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Lemur tail kinase 3 serves as a predictor of patient outcomes and a target for the treatment of ovarian cancer

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Lemur tail kinase 3 (LMTK3) belongs to a family of tyrosine kinases that are known to correlate with tumor grade and patient survival in some cancers. Here, we validated LMTK3 as a specific target and a prognostic biomarker in ovarian cancer (OC). In samples from 204 stage I–II OC patients, immunohistochemical studies revealed a higher cytoplasmic-to-nuclear staining intensity of LMTK3, which correlated with worse overall survival ($p < 0.001$). Efficacy studies utilizing novel LMTK3 binding peptides (LMTK3BPs) showed that all chemosensitive and chemoresistant OC cells were killed without affecting normal cells ($p < 0.005$), with synergistic effects shown following cisplatin and docetaxel treatment. In an orthotopic xenograft mouse model of OC, we saw a 35% tumor reduction in response to intravenous injections of 2 mg/kg LMTK3BP given three times a week for 3 weeks. Furthermore, *in vivo* safety studies showed no signs of toxicity after LMTK3BP treatment, even at doses as high as 40 mg/kg. This study highlights LMTK3 as a predictor of patient clinical outcomes. More importantly, novel LMTK3BPs represent potential safe treatment options, either alone or in combination with therapies, for OC.

INTRODUCTION

Epithelial ovarian cancer is a deadly gynecological cancer that is currently regarded as one of the most common cancer types among women. Due to a lack of early detection biomarkers, it is generally diagnosed at an advanced stage, and it also carries a high risk of recurrence after standard treatment, which consists of surgery and chemotherapy.[1] Unlike other cancers, the survival rate of epithelial ovarian cancer has only improved modestly over the last three decades.[2] Targeted therapies hold the potential to revolutionize the treatment of ovarian cancer and improve patient outcomes. Several potential targeted therapies have been introduced in the clinic, but these have yet to be proven beneficial for all patients.[3] Thus, there is an unmet need to identify new targets to bring novel effective therapies to the clinic.[3]

Lemur tail kinase 3 (LMTK3) has gained attention recently due to the growing evidence of its involvement in many cancers.[4–7] LMTK3 belongs to a family of regulated serine/threonine tyrosine kinases with three structurally related isoforms: LMTK1, LMTK2, and LMTK3. These are localized in the nucleus, cytoplasm, transmembrane, and extracellular space. Both nuclear and cytoplasmic LMTK3 expression correlates with tumor grade and patient survival.[8] Although LMTK3 is not a well-characterized or intensely studied protein, it has been linked previously to several different types of cancers, both as a diagnostic marker (particularly when secreted) and as a potential druggable target.[4] LMTK3 expression was found to be significantly elevated in human non-small cell lung cancer, thus making it a reliable biomarker to screen and predict progression for this deadly cancer.[5] Likewise, serum LMTK3 is a valuable biomarker for predicting the progression and prognosis of colorectal cancer, and polymorphisms in LMTK3 have been shown to serve as prognostic factors for gastric cancer.[5,6]

LMTK3 has also been reported as a therapeutic target for treating cancer patients with type III transmembrane receptor tyrosine kinase KIT mutations, thyroid cancer, and breast cancer. LMTK3 has been linked to endocrine resistance through estrogen receptor α (ERα) activity in breast cancer.[9–13] Specifically, increased levels of LMTK3 have been shown to affect the transcription of genes promoting DNA repair, cell viability, and tumorigenesis in the breast cancer

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cell line MCF-7.[14,15] These studies highlight the potential role of LMTK3 in tumor initiation and progression and the prospect of using it as a diagnostic and therapeutic target. The present study investigates the clinical significance of LMTK3 as a prognostic biomarker of clinical outcomes in ovarian cancer patients and a potential target for safe treatment options. More importantly, we designed LMTK3 binding peptides that specifically target LMTK3. These peptides show potential to be safe treatment options, either alone or in combination with other therapies, for ovarian cancer.

RESULTS

LMTK3 as a prognostic biomarker of clinical outcome

The immunohistochemistry (IHC) results obtained from ovarian cancer patient specimens showed that LMTK3 protein was localized within the nucleus and cytoplasm of tumor cells, with a high percentage of samples (98.5%) showing LMTK3 positivity across all histotypes. Notably, most tumors (67.5%) exhibited strong nuclear LMTK3 staining (Table 1). Clear-cell carcinomas (CCCs) had the lowest proportion of strong nuclear LMTK3 staining (33.3%), while mucinous carcinomas (MCs) had the highest (85.7%). Cytoplasmic staining displayed a similar but less pronounced distribution. Approximately 50.8% of tumors displayed strong cytoplasmic staining, with CCCs exhibiting the lowest degree of strong staining (8.3%) and endometroid carcinomas (EC) showing the highest degree (71.7%). Furthermore, there was a positive correlation between cytoplasmic and nuclear LMTK3 staining (rho = 0.70, $p < 0.001$). Most patients had equivalent nuclear and cytoplasmic staining intensities (69.5%), while 22.3% had stronger nuclear than cytoplasmic staining, and only 8.1% exhibited stronger cytoplasmic staining than nuclear staining.

LMTK3 staining was not significantly correlated with age (nuclear: rho = −0.06, $p = 0.42$; cytoplasmic: rho = −0.003, $p = 0.97$), stage (nuclear, $p = 0.87$; cytoplasm, $p = 0.64$), ploidy (nuclear, $p = 0.74$; cytoplasm, $p = 0.86$), or CA-125 levels (nuclear, $p = 0.54$; cytoplasm, $p = 0.91$). Similarly, the balance between nuclear and cytoplasmic LMTK3 staining did not correlate significantly with age ($p = 0.27$), stage ($p = 0.83$), ploidy ($p = 0.67$), or CA-125 levels ($p = 0.99$). Consequently, it was concluded that LMTK3 staining, particularly in the nucleus, exhibited strong intensity and varied across histotypes but not with patient age or tumor stage.

In univariable analysis, nuclear LMTK3 was associated with favorable prognosis (hazard ratio [HR] 0.71, 95% confidence interval [CI] 0.58–0.88, $p = 0.002$) (Table 2A), while cytoplasmic LMTK3 showed no significant prognostic association (HR 0.85, 95% CI 0.67–1.08, $p = 0.190$). Notably, the balance between cytoplasmic and nuclear LMTK3 staining was prognostic, with patients exhibiting stronger cytoplasmic than nuclear staining having a higher risk of death (HR 3.02, 95% CI 1.53–5.94, $p = 0.001$) (Figure 1; Table 2A). In this group, 75% of patients died of ovarian cancer within the first 5 years after diagnosis. Multivariable analysis was subsequently performed, incorporating nuclear intensity, cytoplasmic intensity, histotype, stage, and age to assess the independent prognostic effects of LMTK3 (Table 2B). The favorable prognostic impact of nuclear LMTK3 in the univariable analysis persisted; however, cytoplasmic LMTK3 did not exhibit statistical significance. The unfavorable prognosis associated with patients exhibiting higher cytoplasmic than nuclear LMTK3 remained significant in the multivariable analysis. In conclusion, nuclear LMTK3 emerged as an independent, prognostically favorable factor, while cytoplasmic staining, especially in the absence of strong nuclear staining, predicted an adverse prognosis (Figure 1). Patients with a higher proportion of cytoplasmic LMTK3 relative to nuclear LMTK3 faced a notably elevated risk of death during the first 1–3 years following diagnosis.

LMTK3-specific binding peptide discovery

We recently identified LMTK3 as the cross-target of the CD11b antibody, which we previously reported to exhibit a remarkable antitumorigenic effect in ovarian cancer cells without affecting normal cells *(16)*. Epitope mapping of CD11b monoclonal antibodies was performed using the Immunosignatures technique.[16] Three different human CD11b antibodies and their respective isotype controls (ab52478 and ab8878, Abcam; MA1-19004, Life Technologies, Carlsbad, CA, USA) were analyzed with a microarray of 330,000 random sequence peptides, as described previously.[16] The pattern of binding to these peptides is the immunosignature of the epitope. Correlation across replicates that was less than R = 0.90 was reprocessed, and analysis was performed using the averaged values from the replicates. Each peptide microarray can detect a 1.21-fold difference in signal on average (alpha = 0.05, beta = 0.20, $N = 3$ replicates), on par with commercial gene expression microarrays, with 100% accuracy in monoclonal antibody classification regardless of the monoclonal antibody tested. The output from the epitope mapping provided 500 sequences ranging in size from 8 to 15 amino acids.

An NCBI protein BLAST search was performed for each peptide sequence identified by epitope mapping, and proteins resulting in an 80%–100% match were selected to form a list of candidate targets. Common epitopes were identified by subtraction of the isotype control epitope sequences. Candidate protein targets were then cross-referenced with the proteins identified by mass spectrometry, leading to the identification of LMTK3. This resulted in the identification of 20 different LMTK3 binding peptides (LMTK3BPs) that range in size between 11 and 12 amino acids. In this study, we tested the safety and efficacy of LMTK3BP #1 and #2, since they displayed the highest affinity for LMTK3. The sequences of the other 18 LMTK3BP are not shown (patent disclosure). Ongoing studies are investigating the safety and tumor cell killing efficacy of these peptides, either alone or in combination with each other.

LMTK3 as a therapeutic target

We utilized the MTT assay for testing the killing efficacy of targeting LMTK3 by a monoclonal antibody, small interfering RNA, and LMTK3BP in four ovarian cancer cell lines (SKOV3, MDAH-2774, A2780, and TOV-21G). Targeting LMTK3 with our novel LMTK3BP #1 or #2 and/or with an LMTK3 antibody induced cell killing in commercially available chemosensitive ovarian cancer cell

Table 1. Distribution of the nuclear and cytoplasmic LMTK3 in the whole cohort and among different histotypes

Variables	CCC	EC	HGSC	MC	Total	*p* Value
Nuclear intensity						
no staining	2 (5.6%)	0 (0%)	1 (1.1%)	0 (0%)	3 (1.5%)	–
low intensity	11 (30.6%)	3 (6.5%)	5 (5.7%)	2 (7.1%)	21 (10.7%)	–
medium intensity	11 (30.6%)	11 (23.9%)	16 (18.4%)	2 (7.1%)	40 (20.3%)	–
high intensity	12 (33.3%)	32 (69.6%)	65 (74.7%)	24 (85.7%)	133 (67.5%)	<0.001
Cytoplasmic intensity						
no staining	0 (0%)	0 (0%)	3 (3.4%)	0 (0%)	3 (1.5%)	–
low intensity	10 (27.8%)	0 (0%)	4 (4.6%)	3 (10.7%)	17 (8.6%)	–
medium intensity	23 (63.9%)	13 (28.3%)	31 (35.6%)	10 (35.7%)	77 (39.1%)	–
high intensity	3 (8.3%)	33 (71.7%)	49 (56.3%)	15 (53.6%)	100 (50.8%)	<0.001
Nuclear versus cytoplasmic intensity						
ncl>cyto	11 (30.6%)	1 (2.2%)	22 (25.3%)	10 (35.7%)	44 (22.3%)	–
ncl = cyto	18 (50%)	41 (89.1%)	61 (70.1%)	17 (60.7%)	137 (69.5%)	–
ncl<cyto	7 (19.4%)	4 (8.7%)	4 (4.6%)	1 (3.6%)	16 (8.1%)	<0.001
Nucleus (%)						
0%–30%	5 (13.9%)	1 (2.2%)	5 (5.7%)	1 (3.6%)	12 (6.1%)	–
40%–70%	3 (8.3%)	1 (2.2%)	2 (2.3%)	2 (7.1%)	8 (4.1%)	–
80%–100%	28 (77.8%)	44 (95.7%)	80 (92%)	25 (89.3%)	177 (89.8%)	0.15
Cytoplasm (%)						
0%–30%	2 (5.6%)	0 (0%)	3 (3.4%)	0 (0%)	5 (2.5%)	–
40%–70%	3 (8.3%)	0 (0%)	2 (2.3%)	0 (0%)	5 (2.5%)	–
80%–100%	31 (86.1%)	46 (100%)	82 (94.3%)	28 (100%)	187 (94.9%)	0.14
Stage						
stage I	30 (83.3%)	32 (69.6%)	49 (56.3%)	21 (75%)	132 (67%)	–
stage II	6 (16.7%)	14 (30.4%)	38 (43.7%)	7 (25%)	65 (33%)	0.02
Ploidy						
aneuploid	29 (85.3%)	26 (60.5%)	65 (77.4%)	18 (72%)	138 (70.1%)	–
near diploid	5 (14.7%)	17 (39.5%)	19 (22.6%)	7 (28%)	48 (24.4%)	–
N/A	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.08
CA125						
<35	13 (36.1%)	13 (28.9%)	17 (19.5%)	10 (35.7%)	53 (26.9%)	–
35–65	8 (22.2%)	7 (15.6%)	17 (19.5%)	8 (28.6%)	40 (20.3%)	–
>65	15 (41.7%)	25 (55.6%)	53 (60.9%)	10 (35.7%)	103 (52.3%)	–
N/A	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.16
Chemotherapy						
yes	0 (0%)	4 (8.7%)	3 (3.4%)	2 (7.1%)	9 (4.6%)	–
no	36 (100%)	42 (91.3%)	84 (96.6%)	26 (92.9%)	188 (95.4%)	–
not available	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.21

Fisher's exact test. CCC, clear cell carcinoma; EC, endometroid carcinoma; HGSC, high-grade serous carcinoma; MC, mucinous carcinoma.

lines (A2780, SKOV-3, and TOV-21G) and their chemoresistant counterparts but not in normal surface epithelial ovarian cells (Figure 2A). Furthermore, cell killing was synergistic with cisplatin and docetaxel treatment (Figure 2B). To demonstrate the specificity of LMTK3 as a target, we utilized LMTK3-specific siRNA; silencing LMTK3 gene expression led to a dose-dependent decrease in cell viability in all ovarian cancer cell lines tested but not in normal epithelial ovarian cells (Figure 3A). Commercially available LMTK3

Table 2. Univariable analysis of LMTK3 expression in ovarian cancer patients

Variable[a]		HR (95% CI)	*p* Value
Nuclear intensity		0.71 (0.58–0.88)	0.002
Cytoplasmic intensity		0.85 (0.67–1.08)	0.190
Cytoplasmic vs. nuclear LMTK3	cytoplasm < nuclear	1.0 (reference)	–
	cytoplasm = nuclear	1.13 (0.72–1.79)	0.598
	cytoplasm > nuclear	3.02 (1.53–5.94)	0.001
Histotype	HGSC	1.0 (ref)	
	EC	0.48 (0.29–0.81)	0.006
	CCC	0.90 (0.55–1.47)	0.675
	MC	0.69 (0.39–1.22)	0.202
Stage	I	1.0 (reference)	–
	II	1.37 (0.94–1.99)	0.104
Age (years)		1.03 (1.01–1.04)	0.001
Variable[b]			
Nuclear intensity		0.55 (0.38–0.79)	0.001
Cytoplasmic intensity		1.40 (0.94–2.07)	0.097
Histotype	HGSC	1.0 (reference)	–
	EC	0.44 (0.26–0.75)	0.003
	CCC	0.75 (0.42–1.33)	0.323
	MC	0.84 (0.47–1.48)	0.541
Stage	I	1.0 (reference)	–
	II	1.58 (1.05–2.38)	0.027
Age (years)		1.03 (1.01–1.04)	0.001

CCC, clear cell carcinoma; CI, confidence interval; EC, endometroid carcinoma; HGSC, high-grade serous carcinoma; HR, hazard ratio; MC, mucinous carcinoma.
[a]Univariable analysis of the prognostic effect of nuclear and cytoplasmic LMTK3 on overall survival within 10 years from diagnosis.
[b]Multivariable analysis of the prognostic effect of nuclear and cytoplasmic LMTK3 on overall survival within 10 years from diagnosis.

ELISA revealed levels of LMTK3 protein in the lysate of SKOV3, A2780, TOV-21G, and TOV112D cell lines (Figure 3B). Caspase-3 activity was significantly increased in response to LMTK3BP #1 and #2 treatment as compared to control treatment ($p < 0.005$; Figure 3C).

To determine binding affinity (Kd) for LMTK3BP, we utilized the Colorimetric Biotin Assay Kit to detect amino terminus biotin-labeled LMTK3BP #1. A plot was constructed for the relationship between increasing concentrations of total protein isolated from the A2780 ovarian cancer cell line with a fixed amount of labeled peptide. The Kd was extrapolated from the tangents of the highest and lowest point of the graph, giving an estimated Kd of 8.9×10^{-5} mM (Figure 4A). LMTK3 biotin-labeled peptide #1 was also shown to bind LMTK3 *in situ*, as indicated by the orange color, which results from the merge between the LMTK3 antibody (red) and LMTK3 biotin-labeled peptide (green; Figure 4B). To test whether LMTK3BP #1 specifically binds to LMTK3, total protein was extracted from two ovarian cancer cell lines (SKOV-3 and TOV-21G). The biotin-avidin detection system was utilized to visualize the resulting band (Figure 4C). A single band of 153 kDa was detected when hybridized with a LMTK3-specific antibody or with LMTK3BP #1 (Figure 4C).

Safety and efficacy of LMTK3BP *in vivo*

The safety of LMTK3BP #1, #2, and #3 was tested in athymic mice administered 2, 10, or 40 mg/kg by intravenous (i.v.) injection 3 times/week for 3 weeks. Additionally, an intraperitoneal injection of 2.5 mg/kg anti-LMTK3 antibody was tested for 7 days in athymic mice. Total body weight, liver and spleen weight, and general mouse behavior were monitored during treatment. Weight loss of >20% of the total body weight and any sign of suffering were the ethical endpoint criteria of the experiment and were not reached in any case. Hematoxylin and eosin sections of the brain, cerebellum, kidney, adrenal gland, heart, lungs, esophagus, stomach, small intestine, liver, pancreas, spleen, ovaries, fallopian tubes, and uterus of the treated mice showed a normal histological patterns (data not shown). No signs of toxicity were detected at any level (Figures 5A and 5B). We then used the A2780 cell line-derived orthotopic xenograft model of ovarian cancer to test the efficacy of LMTK3BP #1 and #2 (peptide #3 as a negative control) *in vivo*. Treatment at a dose of 2 mg/kg i.v. 3 times/week for 3 weeks (Figure 5C) resulted in a 24% and 35% tumor reduction for LMTK3BP #1 and #2, respectively, compared to the control ($p = 0.006$). There was no significant difference in tumor reduction between peptide #3 (negative control) and the vehicle control (Figure 5C).

Proposed mechanism

There was a significant increase in caspase-3 activity when ovarian cancer cells were treated with 10 μg/mL of LMTK3BP #1 and #2 (Figure 4B), suggesting an apoptosis-dependent mechanism. Additionally, recombinant LMTK3 protein treatment of ovarian cancer cells increased the expression of a novel ovarian cancer integrin αV and β1 in a dose-response manner (Figure 6A). We have recently reported the expression and dimerization of integrin αV and β1 subunits in ovarian cancer tissues and cells.[17] The proposed mechanism of survival in epithelial ovarian cancer cells is depicted in Figure 6B.

DISCUSSION

Ovarian cancer is one of the most common cancer types affecting women, posing significant challenges due to the lack of reliable biomarkers for early detection and the development of chemoresistance during treatment. The role of LMTK3 in the pathogenesis of ovarian cancer has been the focus of our *in vitro* and *in vivo* investigations. This study aimed to validate LMTK3 as a specific target and a prognostic indicator for clinical outcomes in ovarian cancer. Here, we demonstrated the presence of the LMTK3 protein in more than 98% of ovarian cancer tissues collected from a cohort of 204 early-stage (stage I–II) ovarian cancer patients representing diverse histological profiles. In normal unprovoked cells, LMTK3 is expressed in the nucleus. When cells experience stress, they release nuclear LMTK3 into the cytoplasm, cell membrane, and/or extracellular space. Our study found LMTK3 protein to be localized within both the nucleus and cytoplasm of tumor cells. Furthermore, our findings

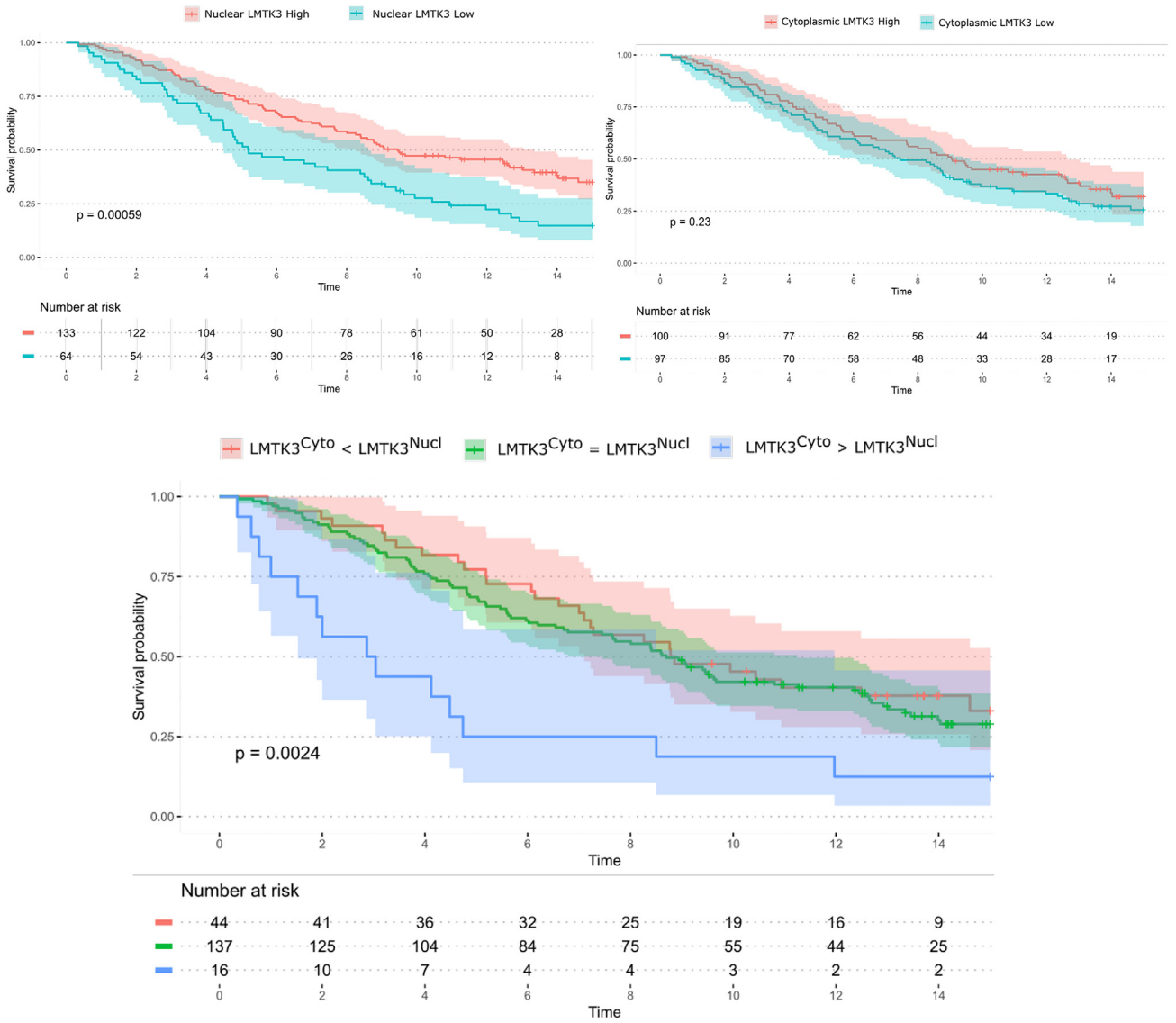

Figure 1. LMTK3 expression as a predictor of overall survival

Nuclear, cytoplasmic, and cytoplasmic:nuclear LMTK3 ratios were established through IHC staining of LMTK3 in 197 human ovarian carcinomas from various stages and histotypes. High cytoplasmic LMTK3 staining significantly correlated with poor prognosis. Significance was determined by one-way ANOVA testing ($p < 0.05$).

revealed a pronounced elevation in the risk of death among ovarian cancer patients who exhibited higher cytoplasmic LMTK3 levels than nuclear LMTK3 levels, particularly within the first few years following diagnosis. Intriguingly, cytoplasmic LMTK3 expression strongly correlated with poorer survival outcomes. Our observations are consistent with previous reports showing a correlation between both nuclear and cytoplasmic LMTK3 expression, tumor grade, and patient survival in breast and colorectal cancer.[6,12,18]

Discovering potent and selective inhibitors of LMTK3 to pharmacologically validate LMTK3 as a novel target for ovarian cancer therapy will have an important translational impact. Our LMTK3BP constructs significantly induced ovarian cancer cell death both *in vitro* and *in vivo*. Additionally, the LMTK3BP retained their efficacy in chemoresistant cells (e.g., docetaxel or cisplatin). The affinity of the LMTK3BP toward their LMTK3 target was found to be very high, and based on published literature, it is expected to serve as a potential target of LMTK3 in patients with ovarian cancer.[4,13,18–20] Our initial data with low doses (2 mg/kg IV) of LMTK3BP #1 and #2 showed a 24% and 35% tumor reduction, respectively. No signs of toxicity were detected, even at a very high dose of 40 mg/kg, which will allow optimization of the dose and administration frequency depending on the stability of the peptides in the serum, which we propose to determine. Targeting LMTK3 with LMTK3BP did not affect normal ovarian cells

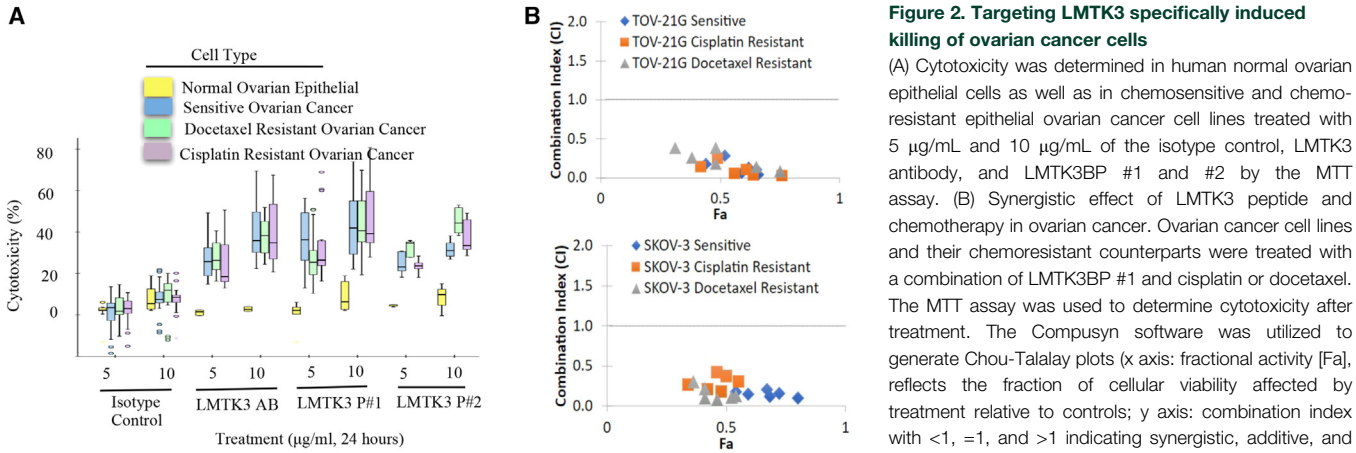

Figure 2. Targeting LMTK3 specifically induced killing of ovarian cancer cells

(A) Cytotoxicity was determined in human normal ovarian epithelial cells as well as in chemosensitive and chemoresistant epithelial ovarian cancer cell lines treated with 5 μg/mL and 10 μg/mL of the isotype control, LMTK3 antibody, and LMTK3BP #1 and #2 by the MTT assay. (B) Synergistic effect of LMTK3 peptide and chemotherapy in ovarian cancer. Ovarian cancer cell lines and their chemoresistant counterparts were treated with a combination of LMTK3BP #1 and cisplatin or docetaxel. The MTT assay was used to determine cytotoxicity after treatment. The Compusyn software was utilized to generate Chou-Talalay plots (x axis: fractional activity [Fa], reflects the fraction of cellular viability affected by treatment relative to controls; y axis: combination index with <1, =1, and >1 indicating synergistic, additive, and antagonistic effects, respectively. Each point represents a different combination of LMTK3BP concentration tested.) Experiments were performed in triplicate.

in vitro and showed a high safety profile *in vivo*, even at higher doses. This additional margin of safety is advantageous when compared to current therapies, which can have significant side effects. For instance, checkpoint inhibitors, expected to be the next significant improvement in ovarian cancer treatment, are effective in only 30% of patients and lead to severe side effects, including dermatologic, gastrointestinal, hepatic, and endocrine toxicity.[3] Furthermore, this high safety profile is particularly important in treating ovarian cancer patients, who often experience recurrent disease, which is refractory to treatment. Determining the effectiveness of LMTK3BP on cells both resistant and sensitive to radiotherapy, like breast cancer, can further improve treatment options to enhance patient health outcomes. Additionally, since LMTK3 is also overexpressed in non-small cell lung, breast, thyroid, gastric, and colorectal cancers, targeting this protein could expand the therapeutic repertoire to other solid tumors.

While LMTK3 was initially identified to have a role in ERα regulation in breast cancer, LMTK3 is now known to fuel tumorigenesis through many diverse mechanisms.[21] LMTK3, also known as LMR3 or AATYK3, belongs to the LMTK (LMR or AATYK) family of kinases, along with LMTK1 (also known as LMR1 or AATK) and LMTK2 (also known as LMR2, BREK, KPI2 or AATYK2). This family of kinases performs a wide range of functions in cell signaling and membrane trafficking, and aberrancies in these proteins are linked to diseases such as cystic fibrosis and cancer.[8] Despite the nomenclature, LMTK3 mainly acts as a serine/threonine kinase, phosphorylating various substrates involved in ERα expression and stability, trafficking, gene regulation, and oncogenesis. LMTK3 is overexpressed in several cancer types, contributing to the progression of the disease.[5,9–14,18,20] The physiological functions of LMTK3 are poorly characterized, as most reports focus on LMTK3 aberrancies in different cancers. Nevertheless, LMTK3 has been studied in normal physiology and proposed to have vital trafficking roles in neurons where LMTK3 knockout profoundly impacts the behavior of mice.[22]

The role of LMTK3 in the initiation and progression of ovarian cancer is not yet understood. We have shown that LMTK3BP directly and specifically targets apoptosis, a major determinant in uncontrolled cell growth in ovarian cancer. Additionally, LMTK3 is reportedly required for cancer cell proliferation and survival but is dispensable in normal ovarian cells, providing a rationale for developing LMTK3-targeted therapeutics.[5,11,19,20] In a recent study, LMTK3 was reported to promote chemoresistance to cetuximab in colorectal cancer via the extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathway *in vitro*.[23] The study concluded that targeting LMTK3 could be a promising therapeutic strategy for effectively treating colorectal cancer. Previous studies reported two compounds, C28 and C36, that exhibited anticancer activity against a variety of cancer cell lines, partly mediated by LMTK3.[24]

We recently identified a novel integrin (αV/β1) that acts as a ligand for Myeloperoxidase (MPO), which is now known to be expressed in all ovarian cancer epithelial cells and tissues but not in normal tissues and markedly inhibits apoptosis of ovarian cancer cells.[25] Our results showed that treatment with LMTK3 protein significantly induces integrin αV/β1 levels in a dose-response manner in ovarian cancer cells. Activated MPO utilizes nitric oxide (NO), produced by inducible NO synthase (iNOS), as a one-electron substrate to generate nitrosonium cations (NO^+). NO^+, a labile nitrosylating species, increases S-nitrosylation of caspase-3, which inhibits apoptosis and, thus, increases survival.[20] We hypothesize that LMTK3 activates αV/β1 integrin, which binds to intracellular MPO and activates MPO, leading to increased survival of ovarian cancer cells.

To conclude, there are currently no drugs or immunotherapy programs specifically targeting LMTK3 in clinical trials for ovarian cancer or other cancers. We designed peptides that target LMTK3 specifically, which manifested high efficacy and safety against ovarian and recurrent ovarian cancer. Based on the results of this study and previous studies, targeting LMTK3 may serve as an effective treatment

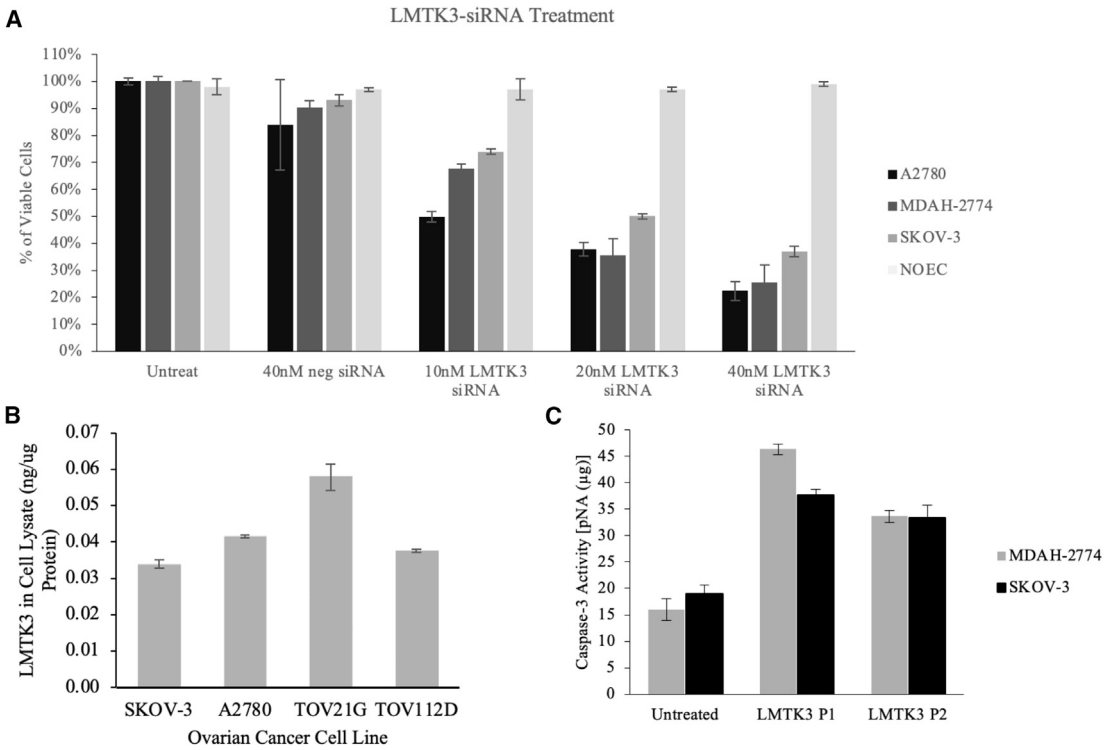

Figure 3. Silencing LMTK3 expression by LMTK3-specific siRNA

(A) Normal and ovarian cancer cells were treated with various doses of siRNA. Viability of cells was determined by the TACS MTT proliferation assay kit. (B) LMTK3 levels in the lysate of ovarian cancer cells. LMTK3 protein levels were measured by commercially available LMTK3 ELISA kits. LMTK3 levels were below the sensitivity range in the lysate of normal epithelial cell lines (normal epithelial ovarian cells and HOSEpiC); thus, they are not depicted in the graph. (C) LMTK3BPs induced apoptosis in ovarian cancer cells. Caspase-3 activity was determined by the Caspase-3 Colorimetric Activity Assay Kit in ovarian cancer cell lines treated with 10 µg/mL of LMTK3BP #1 (P1) and #2 (P2) for 24 h.

approach for this disease.[25–27] Another strength of this study is the potential utilization of LMTK3 as a prognostic indicator for clinical outcomes in ovarian cancer. Ongoing research from our laboratory is currently evaluating the prognostic significance of LMTK3 in a cohort of 270 stage III–IV ovarian cancer patients.

MATERIALS AND METHODS

Study design

We utilized IHC to assess the nuclear and cytoplasmic expression of LMTK3 in formalin-fixed, paraffin-embedded (FFPE) specimens collected from 204 early-stage ovarian cancer patients with various histologies. Univariable analysis of the IHC results was performed to determine the HR and the CI to show whether LMTK3 serves as a prognostic marker of clinical outcomes. Additionally, we designed novel LMTK3BPs that specifically target LMTK3 in both ovarian cancer cell lines and ovarian cancer animal models. Experiments were designed to determine the specificity, affinity, and cell killing efficacy of LMTK3BP.

In vitro studies

IHC analysis

FFPE specimens were collected from pathology labs in Western Sweden. The study encompassed a cohort of 204 stage I–II ovarian cancer patients diagnosed between 1993 and 2007 in Western Sweden, as described previously.[26] Histological classifications (high-grade serous carcinoma [HGSC], EC, MC, and CCC) were updated by a certified pathologist in accordance with current World Health Organization guidelines. Clinical and pathological data were retrieved from the Swedish Cancer Registry, the National Quality Registry, and the Cause of Death Registry. Surviving patients had a median follow-up duration of 14.0 years. The study was conducted according to the Declaration of Helsinki and was approved by the Regional Ethics Committee (approval no. 767-14).

To account for tumor heterogeneity, triplicate 1-mm cores were employed in the preparation of tissue microarrays (TMAs). Four-micrometer FFPE sections were prepared on Dako FLEX IHC microscope slides and desiccated at 60°C. The LMTK3 antibody (antibodies-online.com, ABIN5532611; 1:75 dilution) was optimized using 15 full-face FFPE ovarian tumor samples encompassing various stages and histotypes. Among these 15 samples, one served as a control for the TMA hybridization experiments. IHC was performed on a Dako Autostainer Plus (Agilent Technologies, Santa Clara, CA, USA) using Dako EnVision FLEX visualization systems.[26]

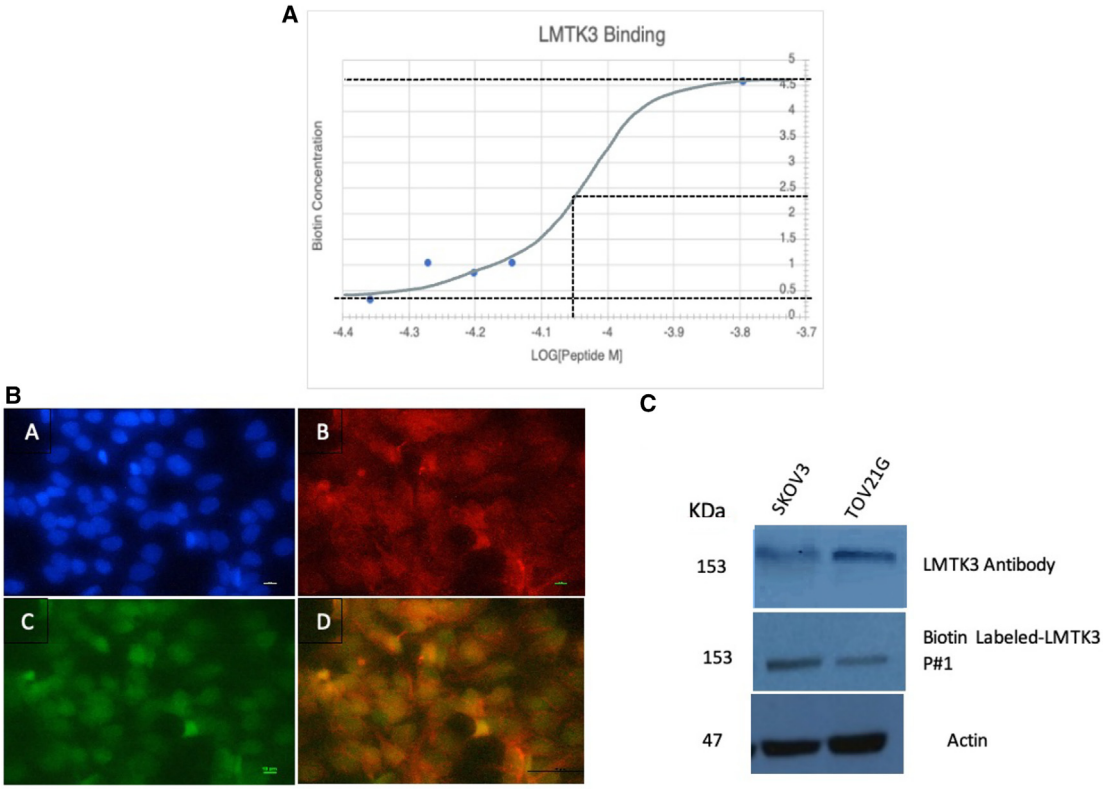

Figure 4. Binding affinity of LMTK3BP

(A) A colorimetric biotin assay was performed with increasing concentrations (3.6, 4.5, 5.4, 6.2, and 18 μg) of N-terminal biotin-labeled LMTK3 peptide #1 and 50 μg of protein isolated from A2780 cells. Using Microsoft Excel, a plot of the relationship between the increasing concentration of the labeled peptide with a fixed amount of protein was constructed, and the Kd was extrapolated from the tangents. (B) Binding of LMTK3 peptide to its target *in situ*. Cells grown in 8-well chamber slides were fixed and stained with DAPI (A), LMTK3 antibody (Invitrogen 1:200) (B), and LMTK3 biotin-labeled peptide 1 (100 μg/mL) (C); (A) and (B) were merged to show LMTK3 cytoplasmic localization (D). Orange/yellow color indicates the co-localization of the LMTK3 antibody and peptide. Images were taken at 40× magnification. (C) LMTK3 antibody and LMTK3BPs bind the same target. Western blot membranes were hybridized with LMTK3 biotin-labeled peptide #1 and LMTK3 antibody, followed by detection with the avidin-biotin complex.

Microscopic evaluation of the immunostained TMA sections was conducted by two certified pathologists (A.K. and E.W.R.). Their evaluation was based on the assessment of nuclear and cytoplasmic staining percentages as well as intensity. Intensity was rated on a scale ranging from 0–3, with 0 indicating negative staining, 1 indicating weak staining, 2 indicating moderate staining, and 3 reflecting strong staining. For each patient, the highest intensity value was used in the analyses. The intraclass correlation coefficient demonstrated good agreement, with a value of 0.69 (0.64–0.74) for nuclear intensity and 0.70 (0.65–0.74) for cytoplasmic intensity. In total, successful analysis was achieved for 197 of the 204 patients.

Ovarian cancer cell lines

The human ovarian cancer cell lines SKOV-3, TOV-21G, TOV112D, and MDAH-2774 were obtained from the American Type Culture Collection (Manasses, VA, USA). The ovarian cancer cell line A2780 and its cisplatin-resistant (1 μM) counterpart were obtained from Sigma-Aldrich (St. Louis, MO, USA). There is no commercially available docetaxel-resistant A2780 cell line. Cells were seeded in 60-mm^2 culture dishes (1.0×10^6) and allowed to rest for 24 h. Chemoresistant cell lines were conferred by exposing parent epithelial ovarian cancer cell lines, SKOV-3 and TOV-21G, to continuous culture in medium containing stepwise increases in either cisplatin (Sigma-Aldrich) or docetaxel (Sigma-Aldrich) over a period of 6 months with a final concentration of 1.5 μM cisplatin or 0.3 μM docetaxel. Upon reaching final concentrations, cells were grown in the absence of chemotherapeutic drugs for 2 weeks, followed by replacement of the drug and verification of resistance by the trypan blue cell viability and MTT cell proliferation assays. Doses were selected based on previously published studies.[25,27] Once confluent, cells were prepared as described above.

Protein extraction from ovarian cancer cells

Total protein from all cancer cell lines was extracted with 1× RIPA buffer and 1× protease and phosphatase inhibitor cocktail (89900, and 1861280, respectively; Thermo Fisher Scientific, Hampton, NH,

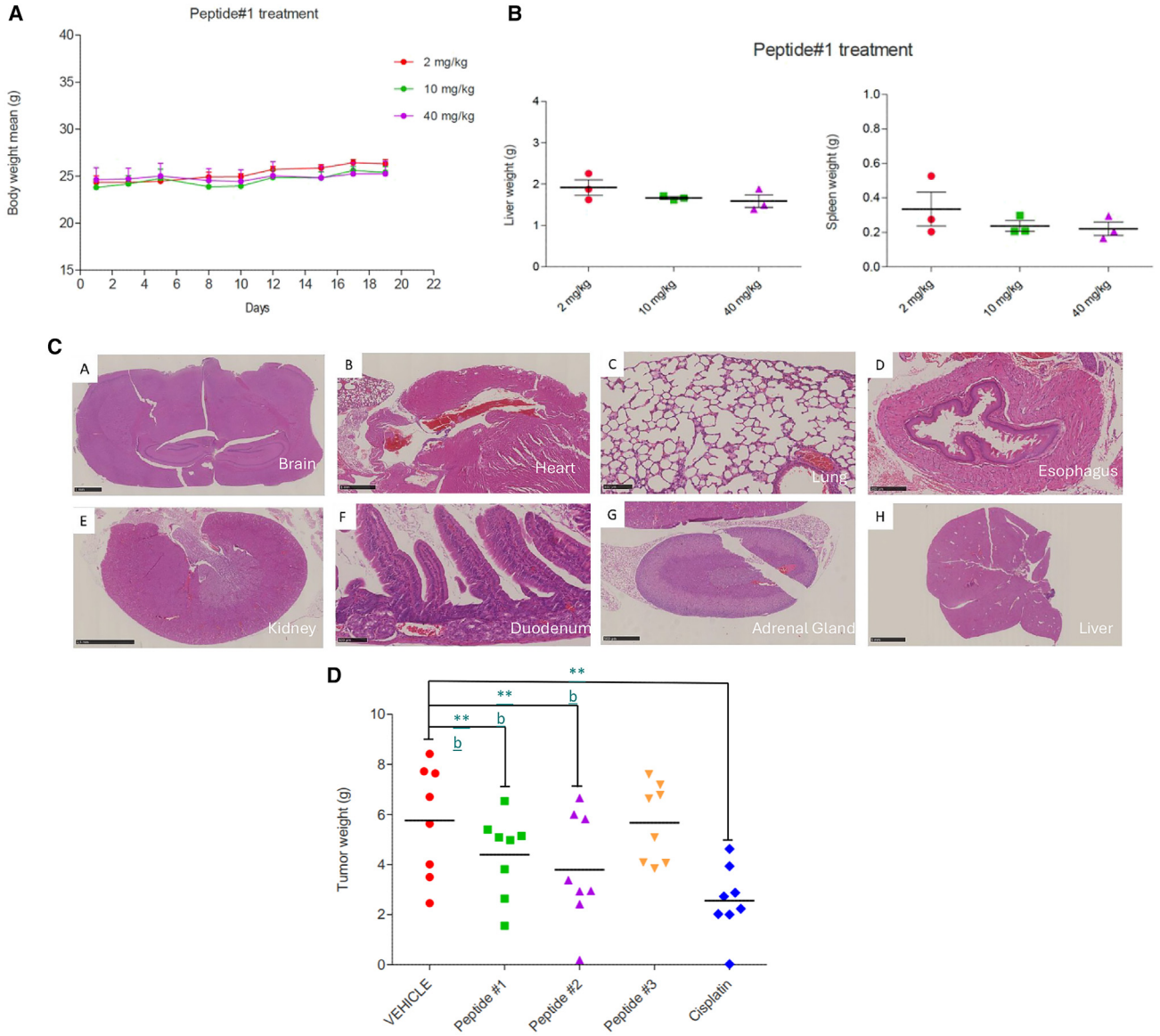

Figure 5. *In vivo* safety and efficacy

(A) The total body weight of mice was determined over 20 days during 2, 10, and 40 mg/kg LMTKBP1 treatment 3 times/week for 3 weeks. (B) Liver and spleen weight of mice treated with 2, 10, and 40 mg/kg LMTKBP1 3 times/week for 3 weeks were determined. (C) Histological appearance of various organs. Representative H&E staining of brain, original magnification 18×; thoracic structures (heart), 8×; lung parenchyma, 200×; esophagus, 100×; kidney, 6×; small intestine and duodenum, 200×; adrenal gland, 50×; and liver, 5×. (D) Tumor weight at sacrifice after 8 doses of vehicle, peptide #1, peptide #2, peptide #3 (negative control), or cisplatin. Bonferroni post hoc test found statistically significant differences between vehicle and peptide #1, vehicle and peptide #2, and vehicle and cisplatin (one-way ANOVA test, $^{**}p = 0.006$).

USA).[28] Protein concentration was determined by the Pierce BCA protein assay (23225, Thermo Fisher Scientific), following the manufacturer's protocol.

LMTK3BP synthesis

LMTK3BP were synthesized by Watson Bio (Houston, TX, USA). A negative peptide was also chosen from the peptide microarray and ordered with a sequence of rGQSHAPNVNPrDaG. All the peptides were of >95% purity and underwent quality control at Watson Bio.

MTT assay with LMTK3BP and LMTK3 antibody

The TACS MTT cell proliferation assay (Trevigen, Gaithersburg, MD, USA) was performed as described previously to determine cell viability and cell cytotoxicity in response to LMTK3 antibody and the two LMTK3BPs and to evaluate the interaction between

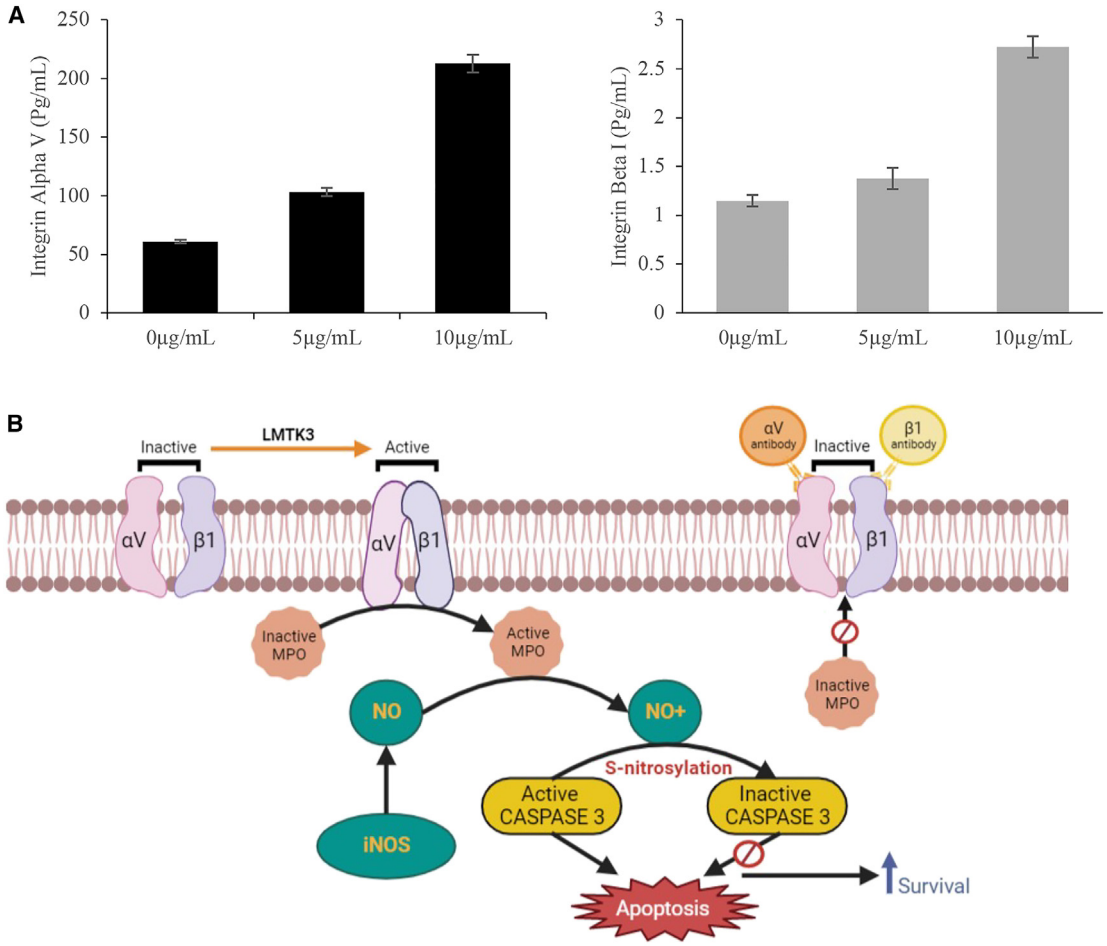

Figure 6. Proposed mechanism of survival in epithelial ovarian cancer cells

(A) Integrin αV and integrin β1 subunit levels were determined with commercially available ELISA kits on the 5 and 10 µg/mL recombinant LMTK3 protein-treated TOV112D cell line. (B) LMTK3-activated αV/β1 integrin, in turn, activates MPO, which produces the one-electron nitrosonium cation from NO. Nitrosylation of caspase-3 leads to its inactivation. NO, nitric oxide; NO^+, nitrosonium cation; iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase.

the LMTK3BPs and chemotherapy, as described previously.[29] Chemosensitive and chemoresistant counterpart ovarian cancer cell lines (A2780, SKOV-3, and TOV-21G) were utilized for this study as described previously.[29] Briefly, cells were seeded into 96-well plates in a fixed volume of 100 µL at a density of 8,000 cells/well. Cells were treated with either 5 or 10 µg/mL of LMTK3 antibody, LMTK3BP #1, or LMTK3BP #2 for 24 h. The LMTK3 antibody isotype was used as a negative control for the LMTK3 antibody experiments, whereas a negative LMTK3BP (described above) was used as a negative control for LMTK3BP experiments. The MTT assay was utilized as described previously.[29] Following incubation, 10 µL of the MTT solution was added to each well and incubated for 2 h, followed by the addition of 100 µL of the detergent solution to each well and incubation for an additional 2–4 h. Absorbance was measured at 570 nm. A blank containing only medium was subtracted from all test samples.

To determine the synergistic effect of LMTK3BPs and chemotherapy in ovarian cancer, TOV-21G and SKOV3 cells and their chemoresistant counterparts were used. Briefly, cells were seeded into 96-well plates in a fixed volume of 100 µL at a density of 8,000 cells/well. Cells were treated with 10 µg/mL of LMTK3BP #1 alone or in combination with cisplatin (1.5 µM) or docetaxel (0.3 µM). After 24 h of treatment, cytotoxicity was determined by the MTT assay as described above. Dose-effect curve parameters were used to calculate combination index values, as described previously.[16] The Compusyn software was utilized to generate Chou-Talalay plots (x axis: fractional activity [Fa], reflects the fraction of cellular viability affected by treatment relative to controls; y axis: combination index with <1, =1, and >1 indicating synergistic, additive, and antagonistic effects, respectively).

Transfection of siRNA for LMTK3

LMTK3-specific siRNAs were designed as described previously.[25] Target sequences were determined by aligning LMTK3 sequences to

an Ambion web-based algorithm. The 21-nt duplex siRNA molecules with 3-dTdT overhangs were resuspended in nuclease-free water according to the manufacturer's protocol (Ambion, Austin, TX, USA). A scrambled control sequence (siRNA-SCR), obtained from Ambion (Silencer Negative Control No. 1 siRNA, 4610), was used to ensure stringent control.

The ovarian cancer cell lines MDAH-2774, SKOV-3, and A2780 and normal epithelial ovarian cells were maintained in DMEM supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin, including 10% heat-inactivated fetal bovine serum at 37°C and 5% CO_2 as described previously.[27] Briefly, for siRNA transfection, cells were grown to a confluence of 30%–40% in 12-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) and transfected with 3 μL NeoFX reagent (1631, Ambion), 2 μL of 20 μmol siRNA, and OptiMEM medium (31985-047, Invitrogen, Waltham, MA, USA) up to a final volume of 100 μL. Neo FX reagent and siRNA were incubated at room temperature (RT) for 10 min and then applied onto 1.0×10^5 cells per well. Transfection mixtures were incubated with cells for 24 h, washed with medium, and incubated for an additional 24 h. The MTT assay was performed as described above.

Caspase-3 colorimetric activity assay

To test the activity of caspases that recognize the sequence DEVD, the caspase-3 colorimetric activity assay (APT165, Chemicon International, Temecula, CA, USA) was utilized as described previously.[19] Briefly, the assay is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA at 405 nm. Comparison of the absorbance of pNA from an apoptotic sample with an uninduced control allows determination of the fold increase in caspase-3 activity. MDAH-2774 and SKOV-3 cells were seeded into 96-well plates in a fixed volume of 100 μL at a density of 8,000 cells/well and treated with 10 μg/mL of LMTK3BP #1 or LMTK3BP #2 for 24 h.

Determination of Kd for LMTK3BP

We utilized the Colorimetric Biotin Assay Kit (MAK171-1KT, Sigma-Aldrich) to detect biotin-labeled LMTK3BP #1 bound to protein as described previously.[24] Briefly, 50 μg of A2780 protein was incubated with increasing amounts of LMTK3BP #1 (3.6, 4.5, 5.4, 6.2, and 18 μg) for 24 h. This colorimetric biotin quantitation kit provides a convenient method for quantitating total biotin concentration and estimating the biotin-protein molar ratio in biotin-protein conjugates. The assay utilizes HABA (4′-hydroxyazobenzene-2- carboxylic acid), a reagent that shows dramatic spectral changes when bound to avidin. Biotin displaces HABA from the HABA/avidin complex, resulting in a decrease in absorption at 500 nm. A plot of the relationship between the increasing concentration of the labeled peptide with a fixed amount of protein was constructed, and the Kd was extrapolated from the tangents of the highest and lowest point of the graph as described.[30]

Determination of binding of LMTK3BP to LMTK3 protein by western blotting

Western blotting was performed as described previously.[25] Briefly, total protein from the TOV-21G and SKOV3 cancer cell lines was extracted with 1× RIPA buffer (89900, Thermo Fisher Scientific), and 1× protease and phosphatase inhibitor cocktail (1861280, Thermo Fisher Scientific). Protein concentration was determined by the Pierce BCA protein assay (23225, Thermo Fisher Scientific), following the manufacturer's protocol. Total proteins (40 μg) were fractionated over 8% SDS-polyacrylamide gel under reducing and denaturing conditions and then transferred overnight at 4°C to 0.45-mm polyvinylidene fluoride membranes. Membranes were washed with TBST 3 × 10 min for 30 min and blocked with 1% BSA in TBST. Biotin-labeled LMTK3BP at a concentration of 20 μg/mL (diluted in 0.1% BSA in PBST) was incubated on the membrane overnight at 4°C. Subsequently, the membranes were washed with TBST 3× for 10 min for 30 min total. The biotin-labeled peptide was visualized with the ABC Peroxidase Staining Kit (32020, Thermo Fisher Scientific), followed by enhanced chemiluminescence (ECL) blotting substrate (32106, Thermo Fisher Scientific). LMTK3 antibody (PA5-38769, Invitrogen) at 1:500 concentration, followed by donkey anti-rabbit horseradish peroxidase (HRP) secondary antibody (SC2313, Santa Cruz Biotechnology, TX, USA), was used to confirm binding to the correct protein. β-Actin at 1:1,000 concentration (SC1614, Santa Cruz Biotechnology) and donkey anti-goat HRP antibody (SC2056, Santa Cruz Biotechnology) were used as loading controls. All bands were detected with ECL blotting substrate (32106, Thermo Fisher Scientific).

Determination of binding of LMTK3BPs to LMTK3 protein by immunofluorescence

Immunofluorescence was performed as described previously.[17] Cancer cells were grown on a Lab-Tek Chamber slide (Sigma Chemicals, St. Louis, MO, USA) overnight at 37°C. The cells were washed briefly with PBS and fixed with 4% paraformaldehyde for 10 min, followed by washing with PBS three times, 10 min each. Cells were permeabilized with ice-cold methanol for 10 min, followed by washing with PBS three times. The cells were then blocked with 10% donkey serum in 0.1% PBST for 1 h. After washing with PBS, cells were incubated with 100 μg/mL biotin-labeled LMTK3BP #1 and 1:200 LMTK3 antibody (PA5-38769, Invitrogen) in 3% donkey serum overnight at 4°C with gentle shaking. The following day, cells were washed with PBS and incubated with 1:150 Avidin-fluorescein isothiocyanate (A2050, Sigma-Aldrich) and 1:200 donkey anti-rabbit immunoglobulin G Alexa 594 (ab150064, Abcam, Cambridge, UK) in 3% donkey serum for 1 h at RT in the dark. Cells were washed with PBS twice, 10 min each, followed by a 5-min incubation with DAPI solution (D1306, Invitrogen). Two final PBS washes were performed for 5 min each. Coverslips were mounted on the slides with a drop of Clarion solution (sc24942, Santa Cruz Biotechnology).

ELISA

The concentration of LMTK3 in the lysate of several ovarian cancer cell lines was determined using a commercially available LMTK3 ELISA kit (OKEH02128, Aviva Systems Biology, San Diego, CA,

USA), following the manufacturer's protocol. The LMTK3 levels were below the sensitivity range in the lysate of normal epithelial cell lines (normal epithelial ovarian cells and HOSEpiC).

Levels of integrin αV in the lysate of TOV112D cells were determined with a commercially available ELISA kit (MBS2886168, MyBioSource, San Diego, CA, USA). Levels of integrin β1 in the lysate of TOV112D cells were also determined with a commercially available ELISA kit (MBS2886562, MyBioSource). The manufacturer's protocol and calculation were followed for both kits. For each ELISA, TOV112D cells were treated with 5 and 10 μg/mL recombinant LMTK3 protein for 24 h. Total protein was collected from LMTK3 untreated and treated cells and subjected to ELISA.

In vivo studies

Safety and efficacy of LMTK3 peptides in vivo

The safety of peptides #1, #2, and #3 (negative control peptide) was tested in athymic mice given 2, 10, or 40 mg/kg i.v. injections 3 times/week for 3 weeks. Additionally, intraperitoneal injection of 2.5 mg/kg anti-LMTK3 antibody (PA5-38769, Invitrogen) was tested for 7 days in athymic mice. Total body weight, liver and spleen weight, and general mouse behavior were monitored during treatment.

For preliminary efficacy testing, an A2780 cell-line-derived orthotopic xenograft mouse model of ovarian cancer was utilized. The mice were intravenously administered 2 mg/kg doses of LMTK3BP #1, #2, and #3 to test their efficacy. Some mice were treated with vehicle or cisplatin to compare the effectiveness of peptides. The mice were killed, and tumor weights were determined after 8 doses of each treatment. All *in vivo* experiments described were performed by Xenopat (L'Hospitalet de Llobregat, Barcelona, Spain).

Statistical analysis

The primary endpoint of this study was the time to death (overall survival) within 10 years from the diagnosis, analyzed using Cox regression without accounting for competing risks in the specimens collected from all patients in the cohort. Nuclear and cytoplasmic LMTK3 intensities were used as continuous variables. The multivariable analysis included histotype, stage, and patient age as covariates. For the construction of Kaplan-Meier plots, the median value served as the cutoff point. To address the strong correlation between nuclear and cytoplasmic staining, we created a combined variable to better assess the independent effects of both types of staining. This combined variable, termed "cytoplasmic:nuclear LMTK3 balance," was categorized into three groups: cytoplasmic staining < nuclear staining, cytoplasmic staining = nuclear staining, and cytoplasmic staining > nuclear staining. The proportional hazards assumption was tested with the Schoenfeld test, and it was found to be violated for the cytoplasmic:nuclear LMTK3 balance variable. Therefore, the HR for this variable should be interpreted as an average over the 10-year follow-up period. The analyses were conducted using R v.4.1.1 and the survival, survminer, and crmprsk packages.

Data were analyzed using a two-way unmatched independent t test in Excel to compare two sample groups, and ANOVA was employed to compare more than two group samples. Values of $p < 0.05$ were considered statistically significant for all analyses.

DATA AND CODE AVAILABILITY

All data were stored on the computer at the corresponding author's main laboratory and can be requested from the corresponding author.

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AUTHOR CONTRIBUTIONS

Conceptualization, G.M.S.; data curation, G.M.S., N.M.F., D.P., A.K., E.W.R., and T.Z.P.; formal analysis, G.M.S., N.M.F., R.M., A.S.T., E.I., D.P., K.H., and A.P.; funding acquisition, G.M.S. and K.H.; methodology, G.M.S., N.M.F., H.S., T.Z.P., D.P., A.K., E.W.R., E.I., K.H., and A.P.; project administration, G.M.S. and K.H.; resources, G.M.S., P.D.-K., K.H., and T.Z.P.; supervision, G.M.S., K.H., P.D.-K., and T.Z.P.; writing – original draft, G.M.S.; writing – review & editing, R.M., G.S., H.S., A.K., E.W.R., E.I., A.S.T., P.D.-K., D.P., T.Z.P., K.H., P.D.G., and N.M.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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