MCL1 and DEDD Promote Urothelial Carcinoma Progression

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Abstract

Focal amplification of chromosome 1q23.3 in patients with advanced primary or relapsed urothelial carcinomas is associated with poor survival. We interrogated chromosome 1q23.3 and the nearby focal amplicon 1q21.3, as both are associated with increased lymph node disease in patients with urothelial carcinoma. Specifically, we assessed whether the oncogene MCL1 that resides in 1q21.3 and the genes that reside in the 1q23.3 amplicon were required for the proliferation or survival of urothelial carcinoma. We observed that suppressing MCL1 or the death effector domain–containing protein (DEDD) in the cells that harbor amplifications of 1q21.3 or 1q23.3, respectively, inhibited cell proliferation. We also found that overexpression of MCL1 or DEDD increased anchorage independence growth in vitro and increased experimental metastasis in vivo in the nonamplified urothelial carcinoma cell line, RT112. The expression of MCL1 confers resistance to a range of apoptosis inducers, while the expression of DEDD led to resistance to TNFα-induced apoptosis. These observations identify MCL1 and DEDD as genes that contribute to aggressive urothelial carcinoma.

Implications: These studies identify MCL1 and DEDD as genes that contribute to aggressive urothelial carcinoma.

Introduction

In 2018, an estimated 81,190 patients will be diagnosed with urothelial carcinomas (1). A total of 20% to 30% of these patients have muscle-invasive disease. Patients with urothelial carcinoma that present with regional (7%) or distant (4%) disease have 5-year survival rates of 34.9% and 4.8%, respectively (1). These survival rates, based on the use of platinum-based chemotherapies, have not changed for the past 20 years (2, 3). Although advances in immunotherapy (e.g., pembrolizumab) have increased survival rates by 3 months in the relapsed setting, less than one-third of patients respond (4, 5), and new therapies are clearly needed.

Several studies have enumerated that amplified genomic regions on chromosome 1q are associated with advanced urothelial carcinoma (6–14). Muscle-invasive urothelial carcinoma tumors harbor high level copy number gain of the 1q22-24 region (6, 7). We and others showed through array comparative genomic hybridization, molecular inversion probe array technology, and multiplex ligation-dependent probe amplification that amplification of 1q23.3 is associated with poor survival and is more prevalent in metastatic samples (11, 13, 14). Several genes including PRRC2C (9), BCL9, CHD1L, MCL1, SETDB1, HIF1B, and PFDN2 (10, 12) have been shown to be recurrently amplified in urothelial carcinoma tumors.

Recently, The Cancer Genome Atlas (TCGA) sequenced urothelial carcinoma tumors from 412 patients and we confirmed that 1q23.3 is focally amplified in urothelial carcinoma and associated with higher disease stage. In addition, 1q21.3 was also found to be recurrently amplified (15). Here we interrogated genes resident in 1q21.3 and 1q23.3 to identify genes involved in the survival or progression of urothelial carcinoma.

Materials and Methods

Cell lines

Cell lines SCABER, HT1376, JMSU, RT112, RT4, HT1197, SW780, and T24 were obtained from the Biological Samples Platform at the Broad Institute of Harvard and MIT (Cambridge, MA) in 2012. UM-JUC10 was provided by the Kwiatkowski laboratory in 2012. TRI-HU1 was provided by the Adam laboratory in 2013. Cells were screened for Mycoplasma by PCR testing. Cell lines used for secondary validation studies (e.g., HT1376, JMSU, RT4, RT112, and TRI-HU1) were confirmed by Fluidigm SNP fingerprinting over the course of the experiments. Cells were kept in culture for maximum of 2 months prior to thawing of a new aliquot to limit genetic drift.
Short hairpin RNA arrayed screen

Lentivirus of individual short hairpin RNA (shRNA) was produced at the Broad Institute of Harvard and MIT (Cambridge, MA) and arrayed in two 96-well plates. Each cell line was optimized for viral toxicity, polynucleotides, puromycin sensitivity, and proliferation rates. Cells were seeded on day –1 in a 384-well plate. Lentivirus was introduced on day 0 and cells were spin infected for 30 minutes. Equal amounts of media with puromycin selection were added on day 1 and cells were monitored over the course of 7 days. At day 7, cells were treated with CellTiter-Glo (Promega) and levels were read on an Envision Plate Reader (PerkinElmer).

Open reading frame vectors

Open reading frames (ORF) for DEDD, F11R, MCL1, and PVRL4 were obtained from the Human ORFeome 8.1 collection at Dana-Farber Cancer Institute (Boston, MA). ARHGAP30 was synthesized by GenScript and sequence verified. We confirmed overexpression of these genes by immunoblot using a V5 antibody.

Anchorage independence by soft agar

In a 6-well plate, agar was prepared with media such that the bottom layer was 0.6% agar and top layer was 0.3% agar. Cells were grown in log phase, counted on a ViCell (Beckman Coulter), and mixed into the top layer. Cells were monitored over 2–4 weeks depending on the cell line. Images were captured on an Olympus SZX9 inverted microscope and analyzed using ImageJ (NIH, Bethesda, MD). Experiments were performed in biological triplicates.

Proliferation studies

After confirmation that cells overexpressed the gene of interest, cells were seeded in technical triplicates in 6-well plates. Plates were read at the specified times and counted on a ViCell. Experiments were performed in biological duplicates for TRT-HU1 and biological triplicates for RT4 and RT112.

BH3 profiling

Cells and supernatant were harvested at log phase and plated into a 384-well plate in the respective growth media. Cells were then treated with the BH3 mimetic and concentration as noted in the figure. Cells were then assessed for MOMP activity as published previously (16). Experiments were performed in biological triplicates.

Compound treatments

Cells were treated with TRAIL, TNFα (R&D Systems), cycloheximide (Cell Signaling Technology), and MK2206 for specified times as noted in the figure legends. These were then assayed either by CellTiter-Glo, CaspaseGlo 3/7 and 8 (Promega) and levels were read at the specified times and counted on a ViCell. Experiments were performed in technical triplicates and were repeated for a minimum of two times for biological replicates.

Immunoblots

Cells were lysed with RIPA Buffer (Cell Signaling Technology) containing protease inhibitors (cComplete, Roche) and phosphatase inhibitors (PhosSTOP, Roche). Antibodies used were as follows: TNF-R1 (H-5; sc-8436; 1:500), NFκB p50 (H-119; sc-7178; 1:500), and beta-actin (C4; sc-47778; 1:10,000) from Santa Cruz Biotechnology, V5/HRP (R961-25; 1:2,000) from Invitrogen, pan-Akt (2920;1:1,000), phospho-Akt (4060; 1:1,000), and FADD (2782; 1:1,000) from Cell Signaling Technology.

In vivo tumor injections and experimental metastases

This research project has been approved under the IRB-approved protocol at Dana-Farber Cancer Institute (Boston, MA). This research project has been reviewed by the Institutional Animal Care and Use Committee and is in compliance with the Animal Welfare Act and the Office of Laboratory Welfare of the NIH (Bethesda, MD). The FIU-Wuc-mCherry-puro vector was introduced into parental RT112 cells (17). Once stable lines were generated, we then introduced the ORFs into these cells and selected the cells with puromycin and blasticidin. Cells were confirmed to have mCherry expression, as a surrogate vector was introduced properly by flow cytometry using the BD F-JFortessa. Cells were subsequently grown in log phase and injected into the tail vein of mice. Female CrTac:NCr-Foxn1-immune (ages 6–8 weeks) were used in this study (Taconic Laboratories). In one experiment, we used 25 × 10⁴ cells (n = 3 mice per ORF) and in the other, we used 5 × 10⁵ cells (n = 5 mice per ORF). Mice were monitored for weight and health over the course of the experiment with BLI imaging performed at every 2-week timepoints.

IHC

All IHC was performed on the Leica Bond Automated Staining platform using the Leica Biosystems Refine Detection Kit with citrate antigen retrieval. Antibodies used include: Ki-67 (CR3M325 clone D20B12; 1:400) from Biocare and cleaved caspase-3 from Cell Signaling Technology (9664 clone Asp175/5A1E; 1:250).

Data availability

All primary data are available from the authors. Noted plasmids in the text are available through Addgene or the Genomics Perturbations Platform at the Broad Institute of Harvard and MIT (Cambridge, MA).

Results

1q21.3 and 1q23.3 are associated with higher disease stage in urothelial carcinoma

Analysis of Affymetrix SNP6.0–segmented copy number TCGA data from patients with urothelial carcinoma identified 37 recurrent focal amplifications and 36 recurrent focal deletions (q < 0.25) based on Genomic Identification of Significant Targets in Cancer (GISTIC) 2.0 analyses (18, 19). We focused our efforts on the amplifications to identify the recurrent-amplified genes that are required for the proliferation or survival of urothelial carcinoma cells. 6p22.3 was the most significantly focal amplicon in urothelial carcinoma with q = 7.03e-82, and harbors SOX4, a master regulator of epithelial–mesenchymal transition (Fig. 1A; ref. 20). The second most significant focal amplification was 1q23.3 with a q-value of 6.28e-56 (Fig. 1A). The 1q23.3 amplicon has a wider GISTIC amplification peak suggesting that a nearby focal amplicon may be a confounding factor. Indeed, 1q21.3 is the sixth most significant focal amplicon with a q-value of 3.52e-28 (Fig. 1A and B).

We then assessed whether any of the 13 clinical features assessed in TCGA correlated with these 37 focal amplifications (21). We found that a copy number gain of 1q21.3 was
correlated with increased pathologic nodal staging ($q = 0.223$; $P = 0.00575$; Fig. 1C). In addition, a copy number gain of 1q23.3 was correlated with age (e.g., older patients were likely to have amplification of 1q23.3; $q = 0.228$) and increased pathologic nodal staging ($q = 0.171$; $P = 0.00255$; Fig. 1D). Patients with a diagnosis of urothelial carcinoma and with increased nodal staging are at higher risk of recurrence and have overall poorer survival (22). Therefore, we concluded that focal amplification of 1q21.3 or 1q23.3 is likely to be associated with and may be a driver of poor outcome in urothelial carcinoma.

The 1q21.3 amplicon encompasses 13 genes (Table 1), including MCL1, an oncogene that was previously identified in a study of 3,131 copy number profiles across a number of cancers (23). MCL1 has been shown to be critical for the regulation of apoptosis in lymphocyte development (24) and found to be overexpressed...
or amplified in follicular lymphoma, diffuse large B-cell lymphoma, and lung and breast cancers but its role in urothelial carcinoma is unknown (23, 25). MCL1 has also been found to be amplified in 11% of cases in a cohort of 35 patients with stage IV urothelial carcinomas (26). The analysis of the urothelial carcinoma TCGA data identified 11.8% of patients (n = 48 of 408 patients) with amplification of MCL1 (Supplementary Table S1; ref. 15).

We previously studied three advanced urothelial carcinoma cohorts and identified a 1q23.3 focal amplicon that was associated with poor survival (13). Within this amplicon, we found five genes that were associated with poor survival; F11R, PFDN2, DEDD, USP21, and PPOX. TCGA studies identified a similar 1q23.3 focal amplicon that included seven genes (two that overlapped with our studies): F11R, TSTD1, USF1, AG30, PVR14, KLHDC9, and PFDN2. These 10 genes spanned chromosome 1 at 160,965,001 to 161,211,218 bp. When we examined this region for any additional genes that were not found in either study, we identified NIT1 and UFC1. We assessed these 12 genes in this study (Table 2). Of these genes, PFDN2 has previously been identified as amplified in urothelial carcinoma urine DNA (12). In addition, PVR14 has been identified to have tumorigenic potential in breast cancer (27). Compared with the 1q21.3 amplicon where MCL1 is the likely gene that leads to tumorigenesis in urothelial carcinoma, 1q23.3 amplicon remains less well studied. Furthermore, the analysis of urothelial carcinoma TCGA showed that the expression of these genes correlated with amplification status (Pearson correlation range 0.2470–0.8794 with associated q-values between 0 and 0.003; Fig. 1E; Supplementary Table S2). Here we focused our efforts on determining the function of MCL1 overexpression in urothelial carcinoma and performed a systematic analysis of the 12 genes identified in the 1q23.3 amplicon.

Urothelial carcinoma cell lines that harbor 1q amplifications depend on MCL1 in 1q21.3 and DEDD or ARHGAP30 in 1q23.3. To validate MCL1 and identify genes required for the proliferation/survival of urothelial carcinoma cells that harbor 1q23.3 amplification, we used RNAi to determine whether MCL1 or genes resident in the 1q23.3 focal amplicon were required for survival (Fig. 2A). We first identified a set of urothelial carcinoma cell lines, which harbored gains or amplifications of 1q23.3 and/or MCL1 based on Affymetrix SNP6.0 arrays or our prior work (Supplementary Table S3; refs. 11, 28). Of cell lines with gains or amplifications of 1q23.3 and/or MCL1, we chose four cell lines, SCABER, U46-UC10, HT-1376, and JMSU for further analyses. We also included five lines that lacked gain or amplification of MCL1 and/or 1q23.3: RF-4, RF-112, HT-1197, SW-780, and T24.

We then designed an arrayed shRNA screen of 81 shRNAs: 71 shRNAs targeting 13 genes (MCL1 in 1q21.3 and 12 genes identified in 1q23.3) and 10 controls (e.g., empty, RFP, Luciferase, and GFP; Supplementary Table S4). After introducing shRNAs targeting these genes into the cells that do or do not harbor amplifications of 1q21.3 or 1q23.3, we measured proliferation/viability by CellTiter-Glo. We confirmed that the expression of the control shRNAs did not lead to significant viability changes in the cell lines (Fig. 2B). We then compared the viability of the cells following RNAi between urothelial carcinoma cell lines with amplification of 1q23.3 and the nonamplified cell lines (Fig. 2C and D). Individual shRNAs were then collapsed to consensus gene dependencies using RNAi gene enrichment ranking (29). MCL1 in 1q21.3, and ARHGAP30 and DEDD in 1q23.3 scored as essential for the proliferation/survival of 1q-amplified cell lines with P < 0.05 (Supplementary Table S5).

MCL1, ARHGAP30, or DEDD is necessary but not sufficient to drive urothelial carcinoma proliferation and anchorage-independent growth

We then performed studies to determine whether the overexpression of MCL1, ARHGAP30, or DEDD induced tumorigenic potential to urothelial cell lines. We first assessed the effects of overexpression in a hTERT-immortalized urothelial cell line, TRTHU1. We used overexpression of HRASG12V, a well characterized oncoprotein that is necessary for tumor formation in urothelial carcinoma, as a positive control (30–32). We then overexpressed luciferase, ARHGAP30, DEDD, or MCL1 in TRT-HU1 (Supplementary Fig. S1A and S1B). We calculated the population doublings by assessing cell counts with trypan blue exclusion. In the TRT-HU1–immortalized bladder cell line, HRASG12V cells doubled 1.50 times faster than luciferase-overexpressing cells. ARHGAP30-, DEDD-, or MCL1-overexpressed cells proliferated at a similar rate to that of luciferase-overexpressing cells (0.86–0.99 ×; Supplementary Fig. S1C). Therefore, expressing ARHGAP30, DEDD, or MCL1 in hTERT-immortalized cells did not affect cell proliferation.

We then overexpressed luciferase, ARHGAP30, DEDD, or MCL1 in urothelial carcinoma cell lines, RT4 and RT112. In RT4, luciferase-overexpressing cells doubled 18.4 times during the time course of this experiment. ARHGAP30 cells proliferated at the same rate (1.04 times) as the luciferase cells, while the cells overexpressing MCL1 or DEDD doubled 1.33 or 1.40 times faster (Fig. 2E). In RT112 cell line, luciferase-overexpressing cells doubled 24.6 times during the time course of the experiment. MCL1 doubled 1.24 times faster, ARHGAP30 doubled 1.28 times faster, and DEDD doubled 1.20 times faster than luciferase (Fig. 2F). In these urothelial carcinoma cell lines, we found that cells overexpressing MCL1 and DEDD proliferated moderately more compared with cells overexpressing luciferase.
We then assessed whether the expression of these genes conferred anchorage-independent growth. We began with the non-urothelial carcinoma TRT-HU1 cells to determine whether overexpression of the gene of interest would lead to colonies in the soft agar in an immortalized urothelial cell line. This cell line harbors a large deletion in the short arm chromosome 9p where CDKNA2 resides but does not form anchorage-independent colonies (30). The expression of HRAS G12V conferred anchorage-independent growth ($P = 0.023$; Supplementary Fig. S1D), but none of the other genes induced anchorage-independent growth (Supplementary Fig. S1D). These findings indicated that the expression of MCL1 in 1q21.3 or ARHGAP30 or DEDD in 1q23.3 failed to induce anchorage-independent growth in an immortalized urothelial cell line.

We then assessed anchorage-independent growth in the urothelial carcinoma cell line, RT112. RT112 harbors FGR3 amplification, forms subcutaneous tumors, and does not harbor gain or amplification of MCL1 or 1q23.3 (32). We asked whether overexpression of the three genes identified from the RNAi screen leads to anchorage independence in RT112. Overexpression of MCL1 ($P = 0.0001$), ARHGAP30 ($P = 0.0054$), and DEDD ($P = 0.0058$) conferred a significant increase in colony formation compared with controls, hCRed, and luciferase (Fig. 2G). Specifically, DEDD overexpression led to an average 2.3-fold increase in colony formation.
colony formation, while MCL1 expression induced a 4.4-fold increase, and ARHGAP30 had a 3.8-fold increase compared with hCRed and Luciferase. These observations indicate that expression of these three genes conferred anchorage-independent growth on RT112 cells.

Taken together, we did not observe a proliferation advantage or anchorage-independent growth of immortalized urothelial cells overexpressing MCL1, ARHGAP30, or DEDD. In urothelial carcinoma cell lines, we saw overexpression of MCL1 or DEDD led to a modest proliferation advantage and anchorage-independent growth.

MCL1 but not DEDD overexpression leads to resistance to intrinsic mitochondrial-dependent apoptosis

On the basis of the loss- and gain-of-function assays, we then focused our efforts on understanding mechanisms by which MCL1 (1q21.3) and DEDD (1q23.3) conferred a fitness advantage in urothelial carcinoma. Prior work has established that MCL1 inhibits apoptosis while DEDD harbors a death effector domain but its role in apoptosis remains unclear (33, 34).

We first assessed the baseline apoptotic potential of these cells when MCL1 or DEDD were overexpressed by measuring caspase-3/7 levels. We found that overexpression of DEDD led to no significant difference to cells overexpressing LacZ ($P = 0.82$) whereas overexpression of MCL1 led to a 24% decrease in caspase-3/7 levels ($P = 3.13e-7$; Supplementary Fig. S2A).

We then began assessing the intrinsic apoptotic pathway. We utilized BH3 profiling assay, which assesses the mitochondrial outer membrane permeabilization (MOMP) induced by an exposure of mitochondria to a panel of synthetic BH3 peptides (35). We expressed LacZ, MCL1, or DEDD in TRT-HU1, RT4, and RT112 bladder cell lines. For each of the cell lines overexpressing LacZ, we established baseline levels of MOMP with various BH3 peptides (Fig. 3). We then compared these profiles with cell lines overexpressing MCL1 or DEDD. When we overexpressed MCL1, we observed decreased MOMP induced by nearly all the BH3 peptides across all three cell lines (Fig. 3A–C). We performed similar BH3 profiling studies with DEDD overexpression across TRT-HU1, RT4, and RT112 cell lines and found no significant changes in MOMP levels after exposure to all BH3 mimetics (Supplementary Fig. S2B–S2D).

These findings suggest that MCL1 is and DEDD is not involved in the intrinsic mitochondrial-mediated apoptotic cascade.

MCL1 or DEDD overexpression leads to resistance to extrinsic apoptosis

We then assessed two extrinsic apoptosis pathways using the RT112 cell line: TRAIL and TNFα-induced apoptosis. We first looked at cell viability as assessed by CellTiter-Glo when LacZ, MCL1, or DEDD were overexpressed and treated with increasing doses of TRAIL. MCL1 has previously been shown to resist TRAIL-induced apoptosis in other cancer types (36). Overexpression of MCL1 leads to resistance to cell death upon TRAIL treatment in urothelial carcinoma. However, we failed to observe resistance to TRAIL-induced apoptosis when DEDD was overexpressed (Fig. 4A).

We then looked at the effects on cell proliferation upon TNFα treatment with CellTiter-Glo. Overexpression of DEDD led to similar resistance to TNFα treatment as MCL1 when compared with luciferase (Fig. 4B). Specifically, upon treatment with TNFα, 63.7% of cells remained alive as compared with no treatment control. A total of 81.2% of cells remained alive when MCL1 was overexpressed ($P = 0.024$) and 79.2% of cells remained alive when DEDD was overexpressed ($P = 0.037$), suggesting both proteins when overexpressed lead to resistance to TNFα-induced cell death. We then assessed the extrinsic apoptotic pathway by measuring caspase-8 activity and downstream caspase-3/7 activity when cells overexpressing LacZ, MCL1, or DEDD were treated with TNFα. Compared with the LacZ control, we found that there...
was a consistent decrease of caspase-8 activation of 54.2% with MCL1 overexpression and a decrease of 26.9% with DEDD overexpression (Fig. 4C). We saw a similar response for activation of caspase-3/7 (Fig. 4D). We further assessed proteins upstream of caspase 8, TNFR1 and NFκB p105/p50 by immunoblotting and failed to observe any differences between cells expressing LacZ and DEDD or MCL1 (Supplementary Fig. S3A).

Figure 4.
MCL1 is a pan antiapoptotic protein while DEDD is a TNFα antiapoptotic protein in RT112. A, MCL1 but not DEDD resists TRAIL-induced apoptosis as measured by CellTiter-Glo. Error bars are SEs of the mean and representative of three technical replicates. AUCs (% viability × ng/mL) listed on the right. Experiments representative of two biological replicates. B, MCL1 and DEDD resist TNFα-induced apoptosis as measured by CellTiter-Glo. Cells were treated with increasing doses with TNFα and viability was measured by CellTiter-Glo after 6 hours. AUCs (% viability × µg/mL) listed on the right. Error bars are SEs of the mean and representative of two biological replicates. C, MCL1 and DEDD resist TNFα-induced apoptosis but to different degrees as measured by activation of caspase-8 activity. Error bars are SEs of the mean and representative of three technical replicates. Experiments representative of two biological replicates. D, Addition of cycloheximide sensitizes DEDD to TNFα at high concentrations. Addition of 1 µmol/L of cycloheximide with increasing concentrations of TNFα for 6 hours leads to the resistance to TNFα-induced apoptosis in both MCL1 and DEDD. At 100 ng/mL, DEDD-overexpressed cells were similarly sensitive as LacZ controls. AUCs (% viability × ng/mL) listed on the right. F, Akt and pAkt (S473) are increased upon TNFα treatment in MCL1 and DEDD. Immunoblots of RT112 cells with or without treatment with TNFα. Immunoblots representative of three biological replicates. G, Addition of MK2206 abrogated DEDD’s and partially MCL1’s ability to resist TNFα-induced apoptosis as measured by CellTiter-Glo. AUCs (% viability × ng/mL) listed on the right. Error bars are SEs of the mean and representative of three technical replicates. Experiments representative of two biological replicates.

TNFα-mediated apoptosis via caspase 8 can be potentiated by a protein synthesis inhibitor such as cycloheximide (37). Cycloheximide treatment eliminates c-FLIP, an endogenous
Here, we saw that although there was no significant increase in baseline Akt levels, but noted an increase in phosphorylated Akt, particularly following TNFα treatment (Fig. 4F). We then introduced LacZ (control), MCL1, and DEDD into these cells. Prior studies in NIH 3T3 cells have shown that Hras-transfected led to experimental metastasis (39, 40). We introduced HRAS<sup>G12V</sup> into RT112 luciferase-expressing cells as positive control. We then injected these cells via the tail vein in immunodeficient mice. We monitored the mice for lung metastasis and other metastatic sites based on BLI measurements. By day 56, we observed a significant difference between BLI measurements of MCL1-, DEDD-, and HRAS<sup>G12V</sup>-overexpressed cells compared with LacZ controls (Fig. 5A and B), but no significant difference between HRAS<sup>G12V</sup> and MCL1 (P = 0.154) or DEDD (P = 0.093).

Furthermore, we found that mice harboring cells overexpressing MCL1, DEDD, or HRAS<sup>G12V</sup> exhibited a significant mortality as compared with mice injected with mice with tumors overexpressing LacZ (Fig. 5C). Specifically, the median survival for cells overexpressing MCL1 was 103.5 days, DEDD 153.5 days, and HRAS<sup>G12V</sup> 119.5 days, while mice with tumors overexpressing LacZ survived to the end of the experiment. The tumors formed at the time of sacrifice were consistent with urothelial carcinoma, and there were no notable morphologic differences between the tumors overexpressing various genes (Fig. 5D). Finally, we assessed baseline apoptosis with cleaved caspase-3 and proliferation with Ki-67 on the lung tumors with overexpression of MCL1, DEDD, and HRAS<sup>G12V</sup>. We failed to find a significant difference between MCL1 or DEDD as compared with HRAS<sup>G12V</sup> showing that the modest proliferative advantage seen in vitro when MCL1 or DEDD is overexpressed (Fig. 2F) is also seen in vivo (Supplementary Fig. S3C–S3D). These findings show that the
overexpression of MCL1 or DEDD in RT112 cells leads to in vivo metastasis.

Discussion

Therapy for advanced urothelial carcinomas remains unchanged for several decades until recent advances with immunotherapy (4, 41). Although these advances have increased survival by 3 months (from 7.4 months to 10.3 months) in the relapsed setting following platinum-based chemotherapy, additional therapy and biomarkers are needed to improve survival. Our initial studies of focal amplicons in advanced urothelial carcinoma identified a region of chromosome 1q23.3. Analysis of a larger cohort of patients in urothelial carcinoma TCGA identified a nearby and potentially confounding focal amplicon on chromosome 1q21.3. We have interrogated both of these regions in this study. We took a targeted approach in 1q21.3 as MCL1, a putative oncogene, was identified by GISTIC to be a potential target gene in this region for patients with urothelial carcinoma. We took a broader and systematic approach to the region of 1q23.3 and assessed the function of 12 genes in this region. We have validated MCL1 as an oncogene in urothelial carcinoma and identified DEDD as a potential oncogene. Both are necessary but not sufficient for tumor maintenance. Suppression of MCL1 or DEDD leads to viability/proliferation defects in urothelial carcinoma cell lines that have gain or amplification of amplicons that harbor these genes. MCL1 or DEDD overexpression provides a modest proliferative advantage, increases anchorage independence by soft agar assay, and has markedly enhanced the ability of urothelial carcinoma cell lines to form lung metastases, similar to the effects of HRASG12V.

MCL1, a putative oncogene in various cancer lineages, has not been functionally tested within urothelial carcinoma although MCL1 overexpression by RNA-seq is seen in 5% of patients in TCGA (n = 412). We found that MCL1 overexpression leads to resistance to both intrinsic and extrinsic apoptotic pathways, similar to effects of MCL1 in other cancer lineages (42, 43).

Studies in 293T cells overexpressing DEDD suggested that DEDD had both apoptotic and antiapoptotic roles (33). Our findings suggest that DEDD overexpression in urothelial carcinoma cell lines enhances total Akt and phosphorylated Akt levels and resists apoptosis (Fig. 6). Although DEDD does not have a role in intrinsic apoptosis as compared with MCL1, we found that it has a role in the extrinsic pathway, specifically through resistance to TNFα-mediated apoptosis. The resistance to TNFα-mediated apoptosis is abrogated when a pan-Akt inhibitor was used (MK2206) which further supports a role for Akt in DEDD overexpression.

We have shown that individually, MCL1 and DEDD are able to confer tumorigenic properties. Furthermore, we have shown that overexpression of DEDD or MCL1 in vivo leads to increased metastatic potential as measured by tail vein injection studies. These findings now identify both DEDD and MCL1 as therapeutic targets in urothelial carcinomas.

Disclosure of Potential Conflicts of Interest

J.L. Guerriero reports receiving commercial research grants from GlaxoSmithKline and Eli Lilly, and is a consultant/advisory board member for GlaxoSmithKline. R. Beroukhim reports receiving commercial research grants from Novartis. A. Letai reports receiving a commercial research grant from Novartis, has ownership interest (including stock, patents, etc.) in Vivid Bioscience, Flash Therapeutics, K-Group Alpha, and Dialytic, is a consultant/advisory board member for Vivid Bioscience, Dialytic, K-Group Alpha, and has provided expert testimony for Abbvie. J.E. Rosenberg reports receiving commercial research grants from Genentech/Roche, Novartis, Astellas, Seattle Genetics, Mirati, AstraZeneca, and Bayer, has received speakers bureau honoraria from Chugai, has ownership interest (including stock, patents, etc.) in Illumina, and is a consultant/advisory board member for BMS, Merck, Genentech/Roche, Inovio, Gritstone, Pharmacyclics, Western Oncology, QED Therapeutics, Bioclin/Ranier, Astellas, Seattle Genetics, Sensei Biotherapeutics, Adict Bio, Bayer, Fortress Biotech, and EnMD-Serono. W.C. Hahn reports receiving a commercial research grant from Deerfield, has ownership interest (including stock, patents, etc.) in KSI Therapeutics, and is a consultant/advisory board member for Thermo Fisher Scientific, MPM Capital, AjuIB, and Paralex. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: A.L. Hong, J.L. Guerriero, J.E. Rosenberg, W.C. Hahn
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.L. Hong, J.L. Guerriero, M.B. Doshi, B.D. Kynnap, W.J. Kim, A.C. Schinzel, R. Modiste, J.E. Rosenberg
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![Diagram of MCL1 and DEDD pathways]

Figure 6.
Summary of effects on urothelial carcinoma cell lines from DEDD overexpression.

from Novartis, has ownership interest (including stock, patents, etc.) in Vivid Bioscience, Flash Therapeutics, K-Group Alpha, and Dialytic, is a consultant/advisory board member for Vivid Bioscience, Dialytic, K-Group Alpha, and has provided expert testimony for Abbvie. J.E. Rosenberg reports receiving commercial research grants from Genentech/Roche, Novartis, Astellas, Seattle Genetics, Mirati, AstraZeneca, and Bayer, has received speakers bureau honoraria from Chugai, has ownership interest (including stock, patents, etc.) in Illumina, and is a consultant/advisory board member for BMS, Merck, Genentech/Roche, Inovio, Gritstone, Pharmacyclics, Western Oncology, QED Therapeutics, Bioclin/Ranier, Astellas, Seattle Genetics, Sensei Biotherapeutics, Adict Bio, Bayer, Fortress Biotech, and EnMD-Serono. W.C. Hahn reports receiving a commercial research grant from Deerfield, has ownership interest (including stock, patents, etc.) in KSI Therapeutics, and is a consultant/advisory board member for Thermo Fisher Scientific, MPM Capital, AjuIB, and Paralex. No potential conflicts of interest were disclosed by the other authors.
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References

9. Huang WC, Taylor S, Nguyen TB, Tomaszewski JE, Libertino JA, Malkowicz SB, et al. KIAA1096, a gene on chromosome 1q, is ampli-
cated and over-
10. Eriksson P, Aine M, Sjodahl G, Staaf J, Lindgren D, Hoglund M. Detailed cation of cytogenetic subgroups and karyotypic pathways in tran-
20. Tiwari N, Tiwari VK, Waldmeier L, Balwierz PJ, Arnold P, Pachkov M, et al. Sox9 is a master regulator of epithelial-mesenchymal transition by con-
24. Opferman JT, Letai A, Beard C, Soricelli MD, Ong CC, Koesmeijer SJ. Development and maintenance of B and T lymphocytes requires anti-


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