## LABORATORY INVESTIGATION

# Immunohistochemical analysis of LRIG proteins in meningiomas: correlation between estrogen receptor status and LRIG expression

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**Abstract** The leucine-rich repeats and immunoglobulin-like domains (LRIG) protein family is comprised of three integral membrane proteins: LRIG1, LRIG2, and LRIG3. LRIG1 is a negative regulator of growth factor signaling. The expression and subcellular localization of LRIG proteins have prognostic implications in primary brain tumors, such as oligodendrogliomas and astrocytomas. The expression of LRIG proteins has not previously been studied in meningiomas. In this study, the expression of LRIG1, LRIG2, and LRIG3 was analyzed in 409 meningiomas by immunohistochemistry, and potential associations between LRIG protein expression and tumor grade, gender, progesterone receptor status, and estrogen receptor (ER) status were investigated. The LRIG proteins were most often expressed in the cytoplasm, though LRIG1 also showed prominent nuclear expression. Cytoplasmic expression of LRIG1 and LRIG2 correlated with histological subtypes of meningiomas ($p = 0.038$ and $0.013$, respectively). Nuclear and cytoplasmic expression of LRIG1 was correlated with ER status ($p = 0.003$ and $0.004$, respectively), as was cytoplasmic expression of LRIG2 ($p = 0.006$). This study is the first to examine the expression of LRIG proteins in meningiomas, and it shows a correlation between ER status and the expression of LRIG1 and LRIG2, which suggests a possible role for LRIG proteins in meningioma pathogenesis.

**Keywords** Meningiomas · LRIG1 · LRIG2 · Estrogen receptor

## Introduction

Meningiomas are the most frequently diagnosed primary spinal or cranial tumors. Although meningiomas are usually benign, their intracranial location can lead to severe and lethal consequences [1]. Additionally, a subset of meningiomas is malignant, with a histologically and/or clinically aggressive phenotype. A recent study demonstrated a large variability in mortality rates among meningioma patients [2]. First-degree relatives of patients with meningiomas have an increased risk of developing the disease, but the etiology remains largely unknown [3]. The only established environmental risk factor for meningiomas is ionizing radiation at both low and high doses [4–7]. There is a 2:1 female-to-male incidence ratio. Breast cancer patients have an increased risk of meningiomas [8]. Epidemiological data have suggested that exogenous estrogens and progesterones may promote meningioma development and/or growth, but these associations are controversial [9]. Taken together, these observations indicate an etiological role for female sex hormones in the growth of meningiomas.

The human leucine-rich repeats and immunoglobulin-like domains (LRIG) gene family is comprised of *LRIG1*, *LRIG2*, and *LRIG3* [10–12]. The LRIG genes encode

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integral membrane proteins consisting of a signal peptide, a leucine-rich repeat domain, three LRIG, a transmembrane domain, and a cytoplasmic tail. It has been suggested that the subcellular localization of the LRIG proteins may be biologically important [13, 14]. *LRIG1*, located at chromosome 3p14.3 [12], encodes a negative feedback regulator of epidermal growth factor receptor (EGFR) signaling [13] that enhances receptor ubiquitination and degradation rates and inhibits signaling [15–17]. EGFR is commonly expressed in meningiomas [18–20]. *LRIG1* has been suggested to be a tumor suppressor gene, and its expression has been linked with a good prognosis and better patient survival in epithelial cancers [21–24]. In prostate cancer, LRIG1 protein expression is regulated by androgen [25], whereas in breast cancer, LRIG1 is regulated by estrogen [22]. *LRIG2* and *LRIG3* are located at chromosomes 1p13 [11] and 12q13.2, respectively [10]. Protein expression of LRIG2 and LRIG3 in the perinuclear area of astrocytoma cells has been associated with better patient survival [26], while LRIG2 has also been associated with poor survival when expressed cytoplasmically in oligodendrogliomas and uterine cervical carcinomas [21, 27]. However, the exact functions of LRIG2 and LRIG3 are currently poorly understood and the impact of subcellular LRIG protein localization on EGFR expression is not known. In meningiomas, the expression profiles of LRIG proteins have not been described. In this study, we used immunohistochemistry (IHC) to evaluate potential associations between LRIG protein expression in meningiomas and histological subtypes, gender, progesterone receptor (PR) status, and estrogen receptor (ER) status.

## Materials and methods

### Study population and tumor specimens

The original material consists of all patients who underwent surgery for intracranial meningioma at the Tampere University Hospital during 1989–1999. First, two patients were excluded from the studies because of their young age (4 and 15 years). Then, all the tumors with enough tumor material for the tissue micro-array blocks were included in the studies. This material of 510 tumors was previously presented in the paper by Korhonen et al. [28]. The present study is based on the same material. However, some of the immunostainings failed or tissue core samples were lost during the section preparation and immunostaining steps. Unfortunately, these cases included all the WHO grade III meningiomas presented in the study by Korhonen et al. All the remaining 409 meningiomas were included in this study. There were too few WHO grade III meningiomas in this study and therefore they were excluded. A total of 399

**Fig. 1** Immunostaining of LRIG proteins in meningiomas. **a** Fibroblastic meningioma that is negative for LRIG1 staining (original magnification, ×400). **b** Fibroblastic meningioma with strong nuclear LRIG1 staining (original magnification, ×400). **c** Meningothelial meningioma with perinuclear (*long arrow*) and strong cytoplasmic (*short arrow*) LRIG1 staining (original magnification, ×600). **d** Meningothelial meningioma with predominantly cytoplasmic LRIG1 staining (original magnification, ×200). **e** Fibroblastic meningioma that is negative for LRIG2 staining (original magnification, ×200). **f** Meningothelial meningioma with cytoplasmic LRIG2 staining (original magnification, ×400). **g** Meningothelial meningioma that is negative for LRIG3 staining (original magnification, ×200). **h** Meningothelial meningioma with cytoplasmic LRIG3 staining (original magnification, ×400)
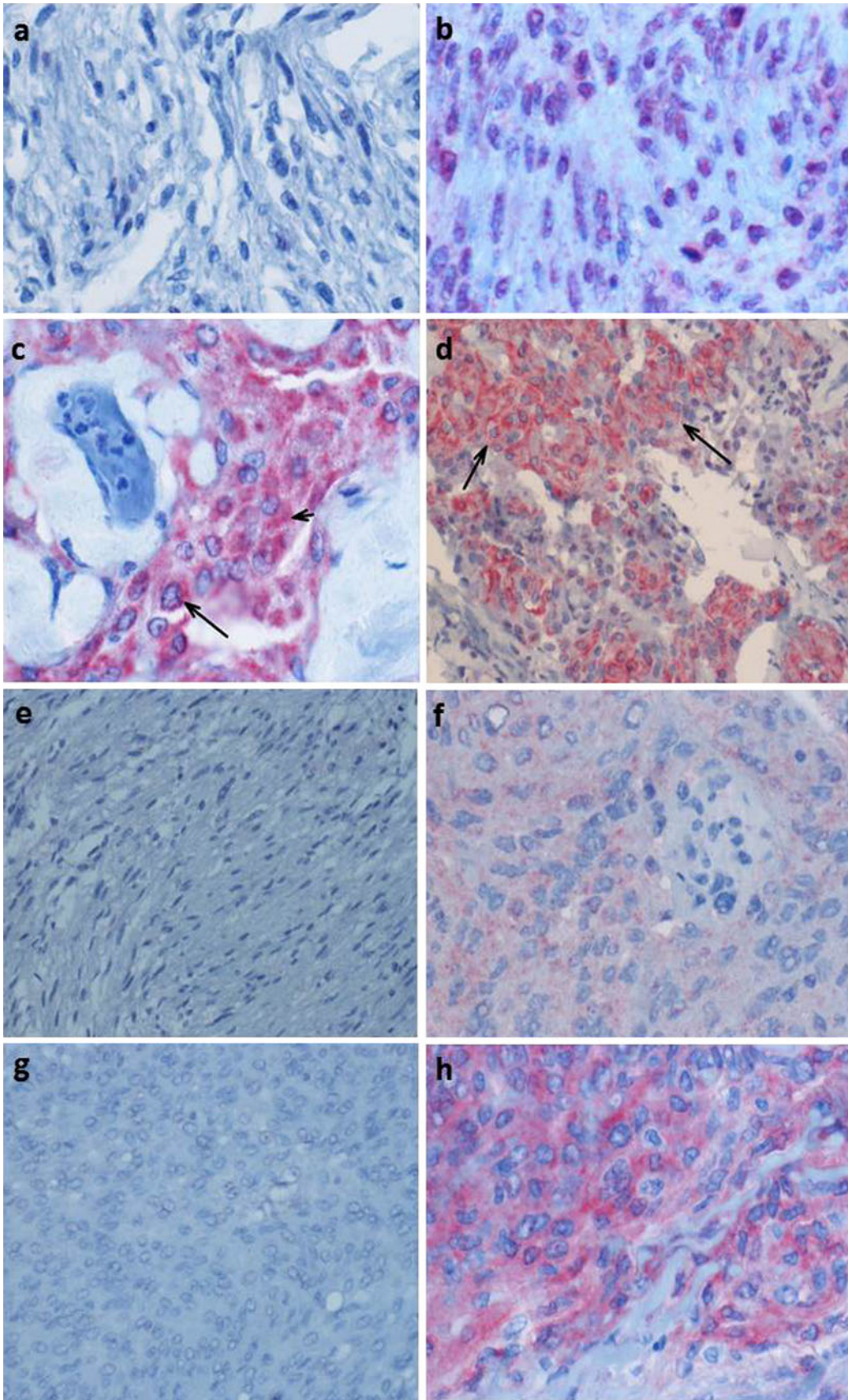
primary and 10 recurrent tumor specimens were included. The study protocol was approved by the Ethical Committee of Tampere University Hospital. The tumors were classified and graded using the World Health Organization (WHO) scheme (grades I–II) [29]. Patient characteristics are summarized in Table 1.

### Immunohistochemistry

Tissue microarrays (TMA) were used for the IHC analysis [30]. The tumor samples were fixed in a phosphate-buffered 4 % formaldehyde solution and processed into paraffin blocks using standard methods. Histologically representative tumor regions of hematoxylin and eosin-stained slides were selected by a neuropathologist (H.Ha.), and corresponding areas were sampled in tissue microarray blocks using a custom built instrument (Beecher Instruments, Silver Spring, MD, USA). One tissue core with a diameter of 600 μm from each tumor was included in the TMA. Polyclonal rabbit antibodies against the cytoplasmic tails of the respective LRIG protein were used for the

**Table 1** Characterization of the meningioma patients included in the tissue microarray (TMA) analyses

| | |
|---|---|
| Total number of patients | 409 |
| Gender (female/male) | 324/85 |
| Age at diagnosis median (years) | 59 (4–84) |
| Female | 60 |
| Male | 57 |
| Histological subypes (WHO 2007) | |
| Meningothelial | 138 |
| Fibrous (Fibroblastic) | 83 |
| Transitional (mixed) | 129 |
| Other benign | 28 |
| Atypical | 31 |
| Tumor grade (WHO 2007) | |
| Grade I | 378 |
| Grade II | 31 |

## a Cytoplasmic expression



## b Perinuclear expression
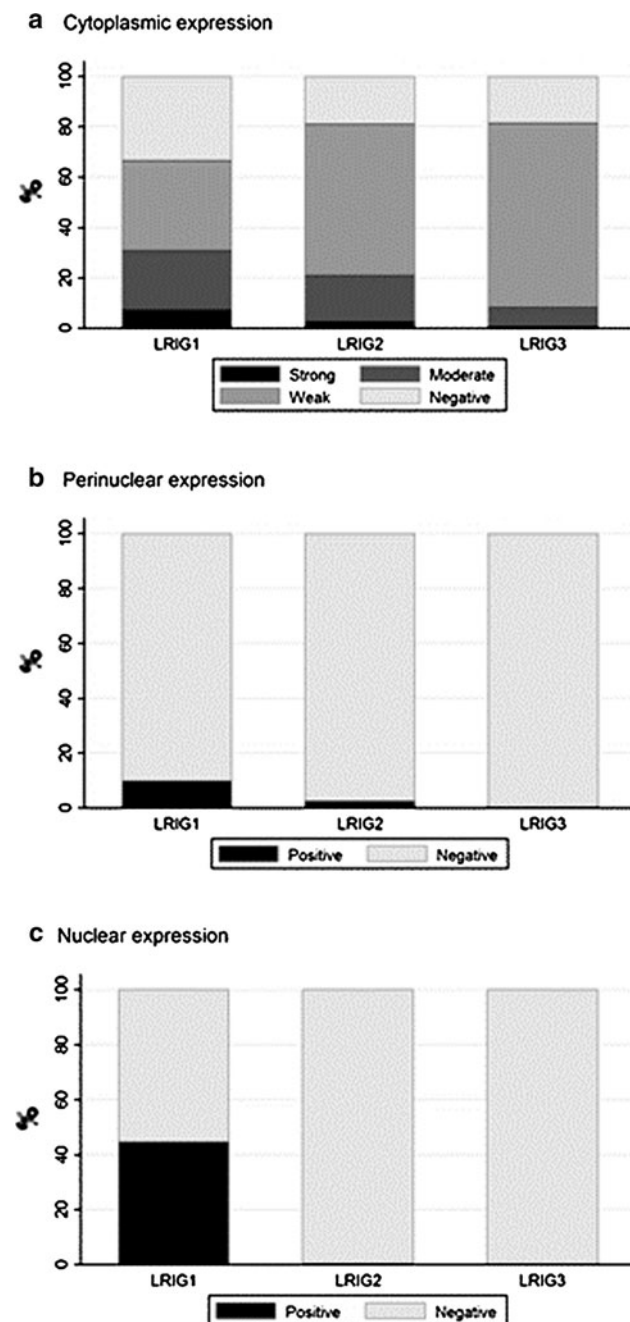


## c Nuclear expression



**Fig. 2** The subcellular localization of LRIG protein immunoreactivity for the 409 meningioma tumors depicted in bar graphs. **a** For cytoplasmic staining, no immunoreactivity was scored as 0, weak as 1, moderate as 2, and strong immunoreactivity as 3. **b** For perinuclear and **c** nuclear staining, no immunoreactivity was scored as 0, and positive immunoreactivity was scored as 1

immunohistochemical labeling of LRIG proteins as previously described [11, 26, 31]. IHC was performed using the Ventana Benchmark system (Ventana Medical System, Tucson, AZ, USA). As a pre-treatment step, tissues were subjected to heat-induced epitope retrieval with the Cell Conditioning 2 solution (Ventana) for 32 min. The stained

TMAs were evaluated by three observers, including one experienced neuropathologist (H.Ha.) and one experienced pathologist (M.E.), and a consensus for each case was determined. Cytoplasmic immunoreactivity was scored in four different categories: 0 for no or very faint immunoreactivity, 1 for weak immunoreactivity, 2 for moderate immunoreactivity, and 3 for intense immunoreactivity. The nuclear and perinuclear immunoreactivities were scored as 0 (negative) for sections with <10 % immunopositive cells or 1 (positive) for sections with 10 % or more immunopositive cells. For IHC of hormone receptors, monoclonal antibodies 6F11, PGR312, and 2F12 were used for PR, ER, and AR, respectively (Novocastra Laboratories, Newcastle, UK). Antigen retrieval was carried out described above. All antibodies were diluted at 1 μg/ml, and detected with a peroxidase-polymer based detection kit (PoerVision + , Immunovision Technologies, Daly City, CA, USA) according to manufacturers' instructions.

### Statistical analysis

Associations between gender, tumor grade, ER status, and PR status and expression of LRIG1, LRIG2, and LRIG3 were evaluated using the $\chi^2$ test. The subcellular distribution of LRIG1, LRIG2, and LRIG3 was evaluated using the Kruskal–Wallis test, and the results are shown as bar graphs. The significance level was set at $p < 0.05$.

### Results

#### Immunohistochemical analysis of LRIG protein expression in meningiomas

LRIG protein expression was analyzed by IHC in 409 meningioma tumor samples collected in a TMA (Table 1). LRIG protein expression was observed in the nuclei, cytoplasm, and perinuclear areas of meningioma cells (Figs. 1 and 2) with occasional immunoreactivity observed in several compartments within individual cells. For example, tumors that displayed perinuclear immunoreactivity often also showed cytoplasmic immunoreactivity. For LRIG1, 67 % of the tumors showed cytoplasmic immunoreactivity, 10 % showed perinuclear immunoreactivity, and 45 % showed nuclear immunoreactivity. LRIG2 showed mostly cytoplasmic immunoreactivity and only rarely perinuclear and nuclear immunoreactivity. LRIG3 immunoreactivity was only observed in the cytoplasm.

LRIG1 and LRIG2 cytoplasmic expression showed a significant correlation with histological subtypes of meningiomas, with expression most frequently observed in the benign subtypes (fibrous and transitional) (Table 2; $p = 0.038$ and 0.013, respectively). No significant correlation was observed

**Table 2** The localization of LRIG protein expression in meningiomas in relation to histological subtypes

| | Histological subtypes | Meningothelial n (%) | Fibroblastic n (%) | Transitional n (%) | Other beningn n (%) | Atypical n (%) | $\chi^2$ tests ($p$) |
|---|---|---|---|---|---|---|---|
| **LRIG1** | | | | | | | |
| Nuclear | Negative | 76 (58.9) | 36 (49.3) | 72 (58.1) | 17 (58.6) | 10 (38.5) | 0.264 |
| | Positive | 53 (41.1) | 37 (50.7) | 52 (41.9) | 12 (41.4) | 16 (61.5) | |
| Perinuclear | Negative | 116 (89.9) | 69 (93.2) | 111 (89.5) | 24 (82.8) | 24 (92.3) | 0.600 |
| | Positive | 13 (10.1) | 5 (6.8) | 13 (10.5) | 5 (17.2) | 2 (7.7) | |
| Cytoplasmic | Negative | 35 (24.1) | 34 (45.9) | 39 (31.5) | 11 (37.9) | 9 (34.6) | **0.038** |
| | Faint | 42 (32.6) | 28 (37.8) | 47 (37.9) | 7 (24.1) | 12 (46.2) | |
| | Moderate | 36 (27.9) | 10 (13.5) | 31 (25.0) | 8 (27.6) | 5 (19.2) | |
| | Strong | 16 (12.4) | 2 (2.7) | 7 (5.6) | 3 (10.3) | 0 (0) | |
| **LRIG2** | | | | | | | |
| Nuclear | Negative | 130 (100) | 75 (98.7) | 126 (100) | 29 (100) | 27 (100) | 0.391 |
| | Positive | 0 (0) | 1 (1.3) | 0 (0) | 0 (0) | 0 (0) | |
| Perinuclear | Negative | 129 (98.5) | 73 (96.1) | 123 (97.6) | 29 (100) | 25 (92.6) | 0.348 |
| | Positive | 2 (1.5) | 3 (3.9) | 3 (2.4) | 0 (0) | 2 (7.4) | |
| Cytoplasmic | Negative | 22 (16.8) | 19 (25.0) | 22 (17.3) | 10 (34.5) | 1 (3.7) | **0.013** |
| | Faint | 77 (58.8) | 51 (67.1) | 74 (58.3) | 16 (55.2) | 16 (59.3) | |
| | Moderate | 27 (20.6) | 4 (5.3) | 29 (22.8) | 2 (6.9) | 9 (33.3) | |
| | Strong | 5 (3.8) | 2 (2.6) | 2 (1.6) | 1 (3.4) | 1 (3.7) | |
| **LRIG3** | | | | | | | |
| Nuclear | Negative | 130 (100) | 80 (100) | 126 (100) | 29 (100) | 26 (100) | N/A |
| | Positive | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | |
| Perinuclear | Negative | 130 (100) | 80 (100) | 127 (100) | 29 (100) | 26 (100) | N/A |
| | Positive | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | |
| Cytoplasmic | Negative | 21 (16.2) | 22 (27.2) | 21 (16.5) | 7 (24.1) | 3 (11.5) | 0.185 |
| | Faint | 95 (73.1) | 56 (69.1) | 96 (75.6) | 21 (72.4) | 19 (73.1) | |
| | Moderate | 13 (10.0) | 3 (3.7) | 10 (7.9) | 1 (3.4) | 3 (11.5) | |
| | Strong | 1 (0.8) | 0 (0) | 0 (0) | 0 (0) | 1 (3.8) | |

Cytoplasmic staining was scored as negative (0) or positive (1, 2, or 3). Nuclear and perinuclear staining was scored as negative (0) or positive (1)

*NA* not applicable

Significant associations ($p < 0.05$) are in bold. Total tumor numbers may vary due to success rates of staining in the TMA analyses

between LRIG3 expression and histological subtypes. A correlation between tumor grade and LRIG3 expression was of borderline significance (data not shown, $p = 0.050$). There was no significant correlation observed between LRIG expression and gender (data not shown).

There was a significant correlation detected between ER status and cytoplasmic and nuclear LRIG1 expression (Table 3; $p = 0.003$ and 0.004, respectively). ER status also correlated with cytoplasmic LRIG2 expression ($p = 0.006$). No relationship between ER status and LRIG3 expression was observed (Table 3).

In this study, we had ten recurrent meningiomas and the statistical analysis did not reveal any association between the LRIG proteins and recurrence (data not shown).

## Discussion

The current study provides the first characterization of the expression and distribution of LRIG proteins in human meningiomas. This tumor type is twice as common in women as it is in men. Female sex hormones (i.e., estrogens) may play a role in the pathogenesis of meningiomas, and estrogen treatment has been proposed as a risk factor. In a large European cohort study, the impact of exogenous hormone use in association with glioma and meningioma risk was analyzed. The study showed an increased meningioma risk for current users of hormones [32]. A population-based case–control study recently conducted in a Finnish population indicated that reproductive factors or

**Table 3** Association between LRIG protein expression and estrogen receptor status

| | Estrogen status | Negative n (%) | Positive n (%) | $\chi^2$ tests (p) |
|---|---|---|---|---|
| LRIG1 | | | | |
| Nuclear | Negative | 102 (48.1) | 88 (63.3) | **0.005** |
| | Positive | 110 (51.9) | 51 (36.7) | |
| Perinuclear | Negative | 193 (90.6) | 122 (87.8) | 0.396 |
| | Positive | 20 (9.4) | 17 (12.2) | |
| Cytoplasmic | Negative | 75 (35.2) | 35 (25.2) | **0.021** |
| | Faint | 82 (38.5) | 46 (33.1) | |
| | Moderate | 42 (19.7) | 45 (32.4) | |
| | Strong | 14 (6.6) | 13 (9.4) | |
| LRIG2 | | | | |
| Nuclear | Negative | 218 (100) | 141 (100) | N/A |
| | Positive | 0 (0) | 0 (0) | |
| Perinuclear | Negative | 215 (98.6) | 137 (96.5) | 0.177 |
| | Positive | 3 (1.4) | 5 (3.5) | |
| Cytoplasmic | Negative | 38 (17.4) | 25 (17.6) | **0.028** |
| | Faint | 141 (64.4) | 78 (54.9) | |
| | Moderate | 38 (17.4) | 31 (21.8) | |
| | Strong | 2 (0.9) | 8 (5.6) | |
| LRIG3 | | | | |
| Nuclear | Negative | 220 (100) | 142 (100) | N/A |
| | Positive | 0 (0) | 0 (0) | |
| Perinuclear | Negative | 221 (100) | 142 (100) | N/A |
| | Positive | 0 (0) | 0 (0) | |
| Cytoplasmic | Negative | 37 (16.7) | 28 (19.7) | 0.433 |
| | Faint | 167 (75.2) | 100 (70.4) | |
| | Moderate | 16 (7.2) | 14 (9.9) | |
| | Strong | 2 (0.9) | 0 (0) | |

Cytoplasmic staining was scored as negative (0) or positive (1, 2, or 3). Nuclear and perinuclear staining was scored as negative (0) or positive (1). Estrogen status was scored as positive (1) or negative (2). Significant associations ($p < 0.05$) are in bold. Total tumor numbers may vary due to success rates of staining in the TMA analyses

the use of exogenous sex hormones affected meningioma risk [33]. Normal meningeal tissues do not express ER [34], and the level of ER expression in meningiomas differs between various studies. In a recent paper, approximately one-third of meningiomas were reported to express ER [35]. Some studies have shown that overexpression of ER is associated with more aggressive clinical behavior of meningiomas [36]. The current study revealed a correlation between ER status and the expression of LRIG1 and LRIG2 proteins in meningiomas. LRIG1 was recently shown to be an estrogen-regulated growth suppressor in breast cancer [22]. Our results suggest that LRIG1 is also regulated by the ER in meningiomas. Less is known about the regulation of LRIG2 expression. However, LRIG2 is

highly expressed in the female reproductive organs, including the uterus and ovaries [11], which also indicates a gender-specific regulation. Whether LRIG1 and LRIG2 function as growth suppressors in meningiomas remains to be determined. LRIG1 negatively regulates the growth stimulatory EGFR family members, and EGFR and ERBB2 are prominently overexpressed in certain meningiomas. Thus, it seems likely that LRIG1 may also function as a growth suppressor in meningiomas. Hoewever, expression of LRIG1 and LRIG2 was not associated with tumor grade, suggesting that the LRIG proteins do not have a clear role in malignant progression from grade I to II meningiomas. In summary, this study is the first to characterize the expression and subcellular distribution of LRIG proteins in meningiomas. ER status correlated with the expression of LRIG1 and LRIG2, which suggests a potential role for LRIG proteins in the pathogenesis of meningiomas, but more studies are needed to confirm this hypothesis.

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