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

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PURPOSE. KIF14 is 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 chromosome 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 Ophtalmol 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. 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.2–7 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.6,9 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.10 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-
real-time PCR system (Prism 7300; ABI). Each reaction included 1 × primer probe mix (TaqMan; ABI), 1 × universal PCR master mix (TaqMan; 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 ΔΔCt 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% H2O2 in H2O (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**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Laterality</th>
<th>Age at Diagnosis (mo)</th>
<th>KIF14 Expression Levels in Tumors Relative to C3 (x-fold)</th>
<th>Differentiation*</th>
<th>Duration</th>
<th>Choroid/Optic Nerve Infiltration</th>
<th>Chemotherapy</th>
</tr>
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<tbody>
<tr>
<td>S1</td>
<td>BLRB</td>
<td>3</td>
<td>48.5</td>
<td>Poor</td>
<td>1 mo</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S2</td>
<td>ULRB</td>
<td>3</td>
<td>19.6</td>
<td>Well</td>
<td>3 wk</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td>ULRB</td>
<td>7</td>
<td>12.5</td>
<td>Well</td>
<td>2 wk</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>S4</td>
<td>BLRB</td>
<td>7</td>
<td>68.5</td>
<td>Well</td>
<td>1 mo</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>S5</td>
<td>BLRB</td>
<td>7</td>
<td>3</td>
<td>No viable cell</td>
<td>3 mo</td>
<td>–</td>
<td>8 cycles</td>
</tr>
<tr>
<td>S6</td>
<td>ULRB</td>
<td>7</td>
<td>64</td>
<td>Poor</td>
<td>1 mo</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S7</td>
<td>ULRB</td>
<td>11</td>
<td>36.7</td>
<td>Well</td>
<td>1.5 mo</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S8</td>
<td>ULRB</td>
<td>15</td>
<td>55</td>
<td>Poor</td>
<td>1 mo</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S9</td>
<td>BLRB</td>
<td>4</td>
<td>27.8</td>
<td>Poor</td>
<td>2 mo</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>S10</td>
<td>ULRB</td>
<td>20</td>
<td>29.8</td>
<td>Poor</td>
<td>3 mo</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S11</td>
<td>ULRB</td>
<td>24</td>
<td>90.7</td>
<td>Poor</td>
<td>1 mo</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>S12</td>
<td>ULRB</td>
<td>24</td>
<td>42.2</td>
<td>Poor</td>
<td>3 mo</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S13</td>
<td>ULRB</td>
<td>24</td>
<td>59.7</td>
<td>Well</td>
<td>1 wk</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S14</td>
<td>ULRB</td>
<td>30</td>
<td>207</td>
<td>Poor</td>
<td>1.5 mo</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>S15</td>
<td>ULRB</td>
<td>30</td>
<td>55.7</td>
<td>Poor</td>
<td>2 mo</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S16</td>
<td>BLRB</td>
<td>30</td>
<td>97</td>
<td>Poor</td>
<td>1 wk</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S17</td>
<td>BLRB</td>
<td>36</td>
<td>137.1</td>
<td>Poor</td>
<td>1 mo</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>S18</td>
<td>ULRB</td>
<td>36</td>
<td>12.1</td>
<td>No viable cell</td>
<td>1 wk</td>
<td>–</td>
<td>6 cycles</td>
</tr>
<tr>
<td>S19</td>
<td>ULRB</td>
<td>36</td>
<td>90.5</td>
<td>Well</td>
<td>18 mo</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>S20</td>
<td>ULRB</td>
<td>36</td>
<td>97</td>
<td>Poor</td>
<td>3 mo</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S21</td>
<td>ULRB</td>
<td>42</td>
<td>103.9</td>
<td>Poor</td>
<td>1 mo</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S22</td>
<td>ULRB</td>
<td>46</td>
<td>16</td>
<td>Poor</td>
<td>2 mo</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>S23</td>
<td>ULRB</td>
<td>48</td>
<td>157</td>
<td>Poor</td>
<td>3 mo</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S24</td>
<td>ULRB</td>
<td>48</td>
<td>207</td>
<td>Poor</td>
<td>2 mo</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>S25</td>
<td>ULRB</td>
<td>48</td>
<td>157</td>
<td>Poor</td>
<td>1 mo</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S26</td>
<td>ULRB</td>
<td>48</td>
<td>128</td>
<td>Poor</td>
<td>1.5 mo</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>S27</td>
<td>ULRB</td>
<td>60</td>
<td>194</td>
<td>Poor</td>
<td>12 mo</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>S28</td>
<td>ULRB</td>
<td>60</td>
<td>68.5</td>
<td>Poor</td>
<td>1 mo</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>S29</td>
<td>ULRB</td>
<td>68</td>
<td>34.2</td>
<td>Poor</td>
<td>24 mo</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>Control</td>
<td>18</td>
<td>1</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
<td></td>
</tr>
</tbody>
</table>

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-cytoplasmatic 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.
**Immunanalysis**

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.

**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. 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...
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). 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).
amino acids (aa), a kinesin motor domain (356-708 aa), and a

KIF14 primary RB samples and to determine the association of human KIF14 during the cell cycle.11

(Fig. 2), the first in situ evidence of KIF14 overexpression in 18-month-old control retina. The protein was seen in the nu-

cleus to aid in spindle formation. During the prophase, the protein regulating cytokinesis 1 (PRC1) and the 900-1649 (the extension amino terminal to the motor domain) bound to KIF14 accumulates at the developing spindle poles and their

port, mitotic spindle formation, chromosome segregation, mid-

body 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 forhead-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 cytoplasmic 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 ob-

served 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 RB9 and is associated with poor prognosis in breast cancer,12 we wanted to confirm the results of the previous study9 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 nu-
cleus 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 localiza-
tion of human KIF14 during the cell cycle.11

The later the median age at diagnosis, the greater the ex-

pression of KIF14 (P = 0.006). We compared the mean in-
crease in KIF14 with laterality, differentiation status, and inva-
sion 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 prolif-
eration and the severity of RB is related to the rate of prolifer-
ation of the tumor, even with known bias in patient’s history, the duration of the disease was calculated for individual pa-
tients 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 correla-
tion was found with laterality, invasion, differentiation status,

and the duration, very high expression of KIF14 in late-pre-
senting 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 chang-
es/abnormal tumor versus median, 1.5 changes/abnormal tu-
mor; P = 0.003) than RBs from children with a young age at enucleation.9 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 tu-
mors 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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