Neocentromeres are functional centromeres formed in chromosome regions outside the normal centromere domains and are found in an increasing number of mitotically stable human marker chromosomes in both neoplastic and non-neoplastic cells. We describe here the formation of a neocentromere in a previously undescribed chromosomal region at 1p32→p36.1 in an oligospermic patient. Cytogenetic GTL banding analysis and the absence of detectable fluorescence in situ hybridisation (FISH) signals using telomeric probes indicate the marker to be a ring chromosome. The chromosome is negative for CBG banding and is devoid of detectable centromeric α satellite and its associated centromere protein CENP-B, suggesting activation of a neocentromere within the 1p32-36.1 region. Functional activity of the neocentromere is shown by the retention of the ring chromosome in 97% of the patient's lymphocytes and 100% of his cultured fibroblasts, as well as by the presence of key centromere binding proteins CENP-E, CENP-F, and INCENP. These results indicate that in addition to CENP-A, CENP-C, and CENP-E described in earlier studies, neocentromere activity can further be defined by CENP-F and INCENP binding. Our evidence suggests that neocentromere formation constitutes a viable mechanism for the mitotic stabilisation of acentric ring chromosomes.

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Normal human centromeres contain an abundance of a tandemly repeated DNA known as α satellite. This DNA is made up of basic units of 171 base pairs that are tandemly repeated into arrays of up to 3-4 megabases on each chromosome.¹ Existing evidence based on the reintroduction of exogenous α satellite DNA into mammalian cells to reconstitute active centromeres suggest that this DNA is likely to have a direct role in centromere function.^{2,3} However, in recent years, a novel class of mitotically stable human marker chromosomes that are devoid of α satellite DNA has been identified. These α satellite negative or anaphoid marker chromosomes contain new centromeres (neocentromeres) that are formed

from apparently non-alphoid DNA sequences on the arms of different human chromosomes.⁴

We report here a new case of neocentromere involving a ring chromosome formed from a previously undescribed region on the short arm of chromosome 1. The ring chromosome was diagnosed in a 38 year old man who presented clinically with oligospermia. We describe the detailed cytogenetic, immunofluorescence, and FISH characterisation of this ring chromosome and discuss our results in light of the emerging neocentromere phenomenon.

Materials and methods

CYTOGENETIC STUDIES AND CELL LINES

Cultured peripheral blood lymphocytes were used for the preparation of metaphase chromosomes for GTL and CBG banding analyses using standard procedures and for FISH experiments. Skin fibroblast and Epstein-Barr virus transformed lymphoblast cell lines were established from the patient for immunofluorescence studies using anti-centromere antibodies or for further cytogenetic analysis.

MICROSATELLITE ANALYSIS

DNA from blood and sperm was extracted with a commercial DNA extraction kit (Nucleon, Amersham) and investigated using polymorphic microsatellites (n=9) located between chromosome bands 1p31.1 and 1p36.23. The proximal to distal order of these microsatellite loci were D1S230, D1S2890, D1S2797, D1S255, D1S234, D1S199, D1S2697, D1S2667, and D1S450. The loci between D1S2797 and D1S2697 were surmised to be present on the rder(1) chromosome. PCR products were analysed on an Applied Biosystems ABI Prism 377 DNA Sequencer.

FISH AND IMMUNOFLUORESCENCE DETECTIONS

FISH was performed according to the manufacturer's instructions using the pan α satellite probe (designated "All Human Centromeres" probe, ONCOR), telomere probe (designated "All Human Telomeres probe, ONCOR), and chromosome 1 specific paint (CAMBIO Ltd). Four different anti-centromere antibodies directed against centromere proteins CENP-B, CENP-E, CENP-F, and INCENP were used in immunofluorescence studies. Anti-CENP-B antibodies were prepared in our earlier study,⁵ anti-CENP-E and anti-CENP-F antibodies were gifts from T J Yen, and anti-INCENP antibody was a gift from W E Earnshaw. The immunofluorescence detection procedure was as described in a previous study.⁶ Images for the pan α satellite, telomere, and chromosome

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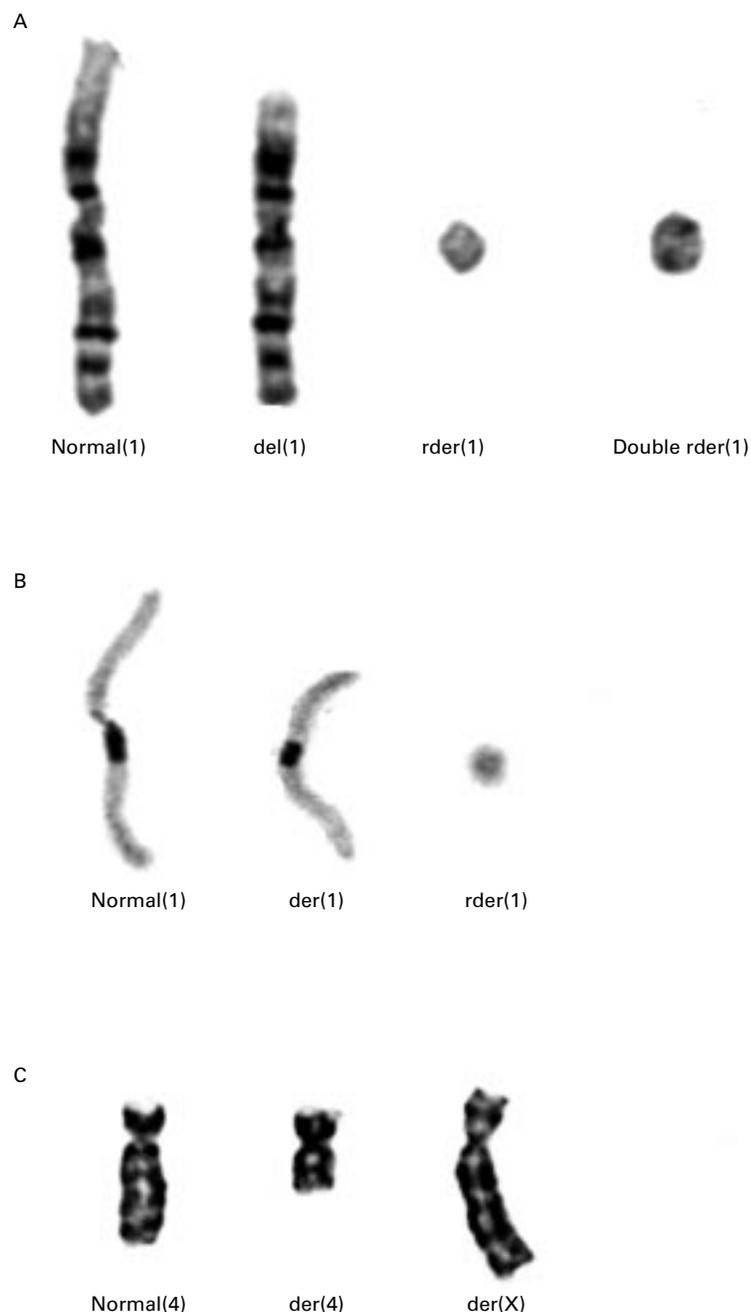


Figure 1 Cytogenetic analyses. (A, B) GTL and CBG banding, respectively, of the patient's lymphocyte chromosomes showing a normal chromosome 1 and the deleted chromosome 1 (*del*(1)), ring chromosome (*rder*(1)), and the occasional double sized *rder*(1). (C) GTL banding of patient's cultured fibroblast chromosomes showing the presence of a reciprocal 4;X translocation in 60% of the cells in addition to the *del*(1) and *rder*(1) chromosomes which are present in all the cells.

1 paint probes were analysed on a Zeiss Axioskop microscope equipped with a Cytovision Image Analysis System (Applied Imaging Ltd), whereas those for anti-centromere antibodies were analysed on a Zeiss Axioskop fluorescence microscope equipped with a $\times 100$ objective and a cooled CCD camera (Photometrics Image Point) controlled by a Power Mac computer.

Results

CYTOGENETIC STUDIES

The patient was a 38 year old man who presented with infertility and oligospermia but

otherwise appeared normal. GTL banding showed a deleted chromosome 1 (designated *del*(1)) in which a segment (band 32-36.1) on the p arm of chromosome 1 was missing in all the 100 cells examined (fig 1A). In addition, an apparently supernumerary, small, ring-like chromosome (designated *rder*(1)) was evident in 97% (97 out of 100) of the cells analysed. In these 97 ring chromosome containing cells, 10 were shown to carry an *rder*(1) chromosome that has doubled in size (fig 1A). Two reasons pointed to this *rder*(1) chromosome having a presumed origin from the deleted 1p32-36.1 segment: (1) its approximate banding morphology is consistent with that of 1p32-36.1, and (2) despite the patient's infertility problem, the patient was phenotypically normal which suggested a more or less balanced karyotype since monosomy of the 1p32-36.1 region accompanied by a trisomy of an unknown but substantial genomic segment would probably have manifested in a more severe phenotype. Confirmation of the chromosome 1 origin of the ring has come from FISH studies below.

CBG banding showed positive staining of the centromeric/pericentric heterochromatins of all the chromosomes except the *rder*(1) chromosome (fig 1B). This result, together with the high level retention of the *rder*(1) chromosome, suggested the presence of a functional centromere that was devoid of centromeric heterochromatic DNA. When the chromosomes of the patient's parents were cytogenetically analysed, these were shown to be normal, suggesting a *de novo* occurrence of the *del*(1) and *rder*(1) chromosomes. The patient's lymphocyte karyotype is therefore 47,XY,*del*(1)(p32p36.1)+*r*(1)(p32p36.1) *de novo*.

Cytogenetic analysis of the patient's fibroblast culture showed the presence of the *del*(1) and *rder*(1) chromosomes in all the 100 cells analysed, although in five of these cells the *rder*(1) chromosome has doubled in size. In addition, a translocation involving the X chromosome and chromosome 4 was observed in 60% of these fibroblast cells (fig 1C), suggesting that the patient may be mosaic for this rearrangement. The patient's fibroblast karyotype is therefore 47,Y,t(X;4)(q23;q13),*del*(1)(p32p36.1)+*r*(1)(p32p36.1)[60]/XY,*del*(1)(p32p36.1)+*r*(1)(p32p36.1) [40].

MICROSATELLITE ANALYSIS

As spermatogenesis may be compromised by ring chromosomes with consequent infertility for most heterozygous males,⁷ it was possible that the presence of the *rder*(1) chromosome in patient AS may explain his oligospermia. We therefore used an array of chromosome 1p specific microsatellite markers to determine the heterozygosity of the *del*(1) and *rder*(1) chromosomes in the sperm DNA sample. As a control, DNA from the patient's blood was used and this indicated all the microsatellite loci (except D1S2697) to be heterozygous. Analysis of sperm DNA showed identical results (data not shown) and gave no evidence of selection against sperm containing the *del*(1) or *rder*(1) or both.

FISH ANALYSES

The origin of the del(1) and rder(1) chromosomes was ascertained by performing FISH on the patient's metaphase preparations using a chromosome 1 paint probe. The result indicated that in addition to the specific painting of the normal chromosome 1, both the del(1) and rder(1) chromosomes were painted (fig 2A), thus confirming their chromosome 1 origin.

FISH analysis of the patient's chromosomes using a pan α satellite probe under low stringency condition showed hybridisation signals on all the chromosomes except rder(1) (fig 2B). When the metaphase chromosomes were hybridised with a telomere probe, positive signals were again observed on all the chromosomes except for the rder(1) chromosome (fig 2C). These results were consistent with the formation of the rder(1) chromosome from an interstitial portion of 1p, followed by rejoining of the sticky ends of the deleted fragments. They further indicated that the rder(1) chromosome has not acquired detectable amounts of exogenous α satellite sequences. These analyses raised the strong possibility that the rder(1) chromosome contained a neocentromere that has been formed from anaphoid DNA sequences within the 1p32-36.1 region.

CENTROMERE PROTEINS

In order to establish the structural and functional integrity of the putative neocentromere on the rder(1) chromosome, the status of various centromere specific proteins was determined by immunofluorescence staining. As shown in fig 2D, use of an anti-CENP-B monoclonal antibody showed variable but detectable levels of CENP-B on the centromeres of all the chromosomes except for the rder(1) and the Y chromosomes. The absence of CENP-B-binding on rder(1) was consistent with the lack of detectable α satellite DNA on this chromosome (see Discussion), whereas the centromeric α satellite of the human Y chromosome is known to have little or no CENP-B box motifs upon which the CENP-B proteins associate.⁸

The other three centromere proteins tested were CENP-E, CENP-F, and INCENP. As shown in figs 2E and F, CENP-E and CENP-F were clearly detected on the rder(1) chromosome, with the immunofluorescence signals being as strong as those seen on the other chromosomes. Compared to the signals for these two proteins, the INCENP signals showed slightly more variability on the different centromeres, with the rder(1) chromosome generally giving the weakest signal (fig 2G). The implications of these results are discussed below.

Discussion

Cytogenetic analyses have identified two apparently unrelated chromosomal rearrangement events in the patient. The first involves an interstitial deletion within the short arm of chromosome 1, leading to the formation of the del(1) and rder(1) chromosomes. This rearrangement is found in 97% of the patient's lymphocytes and 100% of his cultured fibro-

blast cells. In contrast to this, the second rearrangement, which involves a translocation between chromosomes 4 and X, is only detected in the patient's fibroblasts where 60% of the cells carry this aberration in addition to the del(1) and rder(1) chromosomes. It therefore appears that the patient may be mosaic for the 4;X translocation although this awaits confirmation using additional biopsy samples from the patient. At this stage, it is difficult to establish which or whether both of the rearrangements are contributing to oligospermia in the patient since both types of rearrangements may, each on its own, cause such a phenotype. For example, almost all instances of parent to child transmission of ring chromosomes involve the mother as the carrier parent,⁷ suggesting that the presence of a ring chromosome in most male heterozygotes compromises spermatogenesis. Similarly, non-mosaic males with an X;autosome translocation are almost invariably infertile.⁹

The cytogenetic GTL and CBG banding patterns of the del(1) and rder(1) chromosomes, together with the FISH results using a chromosome 1 specific painting probe, indicate the origin of rder(1) from the 1p32-36.1 region. Two lines of evidence suggest that rder(1) is a ring chromosome. The first comes from our failure to detect any telomeric signal which is consistent with the circularisation of a deleted interstitial chromosomal fragment. Secondly, the observation of the occasional double sized ring chromosomes shows structural instability typical of larger rings which form complex ring structures as a result of sister chromatid exchanges.

The high stability of the rder(1) chromosome in both the patient's lymphocytes and his established fibroblast cell line strongly suggests the presence of a functional centromere on the ring chromosome. Proof of this comes from immunofluorescence studies using antibodies against three centromere proteins (CENP-E, CENP-F, and INCENP) that are known to be important for centromere function. CENP-E is a molecular motor that moves chromosomes along microtubules.¹⁰ Although the specific role of CENP-F remains unclear, its apparent interaction with CENP-E suggests a possible role in the proper functioning of a motor complex.¹¹ That both CENP-E and CENP-F are intimately associated with centromere functions is suggested by the observation that these proteins are found on active but not inactive centromeres in dicentric or multicentric chromosomes.¹²⁻¹³ In addition to these two proteins, studies involving overexpression of INCENP mutant proteins in cell culture have shown this protein to be an essential component of the mitotic cell cycle, being involved in prometaphase chromosome congression and cytokinesis.¹⁴ Thus, the demonstration of these three key centromere proteins on the rder(1) chromosome clearly indicates the presence of an active centromere on this chromosome.

At present, it is unclear why the anti-INCENP antibody signals on the rder(1) chromosome are greatly reduced in relation to those on the other chromosomes. Earlier studies have

shown a diffuse localisation of INCENP within the centromeric heterochromatin of normal centromeres.¹⁵ Whether the absence of hetero-

chromatin on the rder(1) chromosome contributes to the severe reduction of centromerically bound INCENP and, if so, what the

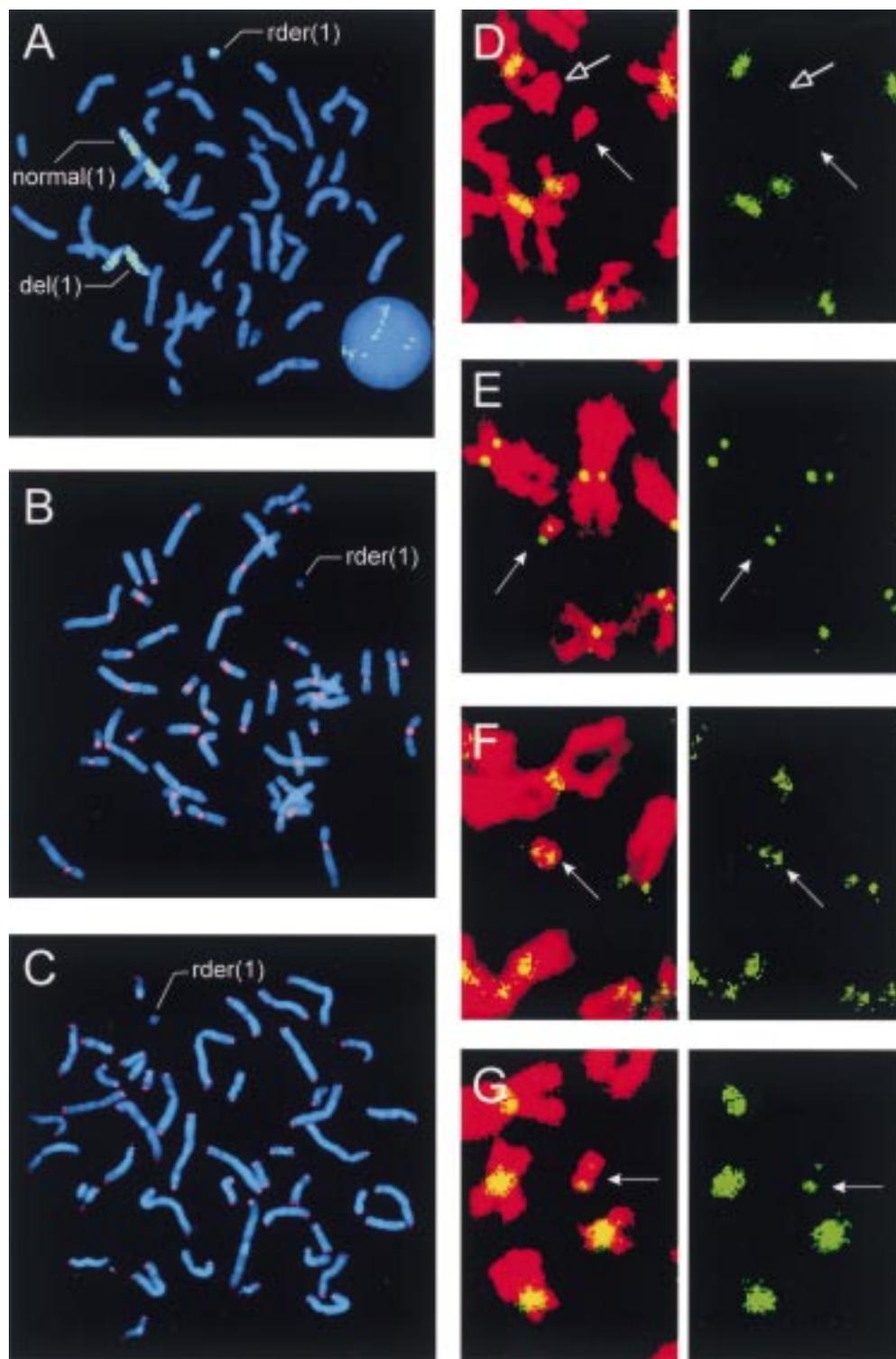


Figure 2 FISH and immunofluorescence studies. (A-C) FISH using chromosome 1 specific paint (green), pan a satellite probe (red), and telomere probe (red), respectively, on a DAPI stained (blue) chromosome background. (D-G) Immunofluorescence detection using antibodies against CENP-B, CENP-E, CENP-F, and INCENP, respectively. In the left hand panel, chromosomes are shown in red while the centromeric antigen specific signals are shown in green (or yellow on a red background). The right hand panel represents split images of the green immunofluorescence signals shown in the left hand panel. Solid arrows indicate the rder(1) chromosome. Open arrow in D points to the CENP-B negative Y chromosome. The detection of two areas of fluorescence on many of the chromosomes (including rder(1)) represents well resolved signals on sister chromatids, rather than implying two different centromeres located on different parts of a chromosome, especially in the case of rder(1). Note the presence of CENP-E, CENP-F, and INCENP, but not CENP-B signals on the rder(1) chromosome.

underlying mechanism is for INCENP binding on a "euchromatic neocentromere", remain to be determined.

Our failure to detect any α satellite DNA on the rder(1) chromosome is consistent with the absence of CENP-B-binding on the rder(1) chromosome since previous studies have established the specific requirement of this protein in binding the CENP-B box sequence motifs on the α satellite DNA.¹⁶ These results indicate that the observed mitotic stability of this chromosome is not associated with the inherent presence or acquisition of any substantial amount of normal centromeric α satellite DNA. Based on this and the above described cytogenetic and immunofluorescence characteristics, it can be concluded that the rder(1) chromosome necessarily contains a functional neocentromere formed from the apparently euchromatic DNA of the chromosome 1p32-36.1 region.

The best characterised anaphoid neocentromere to date has been described for a marker chromosome derived from chromosome 10.^{6, 17-19} This neocentromere forms a distinct primary constriction at the 10q25 region, binds functionally critical centromere antigens CENP-A and CENP-C but not CENP-B, and confers 100% mitotic stability to the patient's lymphocytes and cultured lymphoblasts and fibroblasts. Using the chromosome walking technique, the core functional antigen binding domain of this neocentromere has been localised to an 80 kb DNA.¹⁷ Extensive restriction mapping indicates that the neocentromere DNA is not significantly different from the corresponding DNA of the normal 10q25 region, suggesting a mechanism of neocentromere formation through the epigenetic activation of a normally non-centromeric genomic sequence.^{17, 18} Direct sequencing and detailed computational analyses of the sequence show no distinct homology or structural resemblance to any known eukaryotic centromere.¹⁹ The discovery of an increasing number of putative neocentromeres on other chromosomal sites that carry unrelated, unique genomic sequences⁴ provides further support for the belief that there appears not to be a conserved "magic" primary sequence for the eukaryotic centromeres and that seemingly "ordinary" genomic sequences can be activated to become a functional neocentromere.^{4, 19} The recent identification of a neocentromere in a *Drosophila* minichromosome²⁰ suggests that this phenomenon may be widespread throughout evolution.

In the present study, we have investigated the detailed cytogenetic and antigenic properties of a neocentromere on a ring chromosome formed from a previously undescribed 1p32-36.1 region. We presented evidence that in addition to CENP-A, CENP-C, and CENP-E binding described in earlier studies,^{17, 21} neocentromere activity can further be defined by CENP-F and INCENP binding. Finally, we

have shown that neocentromere formation constitutes a viable mechanism for the mitotic stabilisation of acentric ring chromosomes. Further molecular studies will be necessary to unveil the intricate mechanisms underlying neocentromere formation.

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