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Downregulation and Aberrant Promoter Methylation of *p16INK4A*: A Possible Novel Heritable Susceptibility Marker to Retinoblastoma

PAOLA INDOVINA,[1,2] ANTONIO ACQUAVIVA,[3] GIULIA DE FALCO,[1] VALERIA RIZZO,[1] ANNA ONNIS,[1] ANNA LUZZI,[1] FRANCESCA GIORGI,[1] THEODORA HADJISTILIANOU,[4] PAOLO TOTI,[1] VALENTINA TOMEI,[1] FRANCESCA PENTIMALLI,[2,5] ALESSANDRO CARUGI,[1] AND ANTONIO GIORDANO[1,2,5]*

[1]*Department of Human Pathology and Oncology, University of Siena, Siena, Italy*

[2]*Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, College of Science and Technology, Temple University, Philadelphia, Pennsylvania*

[3]*Department of Pediatrics, Obstetrics and Reproductive Medicine, University of Siena, Siena, Italy*

[4]*Ocular Oncology Unit, Department of Ophthalmology, University of Siena, Siena, Italy*

[5]*CROM-Center of Oncology Research Mercogliano, Avellino, Italy*

RB loss has long been recognized as the causative genetic alteration underlying retinoblastoma but it is increasingly evident that other alterations are required for the tumor to develop. Therefore, we set out to identify additional inheritable susceptibility markers and new potential preventive and therapeutic targets for retinoblastoma. We focused on the *p16INK4A* tumor suppressor gene because of its possible role in retinoblastoma pathogenesis and its involvement in predisposition to familial cancer. *p16INK4A* expression was analyzed in tumor samples from retinoblastoma patients by immunohistochemistry and in peripheral blood cells from both patients and their parents by real-time quantitative reverse transcription-PCR (qRT-PCR). Since promoter methylation is a common mechanism regulating *p16INK4A* expression, the methylation status of its promoter was also analyzed in blood samples from patients and their parents by methylation-specific PCR. A downregulation of *p16INK4A* was observed in 55% of retinoblastoma patients. Interestingly, in 56% of the cases showing *p16INK4A* downregulation at least one of the patients' parents bore the same alteration in blood cells. Analysis of *p16INK4A* promoter methylation showed hypermethylation in most patients with *p16INK4A* downregulation and in the parents with the same alteration in *p16INK4A* expression. The finding that *p16INK4A* was downregulated both in patients and their parents suggests that this alteration could be a novel inheritable susceptibility marker to retinoblastoma. The observation that *p16INK4A* downregulation seems to be due to its promoter hypermethylation opens the way for the development of new preventive and therapeutic strategies using demethylating agents.

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Retinoblastoma, the most common malignant ocular tumor in childhood, has long represented the prototypic example of the genetic predisposition to cancer. Although in the majority of cases no family history is reported, the importance of hereditary factors for this cancer is well established (Vogel, 1979). The familial disease is transmitted as a typical Mendelian autosomal dominant trait. It has been estimated that 60% of cases are non-heritable and 40% are heritable. The majority of heritable cases have sporadic disease (no family history). In fact, in this context the word heritable only means that patients are at risk to transmit the disease to their offspring. The heritable form affects all patients with bilateral retinoblastoma as well as 15% of patients with the unilateral form, is generally multifocal and associated with increased risk of second primary cancers. Non-heritable retinoblastoma is always unilateral, unifocal, and is not associated with risk of other cancers.

Retinoblastoma clinical observations revealed the role of tumor suppressor genes in human cancer and led to the development of the "two hit" model (Knudson, 1971). According to this model, two mutational events or "two hits" are required for tumor onset; therefore, an individual with a germline mutation in a tumor suppressor gene (inherited from an affected parent or occurred de novo in parental germline cells or during embryonal development) will be predisposed to cancer because only another somatic mutation in the same gene will be enough for the tumor to develop.

Biallelic mutations in retinoblastoma tumor suppressor gene (*RB*) have long been recognized as the causative genetic

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*Correspondence to: Antonio Giordano, Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, College of Science and Technology, Temple University, BioLife Science Bldg. Suite 333, 1900 North 12th Street, Philadelphia, PA 19122. E-mail: giordano@temple.edu

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alteration for retinoblastoma (Cavenee et al., 1983; Friend et al., 1986; Fung et al., 1987; Lee et al., 1987; Dunn et al., 1988). The *RB* gene, together with the other members of the *RB* family of tumor suppressors, has a crucial role in cell cycle control, mainly acting as the gatekeeper of the G1–S transition (Giacinti and Giordano, 2006; Sun et al., 2007). The function of RB protein is modulated through changes in its phosphorylation status, which is mediated by cyclin-dependent kinase (CDK)/cyclin complexes (Buchkovich et al., 1989). *RB* inactivation confers a growth advantage and underlies multiple types of human tumors (Friend et al., 1986; Harbour et al., 1988; Lee et al., 1988; Horowitz et al., 1989).

Although mutations in *RB* gene have a central role in the development of retinoblastoma, recent advances in the knowledge of retinoblastoma pathogenesis have revealed that the molecular mechanisms leading to retinoblastoma are more complex than those implied by the two-hit model and involve other genetic or epigenetic alterations that have been long overlooked (Mastrangelo et al., 2007). In particular, it has been shown that in mouse models of retinoblastoma, retinal tumors only develop when *RB* is lost together with at least one other key cell cycle regulatory gene (McPherson and Dyer, 2007). In humans, loss of both copies of *RB* does not lead directly to retinoblastoma, but to the benign precursor lesion retinoma, which commonly progresses to retinoblastoma after further mutational events (Dimaras et al., 2008; Sampieri et al., 2008, 2009). Moreover, a large number of cytogenetic and comparative genomic hybridization (CGH) studies have shown that mutational events affecting *RB* are not the only genomic changes in retinoblastoma (Corson and Gallie, 2007). Finally, *RB* gene mutations have been found in a highly variable percentage of cases, ranging from 10–20% in some reports to 89% in others, suggesting that mutations of this gene are not the only causative genetic alteration for retinoblastoma (Mastrangelo et al., 2007).

Identifying the additional molecular alterations that underlie retinoblastoma pathogenesis is crucial to find new targets for prevention and therapy. The design of novel therapeutic strategies is particularly needed, because, at present, standard treatments are associated with significant toxicities (Dalgard et al., 2008). Therefore, more rationale therapies that selectively target the molecular abnormalities of retinoblastoma are required.

So, we searched for additional inheritable markers of susceptibility that might represent new potential preventive and therapeutic targets for retinoblastoma. A good candidate seemed to be the *p16INK4A* tumor suppressor gene, which encodes a CDK inhibitor that negatively regulates the G1-S transition (Serrano et al., 1993). This protein is able to prevent cell cycle progression by inhibiting the interaction between cyclin D and CDK4 or CDK6. Given its key role in regulating cell cycle, the inactivation of *p16INK4A* can cause the loss of control over cell proliferation and is one of the most commonly observed abnormalities in human cancer (Liggett and Sidransky, 1998). In a recent study, a crucial role of this gene was also suggested in retinoblastoma development (Dimaras et al., 2008). In particular, a high expression of *p16INK4A* in $RB^{-/-}$ retina cells was proposed to block transformation at the stage of the non-proliferative precursor lesion retinoma. Conversely, *p16INK4A* inactivation seemed to promote retinoblastoma development.

Moreover, mutations affecting *p16INK4A* are also involved in the predisposition to familial cancer (Hussussian et al., 1994).

Collectively, the possible role of *p16INK4A* in retinoblastoma pathogenesis and its involvement in predisposition to familial cancer, prompted us to assess whether *p16INK4A* alterations could represent new inheritable susceptibility factors to retinoblastoma. To this end, *p16INK4A* expression was analyzed in tumor samples from retinoblastoma patients by immunohistochemistry and in peripheral blood cells from both patients and their parents by real-time quantitative reverse transcription-PCR (qRT-PCR).

The results revealed a downregulation of *p16INK4A* in about half of the tumor and blood samples from retinoblastoma patients. Furthermore, in most cases with *p16INK4A* downregulation at least one of the parents showed the same alteration in blood cells.

Because promoter hypermethylation is a common cause of reduced *p16INK4A* expression (Gonzalez-Zulueta et al., 1995; Herman et al., 1995; Myohanen et al., 1998), the methylation status of its promoter was evaluated by methylation-specific PCR (MSP). The results showed that *p16INK4A* promoter was hypermethylated in most patients with *p16INK4A* downregulation and an analogous result was observed in the parents with the same alteration in *p16INK4A* expression.

These data suggest that *p16INK4A* downregulation could be a novel inheritable predisposition factor to retinoblastoma. Moreover, the observation that *p16INK4A* downregulation seems to be due to its promoter hypermethylation opens the way for the development of new preventive and therapeutic strategies using demethylating agents.

Materials and Methods

Case selection, tissue processing for histological evaluation, and blood collection

Twenty-nine patients affected by retinoblastoma were enrolled at the Department of Pediatrics of the University of Siena, Italy, and their parents gave informed consent for entrance into the study. In particular, 6 familial (1 unilateral and 5 bilateral) and 23 sporadic (11 unilateral and 12 bilateral) cases were enrolled. Patients' clinical characteristics are reported in Table 1.

Paraffin blocks of 11 pretreatment surgical specimens of retinoblastoma were collected. Tissues were cut and fixed in a buffered 4% aqueous formaldehyde solution, pH 7.4. For conventional histology, 4 μm-thick sections were obtained from representative paraffin blocks and stained with hemalum and eosin, Giemsa, periodic acid-Schiff (PAS), Gomori's silver impregnation, and Feulgen.

Peripheral blood samples were collected from all patients, before they underwent chemotherapy, and from their parents, and seven healthy donors used as normal controls. These samples were subjected to RNA and DNA isolation immediately after collection.

This study was conducted after approval by the Institutional Ethics Review Board.

Immunohistochemistry

Consecutive sections of retinoblastoma tissue cut at 4 μm thickness were subjected to immunostaining. The EnVision™ +/HRP method (Dako, Milan, Italy) was used to visualize immunohistochemical reaction products. Antigen retrieval was achieved by treating the deparaffinized sections with microwaves in 1 mM EDTA, pH 8.0, for 5 min, followed by cooling at room temperature prior to incubation with monoclonal antibodies against p16INK4A (Neomarkers, Fremont, CA), RB (Biogenex, San Ramon, CA), and pRB2/p130 (Neomarkers). All the antibodies were used at a 1:50 dilution. Normal mouse serum was used as a negative control.

RNA extraction and real-time quantitative reverse transcription-PCR

Total RNA was isolated from blood samples of the individuals described above using the RNeasy Minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. *p16INK4A* relative expression was analyzed by real-time qRT-PCR. Five hundred nanograms of total RNA from each sample were reverse transcribed using Transcriptor High Fidelity cDNA Synthesis kit

TABLE 1. Clinical characteristics of retinoblastoma patients

Case number	Age at diagnosis (months)[a]	Sex	Phenotype	Reese-Ellsworth group		Status
				Right eye	Left eye	
1	40	Male	US		Vb	Alive
2	1	Male	BF	Ia	IIa	Alive
3	13	Male	BS	Vb	IIa	Alive
4	1	Female	BF	IIb	Va	Dead[b]
5	3	Female	BS	IIIa	IVa	Alive
6	9	Female	US	Vb		Alive
7	11	Female	BS	Va	IIIb	Alive
8	18	Female	US		Vb	Dead[c]
9	12	Male	BS	Va	Vb	Alive
10	15	Female	BS	IVa	IIa	Alive
11	4	Female	BS	Va	Ia	Alive
12	16	Male	US		Va	Alive
13	1	Male	BS	Ia	Va	Alive
14	5	Male	BS	Vb	Va	Alive
15	3	Male	US		IIIb	Alive
16	20	Female	BS	Va	IIIa	Alive
17	22	Male	BF	IIa	IIb	Alive
18	4	Male	BF	IIa	Ia	Alive
19	4	Male	BS	Va	Vb	Alive
20	76	Female	US		Va	Alive
21	26	Male	BS	IIb	Va	Alive
22	4	Female	BF	IVa	IIa	Alive
23	8	Female	US		Vb	Alive
24	51	Male	US	Vb		Alive
25	15	Female	US	Va		Alive
26	31	Female	US	Vb		Dead[d]
27	16	Female	UF		IIa	Alive
28	3	Female	BS	Va	IIIa	Alive
29	54	Male	US		Vb	Alive

U, unilateral; B, bilateral; F, familial; S, sporadic.
[a]Median age at diagnosis for unilateral cases: 17 months; median age at diagnosis for bilateral cases: 4 months.
[b]Dead for trilateral retinoblastoma.
[c]Dead for a road accident.
[d]Dead for progression of the disease.

(Roche, Mannheim, Germany). The amplification of 1 μl of cDNA was performed in the Opticon 2 real-time PCR cycler (MJ Research, Waltham, MA) using the Fluocycle II SYBR green mix (Euroclone, West York, UK) according to the manufacturer's instructions. The mRNA levels were normalized to those of the housekeeping *HPRT* gene. *p16INK4A* relative expression was calculated by the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). Specific primers were designed across two adjacent exons, using the AutoPrime Program.

Primer sequences:

p16INK4A forward: 5′-GGAAGGTCCCTCAGACATC-3′;
p16INK4A reverse: 5′-GCAGTTGTGGCCCTGTAG-3′;
HPRT forward: 5′-AGCCAGACTTTGTTGGATTTG-3′;
HPRT reverse: 5′-TTTACTGGCGATGTCAATAGG-3′.

The annealing temperature was 64°C.

DNA extraction and methylation analysis

Genomic DNA was extracted from blood samples using Puregene Blood Core kit B (Qiagen) following the manufacturer's instructions.

For methylation analysis of *p16INK4A* promoter, MSP was used. In detail, genomic DNA was subjected to sodium bisulfite modification using the Epitect Bisulfite kit (Qiagen). Treatment of genomic DNA with sodium bisulfite converts unmethylated, but not methylated cytosines, into uracil, producing sequence differences between methylated and unmethylated DNA. Subsequent PCR with primers specifically designed for discriminating between methylated, unmethylated, and bisulfite unmodified DNA was performed. The primer sequences, annealing temperatures, number of PCR cycles, and amplification product sizes have been previously reported (Herman et al., 1996). PCR reactions were performed using 2.5 U of Platinum Taq polymerase (Invitrogen, Carlsbad, CA) and amplification products were analyzed by electrophoresis on 2% agarose gel. Gel images were acquired using the ChemiDoc molecular imaging system.

Statistical analyzes

Statistical analyzes were carried out using Student's *t*-test, χ^2 test, and Dunnett's multiple comparison test.

Results

p16INK4A, RB and pRB2/p130 protein expression in retinoblastoma samples

Immunohistochemical analysis was conducted in 11 tumor samples to investigate p16INK4A expression. Five tumor samples (cases 3, 8, 9, 16, and 21) showed a p16INK4A staining only in a low percentage of tumor cells, ranging approximately 5–40%, whereas the other samples showed a 100% staining (compare representative case 9 with case 20 in Fig. 1A). The p16INK4A staining was mainly cytoplasmic. Adjacent retina cells were negative, except for ganglion cells in the inner part (see representative case 20 in Fig. 1A).

The role of p16INK4A loss in tumor growth largely depends on RB function. In fact, by releasing CDK4/6, p16INK4A loss causes RB constitutive phosphorylation and inactivation, which leads to uncontrolled cell proliferation and tumor progression (Serrano et al., 1993). However, in the absence of RB, p16INK4A loss could promote tumorigenesis by interfering with the activity of other RB family members, such as pRB2/p130 (Dimaras et al., 2008). RB immunohistochemical analysis revealed no staining for this protein in any of the cases analyzed (see representative case 9 in Fig. 1B). Conversely, immunostaining for pRB2/p130, which was conducted in 7 of the 11 tumor samples, including three samples showing a low level of p16INK4A (cases 3, 9, and 21), revealed a strong nuclear staining for pRB2/p130 in all the cases analyzed (see representative case 9 in Fig. 1C). This finding suggests that the

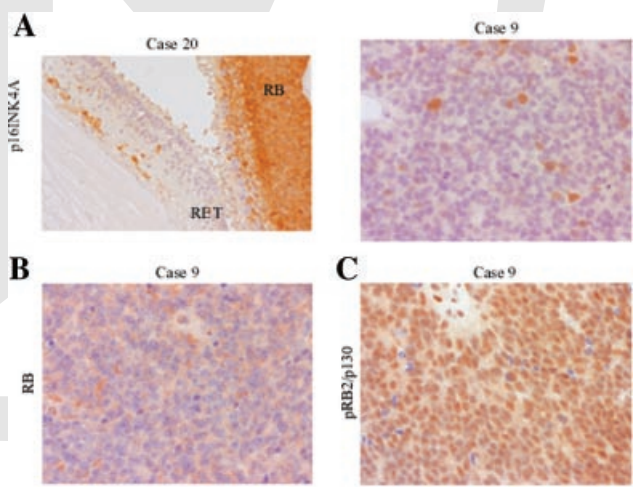

Fig. 1. Representative micrographs of immunostaining for p16INK4A (A), RB (B), and pRB2/p130 (C) in retinoblastoma tumor samples. In A, one representative case with a strong immunostaining for p16INK4A (case 20) and one case in which isolated neoplastic cells are positively stained for this protein (case 9) are shown. Areas of retina (RET) and retinoblastoma (RB) are indicated for case 20. For case 20 in A, a 100× original magnification was used; for case 9 in A, B, and C, a 200× original magnification was used. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

low level of p16INK4A could be important for pRB2/p130 inactivation and tumor growth.

Decreased RNA expression of *p16INK4A* in peripheral blood cells from retinoblastoma patients

p16INK4A RNA expression was measured in peripheral blood cells from 29 patients and seven healthy donors by real-time qRT-PCR. *p16INK4A* RNA expression of both patients and healthy donors was calculated relatively to the same reference sample (a healthy donor who showed a level of *p16INK4A* expression close to the average level in the healthy donor group).

Our results revealed a *p16INK4A* downregulation in 55% of the patients (16/29) with a range of 1.7- to 14-fold decrease (Fig. 2).

The comparison between the mean values of *p16INK4A* expression in patients and healthy donors showed a significant downregulation of this gene in the patient group ($P < 0.05$).

A relationship between the level of *p16INK4A* expression and the clinical and ophthalmoscopic features of retinoblastoma patients (Table 1) was also investigated, but no significant correlation was detected (statistical analysis was conducted using Student's *t*-test and χ^2 test). However, although the difference was not statistically significant, it should be noted that the median age at diagnosis was lower in cases with a *p16INK4A* downregulation with respect to cases not showing this downregulation (10 and 16 months, respectively). Moreover, it should also be noted that the percentage of cases with a *p16INK4A* downregulation was higher among bilateral retinoblastoma patients compared to unilateral patients (64.7% vs. 41.6%).

Decreased RNA expression of *p16INK4A* in peripheral blood cells from parents of retinoblastoma patients

In order to investigate whether the *p16INK4A* downregulation observed in peripheral blood cells from retinoblastoma patients could be inheritable, the analysis of *p16INK4A* expression was extended to patients' parents.

We found that in 56% (9/16) of the cases with *p16INK4A* downregulation at least one of the parents showed the same alteration in peripheral blood cells. However, it should be noted that this percentage could represent an underestimation because RNA from some patients' parents was not available for *p16INK4A* expression analysis. Therefore, some parents with a possible *p16INK4A* downregulation might have been excluded from the analysis.

In Figure 3, we reported the relative *p16INK4A* RNA expression in retinoblastoma patients showing *p16INK4A* downregulation and in their parents. The values represent the means and the standard deviations of three real-time qRT-PCR separate experiments, each conducted in triplicate. For all the families, *p16INK4A* expression was calculated relatively to the same reference sample, as described above.

p16INK4A promoter methylation in peripheral blood cells from retinoblastoma patients and their parents

Because promoter hypermethylation is a common cause of reduced *p16INK4A* expression (Herman et al., 1995; Gonzalez-Zulueta et al., 1995; Myohanen et al., 1998), the methylation status of its promoter was evaluated. These analyses were conducted by MSP using bisulfite modified DNA from peripheral blood cells of retinoblastoma patients and their parents, and healthy donors used as a control.

As can be seen in Figure 4 and Table 2, among the patients with *p16INK4A* downregulation, whose DNA was available for these analyzes (cases 1–12), all except one (case 12) showed some degree of aberrant *p16INK4A* promoter methylation. Conversely, no methylation was observed in patients not showing *p16INK4A* downregulation (cases 19–22). A similar correlation between the methylation status and the level of *p16INK4A* expression was also observed for the patients' parents (Fig. 4 and Table 2). In all the healthy donors, the *p16INK4A* promoter was found unmethylated (Fig. 4).

In addition to primers specifically designed for discriminating between methylated and unmethylated DNA, primers that recognized bisulfite unmodified DNA (wild-type primers) were also used (data not shown). These primers allow to detect an incomplete bisulfite reaction. A weak amplification with

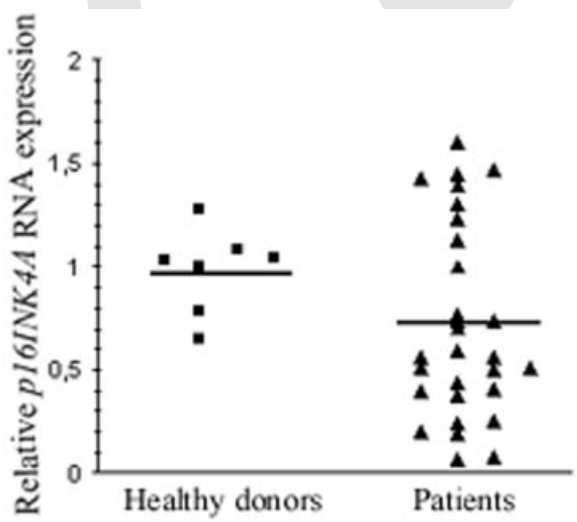

Fig. 2. Relative *p16INK4A* RNA expression in peripheral blood cells from retinoblastoma patients and healthy donors measured by real-time qRT-PCR. The relative expression of each sample is given individually (symbols) and as a mean for each group (lines). The comparison between the mean values of *p16INK4A* expression in patients and healthy donors showed a significant *p16INK4A* downregulation in the patient group ($P < 0.05$). Statistical analysis was conducted using the Student's *t*-test.

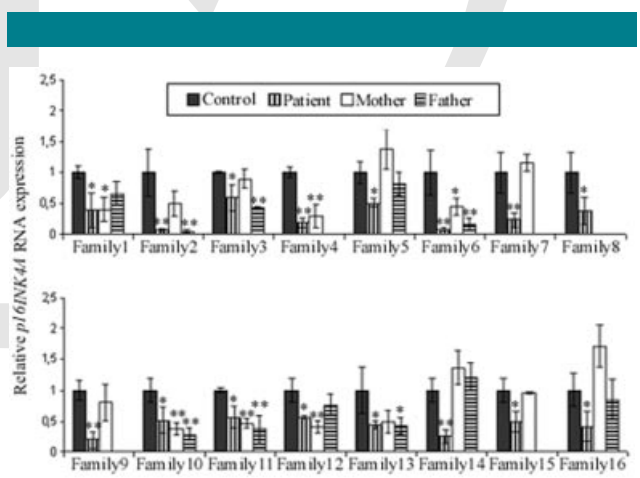

Fig. 3. Histograms reporting the relative *p16INK4A* RNA expression in retinoblastoma patients showing a *p16INK4A* downregulation and in their parents. Asterisks indicate cases showing a statistically significant *p16INK4A* downregulation. Significant $^{*}(P < 0.05)$; very significant $^{**}(P < 0.01)$. Statistical analysis was conducted using Dunnett's multiple comparison test.

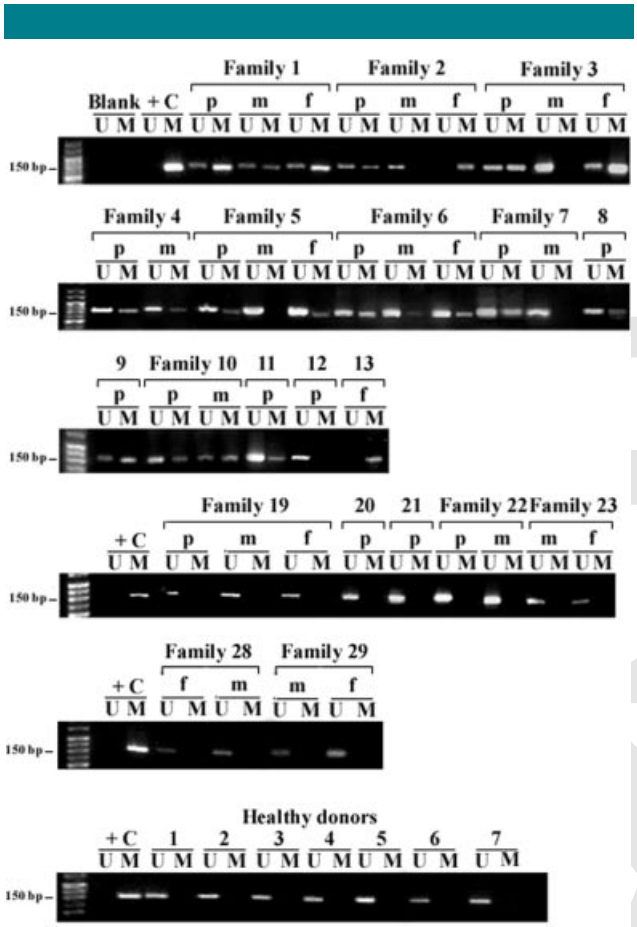

Fig. 4. Methylation-specific PCR of *p16INK4A*. Primer sets used for amplification are designated as unmethylated (U) and methylated (M). A methylated and bisulfite converted human control DNA (EpiTect PCR control DNA; Qiagen), designed as +C, was used as a positive control in all the experiments; p, DNA from retinoblastoma patient; m, DNA from patient's mother; f, DNA from patient's father; 1–7, DNA from healthy donors.

wild-type primers was observed occasionally . However, unmodified DNA was not recognized by the primers specific for modified DNA, and, therefore, did not provide false positive results or interfere with the ability to distinguish methylated from unmethylated allele (Herman et al., 1996).

Discussion

Retinoblastoma clinical studies have greatly improved the understanding of the molecular mechanisms underlying human cancer through the identification of the first tumor suppressor gene and the development of the "two hit" model (Knudson, 1971). Nevertheless, recent advances in the knowledge of retinoblastoma pathogenesis led to a revision of the classical "two hit" model (Corson and Gallie, 2007; Mastrangelo et al., 2007; McPherson and Dyer, 2007; Dimaras et al., 2008; Sampieri et al., 2008, 2009). In fact, molecular mechanisms leading to retinoblastoma are more complex than those theorized in the model and involve an interplay of genetic and epigenetic alterations rather than mutations in a single tumor suppressor gene. The recent finding that *RB* loss does not cause retinoblastoma but only makes it highly likely, encourages further investigations aimed at a detailed understanding of the sequence of molecular events that, starting from RB mutations, lead to malignancy. The molecular alterations occurring during this process could identify good targets for prevention and therapy.

The design of novel therapeutic strategies is particularly important, because, at present, standard treatments are associated with significant toxicities. In fact, although in developed countries the survival rate among children with retinoblastoma is 95%, children who survive this disease suffer serious morbidity (Dalgard et al., 2008). The great majority of children with unilateral disease are treated with enucleation and children with bilateral disease are treated with globe salvage therapies, which are associated with serious toxicities. Therefore, there is currently a great need for the development of improved therapies that minimize adverse effects.

A crucial role of the loss of *p16INK4A* tumor suppressor gene in retinoblastoma progression has been recently suggested (Dimaras et al., 2008). In fact, a high expression of *p16INK4A* in $RB^{-/-}$ retina cells was proposed to prevent retinoblastoma development by blocking transformation at the stage of the non-proliferative precursor lesion retinoma. Conversely, *p16INK4A* inactivation in RB-negative retina cells seemed to promote retinoblastoma development. The finding that *p16INK4A* loss could be a novel molecular alteration promoting retinoblastoma pathogenesis prompted us to analyze its expression in retinoblastoma samples. The results revealed a low p16INK4A expression in 5 of the 11 tumor samples analyzed, whereas in the other samples this protein was detected at a high level in around 100% of cells. This high level of p16INK4A is consistent with the observation that this protein accumulates in RB-negative tumor cells (Parry et al., 1995). The low level of this protein observed in about half of the retinoblastoma samples could be due to an alteration in *p16INK4A* gene that prevented its activation in response to RB loss. It can be hypothesized that the low level of p16INK4A observed in these tumor samples could have contributed to the loss of control over cell proliferation and tumor progression. However, it should be noted that a downregulation of this gene was not observed in all the tumor samples analyzed. It is therefore evident that for retinoblastoma progression, alternative mechanisms not involving *p16INK4A* alterations must exist.

As stated above, a role of *p16INK4A* in retinoblastoma development was previously suggested also by other workers, and a possible mechanism to explain its function was proposed (Dimaras et al., 2008). The product of this gene is a CDK inhibitor that negatively regulates the G1-S transition (Serrano et al., 1993). Cytoplasmic p16INK4A is able to sequester CDK4/6 that would otherwise enter the nucleus and inactivate RB. It can be hypothesized that in the absence of RB, p16INK4A prevent the loss of control of cell proliferation and tumor progression by inhibiting the phosphorylation of the other RB family members, pRB2/p130 and p107 (Dimaras et al., 2008). Therefore, a *p16INK4A* alteration, such as the downregulation reported here could have a role in promoting retinoblastoma tumor growth by leading to pRB2/p130 and p107 constitutive phosphorylation, and, consequently, to the inactivation of their tumor suppressor activity. Immunostaining for pRB2/p130 was performed in three of the five tumor samples showing a low level of p16INK4A. A strong nuclear staining for pRB2/p130 was detected in these samples, suggesting that the low level of p16INK4A could be important for pRB2/p130 inactivation and tumor growth.

In order to assess whether the p16INK4A downregulation observed in some of the tumor samples analyzed could be due to a constitutional and possibly inheritable alteration, *p16INK4A* expression was evaluated in peripheral blood cells from both patients and their parents. This analysis was also extended to other retinoblastoma families, whose tumor samples were not

TABLE 2. Expression and methylation of p16INK4A in peripheral blood cells from retinoblastoma patients and their parents

	Relative *p16* RNA expression[a]				*p16* Methylation		
Family number	Control	Patient	Mother	Father	Patient	Mother	Father
Cases showing *p16* downregulation							
1	1.00 ± 0.10	0.40 ± 0.28*	0.40 ± 0.20*	0.66 ± 0.20	U/M	U/M	U/M
2	1.00 ± 0.38	0.07 ± 0.03**	0.50 ± 0.21	0.04 ± 0.03**	U/M	U	M
3	1.00 ± 0.02	0.59 ± 0.22*	0.90 ± 0.16	0.43 ± 0.02**	U/M	U	U/M
4	1.00 ± 0.08	0.19 ± 0.08**	0.30 ± 0.18**	—	U/M	U/M	—
5	1.00 ± 0.18	0.51 ± 0.07*	1.38 ± 0.31	0.82 ± 0.18	U/M	U	U/M
6	1.00 ± 0.36	0.08 ± 0.03**	0.45 ± 0.13*	0.16 ± 0.10**	U/M	U/M	U/M
7	1.00 ± 0.32	0.24 ± 0.12**	1.15 ± 0.13	—	U/M	U	—
8	1.00 ± 0.32	0.38 ± 0.22**	—	—	U/M	—	—
9	1.00 ± 0.15	0.20 ± 0.14**	0.80 ± 0.28	—	U/M	—	—
10	1.00 ± 0.20	0.51 ± 0.20*	0.37 ± 0.10**	0.28 ± 0.10**	U/M	U/M	—
11	1.00 ± 0.05	0.56 ± 0.17*	0.46 ± 0.08**	0.36 ± 0.23**	U/M	—	—
12	1.00 ± 0.20	0.56 ± 0.03*	0.41 ± 0.10**	0.76 ± 0.17	U	—	—
13	1.00 ± 0.38	0.44 ± 0.08*	0.50 ± 0.18	0.42 ± 0.13*	—	—	M
14	1.00 ± 0.19	0.25 ± 0.10**	1.36 ± 0.28	1.22 ± 0.21	—	—	—
15	1.00 ± 0.20	0.50 ± 0.16*	0.96 ± 0.02	—	—	—	—
16	1.00 ± 0.27	0.41 ± 0.25*	1.36 ± 0.09	0.85 ± 0.32	—	—	—
Cases not showing *p16* downregulation							
17	1.00 ± 0.17	1.00 ± 0.16	—	0.75 ± 0.09	—	—	—
18	1.00 ± 0.24	0.74 ± 0.12	0.42 ± 0.03**	0.69 ± 0.25	—	—	—
19	1.00 ± 0.28	0.74 ± 0.29	1.14 ± 0.27	1.06 ± 0.07	U	U	U
20	1.00 ± 0.54	0.77 ± 0.28	—	—	U	—	—
21	1.00 ± 0.10	1.47 ± 0.40	—	—	U	—	—
22	1.00 ± 0.28	1.60 ± 1.06	1.83 ± 0.95	2.60 ± 0.23	U	U	—
23	1.00 ± 0.28	1.39 ± 0.39	1.62 ± 0.43	0.89 ± 0.14	—	U	U
24	1.00 ± 0.27	0.70 ± 0.46	—	—	—	—	—
25	1.00 ± 0.20	1.45 ± 0.47	1.42 ± 0.18	0.74 ± 0.20	—	—	—
26	1.00 ± 0.19	1.30 ± 0.23	1.00 ± 0.30	—	—	—	—
27	1.00 ± 0.20	1.13 ± 0.58	—	—	—	—	—
28	1.00 ± 0.26	1.23 ± 0.04	1.35 ± 0.16	0.84 ± 0.28	—	U	U
29	1.00 ± 0.35	1.43 ± 0.47	1.58 ± 0.21	0.90 ± 0.23	—	U	U

U, completely unmethylated; U/M, both unmethylated and methylated sequences present; M, completely methylated. Dashes indicate cases not analyzed.
[a]The values represent the means and the standard deviations of three real-time qRT-PCR separate experiments, each conducted in triplicate. The values underlined correspond to the level of *p16INK4A* expression observed in parents with retinoblastoma. Asterisks indicate cases with a statistically significant *p16INK4A* downregulation. Significant *($P < 0.05$); very significant **($P < 0.01$). Statistical analysis was conducted using Dunnett's multiple comparison test.

available, for a total of twenty-nine families and seven healthy donors used as a normal control.

The results revealed a significant *p16INK4A* downregulation in about half of the patients with respect to healthy donors. All but one (case 21) of the five cases with reduced p16INK4A protein expression in tumor samples, presented a *p16INK4A* downregulation also in peripheral blood cells. Moreover, in most cases with *p16INK4A* downregulation in blood cells, at least one of the patients' parents showed the same alteration. The finding that *p16INK4A* expression was reduced in a normal tissue from both retinoblastoma patients and their parents suggests that the *p16INK4A* downregulation could be a novel inheritable predisposition factor to retinoblastoma.

It should be noted that healthy donors and patients were not matched for age, being healthy donors older (aged 24–38) than patients, because of the difficulty to collect healthy donors among children. This observation could raise doubts on the validity of the comparison conducted, especially, if one considers the role of *p16INK4A* in cellular senescence (Mimeault and Batra, 2009). However, the *p16INK4A* downregulation was observed not only in children with retinoblastoma but also in their parents, whose age is approximately comparable with the age of the healthy donor group. Therefore, the differences in *p16INK4A* expression observed between the retinoblastoma patients, their parents, and the healthy donors seem to be correlated with retinoblastoma predisposition rather than with age differences. Furthermore, not all the children analyzed showed a *p16INK4A* dowregulation with respect to the adult healthy donors. In particular, 45% of children with retinoblastoma showed a level of *p16INK4A* expression comparable with that of the healthy donor group. Therefore, the level of *p16INK4A* expression observed in these cases does not seem to depend on age differences.

A relationship between the level of *p16INK4A* expression and the clinical and ophthalmoscopic features of retinoblastoma patients was also sought. Although no significant correlation was detected, a lower median age at diagnosis in cases with a *p16INK4A* downregulation was observed. Moreover, the percentage of cases with a *p16INK4A* downregulation was higher among bilateral retinoblastoma patients compared with unilateral patients. Although the availability of a small cohort of patients, due to the rarity of retinoblastoma, did not allow a statistical confirmation of the observed differences, these preliminary findings suggest that *p16INK4A* downregulation could be more frequently associated with the bilateral form and an earlier disease onset.

Because promoter hypermethylation is a common cause of reduced *p16INK4A* expression (Herman et al., 1995; Gonzalez-Zulueta et al., 1995; Myohanen et al., 1998) to investigate the molecular mechanism underlying the downregulation of this gene, the methylation status of its promoter was analyzed in peripheral blood samples from retinoblastoma patients and their parents. The results showed that *p16INK4A* promoter was hypermethylated in most patients with *p16INK4A* downregulation with respect to healthy donors, who were all negative for *p16INK4A* promoter methylation. Conversely, no methylation was observed in patients not showing *p16INK4A* downregulation. Moreover, a similar correlation between the methylation status and the level of *p16INK4A* expression was also observed for the patients' parents. These findings suggest that *p16INK4A* promoter methylation might be a possible heritable epigenetic risk factor for retinoblastoma. A similar involvement of *p16INK4A* promoter methylation in heritable

predisposition to cancer has been suggested by other researchers (Abbaszadegan et al., 2005). They observed an aberrant *p16INK4A* promoter methylation in most members of a large family with clustering of esophageal squamous cell carcinoma, whereas none of the healthy donors showed this alteration.

In the last few years, the issue of epigenetic modification heritability has aroused great interest. Although inheritance of epigenetic characters has been clearly documented in yeast (Grewal and Klar, 1996; Nakayama et al., 2000), plants (Brink, 1956, 1960), *Drosophila* (Cavalli and Paro, 1998), and, more recently, also in mice (Rakyan et al., 2003; Chong et al., 2007), evidence supporting this type of inheritance in humans is still limited (Chong and Whitelaw, 2004). Generally, it has been assumed that epigenetic modifications are erased during gametogenesis or early embryogenesis to ensure the totipotency of the cells in the developing embryo. Although it cannot be excluded that epigenetic marks at some alleles are not completely erased from one generation to the next (Rakyan et al., 2001; Dobrovic and Kristensen, 2009), it is more generally accepted that the methylation per se is not inherited through the germline but might be the result of an inherited genetic aberration that predisposes to methylation (Dobrovic and Kristensen, 2009). Inheritance of a genetically determined propensity to methylation has been recognized as a possible predisposing factor to cancer (Chan et al., 2006).

On the basis of the above considerations, the inheritance of the *p16INK4A* epimutation (aberrant epigenetic state) described here, is more probably associated with sequence variants that predispose to methylation, rather than to a germline transmission. The identification of sequence variations that could account for a *p16INK4A* methylation propensity will be the subject of a future investigation.

In most cases analyzed in the present work, both methylated and unmethylated DNA was observed. This partial methylation of *p16INK4A* promoter in peripheral blood cells from retinoblastoma patients could correspond to a germline first "hit" of an "expanded two hit model," which includes also epigenetic mechanisms of gene inactivation (Jones and Laird, 1999). This epigenetic first "hit" would inactivate one allele of the *p16INK4A* gene in all cells of the body. The partially methylated retina cells then could acquire a somatic second "hit" (a mutation or a second epigenetic alteration) to progress to cancer. According to this hypothesis, a methylation level of 50% was expected in peripheral blood samples. Nevertheless, deviations from this value were observed in most cases reported here. A similar discrepancy was also noted by other investigators (Chan et al., 2006; Snell et al., 2008). A possible explanation, which was proposed by Chan et al. (2006), might be found in a study examining the inheritance of an epimutation in the flowering plant *Linaria vulgaris* (Cubas et al., 1999). This epimutation is inherited in a stable manner, but is less stable in somatic development during which spontaneous demethylation can occur. Similar somatic demethylation processes could explain the observed deviations from the expected methylation level.

A somatic demethylation process could also explain the finding that some retinoblastoma tumor samples are mosaics consisting of a low percentage of cells expressing p16INK4A and a high percentage of cells not showing this expression. It can be hypothesized that the tumor originates from one retina cell in which both *p16INK4A* alleles are inactivated (one allele by a germline methylation and the other one by a mutation or a second epigenetic alteration), but during the subsequent tumor growth, some cells undergo a demethylation process.

Recently, epigenetic modifications have been increasingly recognized as one of the most common molecular alterations in human neoplasia and they have been associated with the inactivation of tumor suppressor genes and chromosomic instability (Macaluso et al., 2003). Studies on epigenetic changes have led to the development of epigenetic drugs for the treatment of cancer (Graham et al., 2009). In addition to *p16INK4A*, whose hypermethylation is reported in the present article, other aberrantly methylated genes have also been identified in retinoblastoma tumors (Harada et al., 2002; Choy et al., 2005; Tosi et al., 2005). Because these alterations might have an important role in retinoblastoma progression, the potential anticancer properties of demethylating agents should be investigated.

As stated above, a detailed understanding of the sequence of molecular events that starting from *RB* mutations lead to malignancy, could allow the design, not only of new therapeutic strategies but also of preventive approaches. In fact, *RB* loss is insufficient, by itself, for the tumor to develop and an early intervention aimed at halting the subsequent molecular events could prevent the tumor onset. The identification of *p16INK4A* promoter methylation as a new possible epigenetic risk factor for retinoblastoma represents a step forward in understanding the molecular mechanisms contributing to retinoblastoma pathogenesis and in designing new preventive strategies, based on the use of demethylating agents. Moreover, the finding that the *p16INK4A* promoter methylation seems to be a germline and heritable alteration opens up the new possibility of monitoring high-risk families through simple blood sample analysis.

In conclusion, for the first time it is provided evidence of an inherited *p16INK4A* downregulation in retinoblastoma families, suggesting that this alteration might be a possible novel heritable susceptibility marker to retinoblastoma. More importantly, the observation that *p16INK4A* downregulation seems to be due to its promoter hypermethylation opens the way for new preventive and therapeutic approaches.

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