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Liver Protects Metastatic Prostate Cancer From Induced Death by Activating E-cadherin Signaling

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Abstract

Liver is one of the most common sites of cancer metastasis. Once disseminated, the prognosis is poor as these tumors often display generalized chemoresistance, particularly for carcinomas that derive not from the aerodigestive tract. When these cancers seed the liver, the aggressive cells usually undergo a mesenchymal to epithelial reverting transition (MErT) that both aids colonization and renders the tumor cells chemoresistant. *In vitro* studies demonstrate that hepatocytes drive this phenotypic shift. However, the *in vivo* evidence, and the molecular signals that protect these cells from induced death, are yet to be defined. Herein, we report that membrane surface E-cadherin-expressing prostate cancer (PCa) cells were resistant to cell death by chemotherapeutic drugs, but E-cadherin null or those expressing E-cadherin only in the cytoplasm were sensitive to death signals and chemotherapies both *in vitro* and *in vivo*. While cell-cell E-cadherin ligandation reduced mitogenesis, this chemo-protection was proliferation-independent as killing of both EdU+ (or Ki67+) and EdU− (Ki67−) cells was inversely related to membrane-bound E-cadherin. Inhibiting the canonical survival kinases extracellular signal-regulated protein kinases (ERK), protein kinase B (PKB/AKT) and janus kinase (JAK), which are activated by chemotherapeutics in epithelial transitioned PCa, abrogated the chemoresistance both in cell culture and in animal models of metastatic cancer. For disseminated tumors, AKT disruption in itself had no effect on tumor survival but was synergistic with chemotherapy leading to increased killing. *Conclusion*: liver microenvironment-driven phenotypic switching of carcinoma cells and subsequent survival signaling results in activation of canonical survival pathways that protect the disseminated PCa liver micrometastases in a proliferation-independent manner, and that these pathways can be targeted as an adjuvant treatment to improve the efficacy of traditional chemotherapeutics.

Keywords

liver metastases; androgen independent prostate cancer; mesenchymal-epithelial transition; micrometastases; chemoresistance

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Introduction

Most cancer in the liver is metastatic or disseminated cancer, primarily from aerodigestive tract but also prostate and breast carcinomas, for which liver is one of the most common sites of secondary spread (1, 2). This dissemination is a mortal harbinger as the metastatic tumors display a generalized chemoresistance rendering these therapies temporizing at best (3-5). Understanding the basis of drug resilience in the face of therapy is a prerequisite for new, effective approaches.

Androgen-independent prostate cancer (AIPC) grow and survive independently of androgens and display generalized chemoresistance, represent a growing challenge of liver metastases. The outlook for metastatic prostate cancer has not changed in two decades (6-8). Liver metastases of prostate cancer (PCa), though not as common as bone, still presenting as clinically evident macrometastases in 25% patients with hematogeneous tumor spread (9). The median overall survival of AIPC at the state of demonstrable liver metastases is very poor (<6 months) (10). Although several advances have been made in the control of localized prostate tumors (11, 12), we are yet to understand the mechanisms behind chemoresistance of PCa metastasis.

Tumor dissemination generally involves a series of phenotypic switches, defined by the presence of E-cadherin, that allow for ectopic seeding and chemoresistance (13-17) (18, 19). Initially, epithelial-mesenchymal transition (EMT) is associated with autocrine signaling and loss of cell-cell constraints, enabling separated cells to escape through boundary matrices. Tumor cell colonization of the secondary site such as liver, is the most rate-limiting step (20, 21). A reversion of EMT, or mesenchymal to epithelial reverting transition (MErT), aids in the formation of metastatic foci for many cancers (22-25). Hepatocytes are involved in inducing MErT, allowing the tumor cells to survive in the face of an inhospitable micro-environment, and to survive direct challenges such as death cytokines and chemotherapies (26-29). For PCa cells that downregulate E-cadherin via autocrine growth factor signaling, MErT could also be accomplished abrogating epidermal growth factor receptor (EGFR) autocrine signals. Such cells also gained chemoresistance (26, 28, 29). Taken together, these investigations strongly implicated E-cadherin as involved in metastatic chemoresistance. However, only a partial epithelial phenotypic conversion was observed in the prostate and other cancer cells (22), and as such the correlation between E-cadherin re-expression and cell survival remained unknown.

Herein, we established the direct relationship between E-cadherin and death resistance both *in vitro* and in an experimental liver metastases xenograft model. The acquired resistance to chemotherapeutic and cytokine death signals occurred through E-cadherin activation of canonical survival pathways and not solely due to nonproliferative dormancy; this has important implications for the emergence of mortal macrometastases despite therapeutic coverage. Prevention of E-cadherin re-expression decreased hepatic metastases dramatically without affecting the growth of primary tumors. However, the E-cadherin-expressing cells were protected from killing regardless of location. Importantly, targeting the E-cadherin-

triggered survival pathways, at low, ineffective doeses, synergistically augmented chemoresponsiveness of the metastatic tumors.

Materials and Methods

See Supplemental Materials for all Materials and Methods

Mice - intrasplenic inoculations and drug treatments

The AAALAC-accredited Institutional Animal Care and Use Committees of the Veteran's Administration Pittsburgh Health System approved all animal studies and procedures.

Cell lines, fresh human hepatocytes and human colon tumor tissues

The use of all primary cells, cell lines and histopathology slides was approved by the University of Pittsburgh IRB as exempted.

Results

E-cadherin expressing prostate tumor cells are resistant to death signals

Our model has the epithelial-like PCa cells as being resistant to death inducing signals, whether from cytokines or chemotherapies (30). Pro-apoptotic cytokines would be present during the nonspecific inflammatory response triggered by ectopic seeding of the liver, and chemotherapies would be use clinically as adjuvant therapy. Androgen-independent PCa cell lines were used herein as they are representative of the terminal stage of prostate cancer being impervious to androgen ablation therapy. These cells were more sensitive to concurrent treatment with both a death cytokine and a cytotoxic chemotherapy than either used alone (Supplemental Fig. 1A). Therefore, the combination treatment of 1μM camptothecin (CPT) and 100ng/ml TNF-related apoptosis-inducing ligand (TRAIL) was selected for the ensuing experiments.

DU145 cells express only low levels of E-cadherin with few if any on the plasma membrane (DU-L cells). However, upon co-culture with human primary hepatocytes, to replicate the juxtaposition in the liver microenvironment, as subset of these cells re-express demonstrable levels of E-cadherin on their surface (22, 26, 29). Cells, RFP-labeled for tracking, were treated with CPT-TRAIL for 8 hours prior to staining for activated caspase 3 as an indicator of apoptosis. Immunofluorescence (IF) staining found that the membrane E-cadherin expressing DU-L cells were resistant to CPT-TRAIL; cells devoid of E-cadherin, or with expression only in the cytoplasmic were efficiently killed as evinced by cleaved caspase-3 (Fig. 1A). The portion of apoptotic tumor cells was determined by flow cytometry assay of cleaved caspase3 in the RFP-positive population (supplemental Fig. 1B). More tumor cells survived from CPT-TRAIL treatment, concomitant with higher membrane-bound E-cadherin expression levels (Fig. 1B, C and Supplemental Fig. 3A). This death resistance was also found in human primary hepatocytes-driven epithelial-transitioned PC3, another androgen-independent metastatic PCa cell line that has very low to absent levels of E-cadherin (29) (Supplemental Fig. 2).

It is possible that the resistance to treatments resides in hepatocyte metabolism of the agents, though the differential effects on E-cadherin-expressing and -devoid cells in the same culture makes this highly unlikely. To avoid this, we pretreated DU-L cells with the EGFR tyrosine kinase activity inhibitor PD153035 to induce E-cadherin expression (29). IF staining found that very few cells were apoptotic; >90% of DU-L cells challenged with the pro-death signals were undergoing apoptosis; however, >90% of tumors cells re-expressing surface E-cadherin were negative for cleaved caspase3 after CPT-TRAIL treatment (Fig. 2A). Flow cytometry assays consistently showed PD-pretreated DU-L significantly increased membrane-bound E-cadherin expression, concomitant with the reduction of cleaved caspase3 compared to parental cells (Fig. 2B, C and Supplemental Fig. 3B). This was further validated by western blot with PD-pretreated cells also expressing less c-casp3 and its effector cleaved-poly ADP ribose polymerase (PARP) (Fig. 2D).

It is possible that abrogating EGFR activity in and of itself could contribute to the anti-apoptotic or pro-survival signaling leading to the acquired chemoresistance, rather than the phenotypic switch. A spontaneously occuring DU145 subline presenting high basal E-cadherin with surface expression (referred to as DU-H hereafter) was investigated. Both DU-L and DU-H cells were obtained from American Type Culture Collection, and had maintained consistent E-cadherin levels for more than 5 years in our lab, as validated by IF which E-cadherin negative (or positive) membrane staining population was predominant with extremely few exceptions (<0.1%) in DU-L (or DU-H) cell lines. Compared to DU-L, DU-H cells showed dramatically lower levels of cleaved caspase3 (and cleaved PARP) after CPT-TRAIL treatment, as seen either by western blot (Fig. 2E) or IF (Fig. 2F). Decreasing E-cadherin in DU-H cells by shRNA (DU-H-shEcad) to E-cadherin restored the relative chemosensitivity (Fig. 2F). Exogenous expression of wildtype E-cadherin was able to confer chemoresistance, but a dominant negative E-cadherin that fails to achieve intercellular homotypic binding (H2-K^d-E-cadherin fusion protein) did not alter chemoresponsiveness with cells expressing this variant also showing cleaved caspase3 when challenged with death signals (Supplemental Fig. 3C). Taken together, these data directly correlated E-cadherin re-expression on the cell membrane with resistance to induced cell death.

E-cadherin related death resistance is independent of cell proliferation

Chemotherapeutic drugs, as a rule, efficiently kill proliferating cells. As E-cadherin surface expression is related to the cell quiescence noted in dormancy, cell cycle arrest by E-cadherin could be responsible for the observed relative chemoresistance. To determine if this was the underlying cell behavioral mechanism, tumor cell cycling was determined by both 5-ethynyl-2’-deoxyuridine (EdU) incorporation during S-phase and propidium iodide (PI) staining. Compared to DU-L cells, DU-H cells had a significant lower proliferation rate, the difference being more marked with extended culture duration (presumed due to contact inhibition) (Fig. 3A, B). E-cadherin downregulation in DU-H cells resulted in increased EdU incorporation or (Supplemental Fig. 4A). DNA content analyses indicated a slightly higher fraction of DU-L cells in the G2/M phase population than DU-H; and E-cadherin shRNA-mediated downregulation in the DU-H cells increased the fraction of cells in the G2/M phase (Supplemental Fig. 4C). Consistently, after EGFR inhibitor PD153035-pretreatment, E-cadherin expressing DU-L cells also had reduced proliferation compared to cells lacking

surface E-cadherin (Supplemental Fig. 4B). Finally, hepatocyte co-cultured epithelial-transitioned DU-L cells presented reduced EdU incorporation (Fig. 3C). All of above data suggested E-cadherin presence on the cell surface reduced proliferation, consistent with a role for the phenotypic shift in dormancy (30), and this might result in the chemoresistance, even though epithelial-like surface E-cadherin-expressing PCa cells still had over one third of the cells cycling.

To determine whether these non-proliferating cells accounted for the relative chemoresistance, EdU was added before CPT-TRAIL treatment, and co-staining of EdU, E-cadherin, cleaved caspase-3 was performed. Surprisingly, no correlation was found between EdU incorporation and c-caspase-3 presentation; both EdU+ and EdU- cells were protected from CPT-TRAIL treatment when expressing E-cadherin on the cell membrane (Fig. 3D, E). Importantly, co-staining for propidium iodide and cleaved-caspase3 confirmed that both cycling and non-cycling cells underwent apoptosis in the presence of CPT-TRAIL (Supplemental Fig. 4D). All these findings taken together indicated E-cadherin mediated death resistance was cell proliferation independent.

Activation of canonical pro-survival intermediary kinases in epithelial transitioned PCa cells

Given E-cadherin-related death resistance was found to be cell proliferation-independent, we postulated E-cadherin was involved in the activation of survival pathway(s) responding to the chemotherapy. It has been shown Phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB/AKT) and extracellular signal-regulated protein kinases (ERK) were activated by E-cadherin mediated cell-cell junction (31, 32). Moreover, the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is a major anti-apoptotic signaling cascade found to be upregulated in malignancies (33). Jun N-terminal kinase (JNK) and p38 MAPK affect cell survival in a cell context/type-specific manner (34). Herein, to investigate the underlying mechanisms of the noted chemoresistance, the activation levels of all these survival or apoptosis-related intermediary kinases were assessed in the basal state and during chemotherapeutic challenge. In the DU-L cells, western blot data showed the levels of phosphorylated AKT and JAK2 declined upon CPT-TRAIL treatment, consistent with increasing apoptosis (Fig. 4A). On the other hand, p-AKT increased and p-JAK2 was maintained in the face of CPT-TRAIL in DH-H cells. Phosphorylated ERK levels presented a mild elevation at the early stage of killing, but decreased after 2 hours in DU-L; however, p-ERK was consistently activated in DU-H cells. Phosphorylated JNK, a proapoptotic marker, was mildly increased upon drug treatment in DU-L, but this elevation was suppressed in DU-H (Fig. 4A). The similar trends of activation of these intermediary kinases were found in PD-pretreated DU-L cells (Fig. 4B). A minor increase in p-p38 was detected in PD(MErT) DU-L but not DU-H (data not shown). Thus, in the face of surface E-cadherin expression CPT-TRAIL treatment counter-intuitively activated overall pro-survival intermediate kinases.

The involvement of these kinases in survival signaling was supported by directed intervention. Small molecule inhibitors of AKT, JAK or ERK, but not JNK or p38 sensitized the E-cadherin-expressing cells to CPT-TRAIL (Fig. 4C, D). It is not unexpected that the

increase in c-caspase-3 staining was only partial in the face of abrogating only one of the pro-survival given the multipronged survival signaling. Use of multiple AKT, ERK and JAK inhibitors was even more effective in restoring relative chemosensitivity of DU-H cells compared to exposure to the AKT inhibitor alone (Supplemental Fig. 5).

Epithelial transitioned prostate tumor cells present a generalized chemoresistance

To evaluate whether the acquired chemoresistance of epithelial transitioned prostate tumor cells is agent-specific or generalized, two additional chemotherapy drugs with distinct modes of action and metabolism, cisplatin and doxorubicin, were used. Similar to CPT, DU-L cells were more responsive in the face of TRAIL co-exposure (Supplemental Fig. 1C). Similar to the findings with CPT, the E-cadherin-expressing DU-H variant presented significantly lower levels of cleaved caspase-3 than the DU-L cells in the face of exposure to cisplatin or doxorubicin (Fig. 5A, B, C). EdU incorporation showed that this chemoresistance was proliferation-independent as previously found (Fig. 5D). Again, p-AKT/ERK increased and p-JAK was resistant to degradation in DU-H cells in the presence of cisplatin- or doxorubicin-TRAIL (Fig. 5E). This chemoprotection was also noted in the face of docetaxel, a front-line therapy for metastatic prostate cancer. Again, this was cell cycle independent (Supplemental Fig. 6A, B). Moreover, docetaxel triggered increased p-ERK and p-AKT activation in DU-H cells when compared to DU-L cells (Supplemental Fig. 6C). Thus, the chemoresistance is cell-intrinsic to a variety of death signals rather than being specific for selected agents.

Prevention of E-cadherin re-expression decreases liver metastasis

The foregoing demonstrated the potential for this chemoresistance to occur in the micrometastatic niche. This requires validation *in vivo*. Intrasplenic injection of tumor cells has been established as a model of liver metastases (35). We inoculated the spleens of NOD/SCID gamma mice with DU145 or PC3 PCa cells and examined both spleens and livers (36) (Supplemental Fig. 7A, B). Haematoxylin and eosin staining revealed PCa tumor nodules were detectable in the spleen at 3 weeks (data not shown); both micro- and macro-tumor nodules were present in both the spleen and liver by 5 weeks. Interestingly, hepatic tumor nodules of DU-L cells demonstrated increased and membrane-localized E-cadherin expression compared to paired spleen tumor nodules. PC3 liver tumors had increased E-cadherin expression with both cytoplasmic and membrane localization (Fig. 6A).

Our model of tumor metastasis has E-cadherin re-expression promoting metastatic seeding (14, 30). Thus, abrogation of this should limit metastases. This was tested by inoculating the spleens with DU-L or DU-H, with or without E-cadherin shRNA to block endogenous expression, and checking for liver metastases. No obvious changes were noted in the rate or number of splenic nodules (Supplemental Fig. 7C). DU-L ectopic injection induced hepatic metastasis in 13 out of 15 mice, whereas the number of mice with metastatic nodules after DU-H inoculation dropped to 4 out of 10 mice ($P < 0.001$ by chi-squared analysis); the mice that presented the nodules had fewer and smaller ones. Surprisingly, decreased E-cadherin by shRNA in DU-H resulted in liver metastasis in 5 out of 6 mice; but again these mice had fewer and smaller metastatic nodules. This was also noted in DU-L cells in which E-cadherin re-expression was prevented by shRNA; herein this decreased hepatic metastases

number and burden (Fig. 6B, C). Interestingly, DU-H induced hepatic nodules had decreased E-cadherin compared to parental cells but still increased over the DU-L main variant (Fig. 6D). Larger tumor nodules were mainly absent of E-cadherin expression (Fig.6E), suggesting a secondary EMT during metastatic progression as is seen clinically (14, 22). Together, these findings implicate low levels of E-cadherin or EMT as promoting efficient tumor dissemination from the primary sites; on the other hand, a high level of E-cadherin or MErT was needed for efficient metastatic survival and at the micro-metastatic stage.

Increased E-cadherin expression renders tumor nodules resistant to chemotherapies

Whether surface E-cadherin expression protected tumor cells from being killed *in vivo* was assessed by treating the mice with chemotherapy. On the 18th day after intrasplenic inoculation, mice were treated with a taxane, a class of chemotherapy suggested by National Comprehensive Cancer Network for patients with advanced PCa. The agent paclitaxel (PTX) was selected due to prior literature in mice for dosing. Mice weight was recorded prior to each drug injection to monitor for side effects; no obvious weight loss (> 5%) was found in any of the mice. The mice were euthanized on day 30 after 5 rounds of PTX treatment (Fig. 7A). As noted above, DU-H cells were more resistant to PTX and TRAIL than DU-L cells *in vitro* regardless of cell cycle phase (Fig. 7B and Supplemental Fig. 7D). In the splenic tumors of untreated mice, DU-H-derived tumors had lower Ki67 levels compared to DU-L; however, about one-third of the tumor cells were in cycle (Fig. 7C).

After treatment with chemotherapy, DU-L-derived splenic tumor nodules presented central necrosis greatly augmented by PTX; DU-H-derived tumors did not show such, in spite of similar spleen tumor nodule burden (Fig. 7D). Cleaved-caspase 3 staining confirmed increased tumor cell apoptosis in DU-L-derived spleen tumor nodules (the central necrotic area does not stain due to cell death) (Fig. 7E).

The situation in the liver also showed the pattern of E-cadherin expression being inversely linked to PTX-killing. Here in the DU-L-derived tumor nodules expressed E-cadherin. The IHC staining of sister sections revealed that tumors with upregulated E-cadherin present less cleaved-capase-3, whereas tumors with downregulated E-cadherin present more cleaved-caspase 3 in the same liver again showing the discordance between E-cadherin and cell death (Fig. 7F and Supplemental Fig. 8A). Further, E-cadherin shRNA expressing DU-H derived spleen tumors, with reduced E-cadherin levels did not shown increased apoptosis when growing, but this reduction in E-cadherin led to increased chemosensitivity compared to control shRNA (Supplemental Fig. 8B).

AKT-abrogation partially reverses the E-cadherin protection from chemotherapy-induced apoptosis

We next sought to determine whether the chemo-protective effect of E-cadherin related to the activation of pro-survival intermediary kinases. Phosphor-ERK increased in the DU-L derived E-cadherin-expressing hepatic tumor nodules upon chemotherapy exposure. On the other hand, the nodules that did not express E-cadherin failed to show p-ERK staining; this was similar to the staining in the absence of chemotherapy challenge. Phosphor-AKT was undetectable *in vivo*, which could due to transient activation or limited detection levels.

However, both AKT1 and AKT2, two primary AKT isoforms involved in cell survival, increased significantly in E-cadherin-expressing hepatic tumor nodules (Supplemental Fig. 8C, D). Given ERK also acts during induction of EMT, which leads to E-cadherin downregulation and would thus confound analyses, we subsequently disrupted PI3K-AKT kinase cascade by the PI3K inhibitor LY294002 (28). LY294002 itself did not affect the tumor cells, neither by decreasing burden (data not shown) nor by inducing apoptosis. When LY294002 was added to PTX, dramatically more cleaved-caspase3 was noted in the tumor nodules; abrogation of AKT was synergistic with PTX treatment (Fig. 8A, B). This synergistic effect of AKT abrogation was also found in DU-H splenic tumor nodules, which did not respond to chemotherapy (or AKT inhibitor) alone (Fig. 8C). These findings indicated abrogation of PI3K-AKT at least partially reversed E-cadherin related protection *in vivo* in addition to its effect *in vitro*.

Discussion

The liver is the most common site of solid tumors metastases (37). Metastasis of prostate cancer to the liver is not uncommon, being the third most common site after bone and lung, with the incidence of clinically evident liver metastases as high as 25%, with a greater number of patients with clinically silent metastases found at autopsy (1, 9, 38). Importantly the prognosis when these metastases are found is poor at only 6 months (9, 10, 39). Liver metastases are by and large androgen-independent, rendering the main therapy for PCa irrelevant. Unfortunately, these tumors not only present a high potential to spread but also develop chemoresistance (40). Thus, standard chemotherapy is not generally used as disseminated PCa respond poorly (41-43). Given that dissemination has likely occurred by the time of first detection, we need to develop novel treatment approaches that target PCa in the metastatic niche, and that in the liver microenvironment in particular.

The metastatic microenvironment of the liver confers enhanced tumor survival in the face of chemotherapeutic drugs and local death cytokines. The tumor cells are located within a heterogeneous population of cells, which could be involved in the tumor chemoresistance via several mechanisms (44). Hepatic detoxification of the agents does not account for resistance to non-metabolized agents (such as platins) and the fact that the primary nodules often respond while the metastases are resistant. A more promising aspect is that during ectopic seeding the host parenchymal cells induce tumor cells to undergo MErT (22, 26, 27, 29). While this occurs also with other organs, the hepatocyte is striking in its ability to drive this phenotypic switch. Chemoresistance is a relative term that denotes a ‘right-shift’ in the dose-response curve, not an absolute inefficacy of any agent. This reflects the patient situation where dose escalation is limited by usually tight therapeutic indices. The relative chemoresistance could be achieved in E-cadherin positive tumor cells in in vitro 2D culture arguing that this resistance property is intrinsic to the epithelial-like cancer cells (Fig. 2). Resident hepatocytes were only required to induce tumor cells epithelial transition in this model. Once E-cadherin was re-expressed on the surface to establish cell-cell contacts, tumor cells were rendered chemoresistant. Additionally, in mouse xenograft model, DU-H derived splenic tumor nodules strongly resist the induced cell death stimuli (Fig. 7D, E) further implicating the E-cadherin-expressing phenotype in chemoresistance.

Carcinoma cells undergo reversible phenotypic switches between epithelial and mesenchymal through the dissemination progression (14, 17, 45). The mesenchymal phenotype enables the cells to break from the primary lesion and spread, whereas the epithelial phenotype is re-attained in order to efficiently seed and colonize inhospitable tissues. These transitions are dynamics and often partial, with cells in both phenotypes presenting both epithelial and mesenchymal markers. E-cadherin presence in tumor metastases always marks epithelial phenotype (30)., and is categorized as a tumor suppressor based on limiting tumor cell invasion (46-48). At the beginning of spread, epithelial carcinoma cells non-genetically downregulate E-cadherin to allow for migration. Recent evidence suggests that E-cadherin promotes cancer progression and is thus not irreversibly dispensable (49), so that it can be re-expressed to aid in tumor metastatic seeding and survival (23, 26, 50). A similar phenotypic shifting was observed in our mouse xenograft model. DU145 cells expressing E-cadherin (DU-H) grew in the primary splenic site but only poorly metastasized with just four in ten mice showing evidence of liver metastases; interestingly the metastatic nodules of these tumors presented areas absent of E-cadherin implying an EMT to disseminate. The DU-L subline that appears mesenchymal in the spleen disseminated more efficiently, with multiple E-cadherin-expressing nodules evident in the livers (Fig. 6).

In vitro it was easy to validate that surface E-cadherin expression limited killing by death cytokines and chemotherapies (26). This could be due to limited proliferation of the epithelial-like tumor cells or active pro-survival signaling. The novel observation herein was that the protection from killing was independent of proliferative or cell cycling status. Rather, surface E-cadherin-expressing cells were relatively chemoresistant regardless of Ki67/EdU positivity, and the cells lacking E-cadherin were apoptotic even if not cycling. Concomitant with this finding, several intermediary kinases that are known to promote cell survival, ERK and AKT in particular, were activated either basally or in response to death inducers but only in the cells expressing E-cadherin on their surfaces.

This suggested that the MErT phenotypic shift that promotes metastatic seeding and survival could also drive chemoresistance. In the animal model of paired primary (splenic) and metastatic (hepatic) tumor nodules, chemotherapy induced cell apoptosis dependent on the E-cadherin status; there was minimal killing of DU-H tumors in the spleen and DU-L tumors in the liver, tumors with surface E-cadherin. That this chemoresistance was the result of E-cadherin signaling via canonical survival pathways was demonstrated by abrogating one such pathway. We challenged these tumors with low levels of a PI3K inhibitor at doses that did not affect the tumor growth or survival. This was attempted as ‘therapeutic’ levels of such inhibitors that directly limit cancer cell growth or induce apoptosis have proven toxic, while combination with standard chemotherapies show promise of enhancing the effects in early clinical trials. This seemingly ineffective dose greatly augmented the efficacy of the taxane chemotherapy on these metastatic nodules. This finding is distinct from other studies in which such ERK and AKT pathway inhibitors are tested for directed effects. As such therapies are often toxic due to the generalized nature, this low doses treatment regimen as a synergistic adjuvant for standard chemotherapy represents a novel approach to recalcitrant micrometastases.

This leads to the question of relevance to the human situation. We have previously published that E-cadherin can be found on metastatic nodules of PCa even when the primary tumor is essentially devoid of E-cadherin (50, 51). This was particularly evident in small metastases, whereas larger one that became clinically evident appeared to undergo a second mesenchymal transition, or re-EMT (Fig. 6E) (14, 22). Additional evidence for the translation to men with PCa is found in analysis of data from The Cancer Genome Atlas (TCGA) project. Though the correlation did not rise to the level of statistical significance due to the few patients and heterogeneity of primary PCa, E-cadherin expression slightly declined with inclined Gleason score (Supplemental Fig. 9A). Moreover, according to the statistical data based on the primary tumor, the higher E-cadherin level was found in patients with recurrence of local tumors, and lower E-cadherin level was found in patients with distant metastasis (Supplemental Fig. 9B). Such epithelial-mesenchymal plasticity also is noted in other cancers that metastasize to the liver, including breast (14, 18, 22, 23) and colorectal carcinomas (Supplemental Fig. 10). While such expression has been reported in histopathological specimens, as the epithelial phenotype is proposed to be transient during the stage of micrometastasis, the dynamic shifts can only be ascertained in *ex vivo* and animal models.

Having established that E-cadherin contributes, at least in part, to the relative chemoresistance of disseminated cancer, the mechanistic basis was sought. Despite the association between E-cadherin expression and reduced cell proliferation, this could not explain the chemoresistance (Fig. 3). Rather, it appears to involve active signaling. E-cadherin has been shown trigger the intracellular PI3K/AKT and ERK pathway; two pro-survival pathways (52). Our findings confirmed that E-cadherin positive tumor cells resist death by activating overall canonical survival related-kinase e.g. AKT, ERK and JAK (Fig. 4 and 5). Interestingly, this signaling was modulated by the toxic challenge itself. In E-cadherin-expressing cells, the combination of TRAIL with a chemotherapy actually increased activation of the survival pathways, whereas in E-cadherin-negative cells, this same challenge decreased the survival signaling. The general activation of canonical kinases supposedly is induced by upstream factor(s) which could induce robust cellular signaling cascade such as growth factors or secondary messengers. Initial experiments found that the activation was dependent on the intracellular calcium flux but not EGFR (data not shown). Thus, these signaling cascades, all of which have agents in clinical trials, may serve as adjuvant targets to increase the efficacy of known chemotherapies.

Two limitations impact this study. First, prostate cancer disseminates to a multitude of organs in addition to the liver. Lung is also an epithelial organ wherein we have evidence for epithelial transition of mesenchymal carcinoma cells driven by the parenchymal cells *in vitro, in vivo* and in patients (22, 27, 53). However, bone marrow and brain are mesenchymal organs that do not present E-cadherin on their resident cells for homotypic ligation and signaling. Surprisingly, even in these sites, one finds small tumor nodules that express E-cadherin (22, 23). Still, the findings herein need to be validated in other metastatic sites, a project that lies beyond the scope of the present communication.

Second, primary tumors tend to respond, though usually not curative, to chemotherapies despite expressing surface E-cadherin. Thus, there are likely to be other factors in addition to

E-cadherin that contributes to the relative chemoresistance. Suffice to say that much of signaling comes from the micrometastatic microenvironment as tumor cells in a 3D liver tissue display much greater relative resistance (i.e. a further ‘right shift’) than forced expression of E-cadherin in the cancer cells themselves; this was not due to hepatocyte detoxification of the agent as downregulation of E-cadherin in the tumor cells restored full chemosensitivity (54). Discerning these factors or events is the subject of ongoing investigations.

Taken together, our findings indicate that E-cadherin protected epithelial-transitioned prostate tumor cells from chemotherapy or stressed microenvironment in a proliferation-independent manner. E-cadherin mediated general pro-survival kinases activation in tumor cells when challenged with chemotherapy or local inflammatory factors. Hence, inhibiting these intermediate kinases abrogated the chemoresistance of the MErT PCa cells. This suggests that treatment of these signaling nodes could be an adjuvant for standard chemotherapy in disseminated disease to attack the lethal liver micrometastases of androgen-independent prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MErT	mesenchymal to epithelial reverting transition
PCa	prostate cancer
AIPC	androgen-independent prostate cancer
EMT	epithelial-mesenchymal transition
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated protein kinases
PKB/AKT	protein kinase B
JAK	janus kinase
TRAIL	TNF-related apoptosis-inducing ligand
JNK	c-jun N-terminal kinases
PARP	poly ADP ribose polymerase

EdU 5-ethynyl-2’-deoxyuridine

References

1. Neoplastic Diseases (Saunders). W.B. Sauders; philadelphia: 1922. J. E. Tumors of the Prostate..
2. Shah NL, Sanda M. Health-related quality of life in treatment for prostate cancer: looking beyond survival. Support Cancer Ther. 2004; 1:230–236. [PubMed: 18628147]
3. Meads MB, Gatenby RA, Dalton WS. Environment-mediated drug resistance: a major contributor to minimal residual disease. Nat Rev Cancer. 2009; 9:665–674. [PubMed: 19693095]
4. Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene. 2010; 29:4741–4751. [PubMed: 20531305]
5. Wilson TR, Fridlyand J, Yan Y, Penuel E, Burton L, Chan E, Peng J, et al. Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. Nature. 2012; 487:505–509. [PubMed: 22763448]
6. Kim SJ, Uehara H, Yazici S, He J, Langley RR, Mathew P, Fan D, et al. Modulation of bone microenvironment with zoledronate enhances the therapeutic effects of STI571 and paclitaxel against experimental bone metastasis of human prostate cancer. Cancer Res. 2005; 65:3707–3715. [PubMed: 15867366]
7. Mahon KL, Henshall SM, Sutherland RL, Horvath LG. Pathways of chemotherapy resistance in castration-resistant prostate cancer. Endocr Relat Cancer. 2011; 18:R103–123. [PubMed: 21565970]
8. Shah RB, Mehra R, Chinnaiyan AM, Shen R, Ghosh D, Zhou M, Macvicar GR, et al. Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. Cancer Res. 2004; 64:9209–9216. [PubMed: 15604294]
9. Bubendorf L, Schopfer A, Wagner U, Sauter G, Moch H, Willi N, Gasser TC, et al. Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. Hum Pathol. 2000; 31:578–583. [PubMed: 10836297]
10. Wang H, Li B, Zhang P, Yao Y, Chang J. Clinical characteristics and prognostic factors of prostate cancer with liver metastases. Tumour Biol. 2014; 35:595–601. [PubMed: 23959476]
11. Di Lorenzo G, Buonerba C, Autorino R, De Placido S, Sternberg CN. Castration-resistant prostate cancer: current and emerging treatment strategies. Drugs. 2010; 70:983–1000. [PubMed: 20481655]
12. Hotte SJ, Saad F. Current management of castrate-resistant prostate cancer. Curr Oncol. 2010; 17(Suppl 2):S72–79. [PubMed: 20882137]
13. Fischer KR, Durrans A, Lee S, Sheng J, Li F, Wong ST, Choi H, et al. Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. Nature. 2015; 527:472–476. [PubMed: 26560033]
14. Gunasinghe NP, Wells A, Thompson EW, Hugo HJ. Mesenchymal-epithelial transition (MET) as a mechanism for metastatic colonisation in breast cancer. Cancer Metastasis Rev. 2012; 31:469–478. [PubMed: 22729277]
15. Hugo H, Ackland ML, Blick T, Lawrence MG, Clements JA, Williams ED, Thompson EW. Epithelial--mesenchymal and mesenchymal--epithelial transitions in carcinoma progression. J Cell Physiol. 2007; 213:374–383. [PubMed: 17680632]
16. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009; 119:1420–1428. [PubMed: 19487818]
17. Wells A, Chao YL, Grahovac J, Wu Q, Lauffenburger DA. Epithelial and mesenchymal phenotypic switchings modulate cell motility in metastasis. Front Biosci (Landmark Ed). 2011; 16:815–837. [PubMed: 21196205]
18. Thompson EW, Haviv I. The social aspects of EMT-MET plasticity. Nat Med. 2011; 17:1048–1049. [PubMed: 21900919]
19. Thompson EW, Newgreen DF, Tarin D. Carcinoma invasion and metastasis: a role for epithelial mesenchymal transition? Cancer Res. Discussion 5995;2005 65:5991–5995. [PubMed: 16024595]

20. Kienast Y, von Baumgarten L, Fuhrmann M, Klinkert WE, Goldbrunner R, Herms J, Winkler F. Real-time imaging reveals the single steps of brain metastasis formation. Nat Med. 2010; 16:116–122. [PubMed: 20023634]
21. Luzzi KJ, MacDonald IC, Schmidt EE, Kerkvliet N, Morris VL, Chambers AF, Groom AC. Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. Am J Pathol. 1998; 153:865–873. [PubMed: 9736035]
22. Chao Y, Wu Q, Acquafondata M, Dhir R, Wells A. Partial mesenchymal to epithelial reverting transition in breast and prostate cancer metastases. Cancer Microenviron. 2011; 5:19–28. [PubMed: 21892699]
23. Kowalski PJ, Rubin MA, Kleer CG. E-cadherin expression in primary carcinomas of the breast and its distant metastases. Breast Cancer Res. 2003; 5:R217–222. [PubMed: 14580257]
24. Putzke AP, Ventura AP, Bailey AM, Akture C, Opoku-Ansah J, Celiktas M, Hwang MS, et al. Metastatic progression of prostate cancer and e-cadherin regulation by zeb1 and SRC family kinases. Am J Pathol. 2011; 179:400–410. [PubMed: 21703419]
25. Yao D, Dai C, Peng S. Mechanism of the mesenchymal-epithelial transition and its relationship with metastatic tumor formation. Mol Cancer Res. 2011; 9:1608–1620. [PubMed: 21840933]
26. Chao Y, Wu Q, Shepard C, Wells A. Hepatocyte induced re-expression of E-cadherin in breast and prostate cancer cells increases chemoresistance. Clin Exp Metastasis. 2011; 29:39–50. [PubMed: 21964676]
27. Li M, Aliotta JM, Asara JM, Wu Q, Dooner MS, Tucker LD, Wells A, et al. Intercellular transfer of proteins as identified by stable isotope labeling of amino acids in cell culture. J Biol Chem. 2009; 285:6285–6297. [PubMed: 20026604]
28. Ma B, Wells A. The mitogen-activated protein (MAP) kinases p38 and extracellular signal-regulated kinase (ERK) are involved in hepatocyte-mediated phenotypic switching in prostate cancer cells. J Biol Chem. 2014; 289:11153–11161. [PubMed: 24619413]
29. Yates CC, Shepard CR, Stolz DB, Wells A. Co-culturing human prostate carcinoma cells with hepatocytes leads to increased expression of E-cadherin. Br J Cancer. 2007; 96:1246–1252. [PubMed: 17406365]
30. Wells A, Yates C, Shepard CR. E-cadherin as an indicator of mesenchymal to epithelial reverting transitions during the metastatic seeding of disseminated carcinomas. Clin Exp Metastasis. 2008; 25:621–628. [PubMed: 18600305]
31. Pece S, Chiariello M, Murga C, Gutkind JS. Activation of the protein kinase Akt/PKB by the formation of E-cadherin-mediated cell-cell junctions. Evidence for the association of phosphatidylinositol 3-kinase with the E-cadherin adhesion complex. J Biol Chem. 1999; 274:19347–19351. [PubMed: 10383446]
32. Pece S, Gutkind JS. Signaling from E-cadherins to the MAPK pathway by the recruitment and activation of epidermal growth factor receptors upon cell-cell contact formation. J Biol Chem. 2000; 275:41227–41233. [PubMed: 10969083]
33. Vainchenker W, Constantinescu SN. JAK/STAT signaling in hematological malignancies. Oncogene. 2013; 32:2601–2613. [PubMed: 22869151]
34. Wagner EF, Nebreda AR. Signal integration by JNK and p38 MAPK pathways in cancer development. Nat Rev Cancer. 2009; 9:537–549. [PubMed: 19629069]
35. Kuruppu D, Christophi C, Bertram JF, O'Brien PE. Characterization of an animal model of hepatic metastasis. J Gastroenterol Hepatol. 1996; 11:26–32. [PubMed: 8672738]
36. Ding BS, Nolan DJ, Butler JM, James D, Babazadeh AO, Rosenwaks Z, Mittal V, et al. Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. Nature. 2010; 468:310–315. [PubMed: 21068842]
37. Wells, ACY.; Wu, Q. Biology of Metastatic Liver Tumors. Springer; US: 2011.
38. Lamothe F, Kovi J, Heshmat MY, Green EJ. Dissemination of prostatic carcinoma: an autopsy study. J Natl Med Assoc. 1986; 78:1083–1086. [PubMed: 3795287]
39. Pouessel D, Gallet B, Bibeau F, Avances C, Iborra F, Senesse P, Culine S. Liver metastases in prostate carcinoma: clinical characteristics and outcome. BJU Int. 2007; 99:807–811. [PubMed: 17155968]

40. Harris KA, Reese DM. Treatment options in hormone-refractory prostate cancer: current and future approaches. Drugs. 2001; 61:2177–2192. [PubMed: 11772129]
41. Heidenreich A, Bastian PJ, Bellmunt J, Bolla M, Joniau S, van der Kwast T, Mason M, et al. EAU guidelines on prostate cancer. Part II: Treatment of advanced, relapsing, and castration-resistant prostate cancer. Eur Urol. 2013; 65:467–479. [PubMed: 24321502]
42. Shelley M, Harrison C, Coles B, Staffurth J, Wilt TJ, Mason MD. Chemotherapy for hormone-refractory prostate cancer. Cochrane Database Syst Rev. 2006:CD005247. [PubMed: 17054249]
43. van Brussel JP, Mickisch GH. Multidrug resistance in prostate cancer. Onkologie. 2003; 26:175–181. [PubMed: 12771527]
44. Castells M, Thibault B, Delord JP, Couderc B. Implication of tumor microenvironment in chemoresistance: tumor-associated stromal cells protect tumor cells from cell death. Int J Mol Sci. 2012; 13:9545–9571. [PubMed: 22949815]
45. Wells A, Grahovac J, Wheeler S, Ma B, Lauffenburger D. Targeting tumor cell motility as a strategy against invasion and metastasis. Trends Pharmacol Sci. 2013; 34:283–289. [PubMed: 23571046]
46. Frixen UH, Behrens J, Sachs M, Eberle G, Voss B, Warda A, Lochner D, et al. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. J Cell Biol. 1991; 113:173–185. [PubMed: 2007622]
47. Semb H, Christofori G. The tumor-suppressor function of E-cadherin. Am J Hum Genet. 1998; 63:1588–1593. [PubMed: 9837810]
48. Wong AS, Gumbiner BM. Adhesion-independent mechanism for suppression of tumor cell invasion by E-cadherin. J Cell Biol. 2003; 161:1191–1203. [PubMed: 12810698]
49. Rodriguez FJ, Lewis-Tuffin LJ, Anastasiadis PZ. E-cadherin's dark side: possible role in tumor progression. Biochim Biophys Acta. 2012; 1826:23–31. [PubMed: 22440943]
50. Yates C, Wells A, Turner T. Luteinising hormone-releasing hormone analogue reverses the cell adhesion profile of EGFR overexpressing DU-145 human prostate carcinoma subline. Br J Cancer. 2005; 92:366–375. [PubMed: 15655536]
51. Yates C, Shepard CR, Papworth G, Dash A, Beer Stolz D, Tannenbaum S, Griffith L, et al. Novel three-dimensional organotypic liver bioreactor to directly visualize early events in metastatic progression. Adv Cancer Res. 2007; 97:225–246. [PubMed: 17419948]
52. Reddy P, Liu L, Ren C, Lindgren P, Boman K, Shen Y, Lundin E, et al. Formation of E-cadherin-mediated cell-cell adhesion activates AKT and mitogen activated protein kinase via phosphatidylinositol 3 kinase and ligand-independent activation of epidermal growth factor receptor in ovarian cancer cells. Mol Endocrinol. 2005; 19:2564–2578. [PubMed: 15928314]
53. Furukawa M, Wheeler S, Clark AM, Wells A. Lung epithelial cells induce both phenotype alteration and senescence in breast cancer cells. PLoS One. 2015; 10:e0118060. [PubMed: 25635394]
54. Chao YL, Shepard CR, Wells A. Breast carcinoma cells re-express E-cadherin during mesenchymal to epithelial reverting transition. Mol Cancer. 2010; 9:179. [PubMed: 20609236]

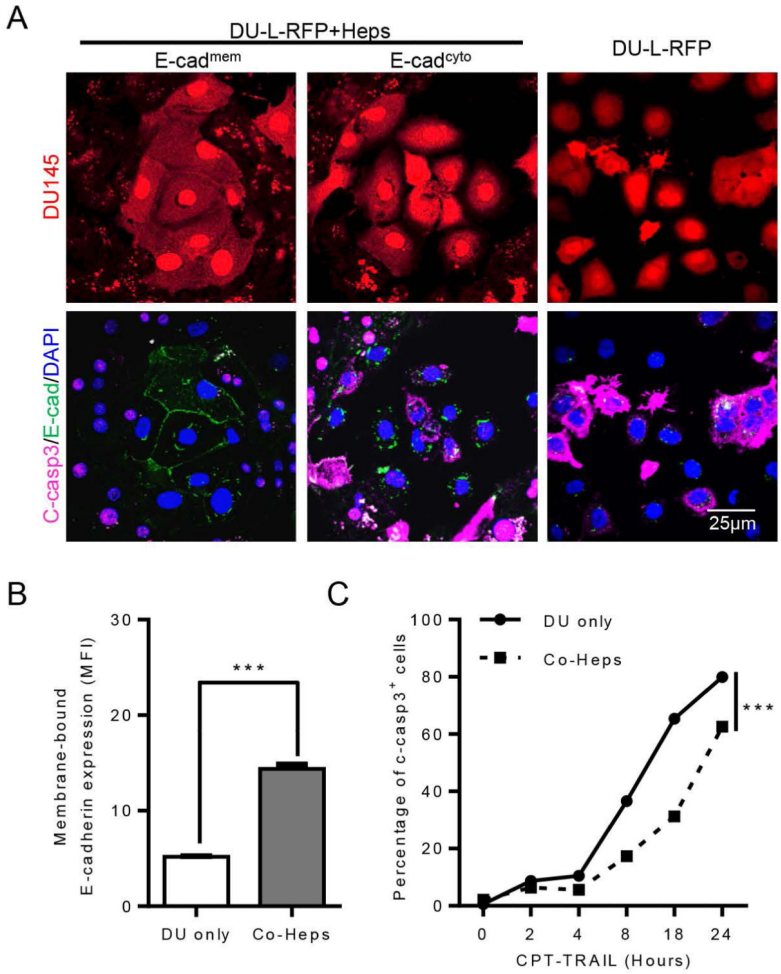

Figure 1.

Hepatocytes increase chemoresistance in DU145 re-expressing E-cadherin. (A) Representative immunofluorescence (IF) images of co-staining cleaved caspase3 (c-casp3, magenta), E-cadherin (E-cad, green), nucleus (DAPI, blue) in RFP labeled DU145-Ecadherin^{low} cells (DU-L-RFP) ± hepatocytes (Heps). Cells were collaborated treated with 1μM camptothecin and 100ng/ml TRAIL (CPT-TRAIL) for 8 hours post 5 days (co-)culture. E-cad^{mem}, membrane-bound E-cadherin; E-cad^{cyto}, cytoplasmic E-cadherin. (B) Mean Fluorescence Intensity (MFI) of E-cadherin membrane expression in DU-L-RFP cells ± hepatocytes post 5 days culture determined by flow cytometry. Student *t*-test, ***, $p<0.001$. (C) Post 5 days culture ± hepatocytes, the percentage of c-casp3 positive DU-L-RFP cells following treatment over a 0-24 hour time course as quantified by flow cytometry. Two-way ANOVA analysis comparing two curves. Data shown as mean ± SD (error bars are contained within the symbols). ***, $p<0.0001$. One representative data out of at least three independent experiments has been shown.

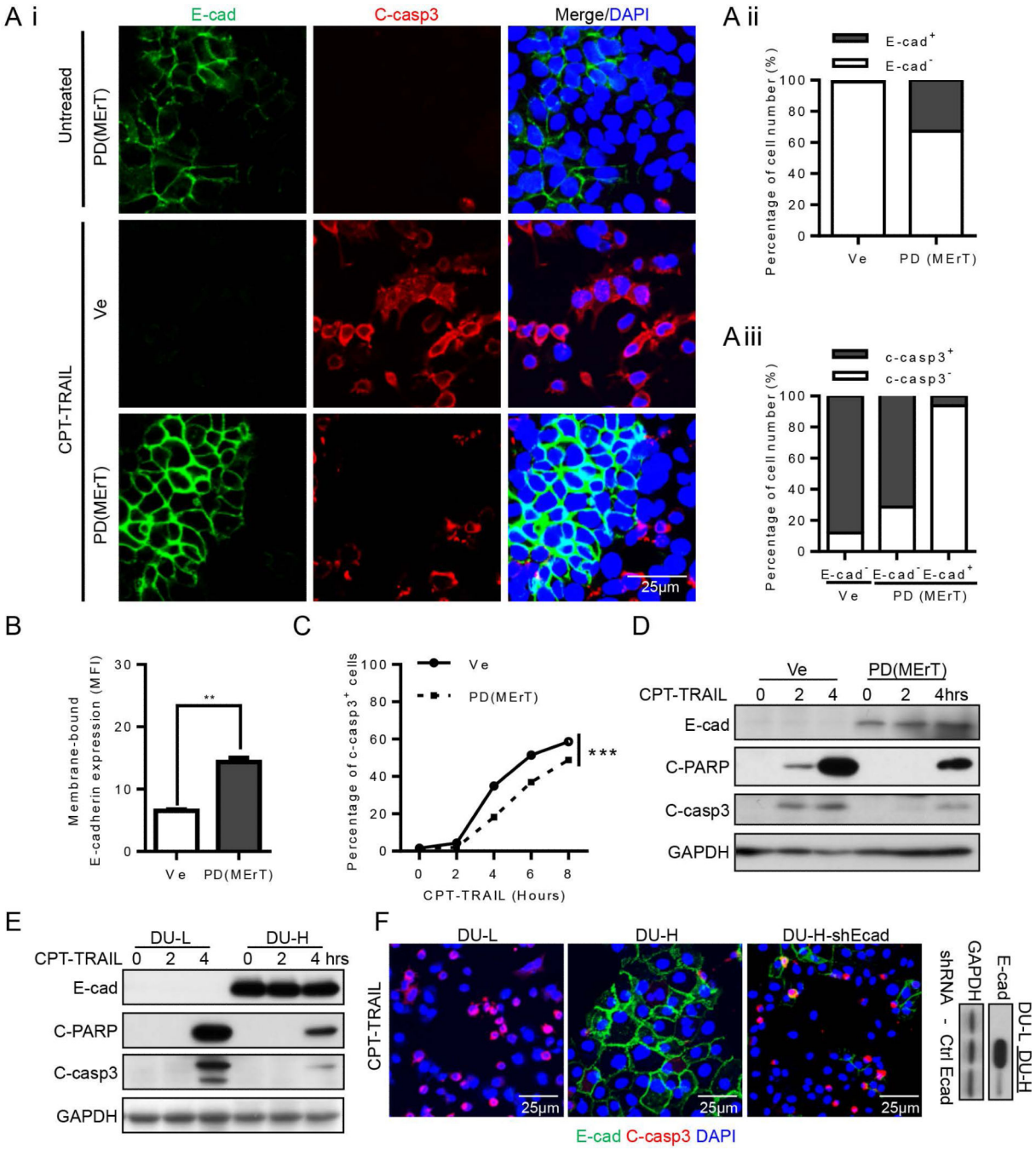

Figure 2.

E-cadherin expressing DU145 cells are resistant to CPT-TRAIL. DU-L cells were pretreated with (PD (MErT)) or without (Ve) 500nM PD153035 for 48 hours to induce MErT for A-D. (Ai) Co-staining of c-casp3 (red), E-cad (green) and nucleus (DAPI, blue) in Ve or PD(MErT)-DU-L cells post 5 hours CPT-TRAIL treatment. (Aii) Quantification of the percentage of cell number of E-cad-null (or in cytosol, E-cad^{-}) and E-cad on the cell membrane (E-cad^{+}). Cell number were determined by counting for 5 fields randomly per stained slide. (Aiii) Quantification of E-cad± and c-casp3± cells in each group. Cell number

were determined by counting for 5 fields randomly per stained slide. (B) MFI of membrane-bound E-cadherin in Ve and PD(MErT)-DU-L cells. Data shown as mean±SD, student *t*-test. **, p=0.002 (C) The percentage of DU-L cells positive for cleaved-caspase-3 (c-casp3^{+}) following treatment with CPT-TRAIL over a 0-24 hour time course as determined by flow cytometry. Data shown as mean±SD. Two-way ANOVA analysis, ***, p<0.0001. (D) Western blot of E-cad, cleaved PARP (c-PARP), c-casp3 in Ve- or PD(MErT)-DU-L cells by CPT-TRAIL, GAPDH as loading control. (E) Western blot of E-cad, c-PARP, c-casp3 in DU-L and DU145-Ecadherin^{high} (DU-H) cells by CPT-TRAIL, GAPDH as loading control. (F) Co-staining of c-casp3 (red), E-cad (green) and nucleus (DAPI, blue) in DU-L, DU-H and DU-H with E-cad shRNA (DU-H-shEcad) cells treated with CPT-TRAIL for 3 hours. Efficacy of intervention is shown by immunoblotting. One representative data out of at least three independent experiments has been shown.

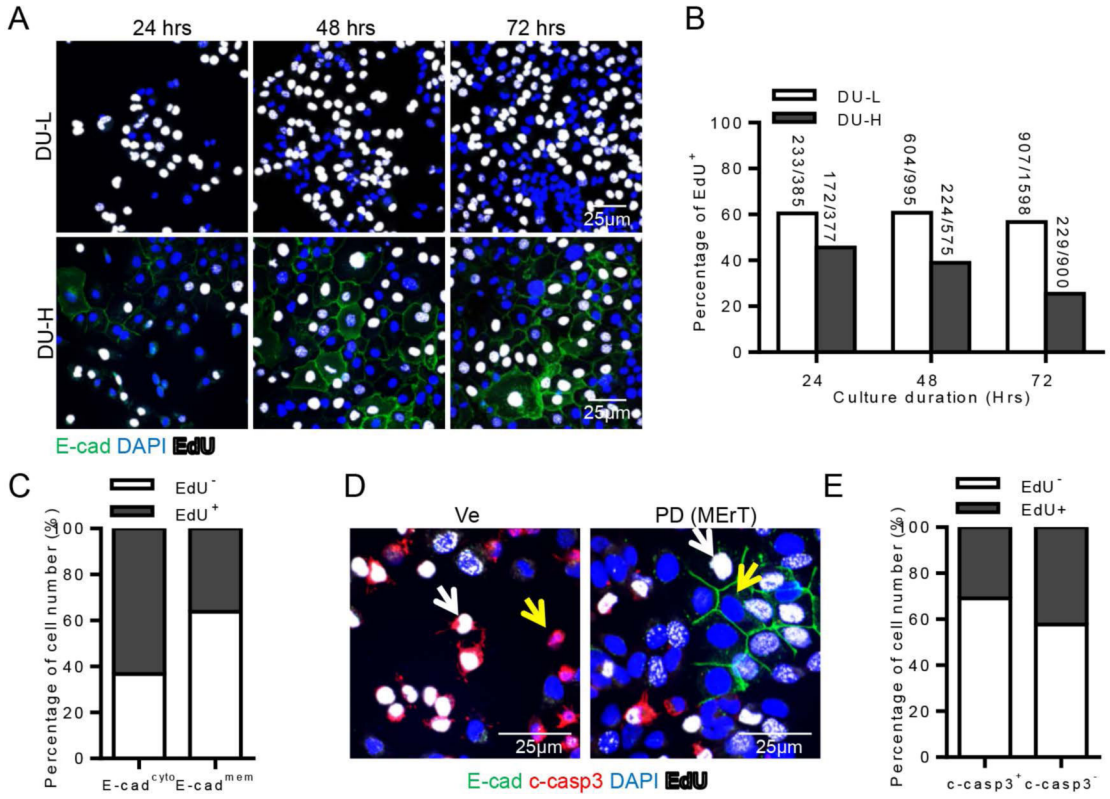

Figure 3.

E-cadherin expression promotes chemoresistance of PCa cells whether proliferating or quiescent. (A) DU-L and DU-H cells were fixed and immunostained after seeded and cultured for 24, 48 or 72 hours. 10μM EdU was added for 6 hours prior to fixation. E-cad: green, EdU: white, DAPI: blue. (B) the percentage of EdU^+ cells in figure 3a were determined by counting for 3 fields randomly per stained slide. (C) The percentage of $EdU^{\pm}$ in DU-L-RFP cells co-cultured with hepatocytes with null or cytoplasmic ($E\text{-}cad^{cyto}$) and membrane-bound ($E\text{-}cad^{mem}$) sub-populations by counting for 13 cancer cell clones. (D) Ve or PD(MErT)-DU-L cells were treated with CPT-TRAIL for 4 hours and 10μM EdU was added simultaneously then fixed. E-cad: green, c-casp3: red, EdU: white, DAPI: blue. White arrow: proliferating cells, yellow arrow: non-proliferating cells. (E) Enumeration and calculation of the percentage of $EdU^{\pm}$ in $c\text{-}casp3^{\pm}$ subgroup by counting for 3 fields randomly per stained slide.

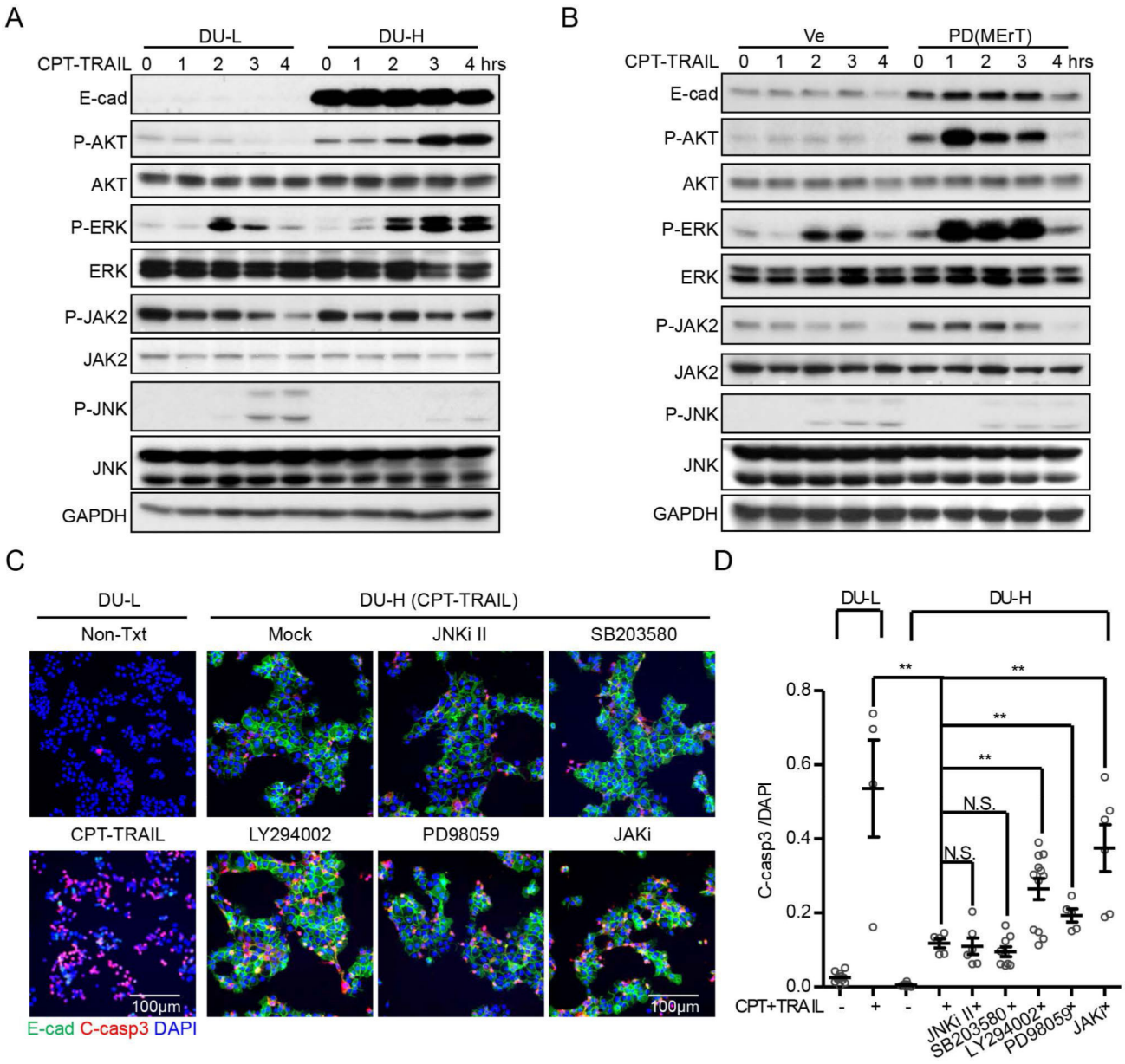

Figure 4.

CPT-TRAIL activates the canonical survival kinases in epithelial tumor cells. Western blot for canonical survival-related intermediate kinases. DU-L and DU-H (A), or PD-induced MErT cells (B) were starved overnight subsequently treated with CPT-TRAIL for indicated time points. (C) DU-H cells were pretreated with kinase inhibitors for 1 hour and washed out before treated with CPT-TRAIL for 4 hours, DU-L ± CPT-TRAIL as controls. Cells were fixed and immunostained with E-cad (green), c-casp3 (red) and DAPI (blue). JNKi II, JNK inhibitor; SB203580, p38 inhibitor; LY294002, PI3K inhibitor; PD98059, MEK1/2 inhibitor; JAKi, JAK inhibitor. (D) C-casp3 (Red) fluorescence density, measured by MetaMorph software, was normalized by DAPI. Paired *t*-test , two tailed p value, **, P<0.01; N.S., non-significant.

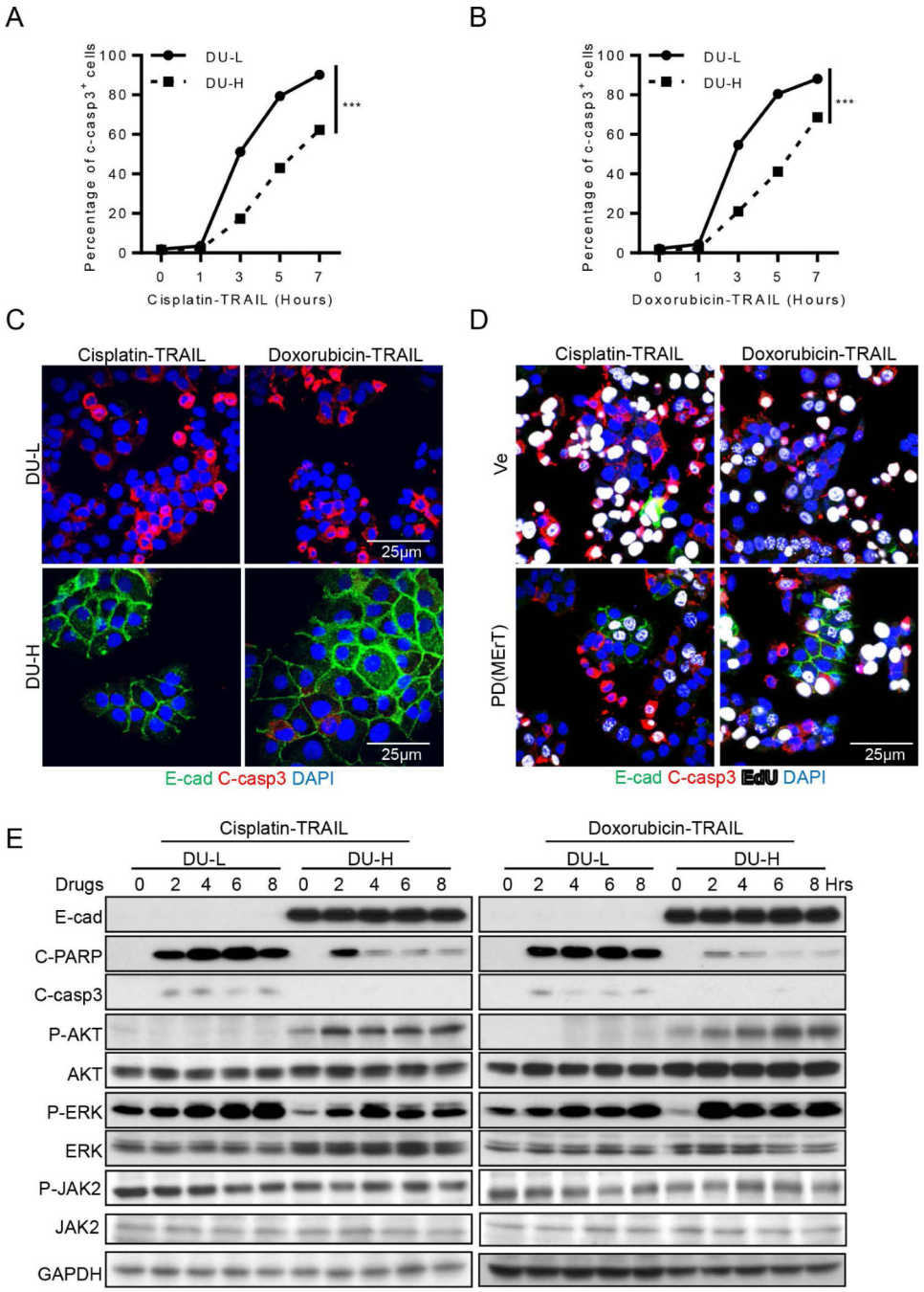

Figure 5.

Epithelial tumor cells resist to cisplatin and doxorubicin. (A), (B) The percentage of c-casp3+ DU-L and DU-H cells post treatment with 20μM cisplatin or 1μM doxorubicin and 100ng/ml TRAIL for indicated time points. Two-way ANOVA analysis, ***, $p<0.001$. (C) Representative pictures of E-cad (green), c-casp3 (red) and DAPI (blue) co-staining for DU-L and DU-H cells post 7 hours treatment with cisplatin or doxorubicin-TRAIL. (D) Representative pictures of E-cad: green, c-casp3: red, EdU: white, DAPI: blue co-staining of Ve or PD(MErT)-DU-L cells post treatment with cisplatin or doxorubicin-TRAIL for 7

hours. 10μM EdU was added for 4 hours prior to treatment. (E) Western blot for c-casp3, c-PARP, p-AKT, AKT, p-ERK, ERK, p-JAK2 and JAK2 for DU-L and DU-H cells treated with cisplatin or doxorubicin-TRAIL for indicated time points.

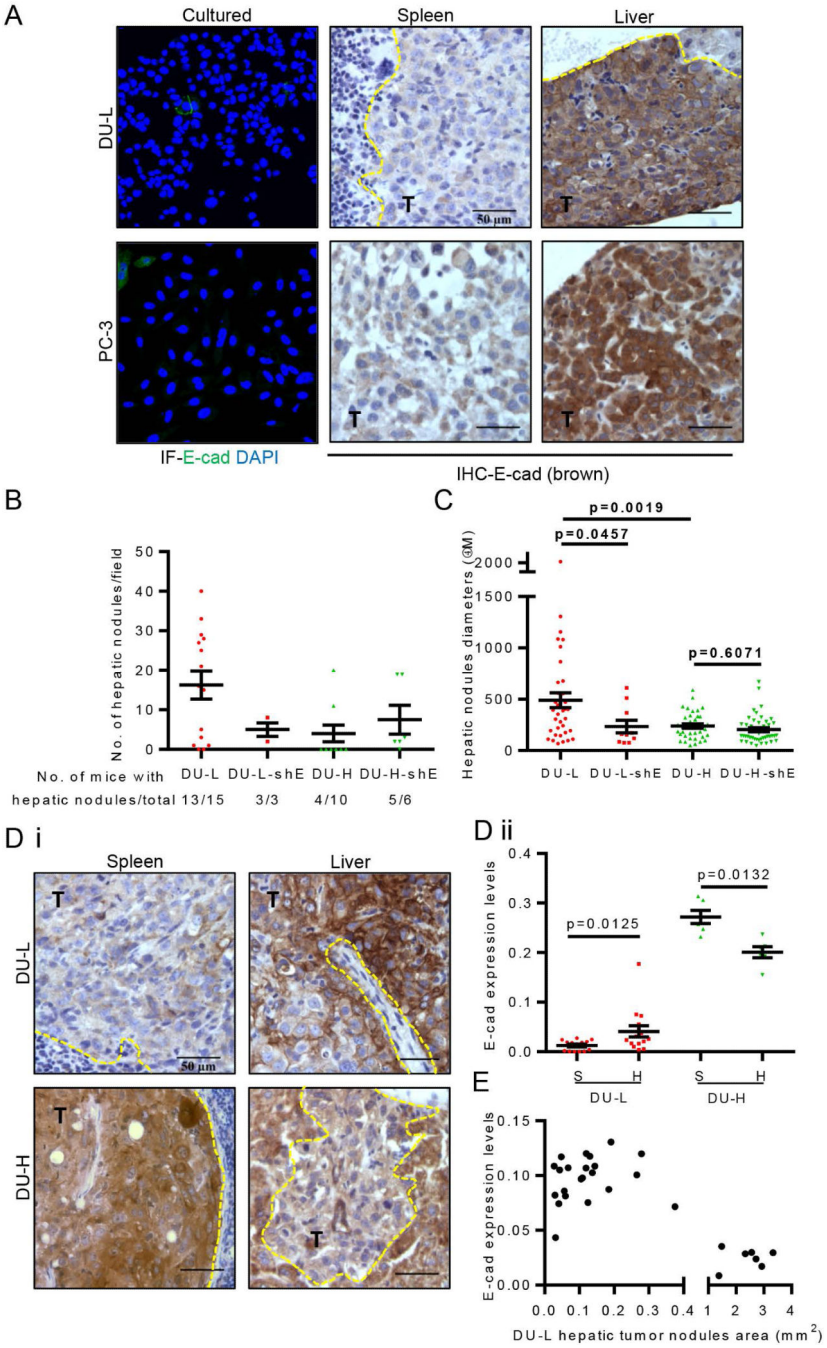

Figure 6.

Re-expression of E-cadherin promotes dissemination to the liver but is metastable as the nodules grow. (A) Representative of E-cad (green) IF staining in DU145 or PC-3 cultured cell line, and E-cad immunohistochemistry(IHC) staining (brown) of DU145 or PC-3 induced splenic and hepatic nodules. (B) Enumeration of hepatic nodules per field (10X magnification), One field was taken per tissue section per mouse. (C) Quantification of hepatic nodules diameters. Each dot represents one tumor nodule. Paired t-test, two-tailed p-value is provided. (Di) Representative E-cad IHC staining of DU-L or DU-H derived splenic

or hepatic tumor nodules. (Dii) Quantification of E-cad expression (quantified with DAB mean values by ImageJ; DAB: 3, 3’ Diaminobenzidine, chromogen for IHC staining) in DU-L or DU-H derived splenic or hepatic tumor nodules. Each dot represents one tumor nodule. Paired t-test, two tailed p values. S, splenic nodules; H, hepatic nodules. (E) Quantification of E-cad expression with DAB value and tumor nodules area. Each dot represents one tumor nodule. T, tumor nodules. Yellow dot line is the tumor nodule's edge. Shown are representative tumor specimens.

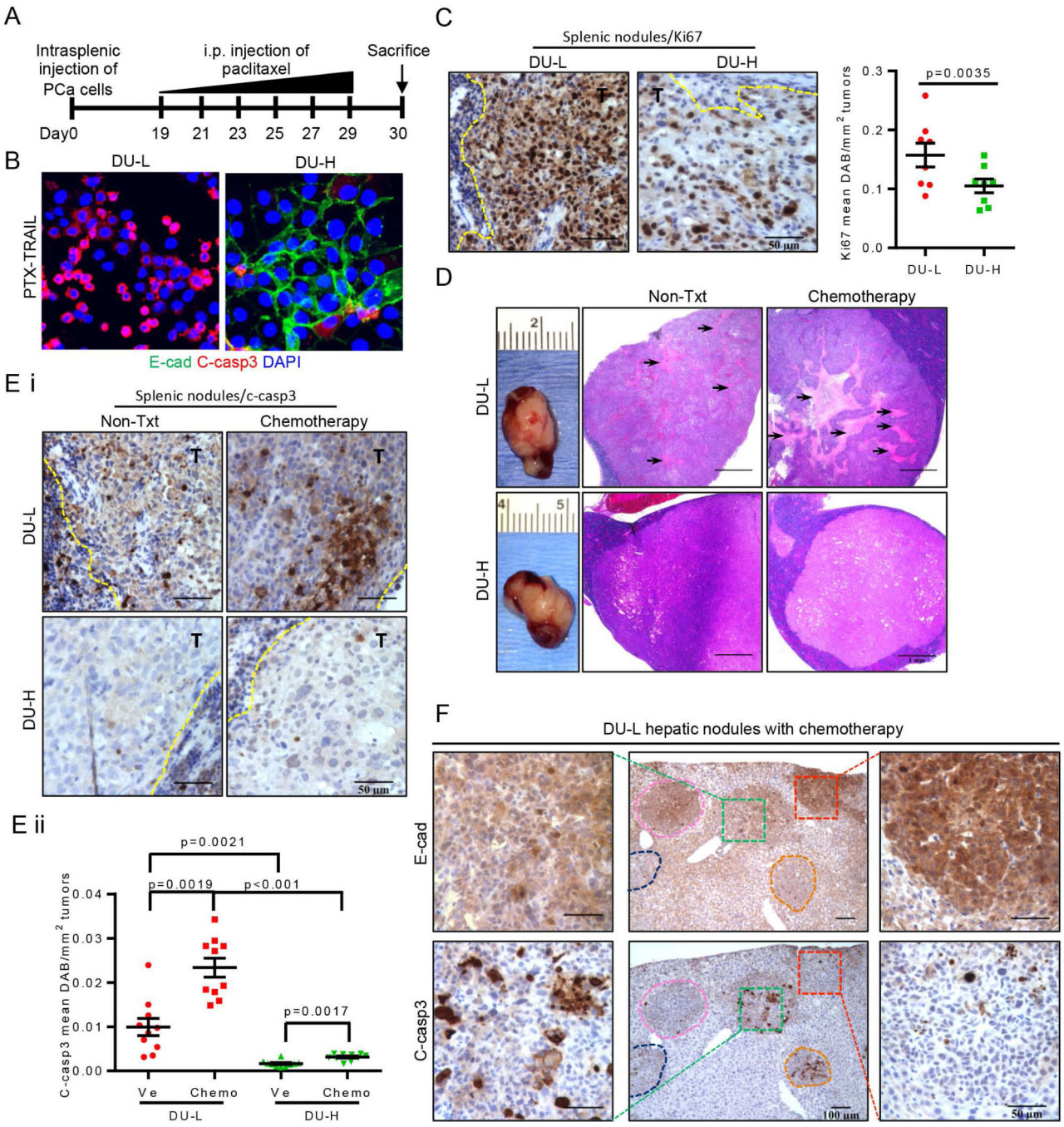

Figure 7.

E-cadherin expression protects the tumor nodules from chemotherapy-induced apoptosis. (A) Schematic of the chemotherapy regimen. (B) Co-staining of E-cad (green), c-casp3(red) and DAPI (blue) in DU-L and DU-H cells after paclitaxel (PTX) and TRAIL treatment. (C) Representative images and quantification of Ki67 expression with DAB mean values in tumors in DU-L or DU-H inoculated spleenPaired *t*-test analysis, two-tailed P value. (D) Representative H&E staining images of DU-L or DU-H inoculated spleen representative images. Arrow: “central necrosis” area. Scale bar=1mm. (Ei) Representative images of c-

casp3 IHC staining in DU-L or DU-H inoculated spleen tumor nodules with or without chemotherapy. (Eii) Quantification of c-casp3 expression with DAB mean values. Paired *t*-test analysis, two-tailed P value. Ve, vehicle control; Chemo, paclitaxel chemotherapy. (F) Representative images of E-cad and c-casp3 on sister sections of the liver. Same tumor on the sister sections is lined by same color. . T, tumor nodule. Yellow dot line is the tumor nodule's edge. Shown are representative tumor specimens.

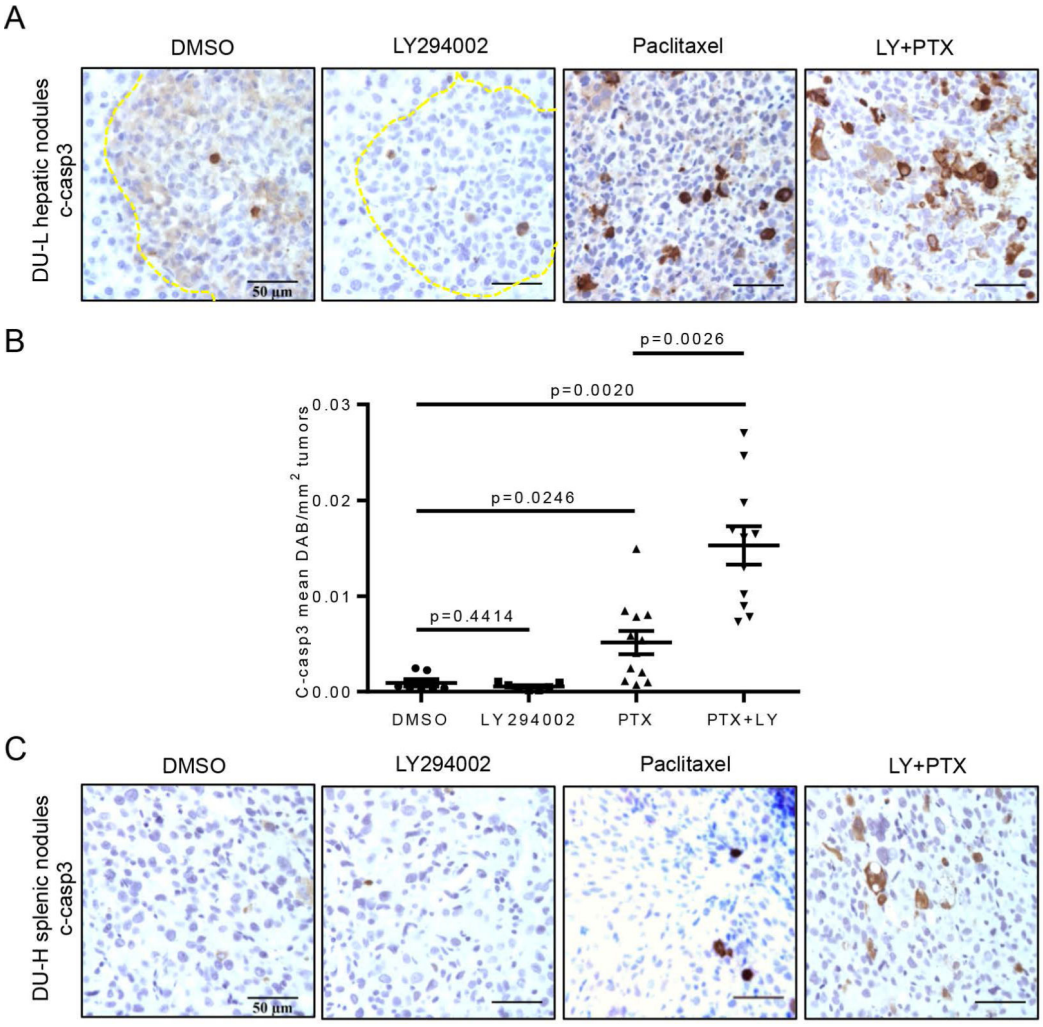

Figure 8.

AKT-abrogation partially reverses the E-cadherin protection from chemotherapy-induced apoptosis. (A) C-casp3 IHC staining (brown) of DU-L hepatic tumor nodules. LY, LY294002; PTX, paclitaxel. Yellow dot line is the tumor nodule's edge. (B) Quantification of c-casp3 expression DAB mean values. Paired *t*-test, two-tailed p value. (C) C-casp3 IHC staining (brown) of DU-H splenic tumor nodules. Shown are representative tumor specimens.