

p38 δ MAPK phenotype: an indicator of chemotherapeutic response in oesophageal squamous cell carcinoma

Carol O'Callaghan^a, Liam J. Fanning^b and Orla P. Barry^a

We recently documented p38 δ differential expression and function in oesophageal squamous cell carcinoma (OESCC). This study expands upon these findings and investigates whether p38 δ status in OESCC can influence response(s) to cytotoxic drugs. The antiproliferative effect of conventional cisplatin and 5-fluorouracil (CF) treatment was compared with the recently reviewed triple regime of cisplatin, 5-fluorouracil and doxorubicin (ACF). p38 δ -positive and p38 δ -negative cell lines were employed using cell-growth and clonogenic assays. Key regulators of intrinsic and extrinsic apoptotic pathways were measured. Wound-healing assays and a Boyden chamber were used to investigate the effect of drug treatments on cell migration. Functional networks were analysed in terms of changes in MAPK expression. p38 δ -negative OESCC is less sensitive to standard CF chemotherapy compared with p38 δ -positive cells. However, following ACF treatment p38 δ -negative cells showed markedly decreased proliferation and cell migration, and increased apoptosis. ACF induced apoptosis through the extrinsic pathway involving Fas activation, caspase-8 and caspase-3 cleavage and degradation of PARP. Loss of mitochondrial membrane potential ($\Delta\Psi_m$) was observed but downregulation of multidomain

proapoptotic proteins, as well as BH3-only proteins, suggests involvement of pathways other than the mitochondrial pathway. Interestingly, induction of p38 and ERK1/2, but not JNK1/2, was observed following ACF treatment. p38 δ -negative OESCC is more resistant to traditional CF treatment compared with p38 δ -positive OESCC. In light of these results, p38 δ phenotyping of tumour tissue may be of considerable value in deciding on an optimal therapeutic strategy for patients with p38 δ -negative OESCC. *Anti-Cancer Drugs* 26:46–55 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Oesophageal cancer is a highly aggressive and fatal malignancy and is the seventh most common cancer worldwide [1]. Oesophageal squamous cell carcinoma (OESCC) is an exceptionally drug-resistant tumour. Although surgery is the best modality in terms of local control [2], outcomes following resection for OESCC remain unsatisfactory because of locoregional and distant failure [3]. Preoperative chemotherapy or chemoradiotherapy with a fluoropyrimidine/platinum combination – that is, a cisplatin and 5-fluorouracil (CF) regimen – has been the standard treatment for locally advanced disease since the 1980s. At present, multimodal therapy is being investigated for different stages of OESCC, even if the tumour is operable [4]. Preoperative chemotherapy with docetaxel plus CF (DCF) has recently been investigated (with or without radiotherapy) with good local control and pathological remission rate being recorded [4, 5]. More recently doxorubicin, cisplatin and 5-fluorouracil (ACF) have undergone a revival, demonstrating higher

response rates than CF treatment, a good safety profile and promising long-term outcomes for patients with highly advanced oesophageal carcinoma [6–8].

The involvement of p38 MAPKs in a variety of pathological conditions is continuing to fuel interest in this particular family of kinases. It consists of four isoforms: p38 α (MAPK14), p38 β (MAPK11), p38 γ (MAPK12) and p38 δ (MAPK13), which to date remains the least studied isoform [9]. The expression of p38 as a family has previously been outlined in oesophageal cancer, as well as in other cancer types [10–13]. We recently outlined for the first time the differential expression of individual p38 isoforms in cancer and in particular OESCC [14,15]. We now know that loss of p38 δ expression in OESCC affords a more sinister phenotype, with increased proliferation, migration and anchorage-independent growth, thus identifying p38 δ as a possible molecular target in OESCC [15].

Advancing our studies a step further we evaluated whether p38 δ status could influence cytotoxic responses to drug treatments in OESCC. We used both negative and positive p38 δ cell lines isolated from patients with OESCC with no prior treatment, as previously outlined by us [15]. Cell viability, wound healing, migration and

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apoptosis were evaluated following standard CF treatment and ACF treatment. To carry out functional networks expression analysis, we also analysed changes in ERK1/2, JNK1/2 and p38 MAPK expression. In conclusion, our study indicates that p38 δ status may be a predictor of response to chemotherapy in OESCC patients.

Materials and methods

Reagents

All chemicals and cell culture reagents were purchased from Sigma Aldrich (Wicklow, Ireland) and primary antibodies from Cell Signalling Technologies (Hertfordshire, UK), unless otherwise stated.

Cell culture

The KE oesophageal squamous cancer cell lines were a kind gift from Professor T. Fujii, Kurume University School of Medicine, Japan [15–18]. Cells were cultured in RPMI-1640 supplemented with 10% foetal calf serum, 100 μ g/ml streptomycin and 100 U/ml penicillin. KE cell line features have been summarized previously by us [15].

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, which depends on the ability of viable cells to reduce MTT to a coloured formazan product, as described previously [19].

Boyden chamber cell migration in-vitro assay

Cells were plated in starvation medium at a density of 3×10^4 cells/well onto a 96-well plate of the upper chamber. The bottom chamber contained 10% foetal calf serum as the chemoattractant. Cells were left to migrate for 24 h through the matrigel filter (8 μ m) and stained as previously described [19].

Wound-healing assay

Cell migration was assessed using an in-vitro wound-healing assay as previously described [20]. Cells migrating into the wound were photographed under a phase-contrast microscope 48 h after wounding. Migration was determined using the ImageJ (National Institutes of Health, Bethesda, Maryland, USA) program as an average closed area of the wound relative to the initial wound area at 48 h after wounding.

Mitochondrial membrane potential ($\Delta\Psi$ m) assay

The decline of mitochondrial membrane potential ($\Delta\Psi$ m) was assessed using a mitochondrial voltage-sensitive dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole carbocyanide iodide (JC-1), according to the manufacturer's instructions [21]. The dye underwent a reversible change in fluorescence emission from red (i.e. aggregate forms of JC-1) to green (i.e. monomer forms of JC-1) as the mitochondrial membrane potential decreased. Briefly, KE cells

were or were not treated with drugs for 24 h. Cells were then washed twice with PBS and loaded with JC-1 for 30 min at 37°C. Images were obtained using an inverted fluorescence microscope (Olympus, Southend-on-Sea, UK). The ratio of red to green fluorescence intensity, which was indicative of a change in $\Delta\Psi$ m, was calculated using the ImageJ software.

Immunoblot analysis

Supernatants used for immunoblotting with specific antibodies, PARP, caspases-3 and 8, Puma, Bak, Bik, Bim, Bid, Bax, p38, phospho-p38, JNK1/2, phospho-JNK1/2, ERK1/2 and phospho-ERK1/2 (New England Biolabs, Hertfordshire, UK), as well as Fas and FasL (Santa Cruz Biotechnology, Santa Cruz, California, USA), have previously been described by us [14,15]. Chemiluminescence detection was performed using SuperSignal WestDura Extended Duration Substrate (Pierce Biotechnology, Rockford, Illinois, USA), and bands were visualized using a Syngene G:Box ChemiXR5 Gel Documentation System (Syngene, Cambridge, UK). Images were quantified using ImageJ software.

Colony formation assay

A colony formation assay determines whether cells can recover from treatment. Following treatment, viable cells were reseeded in fresh media (without drug) in a six-well plate (in triplicate) and allowed to grow for 14 days. Colonies were stained with MTT and subsequently counted using ImageJ software.

Statistical analysis

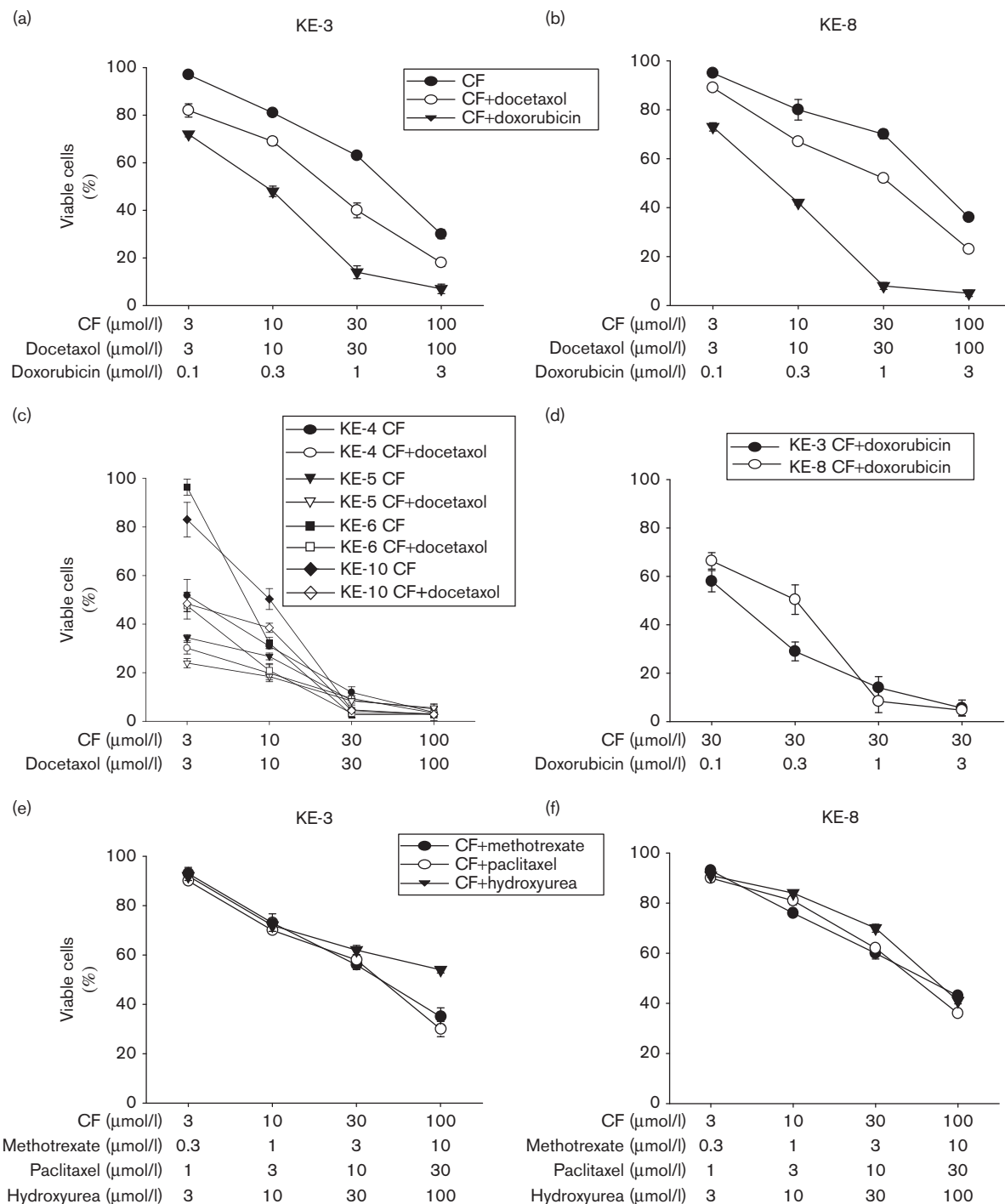
Statistical comparisons were made by analysis of variance with subsequent application of Student's *t*-test, as appropriate. The non-parametric Mann–Whitney *U*-test pairwise comparisons were also performed. As the results obtained using both methods were in agreement, only results for the Student's *t*-test are shown.

Results

p38 δ MAPK-negative OESCC shows decreased sensitivity to chemotherapeutic drugs

Monotherapy is not beneficial in the treatment of patients with oesophageal cancer [22]. Thus, we evaluated the cell viability of KE p38 δ -positive and p38 δ -negative OESCC cell lines (differential p38 δ expression was recently reported by us [15]) following double versus triple drug treatments using a range of concentrations of cytotoxic drugs. Interestingly, KE-3 and KE-8 (p38 δ -negative cell lines) are significantly less sensitive to CF treatment compared with KE-4, KE-5, KE-6 and KE-10 (p38 δ -positive cell lines; Fig. 1a–c). As docetaxel has recently been added to the CF regime [4,5], we investigated the effect of DCF on cell viability. There was a further decrease in KE-3 and KE-8 cell viability (Fig. 1a and b). However, no further reduction in cell viability was observed in the KE p38 δ -positive cell lines upon

Fig. 1



Sensitivity of OESCC to cancer chemotherapeutic drugs is correlated to the presence or absence of p38δ MAPK. (a–f) KE cell lines (KE-3 and KE-8, p38δ negative; KE-4, KE-5, KE-6 and KE-10, p38δ positive) were seeded (2×10^5) in six-well plates, and cell viability was assessed using the MTT assay (as described in the Materials and methods section) at 48 h to determine the sensitivity of each cell line to different drug combinations. (a, b) KE-3 and KE-8 cells were subjected to double treatment with cisplatin (3–100 μmol/l) and 5-fluorouracil (3–100 μmol/l; CF), or triple treatment with cisplatin (3–100 μmol/l), 5-fluorouracil (3–100 μmol/l; CF) and either docetaxel (3–100 μmol/l) or doxorubicin (0.1–3 μmol/l). (c) KE-4, KE-5, KE-6 and KE-10 cells were treated either with cisplatin (3–100 μmol/l) and 5-fluorouracil (3–100 μmol/l; CF), or with cisplatin (3–100 μmol/l), 5-fluorouracil (3–100 μmol/l; CF) and docetaxel (3–100 μmol/l). (d) KE-3 and KE-8 cells were subjected to triple treatment with constant drug concentrations of cisplatin (30 μmol/l) and 5-fluorouracil (30 μmol/l; CF) plus varying drug concentrations of doxorubicin (0.1–3 μmol/l). (e, f) KE-3 and KE-8 cells were subjected to triple treatment with cisplatin (3–100 μmol/l), 5-fluorouracil (3–100 μmol/l; CF) and either methotrexate (0.3–10 μmol/l), paclitaxel (1–30 nmol/l) or hydroxyurea (3–100 μmol/l). Results shown in (a–f) are mean ± SE of three independent experiments. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; OESCC, oesophageal squamous cell carcinoma.

DCF treatment using physiologically relevant drug concentrations (30 μ mol/l; Fig. 1c) [23–25]. As doxorubicin (together with CF) has recently re-emerged as a promising drug treatment strategy for patients with oesophageal cancer [6–8], we also investigated its effects. Interestingly, the greatest loss in KE-3 and KE-8 cell viability was after triple CF plus doxorubicin (ACF) treatment (Fig. 1a and b). Of note, these results are comparable with the loss in cell viability seen in the p38 δ -positive cells upon CF treatment at physiological concentrations (Fig. 1c). Taking the results we obtained with physiological drug concentrations of CF (30 μ mol/l each) a step further, we investigated the appropriate triple ACF drug treatment concentrations for KE-3 and KE-8 cell lines. Using a range of doxorubicin drug concentrations, we ascertained that the best triple ACF concentration using physiological concentrations of all three drugs is 30 μ mol/l each of CF and 1 μ mol/l doxorubicin (Fig. 1d). No significant further decrease in cell viability was observed at a higher doxorubicin drug concentration of 3 μ mol/l (Fig. 1d).

As other chemotherapeutic drug combinations have also been tested for their efficacy in patients with OESCC, namely CF plus methotrexate [26], CF plus paclitaxel [26,27] and CF plus hydroxyurea [28], we also investigated these three additional drug combinations again using a range of different drug concentrations. Interestingly, KE-3 and KE-8 showed less sensitivity to all three triple-drug combinations compared with ACF treatment (Fig. 1e and f). Thus, as the greatest loss in KE-3 and KE-8 cell viability was observed with triple ACF treatment (Fig. 1a, b and d), all subsequent experiments compared traditional CF treatment with ACF treatment.

ACF treatment significantly delays wound healing and migration compared with CF treatment

A key characteristic of cancer cells is their ability to migrate and progress from primary tumours to metastases in distant organs [15]. We examined whether the p38 δ status of OESCCs could influence their wound healing and cell migration following drug treatment. Initially, we identified the highest concentration of drug(s) that does not lead to loss of cell viability – that is, 3 μ mol/l of each drug for CF and 300 nmol/l doxorubicin (data not shown). Double CF treatment of KE-6 and KE-10 (p38 δ -positive cells) for 48 h brought about a significant delay in wound healing, with a 60.0 ± 7 and $66.0 \pm 1.2\%$ loss in wound healing, respectively, compared with untreated cells (Fig. 2a and b). In contrast, CF treatment did not impair the ability of KE-3 and KE-8 cells to migrate into the wound (Fig. 2a and b). Triple ACF treatment, however, significantly delayed the wound-healing ability of KE-3 and KE-8 cell lines, with a 72.8 ± 2.5 and $84.0 \pm 3.9\%$ loss in wound healing at 48 h, respectively. There was a further $15 \pm 5.8\%$ loss in wound healing upon ACF

treatment, compared with CF treatment alone, of KE-6 cells but no significant change in KE-10 cells (Fig. 2a and b). Doxorubicin (300 nmol/l) alone did not have any significant effect on the wound-healing ability of either p38 δ -positive or p38 δ -negative cells (Fig. 2a and b). Further, we compared the migration of p38 δ -positive and p38 δ -negative cells following CF versus ACF treatment using a Boyden chamber assay. Double CF treatment induced a significant (78.7 ± 5.2 and $78.7 \pm 7.8\%$, respectively) decrease in cell migration of KE-6 and KE-10 cells at 24 h; however, no significant change in cell migration was observed upon CF treatment of KE-3 and KE-8 cells (Fig. 2c). In contrast, triple ACF treatment of KE-3 and KE-8 significantly decreased their cell migration by 75.9 ± 2.4 and $81.3 \pm 6.9\%$, respectively (Fig. 2c).

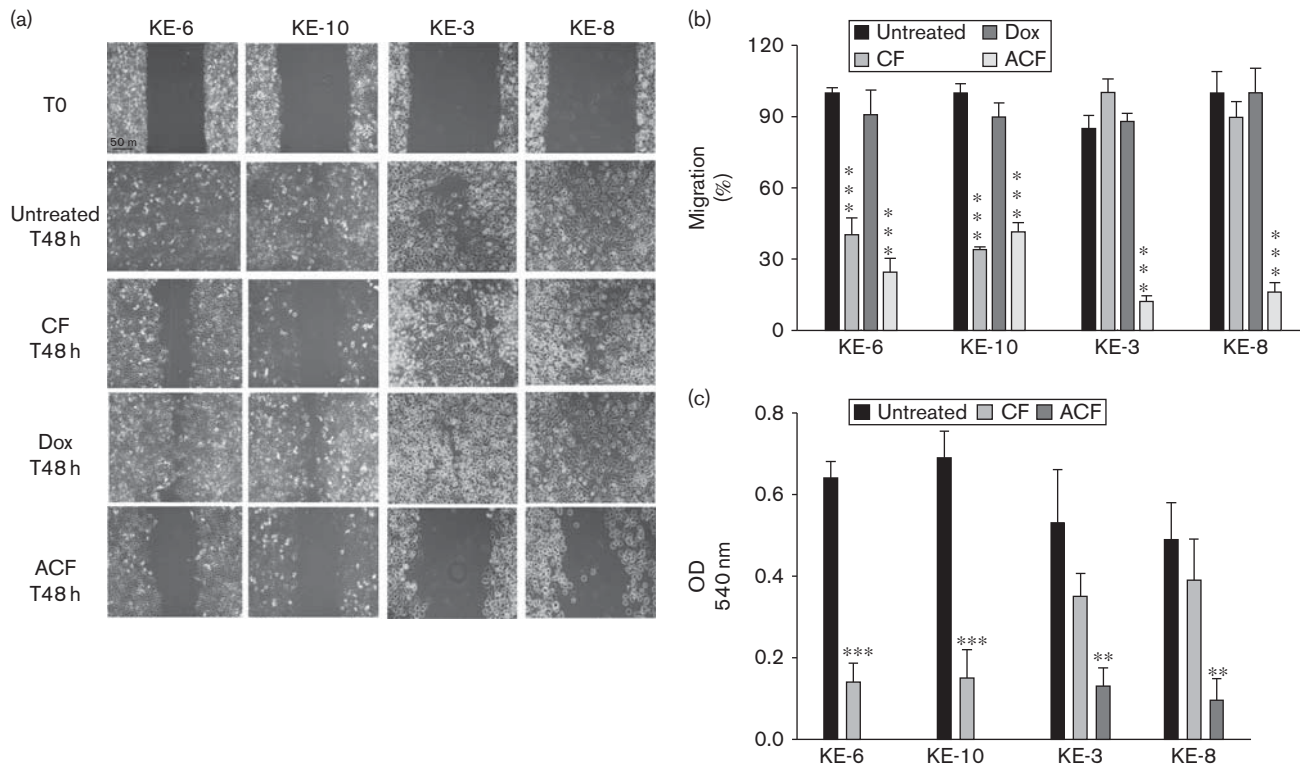
Triple ACF treatment decreases mitochondrial membrane potential and activates extrinsic pathways in p38 δ negative OESCCs

Initially, we investigated the involvement of the mitochondria in OESCC apoptosis using the JC-1 cationic dye. It selectively enters the mitochondria and reversibly changes in colour from red to green as the membrane potential decreases [21]. KE-6 and KE-10 cells show a marked decrease in $\Delta\Psi_m$ following CF and ACF treatment (Fig. 3a and b). Similar losses in $\Delta\Psi_m$ in KE-3 and KE-8 cells were observed only upon ACF treatment (Fig. 3a and b). Doxorubicin alone did not alter the $\Delta\Psi_m$ of KE-6 and KE-10 cells, but it did produce a significant ($P < 0.01$) decrease in the $\Delta\Psi_m$ of KE-3 and KE-8, comparable to that on CF treatment (Fig. 3a and b).

To further investigate the effects of CF-induced versus ACF-induced apoptosis on KE p38 δ -negative cells, intrinsic (mitochondrial) and extrinsic (Fas) apoptotic pathways were investigated. Expression of the relevant apoptosis-related proteins was examined by western blot analysis. To investigate the role of the intrinsic mitochondrial pathway we examined the expression levels of Bcl-2 family members including multidomain proapoptotic proteins Bak and Bax, as well as BH3-only proapoptotic proteins Puma, Bik, Bid and Bim. Interestingly CF treatment downregulated all Bcl-2 proapoptotic members examined, with further reductions in expression being observed in the presence of ACF treatment (Fig. 3c). The mitochondrial apoptotic pathway-related caspase-9 was not cleaved (data not shown).

To investigate whether CF and ACF activate the extrinsic apoptotic pathway, we examined the expression levels of death receptor signalling-related proteins including Fas, caspase-3 and caspase-8 and PARP. Alterations in the expression of Fas and FasL, members of the tumour necrosis family, have been reported previously in OESCC with Fas-activated apoptosis being shown to limit the growth of OESCCs [29,30]. We observed an increase in Fas expression when both KE-3 and KE-8 cells were treated with ACF, but not on CF treatment, implicating the involvement of the extrinsic

Fig. 2



Effect of chemotherapeutic cytotoxic drugs on wound healing and migration of KE p38 δ MAPK positive and negative cell lines. KE-6 and KE-10 cells (p38 δ positive), as well as KE-3 and KE-8 cells (p38 δ negative), were analysed for (a, b) wound healing and (c) cell migration following treatment with cisplatin (3 μ mol/l) and 5-fluorouracil (3 μ mol/l; CF), doxorubicin (Dox; 300 nmol/l) alone or cisplatin (3 μ mol/l), 5-fluorouracil (3 μ mol/l) and doxorubicin (300 nmol/l; triple treatment; ACF). (a) Representative wound-healing images at 0 and 48 h with or without drug treatment. The results shown in (a) are representative of three independent experiments, whereas the results shown in (b) and (c) are the mean \pm SE of three independent experiments. *** P < 0.001, ** P < 0.01, significant changes from control untreated cells.

death pathway. The expression level of FasL was very low in both cell lines, and there was no appreciable change after treatment with CF or ACF (Fig. 3d). We observed that ACF (but not CF) treatment of KE-3 and KE-8 cell lines activated (cleaved) caspase-3 and caspase-8, as well as activated (cleaved) their substrate PARP, producing the 85 kDa proteolytic fragment indicative of caspase activation and apoptosis (Fig. 3d). The extrinsic apoptotic pathway-related caspases-6 and caspase-7 were not cleaved upon CF or ACF treatment (data not shown).

Triple ACF treatment induces p38 and ERK1/2 but not JNK1/2 MAPK activation in p38 δ -negative OESCC

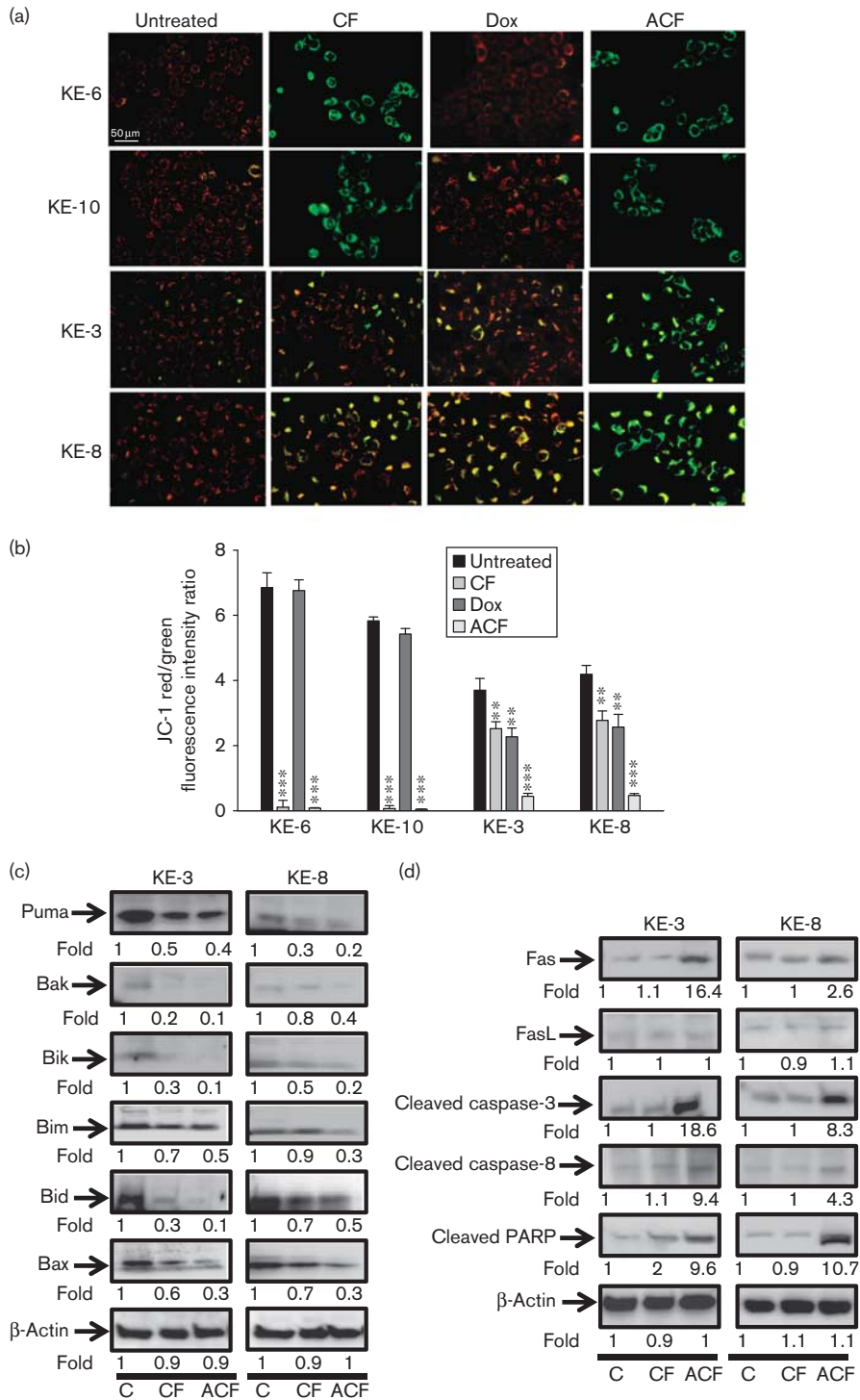
To gain further insight into CF-induced versus ACF-induced cytotoxic effects, we analysed MAPK expression. We observed p38 phosphorylation upon ACF treatment but not CF treatment (Fig. 4a). CF induced ERK1/2 phosphorylation, which was further enhanced with ACF treatment (Fig. 4a). In contrast, JNK1/2 activation was not observed following either CF or ACF treatment (Fig. 4a).

To assess whether p38 and ERK1/2 activation is causal to the actions of our chemotherapeutics we used specific pharmacological inhibitors of p38 and MEK. Interestingly, both SB203590 (20 micromolar) and BIRB 796 (5 micromolar), inhibitors of p38 α and p38 β (but not p38 γ and p38 δ) [24,31] did not prevent the antiproliferative effects of either CF or ACF (Fig. 4b). In fact, in agreement with recent reports (with similar cytotoxic drug combinations), p38 blockade enhanced the cytotoxic effects of ACF treatment significantly (but not CF treatment) at 24 h (Fig. 4b) [24,25]. The enhanced antiproliferative effect of p38 inhibition observed at 24 h with ACF was not obvious at 48 h because of the high level of cell death at this time point (Fig. 4b). The specific MEK inhibitor U0126 (20 micromolar) [32] brought about a significant abolition of the effects of both CF and ACF drug treatments in both cell lines, indicating that ERK1/2 may be directly involved in drug-induced cytotoxicity (Fig. 4b).

Recovery of KE-3 and KE-8 cells following drug treatments

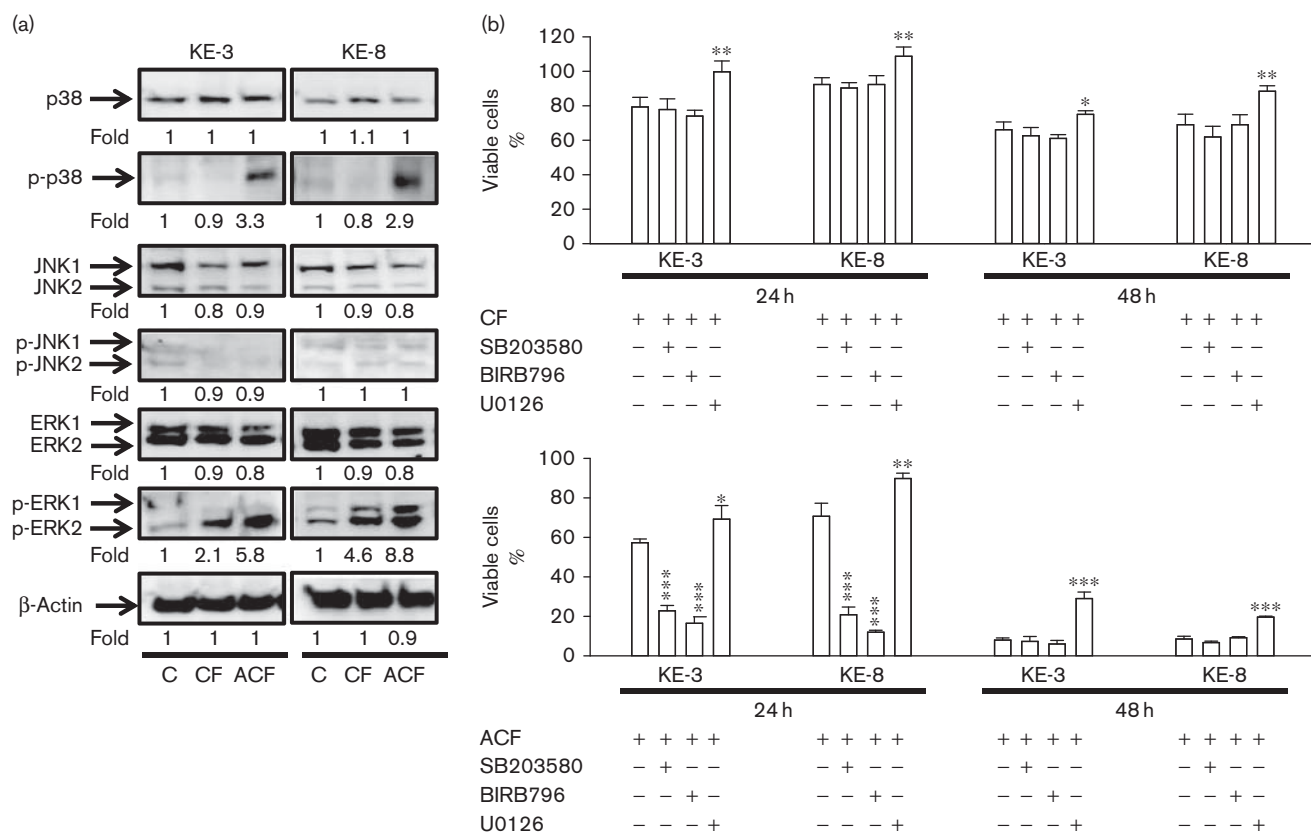
One of the most important parameters for the efficacy of chemotherapeutic drug treatments is the long-term effect

Fig. 3



Effect of chemotherapeutic drug treatment on intrinsic and extrinsic mitochondrial pathways in KE-3 and KE-8 cells. (a) The mitochondrial membrane potential ($\Delta\Psi_m$) of KE cells before and after treatment with chemotherapeutic drugs for 24 h. Representative images (red/green mixed channels) show the changes in mitochondrial membrane potential ($\Delta\Psi_m$) in KE cells, detected using JC-1 dye. Red fluorescence indicates high membrane potential and functional capacity in mitochondria, whereas green cytoplasmic fluorescence is indicative of inactive mitochondria. (b) Decreased red/green fluorescence ratio suggests a decrease in $\Delta\Psi_m$. (c) Whole cell lysates were analysed by western blot analysis of proapoptotic Puma, Bak, Bik, Bim, Bid and Bax, as well as (d) Fas and FasL, cleaved caspase-3 and caspase-8 and PARP in KE-3 and KE-8 cells. The results shown in (a), (c) and (d) are representative of four independent experiments, whereas the results shown in (b) are the mean \pm SE of four independent experiments. *** $P < 0.001$, ** $P < 0.01$, significant changes from control untreated cells.

Fig. 4



MAPK activation in KE-3 and KE-8 cells following chemotherapeutic drug treatment. Western blot analysis of the three different MAPKs (p38 MAPK, JNKs and ERKs) following treatment with cisplatin (30 $\mu\text{mol/l}$) and 5-fluorouracil (30 $\mu\text{mol/l}$; CF), or following triple treatment with cisplatin (30 $\mu\text{mol/l}$), 5-fluorouracil (30 $\mu\text{mol/l}$) and doxorubicin (1 $\mu\text{mol/l}$; ACF) for 24 h. The results shown in (a) are representative of four independent experiments, whereas the results shown in (b) are the mean \pm SE of four independent experiments. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, significant changes from CF-treated or ACF-treated cells.

on cell viability. Thus, we evaluated the effects of CF, doxorubicin alone and ACF treatment on the capacity of KE-3 and KE-8 cells to recover in assays of clonogenic growth. All three different drug treatments had a significant effect on the recovery of both KE-3 and KE-8 cell lines (Fig. 5a and b). However, of note, cell recovery following ACF treatment was never observed, whereas colonies – that is, cell recovery – were observed following treatment with CF or doxorubicin alone (Fig. 5a and b).

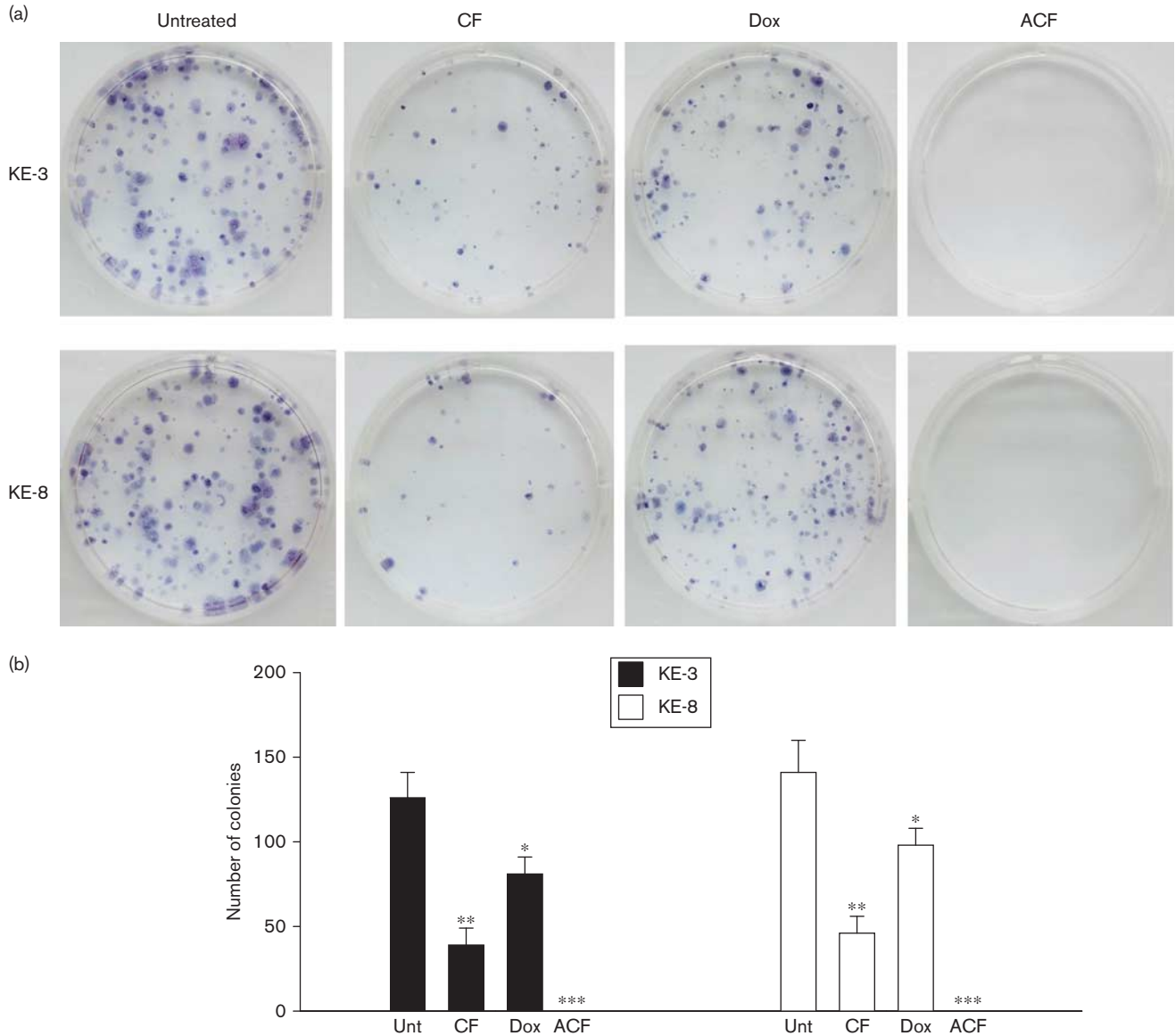
Discussion

In this study, we focused on OESCCs, as this cell phenotype shows differential p38 δ expression [15]. Loss of this important serine/threonine kinase confers greater tumourigenicity and may be a mechanism by which OESCC promotes carcinogenesis [15]. The purpose of this study was to determine whether differences in the OESCC p38 δ phenotype could influence the chemosensitivity of OESCC to conventional CF versus ACF drug combinations.

Single-agent response rates of 10–25% remain poor for oesophageal cancer [22]. Further, the use of double CF is of limited effectiveness in the treatment of patients with OESCC, with respect to improving overall survival time and patient quality of life [3,8]. Triple ACF combination therapy was first reported as far back as 1983 [33], but of late it has sparked renewed interest [4,8]. Doxorubicin is an effective, widely used chemotherapeutic agent in the treatment of a variety of solid tumours and malignant haematologic diseases [34]. There are now reports documenting the usefulness and, importantly, the safety of ACF therapy for the treatment of advanced oesophageal carcinoma [7,8,35]. Despite the absence of phase III clinical trials, ACF is currently used in the clinical setting for treatment of patients with OESCC [8].

All three drugs in this study, cisplatin, 5-fluorouracil and doxorubicin, used as monotherapy or double therapy have been reported to induce intrinsic apoptosis in oesophageal cancer [23,36]. We examined typical markers of both intrinsic and extrinsic apoptotic cell death in our p38 δ -negative cell lines. Although we observed

Fig. 5



Recovery of KE-3 and KE-8 cells following chemotherapeutic drug withdrawal. The ability of p38 δ -negative OESCC cell lines KE-3 and KE-8 to recover after drug withdrawal was assessed with a colony formation assay (clonogenic assay). KE-3 and KE-8 cells were or were not treated (Unt) with cisplatin (30 μ mol/l) and 5-fluorouracil (30 μ mol/l; CF), doxorubicin (1 μ mol/l; Dox) alone or the combination of all three drugs (ACF) for 48 h. Viable, adherent cells were counted and reseeded (1000 cell/well) in a six-well plate (in triplicate) in the absence of drug. (a) Fourteen days later, colonies were stained with MTT. Each well shown is a representative image of nine similar wells (three independent experiments). (b) Colonies were counted using ImageJ software. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, significant changes from untreated cells. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; OESCC, oesophageal squamous cell carcinoma.

mitochondrial depolarization following ACF treatment, there was no subsequent caspase-9 activation. Interestingly, we also observed downregulation of Bcl-2 multidomain, as well as BH-3 proapoptotic proteins. However, decreased expression of these intrinsic pathway proteins is in agreement with a recent report on oxaliplatin-induced apoptosis in squamous oesophageal cancer cell lines [37]. Potentially, the presence of Bcl-2 multidomain and BH3-only proapoptotic molecules may be an important predictor of p38 δ -negative OESCC

response to combination therapy without being directly involved. Our findings suggest that ACF suppresses cell growth in p38 δ -negative OESCC through extrinsic apoptotic pathway activation of the Fas death receptor, with caspase-8 and caspase-3 cleavage and subsequent degradation of PARP.

There are many reports linking MAPK involvement following exposure to mechanistically different chemotherapeutic drugs [38]. However, their suppression

and activation and indeed the absence of any role in apoptosis have been attributed to all three MAPKs [38–40]. Thus, the activation of MAPKs by chemotherapeutic drugs and the subsequent consequences of MAPK activation appear to be very much cell-type specific [39]. Although we observed p38 activation upon ACF treatment, our findings with the p38 pharmacological inhibitors do not support a direct role for p38 MAPK activity in drug-induced cytotoxicity. In contrast, ERK1/2 activation may play a more direct role as its inhibition through MEK can partly reverse the antiproliferative effects of both CF and ACF treatments.

The increased antiproliferative and proapoptotic effects of ACF treatment over CF treatment in our study suggest that the former may be considered as mainstay treatment of patients with p38 δ -negative OESCC. It remains to be investigated whether patients with p38 δ -negative OESCC may benefit more from ACF treatment compared with classical CF treatment, and whether or not ACF treatment can influence overall survival. Further investigations into the mechanistic strategies underpinning p38 δ -negativity related loss in drug sensitivity are warranted. The clonogenic assay is a valuable tool in gauging long-term consequences of single, double and triple chemotherapy in p38 δ -negative OESCC. This assay closely mirrors the clinical situation in which patients are treated with chemotherapeutic drugs in a pulsed rather than a continuous manner. After 2 weeks of treatment, our ACF-treated p38 δ -negative OESCC cells never recovered. Although we observed a statistically significant reduction in colony formation on CF double or doxorubicin treatment, cell recovery was always observed, clearly demonstrating resistance. In general, the MTT assay demonstrated lower cytotoxic activity than the clonogenic assay with these drugs. Of note, a high degree of correlation between both assays is not always apparent, and it may be influenced by both cell type and anticancer drug type [41–43]. Thus, unlike ACF treatment, the limited efficacy of CF or doxorubicin alone could provide an opportunity for cancer persistence in patients with p38 δ -negative OESCC.

In summary, the present study indicates that p38 δ may be a significant predictor of treatment response in patients with p38 δ -negative OESCC. p38 δ genotyping of pretreatment biopsy may potentially be a useful predictor of response to chemotherapy and ultimately prognosis in OESCC patients. Our data support the value of known p38 δ status in the decision process used to inform the optimal treatment of patients with OESCC.

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Conflicts of interest

There are no conflicts of interest.

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