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ORIGINAL ARTICLE

STING pathway expression in low grade serous carcinoma of the ovary: an unexpected therapeutic opportunity?

Running title: Sting expression in low grade serous carcinoma of the ovary

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ABSTRACT

Ovarian carcinoma histotypes are distinct diseases with variable clinical outcomes and response to treatment. There is a need for new subtype specific treatment modalities, especially for women with widespread and chemo-resistant disease. STING (stimulator of interferon genes) is a part of the cGAS–STING pathway that mediates innate immune defence against infectious DNA-containing pathogens and also detects tumour-derived DNA and generates intrinsic antitumour immunity. The STING signalling pathway is suppressed by a number of mechanisms in a variety of malignant diseases and, in some cancers that may be a requirement for cellular transformation. The aim of this study was to use immunohistochemistry to evaluate STING protein expression across normal tissue, paratubal and ovarian cysts, and ovarian tumour histotypes including ovarian carcinomas. Herein we show that the fallopian tube ciliated cells express STING protein, whereas the secretory cells are negative. STING expression differs among ovarian cancer histotypes; low-grade serous ovarian carcinomas and serous borderline tumours have uniform high STING expression, while high-grade serous and endometrioid carcinomas have heterogeneous expression, and clear cell and mucinous carcinomas show low expression. As low-grade serous carcinomas are known to be genomically stable and typically lack a prominent host immune response, the consistently high STING expression is unexpected. High STING expression may reflect pathway activation or histogenesis and the mechanisms may be different in different ovarian carcinoma histotypes. Further studies are needed to determine whether the STING signalling pathway is active and whether these tumours would be candidates for therapeutic interventions that trigger innate immunity activation.

Keywords: STING, innate immunity, low grade serous ovarian carcinoma

INTRODUCTION

Ovarian cancer is the eighth most common cancer in females worldwide, with 295,414 new cases diagnosed in 2018 [1,2]. In the United States, ovarian cancer is the most common cause of death from gynaecological disease, with an estimated 21,750 new cases of ovarian cancer and 13,940 deaths in 2020 [3]. Despite intensive research efforts and accumulating knowledge on the pathogenesis, precursor lesions, as well as prevention strategies and treatment options, these advantages have not significantly improved the prognosis of this lethal malignancy, and further understanding of the pathogenesis and potential therapeutic targets is needed.

Stimulator of interferon genes (STING), also known as transmembrane protein 173 (TMEM173), is a part of cGAS–STING pathway that mediates protective immune defence against infection, detects tumour-derived DNA and generates intrinsic antitumour immunity [4]. cGAS functions to directly detect cytosolic DNA, which then signals STING to induce transcription of type I interferon genes and inflammatory cytokines, essential for successful pathogen elimination [4]. STING signalling protects the cell against various pathogens, as well as from cancer development by promoting antitumour immune responses [5,6]. In cancer, self-DNA from dying tumour cells has been suggested to be an important danger signal that triggers the cGAS–STING pathway to induce interferon [7]. STING signalling can be suppressed through epigenetic silencing or loss-of-function mutations [8]. Additionally, some missense mutants fail to generate cytokines in response to cytosolic DNA or chemotherapy, indicating that suppression of STING signalling may enable precancerous cells to avoid antitumour immune responses [8].

The essential role of STING in immune defence in cancer has raised interest in the cGAS–STING pathway. The development of novel anti-inflammatory and antitumour compounds that specifically target this key signalling pathway has been a research focus during recent years [9]. STING agonists have been developed to function as novel cancer therapeutics, and early phase clinical trials are promising both for STING agonists alone and in combination with checkpoint blockade (reviewed by Flood et al. [10]).

Decreased STING expression has been associated with adverse prognosis in colorectal cancer, hepatocellular cancer, gastric cancer and melanoma [11–15]. In ovarian cell lines and tumour-derived cancer tissue, the STING pathway is suppressed in a majority of cases, either by loss of STING or cGAS expression, epigenetic suppression, or defects in STING translocation [16]. Work focusing on high-grade serous (HGSC) ovarian cancer therapeutics have shown the potential benefit of adding a STING agonist and immune checkpoint blockade to carboplatin chemotherapy to improved survival [17]. In mouse and cell line models, treating *BRCA* deficient HGSC with PARP inhibitors triggered local and systemic antitumour immunity and activation of the cGAS-STING pathway [18], which was further augmented when combined with PD-1 blockade [18,19]. Further on, in an ovarian cancer model, combining oncolytic herpes simplex virus with cisplatin in platinum resistant ovarian cancer cells resulted in the activation of cGAS-STING pathway and induction of an innate immunity [20].

The aim of this study was to evaluate STING protein expression, assessed by immunohistochemistry (IHC), in normal gynaecological tissue, benign ovarian cysts, cancer precursor lesions in the fallopian tube as well as ovarian tumours. Further on, we aimed to evaluate whether STING expression could aid diagnosis or function as a prognostic marker

and whether STING pathway could be targeted, and STING expression used as a predictive marker for targeted therapy in ovarian cancer. To our knowledge, this is the first time STING protein expression has been assessed across different ovarian tumour histotypes, and benign ovarian lesions.

MATERIALS AND METHODS

The expression of STING in normal tissue was evaluated using The Human Protein Atlas [21,22].

These studies have received institutional REB approval.

Cortical inclusion cysts and paratubal cysts

A tissue microarray (TMA) consisting of cortical inclusion cysts (CICs) and paratubal cysts (PTCs) was constructed from formalin-fixed paraffin-embedded (FFPE) ovarian tissue. The TMA consisted of 64 x 2 mm single cores from 29 ovaries with CIC and/or PTC lesions. Cores from fallopian tubes removed as a part of hysterectomy for benign condition were also included.

Ovarian tumour TMAs

We obtained ovarian and endometrial tumours from the OVCARE Tissue Biobank Repository, Vancouver BC, Canada. This study was performed on previously constructed TMAs with duplicate 0.6 mm cores from FFPE tissues. TMAs comprised 566 cases, also including cores from benign proliferative endometrium, fallopian tube and ovary. One of the two cohorts in this study has been previously described [23]. To evaluate the expression of STING in ovarian tumours, we stained for TMEM173/STING on both TMAs. After removing duplicate cases (n=45), cases of endometrial carcinoma (n=21) and cases where

tissue cores were lost or staining was uninterpretable (n=18), 503 cases were entered into this study (supplementary material, Table S1).

Immunohistochemistry

Sections were cut at 4 μm thickness, deparaffinized in xylene, rehydrated through a graded series of ethanol and briefly rinsed in Tris-buffered saline (TBS). TMEM173/STING IHC (antibody HPA038116, Sigma, dilution 1:100) was performed using the Ventana Discovery Ultra automated stainer (Ventana Medical Systems Inc, Tucson, AZ, USA). Calretinin IHC (antibody DAK-Calret-1, IR627, Dako, ready-to-use) was performed using the Dako Omnis automated immunohistochemistry instrument (Agilent Technologies, Santa Clara, CA, USA).

Scoring

The intensity of STING immunostaining was scored negative (0), weak (1), moderate (2) and strong (3), and the frequency of positive tumour cells was assessed as a continuous variable 0-100%. Scoring was performed blinded to the clinical data. An H-score ((1 x %score 1) + (2 x %score 2) + (3 x %score 3), giving a range of 0-300) was calculated for statistical analysis. Calretinin immunostaining percentage was scored semiquantitatively in four categories (0 = 0, 1 = 1-25%, 2 = 25-50%, 3 = 50-75%, and 4 = 75-100%), and intensity in four categories (negative (0), weak (1), moderate (2) and strong (3)); a calretinin score was calculated as intensity x percentage (0-12). Cases with no staining were considered negative. In this work, cases were considered either positive or negative.

Data analysis

All statistical analyses were done using R project for statistical computing (R version 4.0.3.) and survival package was used for survival analysis. Cases with missing values were

removed from analyses and only cases with complete data were considered. For exploratory analysis of STING H-score, quartiles and median were used as cut points. We considered the univariable association between TMEM173/STING (IHC) expression using Chi-Square (or Fisher's Exact, when appropriate) test for categorical biomarker data and Kruskall-Wallis/Wilcoxon rank sum test for continuous biomarker data. Kaplan-Meier plots and Cox regression models were used to assess correlation with survival and observations were randomly censored on December 31st of the fifth year following the year in which they had their surgery. The median H-score of STING expression (H-score 40) was used as a cut-off for survival analysis in histological groups where cases were sufficient. Statistical significance was set at $p = 0.05$ and no attempts were made to adjust for multiple comparisons.

RESULTS

STING expression in non-malignant tissues

A publicly available database, Human Protein Atlas, was used to evaluate STING expression in normal tissue. STING was expressed in ciliated cells of respiratory epithelium, ciliated cells of the fallopian tube, Leydig cells of testis, squamous epithelia in tonsil, basal layer of squamous epithelia (e.g. in cervix and oesophagus), basal cell layer of prostate glands, a proportion of lymphatic cells, and endothelia. Most normal tissues were negative for STING.

In our work, STING was highly expressed in the ciliated cells of normal fallopian tube epithelium. The endothelial lining of lymphatic and blood vessels expressed STING in all tissues examined, as did a subset of lymphocytes. Endometrial glands were mostly STING negative; however, small foci of ciliated metaplasia stained positive (Figure 1).

STING expression in cysts was variable (supplementary material, Figure S1). STING expression was lower in CICs (H-score median 150, IQR 165) than in PTCs (H-score median 300, IQR 150). Some cysts (both PTC and CIC) had a similar staining pattern as the fallopian tube, with staining of ciliated cells. Most of the PTCs had a thin, strongly STING positive epithelium lining the cyst. In CTCs, STING expression varied from negative to strongly positive. There was a strong association between low STING expression and calretinin positivity (H-score median 190 in calretinin positive epithelial lining versus H-score median 60 in calretinin negative epithelium, p=0.014 Mann-Whitney U-test, supplementary material, Figure S2) suggesting that STING expression was low in those cysts with mesothelial lining.

STING expression in ovarian carcinomas

STING expression was evaluated in 503 specimens of which 378 cases were ovarian carcinomas: 55 endometrioid ovarian carcinomas (ENOC), 235 HGSC, 34 low-grade serous ovarian carcinomas (LGSC), 39 clear cell ovarian carcinomas (CCOC) and 16 mucinous ovarian tumours (supplementary material, Table S1). There were significant differences in the expression of STING across the different ovarian tumour histotypes. The H-score boxplots are presented in Figure 2 and photomicrographs of STING IHC in different histotypes in Figure 3. The overall highest expression was observed in LGSC ($p<0.001$) and lowest in CCOC ($p<0.001$) when compared to other histotypes (Wilcoxon rank sum test). Additionally, serous borderline ovarian tumours (SBOT) showed high level STING expression and low expression was observed in mucinous ovarian tumours. HGSC tumours showed varied expression across all samples and no correlation with clinical outcomes.

We observed a significant association between high STING expression and WT1 positivity (H-score: $p<0.001$). Within the CCOC histotype, higher STING expression was associated

with an adverse prognosis whereas, in the other subtypes, STING expression was not associated with survival. *BRCA1* status was available from previous work [24] in 36 HGSC cancers in this study, of which 9 were *BRCA1* mutated (supplementary material, Figure S3). There was a trend towards higher STING expression in the *BRCA1* mutated HGSCs compared with wild-type HGSC, but this did not reach statistical significance (p=0.081).

DISCUSSION

In this study we show that STING protein expression, assessed by IHC, is higher in LGSC and SBOT when compared to most other epithelial ovarian carcinomas. HGSC showed a broad spectrum of staining intensity, whereas ciliated cells, in both normal tissue and ovarian cysts, were positive.

LGSC is a rare ovarian tumour and accounts for 2 – 4 % of all epithelial ovarian cancers and 5 – 10 % of serous ovarian cancer [25,26]. It affects relatively young women, and the disease is often diagnosed at advanced stage when it is associated with poor prognosis. Primary cytoreductive treatment remains the optimal treatment modality [26]. The response to chemotherapeutic agents is modest and there is a need to better understand potential vulnerabilities in the tumour cells that could be targetable.

The mechanism for high STING expression in LGSC remains to be clarified. LGSC is associated with a less immune suppressive landscape and reduced innate immune suppression when compared to high-grade ovarian carcinomas [27]. LGSCs have fewer tumour-infiltrating lymphocytes [28,29] and the expression of B7-H4 (regulating T-cell immunity) has been described to be significantly reduced [30] when compared to HGSC. Yet, the activation of STING-dependent pathway in cancer cells, is the result of tumour-infiltration by

immune cells and modulation of the anticancer immune response. This raises the question of whether high STING expression in LGSC reflects the activation of STING-dependent pathway or could it reflect a disruption in the degradation process i.e. a non-functioning pathway. If not bound to cyclic dinucleotide, STING is usually rapidly degraded. Thus, its presence has been considered a good surrogate for STING activation [31]. Whether or not a potential disruption in the degradation process affects the potential for downstream STING pathway activation is uncertain, but germane to potential therapeutic approaches.

Several antitumoural roles have been described for the cGAS-STING pathway and acute activation of the pathway provides an antitumour effect; however, chronic inflammation by persistent and spontaneous activation of STING has also been speculated to promote tumour growth and metastasis [32] and could explain the high STING expression. However, we note that LGSCs have less impressive immune infiltrates than other histotypes and therefore proximity to inflammation cannot explain the relatively high STING expression in normal ciliated cells, SBOT or LGSC.

One possibility is that high STING expression reflects the histogenesis of LGSC, i.e. is a cell lineage marker. The precursor lesion(s) of LGSC remain debated but recent evidence suggests that they originate from ovarian cortical cysts, benign ovarian serous neoplasms and SBOT although a tubal origin has also been suggested [33–35]. Thus, the STING expression of LGSC could reflect origin from ciliated epithelial cells or cells from that lineage. Supporting this is our observation that, in the inclusion cyst epithelium with a more mesothelial-like immunophenotype, with expression of calretinin, there is reduced STING expression. In this scenario, high STING expression could merely reflect the cell of origin and not activation of the STING pathway. [32]

HGSCs are known to arise from the fallopian tube in most cases and the evolution from secretory cell expansion and loss of ciliated cells [36] through p53 signature lesions and serous tubal intraepithelial lesions to HGSC is a well-described process; however, molecular drivers of these phenomena are incompletely understood. STING signalling may exert a tumour suppressive effect and trigger cytokine production and attract immunosurveillance, and thus suppression of STING could be a requirement for precancerous cells to avoid antitumour immune response and for the cellular transformation process to proceed. Most recently, it has been shown that STING does have tumour suppressive activity and the STING pathway is activated in response to cytosolic tumour DNA. Thus, the expression of STING in a subset of HGSCs could reflect some level of ciliated cell differentiation [37]. In HGSC, a transcriptomic signature characteristic for fallopian tube ciliated cells has been identified and STING was amongst the markers defining ciliated cells, indicating an ability of serous ovarian carcinomas to differentiate into cells that molecularly resemble fallopian tube ciliated cells [38]. The ciliated tumour subtype was highly enriched in the low-grade tumours compared with the high-grade ones. Furthermore, single cell RNA sequencing and IHC of ovarian and endometrial tumours has demonstrated the presence of tumour cells expressing ciliated cell markers [39].

In our work, CCOC had significantly lower STING expression compared to other histotypes, while ENOC showed a similar STING expression profile to HGCS. Both ENOC and CCOC develop from endometriosis, via alterations affecting different genetic pathways [40]. It has been suggested, in the context of endometriosis, that ENOCs are derived from endometriosis cells of the secretory cell lineage, whereas CCOCs could be derived from, or have similarities to, cells of the ciliated cell lineage [41]. This challenges the hypothesis that tumours that have

high STING expression would purely reflect ciliated cell of origin. In the context of endometriosis and adenomyosis, chronic inflammation and upregulated STING expression have been speculated to play a role in eliminating nascent neoplastic cells and the decrease of STING expression potentially leading to emergence of precancerous cells [42]. Therefore, if the STING pathway plays a role in pathogenesis of these cancers, its role is distinct in ENOC and CCOC.

CCOCs are associated with low numbers of tumour-infiltrating lymphocytes and the low expression of STING may be evidence of inactivation of the STING pathway. Interestingly, similarly to our finding, in which ovarian CCOCs are associated with adverse survival, high STING expression is also associated with adverse survival (Human Protein Atlas [21]) in the morphologically similar renal clear cell carcinoma. The mechanism remains unclear.

Overall, it seems likely that STING expression has different roles in different ovarian cancer subtypes and in different contexts. Functional experiments are needed to clarify the role of STING in ovarian carcinomas and to assess the integrity of the cGAS-STING pathway.

In chemoresistent ovarian carcinomas, there is a great need for novel therapeutic strategies, and a dysfunctioning STING pathway could provide a unique opportunity for therapeutic intervention. It has been postulated that STING and cGAS could provide an assay that could help predict the outcome of oncoviral therapy in ovarian cancer [43]. Xia et al hypothesized that, as suppression of STING signalling might be a key requirement for the development of malignant disease, these same defects in STING signalling may render cancer cells highly susceptible to oncolytic viral infections [14,15]. Resistance mechanisms to oncolytic viral therapy often include activation of the STING pathway [14]; therefore tumours, such as

LGSC, that may be defective in STING signalling, could be particularly susceptible to this type of therapeutic intervention.

A dysfunctioning STING pathway can also be targeted with a STING agonist and this has been tested in early clinical trials in HGSC. Our results show that a proportion of HGSCs have low STING expression and STING expression could be used as a predictive marker for STING agonist treatment in HGSC. Additionally, miR-181a targeting of STING has been presented as a therapeutic opportunity [37]. In cells stably overexpressing mature miR-181a, knockdown of STING was sufficient to increase cell proliferation and clonogenic survival. Loss of STING increased the transformation of fallopian tube secretory epithelial cells and miR-181a inhibited this process [37].

In summary, STING expression may reflect different mechanisms in different ovarian carcinoma histotypes. There is variable expression in HGSC and ENOC, while CCOC and MOC show much less expression. As this correlates with the more frequent association of a host immune response in the former than the latter histotypes, expression of STING may reflect pathway activation as part of the host response. LGSCs are distinct in that STING is highly expressed in precursor lesions such as SBOT and CICs, and also in tumour cells, yet there is little evidence of a host immune response and the tumour cells are diploid/near diploid with low mutation burdens. In this setting STING expression may reflect ciliated cell lineage rather than pathway activation. If true, this has potential therapeutic implications as the lack of intact STING signally in LGSC, for example, could render the cells more susceptible to viral oncolytic therapy. The response to chemotherapeutic agents is modest and there is a need to better understand potential vulnerabilities in the tumour cells that could be targetable.

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AUTHOR CONTRIBUTIONS STATEMENT

JH, DRC, AD and DGH conceived the experiments. Optimization and immunostaining of STING was performed by MT and CC. Pathology review and scoring of tumour tissue microarrays was performed by JH. KG and AK provided TMAs. Statistical analysis was performed by SL. The manuscript was prepared by JH. All authors read and reviewed the manuscript.

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FIGURE LEGENDS

Figure 1: Photomicrographs of STING IHC in (A) endometrial glands, (B) endometrial glands with focal of ciliated metaplasia and (C) fallopian tube.

Figure 2: Boxplots of H-scores of STING expression across different tumours ($p<0.001$, Kruskal-Wallis test). OC= ovarian carcinoma, SC= serous carcinoma.

Figure 3: Photomicrographs of different levels of STING expression in low-grade serous carcinoma (LGSC), endometrioid ovarian carcinoma (ENOC), clear cell ovarian carcinoma (CCOC), high-grade serous carcinoma (HGSC) and serous borderline ovarian tumour (SBOT), when present.

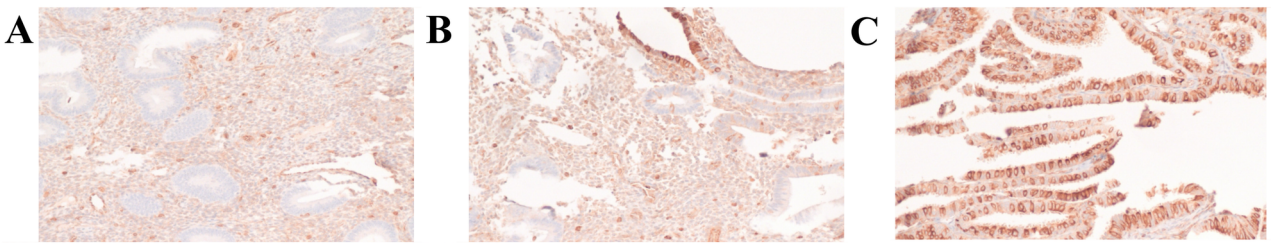
LIST OF SUPPLEMENTARY MATERIAL ONLINE

Figure S1. STING expression in ovarian cortical inclusion cysts and paratubal cysts

Figure S2. STING H-scores in calretinin negative and positive ovarian cortical inclusion cysts and paratubal cysts

Figure S3. H-scores of STING expression in *BRCA1* mutated and wildtype HGSC

Table S1. Histotype distribution of cases on the ovarian tumour TMAs



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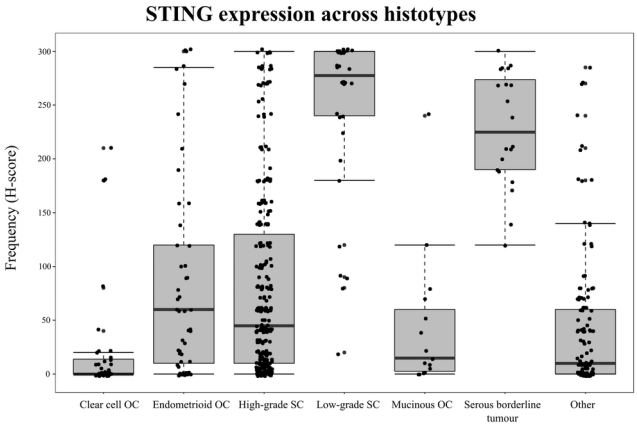

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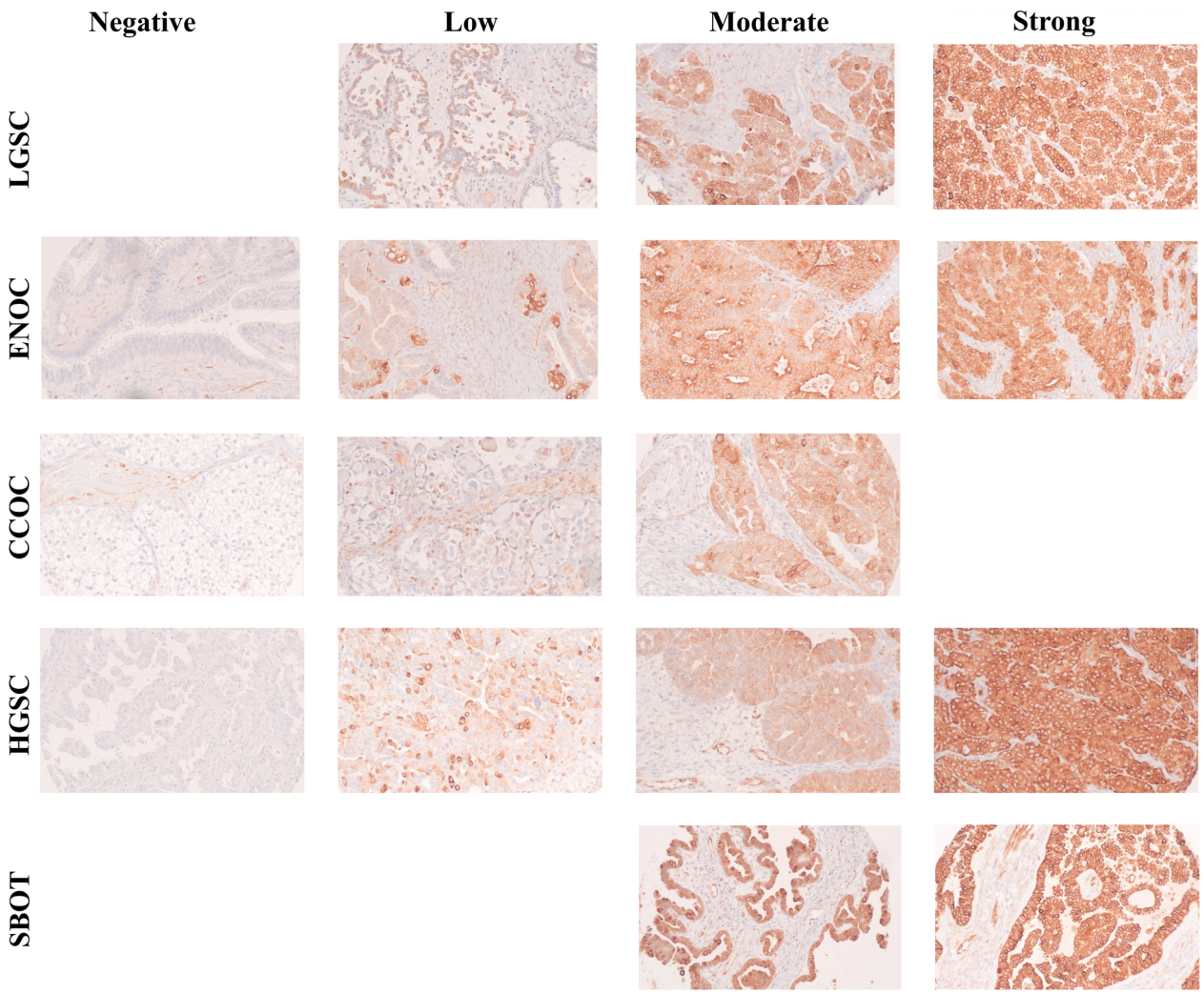

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