FACULDADE DE MEDICINA DA UNIVERSIDADE DE COIMBRA

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P53, MDM2 AND P14ARF IMMUNOHISTOCHEMICAL EXPRESSION IN RETINOBLASTOMA

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Trabalho Final do 6º Ano Médico com vista à atribuição do grau de Mestre no âmbito do Ciclo de Estudos de Mestrado Integrado em Medicina, realizado sob a orientação do Professor Doutor Rui Proença (Faculdade de Medicina da Universidade de Coimbra) e da Professora Doutora Lina Carvalho (Faculdade de Medicina da Universidade de Coimbra).

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List of Abbreviations

CDK – Cyclin dependent Kinase

CpG – Cytosine-phosphate-Guanine

DAB- 3,3- diaminobenzidine tetrahydrochloride

DNA- Deoxyribonucleic acid

EDTA- Ethylenediamine tetraacetic acid

E2F- E2F transcription factor

E2F1- E2F transcription factor 1

HIF- Hypoxia inducible factor 1

HPV 16- Human papillomavirus 16

HUC- Hospitais da Universidade de Coimbra

INK4a/ARF- Alternative reading frame of INK4A gene

MDM2- Mouse Double Minute 2

Mdm2- MDM2 protein

MDMX- Equivalent to MDM4 (Mouse Double Minute 4)

mRNA- Messenger ribonucleic acid

Myc- Myc oncogene

p14- Protein 14

p53- Tumor protein p53

PBS- Phosphate-buffered saline

pRB- Retinoblastoma protein

***Ras*-** Ras oncogene

RB1- Retinoblastoma gene

Abstract

Introduction: Retinoblastoma is the most common primary ocular malignancy in pediatric age. *Knudson* proposed his *two-hit* model, allowing the distinction of retinoblastoma in two major classes: heritable and non-heritable. Retinoblastoma was first considered to arise from a well known mutation in the RB1 tumor-suppressor gene (chromosome 13q14). Currently, evidence supports that biallelic inactivation of RB1 gene is the initiating event, but not sufficient for fully malignant progression [1]. The hypothesis of altered expression of $p14^{ARF}$-MDM2-p53 surveillance pathway components was proposed as an attempt to explain fully retinoblastoma development [2].

Previous studies proposed that $p14^{ARF}$ protein expression was undetectable, in contrast with Mdm2 protein overexpression in retinoblastoma [3].

Objectives: The aim of this study was to evaluate the immunohistochemical expression of p53 pathway components ($p14^{ARF}$, Mdm2 and p53) in order to a better understanding of the molecular pathogenesis and differentiation of retinoblastoma. Additionally, it was attempted to correlate the expression of these proteins with retinoblastoma's heritable pattern, Reese-Ellsworth staging and vital prognosis.

Methods: A cohort of 24 retinoblastoma tissue samples from 22 enucleated cases was obtained from the registry of HUC's Ophthalmic Pathology Laboratory. Clinical records were consulted to collect information including gender, age, heritable pattern, Reese-Ellsworth stage and prognosis. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded retinoblastoma tissue samples using primary antibodies against p53, $p14^{ARF}$ and Mdm2.

Results: Positive p53, $p14^{ARF}$ and Mdm2 expression was obtained in 87.5% (21/24), 87.5% (21/24) and 95.8% (23/24) of the 24 samples, respectively. Overall, p53 protein expression was not positively correlated neither with $p14^{ARF}$ ($p=0.343$) nor Mdm2 expression

(p=1.000). In addition, $p14^{ARF}$ expression was mainly found in tissue samples that were positive for both p53 and Mdm2. Moreover, we did not obtain a positive relationship between p53, $p14^{ARF}$ and Mdm2 expression and the analyzed clinical parameters (heritable pattern, vital prognosis and Reese-Ellsworth staging).

Conclusions: In our study, we obtained 87.4% of positive $p14^{ARF}$ nuclear and nucleolar expression and we even documented the presence of $p14^{ARF}$ overexpression in half of the cases, in opposition to previous reports [3]. According to our results, there was a Mdm2 overexpression in 79.2% of retinoblastoma samples, which supports the hypothesis that MDM2 overexpression may be an important element in retinoblastoma molecular pathogenesis [2,4].

The small cohort of patients involved in this study compromised the final results, which did not show any statistical significance. Further studies need to be performed in order to establish the true prognostic value of these histological markers, using a larger retinoblastoma patient's population.

Key-words: Retinoblastoma, pRB, p53, $p14^{ARF}$, Mdm2, immunohistochemistry

Resumo

Introdução: O retinoblastoma é o tumor maligno intraocular primário mais comum em idade pediátrica. *Knudson* apresentou a proposta do modelo *two-hit*, permitindo distinguir dois grandes grupos de tumores: hereditários e não hereditários. Estabeleceu-se um nexo de causalidade entre a mutação no gene supressor tumoral RB1 (cromossoma 13q14) e o desenvolvimento do retinoblastoma. Evidências actuais sugeriram que a inactivação bialélica do gene RB1 é a lesão iniciadora, mas não é suficiente para a progressão completa do retinoblastoma [1]. Assim, para explicar o desenvolvimento deste tumor, foi proposta a hipótese de uma expressão alterada da via supressora tumoral $p14^{ARF}$-MDM2-p53 [2].

Trabalhos anteriores demonstraram que a expressão da proteína $p14^{ARF}$ era indetectável, ao contrário da Mdm2, que se apresentava sobre-expressa no retinoblastoma [3].

Objectivos: O objectivo deste trabalho foi avaliar a expressão imunohistoquímica dos componentes da via p53 ($p14^{ARF}$, Mdm2 e p53) para melhor compreender a patogenia e diferenciação moleculares do retinoblastoma. Tentou-se também, correlacionar a expressão destas proteínas com parâmetros clínicos dos doentes, nomeadamente o padrão de hereditariedade do tumor, estádio de Reese-Ellsworth e prognóstico vital.

Material e métodos: Foram obtidos 24 cortes histológicos de retinoblastomas de 22 doentes enucleados, provenientes do material em arquivo no Laboratório de Patologia Oftálmica dos HUC. Os seus registos clínicos foram consultados para recolher informação, incluindo idade, género, padrão de hereditariedade, estádio de Reese-Ellsworth e prognóstico vital. O estudo imunohistoquímico foi realizado em cortes histológicos de retinoblastoma incluídos em parafina.

Resultados: Foi obtida positividade da expressão das proteínas p53, $p14^{ARF}$ e Mdm2 em 87,5% (21/24), 87,5% (21/24) e 95,8% (23/24) das 24 amostras de retinoblastomas, respectivamente. Globalmente, a expressão de p53 não se correlaciona positivamente com a

expressão de $p14^{ARF}$ (p=0,343) nem de Mdm2 (p=1,000). Adicionalmente, a expressão de $p14^{ARF}$ foi demonstrada principalmente em amostras de tumores com positividade de expressão para as proteínas p53 e Mdm2, simultaneamente. Igualmente, não foi possível estabelecer qualquer relação entre as expressões das proteínas p53, $p14^{ARF}$ e Mdm2 e os parâmetros clínicos analisados (padrão de hereditariedade, estádio de Reese-Ellsworth e prognóstico vital).

Conclusões: Neste estudo observámos que 87,4% dos casos apresentaram marcação nuclear e nucleolar da proteína $p14^{ARF}$ e, concomitantemente, documentámos a sobre-expressão da desta proteína em metade dos casos, contrariamente a resultados de trabalhos anteriores [3].

De acordo com os nossos resultados, obtivemos sobre-expressão da proteína Mdm2 em 79,2% das amostras de retinoblastomas, o que está de acordo com a hipótese que defende que a sobre-expressão do MDM2 será um elemento importante na patogenia molecular do retinoblastoma [2,4].

A pequena amostra de doentes utilizada neste estudo comprometeu os resultados finais, nos quais não se demonstrou qualquer relação estatisticamente significativa entre os parâmetros considerados. Futuros estudos devem ser realizados no sentido de estabelecer o verdadeiro valor prognóstico destes marcadores histológicos, recorrendo a uma amostra populacional de dimensões superiores.

Palavras-chave: Retinoblastoma, pRB, p53, $p14^{ARF}$, Mdm2, imunohistoquímica.

Introduction

Retinoblastoma is the most common primary intraocular malignant tumor in children, representing roughly 4% of all pediatric malignancies. Most cases occur under the age of 5 years (90%) and the average age of presentation for heritable retinoblastoma is 3 to 18 months, whereas non-heritable retinoblastoma usually presents between 18 to 24 months [5,6].

In 1972, *Alfred Knudson* proposed his *two-hit* model to explain the genetic etiology of retinoblastoma, which allowed its classification in two major groups: heritable and non-heritable. This hypothesis was able to establish a connection between a mutation in the first identified tumor-suppressor gene (RB1, chromosome 13q14) and the development of the tumor. According to *Knudson,* in the heritable retinoblastoma, a mutation in the RB1 gene is inherited via the germline and the second mutation (*second hit*) occurs in somatic cells [5,6].

Heritable retinoblastoma comprises 40% of all cases and the patients are heterozygous for a RB1 mutation. Heterozygous Rb^+/Rb^- individuals only require a single silencing mutation of functioning RB1 allele to originate the *loss of heterozigosity* phenomenon, with subsequent tumor formation. This fact easily correlates with the more precocious age of onset in children with heritable retinoblastoma. Besides, in 90% of these patients, the tumor is bilateral and multifocal, due to the existence of a vast population of heterozygous retinoblasts, susceptible to somatic inactivation of the only functional RB1 allele [6].

Non-heritable retinoblastoma include the other 60% of cases, and the patients are constitutionally RB1 wild-type homozygous (Rb^+/Rb^+), exhibiting acquired somatic mutations in a retinoblast progenitor, in order to originate the tumor cell population. Naturally, such tumors are near universally unilateral, with later ages of onset.

The RB1 gene encodes a phosphoprotein (pRB) which has a tumor suppressor function that plays a central role on the cell cycle regulation. This ability lies mainly on its capacity of arresting cell proliferation in G1, by inhibiting the activity of E2F transcription factors. pRB binds to a number of polypeptides belonging to the E2F family, particularly E2F1, sequestering them and preventing cell cycle progression [6].

Retinoblastoma is one of the few tumors in which the initial genetic mutation is known. Several studies suggested that retinoblastoma bypasses p53 tumor suppressor pathway because it arises from intrinsically death-resistant cells [2]. As the human retinoblastoma express p53 wild type, it was firstly assumed that p53 pathway remained intact. However, *Laurie et al* [2] showed that the tumor surveillance pathway mediated by $p14^{ARF}$-MDM2-p53 is activated after loss of RB1, leading RB^-/RB^- retinoblasts to programmed cell death. This fact implies that the retinoblasts from which retinoblastoma arises must present disruptions in both p53 and pRB suppressor pathways [2].

p53 is described as the "genome guardian" because of its central role in stress response to DNA damage and hyperproliferative signals, in order to control the growth and survival of potentially malignant cells. This response is made possible by the cell cycle arrest in G1-S check-point, in order to trigger a variety of DNA repair mechanisms or induce apoptosis, when such repair is not viable [7].

Another gene activated by p53 *wild-type* is MDM2 (*mouse double minute 2*), which encodes a protein capable of binding to the N-terminal region of p53 and negatively regulate its function. Besides, MDM2 protein (Mdm2) functions as an E3 ubiquitin ligase, which promotes degradation of p53, triggering its nuclear exportation and proteosomal destruction [4].

The product of the alternative reading frame INK4a/ARF locus, $p14^{ARF}$, appears as a fundamental element in the p53 surveillance pathway [8]. $p14^{ARF}$ is a sensor of hyperproliferative signals and acts as an upstream regulator of p53-MDM2 pathway, by binding to Mdm2 and blocking its ubiquitin ligase function. Consequently, its tumor suppressor role resides in the ability to stabilize p53. As it would be expected, focal loss of expression of $p14^{ARF}$ is a common finding in human tumors, reflecting partial silencing of $p14^{ARF}$ gene expression [7].

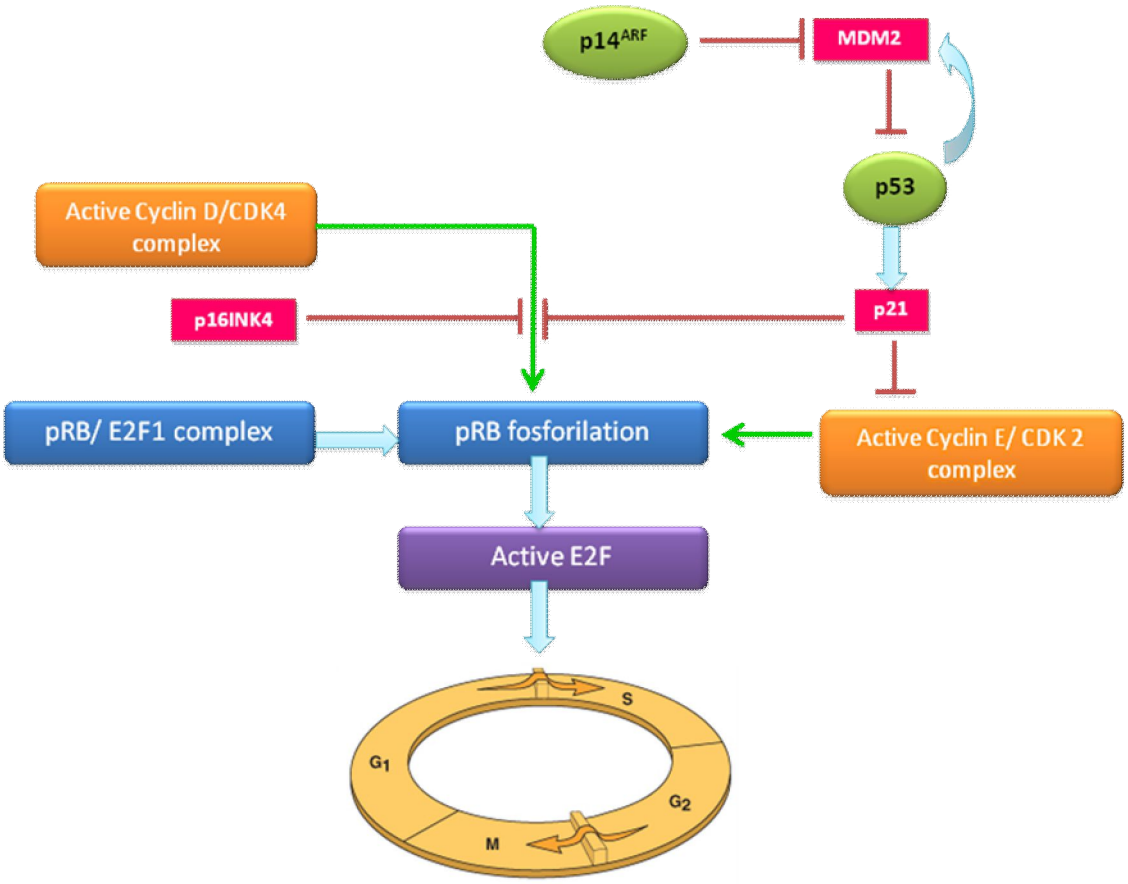

Figure 1: Cyclins and CDKs role in the regulation of G1-S check-point progression. Progression of the cell cycle is dependent upon the release of E2F, which occurs through phosphorylation of pRB. This phenomenon is achieved by the interaction of cyclins with CDKs. In mid/early G1, cyclin D complexes with CDK4, promoting phosphorilation of pRB. In late G1 phase, the complex cyclin E/CDK2 mediates further phosphorilation. The free E2F is then able to act as a transcriptional factor, by binding to gene promoters in DNA. The cell cycle arrest in response to DNA damage or other stimuli is under the regulation of p53. p53 levels are negatively regulated by MDM2, through a feedback loop, under the regulatory control of $p14^{ARF}$. (Green arrows indicate stimulation; red lines indicate inhibition)

The interaction of all the aforementioned proteins has been studied with the purpose to clarify the functional status of various tumor suppressor pathways and the implications of their deregulation in tumor development and progression. Nevertheless, the relationship of $p14^{ARF}$ protein level to Mdm2 and p53 status has not been elucidated in human retinoblastoma. The aim of this study is to evaluate the immunohistochemical expression of p53 pathway components ($p14^{ARF}$, MDM2 and p53) in a cohort of 24 retinoblastoma samples, in order to better understand the molecular pathogenesis of this tumor. Additionally, it will be attempted to correlate the expression of these proteins with retinoblastoma's heritable pattern, Reese-Ellsworth staging and vital prognosis.

Materials and Methods

1. Patients and Tissue Samples

Retinoblastoma tissue samples (24 specimens) were obtained from the records of HUC's Ophthalmic Pathology Laboratory. Those samples consisted on 24 primary retinoblastomas from 22 patients treated with curative intent by enucleation. These patients were followed in HUC's Ocular Oncology Unit and their clinical registries were consulted in order to evaluate the clinical characteristics, evolution and vital prognosis of the disease. Tumor stage for each patient was classified according to the Reese-Ellsworth system, the most popular grouping to predict chances of salvaging the affected eye. The patients' distribution according to their different clinical stage is presented on Table I:

Table I: Distribution and percentage of patients in different Reese-Ellsworth stages

Reese-Ellsworth	No. patients	Percentage of patients (%)
2A	7	31,8
2B	1	4,5
3A	8	36,4
4A	1	4,5
5B	5	22,7

The average age of enucleation was 3.45± 2.49 years and the median was 3 years. Most patients were male (63.6%) and the average age of this group was 4.28± 2.8 years. In contrast, the average age among the female group was 2.0± 1.3 years. 27.3%

of the patients (6/22) showed bilateral retinoblastoma and can be considered to present heritable retinoblastoma. Some of these patients (10/22 and 9/22) underwent chemotherapy and local therapies prior to enucleation (45.5% and 40.9%, respectively). Among the 6 patients presenting bilateral retinoblastoma, two of them had to undergo enucleation of the contralateral eye, as a life saving treatment. The rate of mortality was 13.6% (3/22 patients).

2. Immunohistochemistry

The immunohistochemical study was performed on formalin-fixed, paraffin-embedded retinoblastoma tissue samples. Three-micrometer tissue sections were placed on coated slides and allowed to dry overnight. After deparaffinization and rehydration, antigen unmasking was performed using Module PT (Lab Vision®) for citrate buffer for 25 minutes in p53 antibody, and 40 minutes microwave for EDTA in p14 and Mdm2 antibodies. Endogenous peroxidase activity was quenched using 15 minutes incubation in 3% diluted hydrogen peroxide (H_2O_2). For blocking nonspecific binding, Ultra V Block (Ultra Vision Kit®; TP-015-HL) was applied to the sections and then they were incubated at room temperature, with primary antibodies against p53 (clone DO-7; *DAKO*®) at a dilution of 1:40 for 30 minutes, $p14^{ARF}$ (clone N/A; Imgenex®) at a dilution of 1:40 for 30 minutes, and Mdm2 (clone IF2; Invitrogen®) at a dilution of 1:100 for 60 minutes. After washing with phosphate-buffered saline (PBS), slides were incubated with biotin-labeled secondary antibody (Lab Vision®) for 15 minutes. Primary antibody binding was localized in tissues using peroxidase-conjugated streptavidin (Lab Vision®) and 3,3-diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen, according to manufacturer's instructions. The slides were counterstained with hematoxylin, dehydrated and mounted. In parallel, known positive

and negative controls were used. As positive control for p53, normal skin sections were used. Cervical squamous cell carcinoma and breast fibroadenoma samples were employed as positive control for $p14^{ARF}$. Breast invasive ductal breast carcinoma samples were used as positive controls for Mdm2.

The immunohistochemistry slides were evaluated by an experienced pathologist, Prof. Drª Lina Carvalho, who was blinded to the clinical and pathological features of the patients. The intensity of the staining was graded semi-quantitatively on a three point scale, based on the percentage of immunostained cells. The levels were scored as follows: **0**- 0%; **+** < 25%; **++** 25-75%; **+++** > 75%. Overexpression was defined as more than 75% positive staining cells/nuclei (**+++)**.

3. Statistical analysis

The correlations between immunohistochemical results and clinicopathologic variables were analyzed by the Fisher Exact Test. Since Chi-Square test is not valid for small cohorts of patients, like the one used in this work, the Fisher Exact Test must be used, instead. A p value <0,05 was considered to be significant. All calculations were performed by using EPI Info software 3.5.1.version.

Results

1. p53, p14 ARF and Mdm2 immunohistochemistry expression and immunoscoring

The immunohistochemical expression of p53, $p14^{ARF}$ and Mdm2 was assessed in 24 retinoblastoma samples. Positive p53, $p14^{ARF}$ and Mdm2 protein expression was obtained in 87.5% (21/24), 87.5% (21/24) and 95.8% (23/24) of the 24 samples, respectively. $p14^{ARF}$ expression was considered positive in cases of nuclear and nucleolar staining, in contrast with p53 and Mdm2, in which only nuclear staining was considered. Nucleolar $p14^{ARF}$ expression was present in only one case among the positive $p14^{ARF}$ samples.

Overexpression of p53, $p14^{ARF}$ and Mdm2 was observed in 41.7% (10/24), 50% (12/24) and 79.2% (19/24) of all samples, respectively (Figures 2, 3, 4, 5, 6, 7, 8 and 9)

2. Correlation between p53, p14 ARF and Mdm2 expression

The expression of these proteins was evaluated and correlated in all the 24 retinoblastoma samples. No significant correlation was found between p53 and $p14^{ARF}$ expression ($p = 0.343$), similarly to the inexistence of correlation between p53 and Mdm2 expression ($p=1.000$) (Tables II and III). Equally, the association between $p14^{ARF}$ and Mdm2 expression did not show statistic significance ($p=0.125$) (Table III). We did not find a positive relationship between the intensity of expression of these three proteins.

Table II: Correlation of p53 protein expression with $p14^{ARF}$ and Mdm2 status

	p53 +	p53 -	p- value
Mdm2 +	20	3	1.000
Mdm2 -	1	0	
$p14^{ARF}$ +	19	2	0.343
$p14^{ARF}$ -	2	1	

Table III: Correlation of $p14^{ARF}$ protein expression with Mdm2 status

	$p14^{ARF}$ +	$p14^{ARF}$ -	p- value
Mdm2 +	21	0	0.125
Mdm2 -	2	1	

When evaluating the association between the frequency of $p14^{ARF}$ and p53 and Mdm2 (p53/Mdm2) levels of expression, we observed that the presence of $p14^{ARF}$ staining was more often observed in cases with both p53 and Mdm2 positivity (95%). In contrast, there was no $p14^{ARF}$ expression in both p53 and Mdm2 negative cases (0%) (Table IV).

Table IV: Frequency of $p14^{ARF}$ expression according to different p53 and Mdm2 status

	$P14^{ARF}$ +	$p14^{ARF}$ -	Percentage of expression
$p53^{-}$/ $Mdm2^{-}$	0	0	0%
$p53^{-}$/ $Mdm2^{+}$	2	1	66.6%
$p53^{+}$/$Mdm2^{-}$	0	1	0%
$p53^{+}$/$Mdm2^{+}$	19	1	95%

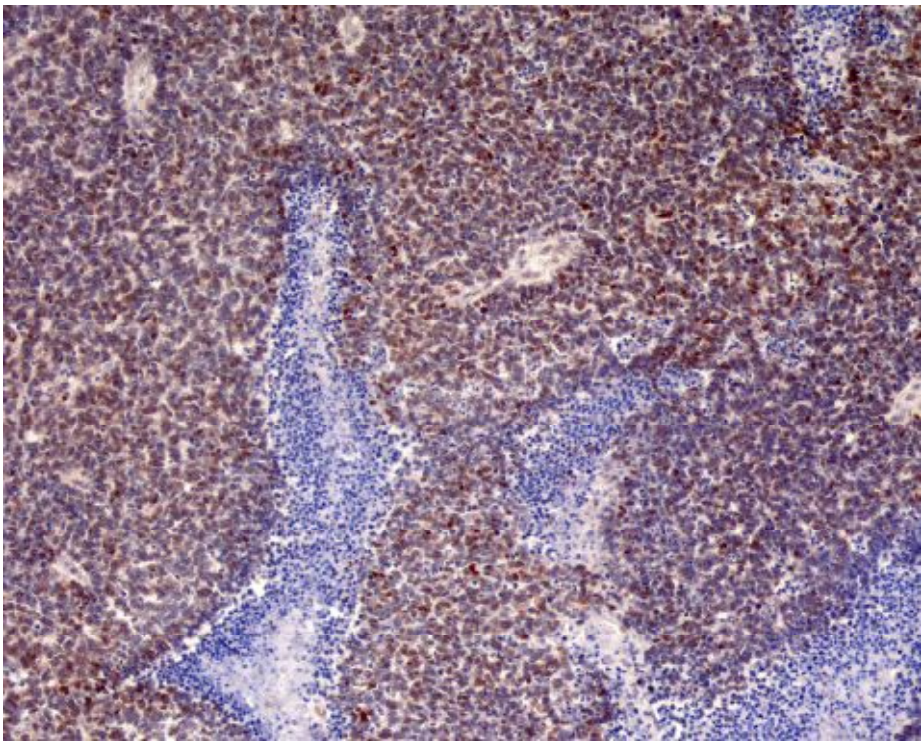

Figure 2: Immunohistochemical staining pattern of p53 protein in retinoblastoma sample (x100): Tumor cells overexpressing p53 protein

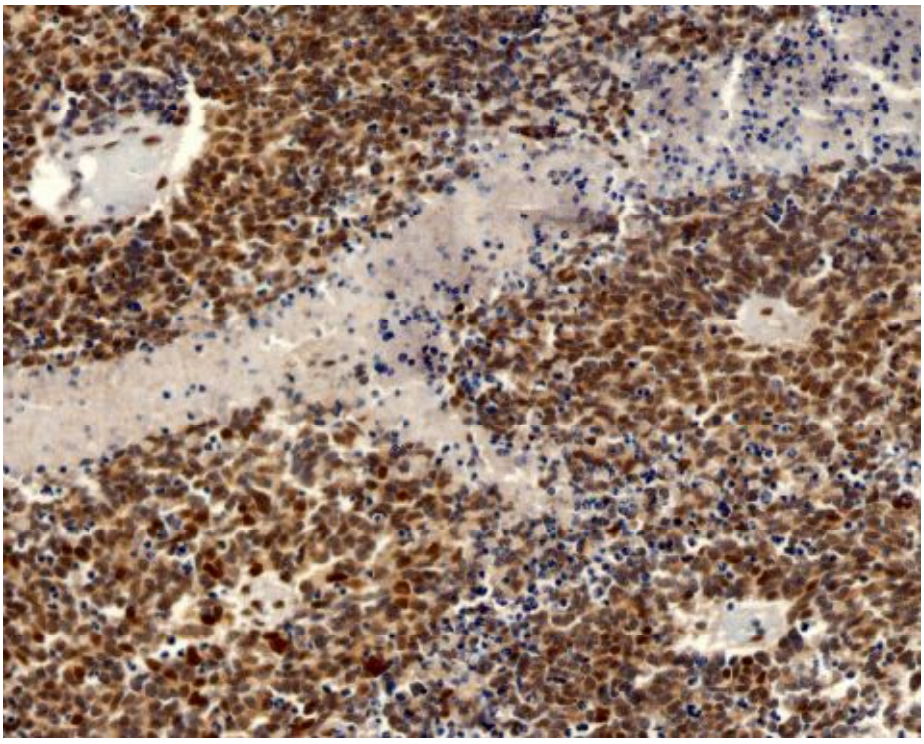

Figure 3: Immunohistochemical staining pattern of p53 protein in retinoblastoma sample (x200): Tumor cells overexpressing p53 protein

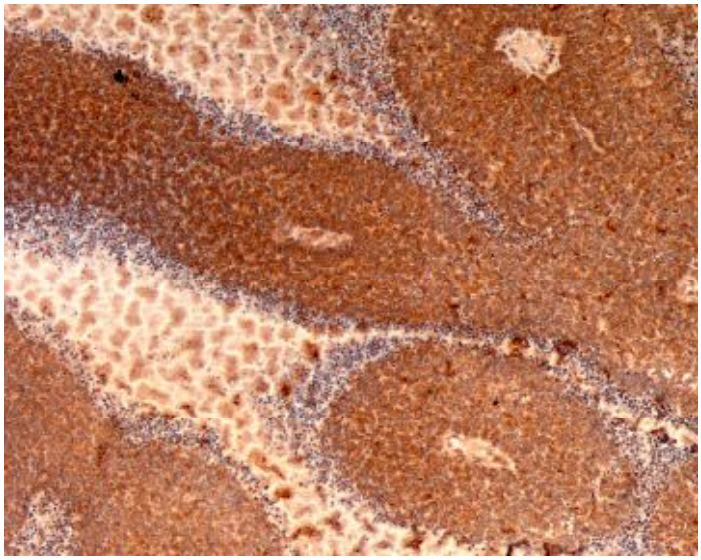

Figure 4: Immunohistochemical staining pattern of $p14^{ARF}$ protein in retinoblastoma sample (x100): Tumor cells overexpressing $p14^{ARF}$ protein with nuclear staining.

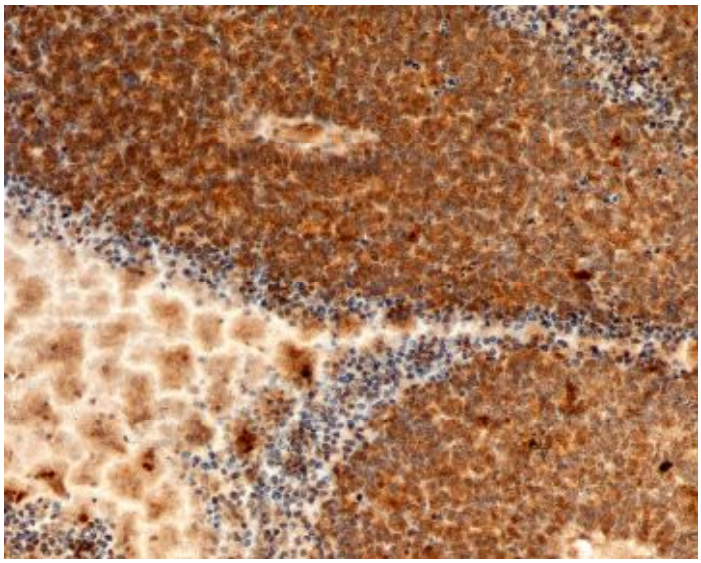

Figure 5: Immunohistochemical staining pattern of $p14^{ARF}$ protein in retinoblastoma sample (x200): Tumor cells overexpressing $p14^{ARF}$ protein with nuclear staining.

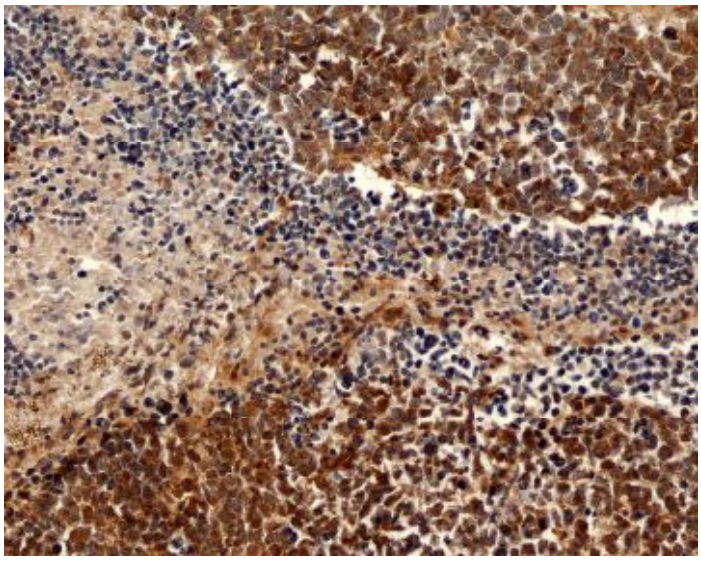

Figure 6: Immunohistochemical staining pattern of $p14^{ARF}$ protein in Retinoblastoma (x200): Tumor cells overexpressing $p14^{ARF}$ protein with nuclear staining.

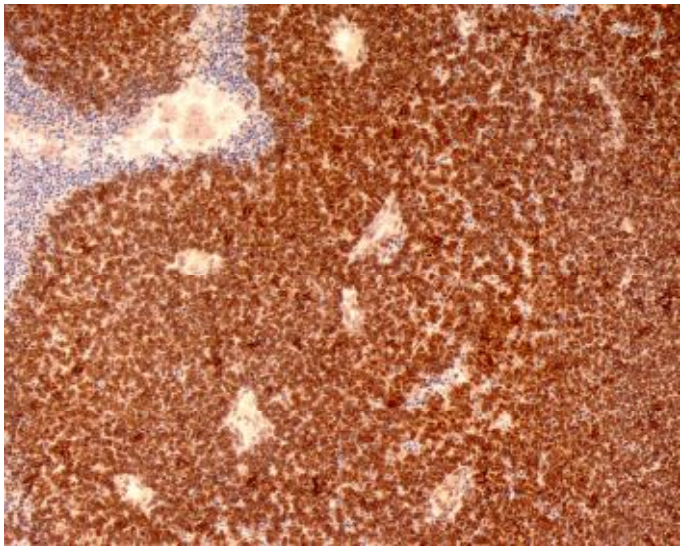

Figure 7: Immunohistochemical staining pattern of Mdm2 in retinoblastoma sample (x100): Tumor cells overexpressing Mdm2 protein with nuclear staining.

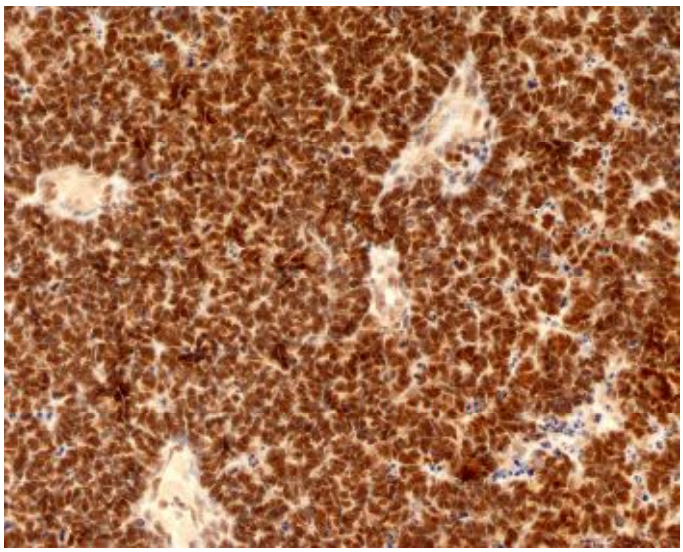

Figure 8: Immunohistochemical staining pattern of Mdm2 in retinoblastoma sample (x200): Tumor cells overexpressing Mdm2 with nuclear staining.

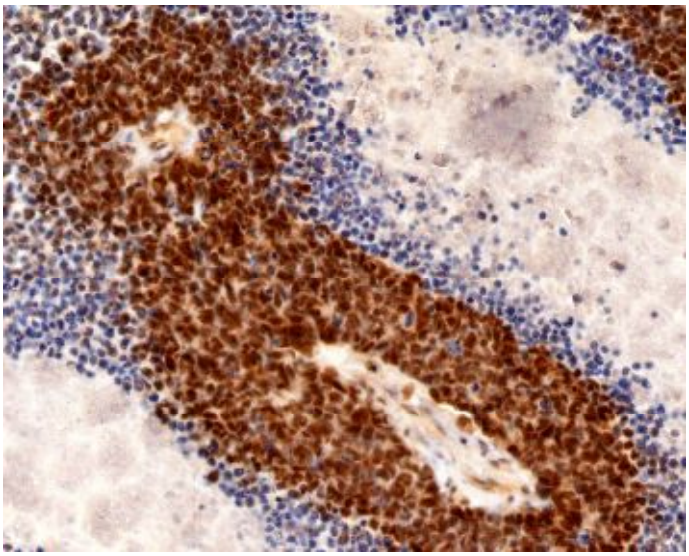

Figure 9: Immunohistochemical staining pattern of Mdm2 in retinoblastoma sample (x200): Tumor cells overexpressing Mdm2 with nuclear staining.

3. Correlation between p53, p14ARF and Mdm2 expression and clinical parameters

Parameters as heritable pattern, vital prognosis and Reese-Ellsworth staging were considered in the 22 patients diagnosed with retinoblastoma. It has been attempted to correlate the expression of p53, p14ARF and Mdm2 with the mentioned variables. The tumor samples of 20 patients expressed p53 protein (90.9%). There was no significant association between p53 positive staining and heritable pattern or vital prognosis. Also, the intensity of p53 staining did not correlate positively with any of the analyzed parameters (Table V).

Table V: Relationship of p53 expression with clinical parameters

	p53 +	p53 -	p- value
Bilateral	6	0	1.000
Unilateral	14	2	
Death +	3	0	1.000
Death -	17	2	

Similar results were obtained concerning p14ARF and Mdm2 expression. Among the 22 considered patients, 19 (86.4%) and 21(95.5%) positively expressed p14ARF and Mdm2, respectively. Again, neither their positive expression nor their intensity of expression correlated with heritable pattern and vital prognosis of the patients (Tables VI and VII).

Table VI: Relationship of $p14^{ARF}$ expression with clinical parameters

	$p14^{ARF}$ +	$p14^{ARF}$ -	p- value
Bilateral	6	0	0.532
Unilateral	13	3	
Death +	3	0	1.000
Death -	16	3	

Table VII: Relationship of MDM2 expression with clinical parameters

	Mdm2 +	Mdm2 -	p- value
Bilateral	6	0	1.000
Unilateral	15	1	
Death +	3	0	1.000
Death -	18	1	

At last, it was attempted to establish a correlation between the expression of these immunohistochemical markers and the clinical Reese-Ellsworth staging of the 22 evaluated patients. Once more, the results obtained failed to demonstrate a statistical significance (p=0.903 for p53, p=0.738 for $p14^{ARF}$ and p=0.766 for Mdm2).

Discussion

Retinoblastoma was first thought to develop primarily from silencing mutations of RB1 alleles, which would cause the inability to arrest cell cycle proliferation. However, studies about the consequences of pRB loss in chimeric mice showed that pRB-deficient retinoblasts tend to undergo p53 dependent apoptosis [9]. Supporting these evidences is the work of *Howes et al,* which demonstrated that loss of pRB induced by expression of HPV-16 E7 oncoprotein resulted in cell death rather than cell proliferation [9].

Mastrangelo et al [1] proposed that loss of RB1 leads to progressive genomic instability, resulting in acquisition of additional mutations that ultimately lead to proliferative retinoblastoma. These authors state that biallelic inactivation of the RB1 gene is the initiating event but it is not sufficient for fully progression. In this new hypothesis, it was suggested that aneuploidy (gains or losses of different regions of the genome or epigenetic alterations) and not RB1 inactivation *per se,* is the initiating event in RB tumor formation [1].

Since retinoblastoma expresses *wild-type* p53, it was firstly assumed that p53 pathway was intact and the status of other components of this pathway was ignored [2]. Furthermore, transactivation of MDM2 gene is achieved by p53 *wild-type* but not by its mutant form [7], which implies that immunohistochemical expression of Mdm2 argues against the presence of p53 mutations. In this study, we confirmed the simultaneous expression of *wild type* p53, results that appear to contradict p53 mutations in retinoblastoma.

RB-deficient cells bypass the G1-checkpoint response and undergo p53 dependent apoptosis [10], which is in agreement with *Nork et al* [11], who showed a

close association between p53 immunoreactive cells and apoptotic cells in retinoblastoma, suggesting that p53 plays a role in regulating cell death [11].

All the previous findings imply that cells from which retinoblastoma arises must also present disruptions in the p53 suppressor pathway, but not in the p53 gene *per se.* Accordingly, *Laurie et al* [2] proposed that inactivation of the p53 pathway promotes the transition from differentiated retinoblastoma cells with amacrine/horizontal cell features to a more immature cell with retinal progenitor cell features [2].

p53 gene is a well known tumor suppressor gene and its product is a transcriptional factor that plays an important role in response to DNA cellular damage. It induces G1/S cell cycle arrest in order to proceed to DNA repair or apoptosis, the latter in case of irreparable damage. Among different kinds of inducing stimuli, hypoxia, DNA damage, oncogene activation and senescence can activate p53-mediated response [7]. Alterations in the p53 suppressor gene pathway are present in more than 50% of all human tumors [12]. Although p53 point mutation is considered to be the most frequent genetic alteration in human cancer [12], this phenomenon does not appear to take place in human retinoblastoma.

According to the work of *Laurie et al* [2] p53 pathway would be subverted in retinoblastoma cells by increased expression of MDMX or MDM2 genes [4]. Mdm2 is a multifunctional protein which negatively regulates p53 in several ways: 1) Mdm2 binds to p53, interfering with its ability to transactivate target genes; 2) It has an ubiquitin ligase activity, which targets p53 to proteosomal degradation; 3) p53 is transported to the cytoplasm by Mdm2 and degradated by cytoplasmic proteosomes [13]. *Chang et al* [14] also contributed to a better understanding of the Mdm2 functions, as they showed that Mdm2 interacts with pRB and promotes its proteosomal

destruction. Furthermore, these authors also demonstrated that Mdm2 binds to pRB and prevents its interaction with E2F1, promoting cell cycle progression [14].

Interestingly, according to *Seville et al* [15], E2F1 release from pRB is associated with cell cycle progression but, above a certain threshold, E2F1 has the ability to trigger apoptosis [15]. A two-threshold model has been proposed to explain E2F1 function. If the first threshold E2F1 level is passed, cells will by-pass the first check-point and proceed in the cell cycle. However, when the second E2F1 level threshold is reached, this transcription factor will switch in order to promote apoptosis [15]. *Pan et al* [15] showed that E2F1 was essential in p53-mediated apoptosis. One model proposed that E2F1 could act through transcriptional activation of INK4A/ARF gene ($p14^{ARF}$ protein), as this gene is a well known E2F1-responsive gene [15].

Other p53 independent MDM2 functions have been described, like the ability to inhibit E2F1-induced apoptosis [16]. The growth promoting and proliferative functions of Mdm2 on E2F1 could be important to further understand MDM2 oncogenic activities [16].

Considering the aforementioned evidence, a new hypothesis for MDM2 oncogenic activity in retinoblastoma can be proposed. Human retinoblastoma cells harbor mutated RB1 gene and *wild-type* p53 gene, resulting in high E2F transcriptional activity [17]. As mentioned above, E2F1 overexpression can induce p53-induced apoptosis, through enhanced INK4A/ARF transcription. Accordingly, MDM2 overexpression would inhibit E2F-induced apoptosis [16], making it impossible for $p14^{ARF}$ to be activated and, consequently, the MDM2-induced p53 degradation would not be inhibited.

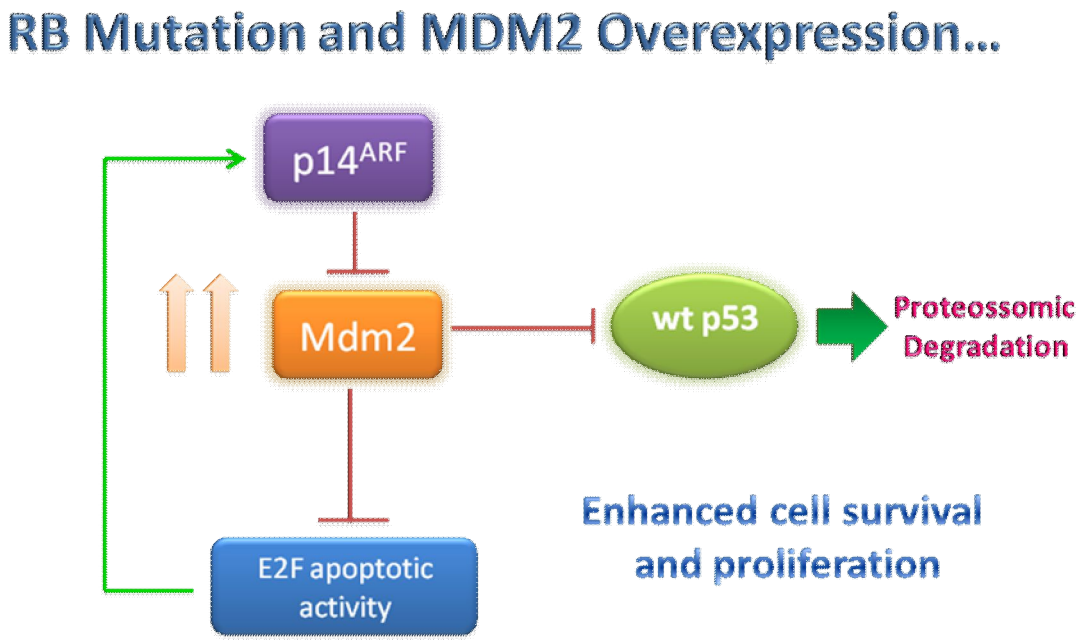

Figure 10: Increased expression of Mdm2 in the absence of RB triggers p53 nuclear exportation and proteosomic activation. Mdm2 overexpression is also able to inhibit E2F1 apoptotic activity, which would occur through $p14^{ARF}$ activation. Consequently, $p14^{ARF}$'s ability to trigger p53-dependent cell cycle arrest would be compromised. (Green arrow indicates stimulation; red lines indicate inhibition)

Overexpression of Mdm2 results from gene amplification, enhanced transcription and/or translation, as has been reported in different tumors [7]. In fact, this gene was originally identified as a highly amplified gene in a transformed tumorigenic fibroblast cell line [12], and amplifications of the MDM2 gene or MDM2 overexpression were reported in 15-36% of human sarcomas, 7-54% of non-small cell lung cancers and 18-36% of esophageal carcinomas [18]. Reflecting these findings, analysis of human retinoblastoma reveals that MDMX and MDM2 genes are amplified in 65% and 10% of the tumors, respectively [2]. *Ying*, G *et al* [3] showed that MDM2 was expressed in all the retinoblastoma samples and cell lines tested [3]. In our work, we obtained Mdm2 overexpression in 79,2% of the 24 retinoblastoma samples. These results confirm previous evidences that MDM2 overexpression may be an important element in retinoblastoma molecular pathogenesis.

Among p53-suppressor pathway gene products is $p14^{ARF}$ which, in theory, is capable of antagonizing all Mdm2 functions. $p14^{ARF}$ constitutively localizes to the nucleolus, whereas p53 and Mdm2 are predominantly nucleoplasmic. Mdm2 sequestration in the nucleolus by $p14^{ARF}$ has been implicated in p53 activation and related to growth inhibitory potential [19]. According to this, we found nucleolar $p14^{ARF}$ expression in one case of our series. However, it is well known that when high levels of $p14^{ARF}$ nuclear expression are present, it becomes very difficult to accurately assess its nucleolar expression [19].

$p14^{ARF}$ tumor suppressor protein acts in order to unleash the p53 apoptotic response and exit from the cell cycle, by binding to Mdm2 and blocking its ubiquitin ligase function. Consequently, its tumor suppressor role relies on the ability to stabilize p53, which accumulates in the nucleoplasm. Three theories arose in order to explain this phenomenon: 1) $p14^{ARF}$ sequesters Mdm2 in the nucleolus preventing p53 export from the nucleus; 2) Mdm2-p53 complex exits the nucleus through the nucleolus and $p14^{ARF}$ interferes with this transport; 3) Ternary complexes of $p14^{ARF}$-Mdm2-p53 can be formed and aggregate to constitute “nuclear bodies” that maintain their transcriptional activity [13].

Although it was not a statistically significant finding, we showed that $p14^{ARF}$ was more frequently expressed in retinoblastoma samples which also positively expressed both p53 and Mdm2. This result suggests an involvement of $p14^{ARF}$ in p53 stabilization, in the presence of Mdm2 positive expression.

Considering $p14^{ARF}$‘s functions, it has been proposed that its loss could be functionally similar to the loss of p53. Consistent with this concept, many human tumors that retain *wild-type* p53, as retinoblastoma does, suffer loss of $p14^{ARF}$ and are unable to activate p53 in response to abnormal signals [19]. Induction of INK4a/ARF is

achieved by hyperproliferative signals from oncogenes such as *Ras*, overexpression of *Myc* and deregulated E2F [20]. Acute RB loss induces $p14^{ARF}$ expression, through the activity of the transcription factor E2F, which provides a link between the pRB and p53 pathways [19]. In contrast, other studies showed that $p14^{ARF}$- induced growth arrest is inhibited by simultaneous inactivation of both p53 and RB, but not by p53 alone. These data imply that $p14^{ARF}$ has a p53 independent activity, mainly through RB pathway. Besides, *Chang et al* [14] suggested an attenuation of $p14^{ARF}$ growth suppression function in case of pRB depletion [14].

Additionally, other p53-independent $p14^{ARF}$ functions have also been reported: vascular regression in the developing eye, cell cycle arrest in murine embryo fibroblasts lacking p53, interaction with other regulatory molecules as topoisomerase I and HIF (*hypoxia inducible factor 1*) [7]. Furthermore, $p14^{ARF}$ is able to suppress growth independently from p53, by delaying S-phase progression by interaction with DNA replication protein A, thus reducing the rate of DNA synthesis [21].

As it would be expected, focal loss of expression of $p14^{ARF}$ is a common finding in human tumors, reflecting partial silencing of $p14^{ARF}$ gene expression. Many reports implicated $p14^{ARF}$ inactivation in the pathogenesis of different human tumors, through homozygous delection, CpG island promoter methylation and less frequently, point mutation [7]. On the contrary, *Laurie N et al* [2] confirmed that expression of $p14^{ARF}$ mRNA was increased 71 to 500 folds in the retinoblastoma tumor samples, in contrast with normal human fetal retinae [2]. Later, *Ying G. et al* [3] showed that $p14^{ARF}$ mRNA levels were dramatically increased in primary retinoblastomas and retinoblastoma cell lines, whereas $p14^{ARF}$ protein expression was undetectable. This finding was proposed to correspond to a post-transcriptional inactivation of $p14^{ARF}$, that would associate to MDM2 and MDMX overexpression in order to trigger retinoblastoma progression. Our

study did not confirm the absence of $p14^{ARF}$ protein expression, since we obtained 87,4% of positive $p14^{ARF}$ nuclear and nucleolar expression in our retinoblastoma series. In fact, we even showed the presence of $p14^{ARF}$ overexpression in half of the cases. These results are supported by the previous description of increased $p14^{ARF}$ mRNA levels and are in opposition to *Ying G. et al* [3] prior reports.

The aforementioned hypothesis of MDM2 overexpression and its interaction with E2F apoptotic functions does not explain the increased levels of $p14^{ARF}$ mRNA and protein. However, E2F1 overexpression is not the only mechanism to stimulate INK4a/ARF transcription and we must also consider other p53-independent functions.

We did not find a significant relationship between p53, $p14^{ARF}$ and Mdm2 expression and the proposed clinical parameters (heritable pattern, vital prognosis and Reese-Ellsworth staging). Therefore, the significance of these proteins as prognostic markers was not recognized. Nevertheless, we should remind that, connected to the relatively low incidence of retinoblastoma, the number of cases available for this study was considerably undersized. Consequently, as the cohort of 22 patients involved in this study was too small, final results did not show statistical significance. Further studies need to be performed in order to establish the true prognostic value of these histological markers, using a larger retinoblastoma patient's population. Additionally, the mechanisms by which $p14^{ARF}$ and Mdm2 interact to facilitate retinoblastoma progression require further analysis, and their functional relevance in oncogenesis provides an interesting target for potential therapeutic agents.

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