Retinoblastoma

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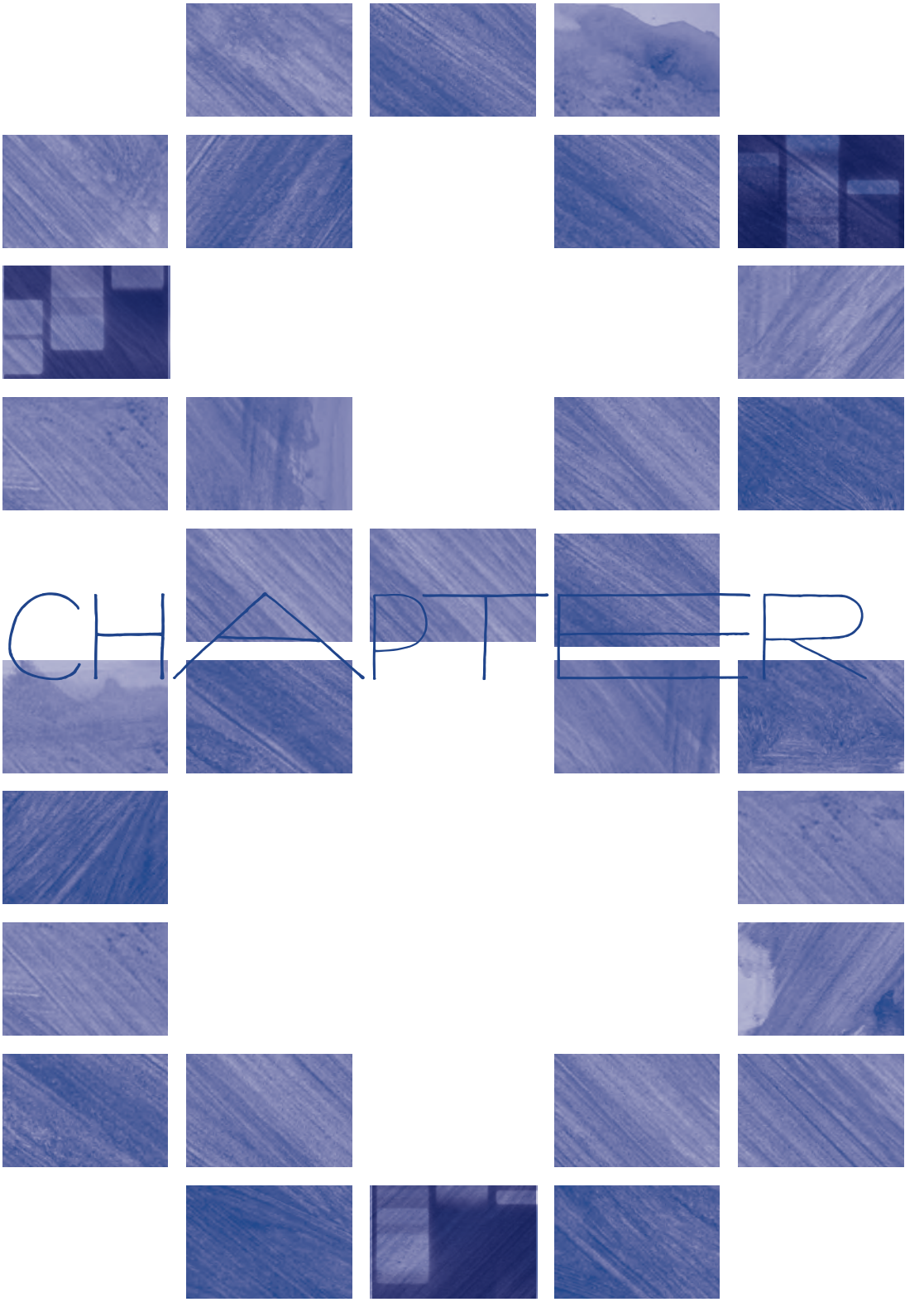
CHAPTER

IVF AND RETINOBLASTOMA REVISITED

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ABSTRACT

Objective

To evaluate the suggested association between in vitro fertilization (IVF), retinoblastoma and tumor methylation characteristics.

Design

Laboratory analysis.

Setting

National Retinoblastoma Center in the Netherlands.

Patients

Retinoblastoma tumors from seven children conceived by IVF or ICSI.

Main Outcome measures

Frozen material from retinoblastoma tumors was tested for mutations in the *RB1* gene and for methylation status of the *RB1* promoter.

Results

For all tumors two causative *RB1* mutations were found. None of the tumors showed hypermethylation of the *RB1* promoter.

Conclusions

Examination of retinoblastoma tumors of seven children conceived by IVF or ICSI did not show hypermethylation of the *RB1* promoter. This demonstrates that an association between IVF or ICSI and retinoblastoma through this epigenetic mechanism is unlikely.

INTRODUCTION

Retinoblastoma is a malignant tumor arising in the developing retina, occurring when both alleles of the tumor suppressor gene *RB1* are lost as an initial step.[1,2] Forty percent of cases are attributed to a germline mutation in the *RB1* gene, followed by a somatic mutation. In 60% of cases retinoblastoma is caused by two somatic mutations in the *RB1* gene. In around 13% of mainly unilateral cases[3,4] one of the inactivating events is an epigenetic mechanism: hypermethylation of the CpG island in the *RB1* promoter region.

Genomic imprinting is an epigenetic process in which only one allele of a gene is functioning while the other allele is silenced, based on parent-of-origin. DNA methylation is one of the major mechanisms in imprinting. Since 2001 there has been concern that in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) might lead to an increasing incidence of imprinting disorders in children conceived by IVF/ICSI. Anteby et al. reported on retinoblastoma in a child conceived by IVF.[5] Since then we and others have reported more cases.[6-10] Two IVF-register based studies did not find an increased incidence of retinoblastoma after IVF, although these studies may have lacked enough power to detect an increased risk.[11,12] More recently, a French group found an increased incidence of retinoblastoma in children conceived by IVF or ICSI.[13] Their cohort of 15,162 children conceived by IVF/ICSI contained 5 retinoblastoma cases, which was 4.5 times higher than expected.

Reports have suggested an association between IVF/ICSI and imprinting disorders, especially Beckwith-Wiedemann syndrome[14,15] and Angelman syndrome.[16] As the expression of retinoblastoma in childhood is influenced by epigenetics, imprinting has also been proposed as the link between IVF/ICSI and a possible increased risk for retinoblastoma.[8,17] In a review of imprinting disorders and assisted reproductive technology, Manipalviratn et al.[18] stated that the methylation status of the tumors of children with retinoblastoma born after IVF was unknown. Because studies on the association of IVF/ICSI and retinoblastoma are inconclusive, it is important to get more insight into a possible link between imprinting and retinoblastoma tumors. Therefore, we tested in the present study the retinoblastoma tissues of the children conceived by IVF/ICSI from our cohort of retinoblastoma patients for mutations in the *RB1* gene.

MATERIALS AND METHODS

Our cohort of retinoblastoma patients has been described elsewhere.[9] Since our 2009 study, two more children, one conceived by IVF and the other by ICSI, have been treated for unilateral, non-familial retinoblastoma. One child was treated with intra-arterial chemotherapy and enucleation of the affected eye had not been necessary, therefore the tumor was not available for DNA testing. The tumor of

the second child treated for retinoblastoma after 2009 was included in the current study. All parents gave full consent for participation in this study, and the study was conducted in accordance with the principles of the Helsinki declaration.
In DNA from frozen tumor material, if available, the *RB1* gene was scanned for mutations by direct sequencing or Denaturing Gradient Gel Electrophoresis (DGGE) analysis. LOH analysis was done with two intragenic markers. Promoter hypermethylation was studied by methylation specific Multiplex Ligation dependent Probe Amplification (MLPA) (MRC Holland, kit ME002). Subsequently, DNA from leucocytes was studied for the mutations detected in the tumor. If no tumor material was available, mutation scanning was performed in DNA from leucocytes. In these cases the *RB1* gene was also studied for large deletions and duplications by MLPA (MRC Holland, kit P047).

RESULTS

The total group of patients conceived by IVF/ICSI and born between 1997 and 2010 who developed retinoblastoma consists of 9 patients. None of the patients had a positive family history. Six patients developed unilateral retinoblastoma; three were treated for bilateral retinoblastoma. Tumor material of two of the unilaterally affected patients was not available for further analysis: as stated above, the eye of one patient was not enucleated and tumor material of the other patient had not been frozen, making DNA-analysis impossible. Analysis was thus done on 7 tumors.
Results are shown in **Table 1**. None of the tumors showed hypermethylation of the *RB1* promoter. In the tumor of two unilaterally affected patients, the first mutation (M1) was a nonsense mutation and the second mutation (M2) showed loss of the wild type allele and therefore loss of heterozygosity (LOH). In the other two unilaterally affected patients both a nonsense mutation and a frameshift mutation were detected. The tumors of the three bilaterally affected patients with a de novo germline mutation, showed LOH as M2 for two tumors and a nonsense mutation as M2 for the third tumor.

Table 1. *RB1* mutation detection in tumors of patients with retinoblastoma, conceived by IVF/ICSI

Patient no.	Gender	Year of Birth	Age at diagnosis (months)	Affected eye(s)	IVF/ ICSI	Germline *RB1* mutation	Tumor *RB1* mutation allele 1	Tumor *RB1* mutation allele 2
1	Female	1997	38	Both	IVF	g.59789A>G/p.Asp286Gly	g.59789A>G/p.Asp286Gly	LOH
2	Male	1999	15	Left	IVF	None	g.76430C>T/p.Arg445X	g.150048_150051dupAACT
3	Female	1998	34	Right	IVF	None	n.p.	n.p.
4	Male	2001	8.5	Both	IVF	g.5904_59708del1AACAG (in mosaic state)	g.59704_59708del1AACAG	LOH
5	Female	1999	32	Left	ICSI	None	g.64348C>T/p.Arg320X	LOH
6	Female	2005	23	Right	ICSI	None	g.160787A>T/p.Lys722X	LOH
7	Female	2005	11	Both	IVF	g.2162C>T/p.Gln35X	g.2162C>T/p.Gln35X	g.59695C>T/p.Arg255X
8	Male	2003	70	Right	IVF	None	g.5457dupT	g.162237C>T/p.Arg787X
9	Male	2010	9	Right	ICSI	None	n.p.	n.p.

ICSI = intracytoplasmic sperm injection, IVF = in vitro fertilization, *RB1* = *RB1* gene, n.p. = examination of tumor material not possible

DISCUSSION

In this study we have examined the mutations of the retinoblastoma tumors of 7 children conceived by IVF/ICSI. We did not detect hypermethylation of the *RB1* promoter in retinoblastoma tumors of children with non-hereditary retinoblastoma conceived by IVF or ICSI. Neither did the tumors of patients with a *de novo RB1* germline mutation show hypermethylation as a second hit. In line with most retinoblastoma tumors the majority (4 out of 7) of the M2 events was LOH.[19] Therefore, this study does not support the suggestion that the association between IVF or ICSI and retinoblastoma may be caused by hypermethylation of the *RB1* promoter as an epigenetic mechanism in the development of a retinoblastoma tumor.

DNA methylation changes during embryogenesis and gametogenesis. In embryogenesis, genome wide methylation is lost in early embryos, except in differentially methylated regions (regions with sex-related methylation), where methylation is maintained.[20] Following the blastocyst stage, *de novo* methylation takes place. In gametogenesis, methylation patterns - including differentially methylated regions - are erased during germ cell proliferation and migration and reestablished in specific sex-related patterns. Male germ cells have completed this imprinting process before meiosis.[21] In female germ cells, reestablishment of methylation is acquired postnatally, in the oocyte growth phase, just before ovulation.[20] The underlying cause of a possible increased risk for imprinting defects acquired during IVF or ICSI is not known. Based on the changes in DNA methylation in the embryo and germ cells described above, several factors have been implicated[22]: the process of ovulation induction, the manipulation of gametes or early embryos and the underlying infertility itself. Furthermore different culture conditions have been associated with imprinting abnormalities in animal studies.[23,24]

In Beckwith-Wiedemann syndrome and Angelman syndrome, an imprinting defect in the germline is the underlying molecular basis in respectively ~50%[25] and ~2-4%[26] of cases. In contrast to these syndromes, there is no known imprinting defect in the germline for retinoblastoma. Hypermethylation of the *RB1* promoter is a retinoblastoma tumor phenomenon. Recently the CpG island in intron 2 of the *RB1* gene (CpG 85) was shown to be differentially methylated. It is methylated on the maternal allele and CpG85 on the paternal allele is unmethylated and can act as a weak promoter of an alternative transcript.[27] This leads probably to a reduced expression of the paternal allele by transcriptional interference of the regular promoter. If this imprint would change during IVF or ICSI, it is difficult to envision a mechanism that would lead to a greater chance of a mutation either in the germline or in somatic retinal precursor cells. To date, no elucidatory theory is known to explain the possible association between retinoblastoma and IVF.

In conclusion, this study demonstrates that hypermethylation of the *RB1* promoter does not function as a mutational event in retinoblastoma tumors of children conceived by IVF or ICSI.

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