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The first *Rb-1* gene promoter germ-line de novo mutation in patient with retinoblastoma

Stanisław ZAJĄCZEK[1], Anna JAKUBOWSKA[1], Grzegorz KURZAWSKI[1], Zofia KRZYSTOLIK[2], Jan LUBIŃSKI[1]

[1] Department of Genetics and Pathology, Hereditary Cancer Centre and
[2] Department of Ophthalmology, Pomeranian Medical University, Szczecin, Poland

Abstract. *Rb-1* gene promoter mutations are very rare events, only three retinoblastoma families with such alterations were reported up to now. Herein, we describe the first case of the *Rb-1* gene promoter germ, line *de novo* mutation in a proband with sporadic unilateral retinoblastoma. All of reported *Rb-1* gene promoter mutations are associated with a reduced penetrance. Together with the literature data it can be roughly estimated that penetration of cases with alteration localised in promoter and similar as in presented case, is at the level of about 60-70%. It seems, that there are some promoter sites more prone for occurrence of mutations.

Key words: de novo mutation, *Rb-1* gene, retinoblastoma.

Introduction

Retinoblastoma is an intraocular malignancy of children and has served as a prototype of hereditary, Mendelian predisposition to cancer. Lack of the human *Rb-1* gene transcription activity and active protein product due to complete inactivation of both copies of *Rb-1* is believed to be essential to generation of most cases of retinoblastomas. It is consistent with the finding that among *Rb-1* mutations detected in retinoblastoma patients known to present time, those generating a stop codon are most frequent category (HARBOUR 1998, LOHMANN 1998).

Rb-1 gene promoter mutations are very rare (FUJITA et al. 1999). Only about 2% (3/192 cases) of the registered constitutional *Rb-1* mutations in retinoblastoma cases are localised in this region (HARBOUR 1998, LOHMANN 1998). In all of these cases they were noted in familial retinoblastomas characterized by a low

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Correspondence: S. ZAJĄCZEK, Department of Genetics and Pathology, Hereditary Cancer Centre, Pomeranian Medical University, Al. Powstańców Wlkp 72, 70-111, Szczecin, Poland.

penetrance of the mutation at pedigree level (SAKAI et al. 1991a, COWELL et al. 1996).

Herein, we describe the first case of germ-line de novo mutation of the *Rb-1* gene promoter.

Patient and methods

Rb-1 gene sequencing including promoter and all exons was performed for DNA from leukocytes of a boy (our RB-10 family) with unilateral retinoblastoma detected at the age of 15 months in the Ophthalmology Department of Pomeranian Medical University. The tumour was unilateral during the follow-up of 96 months.

DNA was isolated from peripheral blood leukocytes from the patient and his first degree relatives using the standard phenol-chloroform protocol.

PCR reactions were performed using conditions and primer sequences described by LOHMANN et al. (1996).

This system did not work well for amplification of the promoter. New pair of primers (T_m = 62°C) was designed:

5`-CCTGGAAGGCGCCTGGACCC-3`,

5`- TCCCCCGCCGGCAACTGAG -3`.

194 bp promoter product was amplified in 100 µL reaction mixture using 50 pmol of each primer, 1 × PCR buffer (10 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$, 50 mM KCl), 0.2 mM of each dNTP, 5% DMSO and 2.5 U Taq polymerase (InGen, POLAND). Thermal cycle conditions were as follows: 2 min. at 94°C; 30 cycles – 45 sec. at 94°C, 30 sec. 62°C, 30 sec. 72°C; final cycle – 10 min. 72°C.

DNA "exon by exon" sequencing was performed according to LOHMANN et al. (1996) as we described earlier (ZAJĄCZEK et al. 1998, 1999).

Results

The *Rb-1* gene sequence analysis of DNA of the patient showed G-to-A substitution 198 bp upstream of the initiating methionine codon (Figure 1A-e). No alterations or polymorphic sites were detected in exons 1-27.

Both parents and four siblings of this child showed no DNA alterations.

Discussion

The promoter is the 5`-region of non-coding gene sequences, localised upstream of the exons. In this part of the gene transcription is initiated by RNA polymerase

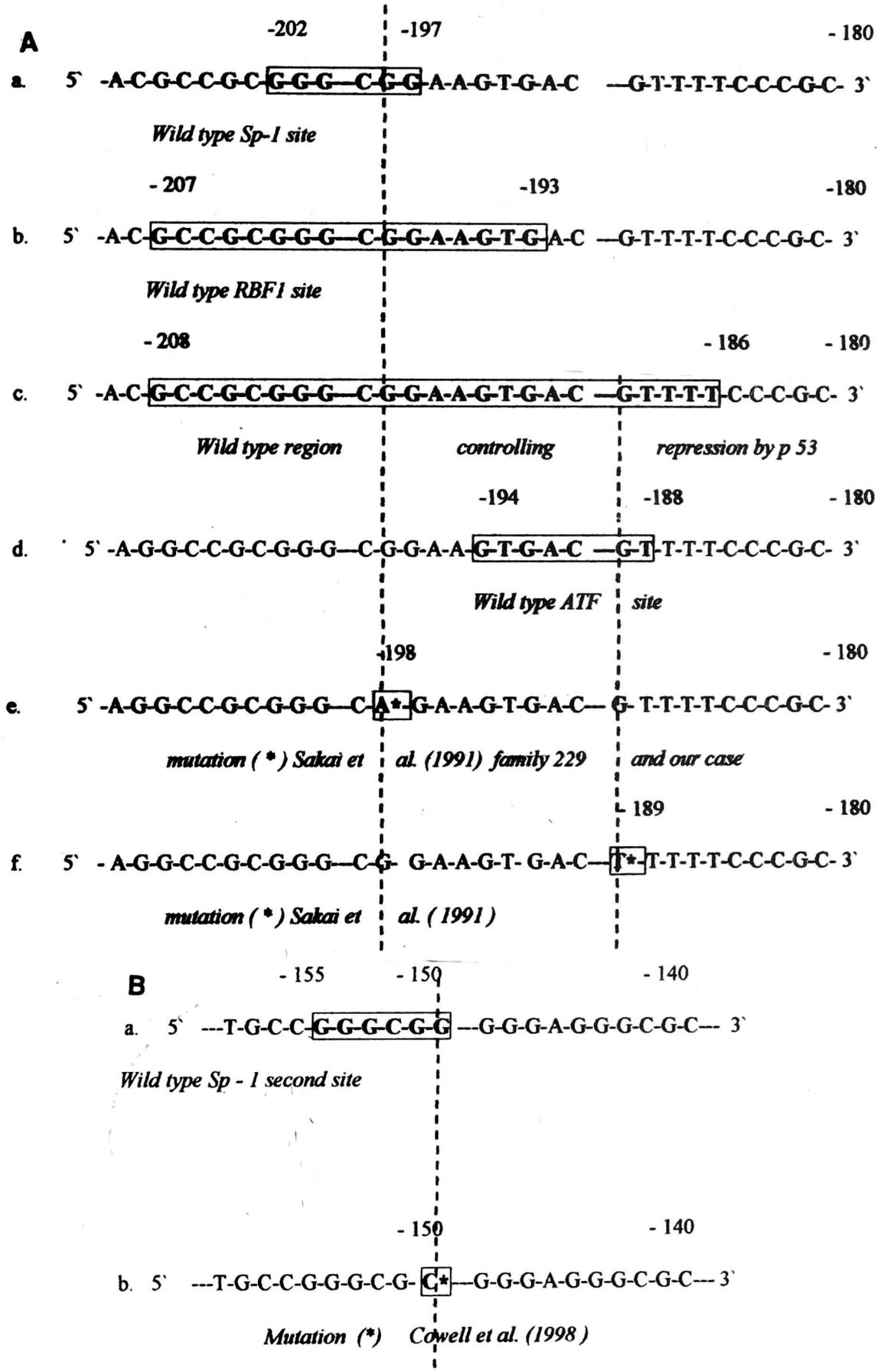

Figure 1. A – Conserved sequences homologous to binding sites of transcription regulatory factors in *Rb-1* gene promoter: a) wild type Sp-1 site (SAKAI et al. 1991a), b) wild type RBF1 site (OSIFCHIN et al. 1994), c) wild type region of repression by p53 (OSIFCHIN et al. 1994), d) wild type ATF site (COWELL et al. 1996) and its known mutations, e) mutation in family 229 and in our case (SAKAI et al. 1991a), f) mutation in family 172 (SAKAI et al. 1991a). B – Second Sp-1 binding site: a) wild type second Sp-1 binding site (COWELL et al. 1996), b) mutation in a case described by COWELL et al. (1996)

Bases are described according to DNA Data (1998) and numbered according to ANTONARAKIS et al. (1998), transcription regulatory factors conserved consensus sites – boldfaced symbols, bases with asterisks – mutated.

binding and regulated by binding of transcription regulatory factors (LEVIN 1997).

Mutations localised in this region are rare. They affect the level of gene activity without altering the quality of gene products and in most cases they are manifested as so-called down-mutations with a decreased transcription efficiency or less frequently, as up-mutations with an increased transcription efficiency (COOPER et al. 1997). A small number of cases with promoter mutations are described among patients with hereditary persistence of foetal haemoglobin (HPFH), thalassemias, haemoglobinopathias, haemophilias, familial combined hyperlipidemias (FCHL), angioedema with C-1 deficiency and retinoblastomas (SAKAI et al. 1991a, COWELL et al. 1996, VERPY et al. 1996, YANG et al. 1996, CRAIG et al.1997, COOPER et al. 1997).

The sequence and functioning of the *Rb-1* promoter are relatively well-known. It is G+C rich localised immediately upstream of the first exon, and contains conservative consensus binding sequences for transcription regulatory factors such as Sp-1 (Figures 1A-a, 1B-a), autoregulatory RBF1 (Figure 1A-b), p-53 (Figure 1A-c), ATF (Figure 1A-d), and differs from other housekeeping genes by lack of TATA and CAAT boxes (HONG et al. 1989, GILL et al. 1994, SAKAI et al. 1991b, COWELL et al. 1996, OHTANI-FUJITA et al. 1994, OSIFCHIN et al. 1994, PARK et al. 1994, SAVOYSKY et al. 1994, EWEN 1998, DONNELLAN, CHETTY 1999).

In the case studied we detected an alteration of the promoter region in a site homologous to binding sequences of a few *Rb-1* gene regulatory factors: Sp-1, ATF and RBF1 (Figure 1A-e); all exon sequences were normal. Neither this nor any other alterations were detected in *Rb-1* constitutive DNA from proband and any of the nearest relatives.

According to our knowledge we are reporting the first case of a germ-line *de novo* promoter mutation of the *Rb-1* gene. On the basis of available data we can diagnose certainly that it is *de novo* alteration, however it is impossible to distinguish whether this mutation occurred as zygotic abnormality or was inherited from mother or father showing mosaicism (SIPPEL et al. 1998).

The spectrum of the constitutional *Rb-1* gene mutations found in retinoblastoma patients is broad, from large chromosomal alterations to mutations detectable only at the DNA level (HARBOUR 1998, LOHMANN 1998). Alterations in the promoter of the *Rb-1* gene were the smallest group and were noted only in three families to date. Two of them were detected by SAKAI et al. (1991a) and one by COWELL et al. (1996).

SAKAI et al. (1991a) described a G-to-T transversion (family 172) (Figure 1A-f) and a G-to-A transition (family 229) (Figure 1A-e) localised respectively 189 and 198 bp upstream from the initiating methionine codon. COWELL et al. (1996) reported a G-to-C change 150 bp upstream from the initiating methionine codon (Figure 1B-b).

All of the above alterations were localised in a region of conserved recognition sequences of the transcription regulatory factors Sp-1, p53, ATF and the autoregulatory sequence RBF1.

In our case the patient showed the same DNA alteration as in family 229 observed by SAKAI et al. (1991a). They proved, using gel-shift assays with nuclear extracts, that binding of two regulatory factors ATF and Sp-1 was diminished.

Generally, known mutations within the *Rb-1* gene are dispersed with a low tendency to show hot-spots of alterations (HARBOUR 1988, LOHMANN 1998). Occurrence of identical changes in 2 of the 4 reported *Rb-1* promoter abnormalities in retinoblastoma patients indicates that mutations could occur more frequently in some particular promoter sites.

Consequences of genomic abnormalities can be fully understood only after performing correlation tests between the clinical presentation of disease and molecular findings. DNA changes may be a pathologic mutations or polymorphism only. Occurrence of identical alteration in the affected patient in our case is an essential confirmation that a change observed by SAKAI et al. (1991a) in family 229 really has a pathological character. Phenotype-genotype correlations are of crucial value also for genetic counselling.

In studied by SAKAI et al. (1991a) family 229, three patients with bilateral tumours, two patients with unilateral disease and three unaffected obligatory carriers were identified. In conjunction with our case, it can be indicated that new putative individuals with an identical molecular constitutional change will have penetrance level of about 66% (6/9) with a similar risk of about 33% of uni- or bilaterality. Obviously the above values are only a very rough preliminary approximation.

By definition in families with low penetrance the risks of retinoblastoma and of the second malignancy are lower as compared to highly penetrant cases. However, the number of such cases reported in literature is extremely low and even fewer of them are diagnosed at molecular level. Thus counselling for particular individuals from families with low penetrance is much less precise at present than for persons from families with the most frequently occurring mutations leading to disease in about 90% of cases.

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