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NEUROPATHOLOGY

Neuropathology 2023 doi:10.1111/neup.12944

Original Article

Increased expression of leucine-rich α-2 glycoprotein 1 as a predictive biomarker of favorable progression-free survival in meningioma

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Most meningiomas, which are frequent central nervous system tumors, are classified as World Health Organization (WHO) grade 1 because of their slow-growing nature. However, the recurrence rate varies and is difficult to predict using conventional histopathological diagnoses. Leucine-rich α-2 glycoprotein 1 (LRG1) is involved in cell signal transduction, cell adhesion, and DNA repair and is a predictive biomarker in different malignant tumors; however, such a relationship has not been reported in meningiomas. We examined tissue microarrays of histological samples from 117 patients with grade 1 and 2 meningiomas and assessed their clinical and pathological features, including expression of LRG1 protein. LRG1-high meningiomas showed an increased number of vessels with CD3-positive cell infiltration ($P = 0.0328$) as well as higher CD105-positive vessels ($P = 0.0084$), as compared to LRG1-low cases. They also demonstrated better progression-free survival (hazard ratio [HR] 0.11, 95% confidence interval [CI] 0.016–0.841) compared to LRG1-low patients ($P = 0.033$). Moreover, multivariate analysis indicated that high LRG1 expression was an independent prognostic factor (HR, 0.13; 95% CI, 0.018–0.991; $P = 0.049$). LRG1 immunohistochemistry may be a convenient tool for estimating the prognosis of meningiomas in routine practice. Further studies are required to elucidate the key role of LRG1 in meningioma progression.

Key words: LRG1, menignioma, pathology, prognosis, progression.

INTRODUCTION

Meningioma is the most common primary intracranial tumor, accounting for 36% of all central nervous system tumors that originate from arachnoid cells. According to the fifth edition of the World Health Organization Classification of the Tumors of the Central Nervous System (WHO CNS5), these tumors are categorized into 15 subtypes and three grades of malignancy.[1] Meningiomas are mostly slow-growing, and their recurrence rate varies from 7 to 25%, thus making their prognosis using conventional pathological diagnosis difficult. A subset of meningiomas contain different genes that regulate tumor growth in vitro and in vivo[2,3]; however, their role as prognostic factors has not been fully revealed.

Leucine-rich α-2 glycoprotein 1 (LRG1) is a member of the leucine-rich repeat protein family, which is involved in cell signal transduction, cell adhesion, and DNA repair. LRG1 is expressed in the hematopoietic stem cells, and after their differentiation into neutrophilic granulocytes,[4] LRG1 is involved in inflammation through activation of the transforming growth factor beta and Smad1/5/8 pathways.[5–7] In fact, serum LRG1 concentration is a biomarker of disease activity in ulcerative colitis and rheumatoid arthritis.[4,5] It also promotes pathogenic neovascularization through endothelial transforming growth factor beta signaling in the presence of CD105 (endoglin).[6]

Studies have shown that LRG1 can be used as a prognosis marker in carcinomas such as hepatocellular carcinoma,[8] ovarian cancer,[9] and non-small-cell lung cancer.[10] Furthermore, our group has reported that LRG1 overexpression is an independent prognostic factor in glioblastomas.[11]

However, the association between clinicopathological features and the expression of LRG1 has never been investigated in meningiomas. In this study, we evaluated the expression of LRG1 in meningioma using immunohistochemistry and validated its role as a prognostic marker.

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Received 21 June 2023; revised 07 September 2023; accepted 11 September 2023.

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MATERIALS AND METHODS

Clinical sample and histology

In this study, tissues from 121 patients with WHO grade 1 and 2 meningiomas were obtained through surgery and stored in the database of the Department of Pathology, Kurume University. Histological analysis was based on the WHO criteria.[1] We used a tissue microarray (TMA) containing each of the 121 specimens in a formalin-fixed paraffin block, where each sample was placed in a 2.5-mm-diameter space. The research ethics committee of Kurume University approved the use of patient materials and clinical information (No. 345), and the study was in accordance with the Declaration of Helsinki.

Evaluation of immunohistochemical staining of LRG1, CD3, CD20, and CD105

Slide-mounted formalin-fixed, paraffin-embedded tissue sections (2.5 μm in thickness) from the TMA were used as previously described.[11] For immunohistochemistry analysis, sections were stained with antibodies against LRG1 (rabbit polyclonal, Sigma-Aldrich, St. Louis, MO, United States; 1:300 dilution), CD3 (Dako, Japan Co, Kyoto, Japan; 1:50 dilution), CD20 (Dako; 1:5 dilution), and CD105 (Dako; 1:50 dilution). To evaluate tumor angiogenesis, CD105-positive vessels were counted.

Definition of immunohistochemical LRG1-score and cutoff value

Two blinded pathologists (MM and MJ) independently evaluated the expression of LRG1. Using the same method as stated previously,[11] the intensity of LRG1 staining of individual tumor cells was graded from 0 to 3 (0, negative; 1, weak or focal positive; 2, moderate; 3, strong and diffuse positive). Based on the results, the study population was divided into two groups: low expression (score 0,1) and high expression (score 2,3) (Fig. 1). For evaluation of tumor angiogenesis, CD105-positive vessels were counted in all the fields of each TMA at high magnification (×400). To evaluate inflammatory cell infiltration, CD3 and CD20-positive lymphocytes were counted in all TMA fields. Cutoff values for high CD105-positive vessel and CD3- and CD20-positive cell infiltration were set above the median value per specimen.

Gene expression profiling

We extracted RNA from formalin-fixed, paraffin-embedded tissue sections of four LRG1-high and four low samples using the RNeasy Micro Kit (Qiagen, Hilden, Germany). This was followed by microarray as described previously.[12] The genes with P-values less than 0.05 and $\log^2$ fold changes of <-1 or >1 were defined as characteristic genes.

Molecular genetic analyses

Genomic DNA was isolated from the same sample as extracted RNA from the relevant FFPE tissue followed by extraction using a GeneRead DNA FFPE Kit (QIAGEN, Hilden, Germany). Telomerase reverse transcriptase (TERT) promoter 5p15.33 was amplified by polymerase chain reaction (PCR) as described previously.[11] No mutation was found, confirming the diagnosis of meningioma grade 1 or 2.

Statistical analysis

The clinicopathological characteristics of the patients were compared using the χ^2-test or Fisher's exact test. Progression-free survival was defined as the time from the date of diagnosis to the date of recurrence or last follow-up. The Kaplan–Meier method was used to estimate progression-free survival distribution, and the log-rank test was performed to evaluate significant differences. Univariate and multivariate Cox proportional regression models were used to evaluate the prognostic factors. Statistical significance was set at $P < 0.05$. JMP version 16.0 was used in all statistical analyses.

RESULTS

Clinicopathological characteristics of patients with meningioma

The clinicopathological characteristics of the 117 patients are summarized in Table 1. The median age was 61 (range 22–85) years. A total of 31 men and 86 women were included. The follow-up period ranged from 0.23 to 114.8 months with a median value of 24.1 months. According to the WHO classification,[1] distribution of histology was fibrous (28.2%), transitional (22.2%), meningothelial (24.8%), microcystic (5.1%), psammomatous (5.1%), angiomatous (4.3%), metaplastic (2.6%), secretory (0.9%), atypical (6.0%), and chordoid (0.9%). Eighty-nine of 117 patients (66.7%) had Simpson grade 1 or 2 in terms of surgical resection completeness,[13] while 50.4% (59/117) of patients had tumors located at the skull base. Twenty-six cases (22.2%) were categorized as LRG1-high.

Clinicopathological comparison between LRG1-high and -low patients

LRG1-high meningiomas showed CD3-positive cell infiltration ($P = 0.033$) and more CD105-positive vessels ($P = 0.008$) in comparison to LRG1 low cases. There were no significant differences in age, sex, histology, Simpson grade,

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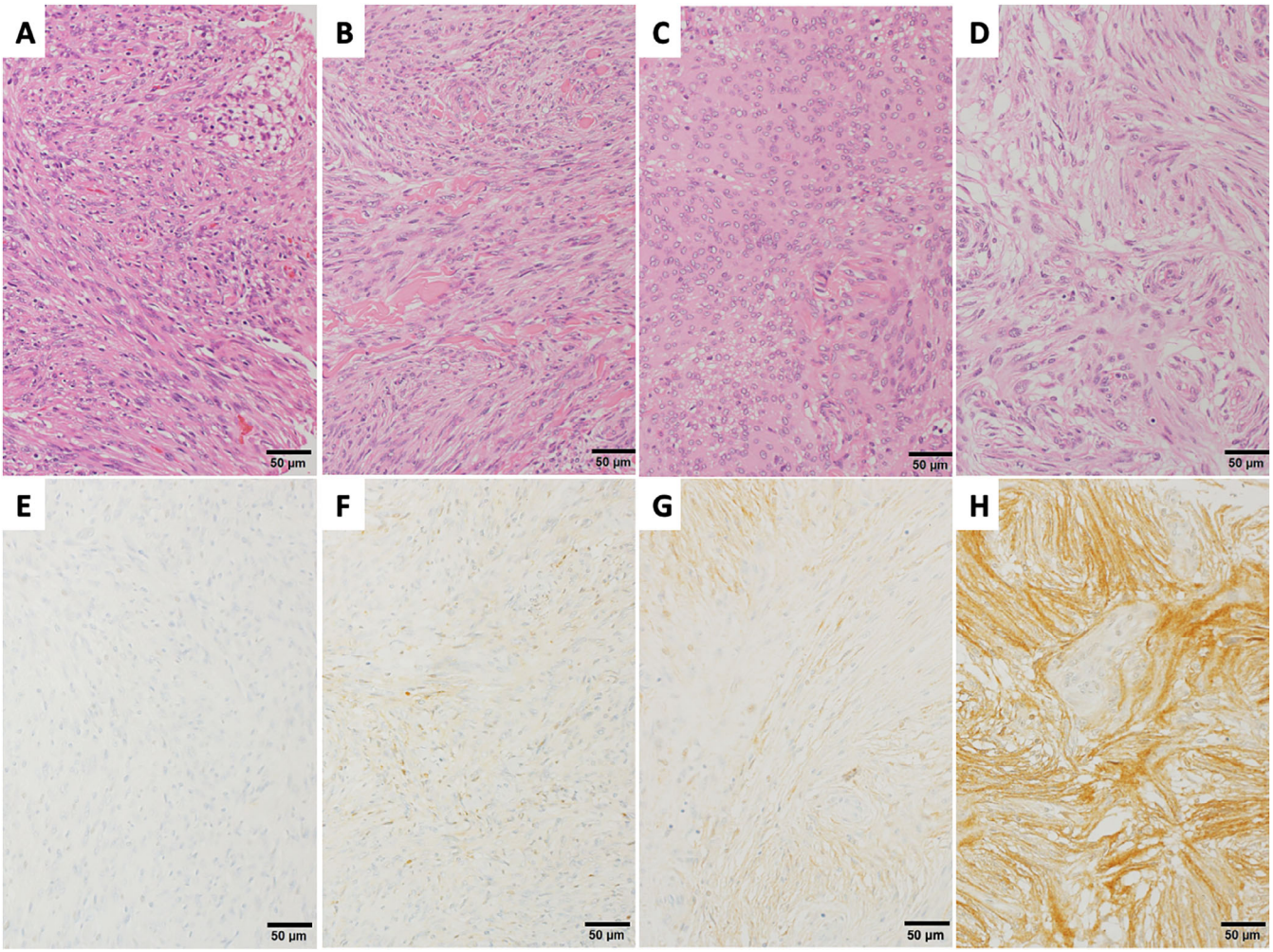

Fig 1 Representative images of meningioma specimen on HE staining (A–D) and leucine-rich α-2 glycoprotein 1 (LRG1) staining (E–H). A transitional meningioma case with LRG1 score 0 (A, E), a fibrous meningioma case with LRG1 score 1 (B, F), a meningothelial meningioma case with LRG1 score 2 (C, G), and a transitional meningioma case with LRG1 score 3 (D, H). Scale bars: 50 μm (A–H).

location, MIB-1 index, and CD20-positive cell infiltration between these two groups (Table 1).

Progression-free survival curves in patients with meningioma according to LRG1 expression

Patients with high LRG1 expression had better progression-free survival compared to LRG1-low patients (log-rank, $P = 0.010$, Fig. 2A). Moreover, grade 1 LRG1-high meningioma showed better progression-free survival in comparison to LRG1-low patients (log-rank, $P = 0.015$, Fig. 2B). Univariate analysis was performed using degree of Simpson grade ≤2 (HR, 0.20; 95% CI, 0.092–0.435; $P < 0.001$), non-skull base (HR, 0.74; 95% CI, 0.348–1.556; $P = 0.422$), and Ki-67 labeling index <4% (HR, 0.40; 95% CI, 0.054–2.923; $P = 0.366$) (Table 2). Multivariate analysis using these variables indicated that high LRG1 expression was an independent prognostic factor (HR, 0.13; 95% CI, 0.018–0.991; $P = 0.049$, Table 2).

RNA microarray expression analysis

Following appropriate normalization and standardization procedures, the data on each chip were compared with each other by using a hierarchical clustering method. In the LRG1-high tumors, 763 probe sets were identified as upregulated or downregulated on a heat map (Fig. 3A). The expression-altered genes were located on the whole genome (Fig. 3B). The first 10 genes showing the highest upregulation or lowest downregulation are listed in Table 3, respectively. Several genes associated with angiogenesis or inflammation were upregulated (Table S1). Pathway enrichment analysis using Metascape[14] showed that LRG1-high specimens contained

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Table 1 Clinicopathological characteristics of patients ($n = 117$)

	All	LRG1		
		Low	High	*P**
Median age, y (range)	61 (22–85)	61 (22–85)	59 (34–85)	0.88
Female	73.5% (86/117)	76.9% (70/91)	61.5% (16/26)	0.135
Histology				
Fibrous	28.2% (33/117)	27.5% (25/91)	30.1% (8/26)	0.761
Meningothelial	22.2% (26/117)	23.1% (21/91)	19.2% (5/26)	
Transitional	24.8% (29/117)	26.4% (24/91)	19.2% (5/26)	
Microcystic	5.1% (6/117)	3.3% (3/91)	11.5% (3/26)	
Psammomatous	5.4% (6/117)	5.8% (5/91)	3.9% (1/26)	
Angiomatous	4.3% (5/117)	3.3% (3/91)	7.7% (2/26)	
Metaplastic	2.6% (3/117)	2.2% (2/91)	3.9% (1/26)	
Secretory	0.9% (1/117)	1.1% (1/91)	0% (0/26)	
Atypical	6.0% (7/117)	6.6% (6/91)	3.9% (1/26)	
Chordoid	0.9% (1/117)	1.1% (1/91)	0% (0/26)	
Simpson grade				
1	27.4% (32/117)	23.1% (21/91)	42.3% (11/26)	0.095
2	39.3% (46/117)	39.6% (36/91)	38.5% (10/26)	
3	16.2% (19/117)	16.5% (15/91)	15.4% (4/26)	
4	17.1% (20/117)	20.9% (19/91)	3.9% (1/26)	
Location				
Skull-base	50.4% (59/117)	51.7% (47/91)	42.3% (11/26)	0.506
Histological findings				
MIB-1 index, % (range)	1.0 (0–20)	1.0 (0–8.0)	1.0 (0–20)	0.516
increased CD105 vessels†	31.6% (37/117)	25.3% (23/91)	53.9% (14/26)	0.008
increased CD3-positive T-cell‡	22.2% (26/117)	17.6% (16/91)	38.5% (10/26)	0.033
increased CD20-positive B-cell§	39.3% (46/117)	37.4% (34/91)	46.2% (12/26)	0.496

Abbreviation: LRG1, leucine-rich α-2 glycoprotein 1. *Fisher's exact test; †≥11.6 (median); ‡≥79.2 (median); §≥6.8 (median).

genes associated with ribosomal proteins, genes regulating apoptosis, and DNA damage response genes mediated by TP53 (Fig. S1). Of note, no-CDKN2A/B homozygous deletion was found. No mutation in *TERT* promoter was identified, confirming the diagnosis of meningioma grade 1 or 2.

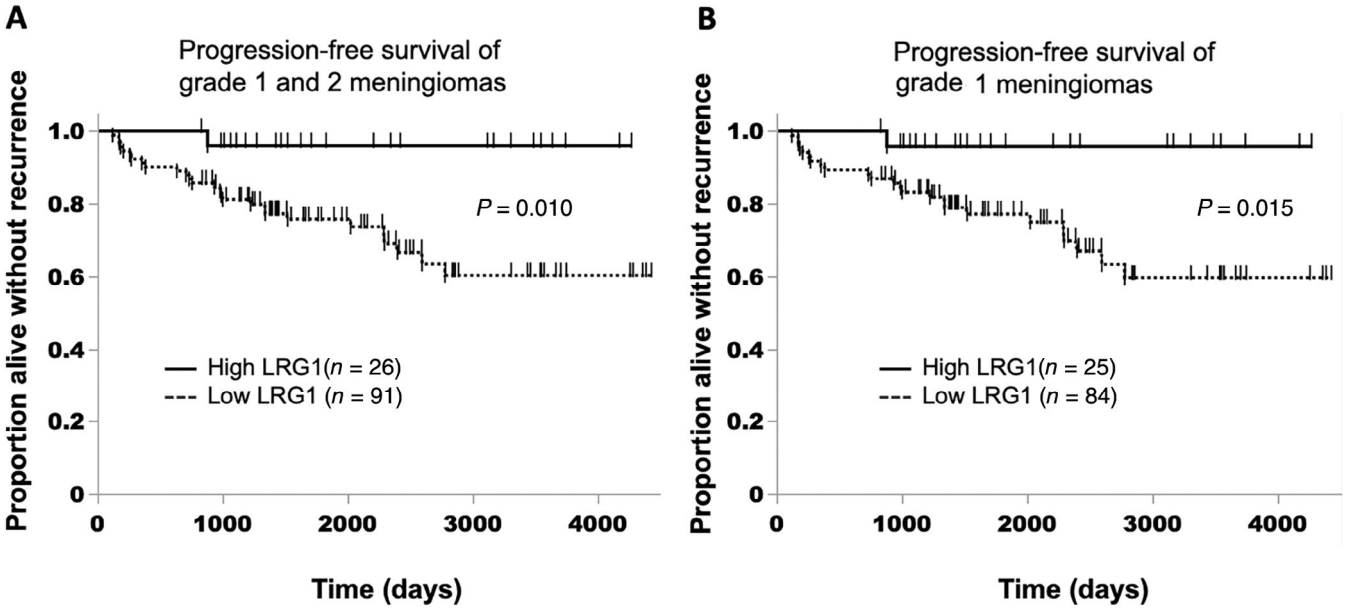

Fig 2 Kaplan–Meier curves of progression-free survival in patients with meningioma. Patients with high leucine-rich α-2 glycoprotein 1 (LRG1) expression had better progression-free survival compared to LRG1-low patients: (A) grade 1 and 2 meningiomas, $P = 0.010$; (B) grade 1 meningiomas, $P = 0.015$.

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Table 2 Prognostic factors affecting the PFS of patients with meningioma

	Univariate			Multivariate		
Variable	HR	95% CI	*P*	HR	95% CI	*P*
Not skull base	0.74	0.348–1.556	0.422			
MIB-1 index <4%	0.40	0.054–2.923	0.366			
CD3 lymphocyte	0.43	0.130–1.432	0.170			
CD105 vessel	0.76	0.352–1.651	0.491			
Simpson grade ≤2	**0.20**	**0.092–0.435**	**<0.001**	**0.22**	**0.099–0.474**	**0.0001**
High LRG1	**0.11**	**0.016–0.841**	**0.033**	**0.13**	**0.018–0.991**	**0.049**

Note: The significant variables has been in bold. LRG1, leucine-rich α-2 glycoprotein 1.

DISCUSSION

In this study, we demonstrated for the first time that high LRG1 expression in patients with low-grade meningioma is an independent prognostic factor for favorable progression-free survival. High LRG1 expression correlated with increased infiltration of CD3-positive T-cells and proliferation of CD105-positive vessels.

The level of expression of LRG1 is associated with progression in various tumors, including colorectal carcinoma, gastric cancer, hepatocellular carcinoma, pancreatic cancer, ovarian cancer, head and neck carcinoma, and glioblastoma.[8–11] Of these, in the case of head and neck squamous cell carcinoma and glioblastomas, high LRG1 expression was a favorable prognostic factor. The

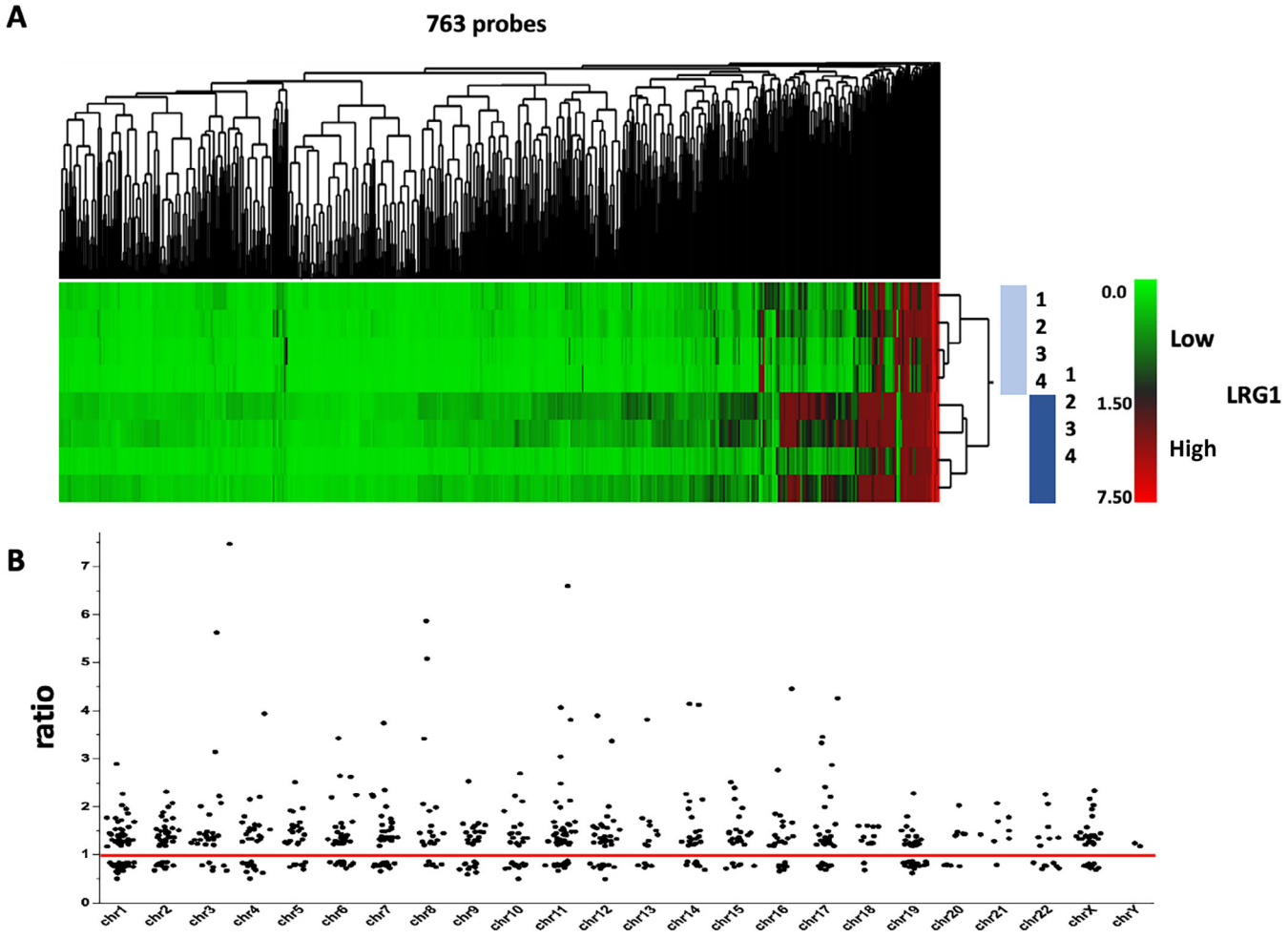

Fig 3 Gene expression profiles and clustering. The gene expression of leucine-rich α-2 glycoprotein 1 (LRG1)-high meningiomas was compared with that of LRG1-low meningiomas by using the hierarchical clustering method (A). In LRG1-high meningiomas, 763 probe sets were identified as upregulated or downregulated (B).

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Table 3 Genes differentially regulated in LRG1 high or low meningioma

	Gene symbol	Gene name	Location	Exp. Value
Up-regulated genes in LRG1 high meningioma compared with LRG1 low meningioma				
1	ETV5	Ets variant 5	3q27.2	7.46226365
2	LDHA	Lactate dehydrogenase A	11p15.1	6.58567127
3	RPL30	Ribosomal protein L30	8q22.2	5.85758845
4	RPL35A	Ribosomal protein L35a	3q29	5.61779313
5	ERI1	Exoribonuclease 1	8p23.1	5.07252592
6	NPIPB4	Nuclear pore complex interacting protein family, member B4	16p12.2	4.44884654
7	GABARAP	GABA(A) receptor-associated protein	17p13.1	4.25306479
8	RPS29	Ribosomal protein S29	14q21.3	4.13068964
9	NDUFB1	NADH dehydrogenase 1 beta subcomplex	14q31.3	4.11329393
10	TAF1D	TATA box binding protein associated factor 1D	11q21	4.05961149
Down-regulated genes in LRG1 high meningioma compared with LRG1 low meningioma				
1	CFAP54	Cilia and flagella associated 54	12q23.1	0.48650797
2	ARHGAP21	Rho GTPase activating protein 21	10p12.1	0.49153549
3	MGST3	Microsomal glutathione S-transferase 3	1q24.1	0.49475796
4	DHX15	DEAH (Asp-Glu-Ala-His) box helicase 15	4p15.2	0.49707456
5	NPR2	Natriuretic peptide receptor 2	9p13.3	0.58995875
6	NBPF15	Neuroblastoma breakpoint family, member 15	1q21.1	0.61717278
7	ZNF418	Zinc finger protein 418	19q13.43	0.61776091
8	SLIT2	Slit guidance ligand 2	4p15.31	0.62028042
9	NCS1	Neuronal calcium sensor 1	9q34.11	0.63012589
10	PPEF2	Protein phosphatase, EF-hand calcium binding domain 2	4q21.1	0.63686081

Abbreviation: LRG1, leucine-rich α-2 glycoprotein 1.

biological roles of LRG1 in tumor aggressiveness are not fully understood, and the mechanism might differ between different types of tumor.

The positive correlation between LRG1 expression and CD3-positive cell infiltration indicated that LRG1 may be associated with the immune milieu of meningiomas. Immune cell infiltration is commonly seen in other tumors, such as glioblastoma, primary central nervous system lymphoma, and metastatic brain tumors.[15–18] Furthermore, several studies have reported a relationship between meningioma and the immune microenvironment, such as CD20 infiltration and PD-L1 expression.[17,18] CD3-positive cell infiltration is controversial, negative correlation between CD3-positive cells and the tumor grade in meningiomas,[18] which may reflect tumor aggressiveness. Similarly, in our study, CD3-positive cell infiltration also showed better progression-free survival.

Moreover, high expression of LRG1 was associated with a greater frequency of CD105-positive vessels. We also performed CD34 immunostaining to detect all blood vessels, and the CD105/CD34 ratio was 5.1%. No association between CD34-positive vessels and LRG1 or histological variants, including angiomatous variants, was identified, suggesting that an increasing number of CD105-positive vessels reflected neoplastic angiogenesis (data not shown). Lower expression of CD105, a marker of angiogenesis, demonstrates better overall survival in several types of neoplasia,[19,20] but no significant relation is observed in meningiomas.[21] High CD105-positive vessels have also been reported to be correlated with a higher Ki-67 index.[21,22] Our study also indicated similar results regarding the CD105 and Ki-67 index ($P = 0.063$, data not shown), but neither was a significant progressive factor.

Molecular genetic profiling of meningiomas has been reported, while similar studies regarding LRG1 are lacking. Next-generation sequencing and methylation analysis have revealed the risk of recurrence of meningiomas. Mutations in genes such as *TRAF7*, *KLF4*, *SMO*, *AKT1*, and chromosome 5 gain were observed in the three benign groups; however, LRG1 and associated genes were not included in that study.[23] We could not detect these genomic abnormalities in gene expression analysis (Table 3, Fig. 3). The decrease of the genes in copy number on chromosome 19, where LRG1 is encoded, is a high-risk loss,[24] indirectly suggesting the relationship between low expression of LRG1 and poor prognosis. Pathway enrichment analysis showed that LRG1-high specimens contained genes associated with ribosomal proteins and DNA damage response genes mediated by TP53 (Table 3). As discussed above, inflammation and angiogenesis may be associated with LRG1, resulting in ribosomal stress[25] and thereby regulating LRG1 expression. We performed immunohistochemical analysis using antibodies against p53 and caspase 3 but did not find a relationship between the expression of LRG1 and these proteins because of the limited number of samples (data not shown).

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Malignant (WHO grade 3) meningiomas were excluded from our study because of the limited availability of samples (only 11 patients). Due to the use of TMA, each single core might not reflect the heterogeneity of LRG1 expression in tumors. LRG1, CD105, and TGF-β were not included in the microarray design. To confirm RNA expression, customized probes and real-time PCR are required in future research. An increased number of samples, such as frozen material and malignant meningioma, are needed to validate the biological function of LRG1 in all meningioma grades. Therefore, it can be concluded that the overexpression of LRG1 is a predictor of good progression-free survival in low-grade meningiomas. LRG1 has been suggested to prevent tumor progression through tumor immunity. Immunohistochemistry for LRG1 might be a powerful prognostic marker that can be easily and rapidly performed in routine practice for low-grade meningiomas with diverse recurrence rates. Further studies may highlight a key role of LRG1 in the oncogenesis and progression of meningiomas, including malignant meningiomas.

AUTHOR CONTRIBUTIONS

Takuya Furuta and Yasuo Sugita were involved in the study conceptualization. Hiroaki Miyoshi and Koichi Ohshima were involved in methodology. Mayuko Moritsubo, Satoru Komaki, and Junko Miyoshi analyzed and investigated the study. Mayuko Moritsubo wrote the original draft. Takuya Furuta, Satoru Komaki, and Kiyohiko Sakata wrote, reviewed, and edited the article. Motohiro Morioka, Koichi Ohshima, and Yasuo Sugita supervised the study. All authors have read and approved the final manuscript. The authors thank Prof. Kenta Murotani for assistance with statistical analysis. This manuscript was formatted and edited by Editage (https://www.editage.com).

ACKNOWLEDGMENTS

This study was supported in part by an Uchimura grant from Kurume University for the Promotion of female researcher, Fukuoka Public Health Promotion Organization Cancer Research Fund (2022-A12), and JSPS KAKENHI Grant Number JP23K14490.

DISCLOSURE

The authors have nothing to disclose.

ETHICS STATEMENT

Approval of the research protocol: Yes.
Informed Consent: Yes.
Registry and the Registration No. of the study/trial: 345 (The Research Ethics Committee, Kurume University).
Animal Studies: N/A.
Research involving recombinant DNA: N/A.

DATA AVAILABILITY STATEMENT

The underlying data can be accessed at reasonable request to the corresponding author.

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SUPPORTING INFORMATION

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Fig. S1. Of the upregulated genes, Metascape[14] automatically reported analyses. Leucine-rich α-2 glycoprotein 1 (LRG1)-high specimens contained genes associated with ribosomal proteins, genes regulating apoptosis, and DNA damage response genes mediated by TP53.

Table S1. Upregulated genes in leucine-rich α-2 glycoprotein 1 (LRG1) high meningioma compared with LRG1 low meningioma.

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