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Replication status as a possible marker for genomic instability in cells originating from genotypes with balanced rearrangements

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Abstract

Most allelic pairs of DNA replicate synchronously during the S phase of the cell cycle. However, some genes frequently replicate asynchronously, i.e. genes on the X chromosome and imprinted genes. Earlier studies demonstrated an asynchronous pattern of replication in some precancerous and invasive squamous carcinoma of the cervix as well as in multiple myeloma. A high rate of asynchronous pattern was found in: (1) lymphocytes of individuals with solid tumors as well as in other malignancies; (2) amniocytes of genotypes with an extra chromosome 13, 18 and 21; (3) lymphocytes of young mothers of a Down syndrome pregnancy. The asynchronic pattern was not locus specific and was found in all loci analyzed. These findings suggested that the mechanism controlling the temporal order of replication could be altered in cells with a genetic predisposition to cancer or aneuploidy. In this study, we found a higher rate of asynchronous pattern in genotypes carrying inversions 2 and 9 and in balanced heritable translocations ($p < 0.01$) and an even higher rate in cases with a *de-novo* balanced translocation. The process of tumorigenesis may begin with a change in cell cycle regulation which includes the duplication, replication and segregation of genetic information. However, it remains unknown whether individuals with balanced chromosome rearrangements are at increased risk of developing cancer later in life.

Introduction

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 during interphase. While an unreplicated DNA sequence reveals a single fluorescent signal (singlet), a replicated sequence gives rise to a duplicated signal (doublet) (Selig *et al.* 1992).

The FISH assay relies on replication-dependent chromatin conformation. Accordingly, the replication status of a locus is inferred from the shape of the hybridization signal obtained at interphase, following FISH with a locus-specific probe. Prior to replication, each identified DNA sequence shows a single dot-like hybridization signal (''singlet''; S), while at the end of replication it assumes a doubled bipartite structure (''doublet''; D) (Mukherjee *et al.* 1992, Selig *et al.* 1992, Boggs

& Chinault 1997). Cells with 1 ''singlet'' and 1 ''doublet'' represent cells in S-phase (designated SD cells), in which only one of the allelic sequences has been replicated. Cells with 2 ''singlets'' (SS cells) represent those in which both sequences are unreplicated, and cells with 2 ''doublets'' (DD cells) represent those in which both sequences have replicated. In an unsynchronized population of replicating cells, the frequency of cells at a given stage expresses the relative duration of that stage. Hence, the frequency of SD cells, out of the total population of cells with 2 hybridization signals, correlates with the time interval (at S-phase) during which the 2 allelic counterparts differ in their replication ''status'' (Dotan *et al.* 2000).

Using this technique, applied to human somatic cells, it has been convincingly demonstrated that alleles of genes which exhibit allele-specific expression (mono-allelic mode of expression), such as imprinted genes (Kitsberg *et al.* 1993, Knoll *et al.* 1994, Gunaratne *et al.* 1995, White *et al.* 1996), genes subjected to X-chromosome inactivation (Boggs & Chinault 1994), replicate asynchronously, whereas alleles which behave in the expected Mendelian manner are expressed concomitantly (biallelic mode of expression) and replicate highly synchronously (Selig *et al.* 1992, Kitsberg *et al.* 1993, Boggs & Chinault 1994).

Cells derived from Down syndrome (DS) fetuses and cells from trisomies 13 and 18 fetuses displayed an asynchronous pattern in the timing of replication of alleles that are unrelated to the additional chromosome. In these cases, the phenomenon is even more pronounced than seen in DS amniocytes, and significantly exceeds the rate of asynchronous replication displayed by the same loci in normal fetuses. In each trisomy, there is a large interindividual variation of anomalies that are not trisomy specific. This may account in part for a non-specific shift in the mode of expression of many loci not present on the extra chromosome, from a biallelic to monoallelic mode (Amiel *et al.* 1998a, 1999) In this study, we evaluated the replication pattern in amniocytes taken from four groups of genotypes with different structural rearrangement: inversion in chromosome 9, inversion in chromosome 2, balanced heritable translocation and *de-novo* translocation. We used a FISH technique with probes for RB-1, 21q22 and p53 which were used in our previous studies (Amiel *et al.* 1997, 1998a, 1999a).

Materials and methods

In our laboratory, we keep amniocytes and leukocytes from pathological cases in fixative. For this study we used cells from this 'library'.

The following sources were used: amniocytes (1–5) and leukocytes (6–9):

Amniocytes:

1. Normal karyotype: 8 cases.
2. Inversion in chromosome 9(p11;q12): 10 cases.
3. Inversion in chromosome 2(p12;q13): 7 cases.
4. Heritable reciprocal balanced translocation: 9 cases.
5. *De-novo* translocation: 5 cases.

Leukocytes:

6. Normal karyotype: 7 cases.
7. Inversion in chromosome 9: 7 cases.
8. Inversion chromosome 2: 4 cases.
9. Heritable reciprocal balanced translocation: 9 cases (all parents of heritable translocation in amniocentesis).

Slide preparation

Glass slides were pre-cleaned for FISH incubation in a concentrated sulphochromic solution, rinsed with distilled water followed by two series of absolute ethanol, and then dried. The stored cell suspensions were washed with a fresh cold 3 : 1 methanol : acetic acid solution and then dropped onto the pre-cleaned slides and air dried.

Probes

Four digoxigenin-labeled commercial probes (Oncor) were used, each identifying a single specific region:

1. RB-1 for chromosome region 13q14 (Oncor-Appligene p5116).
2. 21q22 for chromosome region 21q22 (Oncor-Appligene p5320).
3. p53 for chromosome region 17p13.1 (Oncor-Appligene p5106).

Monocolor FISH

Fresh slide spreads were denatured for 2 min in 70% formamide 2×SSC at 70°C and dehydrated in a graded ethanol series. The probe mix was then applied to air-warmed slides (30 ml mix sealed under a 24 × 50 mm glass cover slip) and hybridized for 18 h at 37°C in a moist chamber. Following 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, Israel). The slides were counterstained in propidium iodide (Sigma, Israel) anti-fade solution and analyzed for simultaneous viewing of FITC, Texas red an DAPI (Chroma).

Cytogenetic evaluation

Following the application on monocolor FISH, 100 interphase cells which showed two hybridization signals were analyzed for each given probe. The cells were classified into three categories according to Selig *et al*. 1992.

1. Cells with two singlets (SS), representing cells where both allelic loci are unreplicated.
2. Cells with two doublets (DD), representing cells in which both allelic loci have replicated.
3. Cells with one singlet and one doublet (SD), revealing S-phase cells where only one of the allelic loci has been replicated.

The samples were analyzed ''blindly''. The pattern of replication for each category was assessed twice by two or three independent readers. The level of synchrony in replication timing was derived from the fraction of SD cells. The scoring results between the different readers and between the repeated analyses did not vary, and we used the mean number for the analyzed categories.

Statistical method

The following statistical tests were used in the analysis of the data presented in this paper. The two-sample *t*-test and non-parametric test were applied for testing differences between the study groups for quantitative parameters. The multiple comparisons (Duncan's method) tests were applied to test quantitative parameters between the study groups. All tests applied were two-tailed and a p value of 5% or less was considered statistically significant. The data were analyzed using the SAS software (SAS Institute Cary North Carolina).

Results

The mean number of cells with synchronous (SS and DD) and asynchronous (SD) patterns of replication (for the three loci) are presented in Tables 1 and 2.

A significantly higher degree of asynchronous replication was demonstrated in the cases with inversion in chromosome 2 or 9 for all the loci analyzed in leukocytes and amniocytes ($p < 0.01$). No interloci difference nor difference between the two genotypes was observed. Similar results were observed in the cases of heritable reciprocal balanced translocation in both cell types (Tables 1 and 2). The highest rate of SD pattern was seen in the genotypes of *de-novo* translocation in the amniocytes (Table 1) for all probes analyzed. This pattern was found at a significantly higher rate than for heritable balanced translocation and inversion genotypes (leukocytes and amniocytes, $p < 0.05$).

The frequencies in which SS and DD cells were observed showed a large intra-genotypic variation (Table 1).

In amniocytes, when using the p53 and RB-1 loci, the frequency of SS cells was lower in the *de-novo* translocation genotype compared with other rearranged genotypes and the control group ($p < 0.05$). It is likely that the increased frequency of SD pattern which was found in these genotypes was 'at the expense' of cells with an SS pattern due to early replication of these loci. It seems that, with all 3 loci analyzed, the frequency of DD cells was lower in the other rearranged genotypes compared with the control group ($p < 0.05$), as suggested here by the increased frequency of SD pattern due to late replication with these loci.

In the cases with familial reciprocal translocation, there was no statistical difference in the frequency of SD pattern between leukocytes (parents) and amniocytes (offsprings) for all probes analyzed. Since there are more dividing cells among amniocytes than in leukocytes, a sig-

Table 1. Replication pattern of control and study groups of the different loci analyzed (mean out of 100 analyzed cells).

Locus/group	Cells in synchronization		Cells in asynchronization
	SS pattern	DD pattern	SD pattern
Control group (N = 8)			
RB-1	67.8 ± 9.7	21.8 ± 8.8	10.5 ± 1.3
21q22	65.9 ± 2.8	23.5 ± 2.7	10.6 ± 1.1
P53	69.0 ± 7.1	20.1 ± 6.5	10.9 ± 0.9
Inversion chromosome 9 (N = 10)			
RB-1	65.5 ± 7.4	13.9 ± 5.2	20.6 ± 2.8
21q22	62.9 ± 6.1	17.1 ± 6.7	20.0 ± 1.3
P53	70.7 ± 4.5	10.7 ± 3.6	18.7 ± 1.9
Inversion chromosome 2 (N = 7)			
RB-1	68.0 ± 5.9	12.5 ± 5.0	19.5 ± 2.3
21q22	65.1 ± 6.0	15.1 ± 7.0	19.5 ± 2.6
P53	64.0 ± 10.8	16.2 ± 10.0	19.8 ± 1.8
Heritable translocation (N = 9)			
RB-1	63.1 ± 8.5	16.8 ± 6.2	20.1 ± 3.2
21q22	60.9 ± 6.6	18.1 ± 7.1	21.0 ± 1.9
P53	64.2 ± 6.5	15.6 ± 6.7	20.2 ± 2.5
***De-novo* translocation** (N = 5)			
RB-1	53.7 ± 7.8	23.0 ± 3.6	23.3 ± 4.2
21q22	63.2 ± 8.1	11.8 ± 6.0	25.0 ± 4.9
P53	49.3 ± 2.1	26.3 ± 4.2	24.3 ± 2.5

nificantly higher proportion of the DD cells (in comparison with SS cells) was found in the amniocytes.

In the leukocytes originating from patients with inversion 2, the frequency of DD pattern was significantly higher for the p53 locus as compared with the two other loci and the control group (i.e. the two alleles replicate earlier). In the leukocytes of the heritable translocation group, the DD pattern was higher with all probes analyzed (i.e. the two alleles replicate earlier).

Discussion

We have previously demonstrated a monoallelic mode of expression in some precancerous and invasive squamous carcinoma of the cervix as well as in multiple myeloma (Amiel *et al.* 1998b, 1999b). These findings suggest that the mechanism controlling the temporal order of replication is altered in cells with a genetic predisposition to cancer. In addition, young mothers of DS offspring showed the same increased unsynchronized replication pattern as older women compared with young women at the same age (Amiel *et al.* 2000).

Table 2. Replication pattern of control and study groups of the different loci analyzed in leukocytes (mean out of 100 analyzed cells).

Locus/group	Cells in synchronization		Cells in asynchronization
	SS pattern	DD pattern	SD pattern
Control group (N = 8)			
RB-1	68.9 ± 7.5	19.9 ± 7.4	11.3 ± 1.1
21q22	71.9 ± 4.9	16.6 ± 5.0	11.6 ± 2.2
P53	73.0 ± 9.0	16.1 ± 8.3	10.9 ± 1.7
Inversion chromosome 9 (N = 10)			
RB-1	59.2 ± 4.5	19.0 ± 4.8	21.8 ± 1.3
21q22	63.4 ± 8.3	15.4 ± 6.9	21.1±2.2
P53	59.1 ± 4.9	18.9 ± 4.3	22.0 ± 2.2
Inversion chromosome 2 (N = 7)			
RB-1	68.0 ± 4.4	11.6 ± 2.9	20.4 ± 1.7
21q22	67.5 ± 4.7	12.7 ± 3.6	19.8 ± 1.5
P53	58.8 ± 8.3	20.3 ± 8.2	20.8 ± 1.0
Heritable translocation (N = 9)			
RB-1	69.5 ± 8.3	10.6 ± 6.5	20.0 ± 2.1
21q22	70.1 ± 5.5	9.2 ± 3.9	20.7 ± 2.1
P53	72.3 ± 4.5	8.6 ± 3.2	19.1 ± 1.6

In this study, we evaluated replication patterns in amniocytes originating from four groups of abnormal genotypes and one group of normal controls. We used this parameter here to determine the instability or the risk of instability of the genomes.

We were able to show a higher rate of asynchronous pattern for genotypes carrying inversions 2 and 9 and for balanced heritable translocations and an even higher rate for the *de-novo* balanced translocations.

What seems to be a 'balanced' *de-novo* translocation can be unbalanced within a single or a few genes which cannot be seen cytogenetically (each observed band contains about 100 genes). Therefore, a *de-novo* translocation can contain an unbalanced genome. We have previously demonstrated that the replication pattern parameter can indicate genomic imbalance (Amiel *et al.* 1998a, 1999a, 2000).

Specific reciprocal translocations and inversions perhaps are the example of how cytogenetic changes pave the way for cancer induction and affect the severity of the disease. These non-heritable genetic abnormalities occur frequently

in hematological malignancies such as leukemia and lymphomas, where they disrupt signaling pathways that enhance cell survival (Look 1998, Rowley 1999).

It is believed that, except for the Philadelphia chromosome, the genetic changes that are observed in the various malignancies are acquired. It is not clear if this apparent cytogenetic instability is the cause or the result of the appearance of the disease (Rowley 1999). It is important to realize that aberrant activation of proto-oncogenes by chromosomal translocations are but one event in the multi-step process of carcinogenesis (Look 1998). There seem to be different mechanisms in the mono-allelic behavior of the different loci and genotypes analyzed here. In the amniocytes, the p53 and RB-1 loci are almost always early replicating ones in the *de-novo* translocation genotype compared with the control group and other rearranged genotypes. All three loci replicate late in all other rearranged genotypes except with the *de-novo* translocation. In most of the leukocytes (taken from cases with rearrangements), one or two alleles seem to replicate earlier. If allele-specific replication indeed reflects allele-specific expression, then, from a functional point of view, asynchronous replication is equivalent to loss of heterozygosity (LOH) – an event that results from the physical loss of an allele brought about either by the loss of a whole chromosome, the logs of a small DNA segment, mitotic recombination or gene conversion – known to accompany the development of cancer (Knudson 1993). In contrast to LOH, however, the assumed event of allele-specific expression that is not associated with a physical loss of an allele but with a loss of function achieved by epigenetic means, retains its reversibility and may play a pivotal role in cancer progression and regression. Alternatively, it may activate a single allele from a normally silent oncogene (Dotan *et al.* 2000).

Most of our analyzed samples were genetically balanced; however, as our data suggests, even a small balanced rearrangement (like the inversions for example) may have an effect on the mode of normal bi-allelic replication and cell cycle progression. There seems to be a similar effect of a heritable translocation genotype on the SD category both in leukocytes (parent) and amniocytes (offspring), but a different effect on cell cycle progression (the DD pattern) in amniocytes, possibly due to the presence of more dividing cells. Possibly, different mechanisms may have an effect on gene replication categories and cell cycle progression. In a previous study in CLL patients, we have demonstrated a direct correlation between the severity of the cytogenetic aberration and level of asynchrony. In a case with trisomy 12, our results showed a correlation between the rate of asynchrony and the aberrant phenotype of the cells, while such a correlation was not seen in a case with deletion of 13q14 (Amiel *et al.* 2001). In the present study, *de-novo* translocation could represent the most severe and unbalanced cytogenetic aberration.

A suggested mechanism for monoallelic expression may originate from differences in the process of genomic methylation, i.e. alterations in normal DNA methylation pathways or activation of DNA demethylation (Ehrlich 2000).

A direct correlation between the overall DNA demethylation (an epigenetic event) and the percentage of rearranged chromosomes has been shown in breast cancer cell lines (Vilain *et al.* 1999). In addition, a loss of imprinting, which is also connected to loss of methylation, has been reported in association with cancer (Jones & Gonzalgo 1997, Cui *et al.* 1998, Randhawa *et al.* 1998). Most cancer-linked DNA methylation abnormalities are probably due to derangement of normal processes for perpetuating methylation patterns, rather than to these alternative mechanisms for generating cancer-associated hypo- and hyper-methylation in the genome (Ehrlich 2000).

Another explanation for the different replication pattern in individuals carrying a structural rearrangement is that they could alter nuclear structure and thus the function of chromosomes. Usually chromosome regions organize nuclear structure and environment (address themselves in sites and condensation patterns most appropriate for their function in the particular cell). Thus, it can explain the contribution of chromosome rearrangements, even in a balanced form to the human pathologic condition (Qumsiyeh 1995, 1999).

It remains unknown whether those individuals with balanced chromosomal rearrangements are prone to tumor genesis more than the normal population but this process of tumorigenesis

may begin with changes in cell cycle regulation, which includes the duplication, replication and segregation of the genetic information (Clurman & Roberts 1998).

To conclude, our data suggest that even minor genomic rearrangements that by conventional cytogenetic techniques seem to be balanced may have an altered replication pattern, leading to genomic instability.

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