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Molecular cytogenetic characteristics of Down syndrome newborns

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Abstract Down syndrome (DS) is a multifactorial disorder with a high predisposition to leukemia and other malignancies. A change in the replication pattern from synchronous in normal genes to asynchronous in DS amniocytes has previously been reported. The objective of this study was to evaluate additional molecular cytogenetic factors which could re-emphasize the high correlation between DS cells and genetic instability. We found a higher rate of random aneuploidy in chromosomes 9 and 18 and a higher rate of asynchronous replication in the subtelomeric region or DS leukocytes than in cells from normal newborns. In addition, the telomere capture phenomenon was observed in the DS leukocytes but not in normal controls. The molecular cytogenetic factors observed in the DS individuals are known to correlate with genomic instability and with predisposition to cancer.

Keywords Down syndrome · Random aneuploidy · Replication pattern · Telomere capture

Introduction

Genomic aneuploidy, defined as an abnormal number of copies of a genomic region, is a common cause of human genetic disorders. This abnormality may arise because of the presence of supernumerary copies of whole

chromosomes (trisomy, triploidy, etc.) or because of the absence of chromosomes (monosomy) (Antonarakis et al. 2004).

Most aneuploidic conceptuses perish in utero, and this is the leading genetic cause of pregnancy loss. Some aneuploidic fetuses survive to term, however (they account for 0.3–0.5% of live births) and the abnormality is a major contributor to the development of mental retardation.

Trisomy 21 may serve as a model for human disorders that result from supernumerary copies of a genomic region. It gives rise to a variety of traits, all of which have variable penetrance and clinical expressivity (Antonarakis et al. 2004). The syndrome carries a 10–30-fold increased risk of developing acute leukemia (Hernandez and Fisher 1996) and an approximately 20-fold increased risk of developing other malignancies (Duesberg et al. 2004).

The aneuploidy-cancer theory proposes that cancer is caused by abnormal dosage of thousands of normal genes. This abnormal dosage of genes is generated by the gain or loss of specific chromosomes or segments of chromosomes (Rasnick and Duesberg 1999; Duesberg and Rasnick 2000; Duesberg and Li 2003; Duesberg et al. 2004). According to this theory, carcinogenesis is initiated by a random aneuploidy which is induced either by carcinogens or arises spontaneously (Duesberg et al. 2000; Fabarius et al. 2002). Chromosomal and genetic instability is proportional to the extent of aneuploidy and to the types of chromosome that are unbalanced (Fabarius et al. 2002). The literature does not demonstrate the existence the higher rate of random aneuploidy in aneuploid individuals, which has been shown in their parents (Amiel et al. 2000; Staessen et al. 1983; Stallard et al. 1981; Stone and Sandberg 1995).

Numerous studies have demonstrated close association between the timing of replication of a given chromosome region during the S-phase and the transcriptional activity of genes in that region. Hence, expressed loci replicate early in the S-phase whereas

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silent genes replicate late (Goldman et al. 1984; Hatton et al. 1988; Holmquist 1987). Recently, using a simple cytogenetic technique based on fluorescence in-situ hybridization (FISH), it became possible to distinguish between replicated and not yet replicated DNA sequences at interphase. When this technique was applied to human somatic cells it convincingly revealed that alleles of genes which exhibit allele-specific expression (monoallelic mode of expression), for example imprinted genes (Kitsberg et al. 1993), genes subjected to X-chromosome inactivation (Boggs and Chinault 1994), and olfactory receptor genes (Chess et al. 1994), replicate asynchronously, whereas alleles which are expressed concomitantly (biallelic mode of expression) replicate highly synchronously (Selig et al. 1992; Amiel et al. 1998a). There is also evidence that alleles of loci associated with cell proliferation (p53, HER-2/neu and c-myc) and chromosome segregation (which normally replicate synchronously) replicate asynchronously in cancer cells (Amiel et al. 1998b, c, 2000).

There are conflicting opinions regarding replication timing of the telomeres in the S-phase. Some suggest they behave like the heterochromatic region and replicate in the late S-phase whereas others are of the opinion they replicate in the early S-phase or randomly during all the S-phase (McCarroll and Fangman 1988; Hultdin et al. 2001; Ofir et al. 2002). Telomeric regions of the human genome are of particular interest because rearrangements of these regions are difficult to identify by conventional chromosome-banding techniques. Recently, with the advent of molecular cytogenetics, for example the FISH technique, it has become possible to investigate the terminus in cytogenetically visible terminal deletions and telomere rearrangements (Meltzer et al. 1993; Ning et al. 1998; Ballif et al. 2000; Amiel et al. 2005a).

In this study we investigated random aneuploidy rates, the replication pattern of telomeres, and telomere capture in newborns with Down syndrome (DS).

Materials and methods

Leukocytes originating from seven DS newborns (up to 1 year of age, who were diagnosed at our institute) were studied. Leukocytes from seven healthy newborns served as controls. Peripheral blood samples were obtained and incubated for short-term culture in F10-supplemented medium at 37°C in a moist chamber for 72 h. The supplemented medium contained 20% FCS, PHA heparin, and 1% antibiotics. After incubation, colchicines (final concentration 0.1 µg mL⁻¹) were added to the cultures for 1 h, followed by hypotonic treatment (0.075 mol L⁻¹ KCl and 37°C for 15 min) and four washes, each with a fresh cold 3:1 methanol–acetic acid solution. The lymphocyte suspensions of the three samples were stored at –4°C.

Probes

Abbott/Vysis Television 19p spectrum green (cat. no. 5J03-18), Television 19q spectrum orange (cat. no. 5J04-19), and Cytocell probes. SNRPN/Imprinting center: red fluorophore with 15qter control probe green fluorophore (cat. no. LPU005) and 13q14.3 deletion probe D13S319-D13 525 red fluorophore 13q telomere-specific (163Cg). Control probe: green fluorophore (cat. no. LPH006). Each probe was used separately.

Specimen pretreatment before co-denaturation

To make the chromosomal DNA accessible for hybridization and to protect the morphology of the chromosomes from the co-denaturation process, pre-treatment according to the Vysis-Abbott procedure for the subtelomeric region was applied.

FISH technique

Fresh slide spreads were denatured for 2 min in 70% formamide/2× standard saline citrate (SSC) at 70°C and dehydrated in a graded ethanol series. The probe mix was then applied to air-warmed slides (30 µL, mix sealed under a 24 mm×50 mm glass cover slips) and hybridized for 18 h at 37°C in a moist chamber. After hybridization the slides were washed in 50% formamide 2× SSC for 20 min at 43°C, rinsed in two changes of 2× SSC at 37°C for 4 min each, and placed in 0.05% Tween 20 (Sigma, Rehovot, Israel). For FISH analysis the slides were counter-stained in DAPI (4',6'-diamidino-2-phenylindole) (Sigma, St Louis, MO, USA) antifade solution and analyzed for simultaneous viewing of FITC (fluorescein isothiocyanate), Texas Red, and DAPI with an image-processing system (Applied Imaging, Santa Clara, CA, USA).

Cytogenetic evaluation

Random aneuploidy: For each cell we recorded the number of hybridization signals. The rate of aneuploidy was inferred from the fraction of cells with one, three, or more hybridization signals per cell (Fig. 1a). The slides were always scored “blindly”.

Replication pattern: Interphase cells with two hybridization signals were analyzed for each probe. The cells were classified into three categories according to Selig et al. (1992).

The samples were scored “blind”. The level of asynchrony in replication timing was derived from the fraction of SD cells. The slides were scored “blindly”.

Fig. 1 **a** A trisomic nucleus (three different, red signals, far apart). **b** A nucleus with four signals. The three upper signals are doublets (two very close, almost touching, signals)

Telomere capture or “translocation” of the telomere in the nucleus

The analysis was performed on interphase nuclei. We compared the numbers of specific loci of the specific chromosome (SNRPN—red) and its telomere (15qter—green). We compared the number of signals of the SNRPN locus to the number of signals of the subtelomeric region of the specific chromosome (green signals). For example the normal appearance is two orange and two green signals (Fig. 2a) whereas abnormal appearance is two orange and three or more green signals (Fig. 2b). For aneuploidy and telomere capture rate, approximately 200 nuclei were analyzed “blindly”.

Statistical analysis

Two two-tailed *t*-test was used to test for quantitative differences between the study groups. A *P*-value of 0.05 or less was regarded as statistically significant. We used Microsoft Excel software.

Results

Patients with DS had a significantly higher rate of random trisomy than the control group (Table 1) for the 13 ($P=0.032$) and 15 ($P=0.0003$) loci (Fig. 3). For the monosomy there was no significant difference between the two loci in the DS and control groups (Table 1).

The SD pattern was significantly higher in the DS patients with the Rb and 13qter loci than in the control group (Fig. 1; $P=7.4\times 10^{-7}$). This was because of both the DD ($P=0.009$) and SS ($P=0.04$) patterns compared with the control group, which means that one allele was replicating earlier than the control allele and one later (Fig. 1; for RB-1, one allele is replicating earlier, whereas for the 13qter one allele is replicating later in the DS newborns). For chromosome 19 telomeres, both had the same pattern of replication—the SD pattern was significantly higher in the DS patient than in the control group (Fig. 4; $P=0.0005$) because of the SS pattern ($P=0.04$). This means that one allele was replicating earlier (Fig. 4).

To estimate the rate of “telomere capture” we compared the number of signals of the SNRPN both with the number of signals of the 15qter region and with the total number of 13q14 signals of the 13qter region (Tables 2, 3). In the controls there were no cells with incompatible numbers between the telomere and the specific locus. In DS patient cells there were incompatible numbers of signals between the two loci and their telomeres for both chromosomes 13 and 15 (Tables 2, 3). The rate of this incompatibility was similar for chromosomes 13 (3.97 ± 1.4) and 15 (5.06 ± 1.7).

Discussion

Assessment of the replication pattern in DS newborns revealed asynchronous replication in the different loci analyzed. We have previously reported (using normal structural alleles p53, HER-2/neu, RB-1, and c-myc) that normal concomitant expression in the expected Mendelian manner, which occurs in normal control cells, is lost in DS amniocytes (Amiel et al. 1998a). Diverse mechanisms may be involved in the mode of replication of the different subtelomeric regions and this could explain the different times of replication during the S-phase of some telomeric regions, as previously reported in the literature (McCarroll and Fangman 1988; Hultdin et al. 2001; Ofir et al. 2002). It seems that the 13qter and 19pter are late-replicating and that the 19qter is replicating earlier in the S-phase, more so for the DD fraction. Our findings also show that different mechanisms are involved in the change from synchronous to asynchronous replication patterns within the different subtelomeric regions that were analyzed.

In contrast, both telomeres of chromosome 19 behaved in the same manner in both subtelomeric regions. In the DS leukocytes one allele replicated earlier. The

Fig. 2 **a** Normal nucleus with the two SNRPN (red) and two 15qter (green) signals. **b** A nucleus with “telomere capture”—two (red) SNRPN signals and three (green) 15qter signals

Table 1 Random aneuploidy in Down syndrome (DS) and control leukocytes

N^a			Mean $\pm$ SD (%)
Chromosome 13			
Control	7	Monosomy	0.33 $\pm$ 0.35
		Disomy	99.74 $\pm$ 0.38
		Trisomy and above	0.26 $\pm$ 0.38
DS	7	Monosomy	0.72 $\pm$ 1.24
		Disomy	96.57 $\pm$ 2.13
		Trisomy and above	2.71 $\pm$ 2.33
Chromosome 15			
Control	7	Monosomy	0.3 $\pm$ 0.29
		Disomy	99.41 $\pm$ 0.53
		Trisomy and above	0.29 $\pm$ 0.39
DS	7	Monosomy	1.45 $\pm$ 1.49
		Disomy	96.15 $\pm$ 1.56
		Trisomy and above	2.77 $\pm$ 1.01

^aNumber of samples

Fig. 3 A cell in the metaphase from a Down syndrome leukocyte, showing 48 chromosomes. The arrows indicate three chromosomes 21 and three chromosomes 13

question whether the mechanism of the loss of synchronous pattern of the subtelomeric region is the same for both telomeres of each chromosome must be studied further, however.

The increased risk of DS individuals of developing leukemia (Hernandez and Fisher 1996) and other malignancies (Duesberg et al. 2004) has been attributed to primary aneuploidy (Trisomy 21) in these individuals, although the actual mechanism has never been clear. According to the aneuploidy-cancer theory, carcinogenesis is initiated by random aneuploidy which is induced either by carcinogens or arises spontaneously (Duesberg and Rasnick 2000).

Fig. 4 Replication pattern for the different loci in Down syndrome and control leukocytes

We observed more random aneuploidy (trisomy and greater) in leukocytes from DS individuals. This finding could support the aneuploidy-cancer theory according to which cancer is caused by abnormal dosage of thousands of normal genes. This abnormal dosage of genes is generated by the increased number of specific chromosomes or segments of chromosomes (Amiel et al. 2005a; Duesberg and Rasnick 2000; Duesberg et al. 2004), as is also in aneuploidy.

We have previously reported that loss of replication control is one of the characteristics of the aneuploid syndromes (DS, Trisomy 13 and 18, etc.) (Amiel et al. 1999). In this study we demonstrated two other aspects of genetic instability:

1 random aneuploidy (the existence of which has been reported for different malignancies); and

2 the telomere capture phenomenon (which was shown to be present in cancer cells) (Amiel et al. 2005a).

It thus seems that these multifactorial disorders arise because of interference with the mechanism and control of gene replication. This supports the hypothesis that “aneuploidic syndromes are likely to reflect an upset in the balance of the regulators that modulate target genes throughout the genome, which in turn will change the phenotype” (Birchler et al. 2005). Recent findings, however, suggest that this balance involves the regulatory system more than changes in the dosage of the target genes, as is often envisaged. A reduced amount of expression from crucial target genes, as a result of regulatory imbalance, might have phenotypic effects in both monosomies and trisomies (Birchler and Newton 1981; Birchler et al. 2001).

Table 2 Mean number of nuclei with incompatibility with the Rb-1 locus and with the 13qter region for Down syndrome patients

Signal number of the Rb locus						
4	3	2	1			
0	0	0.96 ± 1.11	*Monosomic cells with compatibility	Nuclei percentage (%)	1	Number of signals of the 13qter region
0	0	*Disomic cells with compatibility	0.7 ± 0.4	Nuclei percentage (%)	2	
0	Trisomic* cells with compatibility	1.12 ± 2.01	0	Nuclei percentage (%)	3	
*Tetrasomic cells with compatibility 1.40 ± 3.97	0	0.61 ± 0.42	0	Nuclei percentage (%)	4	
				Mean of nuclei with incompatibility (%)		

Table 3 Average number of nuclei with incompatibility, in signal number of the SNRPN locus and signal number of the 15qter region, for Down syndrome patients

Signal number of the SNRPN locus						
4	3	2	1			
0	0	0.47 ± 0.35	*Monosomic cells with compatibility	Nuclei percentage (%)	1	Number of signals of the 15qter region
0	1.5 ± 1.29	*Disomic cells with compatibility	0.94 ± 0.36	Nuclei percentage (%)	2	
0	Trisomic* cells with compatibility	0.99 ± 3.39	0	Nuclei percentage (%)	3	
*Tetrasomic cells with compatibility 1.67 ± 5.06	0	0.27 ± 0.28	0	Nuclei percentage (%)	4	
				Mean of nuclei with incompatibility (%)		

According to some reports total expression in the DS genotype is directly proportional to the number of target gene copies and inversely proportional to the magnitude of the regulatory imbalance. These effects often disappear as a result of dosage compensation. Consequently, the greatest modulation of gene expression in aneuploidies involves the target genes that are not in the aneuploid region, because their copy number is not changed but they are affected by the varied regulators in trans (Devin et al. 1988; Guo and Birchler 1994; FitzPatrick 2005). This can probably explain the loss of the normal replication pattern of the alleles on the non-aneuploidic chromosomes. All the molecular cytogenetic characteristics observed for DS leukocytes have been shown to be highly correlated with genetic instability, cancer predisposition, and cancer phenotype.

To conclude, our findings re-emphasize the great disturbance of the control of gene replication and cell cycle control, chromosome segregation, and telomere function in DS. Further studies are needed to confirm the results in DS and also in other aneuploidic syndromes.

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