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# Therapy-induced E-cadherin downregulation alters expression of programmed death ligand-1 in lung cancer cells

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## Abstract

**Objectives:** Immunotherapy that targets the programmed death-1 / programmed death-ligand 1 (PD-L1) axis has been approved for treatment of non-small cell lung cancer (NSCLC) patients in many countries. However, our current understanding of the role of immunotherapies on NSCLC patients with *epidermal growth factor receptor* (*EGFR*) mutation, following acquisition of resistance to EGFR tyrosine kinase inhibitors (TKIs), is so far unclear. Especially, there is little data on if each acquired resistance mechanism to EGFR-TKIs alters PD-L1 expression status which is employed as an important predictive biomarker for PD-1/PD-L1 targeting agents.

**Materials and methods:** Lung cancer cell lines (HCC827, HCC4006, PC9, H1975, H358, SW900, and H647) and their daughter cells that acquired resistance to EGFR-TKIs or cytotoxic drugs (cisplatin or vinorelbine) were examined. PD-L1 expression was analyzed by immunohistochemistry, immunoblotting, and/or fluorescent imaging. Published microarray data were also employed to evaluate our findings.

**Results and conclusion:** We found correlations between therapy-induced E-cadherin downregulation and decreased PD-L1 expression using our cell lines and published microarray

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Conflict of Interest statement

T. Mitsudomi has received honoraria from AstraZeneca, Chugai, Boehlinger-ingelheim, Pfizer, and Roche; compensation from AstraZeneca, Chugai, Boehlinger-Ingelheim, Pfizer, Roche, and Clovis Oncology for participating in advisory boards; and research funding (through Kindai University Faculty of Medicine) from AstraZeneca and Chugai. F. R. Hirsch has received compensation from Genentech/Roche, Pfizer, BMS, Lilly, Merck, AstraZeneca, Boehringer-Ingelheim and Ventana/Roche for participating in advisory boards; and has received research funding (through the University of Colorado) from Genentech/Roche, BMS, Lilly, Bayer, Amgen and Ventana/Roche. All other authors declare that they have no conflict of interest related to this study.

data. ShRNA mediated E-cadherin knockdown decreased PD-L1 expression in parental cells, and dual immunofluorescent staining of E-cadherin and PD-L1 suggests co-localization of both molecules. We also observed marked downregulation of PD-L1 in cells with E-cadherin downregulation after chronic treatment with vinorelbine. These results indicate a correlation between therapy-induced E-cadherin downregulation and decreased PD-L1 expression, highlighting the importance of re-biopsy after acquisition of resistance to EGFR-TKIs, not only for the evaluation of resistance mechanisms but also for the determination of PD-L1 expression status.

**Keywords**

*EGFR* mutation; Immunotherapy; Acquired resistance; EGFR-TKIs; Epithelial to mesenchymal transition (EMT); Erlotinib

## 1. Introduction

Lung cancers with *epidermal growth factor receptor* (*EGFR*) mutations are one of the most common molecularly defined subtypes of lung cancers [1]. EGFR tyrosine kinase inhibitors (TKIs) demonstrate initial dramatic efficacy in these patients. However, acquisition of resistance to EGFR-TKIs is almost inevitable after a median of approximately 1 year [2]. Therefore, development and optimization of secondary or later treatments is essential to further improve outcomes for lung cancer patients with *EGFR* mutations. T790M-specific EGFR-TKIs, so called 3$^{rd}$ generation EGFR-TKIs, are a promising strategy to overcome the acquired resistance via *EGFR* T790M secondary mutation that accounts for ~50% of resistance mechanisms [3].

Another approach involves immune checkpoint inhibitors that target programmed death-ligand 1 (PD-L1) or programmed death-1 (PD-1) which have shown dramatic and / or durable responses in a subset of lung cancer patients in clinical trials [4–6]. Although high PD-L1 expression is a predictive biomarker for effective treatment with these drugs [7], immunotherapy proves less effective in lung cancers with *EGFR* mutation [8, 9] despite their higher PD-L1 expression status [10–13]. This demonstrates the need for a better understanding of immune marker expression in *EGFR*-mutant tumors, especially PD-L1 expression status after acquisition of resistance to EGFR-TKIs, including the effect of specific acquired resistance molecular mechanisms on PD-L1 expression.

Regulation of PD-L1 expression in tumor cells is complex and affected by several mechanisms including: PD-L1 genomic gains [14], structural variations of the 3’ region of the *PD-L1* gene [15], oncogenic signaling activation such as AKT serine/threonine kinase (AKT) – mechanistic target of rapamycin (mTOR) pathway [16], Janus kinase (JAK) – signal transducer and activator of transcription (STAT) pathway [17], and mitogen-activated protein kinase 1 (MAPK1) – Jun proto-oncogene, AP-1 transcription factor subunit (JUN) pathway [18]. In addition, PD-L1 expression in tumor cells is influenced by a variety of factors such as release of IFN-gamma from T cells in the tumor microenvironment *in vivo* [19]. Therefore, the first step to elucidate the effect of acquired resistance mechanisms to EGFR-TKIs on the expression of PD-L1 would be the comparison of tumor cells between

drug sensitive parental cells and drug resistant isogenic cells in the absence of the tumor microenvironment. To date, we have established several acquired resistance *in vitro* models from *EGFR*-mutated lung cancer cell lines via chronic exposure to EGFR-TKIs [20–23]. This study focuses on analyzing the effect of acquired resistance mechanisms to EGFR-TKIs on the expression of PD-L1 protein employing these *in vitro* models.

## 2. Materials and methods

### 2.1. Cell lines, reagents, and generation of in vitro resistant cell lines

All human lung cancer cell lines used in this study were obtained or established in our previous studies [20–25]. All cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) and 1× penicillin / streptomycin solution (Mediatech, Inc., Manassas, VA) at 37°C / 5% $CO_2$. T790M-specific EGFR-TKI, AZD9291 and cytotoxic agents (vinorelbine and cisplatin) were purchased from Selleck Chemicals (Houston, TX). H1975-AZD cells, SW900-VNR cells, and H647-CDDP cells were developed via chronic, repeated exposure to AZD9291, vinorelbine and cisplatin, respectively, as described previously [20]. All experiments using acquired resistance cells, including the tissue microarray (TMA) preparation, were performed following removal of drug exposure to avoid the direct effects of drugs on PD-L1 expression.

### 2.2 TMA preparation, antibodies and immunohistochemistry (IHC) analysis

Formalin-fixed paraffin-embedded (FFPE) cell blocks were prepared to make a cell line TMA of drug sensitive parental cells and their acquired resistant descendants. Cultured cells were gently harvested using Accutase (Innovative Cell Technologies, Inc., San Diego, CA) and fixed with alcoholic formalin solution for 24 hours. Fixed cells were mixed with melted agarose solution, allowed to solidify, placed in the cassette, and submerged in 70% ethanol. Paraffin-embedding of the agarose cell pellet was performed at our pathology core lab.

The TMA was sectioned at a thickness of 4 μm, and mounted on charged glass slides. All staining was performed on the Benchmark XT automated stainer (Ventana Medical Systems, Inc., Tucson, AZ) or the Link 48 Autostainer (Dako – Agilent Technologies, Carpinteria, CA). Staining for PD-L1 (SP142, Ventana Medical Systems), E-cadherin (anti-E-cadherin (36) Mouse Monoclonal antibody, Ventana Medical Systems), and total-EGFR (2–18C9, Dako-Agilent Technologies) were performed using respective kit systems. Other antibodies were purchased from Cell Signaling Technology (Danvers, MA) and detailed antibody information was summarized in Table 1. The staining platform utilized the Ultraview development reagents (Ventana Medical Systems, Inc.) or the Envision FLEX visualization system (Dako – Agilent Technologies). PD-L1 staining was assessed by the percentage of positive cells. Other specimens were evaluated using the H-score assessment which combines staining intensity (0–3) and the percentage of positive cells (0–100%) as previously described [26].

### 2.3. Antibodies and western blot analysis

Antibodies against E-cadherin, PD-L1, and beta-actin were purchased from Cell Signaling Technology. Total cell lysates were prepared, and immunoblotting conducted as described

elsewhere [22]. Briefly, cells were cultured until sub-confluent, rinsed with phosphate-buffered saline (PBS), lysed in sodium dodecyl sulfate (SDS) sample buffer and homogenized. The total cell lysate (10 μg) was subjected to SDS polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). PVDF membranes were cut to include the indicated sized protein, therefore full-length blots are not available. After blocking with 5% nonfat dry milk, membranes were incubated with primary antibodies, washed with PBS, reacted with secondary antibodies (Cell Signaling Technology), and signals visualized using ECL reagent (Clarity, Bio-Rad, Hercules, CA) and film.

### 2.4. Short hairpin-mediated knockdown

Lentiviral preparations with a short hairpin RNA (shRNA) that specifically target E-cadherin or a non-targeting control were purchased from UCD Genomics Core. Transfection was performed per established protocol using polybrene (Sigma-Aldrich Corporation, St. Louis, MO), and 10 μg/mL puromycin (Invitrogen, Carlsbad, CA) treatment was used to select for the transfected cells.

### 2.5. Confocal image analysis (immunofluorescence)

Cells were seeded in an 8-well Nunc Lab-Tek II Chamber Slide System (Thermo Scientific, Rochester, NY), and treated the day after with PBS or IFN-gamma (Cell Signaling Technology) for 24 hours. Immunofluorescence was performed according to the manufacture's protocol. Briefly, cells were fixed with 4% formaldehyde, blocked with blocking buffer, and incubated with primary antibodies for E-cadherin (mouse monoclonal) or PD-L1 (rabbit monoclonal) at 4°C overnight. After the washing with PBS, cells were incubated with fluorochrome-conjugated secondary antibodies for anti-rabbit and anti-mouse (Cell Signaling Technology). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Microscopy was performed on an EVOS fluorescent microscope (Model FL, Life Technologies, Carlsbad, CA).

## 3. Results

### 3.1. Differential expression of PD-L1 in HCC4006 erlotinib resistant cells

Initially, we screened PD-L1 expression in lung cancer cell lines by immunohistochemistry (IHC), the gold-standard for PD-L1 expression analysis in the clinic, comparing EGFR-TKI acquired resistant descendants to their parent cells in the absence of EGFR-TKIs (Fig 1A-C). HCC827 cells (del E746_A750) and HCC4006 cells (del L747_E749, A750P) harbor *EGFR* exon 19 deletion mutation, and H358 cells harbor *KRAS* G12C mutation but retain EGFR-TKI sensitivity via higher autocrine production of amphiregulin [27]. We found complete loss of PD-L1 expression in HCC4006 erlotinib resistant (ER) cells, while the parent cells showed high PD-L1 expression (IHC positive cells: 0% vs. 95%, respectively, Fig 1B). While HCC827ER cells, with acquired *MET* gene amplification as a resistance mechanism [20], showed slightly decreased PD-L1 expression compared with the parental HCC827 cells (IHC positive cells: 10% vs. 30%, respectively, Fig 1A). H358ER cells, with insulin-like growth factor 1 receptor activation [22], and the parental H358 cells both harbored < 5 % of PD-L1 positive cells (Fig 1C).

As described in our previous report [21], HCC4006ER cells showed marked downregulation of E-cadherin (Fig 1D) and epithelial to mesenchymal transition (EMT) phenotype, and did not harbor any other candidate acquired resistance mechanisms including T790M secondary *EGFR* mutation, *MET* gene amplification, *ERBB2* gene amplification, *CRKL* gene amplification or PTEN downregulation. Microarray analysis data comparing HCC4006ER cells with the parent HCC4006 cells, that was performed in our previous study [28], also identified decreased expression of PD-L1 mRNA. In addition, we observed decreased expression of B7-H4, while sparing the expression of PD-L2, PD-1, and B7-H3 (Fig 1E).

### 3.2. Role of EGFR and downstream signaling in the decreased expression of PD-L1 in HCC4006ER cells

As described above [16–18], the activation of oncogenic signaling pathways positively regulates the expression of PD-L1 [29]. Therefore, we compared the activation of EGFR and these downstream signaling molecules between HCC4006ER cells and HCC4006 parental cells using IHC. As shown in Table 1, H-scores for total EGFR, phosphorylated EGFR (Y1173), and phosphorylated downstream molecules (MAPK, AKT, S6, STAT3, 4E-BP1) are all similar between HCC4006ER cells and HCC4006 parental cells, confirming our previous results which used western blotting analysis [21, 30]. These results encouraged us to analyze the direct relationship between E-cadherin expression and PD-L1 expression in HCC4006 and HCC4006ER cell line models.

### 3.3 PD-L1 expression in the other “EMT-mediated” EGFR-TKI resistant cells

To generalize our findings in HCC4006ER cells, we searched for microarray data from the literature that reported EMT-mediated acquired resistance to EGFR-TKIs in lung cancers. We identified an additional three acquired resistance cell lines with EMT-features and available microarray data [31–33]. As shown in Fig 1F, three cell lines out of four showed decreased expression of PD-L1 mRNA compared with parental cells. HCC827-ER3 cells, the exception, had AXL overexpression together with EMT-features, and these cells harbor comparably higher E-cadherin expression than the other three cells that had decreased PD-L1 expression.

We also established H1975AZD cells (acquired resistant cells to AZD9291 also known as osimertinib) from H1975 lung cancer cells (EGFR L858R plus T790M mutation). We employed H1975 cells because they developed acquired resistance with decreased E-cadherin to irreversible EGFR-TKIs in previous studies [24, 34]. As expected, H1975AZD cells showed decreased expression of E-cadherin, in addition, H1975AZD cells showed decreased expression of PD-L1 by western blot analysis (Fig 2A). We also examined BRC1 cells (*EGFR* exon 19 deletion with T790M; derivative of PC9 cells) and 853#10 cells that were established from BRC1 cells via treatment with afatinib plus cetuximab *in vivo* [23]. Although 853#10 cells showed EMT-like features; with increased fibronectin and slightly decreased E-cadherin, 853#10 cells showed increased PD-L1 expression compared with their parental BRC1 cells (Fig 2B) similar to HCC827-ER3 cells (Fig. 1F). These data suggest that downregulation of PD-L1 is a general phenomenon in lung cancers with the *EGFR* mutation after acquisition of resistance to EGFR-TKIs if they concurrently acquire marked downregulation of E-cadherin.

### 3.4. Effect of E-cadherin knockdown on PD-L1 expression in lung cancer cells with EGFR mutation

To address the relationship between E-cadherin downregulation and PD-L1 downregulation in lung cancer cells with *EGFR* mutations, we performed shRNA-mediated E-cadherin knockdown in HCC4006 parental cells. We observed decreased PD-L1 expression in HCC4006 cells transfected with E-cadherin shRNA compared with non-targeting shRNA transfected control cells (Fig 2C). A similar phenomenon was observed in HCC827 parental cells (Fig 2D), although the magnitude of PD-L1 decrease was not correlated with the magnitude of E-cadherin knockdown. These results indicate a direct relationship between E-cadherin and PD-L1 expression, but the magnitude of PD-L1 downregulation also depends on the other undetermined factor(s).

### 3.5. Effect of IFN-gamma treatment on parental and resistant cells with E-cadherin downregulation

It is also reported that cytokine signaling from the surrounding tumor microenvironment regulates PD-L1 expression in tumor cells [19]. To mimic an immune cell attack, we treated HCC4006 parental cells and HCC4006ER cells with IFN-gamma and measured PD-L1 expression. IFN-gamma treatment induced increased expression of PD-L1 in both HCC4006 and HCC4006ER cells. However, PD-L1 expression was still lower in HCC4006ER cells compared with HCC4006 parental cells after treatment with IFN-gamma (Figs 2E and 3).

### 3.6. Co-localization assay for E-cadherin and PD-L1

To further evaluate the relationship between E-cadherin and PD-L1 in HCC4006 and HCC4006ER cells, we performed a co-localization assay for both proteins using the EVOS cell imaging system (ThemoFisher Scientific). PD-L1 was expressed at the cell membrane as well as in the cytoplasm in HCC4006 cells (Figs 3A-C), consistent with the IHC results (Fig 1B). Upon treatment with IFN-gamma (Figs 3D-F), E-cadherin was internalized as reported previously [35], and membranous PD-L1 expression was dramatically increased. Interestingly, PD-L1 was also internalized together with E-cadherin in HCC4006 parental cells treated with IFN-gamma (Fig 3F), although its biological implication is unclear.

In contrast, treatment with IFN-gamma did not alter expression of E-cadherin in HCC4006ER cells (Figs 3G and H). PD-L1 expression was increased in IFN-gamma treated HCC4006ER cells; however, overexpressed PD-L1 was mainly located in the cytoplasm (Fig 3H).

We have observed partial expression of PD-L1 in HCC827ER cells (Fig 1A). We also found E-cadherin expression in a part of HCC827ER cells (Fig 4A). The co-localization assay for E-cadherin and PD-L1 identified that a subset of cells expressed both proteins, while the other cells did not express either (Figs 4B-D), further supporting the positive relationship between E-cadherin and PD-L1 in lung cancer cells with acquired resistance to EGFR-TKIs.

### 3.7. Chemotherapy induced EMT also decreased the expression of PD-L1

To expand our findings in chemotherapy induced EMT, we treated SW900 cells (lung squamous cell carcinoma) and H647 cells (lung adenosquamous carcinoma), which showed

moderate expression of PD-L1 in our previous research, with cytotoxic agents including cisplatin (CDDP), gemcitabine, docetaxel, or vinorelbine (VNR). We successfully developed CDDP-resistant H647 cells (H647CDDP cells) and VNR-resistant SW900 cells (SW900VNR cells). As shown in Figs 5A and B, SW900VNR cells were spindle shaped with less cell-cell contact compared with SW900 parental cells. In western blot analysis, SW900VNR cells showed markedly decreased E-cadherin expression and decreased PD-L1 expression (Fig 5C), while H647CDDP cells showed increased expression of both proteins (Fig 5D). These results suggest that chemotherapy-induced E-cadherin downregulation has the same effect on PD-L1 expression, in addition to EGFR-TKI mediated E-cadherin downregulation.

## 4. Discussion

EMT has long been recognized as playing a major role in cancer progression and metastasis via providing increased motility and invasive phenotypes to cancer cells. In addition, EMT is also reported as one of the acquired resistance mechanisms to anti-cancer drugs including cytotoxic agents and EGFR-TKIs [21, 28]. However, despite the wide use of the term “EMT”, the definition of EMT is still under debate.

Regarding the relationship between “EMT” and the expression of PD-L1, recent studies have reported increased PD-L1 expression in “*de novo* mesenchymal-type lung cancers” compared with epithelial-type ones [36–40]. However, these studies cannot elucidate the direct relationship between “EMT” and increased expression of PD-L1 due to many confounding factors. Briefly, it is possible that inherent mesenchymal-like lung cancers are distinct from epithelial-like lung cancers in their backgrounds such as patient smoking history or tumor stage that may also affect the expression status of PD-L1.

Therefore, it is not surprising that we found the opposite relationship between E-cadherin downregulation, the best-known hallmark of EMT, and PD-L1 expression status using isogenic cell line models. In this study, we found that acquired resistant cells with marked downregulation of E-cadherin showed decreased expression of PD-L1. We observed two exceptions; HCC827-ER3 cells (Fig 1F) from published microarray data and 853#10 cells (Fig 2B) that was established in our previous study [23] showed increased PD-L1 expression despite their “EMT” features. However, the magnitude of E-cadherin downregulation was quite small in these two cells compared with the other acquired resistant EMT cell lines with PD-L1 downregulation. Therefore, we consider that E-cadherin expression, but not the other EMT features, determines the expression of PD-L1. ShRNA mediated E-cadherin knockdown (Figs 2C and D) and co-localization assays (Fig 3) support this hypothesis. We also observed the same phenomenon in VNR resistant cells with marked downregulation of E-cadherin (Fig 5), suggesting that the downregulation of PD-L1 was not due to long-term inhibition of EGFR.

In this study, together with an extensive review for previous publication, we could not elucidate the molecular mechanism(s) how therapy-induced E-cadherin downregulation can affect the expression of PD-L1. However, the microarray data [28, 33] showed that toll-like receptor 3 (TLR3) and interferon regulatory factor-1 (IRF1), that positively regulate PD-L1

expression [41, 42], were markedly downregulated in erlotinib-resistant HCC4006 cells with E-cadherin downregulation. In addition, TLR3 and IRF1 were downregulated in HCC4006 cells transfected with E-cadherin shRNA compared with those transfected with non-targeting shRNA (data not shown), further suggesting a role of these molecules in the mechanism for PD-L1 downregulation together with E-cadherin downregulation.

Because PD-L1 expression status is the most significant predictive biomarker for candidates receiving anti-PD-1/PD-L1 antibody drugs, our results suggest reduced effectiveness of immunotherapy in patients who develop a marked downregulation of E-cadherin in response to EGFR-TKIs. Although the microarray data suggests slightly higher expression of PD-L2, another ligand for PD-1, in HCC4006ER cells, the expression level of PD-L2 is lower in lung cancers [43] and the role of PD-L2 as a predictive biomarker for anti-PD-1/PD-L1 therapy is currently unclear. We have shown that HCC4006ER cells were insensitive to anti-microtubule agents that are often used as cytotoxic agents in non-small cell lung cancers [28]. These results may indicate that EMT is one of the most intractable cancer status, and treatment strategies that may prevent EMT (or downregulation of E-cadherin), such as upfront polytherapy as recently reported [30, 44, 45], may be needed in future development of treatment strategies.

In this study, we had no data comparing clinical specimens before and after treatment due to the lack of adequate patients samples. We consider that the changes of immune checkpoint marker expression in patients are quite complicated as reported recently [46, 47]. They are affected by both the tumors themselves (including resistance mechanisms as shown in this study) and the microenvironment, which is itself affected by many factors such as tumor cell immunogenicity, patient characteristics, type of chemotherapeutic agents, and the duration of treatment holiday. We consider that detailed *in vitro* analyses provide important fundamental data regarding the role of resistance mechanisms on alteration of immune checkpoint markers.

In this study, we demonstrated that PD-L1 expression decreased in acquired resistant cells with E-cadherin downregulation. Our results support the importance of re-biopsy after acquisition of resistance to EGFR-TKIs, not only for the assessment of resistance mechanisms but also for the evaluation of PD-L1 expression status.

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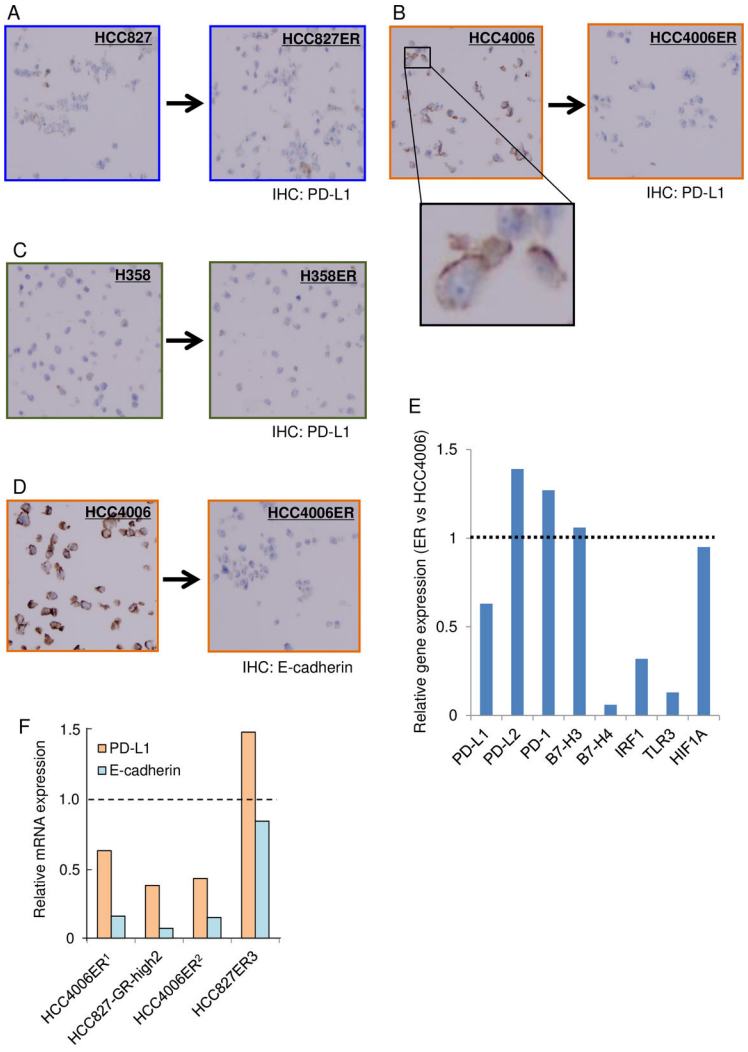



**Figure 1.**

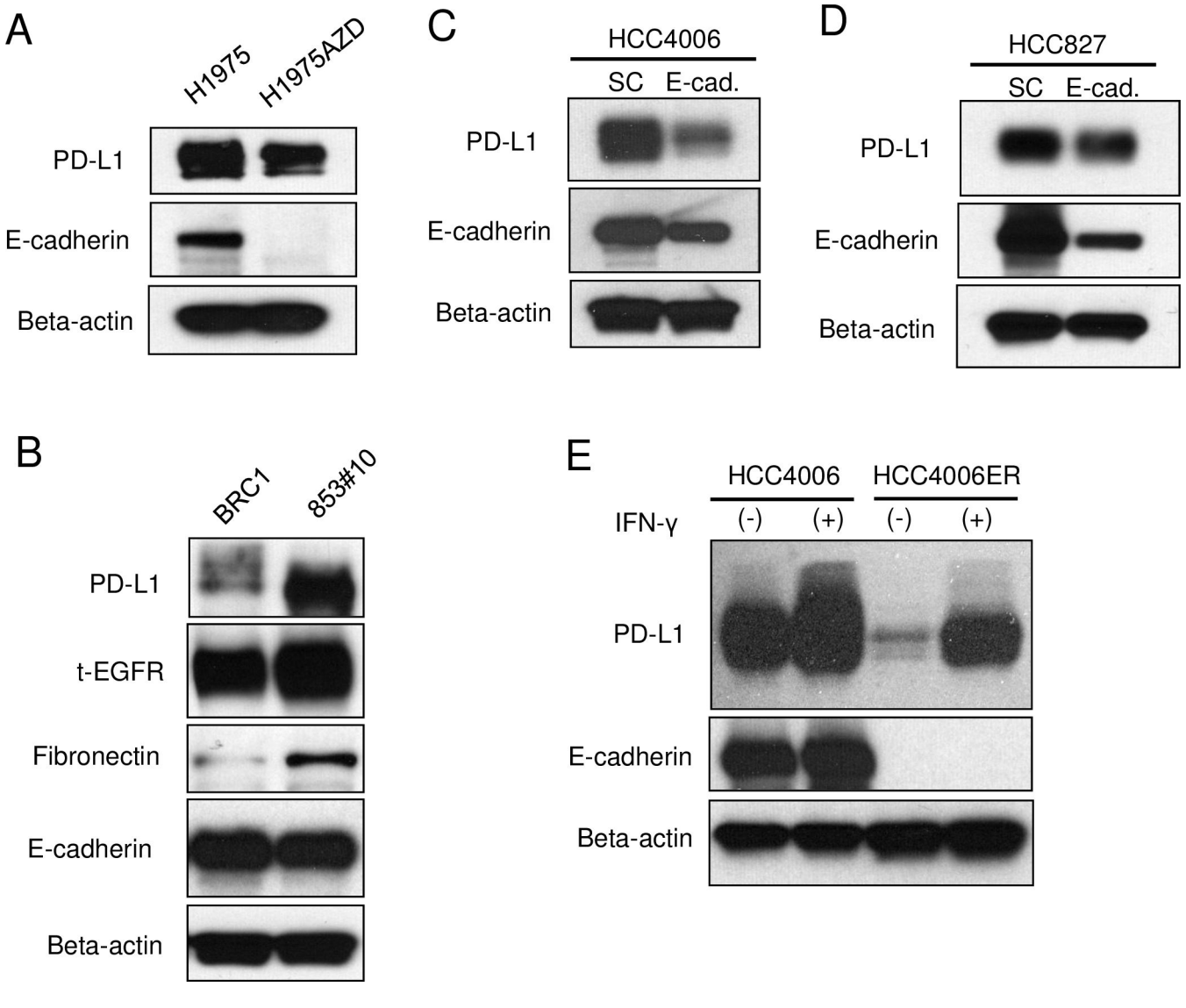



**Figure 2.**

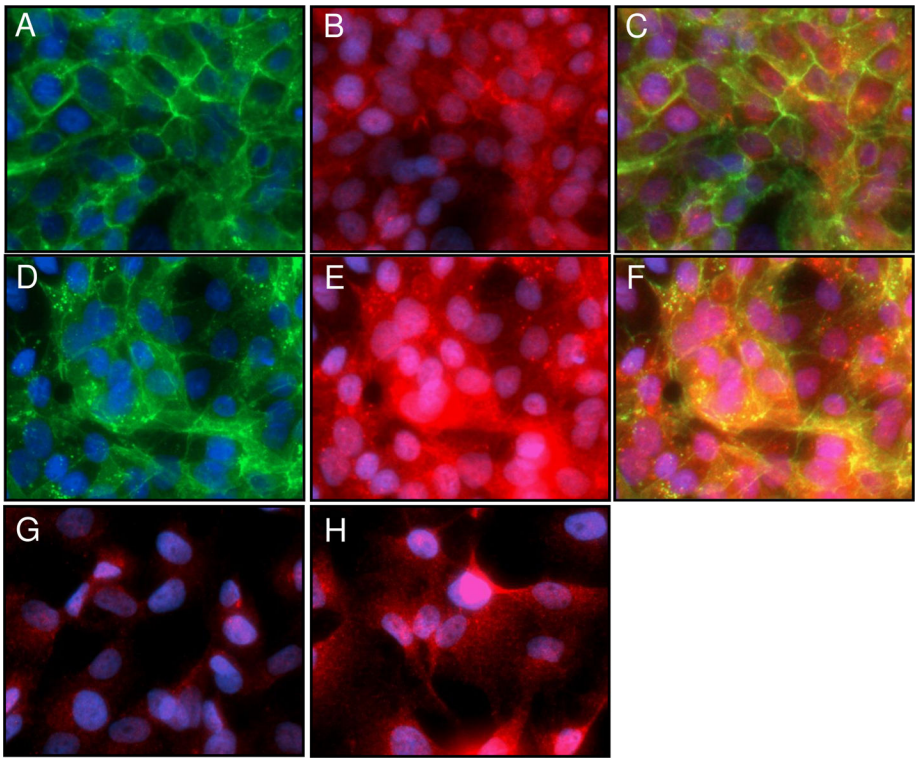
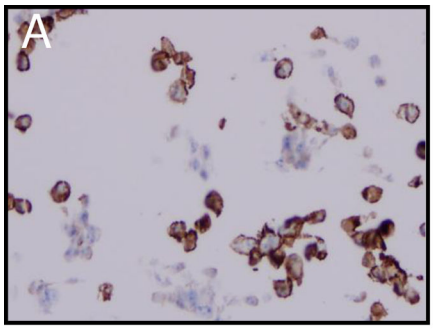



**Figure 3.**




IHC: E-cadherin

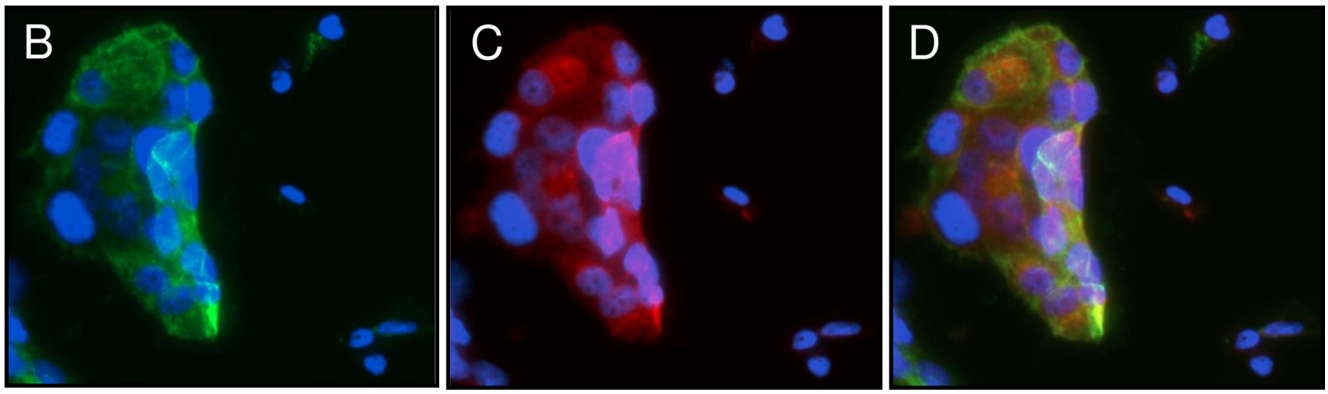



**Figure 4.**

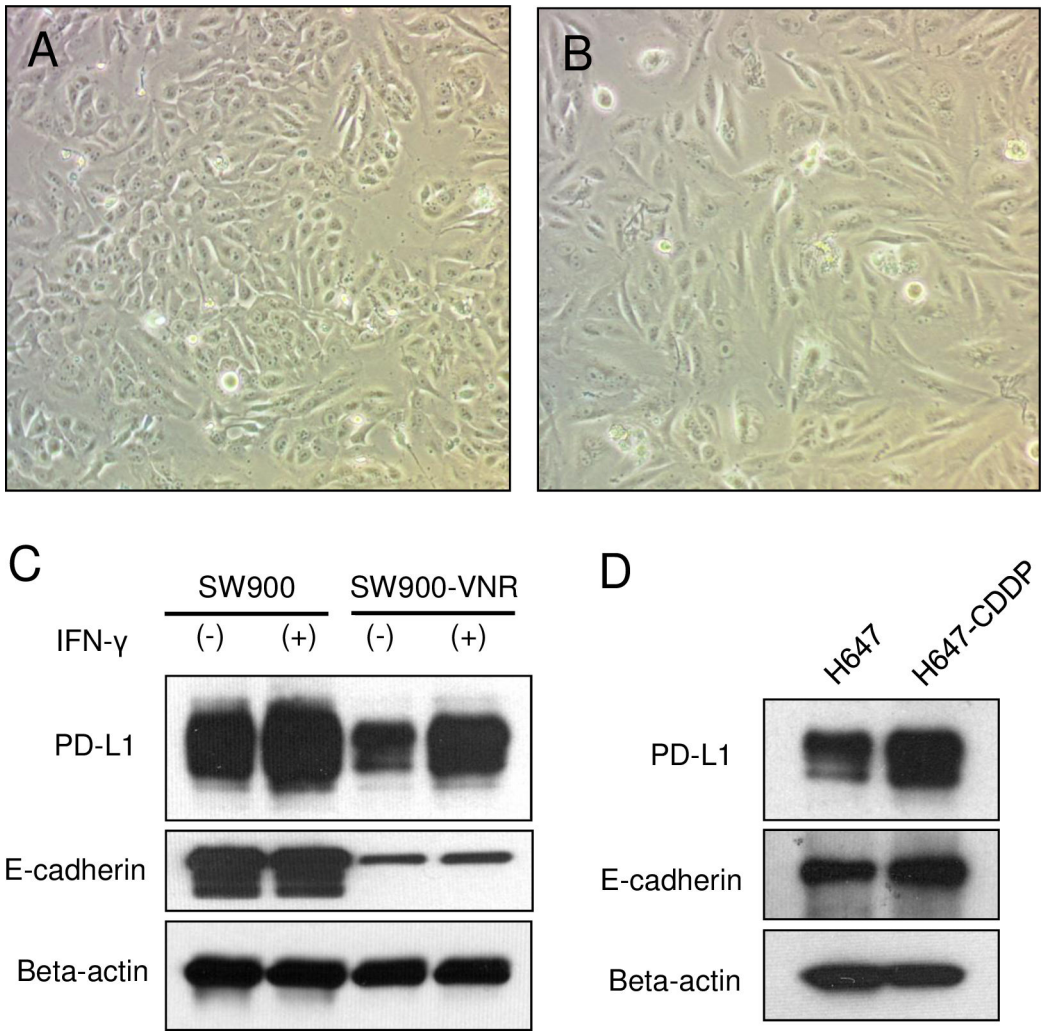



**Figure 5.**

**Table 1**

H-scores for PD-L1, EGFR and downstream molecules

| Molecule | Antibody (dilution) | HCC4006 | HCC4006ER |
|---|---|---|---|
| PD-L1 | SP142 (kit system) | 120 | 0 |
| total EGFR | 2-18C9 (kit system) | 260 | 230 |
| pEGFR | 53A5 (1:250) | 100 | 100 |
| pMAPK | 20G11 (1:100) | 170 | 130 |
| pAkt | 736E11 (1:40) | 0 | <5 |
| pS6 | D57.2.2E (1:400) | 280 | 230 |
| pSTAT3 | D3A7 (1:200) | 8 | <5 |
| p4E-BP1 | 236B4 (1:1000) | 20 | 13 |
| 4E-BP1 | 53H11 (1:1000) | 110 | 110 |