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Analysis of cytogenetic germline changes in Polish patients with retinoblastoma

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Purpose: Retinoblastoma, the most common eye tumor in children, can occur in hereditary or nonhereditary forms. In the hereditary form, various germline alterations, single nucleotide (SNVs) or copy number variations (CNVs) in the *RB1* gene can be detected in patients. The aim of this study was to analyze cytogenetic germline changes in Polish patients with retinoblastoma and to assess whether cytogenetic techniques still have their application in diagnostics for retinoblastoma patients in the era of next-generation sequencing (NGS).

Methods: The results of genetic testing for germline mutations in patients with retinoblastoma performed between 2013 and 2023 were analyzed. In patients with cytogenetic alterations (CNV group, n = 19), the form of disease, age of onset, the first symptom, family history, and the type and extent of cytogenetic changes were verified. Comparative analyses were conducted between the CNV and SNV (n = 83) groups as well as the group of patients with normal genetic test results (n = 126).

Results: Cytogenetic changes were detected in 19 probands. These included: 16 deletions (10 partial and 6 whole gene deletions), 2 duplications, and 1 balanced translocation. Partial gene deletions included from 1 to 16 exons. In the CNV group, bilateral involvement predominated, with strabismus being the most common initial symptom. The mean age of onset was 16.9 months (median = 11 months; IQR, 8–22 months) and was lower in patients with bilateral involvement and partial gene deletions. Statistically significant differences compared to patients with normal genetic test results were observed in terms of laterality, the age of onset, initial symptom, and the family history of retinoblastoma. No such differences were found between the CNV and SNV groups.

Conclusions: Cytogenetic changes constitute a significant part of germline alterations in patients with retinoblastoma. Cytogenetic techniques should still be considered in diagnostic protocols, especially in patients with bilateral involvement and/or positive family history, as well as in parents of patients with CNV.

Retinoblastoma (OMIM 180200; ORPHA, 790) is a retinal tumor that occurs in early childhood, normally before the age of 5. It is the most common eye tumor in children [1,2]. The initial symptoms typically include leukocoria ("white pupil") or strabismus, less commonly redness, pain, or enlargement of the eye. The disease can manifest as unilateral (~60%) or bilateral (~40%). Tumors can present as either unifocal or multifocal [3]. In a small percentage of cases (~3.5% of patients with hereditary retinoblastoma), retinal tumors are accompanied by an intracranial midline primitive neuroectodermal tumor (usually in a pineal gland). This is known as the trilateral form of retinoblastoma [4].

The worldwide incidence of retinoblastoma is estimated to be 1 in 15,000 to 20,000 live births [5-7]. In Poland, the calculated incidence of retinoblastoma was 4.89 per 100,000 live births during the period 2010–2014, corresponding to an incidence of 1 per 20,561 live births [8].

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Retinoblastoma is caused by biallelic pathogenic variants in the *RB1* tumor suppressor gene, located on chromosome 13 (locus 13q14.2). In the non-hereditary form, both alterations are somatic, whereas in the hereditary form, one of the alterations is germline and it is inherited as an autosomal dominant trait. The majority of alterations (80%–84%) are single nucleotide variants (SNVs), while the remaining portion consists of copy number variations (CNVs) [3]. The mutation detection rates are higher in the bilateral form (16.6%–100%) than in the unilateral form (9.5%–56.3%) and largely depend on the diagnostic strategy and methods employed [9]. In rare cases, retinoblastoma may result from somatic amplification of the *MYCN* oncogene [10,11].

Carriers of a germline pathogenic variant in the *RB1* gene are not only at risk of developing retinoblastoma in childhood but also other tumors (e.g., osteosarcoma, soft tissue sarcoma, melanoma) later in life [12,13]. Conducting genetic testing is crucial for genetic counseling, assessing the risk of retinoblastoma in subsequent children, and planning appropriate care for carriers of germinal mutations in the *RB1* gene.

The aim of the study was to analyze changes detected using cytogenetic techniques (classical karyotyping, fluorescence in situ hybridization (FISH), multiplex ligation-dependent probe amplification [MLPA], array comparative genomic hybridization [array CGH]) in Polish patients with retinoblastoma and assess the genotype-phenotype correlation in these patients. Furthermore, we wondered whether, in the era of next-generation sequencing (NGS) technology, which now also allows for the detection of CNVs, cytogenetic techniques still retain their utility in diagnosing patients with retinoblastoma.

METHODS

Data for the study were sourced from the database of patients diagnosed in the Children's Memorial Health Institute genetic counseling clinic due to retinoblastoma. Within this database, we searched for patients with changes involving the *RB1* gene detected using cytogenetic techniques, including classical karyotyping, FISH, MLPA, and array CGH. Further analysis was narrowed down to the years 2013–2023, as since 2013, we routinely began employing the MLPA technique in these patients.

For patients with abnormal cytogenetic test results, we analyzed the form of disease (unilateral, bilateral), age of onset, the first symptom, family history, and the type and extent of cytogenetic changes. For comparative analyses, we also gathered from the above-mentioned database a group of patients with SNVs as well as a group of patients without germline mutations in the *RB1* gene. We sought differences between the groups regarding the laterality, age at onset, first manifestation and family history of retinoblastoma.

Classical karyotyping: Karyotype studies were performed from standard cultured peripheral blood, followed by GTG-banding technique. For each patient, 550-band resolution metaphase chromosomes were analyzed using Cytovision Karyotyping software version 7.4 (Leica Biosystems).

Fluorescence in situ hybridization: Whole chromosome painting probes were hybridized to fixed metaphase chromosomes according to the manufacturer's instructions (CytoCell, Cambridge, UK). Slides were viewed on a Zeiss Axioscop2 fluorescence microscope, and images were captured and analyzed using Cytovision Karyotyping software version 7.4 (Leica Biosystems).

Multiplex ligation-dependent probe amplification: MLPA reaction was performed with DNA extracted from peripheral blood using the SALSA MLPA KIT P047 RB1 (MRC Holland, Amsterdam, The Netherlands). Denaturation, hybridization to probes, ligation, and amplification were performed according to the manufacturer's recommendations. DNA samples with known aberration were included as a control in each reaction's run. The GeneMarker (Soft Genetics, LLC, State College, PA) and Coffalyser.Net (MRC Holland, Amsterdam, The Netherlands) softwares were used for data analysis. Peak ranges 0.7 to 1.3 for loss and gain detection were made.

Array CGH: The whole genome array CGH procedure was performed following the manufacturer's instructions (SurePrint G3 ISCA V2 CGH Microarray Kit; Agilent Technologies, Santa Clara, CA). The 8×60K slides were scanned on a NimbleGen 200 Microarray Scanner (Roche Nimblegen, Madison, WI). Feature extraction and data analysis were performed with Agilent CytoGenomics 5.2.1.4 software (Agilent Technologies, Santa Clara, CA) using default analysis settings. The array CGH results were analyzed with the University of California, Santa Cruz, hg19 assembly.

Assessment of all detected CNVs was performed in accordance with the American College of Medical Genetics and Genomics guidelines. Statistical analysis was performed using Stata 18.0 (StataCorp LLC, College Station, TX). The Mann-Whitney *U* test was conducted to compare the age of onset in different groups. Chi-square analysis was used to examine differences in disease form (unilateral or bilateral), first manifestation (strabismus or leukocoria), and familial history of retinoblastoma (positive or negative); p values < 0.05 were considered statistically significant.

Written informed consent for genetic testing was obtained from each patient or their legal representative before the testing. Consent was also obtained for the anonymous use of results (in connection with clinical data provided in the referral for genetic testing) for scientific purposes.

RESULTS

We identified 19 probands (9 males and 10 females; CNV group) and 7 family members (6 unaffected at the time of genetic testing, 1 with a history of retinoblastoma in childhood) with cytogenetic changes involving *RB1* gene. The mean follow-up period from first to last visit in our center was 101.8 months (median, 116.5; IQR, 34–156 months). The family history of retinoblastoma was positive in 5 probands. The clinical characteristics of patients are presented in Table 1.

In 12 (63.2%) probands, the disease manifested bilaterally. All these patients had a deletion of the entire or part of the *RB1* gene (n = 5 and n = 7, respectively). There was no difference in laterality between patients with partial and

Table 1. Patients characteristics.

ID	Sex	AO (months)	RB phenotype	First sign	Other medical findings	FH	Cytogenetic findings
P1	M	10	bilateral	eye clouding	ASD, obesity	neg.	PGD
P2	F	19	unil., right	strabismus	multiple nevi, thyroid cyst	pos.	balanced translocation
P3	M	42	unil., left	leukocoria	ASD, obesity, dysmorphia	neg.	WGD
P4	F	12	unil., right	strabismus		pos.	duplication
P5	F	22	unil., left	strabismus		neg.	PGD
P6	F	9	bilateral	strabismus		pos.	WGD
P7	F	4	bilateral	leukocoria		neg.	PGD
P8	M	23	bilateral	strabismus	macrocephaly, dysmorphia, pineal glial cyst	neg.	WGD
P9	M	ND	unil., right	ND		neg.	duplication
P10	F	10	bilateral	strabismus		neg.	PGD
P11	M	20	unil., left	strabismus	rhabdoid kidney tumor, DD	neg.	PGD, translocation
P12	M	5	bilateral	nystagmus		neg.	PGD
P13	M	9	bilateral	strabismus	hisiocytosis	neg.	PGD
P14	F	0	bilateral	screening		pos.	PGD
P15	M	2	bilateral	leukocoria		pos.	WGD
P16	F	20	bilateral	red eye	cleft palate	neg.	WGD
P17	F	55	bilateral	strabismus		neg.	WGD
P18	M	8	bilateral	eye asymm.		neg.	PGD
P19	F	35	unil., left	leukocoria	heterochromia	neg.	PGD

AO – age of onset; ASD – autism spectrum disorder; asymm.- asymmetry; DD – developmental delay; F – female, FH – family history; ID – patient's identification; M – male; neg. – negative; ND – no data; P – patient; PGD – partial gene deletion; pos. – positive; unil. – unilateral; RB – retinoblastoma; WGD – whole gene deletion

whole gene deletion ($p = 0.55$), in both of these subgroups, the bilateral form predominated (70% and 83.3%, respectively).

In total, we identified 16 probands with a deletion (6 whole gene deletions, 10 partial deletions, including 1 as a result of translocation), 2 with a duplication, and 1 with a balanced translocation between chromosome 13 and 16. Partial gene deletions included from 1 to 16 exons. Detected CNVs are illustrated in Figure 1, and detailed data on cytogenetic testing results are presented in Appendix 1.

Detailed data regarding the exact age of onset were available for 18 probands. One proband was an adult at the time of genetic testing and did not know the age of onset. The average age of onset was 16.9 months (median, 11 months; IQR, 8–22 months) and was lower in patients with bilateral involvement compared to those with unilateral involvement (12.9 and 25 months, respectively; $p = 0.03$).

The most common initial symptom was strabismus (n = 9), followed by leukocoria (n = 4). One patient was diagnosed during screening due to a family history of the condition. Other initial manifestations included clouding of the eye, poor eye contact, nystagmus, eye redness and swelling, as well as eye asymmetry.

Patients from the CNV group were compared with SNV patients (n = 83; male = 50.6%) and patients with normal genetic test results (n = 126; male = 55.6%). Comparing the CNV group to the SNV group, we did not find statistically significant differences regarding the disease type (unilateral versus bilateral; $p = 0.098$) or age of onset ($p = 0.073$). However, in comparison to the group with normal genetic test results, bilateral disease manifestation occurred significantly more often in the CNV group ($p < 0.001$), and patients in this group were significantly younger at the time of onset ($p = 0.039$). Furthermore, when comparing the CNV group to the group with normal genetic test results, we found differences regarding the initial symptom (in the CNV group, strabismus was the more frequent symptom; $p=0.016$) and family history (in the CNV group, family history was significantly more often positive; $p < 0.001$). There were no such differences

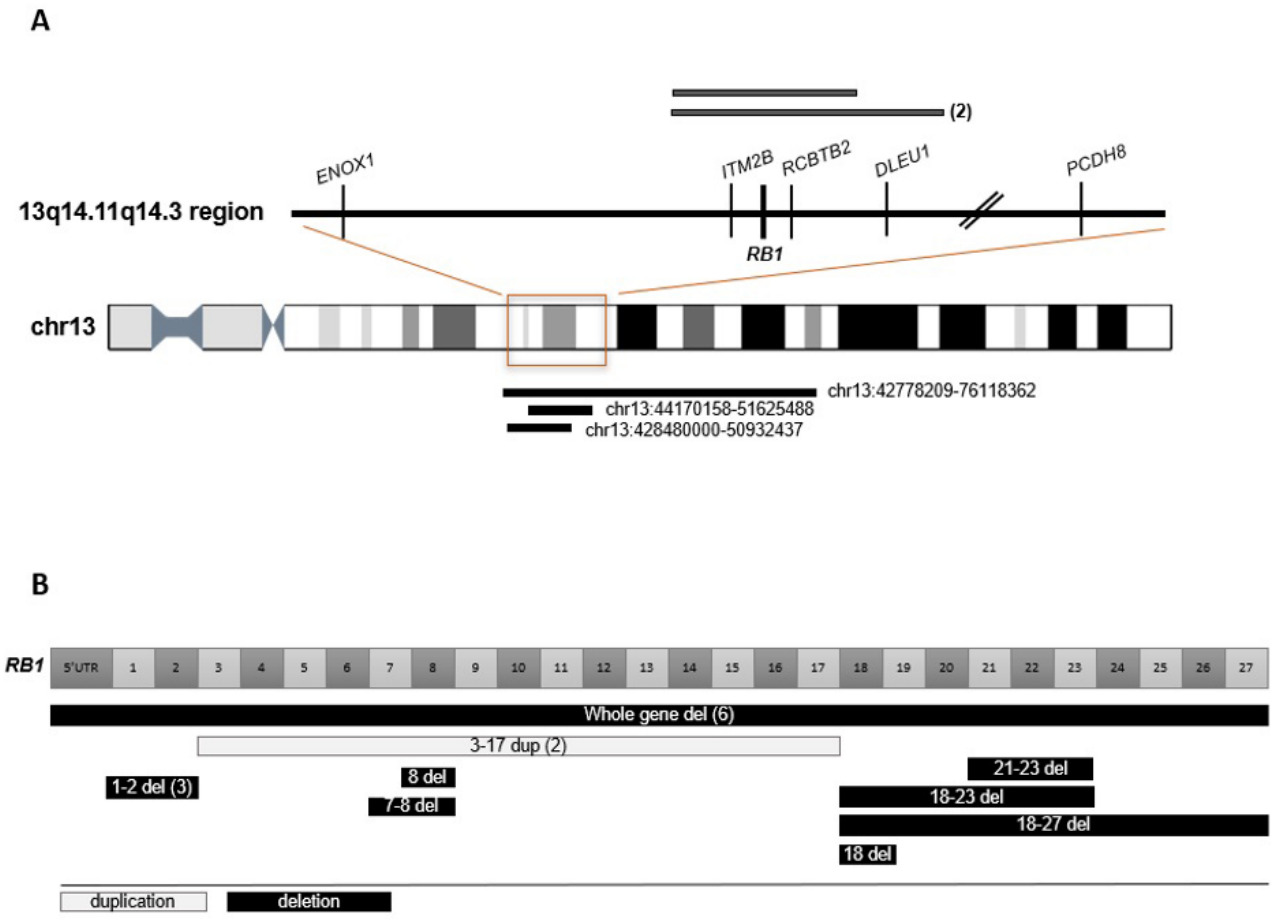

Figure 1. Graphical representation of the extent of *RB1* CNV identified in patients with retinoblastoma. The number of patients with the indicated change is given in brackets. **A**: Gross deletions and gains encompassing *RB1*. Horizontal black bars represent the extent of the deletion identified in array CGH analysis, and gray bars represent the deletions within the 13q14.2 region identified by MLPA. **B**: Intragenic *RB1* deletions and gains at the exonic level.

between the CNV and SNV groups (*p* values 0.154 and 0.328, respectively).

DISCUSSION

According to the literature, the frequency of copy number variations (CNVs) in patients with retinoblastoma ranges between 8.1% and 24.8% [14-20]. In our cohort, we obtained a similar result by detecting cytogenetic changes in 19 out of 228 patients (8.3%). Cytogenetic changes constituted 18.6% of all alterations detected in examined patients (8.2%–22.8% in other cohorts from literature; Table 2).

The most commonly detected alterations were partial *RB1* deletions (n = 10), ranging from 1 to 16 exons. According to literature data, patients with whole gene deletions larger than ~1 Mb exhibit a milder phenotype (including more frequent unilateral presentation) and reduced penetrance [21]. In our cohort, both in patients with partial and whole gene deletions, the bilateral form predominated (70% and 83.3%, respectively). Similarly to Mitter et al. [21], we did not find differences in the age of onset between these subgroups.

Duplications are a rare form of CNV in patients with retinoblastoma. Such alterations were noted in only isolated cases in the analyzed literature [14,15,17-19], Table 2. We identified two unrelated families with a duplication spanning exons 3 to 17. In both families, the disease presented unilaterally, and the duplication was also detected in other family members (both affected and healthy). The evaluation of the pathogenicity of the identified duplications requires further studies to determine their molecular characterization and function. Due to the rarity of this CNV form in retinoblastoma cases, we are going to describe these two families in a separate paper.

It is worth emphasizing that, in rare cases, deletions and duplications involving the *RB1* gene may occur as a result of balanced translocations in a parent. Therefore, classical cytogenetic studies on the parents of patients with CNV are recommended to accurately determine the risk of recurrence of retinoblastoma in siblings [22,23].

In our center, during the analyzed period, classical karyotyping was performed on 18 patients with retinoblastoma. Two deletions encompassing the *RB1* gene were detected: whole gene deletion in patient 3 and partial gene deletion due to translocation in patient 11. Additionally, a translocation between chromosomes 13 and 16 was detected in family nr 2 (a daughter with unilateral retinoblastoma and a mother with bilateral manifestation). Deletions and duplications at breakpoints were ruled out using MLPA and array CGH. *RB1* gene sequencing did not reveal the presence of SNV. Further mapping of breakpoints is needed to establish whether the presence of translocation in two symptomatic individuals within the family has a significant role in the pathogenesis of the disease.

Literature reports on balanced translocations in patients with retinoblastoma are scarce, and most of them were published over 20 years ago [24-30]. These translocations involved both autosomes (chromosomes 2 [24], 4 [27] or 5 [30]) and the X chromosome [25,26,28,29]. Among more recent publications, we found a report of a girl with unilateral retinoblastoma and a suprasellar primitive neuroectodermal tumor, in whom a balanced translocation between chromosomes 11 and 13 was identified [31].

In 2019, Tsutsumi et al. described a female patient with retinoblastoma and severe intellectual disability carrying an X;13 balanced translocation without rearrangement in the *RB1* gene [32]. Based on the results of the conducted analyses, the authors concluded that the retinoblastoma in the patient was associated with the inactivation of the *RB1* gene, while the remaining symptoms stemmed from functional disomy of Xq28. Such a mechanism (functional monosomy of 13q14 due to the spreading of inactivation of the translocated X chromosome segment) has been previously discussed in the literature [25,26,28].

In 2021, Davies et al., using whole genome sequencing techniques for retinoblastoma tumors testing, demonstrated that damage to the *RB1* gene can occur as a result of balanced rearrangements [10]. None of the analyzed publications from Table 2 considered balanced translocations as a predisposing change for the occurrence of retinoblastoma.

Table 2. Copy number variations (CNVs) in RB patients from literature and in our cohort.

Various	Akdeniz 2023 [14]	Albrecht 2005 [15]	Kugalingam 2023 [17]	Lan 2020 [18]	Mendonça 2022 [19]	Taylor 2007 [20]	Our cohort
Patients information							
Pts tested for CNV	136	129	31	117	159	165	228
Bil. / trilat.	47 / 3	57	6	-	39	102	86
Unilateral	84	72	25	-	117	21	142
CNV statistics							
All CNV	13	32	4	12	19	19	19*
CNV in affected (%)	12/136 (8.8)	32/129 (24.8)	4/31 (12.9)	12/117 (10.3)	19/159 (11.9)	18/128 (14.1)	19*/228 (8.3)
CNV / all alterations (%)	13/57 (22.8)	-	4/49 (8.2)	12/75 (16.0)	-	19/165 (11.5)	19*/102 (18.6)
Type of CNV							
WGD (%)	5 (38.5)	14 (43.8)	1 (25)	5 (41.7)	10 (52.6)	4 (21.1)	6 (31.6)
PGD (%)	7 (53.8)	17 (53.1)	2 (50)	6 (50)	8 (42.1)	15 (78.9)	10 (52.6)
Duplicat. (%)	1 (7.7) ex 4–17	1 (3.1) ex 1–2	1 (25) ex 27	1 (8.3) ex 3–5	1 (5.3) ex 12–17	-	2 (10.5) ex 3–17
Laterality in CNV patients							
Unilateral	5	6	-	2	5	1	7
Bil. / trilat.	6 / 1	26	-	10	13 / 1	15	12

Bil. – bilateral; CNV – copy number variation; duplicat. – duplication; PGD – partial gene deletion; pts – patients; RB – retinoblastoma; trilat. – trilateral; WGD – whole gene deletion

Our work has both strengths and weaknesses. A strong aspect of this study is the large number of retinoblastoma patients included for preliminary searches. Children's Memorial Health Institute, as a leading center in the country, provides care for patients from all provinces. All cytogenetic analyses were conducted at our center. We emphasized the need to take into account the possibility of balanced translocations in retinoblastoma patients, which the MLPA technique or targeted NGS panel cannot detect. The limitations of the study are related to its retrospective nature (missing data, lost to follow-up). In patients where Sanger sequencing was performed, mosaic variants may have gone undetected, potentially leading to misclassification into the group with normal genetic test results instead of the SNV group. In this group of patients, noncoding regions near or within *RB1* may be an additional reason for misdiagnosis, as they will not be detected using study techniques. The small sample size of the study group can also be considered a limitation of the work, but it is important to remember that retinoblastoma is a rare disease, and cytogenetic changes constitute only part of germline alterations detected in patients with retinoblastoma. In our study, only blood samples were tested, which is a limitation for somatic constitutional mosaicism detection. Ectodermally derived tissues, such as a skin fibroblast, buccal or retinal tissue itself, would be a better source for testing for tissue-limited mosaicism. However, these are not standardly collected for *RB1* tests in our center. Genetic testing on tumor tissue was not conducted in our patients.

Prospects for the future: the continuous development of diagnostic techniques gives hope that, for patients with retinoblastoma as well, diagnostics will be conducted with greater sensitivity and precision. Thanks to techniques such as long-range nanopore-based testing, it will be possible to detect SNVs, CNVs, and all types of SVs (e.g., translocations, inversions, insertions, noncoding variants), as well as mosaicism at low variant allele frequencies (VAF), with detailed phasing of variants and precise identification of breakpoints. RNA sequencing of *RB1*-expressing tissue should be considered for future studies to identify functional changes in the gene leading to genetic diagnosis. For several years, research has been underway on the use of aqueous humor liquid biopsy to obtain material for diagnostic and prognostic studies in patients with retinoblastoma, and the results have been promising [33].

Conclusions: The results of our study confirmed that cytogenetic changes constitute a significant part of the alterations detected in patients with retinoblastoma. Although NGS currently enables the detection of CNVs, relying solely on this technique (gene-targeted panels) may result in balanced translocations not being detected. Therefore, classical cytogenetic techniques should still be employed, especially in patients with bilateral retinoblastoma and/or a family history of the disease. Moreover, karyotyping is recommended for the detection of balanced translocations in the parents of patients with CNV.

APPENDIX 1. SUPPLEMENTARY TABLE 1. DETAILED DATA ON CYTOGENETIC TESTING RESULTS

To access the data, click or select the words "Appendix 1."

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