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Expression of $p14^{ARF}$, *MDM2*, and *MDM4* in human retinoblastoma

Ying Guo [a], Sanja Pajovic [a], Brenda L. Gallie [a,b,c,d,*]

[a] Division of Applied Molecular Oncology, Ontario Cancer Institute/Princess Margaret Hospital, University Health Network, Room 8-415, 610 University Avenue, Toronto, ON, Canada
[b] Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada
[c] Department of Molecular and Medical Genetics, University of Toronto, Toronto, ON, Canada
[d] Department of Ophthalmology, University of Toronto, ON, Canada

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ABSTRACT

It is still not clear whether the p53 pathway is altered in retinoblastoma development. We assessed the expression of the p53 pathway genes $p14^{ARF}$, *mouse double minute 2* (*MDM2*), and *mouse double minute 4* (*MDM4*) in human retinoblastoma compared to normal retina. Primary human retinoblastomas, retinoblastoma cell lines and normal retinas were assessed for $p14^{ARF}$ and *MDM4* mRNA by quantitative RT-PCR. $p14^{ARF}$, MDM2, and MDM4 protein were measured by immunoblot and immunohistochemistry. Compared to retina, $p14^{ARF}$ mRNA expression was notably increased in retinoblastoma but $p14^{ARF}$ protein was undetectable. MDM2 and MDM4 proteins were expressed in 22/22 retinoblastomas. MDM2 was expressed in 3/10 retinas tested, and MDM4 in 10/10 retinas. The expression level of MDM2 protein in retinoblastomas and retina was comparable, while MDM4 protein was overexpressed in one retinoblastoma cell line Y79 and two primary retinoblastomas. We observe that overexpression of MDM2 and MDM4 is not a necessary step in retinoblastoma development. However, loss of detectable $p14^{ARF}$ protein and resultant lack of functional inactivation of these p53 inhibitors may contribute to retinoblastoma development by constitutive inhibition of p53.

$p14^{ARF}$ (human)/$p19^{ARF}$ (mouse) is an important tumor suppressor and its expression is lost in many cancers [1–4]. $p19^{ARF}$-null mouse embryo fibroblasts can be transformed by a single oncogene such as *c-Myc* or *RasV12*, and $p19^{ARF}$-null mice spontaneously and rapidly develop different types of cancer in their first year of life [5]. Although the mechanism is still not very clear, in general $p14^{ARF}$ prevents tumor formation through the MDM2–p53 tumor surveillance pathway [6,7]. In cells with an intact $p14^{ARF}$–MDM2–p53 pathway, aberrant mitogenic signals and inactivation of the retinoblastoma tumor-suppressor gene (*RB1*) induce expression of $p14^{ARF}$ [8–12], which in turn antagonizes the ubiquitin ligase activity of MDM2, stabilizes p53 and triggers p53-dependent G1 phase arrest or apoptosis [13–16]. Abrogation of the $p14^{ARF}$–MDM2–p53 tumor surveillance pathway allows mutant cells to bypass apoptosis and proliferate continuously, thereby increasing susceptibility to cancer. Although inactivation of this tumor surveillance pathway, either by loss of $p14^{ARF}$ or *p53*, or by overexpression of *MDM2*, has been reported in various tumor types [2], it is unclear whether these genes are involved in retinoblastoma development. It has been shown previously that *p53* gene and protein remain normal in retinoblastoma [17,18]. However, a recent report showed that *MDM2* and a similar gene *MDM4* [19] were amplified frequently in retinoblastoma, and that the consequent overexpression of *MDM2* and *MDM4* blocked the function of p53 in retinoblastoma cells [12]. These findings suggested that at least in some retinoblastoma, amplification and overexpression of *MDM2* or *MDM4* could compromise p53 function, which may contribute to tumorigenesis.

To elucidate their roles in retinoblastoma development, we compared expression of $p14^{ARF}$, *MDM2*, and *MDM4* in normal retina and retinoblastoma. In contrast to what was previously reported [12], we found that although $p14^{ARF}$ message was notably increased in retinoblastoma compared to retina, $p14^{ARF}$ protein was undetectable. We also show that MDM2 and MDM4 were expressed in all retinoblastoma samples tested. MDM2 protein was expressed at levels comparable to human retina, while MDM4 protein was overexpressed in one retinoblastoma cell line and two primary retinoblastomas.

Materials and methods

Specimen collection. Control normal human retinas (Table 1) were obtained from the enucleated eyes of cornea donors in the Eye Bank of Canada, with the University Health Network Research Ethics Board (REB) approval for anonymous use of discarded

* Corresponding author. Address: Division of Applied Molecular Oncology, Ontario Cancer Institute/Princess Margaret Hospital, University Health Network, Room 8-415, 610 University Avenue, Toronto, ON, Canada M5G 2M9. Fax: +1 416 946 4619.
E-mail address: gallie@attglobal.net (B.L. Gallie).

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Table 1
Samples used in this study

Sample ID	Patient/donor age	Patient/donor gender	IIRC group
Retinoblastoma			
RB1904	2 months	Female	D
RB1935	2.5 years	Male	D
RB2115	1 year	Male	D
RB2124	3 years	Female	Not available
RB2132	4 years	Male	D
RB2136	3 months	Male	D
RB2146	1 year	Female	D
RB2175	3 years	Female	Not available
RB2180	1 year	Male	D
RB2181	1 year	Female	D
RB2238	1 year	Female	D
RB2240	2 years	Male	D
RB2244	4 months	Female	D
RB2252	2 years	Female	Not available
RB2253	4 months	Male	D
RB2280	2.5 years	Female	E
RB2284	3 years	Female	D
RB2294	2 years	Male	D
RB2527	3 years	Female	D
RB2667	1 year	Male	D
RB2716	2 years	Male	E
Normal retina			
HR9	53 years	Female	NA
HR18	14 months	Female	NA
HR20	2 years	Male	NA
HR47	13 years	Female	NA
HR50	41 years	Female	NA
HR58	12 days	Male	NA
HR125	32 weeks	Male	NA
HR130	15 years	Male	NA
HR131	7 years	Male	NA
HR141	4 days	Female	NA

NA, not applicable.
IIRC, International Intraocular Retinoblastoma Classification [36].

specimens. Retinoblastoma tumor samples (Table 1) were obtained from eyes removed as part of therapy with approval of REB of the Hospital for Sick Children and the University Health Network. Research studies were performed only after all clinical tests were complete. Retinoblastoma cells lines Y79 [20] and WERI-RB1 [21] were obtained from their originators; cervical carcinoma cell lines C33A and HeLa were purchased from American Type Culture Collection (Manassas, VA, USA).

Quantitative RT-PCR. Total RNA was prepared using TRIzol reagent (Invitrogen, Burlington, ON, Canada) and cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and random primers. Gene specific primers *p14^{ARF}* (product ID:Hs99999_ml), *MDM4* (product ID:SRGHs00159092_ml) and *TBP* (product ID:Hs99999910_ml) were designed using Primer Express software (Applied Biosystems, Foster City, CA). Quantitative RT-PCR was performed in triplicate using ABI 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). The data were normalized to *TBP* expression and analyzed by SDS2.1 software (Applied Biosystems, Foster City, CA).

Immunoblot. Cells were lysed with 1× whole cell extraction buffer (20 mM Hepes, PH 7.4, 100 mM KCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 7% glycerol, 1 mM NaF, 0.2 mM DTT, 0.2 mM PMSF, 1% NP40, 5 μg/ml aprotonin, and leupeptin). Samples (30 μg protein per lane) were separated by electrophoresis on 10–20% gradient SDS–PAGE gels (Bio-Rad, Hercules, CA) and were transferred to Immobilon polyvinylidine difluoride membrane (Bio-Rad, Hercules, CA). Proteins were detected using anti-MDM2 mouse monoclonal antibody (1:200, Calbiochem, San Diego, CA), anti-MDM4 rabbit polyclonal antibody (1:500, Bethyl, TX) and anti-ARF rabbit polyclonal antibody (1:200, Abcam, Cambridge, MA), followed by either goat anti-mouse IgG or goat anti-rabbit IgG-horseradish peroxidase conjugated secondary antibody (1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were visualized using enhanced chemiluminescence reagents (Amersham, Little Chalfont, United Kingdom). β-Tubulin mouse monoclonal antibody was used as a loading control. (1:1000, Sigma Chemical Co., St. Louis, MO).

Immunohistochemistry. Formaldehyde-fixed, paraffin-embedded (FFPE) sections (7 μm) of human retinas and retinoblastomas were deparaffinized and rehydrated followed by antigen retrieval by heating. HeLa cells, used as positive control for p14^{ARF} protein were fixed with 4% PFA. Specimens were blocked with 10% commercial blocking solution (DAKO, Mississauga, ON, Canada). Sections were stained for 1 hour with anti-p14^{ARF} rabbit polyclonal antibody (Abcam, Cambridge, MA), followed by 1-h incubation with biotinylated anti-rabbit immunoglobulin (Santa Cruz Biotechnology, Santa Cruz, CA) and streptavidin-conjugated Alexa 594 (Invitrogen, Burlington, ON). Images were captured using a Coolsnap camera attached to a Leica DMLB microscope and processed using Photoshop 7.0 software.

Results

p14^{ARF} mRNA is markedly elevated in retinoblastoma

To determine if *p14^{ARF}* expression was induced in retinoblastoma, we first measured *p14^{ARF}* mRNA expression by quantitative RT-PCR in 6 normal retinas, 2 retinoblastoma cell lines, and 14 retinoblastoma primary tumors. Compared to normal retinas, all primary retinoblastomas and retinoblastoma cell lines displayed a prominent increase in *p14^{ARF}* mRNA (Fig. 1A and data not shown). Levels of *p14^{ARF}* mRNA in primary retinoblastomas were 500- to 1000-fold higher than in normal retinas (Fig. 1A). Surprisingly, *p14^{ARF}* mRNA expression in retinoblastoma cell lines was lower than primary retinoblastomas, but was still approximately 50-fold higher than in normal retinas.

p14^{ARF} protein is undetectable in retinoblastoma

The striking elevation of *p14^{ARF}* mRNA in retinoblastoma compared to retina prompted us to measure p14^{ARF} protein levels. Protein lysates from 20 primary retinoblastomas and 2 retinoblastoma cell lines were tested for expression of p14^{ARF} protein expression by immunoblot, and 6 retinoblastomas were immunostained with p14^{ARF} antibody. In contrast to the mRNA results, by both immunoblot and immunohistochemistry, p14^{ARF} protein was undetectable in both primary retinoblastomas and retinoblastoma cell lines, similar to normal retinas (Fig. 1B and data not shown). As expected, we detected p14^{ARF} protein by immunoblot and immunofluorescence in the positive controls C33A and HeLa cell lines (Fig. 1B and C and data not shown).

MDM2 and MDM4 are expressed in retinoblastoma

We examined the expression of MDM2 and MDM4, two inhibitors of p53 activity. *MDM4* mRNA was detected in all (100%) of normal retinas (6/6), retinoblastoma cell lines (2/2), and primary retinoblastomas (14/14) tested (Fig. 2A and data not shown). MDM4 protein was detected in 100% of primary retinoblastomas (20/20) and retinoblastoma cell lines (2/2) tested. For most retinoblastomas, MDM4 protein was expressed at levels comparable to unaffected retinas, but was overexpressed in one retinoblastoma cell line Y79 and two primary retinoblastomas (Fig. 2B and data not shown). MDM2 protein was detected in 30% (3/10) of human retinas and 100% of retinoblastomas (20/20) and retinoblastoma cell lines (2/2) (Fig. 3 and data not shown). MDM2 protein expression

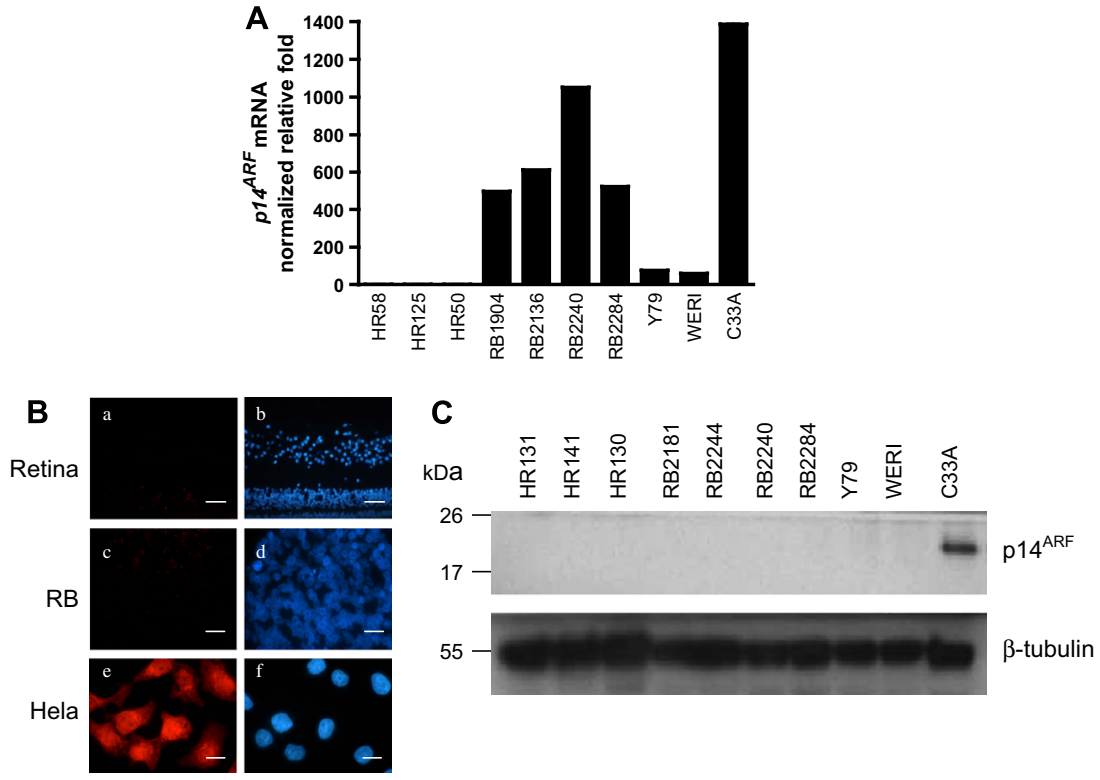

Fig. 1. Retinoblastoma highly expresses $p14^{ARF}$ mRNA but not protein. (A) Quantitative RT-PCR analysis of $p14^{ARF}$ was performed on mRNA isolated from 6 normal human retinas, 14 retinoblastomas, and 2 retinoblastoma cell lines (3 normal retinas (column 1–3), 4 primary retinoblastoma tumors (column 4–7), and 2 retinoblastoma cell lines (column 8 and 9) are shown). C33A, a human cervical cancer cell line, was used as positive control (column 10). Reactions were run in triplicate and calibrated to normal retina HR58 (column 1), using *TBP* as an endogenous control. Data shown are means (standard deviations were all too small ($\leqslant$0.36) to be shown). (B) Immunohistochemistry of $p14^{ARF}$. Normal retina (a,b), retinoblastoma (RB) tumor RB2115 (c,d), and HeLa cell (e,f), used as positive control, were stained with $p14^{ARF}$ specific Ab (red) and DAPI (blue). Scale bars in (a) and (b) represent 40 μm and in (c)–(f) 10 μm. (C) Immunoblot analysis of $p14^{ARF}$ was performed on normal retinas (lanes 1–3), primary retinoblastoma tumors (lanes 4–7), and retinoblastoma cell lines (lanes 8 and 9) and C33A was used as positive control (lane 10). Upper panel $p14^{ARF}$, lower panel β-tubulin. (For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

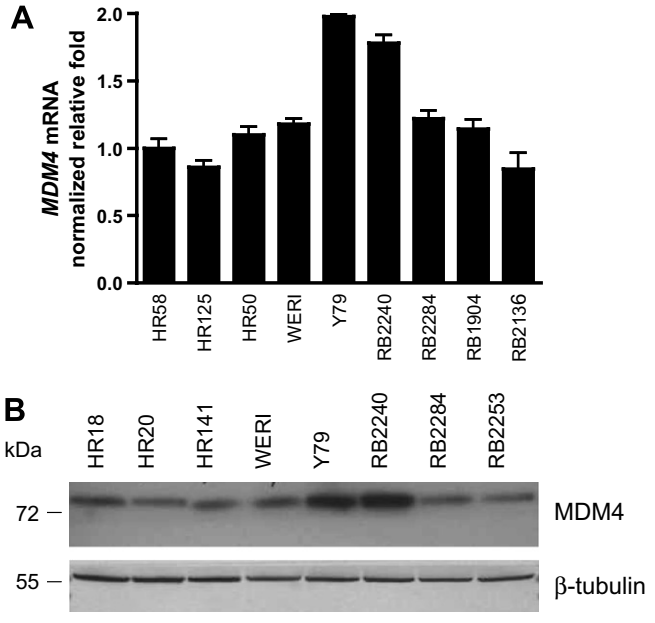

Fig. 2. Retinoblastoma and normal retina express MDM4. (A) Quantitative RT-PCR analysis of MDM4 expression in normal retinas (columns 1–3), retinoblastoma cell lines (columns 4 and 5), and primary retinoblastoma tumors (columns 6–9). Data shown are means ± standard deviation. (B) Immunoblot analysis of MDM4 in normal retinas (lanes 1–3), retinoblastoma cell lines (lanes 4 and 5), and primary retinoblastoma tumors (lanes 6–8). Upper panel MDM4 and lower panel β-tubulin.

levels in retinoblastomas and RB cell lines were not elevated compared to normal retinas.

Discussion

It should be noted that in the current study we compared expression of $p14^{ARF}$, MDM2, and MDM4 in normal retinal tissues, tumor samples (both are *ex vivo*) and retinoblastoma cell lines (*in vitro*), therefore it is possible that some results might reflect these various origins of tissue, rather than the true biology. As well, the cell or origin of retinoblastoma is not known, and post-natal human retina is only an approximation.

Though loss of *RB1* function is regarded as the first pivotal step in retinoblastoma development, numerous studies have suggested that additional genetic alterations are required for full malignant transformation of retina to retinoblastoma [22,23]. In many tissues, *RB1* loss induces high $p14^{ARF}$ expression [8,24], which in turn inhibits MDM2 and activates p53-dependent cell arrest and/or apoptosis [2,11]. Earlier studies suggest that *p53* gene and protein remained normal in retinoblastoma [17,18]. Therefore, it is interesting to speculate that the other members of $p14^{ARF}$–MDM2–p53 tumor surveillance pathway could be altered in retinoblastoma to allow these *RB1* mutant cells to proliferate unchecked.

In one cohort of samples, levels of $p14^{ARF}$ mRNA in retinoblastoma were dramatically higher than in normal retina [12]. Consistent with these results, we also found marked elevation of $p14^{ARF}$ mRNA in retinoblastomas. However, this high $p14^{ARF}$ mRNA expression is not matched by protein, which was undetectable in retinoblastomas. The increase of $p14^{ARF}$ mRNA in retinoblastomas

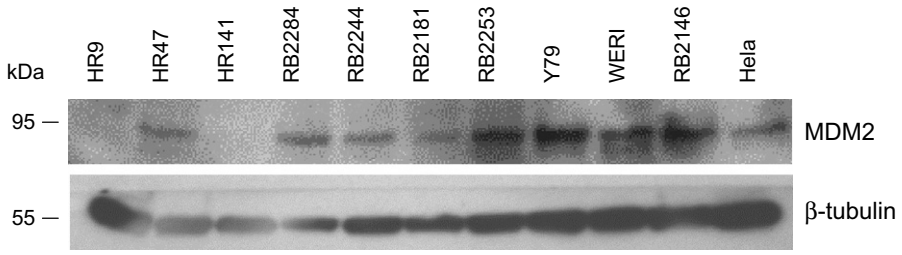

Fig. 3. MDM2 protein is expressed in all retinoblastomas and some normal retinas. Immunoblot analysis of MDM2 protein expression in normal retinas (lanes 1–3), primary retinoblastoma tumors (lanes 4–7 and 10) and retinoblastoma cell lines (lanes 8 and 9). HeLa cell was used as positive control (lane 11). Upper panel MDM2 and lower panel β-tubulin.

may reflect an intact initial $p14^{ARF}$ response to *RB1* loss in these tumor cells. The discrepancy between mRNA and protein expression of $p14^{ARF}$ suggests that a post-transcriptional mechanism actively blocks $p14^{ARF}$ function in retinoblastoma. Two mechanisms have been identified so far for the loss of $p14^{ARF}$ expression in a variety of tumors: methylation of a CpG island in the *ARF* promoter and biallelic deletion of the entire *ARF* locus [25–28]. The post-transcriptional silencing of $p14^{ARF}$ in retinoblastoma as reported here adds another novel mechanism for the loss of $p14^{ARF}$ expression in tumors. The molecular details of this novel mechanism and its role in development of retinoblastoma and other tumors need further study.

MDM2 is a well-studied oncogene that is able to inactivate p53 by proteosome-dependent degradation [29,30]. $p14^{ARF}$ segregates MDM2 from p53 and antagonizes the E3 ubiquitin protein ligase activity of MDM2, thus blocking inactivation of p53 [6,13–16]. *MDM2* is overexpressed in 5–10% of human tumors, potentially resulting in disruption the $p14^{ARF}$–MDM2–p53 pathway [2,11]. We show that the majority of normal retinas (70%) did not express MDM2, but MDM2 was expressed in all the retinoblastoma samples and cell lines tested. The absence of detectable $p14^{ARF}$ protein in our samples suggests that MDM2 protein in retinoblastoma may be unsuppressed and thereby constitutively block p53 tumor surveillance and promote retinoblastoma development. This hypothesis is supported by recent studies showing that nutlin, a small molecule inhibitor of MDM2, can reactivate p53 function and induce cell death of retinoblastoma cells [12,31].

MDM4 was identified as a p53 binding protein with similar structure to MDM2 [19,32] that can antagonize the transcriptional activity of p53 [19]. Amplification and overexpression of *MDM4* has been detected in some primary tumors and cell lines, especially in tumors with wild type *p53* [33,34]. Therefore, *MDM4* may contribute to tumorigenesis as an oncogene. One study showed amplification of *MDM4* in 3/7 fresh and in 13/49 paraffin-embedded retinoblastoma tumors, however overexpression of MDM4 protein could only be detected in some primary retinoblastomas [12]. We found the expression levels of MDM4 protein in most retinoblastoma and normal retinas were similar. Furthermore in our cohort of retinoblastoma samples we detected gain of MDM4 in 2/4 samples and only in combination with the more highly and frequently gained/amplified oncogene *KIF14*, located close to MDM4 on 1q [35]. To what extent *MDM4* is amplified and overexpressed in retinoblastoma and whether MDM4 plays a major role in retinoblastoma development needs further study.

In summary, $p14^{ARF}$ was overexpressed at mRNA level but the protein remained undetectable in retinoblastomas compared with normal retinas. MDM2 was expressed in all retinoblastomas but only in 3 of 10 normal retinas. MDM4 was expressed in both retinoblastomas and normal retinas and for most retinoblastomas was not overexpressed. Our findings suggest that post-transcriptional inactivation of $p14^{ARF}$ may contribute to tumorigenesis of retinoblastoma by unleashing MDM2 to constitutively inhibit p53.

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