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Constitutional chromosome instability in families with patients affected by sporadic non-hereditary retinoblastoma

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Abstract: It has been suggested that chromosome instability is a constitutional factor which may be characteristic of families with an increased risk for the development of malignancies. In this study spontaneous chromosome aberrations, sister chromatid exchanges (SCEs) and chromosome sensitivity to bleomycin were analysed in cultured lymphocytes from 13 families with sporadic non-hereditary unilateral retinoblastoma patients. The significantly increased chromosome instability was detected only by the bleomycin test. Higher mean values of breaks/cell were found in all groups of relatives: patients, parents and siblings.

Key words: bleomycin test, chromosome instability, sporadic retinoblastoma.

Introduction

A large body of literature points to the possible involvement of chromosome instability in carcinogenesis (HEIM et al. 1989, WIENCKE, SPITZ 1994, TZANCHEVA, KOMITOWSKI 1997, TONCHEVA, NACHEVA 1998). It was found that for some types of tumours the affected persons and their relatives have an increased level of spontaneous chromosome breakage and rearrangements as well as elevated susceptibility to chromosome breakage induced by various mutagens. Evidence of chromosome instability exists for patients from families with diseases characterised by an increased hereditary predisposition to malignancies such as the so-called chromosome instability syndromes (MEYN 1997), Lynch syndrome (KŁADNY et al. 1996), Li-Fraumeni syndrome (BOYLE et al. 1998),

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Peutz-Jaeghers syndrome (RICHARD et al. 1994), multiple endocrine neoplasia (SCAPPATICCI et al. 1991) and other syndromes (DELHANTY et al. 1983, HEIM et al. 1989, TZANCHEVA, KOMITOWSKI 1997, TONCHEVA, NACHEVA 1998). Moreover, constitutional chromosome instability has been reported in patients with different types of sporadic neoplasias such as cancers of the breast (BARRIOS et al. 1991), lung (WU et al. 1995), colon (RICHARD et al. 1994) or head and neck (SCHANTZ et al.1990, CLOOS 1996). HSU (1983) and HSU et al. (1989) suggests also that in some families and neoplasia types, chromosomal instability may be preferentially connected with different reactions to environmental carcinogens/mutagens. In several other tumour types it is difficult to conclude that chromosome instability is associated with tumour development (HEIM et al. 1989). This is mainly due to lack of methodological consistency of the reports. In many of them the studied groups are genetically heterogeneous or small and do not include appropriate control populations. Such a situation exists also in the case of retinoblastoma.

Retinoblastoma is a malignant childhood tumour, which is familial or sporadic. All familial and sporadic bilateral tumours are hereditary (NEWSHAM et al. 1997). Around 10-20% of sporadic unilateral cases are also hereditary which has been proven by the follow-up of families and/or detection of constitutional *RB-1* gene mutations at DNA level (LOHMANN et al. 1997). There are some suggestions that genomic instability in patients with hereditary and sporadic retinoblastomas is different (KNIGHT et al. 1979). However, up to now it is difficult to conclude definitively about constitutional chromosome instability in different groups of retinoblastomas, because all of the published results were obtained by analyses of genetically heterogeneous (without precisely determined occurrence of constitutional *RB-1* gene mutations), short series of cases and without appropriate age- and sex-matched controls.

Studies of chromosome instability in retinoblastoma patients have been performed using different tests such as determination of the levels of spontaneous chromosome aberrations, sister chromatid exchanges (SCEs) and induction of chromosome aberrations by variable mutagens. Unfortunately, there are significant differences between experimental conditions applied by particular authors, which additionally reduces the reliability of the conclusions that might be drawn.

Herein, we present the results of studies on constitutional chromosome instability examined by simultaneously performed tests for spontaneous chromosome aberrations, SCEs and bleomycin sensitivity in a group of 13 families with sporadic unilateral retinoblastomas and healthy age- and sex-matched control individuals. The non-hereditary nature of the investigated cases was determined not only by pedigree and clinical data analysis, but additionally by exclusion of *RB-1* gene constitutional mutations throughout DNA sequencing and Southern blot analyses (ZAJĄCZEK et al. 1999).

Material and methods

Patients

Initially 17 families with clinically sporadic unilateral retinoblastomas were taken into consideration (ZAJĄCZEK et al. 1998). After DNA analysis four families were excluded from the studies, because constitutional de novo *RB-1* gene mutations were detected (ZAJĄCZEK et al. 1999). Thus, the investigations finally involved the members of the 13 families (subgroups: 13 patients, 25 parents: 12 fathers, 13 mothers, and 12 siblings) with children affected by non-hereditary sporadic retinoblastoma referred to the Ophthalmologic Department, Pomeranian Medical University, Szczecin.

Control experiments were performed on different healthy individuals without tumours within first degree relatives, paired-matched in terms of age, sex and smoking. In children blood samples were taken as a part of routinely performed laboratory procedures prior to the surgery because of phimosis, strabismus or hernia. Experimental conditions were approved by the Ethical Committee of our University.

In the investigated groups only five parents (3 males + 2 females among 25 persons) were recognized as smokers. The so-called smoke indexes of the smokers and of matched control persons did not differ significantly (data not shown). Both the studied and control persons were healthy and did not receive any medical treatment (excluding the above-mentioned in controls) during at least the past month.

Methods

For all experiments peripheral blood was immediately cultured after PHA stimulation for 72 hrs in RPMI supplemented with 20% FCS. Metaphases were harvested after 2 hrs colcemide (GIBCO, conc. 0.2 $\mu g\ mL^{-1}$ of culture) block.

Spontaneous chromosome aberrations

Chromosome preparations were made by the standard Giemsa staining after routinely performed hypotonic treatment and fixation (0.075 M KCl, methanol: glacial acetic acid 3 : 1, 4°C). Slides were coded and 100 consecutive metaphases were analysed under oil immersion. Only metaphases with 46 centromeres and without chromosomes overlap, pulverisation or obscuring debris were recorded.

Structural aberrations of chromosomes were recorded according to the standard classification (BUCKTON, EVANS 1973, ISCN 1985, TAWN, HOLDSWORTH 1992). Aberrations were identified as chromatid and chromosome type and gaps were excluded. Chromatid breaks, single chromatid fragments and chromosome breaks and fragments were scored as single break-point aberrations. Translocations, dicentrics and other exchange type aberrations were scored as two break-point aberrations.

In each case the percentage of the metaphases with aberrations and mean breaks per cell number were determined.

Sister Chromatid Exchanges

5-BrdU was added at a final concentration of 10 mg mL^{-1} for the last 48 h of incubation and the cultures were kept in the dark. Then the cultures were harvested, stained for 12 min. in 0.5 mg mL^{-1} solution of the Hoechst 33258 fluorochrome, exposed to bactericidal UV light lamp for 20 min. in sodium phosphate buffer (0.3 M, pH 7.0) and stained with 4% Giemsa (Gurr, Improved R 66, pH 6.8) according to PERRY and WOLFF (1974).

The slides were randomised and coded. In each case 50 consecutive, well spread second division metaphases were scored. The results were expressed as:
(1) total average number of SCEs per cell in all the scored cells;
(2) high frequency cell SCEs (HFC): SCE per cell in 10% of the cells with the highest number of SCEs in the analysed case;
(3) SCEs in cells with a basic SCE level (BLC): SCEs per cell in 90% of cells after exclusion of 10% HFC.

Proliferation of the cultures measured by the mean Proliferation Index according to PRESTON et al. (1987) did not differ essentially between the studied and control groups (data not shown).

Bleomycin sensitivity test

Chromosome sensitivity to bleomycin was measured according to HSU et al. (1989). Bleomycin (Nippon-Kayaku, Japan) was added 5 hrs before the end of the culture at a concentration of 0.03 IU mL^{-1}. Conventional harvesting and Giemsa staining were made. Reading of coded slides was done with 2 × 50 metaphases from two separate simultaneously cultured bottles. All chromatid aberrations (excluding gaps) were scored as one break-point, each chromosome break/fragment and exchange type aberration as two break-points. The frequency of break-points was expressed as breaks per cell (b/c).

Following the guidelines of HSU (1983) and HSU et al. (1989), hypersensitivity to bleomycin was recognised in individuals with values higher than mean b/c + 1 SD in controls.

Statistical analysis

Statistical analysis was performed by the Mann-Whitney *U*-test, using "Statistica 5.0" (1997) software.

Results

The results are summarised in Table 1. The mean numbers of spontaneous structural aberrations of chromosomes in 72 h cultures of peripheral blood lympho-

Table 1. Constitutional chromosome instability in studied sub-groups (patients with sporadic non-hereditary retinoblastomas and their first-degree relatives) compared to the paired-matched controls

Studied sub-groups and their controls		Age (years)	Sex	Spontaneous chromosome aberrations		Sister chromatid exchanges (SCE)			Bleomycin test (break/cell)
				break per cell	cells with aberrations (%)	total[1]	BLC[2]	HFC[3]	
Retinoblastoma patients	mean	5.84	9 F.	0.0215	1.8461	7.0300	6.4553	11.7230	0.5638
n = 13	SD	3.85	4 M.	0.0167	1.2142	1.6350	1.5576	2.3259	0.2564
Paired-matched controls	mean	5.70	9 F.	0.0269	2.3076	6.3169	5.7541	10.9769	0.2453
n = 13	SD	3.79	4 M.	0.0271	1.7974	0.7814	0.7399	1.5717	0.0701
				P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.001
Retinoblastoma patient parents	mean	31.18	13 F.	0.0194	1.7576	7.1397	6.4770	12.3424	0.4948
n = 25	SD	5.12	12 M.	0.0164	1.6208	1.3035	0.8505	2.4432	0.1475
Paired-matched controls	mean	30.74	13 F.	0.0227	1.9697	7.0994	6.3970	12.1212	0.3479
n = 25	SD	4.57	12 M.	0.0187	1.5709	0.7266	0.9137	1.3564	0.1057
				P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.001
Retinoblastoma patient siblings	mean	7.10	7 F.	0.0221	2.0000	7.6160	7.1736	12.9650	0.5610
n = 12	SD	5.60	5 M.	0.0079	0.8520	1.1242	1.5850	1.5378	0.1751
Paired-matched controls	mean	6.80	7 F.	0.0212	2.0830	6.3852	5.8503	10.6518	0.2219
n = 12	SD	6.22	5 M.	0.0086	0.9000	0.5226	0.4400	1.3572	0.0756
				P > 0.05	P > 0.05	P < 0.02	P < 0.05	P < 0.01	P < 0.001

[1] mean number of total SCEs per cell
[2] BLC – SCEs per cell in 90% of cells after exclusion of the cells with the highest number of SCEs
[3] HFC – SCEs per cell in 10% of the cells with the highest number of SCEs

cytes, expressed as the number of breaks/cell (b/c) and percentage of cells with aberrations did not differ significantly between all the studied groups and their controls.

SCE levels, expressed as the mean number of total SCEs per cell and mean number of SCEs in BLC and HFC, were generally similar when the studied groups were compared to controls, except for slightly increased SCEs (total, BLC and HFC) levels in siblings of the retinoblastoma patients.

Results of bleomycin tests showed an increased susceptibility in all the studied groups.

Mean numbers of b/c in particular groups and their controls were as follows: children with retinoblastoma 0.5638 ± 0.2564 vs. 0.2453 ± 0.0701 in controls, parents 0.4948 ± 0.1475 vs. 0.3479 ± 0.1037 in controls, and siblings 0.5610 ± 0.1750 vs. 0.2219 ± 0.0756 in controls.

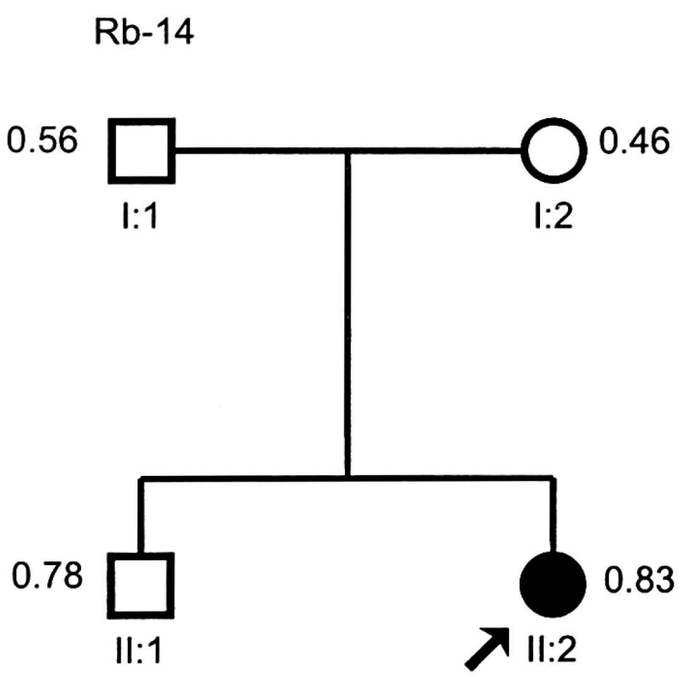

Figure 1. An example of the pedigree of the family in which all relatives are hypersensitive to bleomycin

The level of b/c above which hypersensitivity to bleomycin could be recognised under our experimental conditions was 0.3701 for children (mean b/c value + 1 SD in control group = 0.2453 + 0.0701) and 0.4536 for adults (0.3479 + 0.1057).

The percentage of hypersensitive individuals in particular groups was as follows: retinoblastoma patients ~ 92% (12/13) vs. ~ 15% in controls (2/13), parents of the retinoblastoma patients ~ 68% (17/25) vs. ~ 12% (3/25) in controls, and patients' siblings ~ 73% (10/12) vs. ~ 23% (3/12) in controls.

In 12 out of 13 families it was possible to study parents and at least one child. In six of these families all relatives were hypersensitive to bleomycin (see example in Figure 1). In five families only one person was not showing hypersensitivity to bleomycin. In one family nobody was hypersensitive.

Discussion

In the studied groups we did not find any increased levels of spontaneous chromosome aberrations and SCEs in cultures of peripheral blood of patients with sporadic unilateral retinoblastomas, their parents and siblings. However, results of our studies suggest that in the examined families there is an increased chromosome instability, as measured by the bleomycin sensitivity test in each investigated group (patients, parents, siblings) in comparison with paired matched control groups.

Spontaneous chromosome aberrations in patients with sporadic unilateral retinoblastomas were studied by CZEIZEL et al. (1974), HERAS, COCO (1987) and de NUNEZ et al. (1984), who studied 9, 9 and 10 cases respectively. Additionally three patients with unilateral retinoblastomas were examined by KNIGHT et al. (1979), although she did not report their cancer family history. CZEIZEL et al. (1974) and de NUNEZ et al. (1984) found an increased level of spontaneous chromosome aberrations. Similarly to our study KNIGHT et al. (1979) and HERAS, COCO (1987) did not detect this type of changes.

Analysing the results published by CZEIZEL et al. (1974) we found that his scoring system was different from the currently accepted rules – he was scoring gaps and only 12-55 metaphases/case. Differences between our results and those obtained by de NUNEZ et al. (1984) may be additionally due to a very low number of individuals with aberrations detected by those researchers.

Many authors have reported high SCE frequencies in patients with various types of neoplasias (WIENCKE, SPITZ 1994, DHILLON et al. 1996). Up to now the spontaneous SCE level in patients with sporadic unilateral retinoblastomas was evaluated only in three cases reported by ABRAMOVSKY-KAPLAN and JONES (1984). These authors did not find increased SCE level in cancer patients.

Current evidence indicates that generalised – that is detected with any assay – chromosome instability is not a common feature of cancer susceptibility (HEIM et al. 1989, WIENCKE, SPITZ 1994). Thus, it is not surprising that in our patients and their relatives we did not detect elevated levels of spontaneous chromosome aberrations and spontaneous SCE (except for a very low level of alterations in siblings), but we were able to show an increased sensitivity to bleomycin.

For the investigation of latent or "hidden" chromosome instability, mutagen sensitivity assays can be used (HSU 1983, WIENCKE, SPITZ 1994, TZANCHEVA, KOMITOWSKI 1997, TONCHEVA, NACHEVA 1998). There are several cytogenetic tests of in vitro mutagen sensitivity. HSU (1989) has developed a relatively simple

assay in which the chromosomal alterations (mostly simple chromatid and chromosome breaks) are induced in vitro by exposure of cells at G_2 to the radiomimetic drug bleomycin. Using this test Hsu and his group were able to show an increased bleomycin sensitivity in patients with thyroid, colorectal, lung, head and neck and other cancers (CHERRY, HSU 1983, SCHANTZ et al. 1990). Bleomycin causes DNA damage by hydroxyl radicals induced via Fenton's reaction and, possibly, directly by its activated form. Due to bleomycin activity apurinic/apyrimidinic sites and single/double DNA strand breaks are formed (UMEZAWA et al. 1984, STUBBE, KOZARICH 1987). The chromosome breakage, which is visible as an endpoint in the bleomycin sensitivity assay, represents a fraction of the total mutation output.

Bleomycin induced chromosome breakage in lymphocytes of only two patients with sporadic unilateral retinoblastomas was examined by CHAUM et al. (1984). These authors did not detect any increased number of aberrations, but they used different experimental conditions than those used in our study, with an around 10 times lower concentration of bleomycin and the time of incubation with the mutagen shortened from 5 to 4 h.

It seems that bleomycin-induced b/c level can be dependent on experimental conditions and population factors. It is difficult to explain why mean b/c values in various laboratories studying control groups of adults with the use of the Hsu test are different. In studies performed by HSU et al. (1989) mean b/c for adults was ~ 0.6, whereas in our control group for adults it was ~ 0.35. It cannot be excluded that the lower b/c values in our series are caused by a younger age of adults in our controls.

It has been suggested that bleomycin sensitivity is a genetic constitutional feature which might be inherited as an autosomal dominant trait, although investigations of families were up to now limited to medullary thyroid cancer and dyskeratosis congenita (CHERRY, HSU 1983, NING et al. 1992).

Results of our studies suggest that an increased bleomycin sensitivity is a characteristic feature of families with sporadic unilateral retinoblastoma. It may be related to hereditary and/or epigenetic factors. Further investigations are needed in order to identify environmental factors which could influence responses to bleomycin. It has been shown that smoking does not belong to this group (HSU et al. 1989, CLOOS 1996). Interestingly we observed in vitro normalisation of increased bleomycin sensitivity by sodium selenite (KŁADNY et al. 1996).

Bleomycin is a radiomimetic. Thus, it is surprising that contrary to our results obtained with bleomycin, HERAS, COCO (1987) did not detect any increased level of chromosome aberrations after X-ray irradiation of lymphocytes from patients with unilateral sporadic retinoblastomas. In our opinion the most reasonable explanation of this discrepancy is that X-ray exposure was done at the G_0 and in our investigations bleomycin was added at the G_2 phase. Thus cells irradiated by HERAS and COCO had a better opportunity to repair the induced damages before mitosis.

In addition to the above studies analyses of mutagen-induced aberrations in patients with sporadic unilateral retinoblastoma were performed by DER-SARKISSIAN et al. (1982) and ABRAMOVSKY-KAPLAN, JONES (1984). SCE levels were not increased after exposure to X-rays in three cases studied by ABRAMOVSKY-KAPLAN and JONES (1984) and Mitomycin C in four cases reported by DER-SARKISSIAN et al. (1982).

Other non-cytogenetic assays measuring mutagen sensitivity were not performed to date in any series of patients with sporadic unilateral retinoblastomas.

In summary, results of our studies indicate that particular types of genomic instability, such as those, which are expressed by an increased chromosome sensitivity to bleomycin, can play an essential role in the development of sporadic retinoblastomas.

Further investigations of a higher number of cases are needed in order to verify our results and then to elucidate the molecular mechanisms of the observed phenomenon. Recognition of these mechanisms might have important consequences for chemoprevention, early diagnosis and treatment of retinoblastoma.

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