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Lenalidomide suppresses VEGF-dependent angiogenesis and cholangiocarcinoma growth in an *in vivo* mouse model

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ABSTRACT: Cholangiocarcinoma (CCA) is an aggressive cancer of the bile duct and is a major health concern in Thailand. Owing to its heterogeneous nature and clustering of patients in limited resource areas, advancements in treatment options are limited. Our group previously identified that CCA cells derived from Thai patients could release high levels of vascular endothelial growth factor (VEGF). Although these cells responded well to the anti-VEGF therapy bevacizumab, the induction of hypoxia-inducible factor 1 alpha (HIF1α) was a potential concern. Therefore, lenalidomide with potent anti-VEGF and anti-HIF1α activities was selected to test anti-CCA activity. The effects of lenalidomide were investigated both *in vitro* and *in vivo*. Tumor volume, tumor weight, and CD31-immunohistochemistry staining were used to determine tumor growth and angiogenesis. The direct anti-proliferative effects of lenalidomide were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide and colony-forming assays. The cytokines in the conditioned media (CM) of untreated and lenalidomide-treated cells were measured using a cytokine array. VEGF in the CM of cells was confirmed by enzyme-linked immunosorbent assay. The anti-CCA effects of lenalidomide were demonstrated *in vivo*, as evidenced by the reduced tumor volumes, tumor weights, and peritumoral and intratumoral vascularization. Decreased CD31-positive areas in tumors from the lenalidomide-treated group confirmed its antiangiogenic effects. Although lenalidomide directly inhibited VEGF production in CCA cells, it had no direct effect on CCA cell proliferation. The findings suggested that the anti-CCA effect of lenalidomide is VEGF-dependent; moreover, the safety of lenalidomide warrants further investigation. Ultimately, this study proposed lenalidomide as a potential treatment option for CCA.

KEYWORDS: cholangiocarcinoma, lenalidomide, vascular endothelial growth factor, angiogenesis

INTRODUCTION

Cholangiocarcinoma (CCA), originating from the bile duct lining cells, is a leading health problem in the East Asia Region and the Greater Mekong Subregion, including Thailand [1]. The increasing incidence of intrahepatic CCA worldwide has raised global concerns [2]. Owing to its highly heterogeneous nature, with no known pathognomonic signs or specific diagnostic markers, patients are commonly diagnosed at an advanced stage, which further complicates treatment planning [3]. Given the limited treatment options available, which considerably affect the patients' quality of life, early detection methods and improved treatment options are essential to enhance prognosis and outcomes [4, 5].

Targeted therapy is recommended for cancer treatment, including CCA; however, the efficacy of such therapy largely depends on identifying genetic alterations within specific subgroups [6, 7]. CCA cells produce functional vascular endothelial growth factor (VEGF) [8]. The combination treatment of bevacizumab, an antiangiogenic drug, with acetazolamide, a carbonic anhydrase inhibitor, has been proposed for preventing hypoxia-induced adverse effects of anti-VEGF therapy. Lenalidomide is an immunomodulatory imide drug (IMiD) that is routinely used for treating multiple myeloma and myelodysplastic syndrome [9, 10]. The antiangiogenic effects of lenalidomide include inhibition of VEGF production, suppression of endothelial migration, and suppression of vascular formation [11–13]. Modulation of inflammatory cytokine production is another advantage [14–16]. Moreover, VEGF exerts direct growth-inhibitory and apoptosis-inducing effects on cancer cells [17, 18]. Moreover, lenalidomide has shown remarkable potential in suppressing hypoxia-inducible factor 1 alpha (HIF1α) expression and HIF1α-dependent neovascular formation [13]. Hence, this research aimed to investigate the effects of lenalidomide treatment on CCA, keeping in mind its anti-angiogenic-induced HIF1α [8]. The effects of lenalidomide on CCA cell proliferation,

colony formation, and cytokine production were investigated *in vitro*. Anti-CCA growth and angiogenesis were demonstrated using an *in vivo* model. The study findings were anticipated to provide potential insights into the future clinical application of lenalidomide for CCA.

MATERIALS AND METHODS

Cell lines and reagents

KKU-213A and KKU-213B cell lines were derived from a patient with CCA in Thailand [19] and also obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The cells were cultured in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% (v/v) fetal bovine serum (HyClone Laboratories, Inc., Logan, UT, USA), 100 µg/ml streptomycin, and 100 U/ml penicillin in a humidified incubator at 37 °C supplemented with 5% carbon dioxide. Lenalidomide was purchased from Bristol-Myers Squibb (Lawrenceville, NJ, USA).

MTT assay and colony formation assay

The effect of lenalidomide on CCA cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 3×10^3 cells were seeded into a single well of a 96-well plate. After overnight incubation, the cells were treated with 0, 2.5, 5, 10, and 100 µM lenalidomide for 72 h. MTT was added to achieve a 0.5 mg/ml final concentration. Formazan crystals were dissolved in acid isopropanol, and the optical density at 595 nm (OD_{595}) was determined using an iMark microplate reader (Bio-Rad, Hercules, CA, USA). The percentage of cells was calculated from (OD_{595} of treated cells/OD_{595} of untreated cells) ×100. Data from three independent experiments were presented as mean ± SD.

To determine the effect of lenalidomide on colony formation, CCA cells were seeded at a density of 100 cells/well in 12-well plates. Lenalidomide was supplemented to get the final concentration of 1–100 µM, and cells were cultured for an additional 10 days; then, fixed with 4% (v/v) paraformaldehyde. For visualization purposes, 0.5% (w/v) crystal violet was added, and colonies larger than 1 mm were counted. The number of colonies per well was presented as mean ± SD of three independent experiments.

Cytokine secretion

Cytokines in conditioned media (CM) from KKU-213A in untreated and treated with 10 µM lenalidomide conditions were detected using the Human Cytokine Array C3 kit (Ray Biotech, Peachtree Corners, GA, USA). Signals were detected and quantified as previously described [20].

VEGF enzyme-linked immunosorbent assay (ELISA)

VEGF levels in CM obtained from KKU-213A treated with 0, 0.1, 1, and 10 µM lenalidomide were measured using Human VEGF ELISA kit (Invitrogen, Frederick, MD, USA) according to the manufacturer's instructions. The OD_{450} was determined using a microplate reader, and the amount of VEGF in the CM was calculated using a standard curve.

Lenalidomide effects on cell growth and angiogenesis *in vivo*

One million KKU-213A cells were injected subcutaneously into both flanks of BALB/c nude Rag-2/Jak3-deficient (Nude RJ) mice ($n = 10$/group) [21]. Treatment with lenalidomide. resuspended in sterile 0.5% carboxymethyl cellulose at 6.25 mg/ml and administered at 50 mg/kg/mouse/day intraperitoneally, was initiated on day 3 [18]. Tumor volume was calculated as previously described [22]. On day 10, the tumors were harvested, weighed, and cryopreserved in OCT medium (Leica Microsystems, Tokyo, Japan).

The mice were monitored in an institutional animal research facility according to the guidelines and the experimental protocols approved by the Kumamoto University Institutional Animal Care and Use Committee, Kumamoto, Japan.

Immunohistochemistry staining of CD31 angiogenic marker

Immunohistochemistry was performed as previously described [8]. The Vectastain Elite ABC standard kit (Vector Laboratories, Burlingame, CA, USA) and the Histofine® DAB substrate kit (Nichirei Bioscience, Tokyo, Japan) were used for signal amplification and detection. The following antibodies were used: anti-CD31/PECAM-1 (MEC13.3) from Biolegend (San Diego, CA, USA) and biotinylated goat anti-rat IgG from Vector Laboratories.

CD31-positive areas were captured using a BZ-8100 Biozero fluorescence microscope (Keyence, Osaka, Japan) (8–10 fields/sample using a 20 × objective lens). Positive signals were quantified using a BZ-II Analyzer (Keyence). The data were presented as percentages of CD31-stained areas [8].

Statistical analysis

Student's t-tests were performed using GraphPad Prism software version 8 (GraphPad Software, Inc., San Diego, CA, USA) to determine statistical differences between groups. A p-value < 0.05 indicated statistical significance.

RESULTS

Effects of lenalidomide on CCA growth and suppression of angiogenesis *in vivo*

It was previously demonstrated that CCA cells exhibit angiogenesis-promoting properties through the production of VEGF [8], and the significant possible adverse effects of the antiangiogenic treatment, bevacizumab, are hypoxia-related. The strong antiangiogenic effects of lenalidomide and its superior endothelial suppressive effects under hypoxic conditions highlighted the need to investigate its anti-CCA effects. KKU-213A CCA cells were subcutaneously xenografted into the Nude RJ mice. Lenalidomide was initiated on day 3 after CCA transplantation, when the tumors were measurable. Lenalidomide was intraperitoneally injected once daily at a dose of 50 mg/kg/day for 1 week. The experiment was terminated owing to the substantial effects of lenalidomide on mouse body weight (Fig. 1A) and tumor growth (Fig. 1B,C). In the lenalidomide-treated group, the body weights decreased by approximately 20% (23.2±1.4 g on day 0 vs. 21.2±1.3 g on day 10, $p < 0.05$), and the tumor volumes and tumor weights were reduced by approximately 60–65% (tumor volumes: 154.6±79.5 mm^3 in the control vs. 56.1±32.5 mm^3 in the treated group, $p < 0.01$; tumor weights: 80.2±36.3 mg vs. 30.0±13.8 mg, $p < 0.01$).

To confirm the potent antiangiogenic effects of lenalidomide, peritumoral and intratumoral vascularization were determined. Reduced vascularization was observed grossly and microscopically (Fig. 1D–F). The percentage of the intra-tumoral CD31-positive area was significantly lower in the lenalidomide-treated group than in the control group (Fig. 1F, 10.9±2.5% in the treated group vs. 4.6±0.8% in the control group, $p < 0.01$).

Effects of lenalidomide on CCA cell growth

Lenalidomide exerts a direct effect on cell growth in colorectal cancer cells [18]. Therefore, the direct growth-inhibitory effect of lenalidomide was analyzed using the MTT assay. Two patient-derived CCA cell lines, KKU-213A and KKU-213B, were selected as the models. The results demonstrated that lenalidomide at 2.5 and 100 µM statistically reduced KKU-213A cell numbers but no statistically differences were detected when cells were treated with 5 and 10 µM lenalidomide. No statistically alterations in KKU-213B cell numbers were detected. Owing to the minimal changes observed in lenalidomide-treated KKU-213A and no dose-dependent effects observed, the authors concluded that lenalidomide had no significant direct effect on the proliferation of CCA cells (Fig. 2A). A colony formation assay was performed to confirm the MTT results. KKU-213A and KKU-213B cells were treated with 1–100 µM lenalidomide for 10 days. After fixation and staining, the colony numbers were compared between the groups. The results showed that lenalidomide did not affect the efficiency of CCA colony formation (Fig. 2B).

Effects of lenalidomide on VEGF production in CCA cells

Lenalidomide exhibits cytokine modulatory effects [14–16]. The anti-CCA properties, mainly antiangiogenesis, of lenalidomide have been demonstrated in a mouse model. Therefore, the effect of lenalidomide on cytokine production in KKU-213A CCA cells was evaluated using a cytokine array. Of the 42 cytokines analyzed, lenalidomide reduced the levels of 16 cytokines ($\leqslant$ 0.8 folds) and increased those of two cytokines ($\geqslant$ 1.2 folds). Reductions in angiogenin (ANG), epidermal growth factor (EGF), growth-regulated alpha protein (GROa), insulin-like growth factor I (IGF-1), Interleukin-(IL)-1β, IL-2, IL-13, IL-15, leptin, monocyte chemoattractant protein (MCP)-2, MCP-3, platelet-derived growth factor (PDGF), stem cell factor (SCF), stromal cell-derived factor-1 (SDF-1), transforming growth factor (TGF)-β1, and VEGF, and inductions in macrophage colony-stimulating factor (M-CSF) and macrophage inflammatory protein (MIP)-1δ were observed (Fig. 3A,B).

Because the antiangiogenic effects of lenalidomide were of prime concern, the direct effects of lenalidomide on VEGF production in CCA cells were performed using VEGF ELISA. The KKU-213A CCA cells were treated with 0.1–10 µM lenalidomide. The results revealed that lenalidomide suppressed VEGF production and secretion by KKU-213A cells in a dose-dependent manner (Fig. 3C).

DISCUSSION

CCA is a significant health concern in Thailand. Given its diverse clinical and histological presentations and genetic backgrounds, diagnosis and treatment of CCA remain challenging [4, 5, 7, 23]. Radical surgical intervention is considered the mainstay curative treatment for CCA; however, the number of candidates eligible for curative outcomes are limited [24]. Therefore, alternative therapies for CCA are urgently needed. There have been several efforts to discover practical solutions; nonetheless, the outcomes have been inappreciable [4, 5]. Our previous study reported the production and the secretion of VEGF from cholangiocarcinoma cells and the benefits of anti-VEGF treatment in suppressing cancer growth and angiogenesis [8]. However, hypoxia-induced adverse effects remain a substantial concern. Therefore, lenalidomide, previously demonstrating antiangiogenic effects under both normoxic and hypoxic conditions [13], was considered a potential candidate for CCA treatment. The anti-CCA effects of lenalidomide were demonstrated using an *in vivo* xenograft model. Inhibition of neovascularization primarily contributed to its tumor-suppressive effects

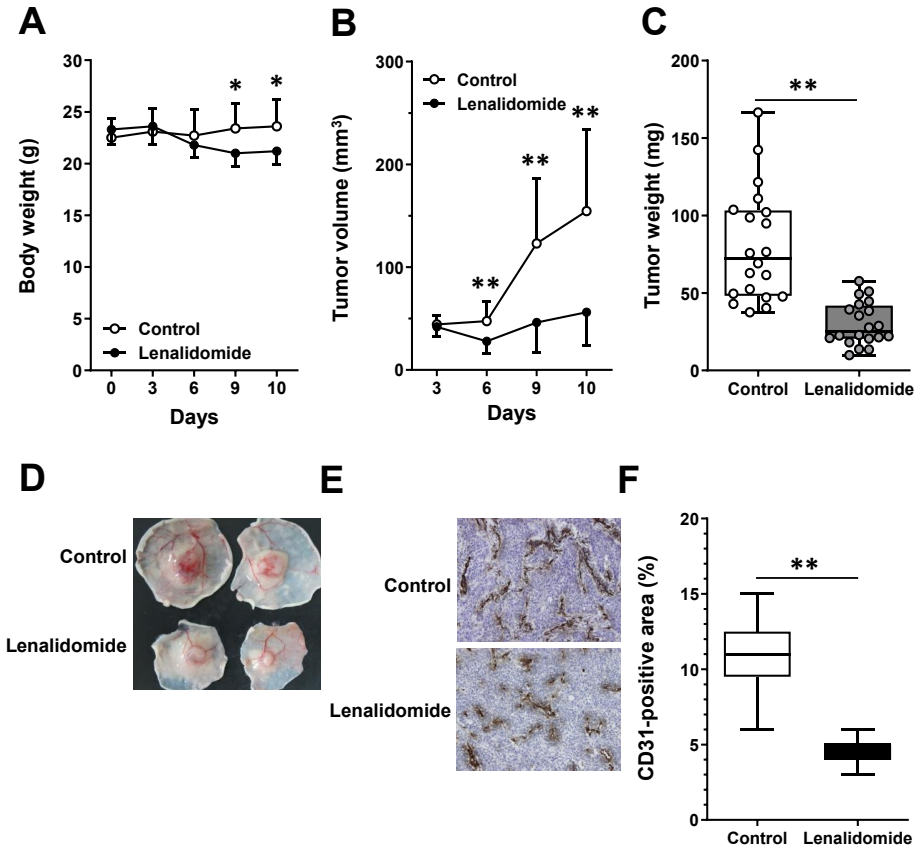

Fig. 1 Effects of lenalidomide treatment on cholangiocarcinoma (CCA) growth in the xenotransplanted mice. (A), Mouse body weights; (B), tumor volumes; (C), tumor weights, each dot representing an individual sample; (D), gross observation of peritumoral vascularization; (E), immunohistochemistry staining for evaluating the CD31-stained area; (F), percentage of CD31-positive area. Means ± standard deviation (SD) was shown in 1A and 1B. Box and whisker plots with the minimum to maximum ranges were used in 1C and 1F. * $p < 0.05$, ** $p < 0.01$.

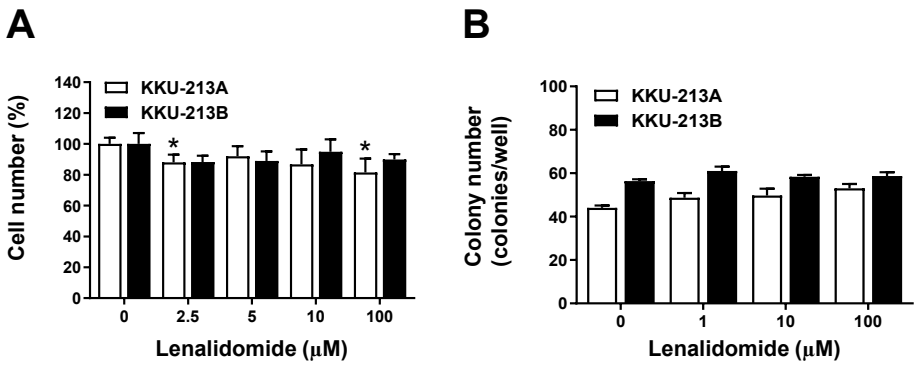

Fig. 2 Effects of lenalidomide on CCA cell proliferation. (A), Anti-proliferative effects of lenalidomide on two CCA cell lines; (B), colony-forming ability of lenalidomide-treated and untreated cells. The data were presented as means ± SD from three independent experiments. * $p < 0.05$.

with no significant effects on CCA cells or colony numbers. Moreover, lenalidomide treatment had a dose-dependent effect on VEGF production and secretion. Collectively, the results of the current study suggested the potential use of IMiD, lenalidomide, as an antiangiogenic agent for CCA treatment.

Lenalidomide substantially affects solid tumor growth and metastatic potential in colorectal and lung cancers [17, 18]. The direct effects of lenalidomide on cell growth were not observed in CCA, as comparably

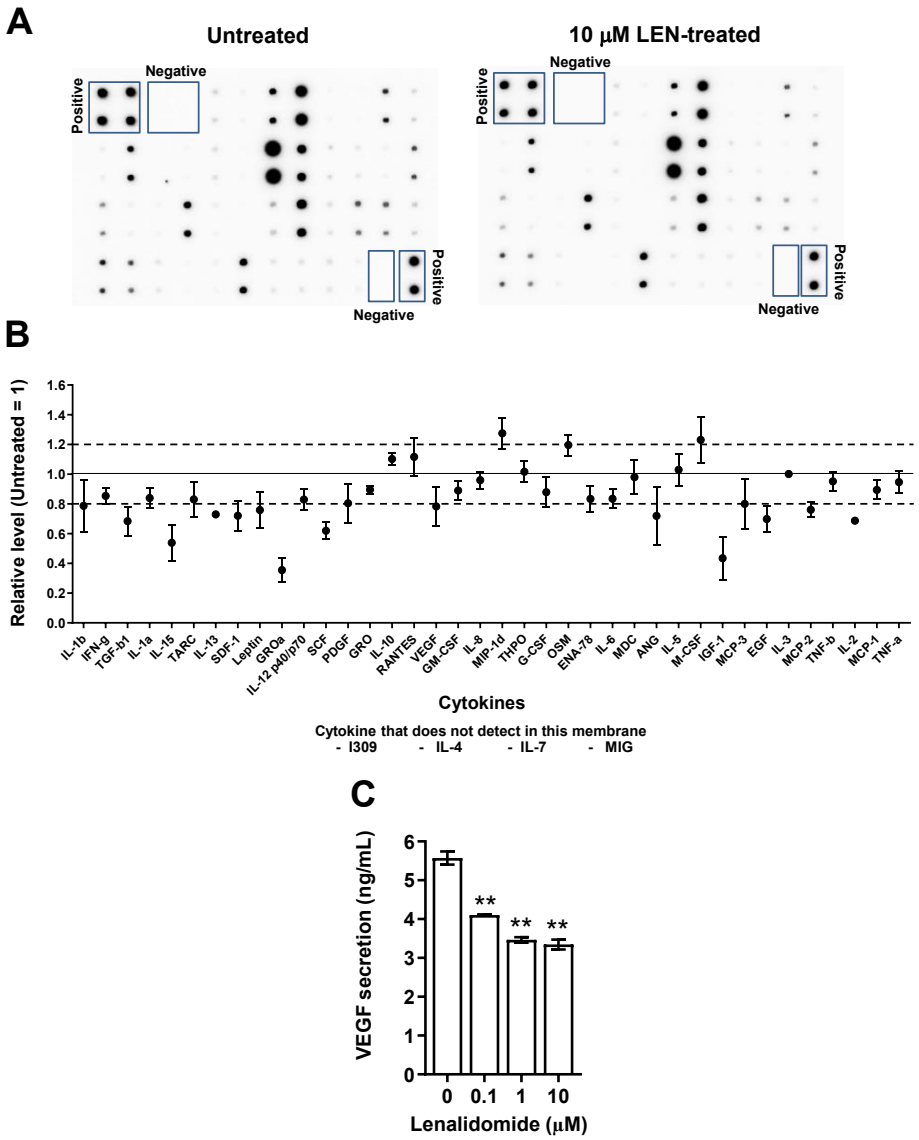

Fig. 3 Effects of lenalidomide treatment on CCA cytokine production. (A), Releases of cytokines in untreated and lenalidomide-treated (LEN-treated) conditions with Positive = positive control spots and Negative = negative control spots; (B), altered cytokines in lenalidomide-treated conditions compared with the untreated controls, with solid lines representing equal density between the two groups, and dash lines representing the range of cytokines higher ($\geqslant$ 1.2-fold) and lower ($\leqslant$ 0.8-fold) levels; (C), VEGF secretion levels in CCA-CM. Data were presented as means ± SD. ** $p < 0.01$.

observed in triple-negative breast cancer [25]. Hence, lenalidomide treatment had minimal effects on breast cancer cell proliferation and apoptosis induction but potentiates the anti-breast cancer effects of cisplatin.

Because the direct effects of lenalidomide on CCA cell proliferation were not observed in this study, its immunomodulatory effects were focused, particularly via alterations in cytokine production. Decreases in ANG, EGF, GROa, IGF-1, IL-1β, IL-2, IL-13, IL-15, leptin, MCP-2, MCP-3, PDGF, SCF, SDF-1, TGF-β1, and VEGF; but increases in M-CSF and MIP-1δ were identified in the study. Alterations in IL-1β, IL-6, and IL-10 from lipopolysaccharide-induced peripheral blood mononuclear cells and suppression of VEGF production from hepatocellular carcinoma have been reported [11, 14]. Our findings elucidated a consistent reduction in IL-1β and VEGF levels. Although a slight reduction in IL-6 and minimal induction of IL-10 were observed in our model, these changes did not meet the predefined cut-off values for this study ($\leqslant$ 0.8- or $\geqslant$ 1.2-fold). However, the functional involvement of other altered cytokines remains to be elucidated; and

a suitable CCA model that incorporates the influence of the tumor microenvironment (TME) could offer a more comprehensive understanding of this complex disease. Notably, the lenalidomide dose used in the current study (50 mg/kg/day) was based on a previous report [18], which resulted in considerable side effects in mice, highlighting the need for dose optimization or schedule adjustments to mitigate these adverse effects [26]. It's worth noting that the current study had some limitations that should be taken into consideration. The immune cells in the *in vivo* model were compromised; thus, the effects of lenalidomide on CCA and immune cells in TME could not be evaluated. In addition, the subcutaneous transplantation of CCA cells did not reflect the natural environment, and the effects of tumor regression could not be accurately measured [27].

In summary, our findings elucidated the antiangiogenic effects of lenalidomide on CCA cells, mainly via VEGF production and secretion. These findings advocated the use of lenalidomide for CCA treatment with additional study on dosage optimization.

CONCLUSION

This study demonstrated the VEGF-dependent antiangiogenic effects of lenalidomide on CCA cells *in vivo* on the reduction of VEGF production and secretion and suggested using lenalidomide as an immunomodulatory imide drug (IMiD) for CCA treatment.

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