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Investigating the Therapeutic Potential of Targeting LRRC15 in Endometrial Cancer

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Investigating the Therapeutic Potential of Targeting LRRC15 in Endometrial Cancer

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Abstract

Endometrial cancer is the most common gynecological malignancy and accounts for 6% of all cancers in women. [1] In 2018, there were an estimated 63,280 new cases of endometrial cancer resulting in 11,350 deaths.[2] In advanced stages, aggressive forms of endometrial cancer are able to invade the peritoneum and metastasize to the omentum and bowel.[1,3,4,5] Shridhar *et al.* have identified leucine rich repeat containing 15 (LRRC15), a protein involved in cell adhesion and interactions with the extracellular matrix (ECM), as a potential therapeutic target due to its significantly higher expression in ovarian cancer tumors that have metastasized to the omentum and bowel when compared to their matched primary tumor counterparts. Additionally, Shridhar *et al.* have shown that LRRC15 is capable of associating directly with β1-integrin and promotes invasion and metastasis through the activation of focal adhesion kinase (FAK) in ovarian cancer. In this study, knockdown of LRRC15 in endometrial cancer cell lines lowered the expression of proteins within the FAK signaling pathway, decreased cancer cell adhesion, and decreased cell migration *in vitro.* Conversely, induced overexpression of LRRC15 led to an increase in expression of proteins in the FAK pathway and an increase in cell adhesion. Treatment of endometrial cancer cell lines with the therapeutic agent ABBV-085, a drug antibody conjugate which targets cells expressing LRRC15, was tested *in vitro* and demonstrated high specificity compared to the control with an IC_{50} between 0.1-1.0 nM. Overall, our results from LRRC15 knockdown and ABBV-085 treatment demonstrate that targeting LRRC15 may be therapeutically beneficial in endometrial cancer.

Introduction

In a recent unpublished study of patients with metastatic ovarian cancer, Shridhar *et al.* compared protein expression signatures of primary tumors with their metastatic counterparts taken from either the bowel or omentum. Shridhar *et al*. chose to analyze leucine rich repeat containing 15 (LRRC15) due to its increased expression in ovarian metastatic lesions compared to primary lesions (unpublished data). Furthermore, Shridhar *et al*. discovered that LRRC15 associates with β1-integrin and may play a role in ovarian cancer metastasis by facilitating integrin signaling and the activation of focal adhesion kinase, or FAK (unpublished data).

In advanced stages, endometrial cancer can metastasize to the omentum and bowel.[1,3,4,5] Approximately 3-11% of endometrial cancer tumors result in malignant bowel obstruction (MBO) and 5.9% metastasize to the omentum.[3,4] Given that endometrial cancer also expresses LRRC15, we decided to elucidate the role of LRRC15 in endometrial carcinoma. In addition, we decided to test the specificity of the drug antibody conjugate, ABBV-085, given that it targets cells expressing LRRC15.[6]

Background

Endometrial Cancer Diagnosis and Treatment

Approximately 2.9% of women will be diagnosed with endometrial cancer during their lifetime and it is most commonly diagnosed in women ages 45-75.[2] There is no general screening test for endometrial cancer because it will mainly detect low-risk tumors, and so it is unlikely to decrease mortality .[7] Instead, endometrial cancer is most commonly diagnosed by an endometrial biopsy in symptomatic patients.[8] A noninvasive procedure like transvaginal ultrasonography (TVU) is not recommended for diagnosing asymptomatic patients given its limited accuracy.[8,9] The most common symptom of endometrial cancer is abnormal vaginal bleeding such as when patients report post-menopausal vaginal bleeding.[7,8] Gregory Robertson, a gynecological oncologist, recommends educating women on the importance of investigating vaginal bleeding especially if they have a history of taking tamoxifen or a family history of hereditary non-polyposis colon cancer (HNPCC).[10] Tamoxifen is a nonsteroidal hormone that has antiestrogen effects in breast tissue and estrogen-like effects in endometrial tissue that is used to effectively treat breast cancer.[11] Patients taking tamoxifen for more than 5 years experienced a 4.06 fold increase in the odds of developing endometrial cancer compared to nonusers.[11] HNPCC is a mendelian dominant syndrome characterized by germline mutations in various mismatch repair genes.[7] This syndrome leads to an individual having a significantly greater risk of developing a variety of cancers throughout their lifetime.[12] Patients diagnosed with HNCPCC have a high chance of developing colon cancer (40-60%), endometrial cancer (40-60%), and ovarian cancer (12%).[12]

Endometrial cancer is initially staged and treated using surgery.[8] Surgery involves a total hysterectomy and bilateral salpingo-oophorectomy.[7] In selective cases, a pelvic or para-aortic lymphadenectomy may be performed.[8] Patients that are not surgical candidates may receive supplemental radiation therapy.[8] Also, patients with high-intermediate risk disease are treated with adjuvant radiotherapy (external beam or vaginal brachytherapy) as an effort to reduce local tumor recurrence.[8] Adjuvant therapy using whole pelvic radiotherapy decreased the risk of recurrence in patients with intermediate disease but did not significantly improve survival.[13] Patients with low grade stage IA and IB disease are optimally treated with hysterectomy alone.[14] In addition to radiotherapy, adjuvant chemotherapy and hormone therapy are also available. Chemotherapy is primarily used in patients with metastatic disease.[7] Platinum-based compounds, anthracyclines, and taxanes all have response rates greater than 20% when used as single agents.[8] When used in combination, drugs such as doxorubicin, cisplatin and paclitaxel have produced response rates of up to 57% although this kind of multiple agent therapy is prone to cause negative side effects such as peripheral neuropathy.[15]

Given the adverse side effects of chemotherapy and the hormonal sensitivity of endometrial tissue, hormone therapy has been suggested as a possible treatment

for endometrial cancer.[16] Hormone therapy has mild side effects such as edema and weight gain although there is an increased risk of venous thromboembolism.[16] Megestrol acetate is a progestin that was approved for the palliative treatment of recurrent, metastatic endometrial cancers decades ago but has had limited efficacy.[17] Treatment with 50 mg of Megestrol acetate three times a day produced an overall response rate of 14% in patients with recurrent endometrial cancer.[18] A Gynecologic Oncology Group (GOG) study evaluated the combined hormonal strategy of alternating tamoxifen and megestrol acetate based on the hypothesis that tamoxifen increases the expression of progesterone receptors and thereby increases the efficacy of megestrol acetate.[17] Megestrol acetate at 80 mg twice daily every 3 weeks, alternating with tamoxifen 20 mg twice daily every 3 weeks, was associated with an overall response rate of 27% which decreased as the grade of disease increased.[19]

Type I vs. Type II Endometrial Cancer

Endometrial cancer can be divided into two subtypes: type I and II. Type I endometrial carcinoma typically occurs in premenopausal and younger post-menopausal women whereas type II typically occurs in older postmenopausal women.[20] Type I endometrial carcinoma has an endometrioid morphology and is moderately to well differentiated.[7] Type I makes up around 80% of endometrial cancer and is characteristically preceded by endometrial hyperplasia.[7,8] Type I endometrial carcinoma is associated with unopposed estrogenic stimulation and is relatively low grade with a good prognosis.[7,8] Around 10% of women with endometrial cancer have type II endometrial carcinoma, which is poorly differentiated and heterogeneous.[8] The morphology of type II endometrial cancer consists of the high-grade endometrioid morphology along with non-endometrioid morphologies such as serous papillary and clear cell.[7,8] Type II endometrial carcinoma is not driven by estrogen and is associated with endometrial atrophy.[7] Type II endometrial carcinoma carries a poor prognosis with a high risk of recurrence and metastatic disease.[7] Type II endometrial carcinoma has a much lower mean survival time than type I (24.72 months vs. 113.68 months).[21]

In addition, type I and II endometrial cancer have characteristic molecular profiles. Type I endometrial carcinoma is associated with mutated or unexpressed phosphate and tensin homolog protein (PTEN) resulting in constitutive activation of the Akt and mTOR pathways.[8,17] Type I endometrial carcinoma is also associated with mutations in the KRAS oncogene.[7,20] In contrast, type II endometrial carcinoma is associated with alterations in the TP53 and p16 gene and overexpression of the ErbB2 (Her-2/neu) gene.[8,22,23] Approximately 90% of serous endometrial carcinomas, a subtype of type II endometrial carcinoma, harbor a mutation in TP53 and 18% exhibit positive immunohistochemical (IHC) expression of Her-2/neu.[24,25] In reality, the characterization of endometrial cancer tumors can be complex because a given lesion can display characteristics of both type I and type II endometrial carcinoma.[8] This can make distinguishing between type I and II endometrial carcinoma a challenge in clinical practice.[22] Wei *et al.* suggest the using

a panel of immunohistochemical markers to help assist with the classification of ambiguous tumors.[22]

Endometrial Cancer Metastasis and Recurrence

Most women are diagnosed with endometrial cancer in an early stage, which is associated with a good prognosis.[17] Approximately 67% of all endometrial cases are localized disease, which has a 5-year progression-free survival of 94.9%.[2] However, advanced metastatic disease occurs in around 9% of endometrial cancer cases and has a 5-year progression free survival of 16.3%.[2] In particular, uterine papillary serous carcinoma (UPSC), an aggressive subtype of type II endometrial carcinoma, accounts for over 50% of recurrences and deaths caused by endometrial cancer even though less than 10% of endometrial cancers are UPSC.[25,26] The 5-year overall survival rate for women with UPSC is 46%.[27] Endometrial carcinoma usually spreads through lymphatic vessels to the pelvic and para-aortic lymph nodes and by local invasion to the ovaries and the tissue surrounding the uterus.[28] Endometrial carcinoma can also spread via the bloodstream to various organs but this is less common.[28]

In general, as the level of differentiation decreases and depth of myometrial invasion increases, the grade of the tumor increases.[29] In a GOG study performed by Creasman *et al*., 78% of Grade 1 tumors had only endometrial or superficial muscle involvement whereas 58% of Grade 3 tumors had mild or deep muscle invasion.[29] However, the author did find some cases (7-10%) where Grade 3 lesions had only endometrial invasion and Grade 1 lesions had deep muscle invasion.[29] Importantly, there is a large positive correlation between depth of invasion and nodal metastasis with only 1 % of lesions that penetrated the endometrium alone having nodal metastasis (pelvic or para-aortic).[29] Conversely, lesions with deep muscle invasion metastasized to the pelvic lymph nodes with a frequency of 25% and the para-aortic lymph nodes with a frequency of 17%.[29]

Even though endometrial cancer has an overall 5-year survival rate of 81.1%, endometrial cancer recurs in approximately 13% of cases after initial treatment.[2,30] Aalders *et al.* define recurrence as regrowth of the endometrial cancer after an apparently complete remission that last for 3 months following primary treatment.[31] In a study conducted by Aalders *et al.* on patients with recurrent endometrial carcinoma, 50% of patients had local recurrence (tumors maintained within pelvic structures including lymph nodes below pelvic brim), 28% had distant metastasis, while 21% had both.[31] In addition, 34% of all recurrences were detected within one year whereas 76% were detected within three years of initial treatment.[31] Typical sites of recurrent endometrial carcinoma include the pelvic and para-aortic lymph nodes, vagina, peritoneum, and lungs whereas atypical sites such as intra-abdominal organs, bones, brain, abdominal wall, and muscle have also been observed.[1] A study conducted by Sohaib *et al.* on patients with recurrent endometrial carcinoma found the highest incidence of relapse in lymph nodes (46%), vagina (42%), peritoneum (28%), and lung (24%).[32]

Role of Integrins, FAK, and SRC in Metastasis

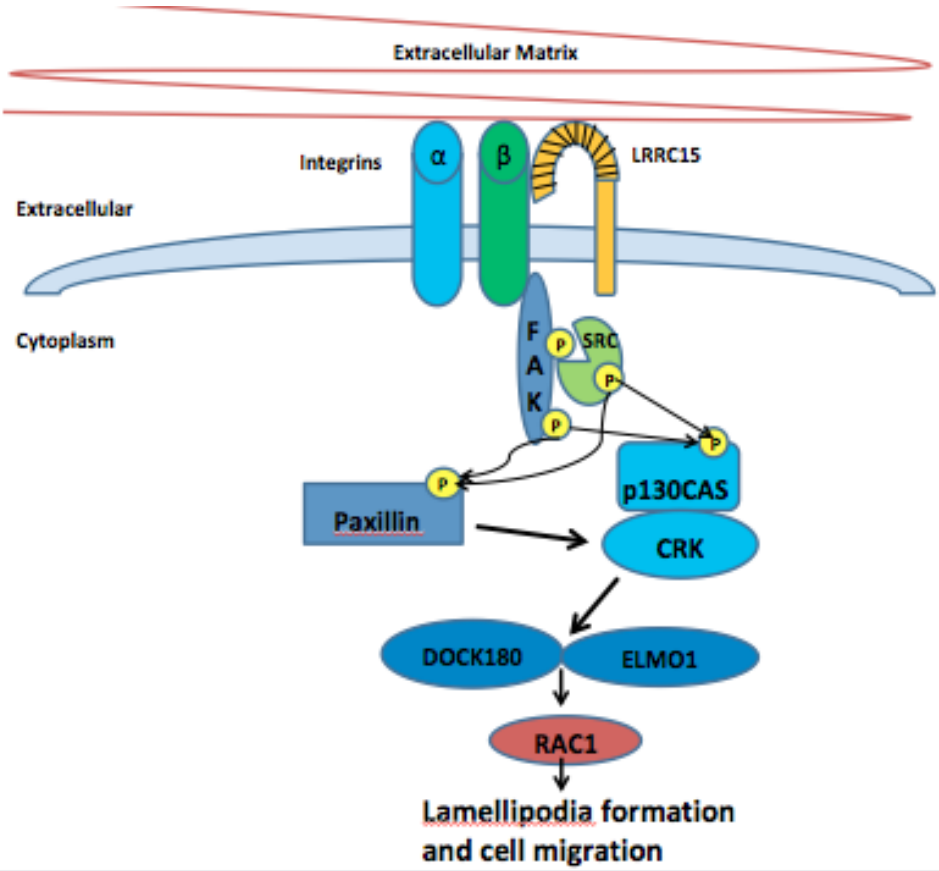

Figure 1. Integrin-mediated activation of FAK/SRC signaling pathway. Integrins binding to ligands of the ECM promote focal adhesion formation and FAK activation. FAK forms a complex with SRC and phosphorylates downstream effectors eventually leading to Rac1 activation and lamellipodia formation. Figure adapted from Huveneers and Dannen (2009) to reflect the presence of LRRC15.

Integrins play a role in tumor cell adhesion and have been implicated in tumor cell metastasis.[33] Integrins are a family of transmembrane glycoproteins that form non-covalent heterodimers made up of an α and β subunit.[33,34] There are 18 α- and 8 β-integrins that combine to form 24 canonical α/β receptors.[35] Integrins mediate cell adhesion and directly bind components of the extracellular matrix (ECM), including fibronectin, vitronectin, laminin, and collagen, thereby providing anchorage for cell motility and invasion.[33] Integrins binding to extracellular ligands initiate a clustering of cytoskeletal proteins, which form into what is known as a focal adhesion.[36] Importantly, focal adhesion kinase (FAK) localizes with integrins in focal adhesions and interactions between FAK and integrins play an important role

in cell adhesion.[36] Integrin-mediated adhesion induces the autophosphorylation of FAK at tyrosine 397, creating a binding site for the SH2 domain of the kinase SRC, which in turn phosphorylates FAK at the following tyrosine residues: Y576, Y577, Y861 and Y925 **(Figure 1)**.[37,38] SRC is activated by phosphorylation at Y416 and promotes maximal FAK kinase activity and allows FAK to bind other proteins.[37,38] The FAK-SRC complex then phosphorylates the adaptor proteins paxillin and p130CAS (hereafter, CAS).[37] Phosphorylation of paxillin creates SH2 binding sites for and promotes activity of the adaptor protein Crk.[39] The activated FAK-SRC complex recruits and phosphorylates CAS, which also interacts with Crk.[36]

CAS and Crk further assemble into a complex, which results in the activation of Rac downstream and leads to cell migration.[40] CAS/Crk activates Rac through the cooperation between DOCK180 and ELMO1, which promotes the formation of membrane protrusions.[38,41] Rac works with the protein Cdc42 at the leading edge of cells in the formation of lamellipodia and filopodia, respectively.[38,42,43,44] Lamellipodia and filopodia are protrusions that come out from the cell.[45] Lamellipodia are thin extensions of the cell's periphery made up of unipolar actin filaments whereas filopodia are spikey bundles of actin filaments that are within or extend from lamellipodia.[45] Lamellipodia are important because they are what allows the cells to "crawl" forward.[45] Lamellipodia protrude outward from the leading edge of the migrating cell and form new contact sites.[45] Then with the help of other Rho GTPase family members, new contact sites are initiated and turned over and focal adhesion sites are developed.[45] Rac and Cdc42 activation leads to the activation of the WAVE and WASP family of proteins, which leads to the activation of the Arp2/3 complex. Activation of the Arp2/3 complex serves to initiate new actin filament formation, which pushes the leading edge forward and increases cell motility.[36,46,47] Overall, the activation of Rac and other Rho GTPase family members leads to cell adhesion and protrusion which are required events in cell migration for they promote cell spreading that can later lead to metastasis.[36]

Role of LRRC15 in Cancer

Leucine rich repeat 15 (LRRC15) (also known as hLib) is a 581 amino acid type I transmembrane protein with an extracellular domain that contains fifteen leucine rich repeats (LRRs).[6,48] LRRC15 belongs to the leucine rich repeat superfamily, which is involved in cell-cell and cell-extracellular matrix interactions.[49] LRRC15 has no obvious intracellular signaling domains and was first identified as a protein induced by B-amyloid in rat astrocytes.[6,48,49] LRRC15 has been found to have low expression in most normal tissues but Northern blot analysis shows high LRRC15 mRNA expression in the placenta.[48] Using RNA in situ hybridization, Reynolds *et al*. found that LRRC15 expression was specifically in the cytotrophoblast cell layer, which is the invasive layer of the placenta.[6,50] Immunohistochemical (IHC) analysis has further demonstrated normal LRRC15 expression restricted to particular areas such as hair follicles, tonsil, stomach (cardia and pylorus regions only), spleen (peritrabecular region), osteoblasts, and sites of wound healing.[6]

LRRC15 overexpression has been associated with aggressive disease in multiple tumor types including breast, ovarian, and prostate cancer and IHC analysis demonstrates that LRRC15 is highly prevalent in breast cancer, head and neck cancer, non-small cell lung cancer, and pancreatic cancer with a majority of LRRC15 being expressed in the stroma.[6,51,52,53,54] Interestingly, IHC analysis performed by Purcell *et al.* did not detect LRRC15 expression in any prostate samples even though high LRRC15 mRNA expression was associated with aggressive prostate cancer .[6,54] LRRC15 was found to be highly expressed on cancer associated fibroblasts located in the tumor stroma of multiple tumor types and cancers associated with mesenchymal origin (sarcoma, glioblastoma, and melanoma).[6] LRRC15 expression was also shown to be regulated by transforming growth factor beta (TGFB) in normal human lung fibroblasts.[6] A different study using Desmoplastic small round cell tumor, a highly aggressive tumor arising from the peritoneum, shows LRRC15 can be induced by the chimeric EWS–WT1(+KTS) transcription factor and that LRRC15 colocalizes with F-actin at the leading edge of migrating cells.[50]

On a molecular level, little is known about the function of LRRC15 in normal and cancerous tissue. Research using bone marrow-derived mesenchymal stem cells demonstrates that LRRC15 functions as a repressor of NFκB signaling by promoting the nuclear exclusion of p65.[55] As previously mentioned, LRRC15 has also recently been shown to associate with β1-integrin in ovarian cancer. To date, little is known about the role of LRRC15 in endometrial cancer.

Results

LRRC15 Expression in Endometrial Cancer

Using tissue microarray (TMA) analysis, we found that LRRC15 expression was pronounced in many samples of uterine papillary serous carcinoma (UPSC) although some tumors displayed little LRRC15 expression (data not shown). Interestingly, we discovered that LRRC15 expression could be found in the tumor stroma, cells, and both in UPSC tumors **(Figure 2A).** Next, we used Western blot analysis to determine LRRC15 expression in several endometrial cancer cell lines. We found that LRRC15 was expressed in both type I and type II endometrial cancer cell lines **(Figure 2B)**. LRRC15 was expressed in the type I cell lines Ishikawa and RL-95, and in the type II cell lines SPAC-1L and HEC155 **(Figure 2B and Figure 3B)**. In contrast, the type II cell line HEC-1A did not show any LRRC15 expression **(Figure 2B)**.

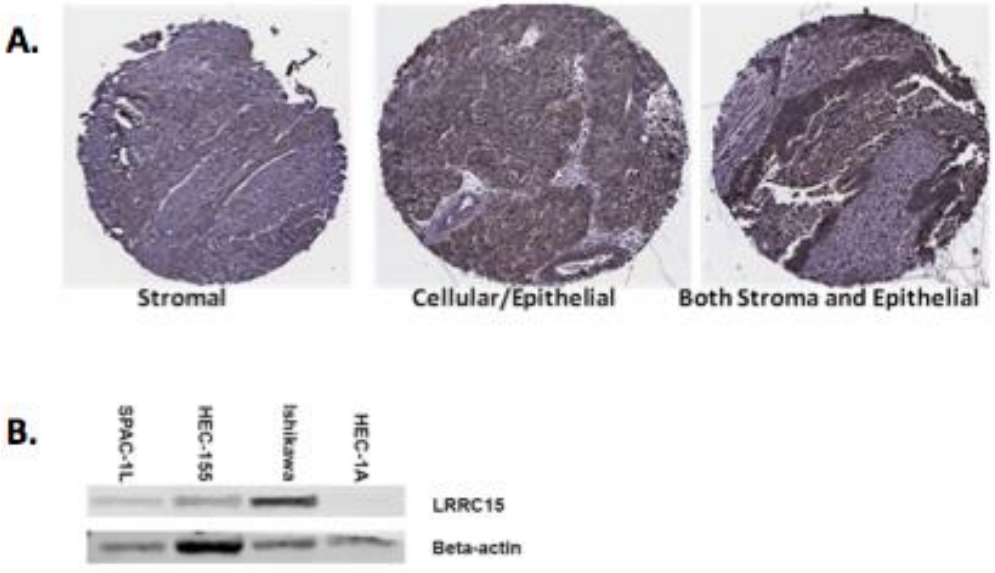

Figure 2. Expression of LRRC15 in endometrial cancer. (A) Tissue microarray was used to analyze LRRC15 expression (brown stain) in uterine papillary serous carcinoma . **(B)** LRRC15 expression was also analyzed using western blotting in several endometrial cancer lines.

LRRC15 Knockdown Decreases FAK and SRC Signaling

Using Western blot analysis, we examined the expression of protein members in the FAK/SRC signaling pathway after transient LRRC15 knockdown with two short-hairpin RNA constructs (sh1 and sh2) in two endometrial cancer cell lines. In the Ishikawa and RL-95 cell lines, LRRC15 expression was reduced by both sh1 and sh2 with sh2 demonstrating more knockdown **(Figure 3)**. Moderate LRRC15 knockdown by sh1 in Ishikawa and RL-95 cells resulted in reduced phosphorylation of FAK at the Y397 residue and SRC at the Y416 residue while having a varied effect on the expression of total FAK and total SRC protein levels **(Figure 3)**. Moderate LRRC15 knockdown by sh1 also led to a slight decrease in Rac1 expression in Ishikawa and RL-95 cells **(Figure 3)**. The more pronounced LRRC15 knockdown by sh2 in Ishikawa and RL-95 cells resulted in a greater reduction in expression of phosphorylated FAK (Y397), phosphorylated SRC (Y416), and Rac1 **(Figure 3)**. In the Ishikawa cell line, LRRC15 knockdown by sh2 also reduced the expression of total protein levels **(Figure 3)**.

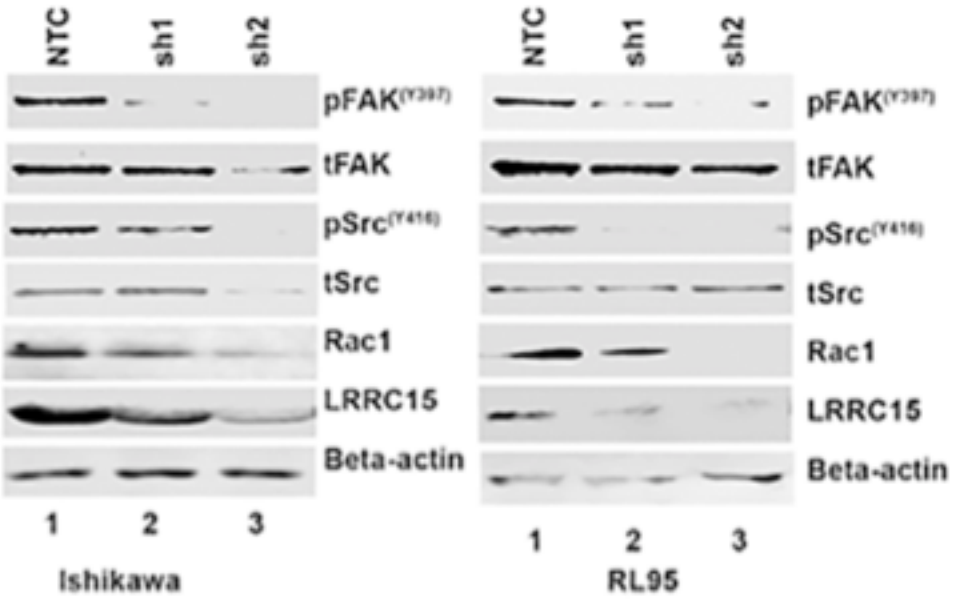

Figure 3. Effect of LRRC15 KD on FAK/SRC signaling in endometrial cancer. Expression of proteins in the FAK/SRC pathway was assessed by Western blot after transient knockdown of LRRC15 in Ishikawa and RL-95 using two short-hairpin RNA constructs (sh1 and sh2).

Induced Expression of LRRC15 Increases FAK and SRC Signaling

Given that HEC-1A cells do not express LRRC15 we also wanted to analyze the effect inducing LRRC15 overexpression has on protein expression within the FAK/SRC signaling pathway. HEC-1A cells underwent transient induction of LRRC15 for 6 hours using a plasmid vector. Western blot analysis shows that the empty vector cells do not express LRRC15 and show minimal expression of phosphorylated FAK (Y397) and SRC (Y416) while expressing reduced levels of total proteins **(Figure 4)**. Empty vector cells also had reduced Rac1 expression compared to LRRC15 expressing cells **(Figure 4)**. Hec-1A cells with induced LRRC15 overexpression demonstrated a slight increase in expression of phosphorylated FAK (Y397) and SRC (Y416) while displaying a prominent increase in the expression of Rac1 and total FAK and total SRC **(Figure 4)**.

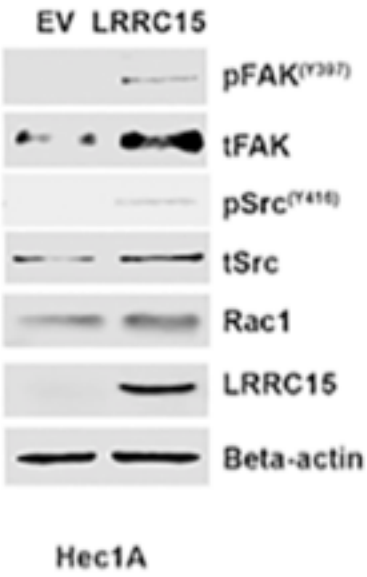

Figure 4. Effect of induced overexpression of LRRC15 on FAK/SRC signaling in endometrial cancer. Expression of proteins in the FAK/SRC pathway was assessed by Western blot after transient induction of LRRC15 into HEC-1A cells using a plasmid vector.

LRRC15 Expression Influences Cell Adhesion

Cell adhesion of LRRC15 knockdown and LRRC15 overexpressing endometrial cancer cells was assessed by cell adhesion assay using Cell Tracker™ Working Solution. Induced overexpression of LRRC15 in HEC-1A cells led to a greater amount of cell adhesion compared to the empty vector, as indicated by a greater amount of fluorescence **(Figure 5A)**. LRRC15 knockdown in HEC155 cells using sh1 (sh3382) and sh2 (sh5675) resulted in a slight decrease in cell adhesion when compared to control **(Figure 5B)**. LRRC15 knockdown in Ishikawa cells using sh1 and sh2 had an even more pronounced decrease in cell adhesion indicated by the decrease in fluorescence compared to control **(Figure 5C)**. Importantly, LRRC15 knockdown and upregulation was confirmed using Western blot analysis.

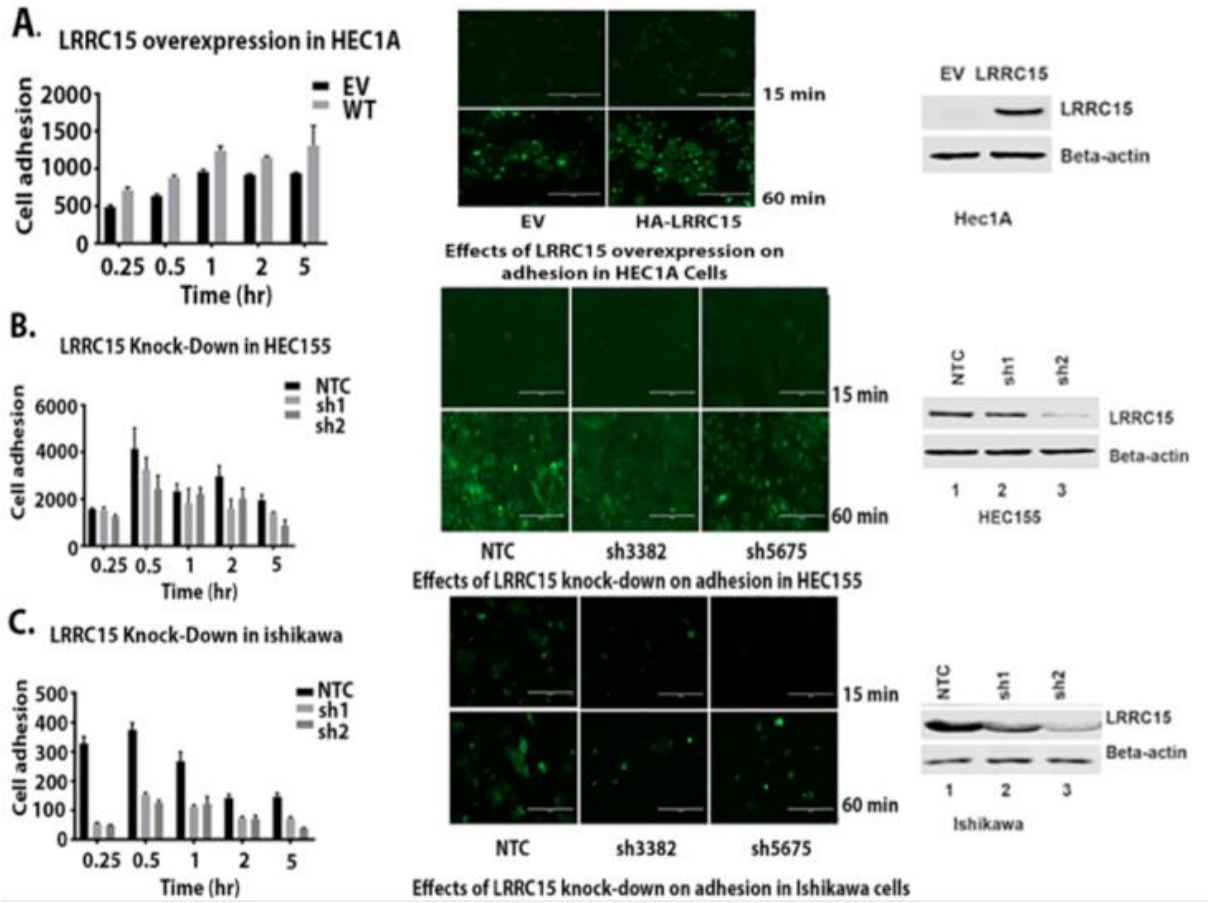

Figure 5. Analyzing the role of LRRC15 in endometrial cancer cell adhesion. Cell adhesion assays using Cell TrackerTM Working Solution (10 uM) were performed. Representative pictures of GFP florescence were taken at the 15 minute and 1 hour time points using an EVOS FL microscope and LRRC15 knockdown and upregulation was confirmed by western blot. Cell adhesion was measured with readings taken using the SYNERGY multi-mode reader at different time points**. (A)** Cell adhesion after upregulation of LRRC15 in HEC-1A cells. **(B)** and **(C)** show cell adhesion after knockdown of LRRC15 in HEC-155 and Ishikawa cell lines, respectively.

LRRC15 Knockdown Decreases Cell Migration

Cell migration of LRRC15 knockdown endometrial cancer cells was assessed using scratch assays. Ishikawa cells underwent transient LRRC15 knockdown using sh1 and sh2 with sh2 resulting in less LRRC15 expression **(Figure 6C)**. After knockdown, the cells were allowed to grow until fully confluent. Then, a scratch assay was performed. Given that our scratch assay results are hard to evaluate with the naked eye **(Figure 6A)**, we used computational analysis to help assist with calculating percent migration. LRRC15 knockdown using both sh1 and sh2 resulted in a slight decrease in cell migration in the Ishikawa cell line when compared with control although the error bars overlap **(Figure 6B)**. Cell migration assays were not

performed in LRRC15 overexpressing HEC-1A due to its slow proliferation rate when grown in culture.

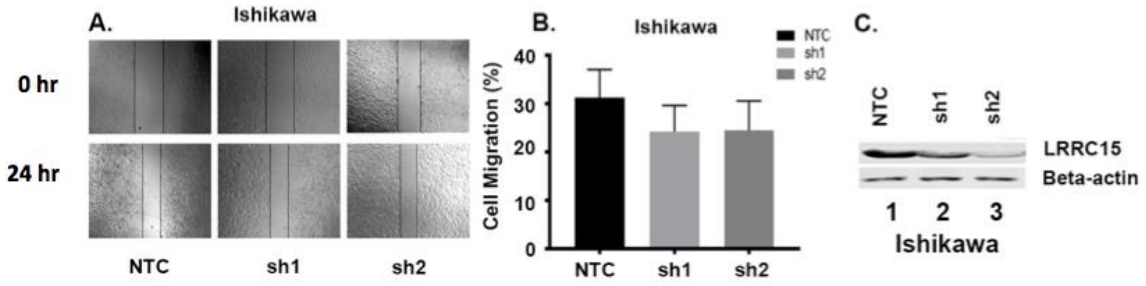

Figure 6. Effect of LRRC15 KD on Ishikawa cell migration. In **(A)** and **(B)** cell migration of Ishikawa cells after LRRC15 knockdown was analyzed using a scratch assay. Migration was measured after 24 hours. **(C)** LRRC15 knockdown in Ishikawa cells was confirmed by Western blot.

ABBV-085 Exhibits High Specificity Towards LRRC15 Expressing Endometrial Cancer Cells

ABBV-085 is a cell permeable drug antibody conjugate that targets cells expressing LRRC15 and is conjugated to the potent anti-mitotic monomethyl auristatin E (MMAE) molecule via a cleavable valine-citrulline linker.[6,56] The linker allows the drug to be stable in the extracellular fluid but become activated by cathepsin B when it enters a tumor cell.[56,57] MMAE cannot be used as a single agent due to its high toxicity and thus must be attached to a monoclonal antibody that will direct it specifically to tumor cells. Specifically, ABBV-085 contains a ratio of two MMAE molecules per antibody known as an E2 conjugation.[6] ABBV-085 uses an LRRC15-specific monoclonal antibody to localize the MMAE payload to the LRRC15-rich stroma and tumor cells where the MMAE payload can diffuse into nearby cancer cells and induce toxic antimitotic effects leading to tumor death.[6] In this study, we decided to test the specificity of ABBV-085 towards LRRC15-expressing SPAC-1L cells. SPAC-1L cells were used for this experiment due to their fast proliferation rate and ability to grow well in culture. In order to test the specificity of ABBV-085, SPAC-1L cells were additionally treated with a set of three controls: Isotype MMAE, Isotype AB, and M25. Isotype MMAE is a drug antibody conjugate between a nonspecific antibody and MMAE. Isotype AB is the monoclonal LRRC15 antibody portion of ABBV-085 and is without MMAE. M25 is simply a nonspecific antibody. SPAC-1L cells were treated with a range of doses (0-100 nM) of ABBV-085, Isotype MMAE, Isotype AB, and M25 for 48 hours. The media was then changed and cell viability was determined at the 72 hour time point using an MTT assay. Our results show that ABBV-085 demonstrates high specificity towards SPAC-1L tumor cells compared to control. ABBV-085 had an IC_{50} between 0.1 nM and 1.0 nM compared

to the IC_{50} of Isotype MMAE which was 1000 nM (data not shown) **(Figure 7)**. Isotype AB and M25 did not decrease cell viability by 50% at doses up to 1000 nM (data not shown).

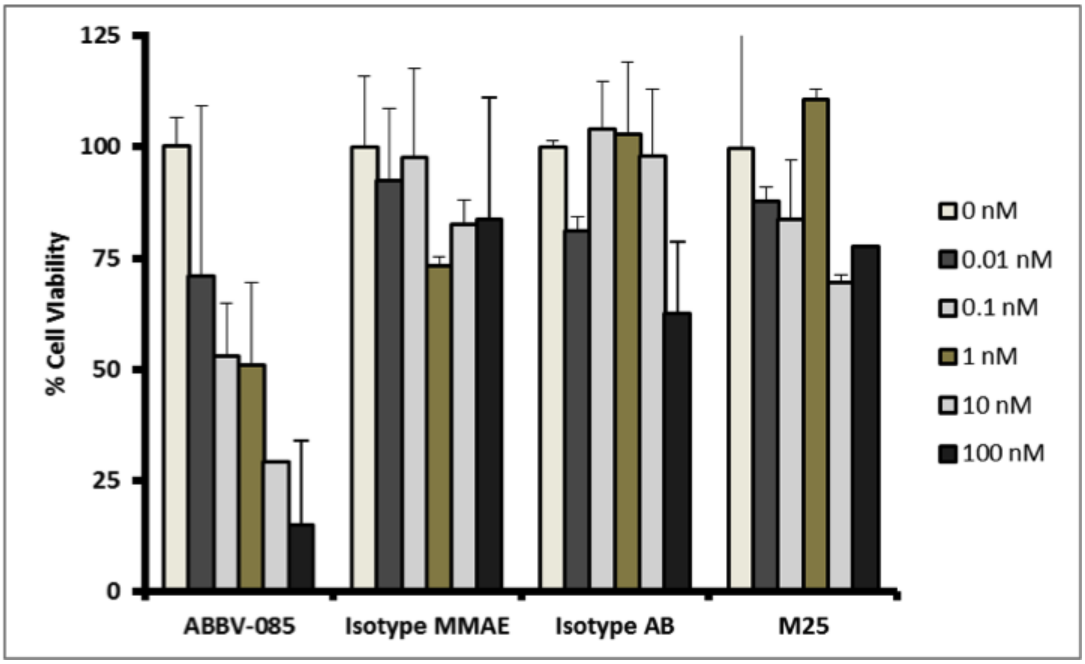

Figure 7. Specificity of ABBV-085 in SPAC-1L cells. SPAC-1L cells were treated with a range of doses of ABBV-085, Isotype MMAE, Isotype AB, and M25 every 48 hours. After 72 hours, cell viability was determined using an MTT assay.

Discussion

LRRC15 expression has been shown to be highly expressed in aggressive tumors and has been found at the leading edge of migrating cells.[6,50,51,52,53,54] We demonstrate that LRRC15 is expressed in both the stroma and cells within uterine serous papillary carcinoma tumors and is expressed in several type I and II endometrial cancer cell lines. This data suggests that LRRC15 may play a role in both types of endometrial cancer. Furthermore, our results indicate that LRRC15 promotes endometrial cancer cell adhesion and migration *in vitro*. We demonstrate that knockdown of LRRC15 in endometrial cancer cell lines results in decreased protein expression of members in the FAK/SRC signaling pathway. We also demonstrate that LRRC15 knockdown decreases cell adhesion and migration in endometrial carcinoma *in vitro*. We suggest further cell migration experiments using a type II endometrial cancer cell line such as HEC155 given we only studied Ishikawa cell migration *in vitro*. In addition, we show that inducing LRRC15 overexpression in HEC-1A cells resulted in an increase in cell adhesion and FAK/SRC signaling. This agrees with previous unpublished work by Shridhar *et al.*

showing that LRRC15 promotes FAK/SRC signaling in ovarian cancer and suggests that LRRC15 may play a role in integrin-mediated adhesion in multiple cancer types.

Shridhar *et al.* also demonstrated that LRRC15 associates with β1-integrin in ovarian cancer, which is something we have yet to investigate in endometrial cancer. Interestingly, endometrial cancer metastasis is associated with a general decline in integrins where the loss of α2β1 integrin was highly associated with lymph node metastasis and the loss of α6β4 integrin was highly associated with increased tumor grade.[58] Conversely, a different study found the presence of αVβ6 to be abundant in 42% of endometrial carcinomas and upregulation of αVβ6 was associated with increased grade and metastasis.[59] A study using HEC-1A, Ishikawa, and AN3CA endometrial cancer cell lines found that the α4β1, α5β1, and α6β1 integrin heterodimers mediate adhesion as well as migration into the artificial matrix matrigel.[60] Therefore, our results demonstrate the therapeutic potential of LRRC15 knockdown in reducing cell adhesion and migration in endometrial cancer cell lines but more work is needed to elucidate protein associations with LRRC15.

Furthermore, work by Purcell *et al.* has shown promising results in using ABBV-085 to treat multiple types of solid tumors.[6] Similarly, our initial experiments using ABBV-085 in the treatment of endometrial cancer *in vitro* have demonstrated that ABBV-085 exhibits high specificity towards the SPAC-1L cell line. We suggest further work should be done using ABBV-085 to treat other endometrial cancer cell lines *in vitro* and *in vivo*. Given that LRRC15 was expressed in both the stroma and the cells of uterine serous papillary carcinoma tumors, ABBV-085 offers exciting promise for delivering its MMAE payload to endometrial cancer tumors.

Conclusion

Overall, our results demonstrate that LRRC15 plays an important role in endometrial cancer cell adhesion and migration. Knockdown of LRRC15 led to a decrease in FAK/SRC signaling, cell adhesion, and migration suggesting that LRRC15 could be a potential therapeutic target for molecular inhibitors that could be utilized to treat endometrial cancer. Furthermore, initial experiments using the drug antibody conjugate ABBV-085 demonstrated high specificity towards an endometrial cancer cell line. Therefore, our results present two possible methods for using LRRC15 as a therapeutic target in endometrial carcinoma.

Materials and Methods

Cell lines

Ishikawa, RL-95, SPAC-1L, HEC155, and HEC-1A endometrial cancer cell lines were provided by Viji Shridhar from the Mayo Clinic in Rochester, MN.

Transfection

Cells were plated in a 6-well plate (300,000 per well) and incubated 24 hours. Transient transfection of LRRC15 knockdown shRNAs (sh1/sh3382 and sh2/sh5675) and upregulation of LRRC15 were then performed. Preparation was carried out in 1.5 ml centrifuge tubes pipetting 2 µg of DNA per well into 300 µl of Opti-mem medium in each tube. Lipofectamine 2000 was added in a 1:2 µl/ µg ratio compared to the concentration of DNA. The tube was vortexed and incubated at room temperature for 20-30 minutes. The media on the cells was changed from normal to Opti-mem and 1 ml of the contents of the tubes was pipetted into a specified well on the 6-well plate. After 6 hours of incubation, the medium was changed back to normal and the cells were allowed to grow. For a given 6-well plate, 2 wells were designated NTC, 2 wells would receive sh1, and 2 wells would receive sh2. All experiments except for the migration assay were started immediately after transfection. For the migration assay, cells were allowed to grow until confluence and the amount of time to confluence varied between the different cell lines.

Migration assay

Cells were allowed to grow until confluent in a 6-well plate. Scratch assays were performed using a micropipette tip. Initial pictures were taken of each scratch using the EVOS FL microscope. After 24 hours, pictures were taken again and differences were calculating using computational analysis.

Cell adhesion

Cell adhesion of LRRC15 knockdown and LRRC15 overexpressing endometrial cancer cells was assessed by cell adhesion assay. 96-well plates were plated with 0.1 mg/ml of collagen and incubated for 24 hours. Next, the 96-well plates were plated with approximately 8,000 cells/well and incubated 24 hours. 10 µM Cell Tracker™ Working Solution was added to the medium and the cells were incubated for 30 minutes at 37 °C. Next, the Cell Tracker™ Working Solution was removed and the medium was changed. Cell adhesion was measured using raw data readings from the SYNERGY multi-mode reader taken at various time points up to five hours. Representative pictures of GFP florescence were taken at the 15 minute and 1 hour

time points using an EVOS FL microscope for when cells adhered they gave off green fluorescence. Three replicates were performed and error bars represent standard deviation of the mean.

Western blotting

Cells were harvested for Western blotting using trypsin and subsequently pelleted. Approximately 100 µl of lysis buffer was added to the pellet and incubated on ice for 30 minutes. Next, the sample was spun in a centrifuge at 4 °C at 13,000 rpm for 10 minutes. The protein concentration was then calculated using a standard. The amounts of lysate, lysis buffer, and 30 µl of 4x loading dye were subsequently determined and measured into an Eppendorf tube. Once in the tube, the contents were mixed and placed on a hot plate for 5 minutes. A gel electrophoresis chamber was set up with running buffer and the gel was loaded with 5 µl of standard in one well and 35 µl of sample in subsequent wells. The gel was run at 150V for one hour. Then the gel was transferred to a PDVF membrane and incubated in 5% blocking milk for one hour. Primary antibody was added to BSA in a 1:1000 ratio. The primary antibody solution was added to the membrane overnight where it incubated at 20 °C. The next morning secondary antibody was added to the blocking milk in a 1:2000 ratio. This secondary antibody solution was placed on the membrane for one hour and then the membrane was imaged using a fluorescent imager. Beta-actin was used as a control.

ABBV-085 Treatment

SPAC-1L cells were plated in 96-well plates with 5,000 cells/well and incubated for 24 hours. Next, the wells were treated with varying concentrations (0-1000 nM) of a respective drug (ABBV-085, Isotype MMAE, Isotype AB, or M25) for 48 hours. The media was then changed back to normal and at the 72 hour time point cell viability was determined by MTT assay.

Statistical Analysis

Limited statistical analysis was performed for our study primarily because the raw data is being stored at the Mayo Clinic, and I do not have direct access to it. The error bars in our bar graphs represent the standard error of the mean of the data and each bar graph was constructed using three replicates. If I were going to perform statistical analysis I would use two-tailed unpaired t-tests and consider p values less than 0.05 as significant.

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