

Recapitulation of the Roberts syndrome cellular phenotype by inhibition of *INCENP*, *ZWINT-1* and *ZW10* genes

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Abstract

Roberts syndrome is an autosomal recessive disorder characterised primarily by symmetric reduction of all limbs and growth retardation. Patients have been reported to have premature separation of heterochromatin regions of many chromosomes and abnormalities in cell cycle. Given the rarity of the syndrome, the linkage analysis approach is not suitable to identify the responsible gene. In this work, a cell line derived from a patient affected by Roberts syndrome was characterized by cell biology and molecular cytogenetics, including comparative genomic hybridization and spectral karyotype. No recurrent chromosomal rearrangements were identified. Thereafter, based on the fact that premature chromatid separation is a reliable marker of the disease, we used antisense oligonucleotide technologies to inhibit six genes involved in various steps of the correct chromosome segregation, such as chromosome cohesion, kinetochore assembling, spindle checkpoint and spindle formation. We found that the inhibition of *INCENP*, *ZWINT-1*, *ZW10* genes results in the appearance of mitotic cells characterised by centromere separation, chromosome aneuploidy and micronuclei formation. In addition, *INCENP*, *ZWINT-1*, *ZW10* antisense-treated chromosome morphology was very similar to that of Roberts chromosome when analysed by atomic force microscopy. We concluded that *INCENP*, *ZWINT-1*, *ZW10* gene inhibition results in cellular phenocopies of Roberts syndrome. Taken together, these findings support a possible role of these genes in the pathogenesis of Roberts syndrome.

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1. Introduction

Roberts syndrome (MIM 268300) is a rare autosomal recessive disorder that has been documented in about 100 cases in the literature. It is characterised by tetraphocomelia (symmetrical limb reduction), cleft lip and palate, and severe growth and mental retardation. About 50% of patients have atrial and ventricular septal defects and kidney malformations. These cardiac and renal malformations are

the most common contributing factors to the premature death of patients. Cells from Roberts syndrome patients exhibit a characteristic abnormality of their constitutive heterochromatin, described as precocious centromere separation and puffing of the peri- and paracentromeric regions (Van den Berg and Francke, 1993). Chromosome puffing is most obvious at the large heterochromatic regions of chromosomes 1, 9 and 16 while several other chromosomes may display a railroad track appearance due to the absence of a constriction at the centromere. Centromere separation can be complemented by fusion of Roberts cell with wild-type cells suggesting that the involved protein(s) acts in trans (McDaniel et al., 2000). The function of this protein is conserved in mammals, since the defect is rescued when affected cells are fused with normal cells from rat, mouse and hamster (Dev and Wertelecki, 1984; Krassikoff et al., 1986).

Abbreviations: DMEM, Dulbecco's minimal essential medium; FCS, Foetal calf serum; PD, Population doubling; TUNEL, TdT-mediated dUTP nick end labelling; FITC, Fluorescein isothiocyanate; SKY, Spectral karyotyping; CGH, comparative genomic hybridization; AFM, Atomic force microscopy.

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Mitosis in Roberts cell is abnormal in metaphase duration and anaphase progression. In particular, an increased level of lagging chromosomes or prematurely advancing toward the pole was found in patients' cells when compared to normal ones. In addition, cells show chromosome aneuploidy, micronuclei formation, poor growth, reduced plating efficiency and lower density for confluent cultures, all features that could be compatible with a defect in late mitosis (Jabs et al., 1991). Taken together, these observations suggest that Roberts syndrome is a human mitotic mutation syndrome, leading to secondary developmental defects. In fact, it has been hypothesized that the prolonged cell cycle rate of Roberts syndrome cells could account for the slow pre- and post-natal growth as well as the developmental abnormalities by reducing the number of cells in developing tissue leading to alterations in developmental patterns and dysmorphic changes (Tomkins and Siskin, 1984).

Premature centromere separation is the hallmark of disease both in pre- and post-natal diagnoses but until now no attempt has been made to recognize the molecular basis underlying Roberts syndrome. In addition, the rarity of the disease and difficulties in maintaining cell lines from Roberts patients has precluded mapping of the responsible gene, leaving the candidate gene approach as the only method of gene identification. In this paper, a Roberts syndrome cell line was characterised by classical and molecular cytogenetic techniques, but these approaches did not allow the identification of chromosomal rearrangements that may pinpoint candidate regions. On the other hand, caffeine treatment showed that a significant fraction of cells are blocked in the G₂ phase suggesting that the defect associated with the syndrome may affect the function of proteins involved both in G₂/M transition and centromere function. Based on this finding, we attempted to identify possible candidates by a novel approach based on specific gene inhibition by antisense oligonucleotide technology. We studied the effect of the inhibition of six genes involved in correct chromosome segregation through different pathways, namely *ARP1*, *BUB1*, *INCENP*, *SMC1*, *ZWINT-1*, *ZW10*. Our results showed that inhibition of three of these genes, namely *INCENP*, *ZWINT-1* and *ZW10*, generates cellular phenocopies of Roberts syndrome cells, i.e., cells characterised by premature centromere separation, aneuploidy, and micronuclei. Morphology of antisense treated chromosomes analysed by atomic force microscopy was very similar to Roberts chromosome. Taken together, this evidence supports a possible role of *INCENP*, *ZWINT-1* and *ZW10* in Roberts syndrome.

2. Materials and methods

2.1. Cell culture

D8629M Roberts syndrome cell line, obtained from Galliera Genetic Bank (Genoa, Italy), was cultured in RPMI

(Gibco, BRL) supplemented with 15% foetal calf serum (FCS), glutamine and antibiotics in a humidified 5% CO₂ atmosphere.

For antisense inhibition experiments, two normal primary human fibroblast cell lines growing in Dulbecco's minimal essential medium (DMEM, Gibco BRL) supplemented with 10% FCS and antibiotics in a humidified 5% CO₂ atmosphere were used.

2.2. Cell growth analysis and caffeine treatment

The population doubling (PD) was calculated as $\log_2(D/D_0)$ where D is the density of cells when harvesting and D_0 is the density of cells when seeding. Roberts cells were treated with Caffeine (2.2 mM, Sigma) for the last 2 h of cell culture.

2.3. Apoptosis assay

TdT-mediated dUTP nick end labelling (TUNEL) assay for DNA fragmentation was done using an "In Situ Death Detection" kit (Roche) according to manufacturer's recommendations. At least 500 cells were analysed for each individual treatment.

2.4. Antisense treatment

For inhibition studies, three oligodeoxynucleotides were synthesized: antisense oligomers designed as a complementary sequence at the 5' end of the coding region, sense oligomers from the identical region and scrambled oligomer with the same nucleotides used for the antisense oligomer. Cells were treated with 40 µg/ml of each antisense, sense and scrambled oligonucleotides (diluted in DMEM) for 24 h and additional 20 µg/ml for a further 24 h. One hundred cells were analysed for each individual treatment.

2.5. *INCENP*, *ZWINT-1* and *ZW10* expression by RT-PCR analysis

Total RNA was extracted from cells exposed to *INCENP*, *ZWINT-1* and *ZW10* antisense oligonucleotides by the SV Total RNA Isolation System (Promega) and used for cDNA synthesis with random hexamers. The presence of *INCENP*, *ZWINT-1* and *ZW10* transcripts was analysed by RT-PCR performed combining both a variable number of amplification cycles and different dilutions of primers. HPRT was used as an internal standard.

2.6. Cytogenetic analysis

Exponentially growing fibroblasts were treated with colcemid (0.05 µg/ml, Gibco BRL), harvested, incubated with KCl 0.075 M, and fixed in methanol:acetic acid 3:1. Slides were scored to search chromosome metaphases with centromere separation. In addition, chromosome prepara-

tions were G-banded according to the trypsin digestion procedure and aneuploidy cells, i.e. any variation from diploid number, or rearranged chromosomes were scored by direct microscopic examination.

2.7. Fluorescence *in situ* hybridization

In situ hybridization with a biotin-labelled probe recognizing all the human centromeres (Appligene Oncor) was performed according to a published protocol (Musio et al., 1996). Briefly, slides were pre-treated with 10% pepsin in 10 mM HCl for 10 min at 37 °C. After hybridization, the slides were washed in 50% formamide/2×SSC for 5 min and three times in 2×SSC/0.05% Tween20 for 5 min each. To reduce the background, the slides were pre-incubated for 10 min at room temperature in 100 µl of blocking buffer (4×SSC, 0.05% Tween20 and 5% non-fat dry milk). The biotin-labelled probe was detected by incubating the slides in fluorescein isothiocyanate (FITC)-avidin conjugate. DNA was counterstained with 0.1 µg/ml propidium iodide.

2.8. Spectral karyotyping

Spectral karyotyping (SKY) was performed as described (Schrock et al., 1996). After *in situ* hybridization, images were acquired using an epifluorescence microscope (DMRXA, Leica) connected to an imaging interferometer (SD200, Applied Spectral Imaging) and analyzed using SKYView v1.6 software (Applied Spectral Imaging).

2.9. Comparative genomic hybridization

For comparative genomic hybridization (CGH), DNA was prepared using high salt extraction and phenol purification and labeled by nick-translation using biotin-11-dUTP (Roche). Biotin-labeled DNA and digoxigenin-labeled normal donor DNA (sex-matched) was co-hybridized to sex-matched normal human lymphocyte metaphase chromosomes. Images were acquired with a Leica DMRXA epifluorescence microscope (Leica) using fluorochrome-specific filters (Chroma Technologies). Quantitative fluorescence imaging and CGH analysis was performed using Leica CW4000CGH software (Leica Microsystem Imaging Solutions). Further details for CGH and SKY protocols can be found at <http://www.riedlab.nci.nih.gov/>.

2.10. Atomic force microscopy (AFM)

To obtain an image of the chromosome surface, we used an AFM specifically designed for cytological and histological studies. The operating principle of AFM is described elsewhere (Musio et al., 1994; Mariani et al., 1994). Briefly, an ultrasharp tip scans the sample surface and moves up and down following the surface corrugations. The tip displacement is converted to an electric signal and used to draw topographic maps of the sample. The AFM is a non-invasive

instrument; it operates on samples which do not require any kind of treatment and can produce information supplementing that given by traditional microscope methods. In addition, it allows a three-dimensional reconstruction of sample and provides a height profile with unprecedented resolution.

3. Results and discussion

3.1. Analysis of cell growth in normal and Roberts cells

Roberts syndrome represents an enigma for cell biologists and is of particular interest because its characterization may provide new insights into mechanisms of normal mammalian cell cycle regulation. In order to obtain information on the pathogenesis of this disease, we used a control cell line and D8629M Roberts cells, for analysing their growth curve. Normal fibroblasts showed normal growth with a peak at 96 hours and a doubling time of 19 h. On the other hand, D8629M cells showed an abnormal response, with a scanty increase at 72 h followed by a rapid decrease (Fig. 1), and a doubling time higher than 50 h. In addition, they showed lower cell density at confluence compared to normal fibroblasts (data not shown). A similar trend was found when both apoptosis and micronuclei formation time course were analysed. In Roberts cells the number of both micronuclei (Fig. 2a) and apoptotic cells (Fig. 2b) showed a progressive increase with time and their frequencies were significantly different from normal fibroblasts. These observations suggest that a proportion of Roberts cells is continually being lost in culture and this may explain reduced growth we observe in culture. To gain further insight into Roberts cell cycle, we treated D8629M cells with caffeine. Caffeine treatment led to an increase of mitotic index suggesting that a significant proportion of cells are blocked in the G₂ phase ($P=0.0011$, Table 1). When the G₂ checkpoint was abrogated by caffeine treatment, cells were forced into the M phase but their chromosomes were tightly packed or covered by nuclear membrane

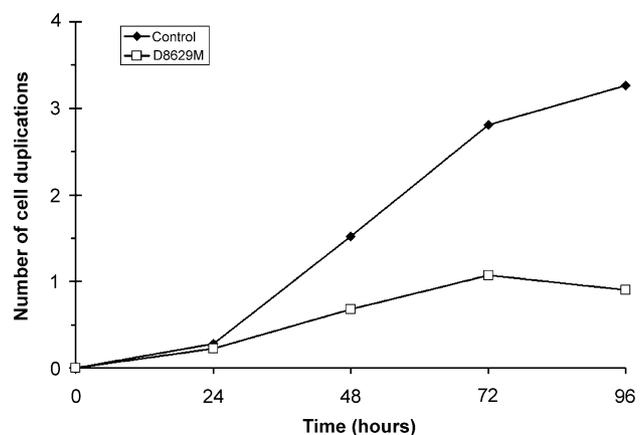


Fig. 1. Growth curve of D8629M and reference cell lines.

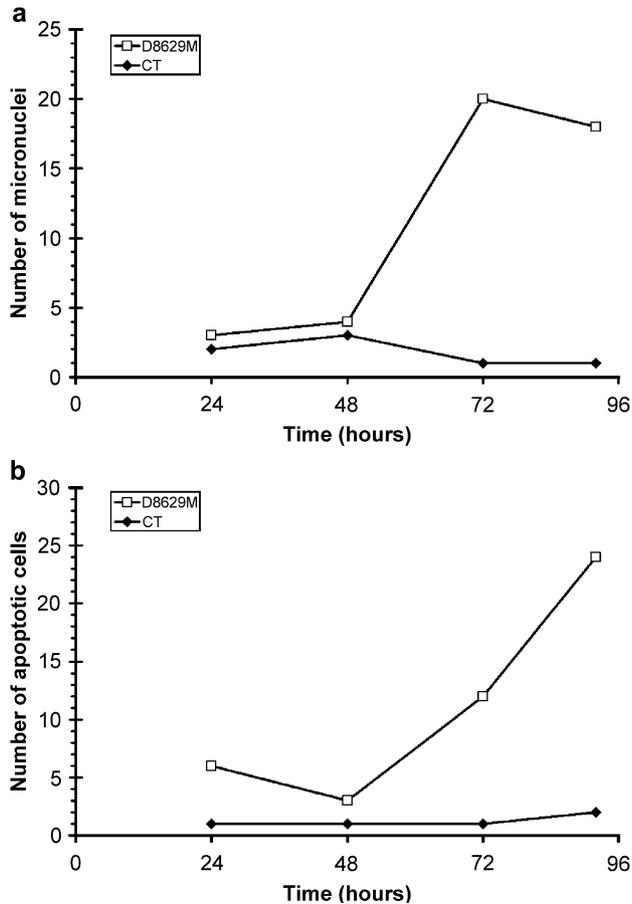


Fig. 2. Time course of micronuclei (a) and apoptotic (b) in D8629M cells and control cells. Five hundred cells were analysed.

(data not shown). Taken together, these findings suggest that a defect in the last mitotic steps activates a checkpoint and that its abrogation by caffeine allows cells to proceed to anaphase where apoptosis occurs.

3.2. Cytogenetic studies with SKY and CGH

In order to identify chromosomal rearrangements, which may help to identify the region where the responsible gene could be located, Roberts cells were analysed by both classical and molecular cytogenetics such as SKY and CGH. Karyotype of Roberts cells showed 58% of aneuploidy, with chromosome numbers ranging from 38 to 91, while no evidence of spontaneous structural chromosomal instability was evidenced (data not shown). These data were

Table 1

Mitotic index of D8629M cells after caffeine treatment

Treatment	Mitotic index ^a
Control	3.33±0.57
Caffeine	15.66±3.05

^a At least 1000 cells were analysed. Means of three independent experiments.

confirmed by more sensitive analysis. The SKY technique showed no chromosome rearrangements, i.e. chromosome translocations or other structural defects (Fig. 3a), except a duplication in the 14p11.1–11.2 chromosomal band (Fig. 3b) detected by CGH. No obvious candidate gene is located on this region when in silico analysis was performed (not shown). The significance of this data is unclear at present, since only the analysis of other Roberts syndrome cell lines will confirm the specificity of this finding. CGH analysis of the D8629M cell line showed trisomy for chromosome 7 (Fig. 3b). However, gain of chromosome 7 in cultured cells has previously been reported and is a common pseudomosaic finding in cultured amniocytes (Tomkins et al., 1979; Parry et al., 1986).

3.3. Antisense inhibition of genes in kinetochore assembly and function

Caffeine treatment suggests that the defect associated with Roberts syndrome may affect the function of proteins that are transiently associated with centromere and are involved in spindle checkpoint. For this reason, we have used antisense technology to inhibit six genes involved in chromosome segregation to see whether the downregulation of their protein products recapitulates the cytogenetic hallmarks of Roberts syndrome cells. Two human normal primary fibroblast cell lines were treated with antisense oligonucleotides against genes known to be involved in different steps or pathways of the correct chromosome segregation, such as chromosome cohesion (*SMC1*), spindle checkpoint (*BUB1*, *INCENP*, *ZWINT-1*, *ZW10*) and spindle formation (*ARPI*). Antisense oligonucleotides used to inhibit *INCENP*, *ZWINT-1* and *ZW10* genes are reported in Table 2 and their efficacy was evaluated by analysis of transcripts. In fact, a down-regulation of *INCENP*, *ZWINT-1* and *ZW10* transcription was shown to occur in antisense-treated cells because by reducing the primer concentration specific bands were seen only in control cells (Fig. 4a–c). The effectiveness of *ARPI*, *BUB1* and *SMC1* inhibition is shown elsewhere (Musio et al., 2003). Antisense inhibition

Table 2

Antisense and scramble oligonucleotide sequences used for human fibroblast treatments

	Antisense oligonucleotide ^a	Scrambled oligonucleotide
INCENP	GGG GTG GGT GAA GAA GGT GC	ATG AGG GAG TGT GCG AGG GG
ZWINT-1	CCT CTG TCT CCG CTG CCT CC	TGC CTC CTC GCC CGT TCC TC
ZW10	ACC TCA CTC TCT ATC CT	TTC AAA CCT TCC CTC TC

^a Sense oligonucleotides are the reverse/complement of antisense oligonucleotides.

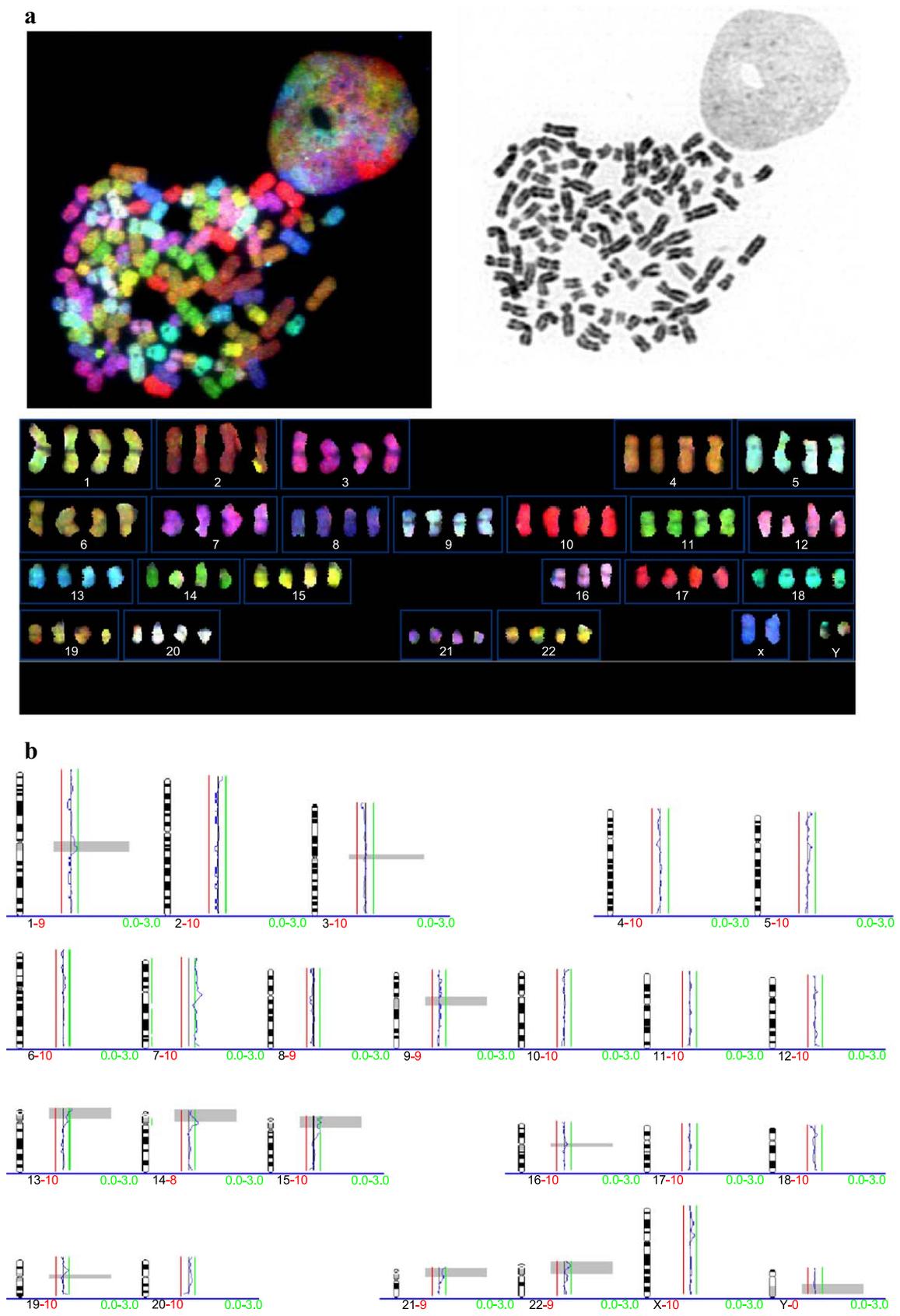


Fig. 3. SKY (a) and CGH analysis (b) of D8629M cells.

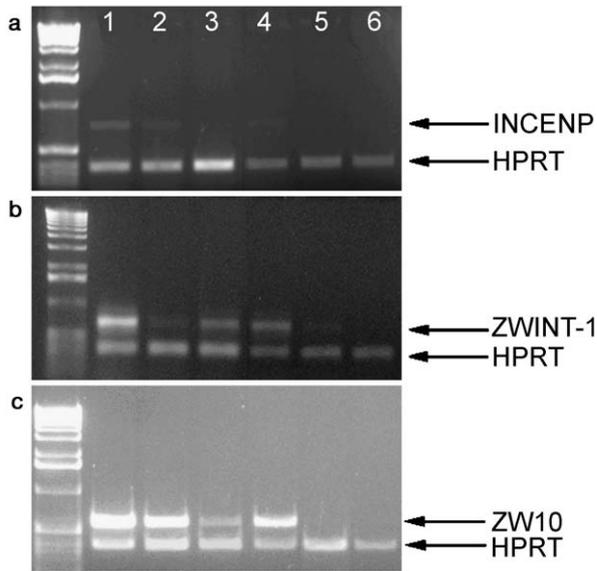


Fig. 4. Effect of antisense oligonucleotide treatment on *INCENP* (a), *ZWINT-1* (b) and *ZW10* (c) (lanes 4–6) and control cells (1–3).

of most genes investigated in this study led to chromosome aneuploidy to different extents (Table 3), providing evidence that these genes play an important role in maintaining the integrity of the genome in human cells as well as other systems. No specific chromosome loss or gain was detected by G-banding suggesting that aneuploidy was random (data not shown). These data are discussed in detail elsewhere (Musio et al., 2003).

Of note, in addition to chromosome aneuploidy, inhibition of *INCENP*, *ZWINT-1* and *ZW10* led to premature centromere separation in the presence of a short treatment (1 h) with colcemid, a spindle poison, whereas no centromere separation was observed either in control cells or in cells treated with antisense oligonucleotides against the other genes tested (Table 2). Moreover, from 9% to 13% of cells and from 5% to 14% showed this characteristic cytogenetic feature in subjects 1 and 2, respectively (Table 3).

Under optical microscopy, untreated chromosomes showed a normal morphology with a clear primary constriction (centromere) separating the two arms (Fig. 5a). On the

contrary, most antisense-treated metaphase chromosomes have separated roadlike straight chromatids with a railroad track appearance (Fig. 5b). In addition to aneuploidy and centromere separation, inhibition of *INCENP*, *ZWINT-1* and *ZW10* also caused the formation of micronuclei ranging from 9% to 20%. The chromosome presence inside the micronuclei was shown by fluorescence in situ hybridization using a pancentromeric probe (data not shown) confirming that micronuclei formation is a direct result of abnormal chromosome segregation and lagging chromosomes. In humans, all these features are reminiscent of Roberts syndrome.

3.4. Atomic force microscopy studies

To gain insight into the chromosome structures after antisense treatment, chromosomes were analysed by atomic

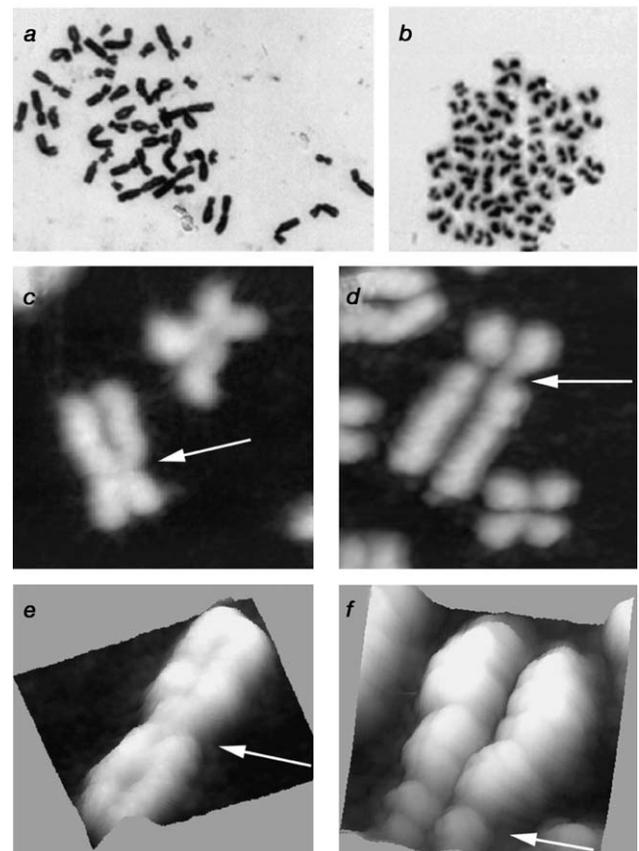


Fig. 5. Effect of gene inhibition by antisense treatment on normal human chromosomes. (a) Untreated normal metaphase analysed by optical microscopy. All chromosomes show a normal morphology where a distinctive centromere separates the two arms. (b) Chromosomes analysed by optical microscopy after *INCENP* antisense treatment. Most chromosomes show a premature centromere separation with a railroad track appearance. (c,e) Untreated chromosomes imaged by AFM. Sister chromatids are linked at centromere level (arrows). (d) Sister chromatids are clearly separated after *INCENP* antisense treatment (arrows). Similar results were obtained with *ZW10* and *ZWINT-1* antisense treatments. (f) Roberts chromosome showing chromatid separation.

Table 3
Aneuploid cells and centromere separation after oligonucleotide antisense treatments

	Subject 1		Subject 2	
	Aneuploidy cells	Centromere separation	Aneuploidy cells	Centromere separation
Control	2	0	4	0
ARF1	24	0	31	0
BUB1	15	0	12	0
INCENP	20	9	23	14
SMC1	26	0	31	0
ZW10	17	13	27	6
ZWINT-1	22	11	18	5

force microscopy (AFM) without Giemsa staining or banding techniques. While in normal metaphases chromosomes are discrete with defined morphology and well-recognized centromeric regions as shown in Fig. 5c and e, antisense-treated chromosomes, on the contrary, showed a separation at centromeric regions (Fig. 5d) with an appearance very similar to Roberts chromosome (Fig. 5f). Moreover, AFM images show that sister chromatids were completely separated and no structures or elements were evident between them.

Our findings emphasize that inhibition of *INCENP*, *ZWINT-1* and *ZW10* genes generates an in vitro phenotype very similar to that of Roberts syndrome, i.e. characterised by centromere separation with a morphology close to that of Roberts syndrome when compared by AFM, chromosome aneuploidy and micronuclei formation, providing for the first time a possible molecular basis underlying this disease. However, a preliminary analysis of the structure of these three genes performed by RT-PCR mediated amplification of their transcripts showed no difference between Roberts and control cells (data not shown). While this finding excludes the presence of gross abnormalities, it does not rule out the possibility of more subtle alterations whose identification requires complete sequence of the gene (work in progress).

Caffeine treatment leads cells to escape G₂ block and to entry into M phase suggesting that protein(s) involved may be transiently associated with centromere. *INCENP*, *ZWINT-1* and *ZW10* genes are passenger proteins which associate with the centromere transiently during the cell cycle, usually between the late G₂ and early prophase, before dissociating prior to the completion of mitosis. *INCENP* relocates from the centromere to the spindle midzone during metaphase–anaphase transition (Adams et al., 2001; Earnshaw and Cooke, 1991). Interestingly, *INCENP* interacts with the heterochromatin protein 1 (HP-1), a chromosomal adapter protein acting as a “docking site” for *INCENP* (Mackay et al., 1998; Bannister et al., 2001; Lachner et al., 2001). *ZW10* and *ZWINT-1* interact with each other and this interaction is a prerequisite for *ZW10* localization to kinetochore at prometaphase. In addition, *ZW10* is thought to play a role in the recruitment of dynein to the kinetochore and is an essential component of the mitotic checkpoint (Chan et al., 2000; Starr et al., 2000). In *zw10 Drosophila* null mutants, sister chromatids are separated in the presence of a microtubule-disrupting agent, suggesting a malfunction of the mitotic checkpoint. Microtubule interaction and heterochromatin integrity are under the control of an elaborate checkpoint system whose failure may cause both centromere separation and chromosome aneuploidy.

In conclusion, we suggest that *INCENP*, *ZWINT-1* and *ZW10* or other kinetochore-associated genes not investigated here, may play a role in Roberts syndrome pathogenesis because their inhibition led to a cell phenotype similar to Roberts cells. This evidence supports the notion that Roberts

syndrome is a mitotic-defective mutant and further investigation of proteins involved in kinetochore assembly and function may finally allow us to identify the molecular basis of the Roberts syndrome genetic defect.

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