Leucine-Rich α-2-Glycoprotein-1 (LRG-1) Expression in Retinoblastoma

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Purpose. Retinoblastomas’ growth rate is dependent on their ability to induce neovascularization. Leucine-rich α-2-glycoprotein-1 (LRG-1) was recently reported to be upregulated in human retinoblastoma and to correlate it with clinical and histopathologic parameters and to assess how its expression correlates with vascular endothelial growth factor (VEGF) expression.

Methods. LRG-1 expression was immunohistochemically evaluated in 34 retinoblastoma sections. Immunofluorescence for LRG-1/VEGF-A, LRG-1/TGF-β1/CD31, and LRG-1/Ki67 was performed. Quantitative RT-PCR analysis for the expression of LRG-1 was also done.

Results. LRG-1 was found to be extensively and robustly expressed in retinoblastoma tumors (88%) irrespective of the degree of invasiveness, differentiation, iris neovascularization, and anterior segment involvement. LRG-1 immunoreactivity was predominately observed in the central tumor vasculature and in the surrounding rim of ischemia. The higher frequency of LRG-1 expression in the presence of optic nerve infiltration, vitreous seeding, and necrosis was not statistically significant. Colocalization was observed between LRG-1 and VEGF-A staining, and no difference in their counts was detected. Quantitative RT-PCR analysis showed that LRG-1 gene expression was significantly upregulated (4.8-fold increase, \( P = 0.01 \)).

Conclusions. LRG-1 was highly expressed in human retinoblastoma sections, thus providing new insights into the molecular mechanism of retinoblastoma pathogenesis, and suggests a possible new therapeutic target. LRG-1 is a novel oncogene-associated protein shown to be vital to the progression of human cancers. Inhibiting tumor vasculature is progressively evolving as a target in anticancer therapy.

Keywords: retinoblastoma, leucine-rich α-2-glycoprotein-1, vascular endothelial growth factor, angiogenesis, tumor necrosis
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LRG-1 Expression in Retinoblastoma

Formalin-fixed paraffin-embedded sections of retinoblastoma from 34 patients who underwent enucleation at the Department of Ophthalmology of the Hadassah-Hebrew University Medical Center between 1999 and 2015 were investigated. Patients' demographic data, including sex, age at enucleation, eye involved, laterality of retinoblastoma, and previous therapy, were collected. Histologic features like tumor necrosis, calcifications, iris neovascularization, vitreous seeding, and infiltration into the optic nerve, choroid, subretinal space, or anterior segment was documented. The type of the tumor tissue sections was approved by the Institutional Review Board (Helsinki committee) for experiments in human subjects. The research was conducted in accordance with the Declaration of Helsinki.

**Immunohistochemistry for the Detection of LRG-1**

Formalin-fixed and paraffin-embedded retinoblastoma specimens were cut into 5-μm sections. Before deparaffination, sections were heated for 20 minutes at temperature of 60°C. Paraffin was removed from the sections, which were then rehydrated through a series of absolute xylene (100%) (three times, each time for 5 minutes) and graded ethanolns, washed in double-distilled water (DDW) for 2 minutes, and then rinsed in Tris-buffered saline (TBS; pH 7.6) with 0.1% Tween-20 added. The slides were heated by pressure cooker for 20 minutes in 10 mM citrate buffer (pH 6.0). After cooling the slides for 20 minutes, they were washed three times in TBS plus 0.1% Tween-20 for 5 minutes. Nonspecific binding was blocked by incubating the slides in blocking solution (Abcam, Tel Aviv, Israel) for 30 minutes. For incubation with the rabbit anti-human LRG-1 monoclonal antibody (0.2 mg/mL; Sigma-Aldrich Corp., St. Louis, MO, USA) at 1:250 or with the mouse anti-human VEGF-A monoclonal antibody (100 µg/mL; Biotest, Dreieich, Deutschland) at 1:500. All immunohistochemical samples were stained with Mayer's hemalum diluted 1:1 in distilled water for 10 seconds, blued under running tap water, and mounted (Crystal Mount; Biomedia Corp., Foster City, CA, USA). Negative controls were treated the same way using the rabbit IgG-isotype control (10 mg/mL; Novus, Littleton, CO, USA) at 1:100. All immunohistochemical samples were graded as being positive or negative. No measure of staining intensity or percentage of stained cells was recorded for immunohistochemical findings.

**Immunofluorescence**

Direct immunofluorescence on retinoblastoma sections was performed to assess immunoreactivity of the following combinations: LRG-1/VEGF-A, LRG-1/TGF-β1/CD31, and LRG-1/Ki67.

To identify LRG-1, VEGF-A, TGF-β1, CD31, and Ki67-positive cells, immunofluorescence staining was performed. Before deparaffination, sections were heated for 20 minutes at temperature of 60°C. Paraffin was removed from the sections, which were then rehydrated through a series of absolute xylene (100%) (three times, each time for 5 minutes) and graded ethanolns, washed in DDW for 2 minutes, and then rinsed in TBS (pH 7.6) with 0.1% Tween-20 added. The slides were heated by pressure cooker for 20 minutes in 10 mM citrate buffer (pH 6.0). After cooling the slides for 20 minutes, they were washed three times in TBS plus 0.1% Tween-20 for 5 minutes. Nonspecific binding was blocked by incubating the slides in 10% normal rabbit serum (Dako) for 30 minutes, followed by incubation with one of the following: the rabbit anti-human LRG-1 monoclonal antibody (0.2 mg/mL; Sigma-Aldrich Corp.) at 1:250; the mouse anti-human VEGF-A monoclonal antibody (100 µg/mL; Biotest) at 1:500; the mouse anti-human TGF-β1 (1 mg/mL; Novus) at 1:100; the sheep anti-human CD31 (100 µg/mL; R&D Systems, Minneapolis, MN, USA) at 1:250; the mouse anti-human Ki67 (0.5 µg/mL; Eldan, Petah Tikva, Israel) at 1:100 overnight at 4°C. The following morning the sections were washed with PBS 1%. Then the secondary antibodies were added: Cy-2-conjugated antibody against LRG-1 (goat, anti-rabbit) (2 mg/mL at 1:250; Abcam); Cy-3-conjugated antibody against VEGF-A (donkey, anti-mouse) (0.5 mg/mL Abcam) in the sections in which LRG-1 and VEGF-A were immunostained.

In the sections in which LRG-1, TGF-β1, and CD31 were immunostained, the secondary antibodies included Cy-2-conjugated antibody against TGF-β1 (goat, anti-mouse) (2 mg/mL at 1:400; Abcam), Cy-3-conjugated antibody against LRG-1 (donkey, anti-rabbit) (2 mg/mL at 1:250; Abcam), and Alexa-Fluor 647-conjugated antibody against CD31 (donkey, anti-sheep) (2 mg/mL, at 1:400; Abcam).

In the sections in which LRG-1 and Ki67 were immunostained, the secondary antibodies included Cy-2-conjugated antibody against Ki67 (donkey, anti-mouse) (0.5 mg/mL at 1:400; Abcam) and Cy-3-conjugated antibody against LRG-1 (goat, anti-rabbit) (2 mg/mL at 1:250; Abcam). Negative controls were treated the same way, omitting the incubation with the primary antibody.

All sections were washed with PBS 1% and 4′,6-diamidino-2-phenylindole, and mounting liquid was added. Antifade/gel-mounted sections (Vector Laboratories) were examined at 60X magnification in oil immersion using an E2000U three-laser confocal microscope equipped with C1 software (LESM710; Carl Zeiss AG, Oberkochen, Switzerland).
Quantitative RT-PCR
Total cellular RNA was isolated from five formalin-fixed, paraffin-embedded tissue sections of four eyes with retinoblastoma and one normal eye after exenteration for orbital cancer, using 2 mL TRI Reagent (cat no. 90102331; Bio Lab, Jerusalem, Israel) for each cubic centimeter of tissue. The samples were homogenized for 5 minutes at a room temperature of 25°C. Chloroform at a volume of 0.2 mL (cat no. 03080521; Bio Lab) was added to each sample, incubated for 15 minutes at room temperature, and centrifuged (220 g) for 15 minutes at 4°C. For RNA precipitation, the supernatant in each sample was transferred to new Eppendorf tubes, 0.5 mL isopropanol (cat no. 16260521; Bio Lab) was added and left for 10 minutes at 25°C, then it was centrifuged (16,128 g) for 10 minutes at 4°C. The supernatants were removed, and 1 mL ethanol (75%) was added to the pellet and centrifuged (6300 g) for 5 minutes. The pellets were dried in air at room temperature for 15 minutes, 50 lL diethyl pyrocarbonate was added, and the samples were heated for 10 minutes at 55°C. Preparation of cDNA was performed using high-capacity cDNA isolation kit (cat no. 1406197; R&D Systems). RT-PCR was performed for the quantification of the gene expression that encoded LRG-1 (cat no. AB-4331182; rhenium) compared to GAPDH as a housekeeping gene by using Taqman Fast Advanced Master Mix (cat no. 4371130; Applied Biosystems, Foster City, CA, USA). Student’s t-test was performed to determine statistical significance between test groups.

Evaluation of Staining Pattern and Statistical Analysis
LRG-1 and VEGF-A immunohistochemical staining of retinoblastoma sections was recorded by an observer who was masked to the clinical data at the time of evaluation. Generally, the 34 cases of retinoblastoma were divided into positive and negative tumors. LRG-1 and VEGF-A staining was evaluated on the basis of the percentage of positive cells: For more than 10% of the cells, staining was graded as positive, and if there was no detectable staining or <10% of cells were stained, staining was graded as negative.12 For documentation, the slides were examined with a digital camera (DP70; Olympus, Melville, NY, USA) and photographed. The correlation between LRG-1/VEGF-A expression and the association with clinical and histopathologic parameters described previously were investigated.

Statistical analysis was performed using software (SPSS version 24.0; SPSS, Inc., Chicago, IL, USA). Measures of continuous parameters as age and follow-up time were summarized using means, standard deviations, median, and range. Fisher’s exact test was applied to compare the presence of categorical parameters (anterior segment, vitreous, choroidal, subretinal, optic nerve infiltration, etc.) between eyes with positive or negative staining of LRG-1 and VEGF-A. Logistic
regression was performed for multivariate analysis. Correlation between staining and high-risk features was evaluated using the Spearman coefficient. A P value of less than 0.05 was considered significant.

RESULTS

Thirty-four eyes of 34 patients with retinoblastoma were included in the study. Nineteen eyes belonged to male patients (56%). Mean (±SD) age at enucleation was 33.4 (±27.6) months (median 24 months; range, 5–109). For twenty-one (62%) patients, enucleation was performed at less than 24 months of age. Twenty-nine (85%) patients had unilateral disease, and five (15%) had bilateral involvement. Included were 20 (59%) left eyes and 14 (41%) right eyes. Mean (±SD) follow-up period was 82.4 (±67.8) months (range, 18–207).

For 27 patients (79%), it was primary enucleation, whereas for seven patients (21%) it was post chemotherapy (carboplatin, vincristine, and etoposide). Following enucleation, there was no further information on 10 patients; however, for the remaining 24 patients, 22 (92%) were alive at the last follow-up visit.

Optic nerve infiltration was observed in 14 eyes (41%) and choroidal invasion in nine eyes (26.5%). Eleven eyes (32%) demonstrated tumor cells in the subretinal space, and 15 eyes had vitreous seeding (44%). Anterior segment involvement was observed in three eyes (9%), and iris neovascularization was noted in 17 eyes (50%). Pathologic high-risk features (HRF) were defined according to the Children’s Oncology Group and the International Retinoblastoma Staging Group and included the presence of both optic nerve and choroidal invasion. In total, five (15%) eyes had HRF. Necrosis was noted in 30
retinoblastoma sections (88%), and 22 had calcifications (65%). Twenty retinoblastoma sections showed the presence of rosettes (59%). Positive LRG-1 immunoreactivity was noted in 30 of the retinoblastoma sections (88%) of 15 male and 15 female patients (Table; Fig. 1). The remaining four negative LRG-1 sections belonged to male patients ($P = 0.081$). Mean age at enucleation for the LRG-1-positive tumors was 33.7 months, whereas it was 31.3 months for the LRG-1-negative tumors. Optic nerve involvement was noted in 13 of the LRG-1-positive tumors (43%), while it was noted in one of the LRG-1-negative tumors (25%); this, however, did not reach statistical significance ($P = 0.44$). Choroidal invasion was noted in seven of the LRG-1-positive tumors (23%) and in two of the LRG-1-negative tumors (50%). Subretinal infiltration was seen in 10 of the LRG-1-positive tumors (33%) and in one of the LRG-1-negative tumors (25%). Vitreous seeding was recorded in 15 of the LRG-1-positive tumors (50%) and in none of the LRG-1-negative tumors ($P = 0.08$). Iris neovascularization was noted in 15 of the LRG-1-positive tumors (50%) and in two of the LRG-1-negative tumors (50%). Anterior segment involvement was noted in two of the LRG-1-positive tumors (7%) and in one of the LRG-1-negative tumors (25%). Necrosis was noted in 27 of the LRG-1-positive tumors (90%) and in three of the LRG-1-negative tumors (75%) ($P = 0.61$). Calcifications were noted in 19 of the LRG-1-positive tumors (63%) and in three of the LRG-1-negative tumors (75%). All seven eyes that were enucleated post chemotherapy showed LRG-1 positivity. LRG-1 immunoreactivity was also observed in nonneoplastic tissue such as iris, pars plica, choriocapillaris, optic nerve, and ganglion cell layer (Fig. 2).

Positive VEGF-A immunoreactivity was observed in 28 of the 33 (in one tumor additional slides were unavailable) stained retinoblastoma sections (85%). Immunofluorescence showed strong colocalization between LRG-1 and VEGF-A staining, particularly observed in the central tumor feeder vessels and in the ischemic areas at the periphery of the tumoral sleeves (Figs. 3, 4). Colocalization was also observed between LRG-1, CD31, and TGF-$\beta 1$ (Fig. 5) and between LRG-1 and Ki67 (Fig. 6).

There was no difference in counts between VEGF-A- and LRG-1-positive cells on immunofluorescence images ($n = 3, P = 0.49$). There was no correlation between positive LRG-1 and VEGF-A staining ($P = 0.57$, Spearman coefficient). No correlation was observed between LRG-1 or VEGF-A and HRF.

Quantitative RT-PCR analysis of the expression of LRG-1 in formalin-fixed, paraffin-embedded tissue sections of four eyes with retinoblastoma and one normal eye after exenteration for orbital cancer showed that LRG-1 gene expression was significantly upregulated compared to normal tissue (Fig. 7) (4.8-fold increase, $P = 0.01$).

**DISCUSSION**

In the present study, LRG-1 was found to be highly expressed in human retinoblastoma sections (88%), thus providing new insights into the molecular mechanism of retinoblastoma pathogenesis and suggesting a possible new therapeutic target. To the best of our knowledge, this is the first study that analyzes the expression of LRG-1 in human retinoblastoma. LRG-1 is a novel oncogene-associated protein shown to be vital to the progression of human cancers. Studies have demonstrated that LRG-1 overexpression is associated with several types of tumors and could be regarded as a diagnostic marker of cancers. Elevated expression of LRG-1 was frequently accompanied with worse malignant phenomenon, such as larger tumor size, more advanced tumor stage, poorer tumor differentiation, and more vascular invasion. In endometrial cancer, LRG-1 expression was associated with tumor stage and lymphatic metastasis and was an independent prognostic factor for overall survival.
LRG-1 expression did not correlate with HRF of retinoblastoma such as choroidal and optic nerve invasion. It was found to be extensively and robustly expressed in retinoblastoma tumors irrespective of the degree of invasiveness, differentiation, iris neovascularization, and anterior segment involvement. LRG-1 immunoreactivity was predominantly observed in the central tumor blood vessels and in the surrounding rim of ischemia. It showed colocalization with CD31 (used primarily to demonstrate the presence of endothelial cells in histologic tissue sections). It also colocalized with Ki67 cells, suggesting possible upregulation by actively proliferating tumor cells.

Despite the lack of statistical significance, LRG-1 was observed more in retinoblastomas with necrosis. Wang et al. previously reported on the upregulation of LRG-1 transcript levels during the ischemic proliferative phase of oxygen-induced retinopathy (OIR), which represents a model of hypoxia-driven retinal angiogenesis. The pattern of LRG-1 up- and downregulation in OIR mirrored the expression of the hypoxia-responsive genes VEGF-A and Apln (apelin) and its receptor Aplnr. Also as mentioned by Wang et al., an upregulation of LRG-1 levels was noted in human vitreous samples of patients with PDR, again demonstrating the relation between hypoxia/ischemia and LRG-1 overproduction.

VEGF-A expression in the present study did not show a statistically significant correlation with HRF ($P = 0.06$). However, Garcia et al. demonstrated that HRF retinoblastoma tumors elicited more angiogenesis than non-HRF tumors as they expressed more VEGF, especially in the tumor and iris. Similarly, Areán et al. reported on a positive correlation
between VEGF staining intensity and time of progression and mitotic and apoptotic indexes. Vitreous seeding was observed in LRG-1-positive tumors and was not seen in LRG-1-negative tumors. Not many years ago, the lack of vitreous seed control was often the limiting factor in globe salvage as they were the least responsive to intravenous chemotherapy and intra-arterial chemotherapy. This is sequential to their location in the vitreous remote from blood supply, and thus they rely on diffusion of chemotherapy into the vitreous gel from the retina. Prognosis of vitreous seeds-harboring eyes has improved significantly following the introduction of intravitreal melphalan, with complete control relatively early in the course of therapy. Despite the lack of ocular complications in some studies, other publications reported on the occurrence of vision-threatening complications such as vitreous hemorrhage or serous retinal detachment with intravitreal chemotherapy. With the knowledge obtained in the index study on the strong expression of LRG-1 in vitreous seeds, it is plausible to consider LRG-1 as playing an important role in their development and progression by providing vascular supply and may possibly play a potential role in their regression by LRG-1 blockade. No increased expression of LRG-1 was noted in subretinal or anterior chamber seeds.

LRG-1 was recently reported to promote angiogenesis by modulating endothelial TGF-β signaling. In the present study, LRG-1 showed colocalization with TGF-β1. LRG-1 binds directly to the TGF-β accessory receptor endoglin (CD105), which in the presence of TGF-β1 results in promotion of the proangiogenic Smad1/5/8 signaling pathway. Endoglin expression in retinoblastoma was studied by Garcia et al. It was found to be expressed in low levels in the HRF and non-HRF tumors and in the nonneoplastic retina. Its significant expression, however, was observed in the iris neovascular membranes of HRF retinoblastomas. The authors did not observe a significant level of coexpression between VEGFR-2 and endoglin (CD105). Similarly, in the index study, there was no correlation between LRG-1 and VEGF-A expression. The lack of correlation between endoglin/VEGFR-2 and LRG-1/VEGF-A expression is probably due to separate/parallel proangiogenic signaling pathways activated in the tumor microenvironment: LRG-1 acting by binding to endoglin and activating the proangiogenic Smad1/5/8 pathway and VEGF-A acting through VEGFR-2 (which is the principal mediator of VEGF-A effects on endothelial cells) and inducing proliferation via activation of the classical extracellular signal-regulated kinases pathway.

The concept of angiogenesis inhibition in retinoblastoma has already been tried. The fact that VEGF level is highly expressed in retinoblastoma and is correlated with the aggressive disease features such as optic nerve invasion and poor patient outcome, as well as the observation that VEGF was highly expressed in residual tumor cells after neoadjuvant chemotherapy correlating to local invasion and worse prognosis, triggered researchers and clinicians to try anti-VEGF therapy both experimentally and clinically. Lee et al. evaluated the antiangiogenic effects of bevacizumab in a coculture of a Y79 human retinoblastoma cell line and a human umbilical vein endothelial cell line. Also, Y79 xenotransplanted nude mice were treated with bevacizumab. The authors reported that bevacizumab induced a 75% reduction in the growth of the retinoblastomas without producing significant systemic toxicity. Also, demonstrated that bevacizumab could inhibit differentiation of retinoblastoma cells without affecting cellular viability. Hou et al. evaluated the efficacy of intravitreal carboplatin plus bevacizumab in 11 patients with refractory retinoblastoma and concluded that it was a relatively safe, effective treatment for refractory retinoblastoma, but ineffective for recurrent tumor. Magacizumab, a monoclonal antibody that specifically targets LRG-1 and prevents the formation of abnormal blood vessels, is now being developed and is currently tried in phase 1 and phase 2a clinical trials to assess whether the drug is safe for humans and whether it will halt the disease in AMD patients who are no longer responsive to the standard anti-VEGF treatment (Greenwood J., The pathogenic role of LRG1 in ocular neovascularization: from discovery to targeted therapy, EVER-Acta lecture presented at EVER Meeting, October 6, 2016). This approach targets a novel pathway involved in pathologic angiogenesis, and if proven safe in humans, then its use may have important implications for other areas of medicine, including retinoblastoma in which it is highly expressed.

Finally, recent advances in antiangiogenic therapies for solid malignancies have improved patient survival and thus validated the tumor vasculature as a target in anticancer therapy. While some have shown activity as monotherapies, most clinical trials to date indicate that they are most effective when piggybacked onto traditional therapies, especially chemotherapy. They may produce the chemosensitizing effects by normalizing tumor vasculature, thus enabling efficient delivery of antitumor drugs, by preventing rapid tumor cell repopulation, and by augmenting the antivascular effects of chemotherapy.

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**References**


