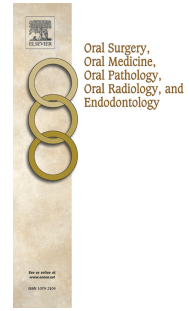# Accepted Manuscript

LGR5 expression in oral epithelial dysplasia and oral squamous cell carcinoma

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Objective.

LGR5 is pivotal to oral cavity development and is implicated in epithelial malignancy whereby stimulation of LGR5 potentiates canonical Wnt signalling. This investigation tested our hypothesis of a correlation between LGR5 expression and the severity of oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC).

Study Design.

Immunoreactive LGR5 protein expression was quantified in 342 tissue samples ranging in disease severity from normal through mild and moderate/severe OED to OSCC.

Results.

LGR5 was restricted to the basal layers for normal tissues, projected to the stratum granulosum in severe dysplasia, was intense in carcinoma nests of well differentiated OSCC, but uniformly diffuse throughout poorly differentiated OSCC. Median LGR5 immunoreactivity index scores increased with disease severity: mild dysplasia = 1 < moderate/severe dysplasia = 2.5 < OSCC = 6.

Conclusion.

Inclusion of LGR5 in a panel of immunohistochemical biomarkers may improve identification of increased potential for malignancy in OED.

Clinical Significance (40 words)

This is the first report of increased LGR5 receptor IHC stain intensity correlating with disease severity for Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma. LGR5 immunostaining (perhaps combined with p53 staining) may improve prognostic evaluation of oral malignant transformation.

Introduction

Oral squamous cell carcinoma (OSCC) accounts for 90% of oral malignancy and with an annual incidence of 275000 it ranks as sixth most common cancer worldwide. Known risk factors include habitual exposure to alcohol, tobacco and betel quid, and optimal clinical intervention occurs prior to malignancy when patients present with oral potentially malignant lesions (OPML).[1]

Molecular profiling of high incidence malignancies has disclosed gene products with potential application for OPML and OSCC disease stratification. Of particular interest are stem cell associated molecular markers with proven relevance to rectal cancer,[2] since rectal and oral tissues share features of embryologic development involving endodermal and ectodermal derived stem cells [3] that persist throughout adulthood and may contribute to neoplasia.[4]

The putative cancer stem cell marker LGR5 (leucine-rich repeat-containing G-protein coupled receptor 5) is elevated in colorectal cancer [5] and plays a pivotal role in embryologic development of the oral cavity where it orchestrates formation of the tongue from the foregut endoderm.[6, 7] Ablation of LGR5 is lethal in-utero but otherwise would cause complete ankyloglossia whereby the tongue does not separate from the floor of mouth.[6] Figure 1 summarises the integration of LGR5 with the canonical Wnt signaling pathway. The functional agonists for LGR5 are four small secreted proteins that comprise the R-spondin family (RSPO1-4) which act synergistically with Wnt agonists during embryogenesis and oncogenesis to modulate cellular proliferation, differentiation and to maintain the stem cell phenotype.[8-10]

Given that LGR5 activation potentiates Wnt signalling [8] to drive stem cell maintenance [10] and oncogenesis,[9] and that LGR5 is elevated in colorectal cancer [5] and is pivotal to oral tissue embryogenesis, we hypothesise a correlation between LGR5 expression andthe severity of oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC). This study compared LGR5 immunoreactive protein expression in potentially malignant and cancerous oral lesions from 342 formalin fixed paraffin embedded (FFPE) oral biopsies bearing a spectrum of disease from normal through mild and moderate/severe dysplasia to oral squamous cell carcinoma (OSCC).

Materials & Methods

Patient samples: 342 FFPE specimens: 109 normal mucosa, 43 mild dysplasia (MD), 31 moderate to severe dysplasia (SD), and 159 oral squamous cell carcinoma (OSCC) were obtained from 314 patients: 173 male, 130 female and 11 gender unknown; mean (standard deviation) age 55 (18) years. Diagnosis was confirmed retrospectively by an oral pathologist (CSF) according to the World Health Organization (WHO) classification system.[11] The study received ethical review and approval from Hospital and University Human Research Ethics Committees (HREC/10/QRBW/336 and UQ/2007001478) and was run in accordance with the principles outlined by the Declaration of Helsinki.

Immunohistochemistry (IHC): Performed as described previously.[12] Briefly, 5-μm sections were incubated overnight at 4°C with rabbit monoclonal anti-LGR5 (#LS-C105455, Lifespan Biosciences Inc, WA, USA) diluted 1:1000 in Background Sniper (#BS966M, Biocare Medical, Concorde, CA, USA). Staining utilised MACH 2 Universal HRP-Polymer (#M2U522, Biocare Medical) with Betazoid DAB chromogen (#BDB2004L, Biocare Medical), CAT haematoxylin (#CATHE-M, Biocare Medical) and Leica CV Mount (Leica, Nussloch, Germany). Positive staining controls (colon cancer) are available as Supplementary Online Material. Negative staining controls were also conducted.

Scoring: Immunoreactivity was evaluated semi-quantitatively as previously described [13,14] with reference to published guidelines [15] by two examiners (LPP and AGM) and confirmed independently by a third examiner (AAAM) all of whom were trained and assessed by an oral pathologist (CSF). Colon cancer tissues were used as positive staining reference material, and reference photomicrographs and slides were used by the examiners to ensure consistency of stain intensity scoring. Ten random fields were scored at ×400 magnification, counting >500 cells. Cells were firstly evaluated as positive or negative for LGR5, then positive cells were graded for intensity of immunoreactivity. Stain intensity was scaled: 0 = no stain, 1 = weak intensity, 2 = moderate intensity, 3 = strong intensity. The percent positively stained epithelial cells were calculated (numerator = number of positively stained cells; denominator = total number of cells observed) and categorised: 0% positive= score 0; 1-25% positive = score 1; 25-49% positive = score 2; 50-74% positive= score 3; and 75-100% positive = score 4. The final index score, which ranged from 0 to 12, was the product of categorised percentage positive score and the scaled stain intensity score.

Statistical analysis: Non-parametric analysis and presentation used IBM SPSS Statistics V.20 software (IBM Corporation, Armonk, NY, USA) and the R computing language ([http://www.R-project.org]). Median (Q2) and inter-quartile range (IQR) have been presented. The proportion of positively stained sections has been reported for each pathology (numerator = number of positively stained sections; denominator = total number of sections observed). Immunolocalisation score data for disease groups were compared to normal using Mann-Whitney U tests with Bonferroni correction for n=3 comparisons (*p<0.033; ***p<0.003).

Results

LGR5 antigen stain intensity in normal, dysplastic and OSCC biopsies

LGR5 immunoreactivity is illustrated through representative photomicrographs (Figure 2 A - E) and a graphical representation (Figure 2 F).

The proportion of sections staining positively for LGR5 was lowest for normal tissues (63% positive) then increased with disease severity from mild dysplasia (67% positive) through moderate/severe dysplasia (74% positive) to OSCC (89% positive). Similarly, LGR5 antigen stain intensity increased with disease severity, a trend that was clearly evident from the immunoreactivity index scores: mild dysplasia (median score = 1; IQR = 0 to 5.3) < moderate/severe dysplasia (median score = 2.5; IQR = 0 .5 to 8) < OSCC tissue (median score = 6; IQR = 2 to 8.6) (Figure 2F). The pattern of LGR5 immunostaining showed marked differences between sample groups (Figure 2 A to E). LGR5 was chiefly restricted to the basal layers in normal tissues, sometimes projecting into the stratum spinosum. Greater intensity LGR5 immunostaining throughout the stratum spinosum to the stratum granulosum typified more severe cases of dysplasia. Well differentiated OSCC samples expressed LGR5 at the perimeter of carcinoma nests but not in the keratinised central areas, while moderate to poorly differentiated OSCC specimens exhibited LGR5 diffusely throughout the carcinoma cells (Figure 2 D & E).

Discussion

This study analysed LGR5 protein expression in 342 FFPE oral biopsies bearing a spectrum of disease ranging from normal through mild and moderate/severe dysplasia to oral squamous cell carcinoma (OSCC) to test our hypothesis of a correlation between LGR5 expression and the pathology grading of these lesions. This hypothesis was supported by demonstration of elevated LGR5 expression in OSCC and moderate/severe dysplasia relative to normal oral tissues (statistically significant; Mann-Whitney) and the positive trend between LGR5 immunoscore data and diagnostic grading of OPML severity. Previously, using a sub-group of these biopsies (n=100 specimens: 18 normal, 27 mild dysplasia, 8 moderate dysplasia, 47 OSCC) we demonstrated an equivalent positive trend between cytoplasmic and nuclear β-catenin immunoscore data and the diagnostic grading of OPML and OSCC.[14] Cytoplasmic and nuclear accumulation of β-catenin is indicative of signal transduction via the canonical Wnt signalling pathway (figure 1),[10] and the mutually supportive findings of these discrete IHC studies indicate progressive dysregulation of canonical Wnt signalling in oral malignant transformation. The findings of this study suggest potential application of LGR5 expression analysis to the diagnostic grading of OPML and OSCC and further supports the hypothesised involvement of cancer stem cell marker positive cell populations in tumour formation.[4]

LGR5 plays a definitive role in the embryologic development of oral tissue [6, 7] via its highly influential mechanism of action: potentiation of the canonical Wnt signalling pathway.[8-10] Early genetic aberrations in OSCC impede p53 and the pRb pathway to promote cell immortalisation.[16] Recently, p53 (and its transcriptional target, miR-34) has been demonstrated to be a potent suppressor of Wnt signalling.[17] This may explain elevated Wnt signalling in tumours lacking p53,[18] and provide a mechanism by which loss of p53 activity can promote Wnt signal driven clonal expansion during cancer progression.[19] Cells with elevated LGR5 expression and potentiated Wnt signal response[14] may exhibit increased neoplastic potential following loss of p53 tumour suppressor activity. The pathway shown in figure 1 is currently untested and the association between elevated LGR5 expression and oral potentially malignant lesion severity shown here does not indicate causality of malignant transformation. It is, therefore, too speculative to suggest that a panel of molecular biomarkers combining LGR5 with p53 or miR-34 may provide a means to identify

increased potential for malignancy in OPML, although further research into this translational opportunity is warranted.

This is the first demonstration of elevated LGR5 expression in OSCC and OPML. This result corresponds with our prior report of intracellular β-catenin accumulation in OPML and OSCC,[14] with prior reports of elevated LGR5 expression in colorectal cancer [5] and with the commonality between rectal and oral tissue embryologic development.[3] We suggest that LGR5 overexpression may contribute to clonal expansion during OSCC development via a mechanism that involves LGR5 mediated potentiation of Wnt signaling,[8-10] p53 tumour suppressor mediated inhibition of Wnt signaling,[17] and abrogation of p53 tumour suppressor activity as an early event in OSCC development.[16]

Figure Legends

Figure 1 Simplified schematic of the canonical Wnt signalling pathway showing potentiation of signal transduction by LGR5 receptor co-stimulation and the quiescing influence of p53 tumour suppressor. **Resting State:** β-catenin (β-Cat) is the principal effector protein of the canonical Wnt pathway. It is constitutively synthesised and, in the absence of Wnt signalling, β-catenin undergoes proteolysis by a "destruction complex" of cytoplasmic proteins that includes tumour suppressors (Axin and APC), Ser/Thr kinases (GSK-3 and CK1), protein phosphatases (PP2A), and a ubiquitin ligase (β-TrCP). **Signalling Event:** Wnt signal instigation involves interaction of plasma membrane bound receptors (Frizzled and Lrp5/6) with extracellular Wnt agonist. Consequent phosphorylation events recruit the "destruction complex" to the membrane where it is disabled; intracellular β-catenin accumulates. Cytoplasmic β-catenin enters the nucleous to form a complex with T-cell transcription factor (Tcf) which drives the transcription of numerous cell regulatory Wnt target genes. **Signal Potentiation:** Stimulation of membrane bound LGR5 receptor by its agonist (R-spondin) significantly amplifies Wnt signal instigation. **Pathway Quiescence:** Transcription of microRNA-34 (miR-34) is promoted by tumour suppressor protein p53. Interaction of miR-34 with near-complementary target sites in the 5' and/or 3' untranslated regions of Wnt target gene mRNA transcripts imposes post-transcriptional repression upon numerous Wnt targets. Note: this pathway has not yet been investigated with direct reference to OSCC.

Figure 2 LGR5 immunostaining in oral epithelial dysplasia and oral squamous cell carcinoma. Representative immunostaining of LGR5 for normal (A; final score 2), mild dysplasia (B; final score 3), moderate-severe dysplasia (C; final score 6) well differentiated OSCC (D; final score 9) and poorly differentiated OSCC (E; final score 12) (original magnification x 100). (F) Box and Whisker plots of LGR5 immunoscoring grouped by histopathology in normal oral mucosa, mild dysplasia (MD), moderate/severe dysplasia (SD) and OSCC cases showing median (bold line), 25th to 75th percentiles (box) and outliers (circles); * p<0.03, *** p<0.003 (Mann–Whitney U test). (G and H) show low power (original magnification x 50)

haematoxylin & eosin sections of well differentiated and poorly differentiated OSCC shown in (D) and (E) respectively.

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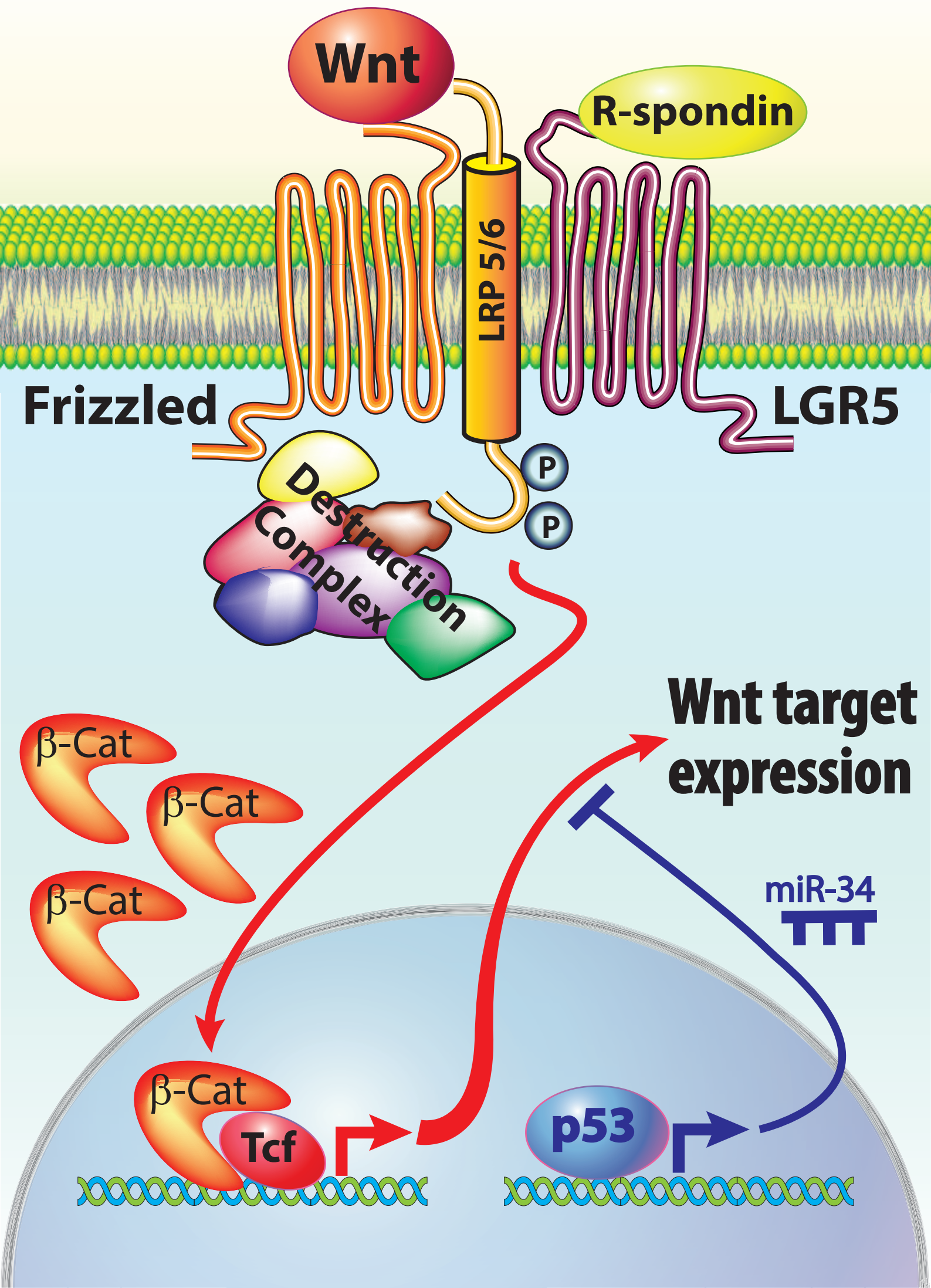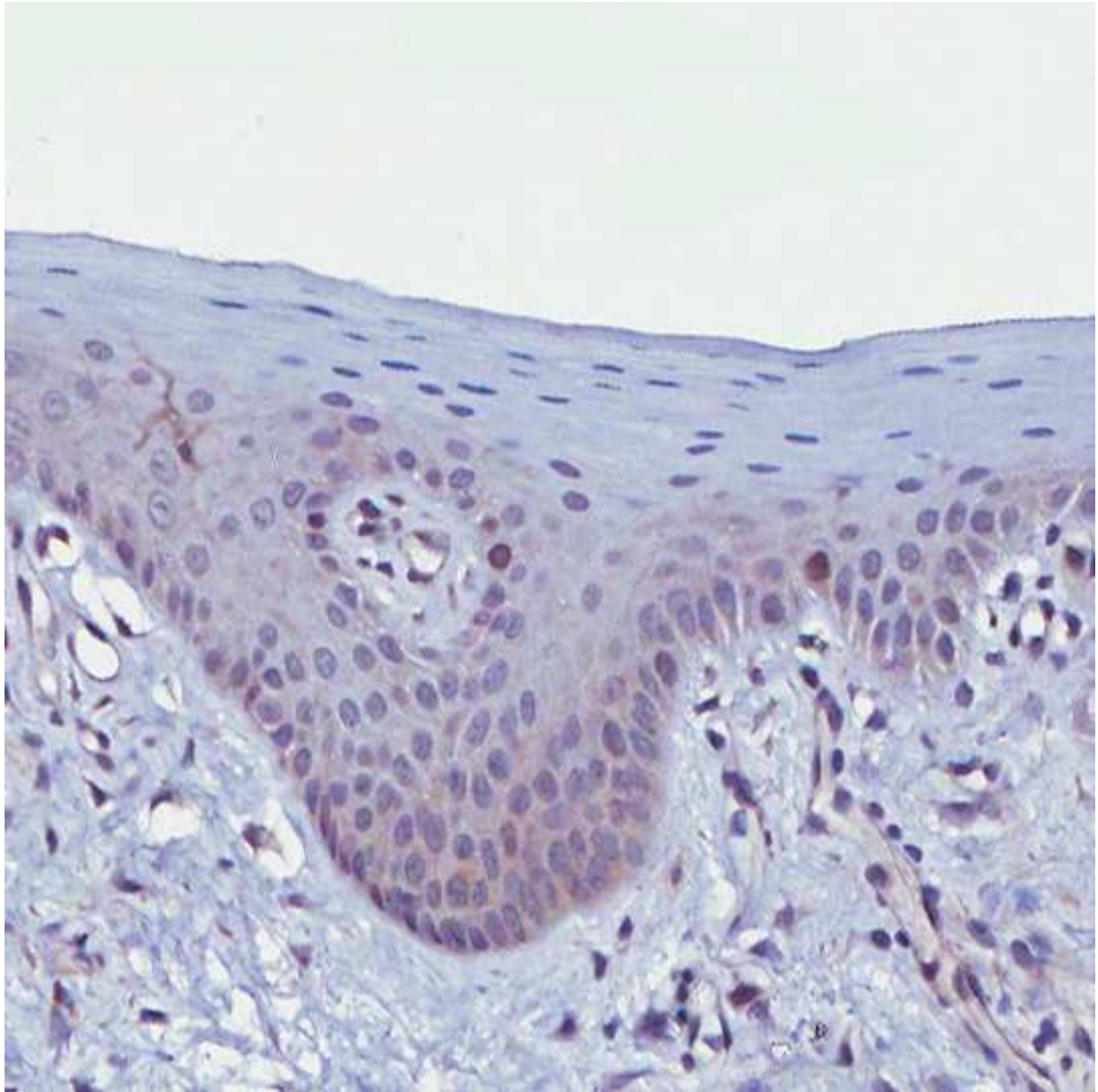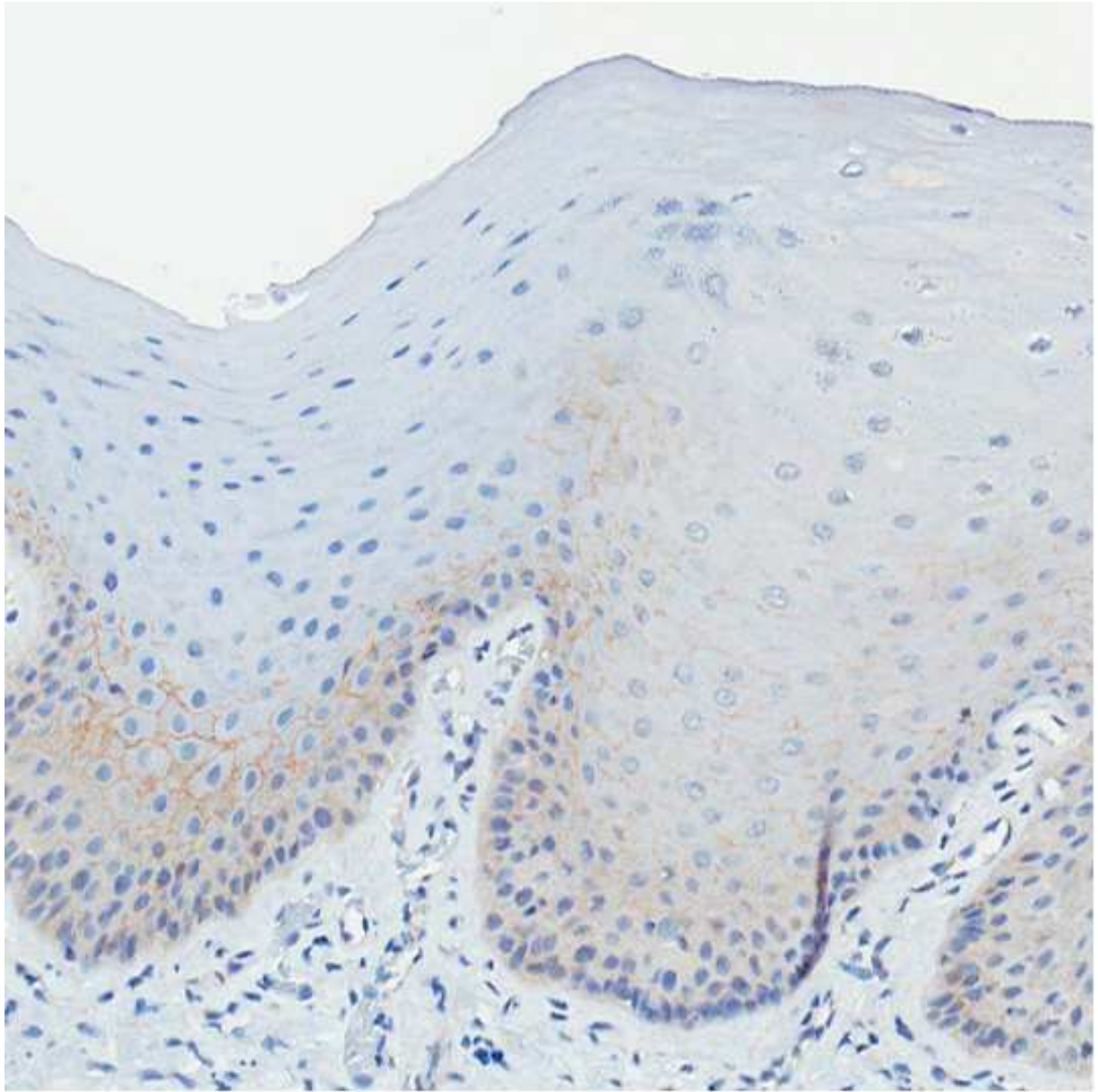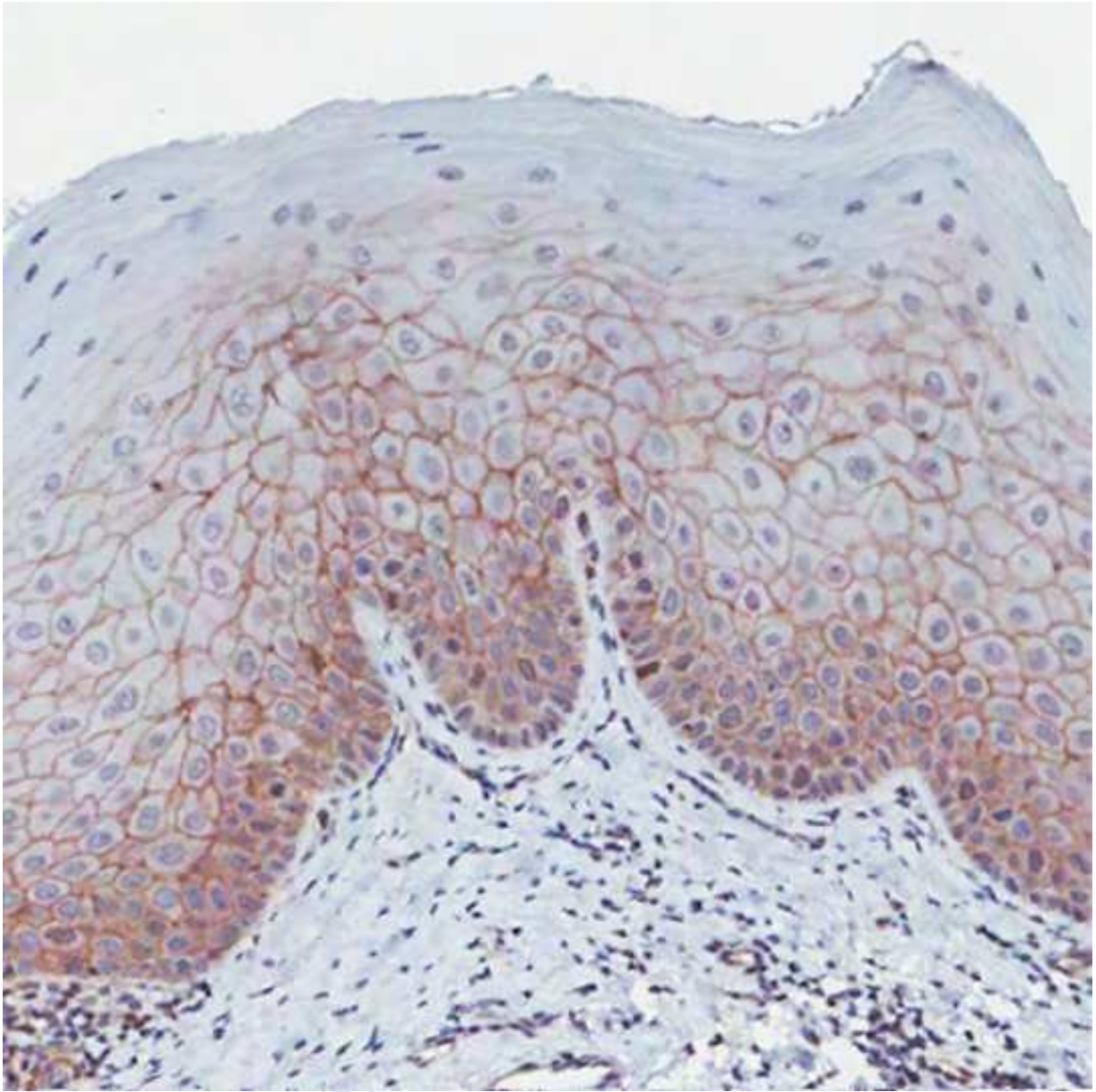
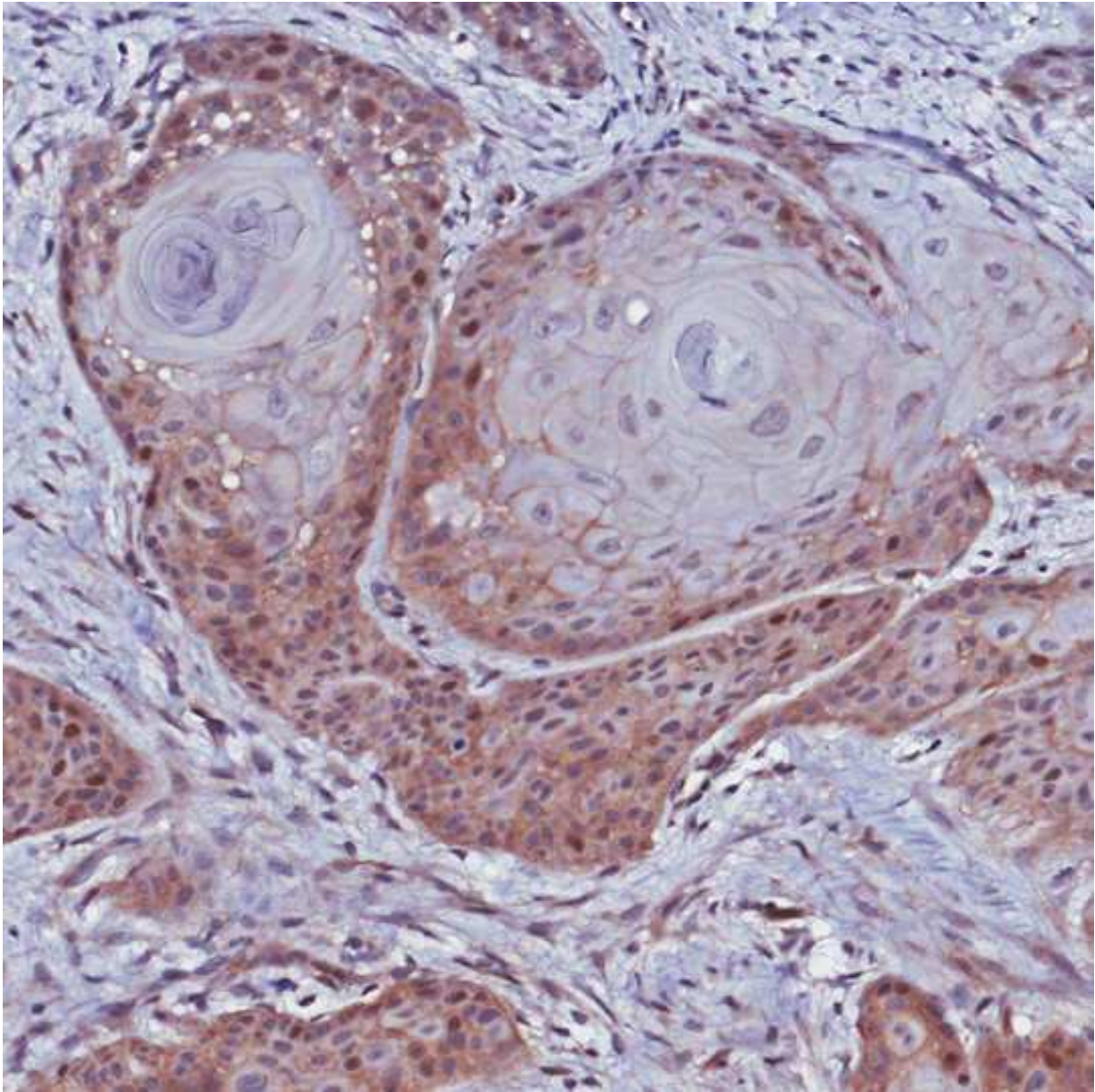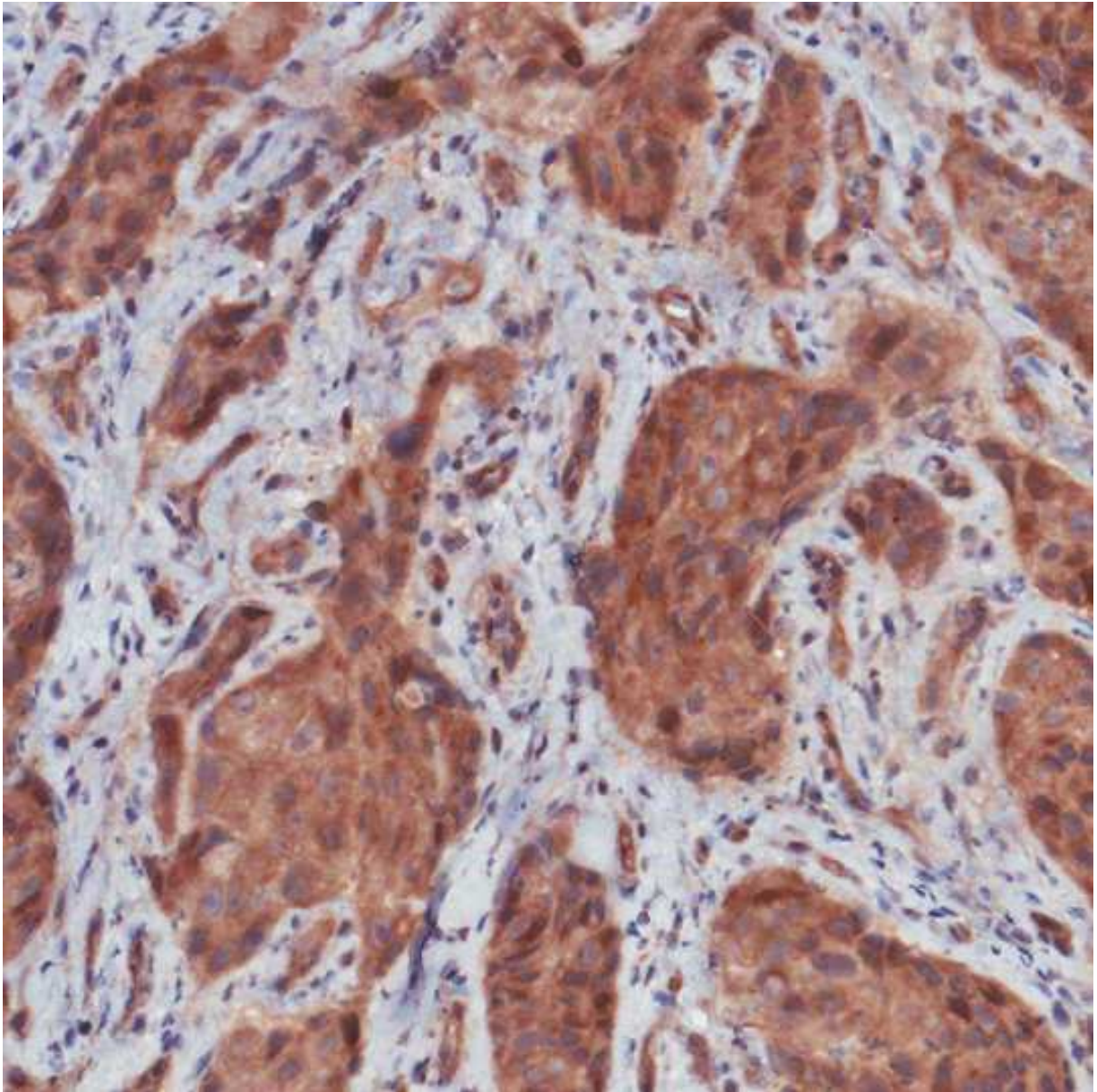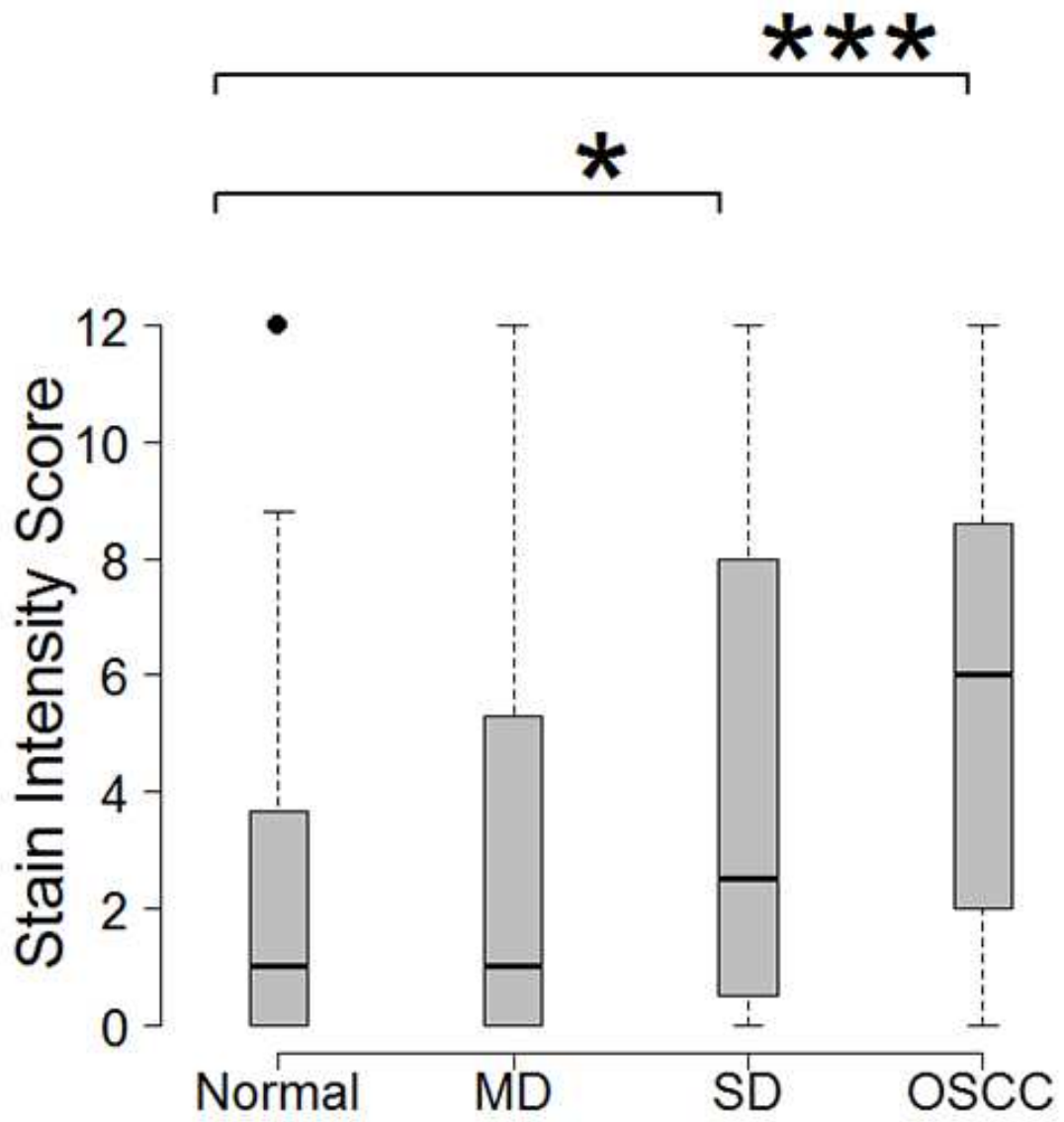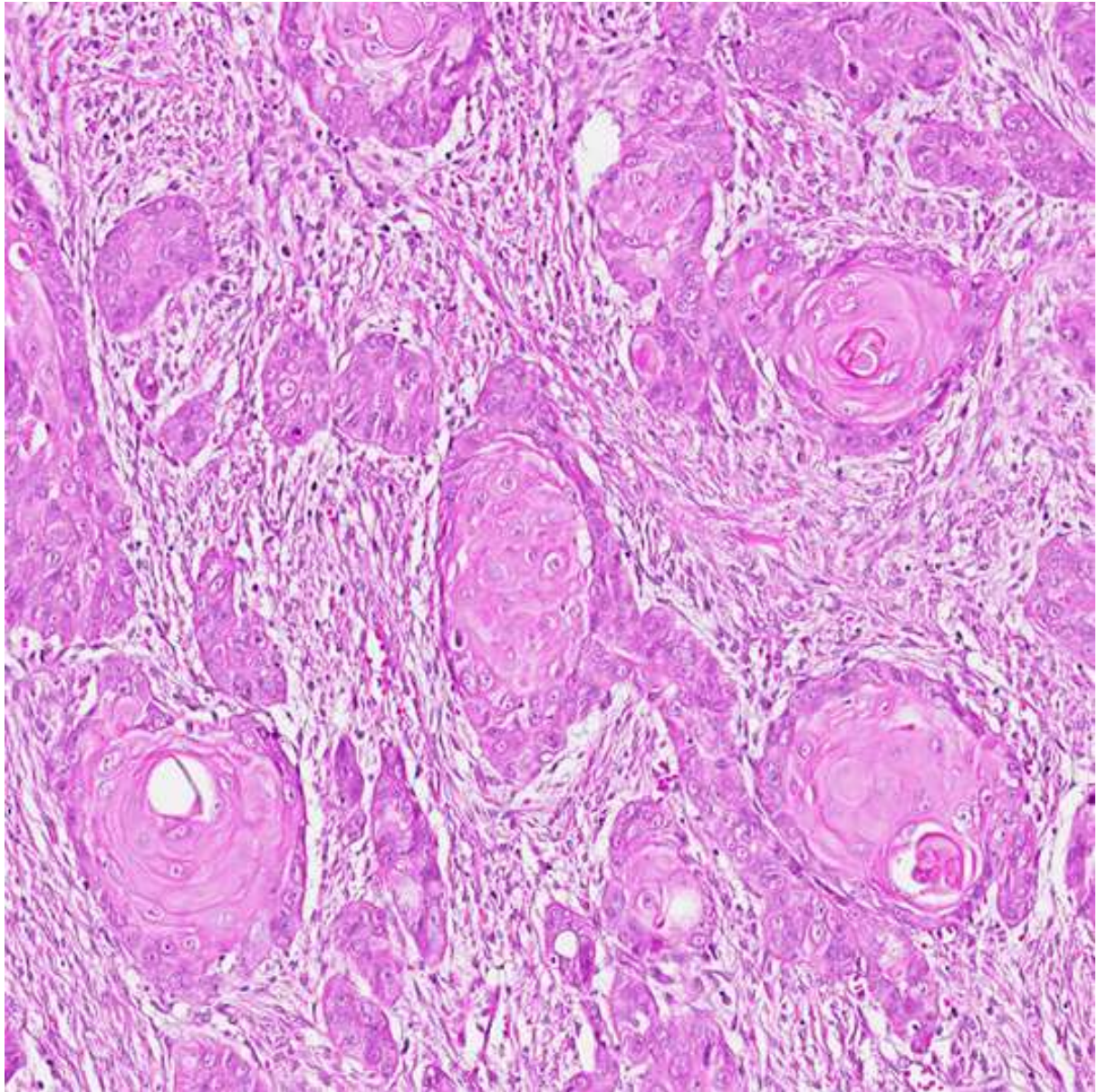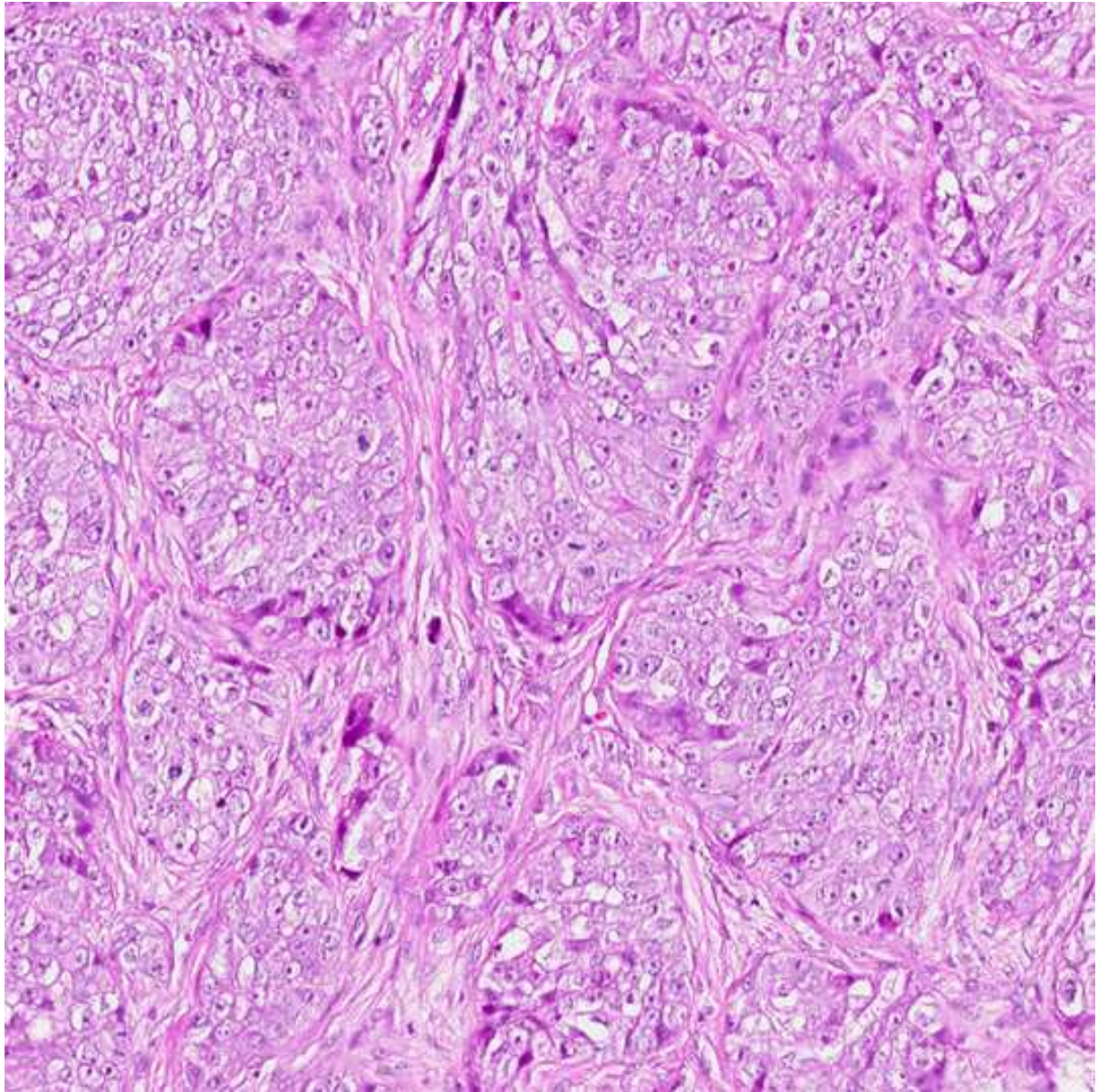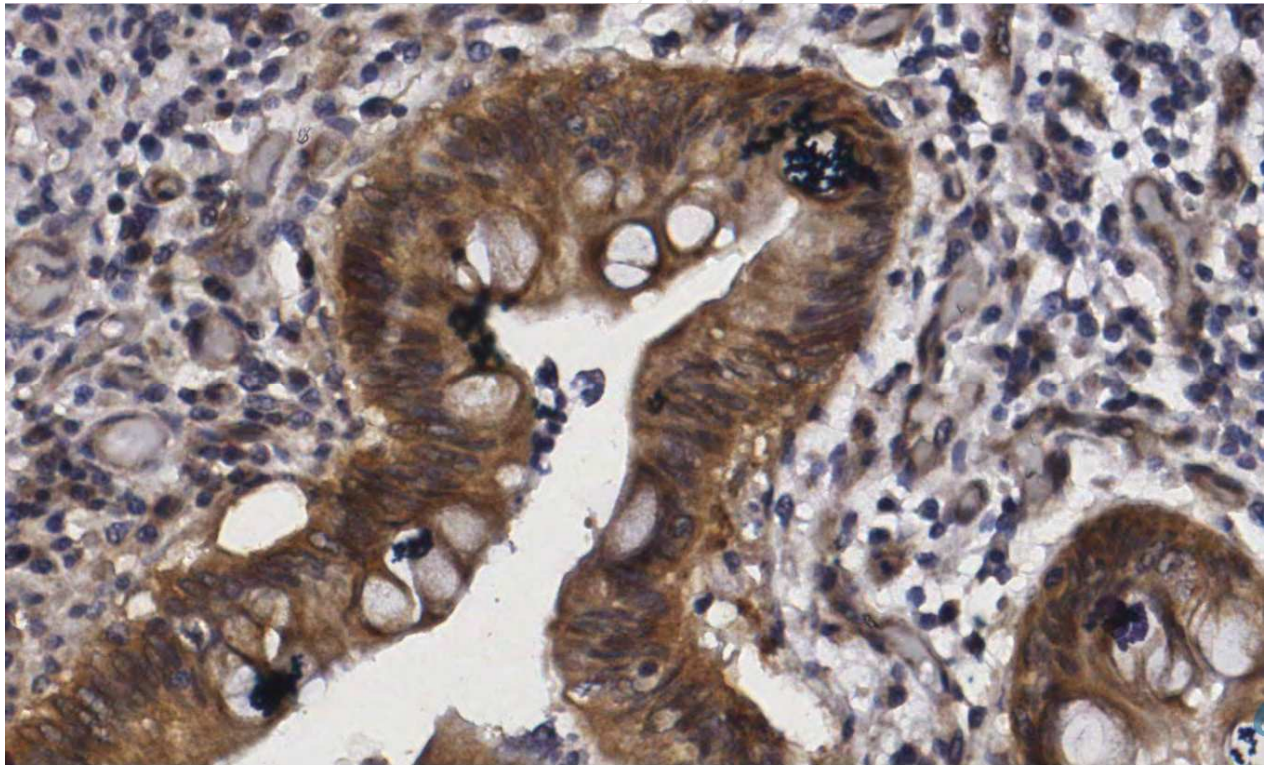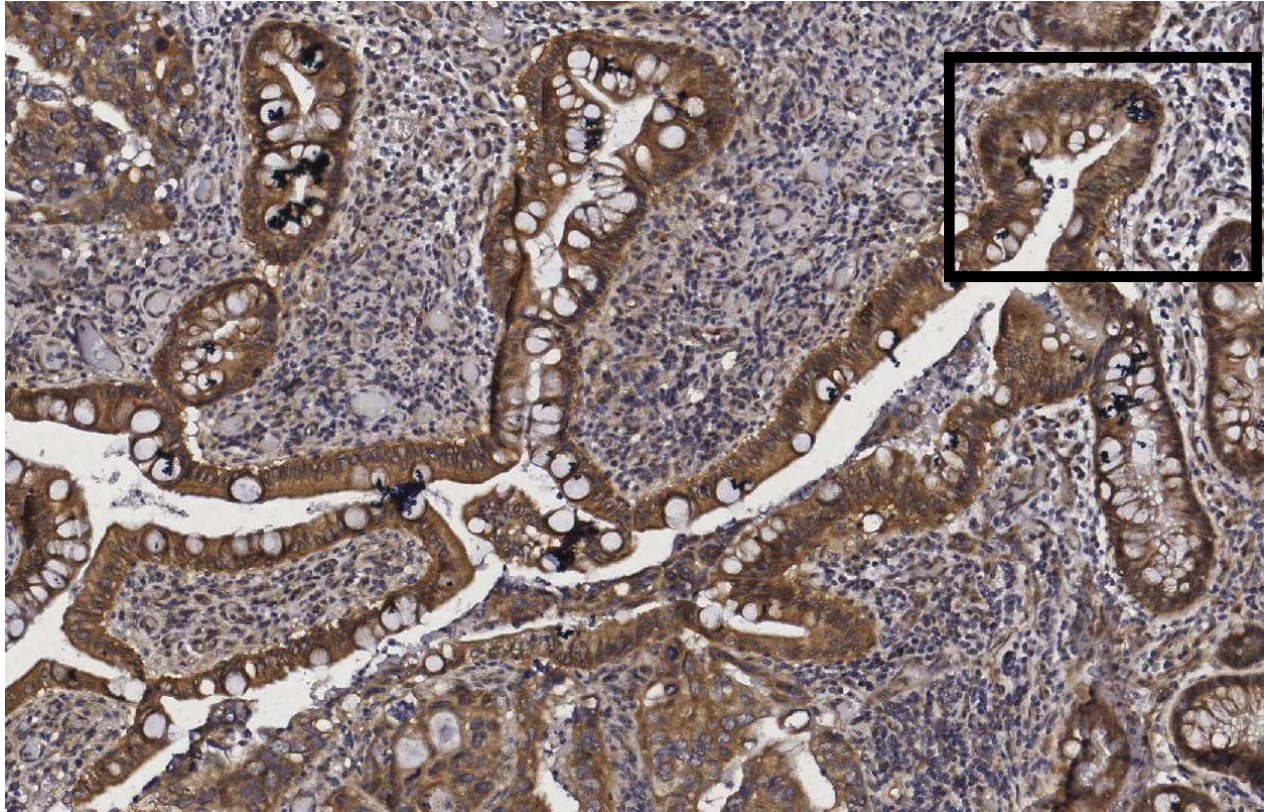**Supplementary Figure:**

Positive control immunostaining for LGR5 in a FFPE section of colon cancer.