High Expression of *KIF14* in Retinoblastoma: Association with Older Age at Diagnosis

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Purpose. *KIF14* a mitotic kinesin gene plays an important role in cytokinesis. Deregulation of *KIF14* may be a pathway of tumor progression and results in decreased patient survival as seen in breast tumors. Recently, *KIF14*, a possible gene that drives gain of chromosome arm 1q (the most commonly gained chromosomal region in retinoblastoma), has been shown to be a strong oncogene candidate overexpressed by more than two orders of magnitude in retinoblastoma. This study was conducted to quantify the expression of *KIF14* in human retinoblastoma tumors and correlate it with disease phenotype.

Methods. *KIF14* expression was examined by using real-time RT-PCR in 30 retinoblastoma tumors with age at diagnosis between 3 and 68 months. Two 18-month-old, three adult (55–62 years), and three fetal (one 18 weeks' and another pooled retina of 18 and 20 weeks' gestation) retinas were used as the control. *KIF14* expression was normalized to the housekeeping control gene *TBP* and compared with that in an 18-month-old control retina. The protein expression was confirmed in tumor cells by immunohistochemistry and phenotypic correlation was performed.

Results. *KIF14* was expressed between 3- and 207-fold greater than 18-month-old retina in 30 retinoblastoma tumors ($P < 0.0001$). Immunohistochemistry revealed KIF14 localization to both nucleus and cytoplasm of tumor cells. *KIF14* mRNA overexpression correlated significantly with older age at diagnosis ($P = 0.006$). There was no association with differentiation, invasion, or duration of the disease with *KIF14* overexpression.

Conclusions. Overexpression of *KIF14* was confirmed in primary human retinoblastoma and showed that patients with an older age at diagnosis express significantly higher levels of *KIF14*. (*Invest Ophthalmol Vis Sci.* 2007;48:4901–4906) DOI: 10.1167/iovs.07-0063

The two-hit hypothesis describes the rate-limiting event in the initiation of retinoblastoma (RB).[1] Identification of mutations in addition to the initiating two hits in the *RB1* gene is imperative for understanding the molecular pathogenesis of malignant transformation and progression of the tumor.[2] Apart from the *RB1* mutations in chromosome arm 13q, genomic gains and losses in other chromosomes have been identified by comparative genomic hybridization (CGH) in RB tumors.[3–7] The minimal region most frequently gained was 1q31, present in approximately 50% of all tumors. A chromosomal gain at arm 1q is also found in many other cancers.[8] Two CGH studies showed that gains in the 1q region were restricted to more advanced tumors in older children.[4,6] Recently, *KIF14*, a possible gene that drives the 1q gain and lays in a 3-Mbp minimal region of gain, has been shown to be a strong oncogene candidate that is overexpressed by more than two orders of magnitude in RB and also is overexpressed in breast and lung cancers and medulloblastoma cell lines.[9] The *KIF14* locus is gained or amplified in 62% of primary RB, and *KIF14* gain is likely to be an early genomic event in RB development.[10]

KIF14 is a mitotic kinesin.[11,12] *KIF14* gene expression is regulated during the cell cycle, and the level of *KIF14* correlates with mitotic progression. *KIF14*, along with the microtubule-bundling protein PRC1 and citron kinase, with which it interacts, plays an important role in cytokinesis during midbody formation and completion of cytokinesis.[12] RNA interference-mediated silencing of *KIF14* disrupts cell cycle progression due to deficient midbody cleavage, leading to the formation of binucleated cells.[11] Deregulation of *KIF14*, such as overexpression, may be a pathway of tumor progression and results in decreased patient survival, as seen in breast tumors.[13]

In this study, we looked at the expression of *KIF14* in a large series of RB and correlated the results with clinical disease phenotypes to understand the role of this gene in RB progression. There was an increase of 3- to 207-fold in *KIF14* gene expression in thirty tumor samples compared with 18-month-old control retina. *KIF14* mRNA expression increased with patient's age at presentation ($P = 0.006$).

Materials and Methods

Clinical Samples

The study adhered to the guidelines in the Declaration of Helsinki. This study was conducted at the Medical Research Foundation and Vision Research Foundation, Sankara Nethralaya, India, and was approved by the institutional ethics boards. Informed consent was obtained from the parents for the research use of RB tumor samples obtained from enucleated eyes removed as a part of treatment. Normal human retinas were obtained from the C. U. Shah Eye Bank (Sankara Nethralaya Medical Research Foundation, Chennai), and the Lions Eye Bank, (Re-

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gional Institute of Ophthalmology, Chennai), after proper approval and examination of the eyeball under the microscope. Fetal retinas were commercially purchased (Advanced Bioscience Resources, Inc., Alameda, CA). We examined *KIF14* expression using real-time RT-PCR in 30 RB tumors with age at diagnosis between 3 and 68 months. Two 18-month-old, three adult (55–62 years), and three fetal retinas (one 18 weeks' and another pooled retina of 18 and 20 weeks' gestation) were used as the control.

RNA Extraction and Reverse Transcription

Total RNA was extracted from tumors and normal healthy retinas by the guanidine isothiocyanate and chloroform method (TRI Reagent; Sigma-Aldrich, Bangalore, India). All RNA samples were treated with DNase (Turbo; Ambion, Genetix Biotech Asia Pvt. Ltd., Chennai, India). For all samples, 1 μg of total RNA was used to synthesize first-strand cDNA with reverse transcriptase (SuperScript II; Invitrogen, Joyvel, Chennai, India) and random primers.

Real-Time RT-PCR Analyses

Gene expression assays for *KIF14* (Hs00978216_m1; *Taq*Man) and two endogenous controls, *TBP* (Hs99999910_m1) and *HPRT*, (Hs99999909_m1) were obtained from Applied Biosystems (LabIndia, Chennai, India). Quantification of gene expression was performed in triplicate in a 20-μL volume in 96-well plates on a real-time PCR system (Prism 7300; ABI). Each reaction included 1× primer probe mix (*Taq*Man; ABI), 1× universal PCR master mix (*Taq*Man; ABI), and 100 ng of cDNA. Cycling conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 sec at 95°C, plus 1 minute at 60°C. Commercial software (SDS ver. 1.3; ABI) was used to calculate $\Delta\Delta$Ct relative expression values for *KIF14* and *HPRT* normalized to the *TBP* endogenous control and calibrated to an 18-month-old normal control retina.

Immunohistochemistry

Paraffin-embedded sections of RB tumor and nonneoplastic retina from a 56-years-old donor eyeball (5 μm thick) were dewaxed and rehydrated. Antigen retrieval was performed by the pressure-cooker method in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% H_2O_2 in H_2O (10 minutes), and the slides were incubated with rabbit polyclonal affinity-purified anti-*KIF14* antibody (BL358, 1:75 in Tris buffer [pH 7.6]; Bethyl Laboratories, Genuine Chemical Corp., New Delhi, India). Immunostaining was performed using the labelled streptavidin–biotin visualization system (LSAB)-horseradish peroxidase system ($LSAB^+$ system; DakoCytomation, Glostrup, Denmark). The reaction was revealed by 3,3′-diaminobenzidine tetrahydrochloride (DakoCytomation) and counterstained with hematoxylin. For the negative control, the immunostaining was done without primary antibody.

TABLE 1. Phenotype of RB Samples with Their *KIF14* mRNA Expression Levels

Sample	Laterality	Age at Diagnosis (mo)	*KIF14* Expression Levels in Tumors Relative to C3 (x-fold)	Differentiation*	Duration	Choroid/Optic Nerve Infiltration	Chemotherapy
S1	BLRB	3	48.5	Poor	1 mo	+	−
S32	ULRB	3	19.6	Well	3 wk	−	−
S2	ULRB	7	12.5	Well	2 wk	−	−
S3	BLRB	7	68.5	Well	1 mo	−	−
S7	BLRB	7	3	No viable cell	3 mo	−	8 cycles
S12	ULRB	7	64	Poor	1 mo	+	−
S27	ULRB	11	36.7	Well	1.5mo	+	−
S35	BLRB	14	36.7	Well	11 mo	+	−
S21	ULRB	15	55	Poor	1 mo	+	−
S14	BLRB	18	27.8	Well	6 mo	−	−
S26	ULRB	20	29.8	Poor	3 mo	+	−
S31	ULRB	24	90.7	Poor	1 mo	−	−
S29	ULRB	24	42.2	Poor	3 mo	+	−
S23	ULRB	24	59.7	Well	1 wk	+	−
S6	ULRB	30	207	Poor	1.5mo	−	−
S16	BLRB	30	55.7	Poor	2 mo	+	−
S34	BLRB	30	97	Well	1 wk	+	−
S30	ULRB	36	137.1	Poor	1 mo	−	−
S13	ULRB	36	12.1	No viable cell	1 wk	−	6 cycles
S10	ULRB	36	90.5	Well	18 mo	−	−
S9	ULRB	36	97	Poor	3 mo	+	−
S36	ULRB	42	103.9	Poor	1 mo	+	−
S18	ULRB	46	16	Poor	2 mo	−	−
S11	ULRB	48	137	Poor	3 mo	+	−
S19	ULRB	48	207	Well	2 mo	−	−
S20	ULRB	48	157	Poor	1 mo	+	−
S24	ULRB	48	128	Poor	1.5mo	−	−
S33	ULRB	60	194	Poor	12 mo	+	−
S22	ULRB	60	68.5	Poor	1 mo	−	−
S28	ULRB	68	34.2	Poor	24 mo	+	−
C3	Control	18	1	Nil	Nil	Nil	Nil

ULRB: unilateral retinoblastoma; BLRB: bilateral retinoblastoma.

* Poorly differentiated cells: high nuclear-to-cytoplasmic ratios and high mitotic indices (pseudorosettes and Homer-Wright-rosettes were found in such areas). Moderately differentiated cells: moderate nuclear-to-cytoplasmic ratios, moderate mitotic indices, and possible pseudorosettes and Flexner-Winter Steiner rosettes. Well-differentiated cells: Low nuclear-to-cytoplasmic ratios, low mitotic indices, and the presence of Flexner-Winter Steiner rosettes and florets.

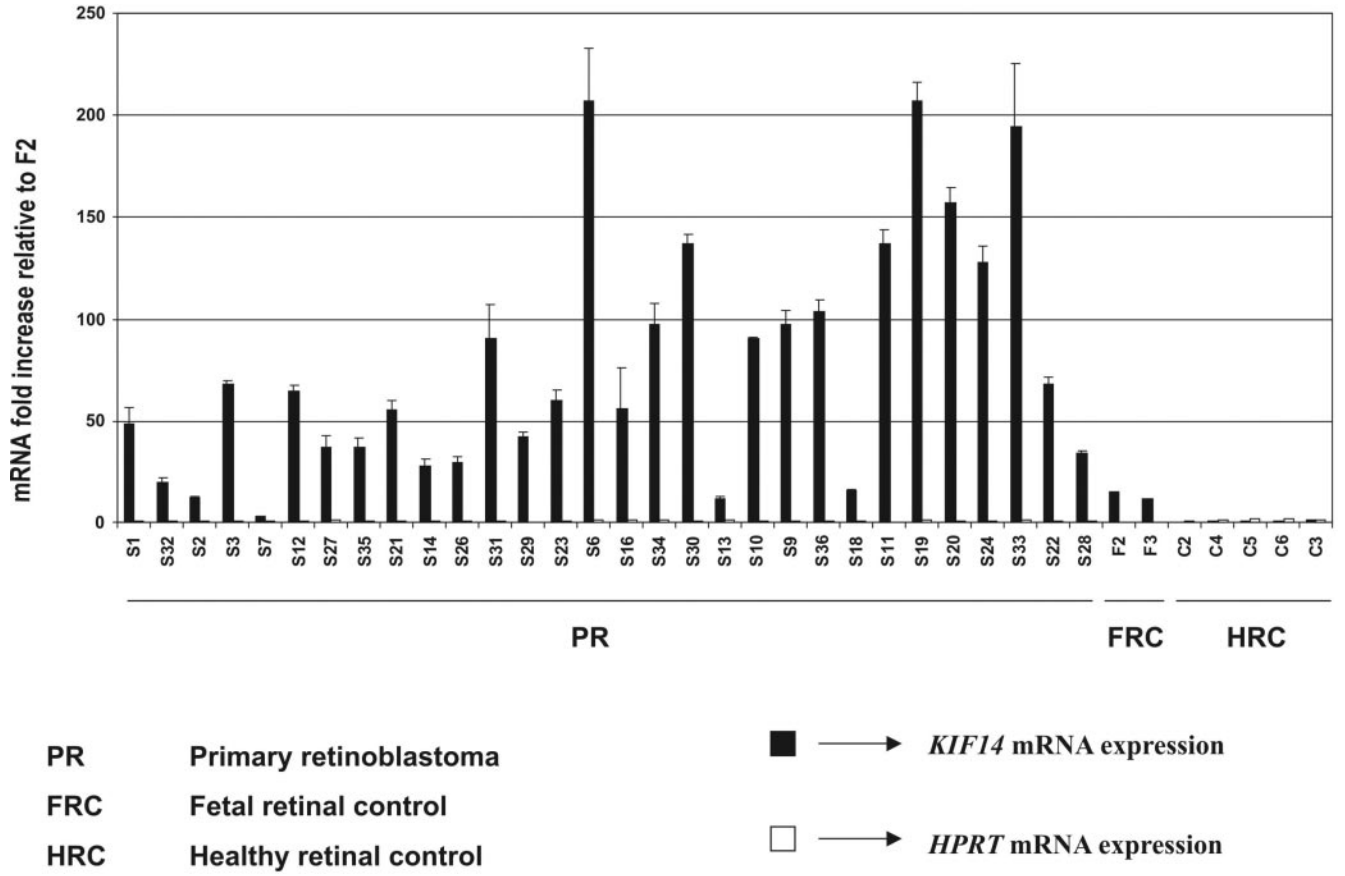

FIGURE 1. *KIF14* mRNA expression in retinoblastoma. *KIF14* mRNA expression in retinoblastomas, fetal retinas, and adult control retinas calibrated to 18-month-old normal control retina (*bar C3*). Error bars represent SE of the relative expression levels, normalized against a TBP internal control and calibrated to C3. *HPRT* was included as the negative control. Subjects S7 and S13 had chemotherapy before enucleation. The RB samples along the *x*-axis are arranged according to age at diagnosis.

Immunoanalysis

Evaluation of immunostaining in tumor cells was objectively performed by two investigators (JM, KM). Randomly, 10 tumor fields were scanned for protein expression under 40× magnification, and the percentage of positive tumor cells was noted for each field. Finally, the average expression was calculated from the 10 values for the entire slide. Depending on the percentage of positive cells, four categories were established: 0, no positive cells; 1+, positive cells in less than one third; 2+, positive cells in 33% to 67% of the cells; and 3+, positive cells in more than 67% of the total tumor cell population.[14]

Phenotypic Assessment

Age at diagnosis, laterality, differentiation, invasion of the tumor into the choroid and optic nerve and the duration of the disease were considered for phenotypic correlation (Table 1). Duration of disease is defined as the period between identification of symptoms to the time of enucleation.

Statistical Analysis

The data were analyzed for the association between different variables and increase in *KIF14*. The median increase (61.85-fold) was used to divide the cohort into two groups. Multivariate logistic regression was performed to study the effects of age, age group (based on median age of 30 months), duration of the disease, laterality, differentiation, and choroidal/optic nerve invasion on the increase in expression of *KIF14*. All analyses were performed on computer (SPSS ver. 13; SPSS, Chicago, IL), and $P < 0.05$ was considered significant.

RESULTS

Real-Time mRNA Quantification of KIF14 Expression Levels in RB Samples and Control Retinas

When normalized against the housekeeping gene *TBP*, *KIF14* showed 3- to 207-fold greater expression ($P < 0.0001$) in all the RB samples compared with 18-month-old control retina (C3; Fig. 1). Median expression was 61.8-fold higher in RB samples than in 18-month-old control retina. Some variability in the expression of the housekeeping gene *HPRT* (included as a negative control) when normalized against *TBP* was noted (up to fivefold) between RB samples and healthy control retinas. This variability is similar to that reported in a previous study.[9] However, the increased expression of *KIF14* was far higher than the variability noted. Proliferative fetal control retinas showed an overexpression of 12- to 14-fold on comparison with 18-month-old control retina, which is far less than the mean expression in tumor samples. Even though there were no viable cells in the immunohistochemistry report of two samples treated with chemotherapy before enucleation (S7 and S13), good quantity and quality RNA was extracted from a large amount of tumor tissue taken (400–500 mg) from the enucleated eyeballs of these patients.

Immunoreactivity of KIF14 in the Nonneoplstic Retina and RB Tumor Cells

No immunoreactivity for KIF14 was noted in the control healthy retina (Fig. 2B), whereas the expression was localized

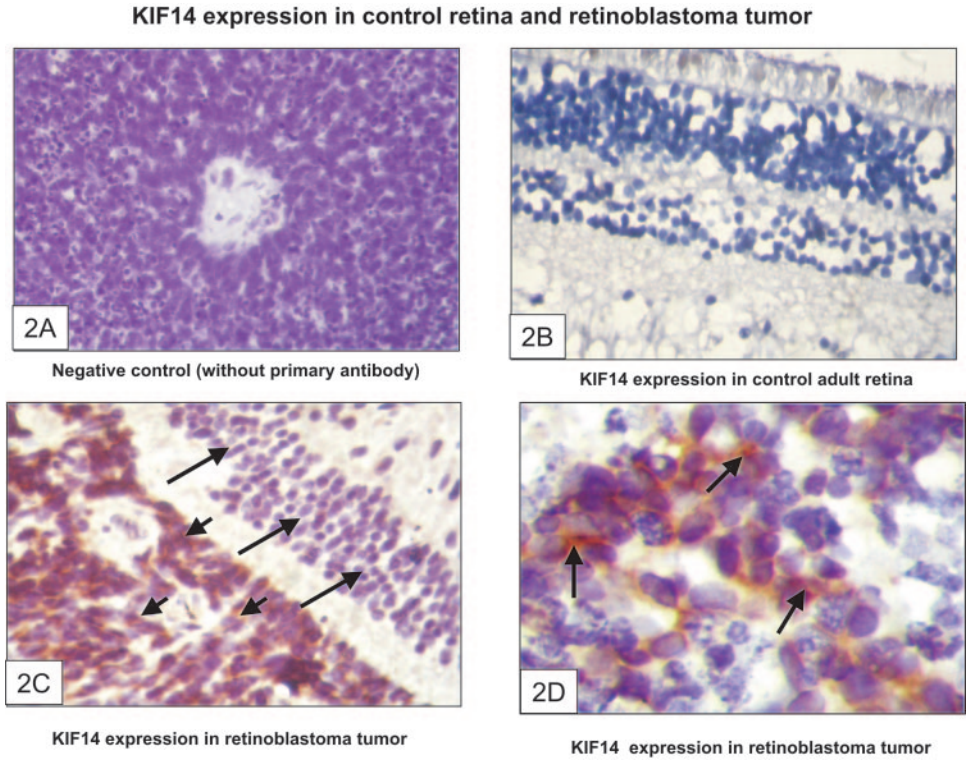

FIGURE 2. Immunohistochemistry of KIF14 protein expression and localization in retinoblastoma tumor. (**A**) Negative control without primary antibody. (**B**) No immunoreactivity of KIF14 in nonneoplastic retina. (**C**) Positivity of *KIF14* in the tumor cells arising from the inner nuclear layer (*down arrows*) and the absent staining in the outer nuclear layer (*up arrows*) of the retina. (**D**) Positive cytoplasmic expression (*arrows*) of *KIF14* in the tumor with choroidal invasion. All are 3,3′-diaminobenzidine staining with hematoxylin counterstain. Magnification, ×40.

to the nucleus and cytoplasm in tumor cells (Figs. 2C, 2D). All six stained tumors showed heterogeneous expression of KIF14 with 2+ staining.

KIF14 mRNA Expression Levels in RB Samples and Phenotype Correlation

A phenotype correlation was attempted with the KIF14 mRNA expression levels as shown in Table 1. Of the 30 patients, 23 had unilateral RB and 7 had bilateral RB. Two patients had chemotherapy before enucleation and were excluded from further analysis. The age at diagnosis showed a significant positive correlation ($r = 0.509$; $P = 0.006$; Fig. 3) with an increase in *KIF14*. Multivariate analysis of different variables on the increase in *KIF14* is shown in Table 2. Multivariate logistic regression analysis adjusted for the effect of laterality, duration of disease, choroidal/optic nerve infiltration and differentiation status on relative *KIF14* expression, and showed a significant effect of age at diagnosis ($P = 0.005$), odds ratio 36.8 (95% CI: 2.29–682.8).

Tumors that were used for the study were in advance stages of malignancy (stage D or E of the international classification for intraocular RB).[15] The advanced disease prevented the analysis of *KIF14* levels in early versus late or advanced tumors to assess *KIF14* levels and progression of the tumor. Of note, *KIF14* levels in two patients treated with chemotherapy before enucleation were 3- and 12-fold greater than in normal retina, far less than in untreated RB tumors (Figs. 1, 3).

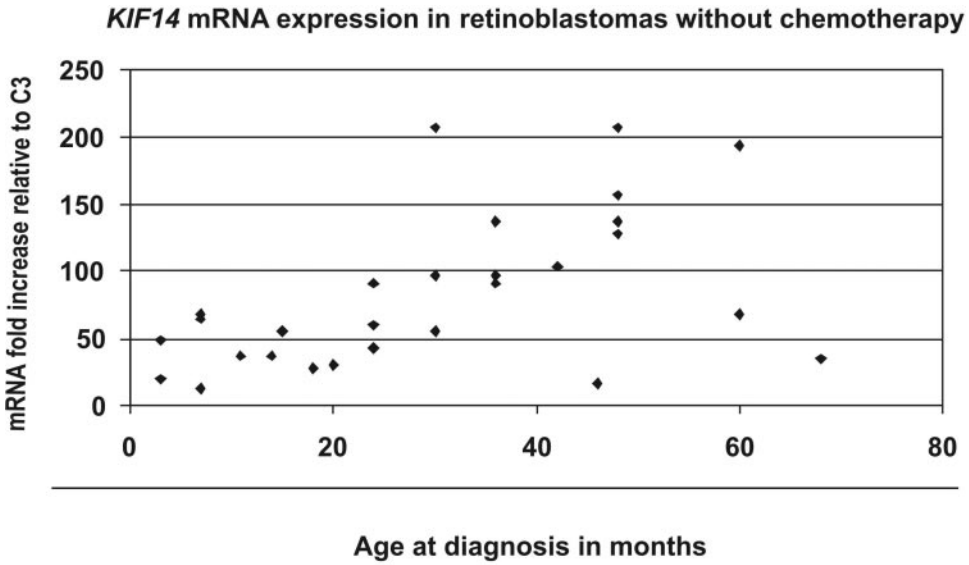

FIGURE 3. Scatterplot showing the increase in relative KIF14 mRNA expression against age at diagnosis ($r = 0.509$; $P = 0.006$).

TABLE 2. Multivariate Regression Showing the Effect of Different Clinical Variables on Increases in *KIF14* mRNA

Variable	Odds Ratio	95% CI	*P*
Age at diagnosis	1.06	1.004–1.11	**0.036**
Age group (at diagnosis) (≤30 mo; >30 mo)	13.33	2.2–81.2	**0.005**
Duration	0.99	0.995–1.004	0.73
Laterality	0.11	0.01–1.16	0.06
Differentiation	2.36	0.49–11.45	0.29
Choroidal/optic nerve invasion	2.57	0.54–12.17	0.23

*Significant differences ($P < 0.05$) are highlighted in bold.

DISCUSSION

Kinesins are a superfamily (45 members) of motor proteins with a wide range of cellular functions, including vesicle transport, mitotic spindle formation, chromosome segregation, midbody formation, and cytokinesis completion.[16,17] They are defined by the motor domain position at the N terminus (N type), C terminus (C type), and internal region (I type).[18]

KIF14 is a mammalian kinesin classified as an N-type kinesin 3 family member containing an N-terminal extension (1-356 amino acids [aa]), a kinesin motor domain (356-708 aa), and a forkhead-associated domain containing stalk and tail region (800-1649 aa). The *KIF14* motor domain exhibits microtubule-dependent ATPase activity.[11] The 1-356 aa region of *KIF14* (the extension amino terminal to the motor domain) bound to the protein regulating cytokinesis 1 (PRC1) and the 900-1649 aa region binds citron kinase to guide spindle formation and effective cytokinesis.[12] KIF14 is localized to the cytoplasm during the interphase of cell division and redistributes to the nucleus to aid in spindle formation. During the prophase, KIF14 accumulates at the developing spindle poles and their associated microtubules; a similar distribution has been observed in cells throughout the metaphase. In contrast, during the anaphase, KIF14 accumulates at the spindle midzone, and appears increasingly concentrated at the midbody during the telophase.[11]

As *KIF14* is overexpressed in RB[9] and is associated with poor prognosis in breast cancer,[12] we wanted to confirm the results of the previous study[9] in a much larger cohort of primary RB samples and to determine the association of *KIF14* expression with clinical variables in patients with RB. All the tumor samples taken for the study showed an overexpression of the *KIF14* gene between 3- and 207-fold greater than the 18-month-old control retina. The protein was seen in the nucleus and cytoplasm of tumor cells by immunohistochemistry (Fig. 2), the first in situ evidence of KIF14 overexpression in cancer. This confirms the earlier finding of subcellular localization of human KIF14 during the cell cycle.[11]

The later the median age at diagnosis, the greater the expression of *KIF14* ($P = 0.006$). We compared the mean increase in *KIF14* with laterality, differentiation status, and invasion of the tumor, to understand why with later age at diagnosis tumors tend to have very high expression of *KIF14*. However, we did not find any statistical significance between different subtypes. As *KIF14* probably has a role in cell proliferation and the severity of RB is related to the rate of proliferation of the tumor, even with known bias in patient's history, the duration of the disease was calculated for individual patients and correlated with the relative *KIF14* expression. No significant variability in *KIF14* expression was noted with the increase or decrease in duration of the disease. As no correlation was found with laterality, invasion, differentiation status, and the duration, very high expression of *KIF14* in late-presenting patients may be due to the more predisposing genomic instability (possibly assisted by *KIF14*) required for tumor progression in older patients than in patients with early-onset tumors.[4] A CGH study on 66 RB tumor samples showed more frequent and more complex abnormalities (median, five changes/abnormal tumor versus median, 1.5 changes/abnormal tumor; $P = 0.003$) than RBs from children with a young age at enucleation.[6] In that study gains of all of 1q, 2p, 17q, of the entire chromosome 19, and losses of 16q were restricted to the older age group. These results suggest that the progression of RBs from older patients follows mutational pathways different from those of younger patients.

The relative *KIF14* expression in chemotherapy-treated tumors was low compared with the median expression of all RB samples, suggesting that decreasing *KIF14* is a response of the tumors to chemotherapy, probably associated with decreased proliferation. More samples treated with chemotherapy are needed to confirm this finding.

To conclude, we confirmed the overexpression of *KIF14* mRNA and protein in primary RB, which may be one of the additional required components in RB progression. Further, we showed that patients with older age at diagnosis have a statistically significant high expression of the *KIF14* gene. This is the first report to associate *KIF14* mRNA overexpression with older age at diagnosis and the first report on *KIF14* gene quantification in Indian RB samples.

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