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## Original Paper

# Oncogenic Ras Modulates Epidermal Growth Factor Responsiveness in Endometrial Carcinomas

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**Since the majority of endometrial carcinomas do not contain any detectable *ras* mutations, the precise contribution of aberrant Ras function, if any, to endometrial carcinoma development remains to be determined. Since there is considerable evidence that Ras transformation is associated with a decreased requirement for growth factors, we compared the growth response of endometrial carcinoma cells harbouring wild-type (Ishikawa cells) or mutated (HHUA cells) K-*ras* to epidermal growth factor (EGF). K-*ras* mutation did not significantly affect the level of the EGF receptor (EGFR) expressed in these carcinoma cells. EGF could stimulate the growth of Ishikawa, but not HHUA cells. Furthermore, EGF caused elevation of Ras-GTP levels in Ishikawa, but not HHUA cells. However, the introduction of mutated, but not normal, K-*ras* into Ishikawa cells rendered them non-responsive to EGF growth stimulation. Thus, the presence of mutated K-*ras* alone modulated the growth response of endometrial carcinoma cells to EGF. An inhibitor of the EGFR tyrosine kinase activity could prevent soft agar colony formation of Ishikawa cells, but not HHUA or mutant K-*ras*(12V)-transfected Ishikawa cells. Taken together, these results suggest that mutated K-*ras* causes a loss of responsiveness to EGF stimulation and that EGFR function is dispensable for the growth of mutant Ras-positive endometrial carcinoma cells.** 

**Key words: endometrial carcinoma, Ras, signal transduction, EGF receptor**
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## INTRODUCTION

MUTATED FORMS of the three human *ras* genes (H-, K- and N-*ras*) have been observed in approximately 30% of all human cancers [1]. However, the frequency of mutations is quite variable in different neoplasms. For example, whereas 50% of colorectal and 90% of pancreatic carcinomas have been found to contain predominantly mutated forms of K-*ras*, 40% of acute chronic myelogenous leukaemias contain mutated forms of predominantly N-*ras*. In contrast, although 50% of thyroid tumours possess mutated *ras* sequences, the frequency is distributed between H-, K- and N-*ras*. Finally, mutated *ras* genes are infrequently seen (< 5%) in breast and prostate carcinomas. Thus, both the frequency of *ras* mutations, as well as the specific *ras* gene involved, vary significantly between different types of tumours [2].

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Since tumour progression involves multiple genetic steps, the contribution of *ras* mutations to tumour development has not been precisely defined. However, the demonstration that homologous recombination knockout of the mutated K-*ras* allele in colorectal carcinoma cells results in a decreased *in vitro* and *in vivo* growth potential supports the essential role of *ras* mutations in the maintenance of the malignant phenotype of colorectal carcinoma cells [3]. However, because mutations in K-*ras* are associated with only 20% of human endometrial carcinomas [4–7], it is not clear whether aberrant Ras function is critical for the development of these tumours. While it is clear that endometrial carcinoma development can occur in the absence of mutations in *ras*, the contribution of aberrant Ras function to mutant Ras-positive tumours is not well understood.

Ras proteins are guanine nucleotide-binding proteins which control signal transduction pathways that regulate cell growth and differentiation. Ras proteins function as biological

switches that cycle between active, guanine triphosphate (GTP)-bound and inactive, guanine diphosphate (GDP)-bound states [8]. This GDP/GTP cycle is controlled by two classes of regulatory proteins. The low basal rate of GTP hydrolysis Ras is stimulated by GTPase activating proteins (GAPs; p120- and NF1-GAP), whereas Ras GDP/GTP exchange is stimulated by guanine nucleotide exchange factors (GEFs; SOS and RasGRF) [9]. Mutations that activate Ras transforming potential (substitutions at amino acids 12, 13 or 61) result in mutant proteins that are unresponsive to GAP stimulation. Consequently, mutant Ras persists in a chronic, GTP-bound state, leading to constitutive and deregulated stimulation of growth regulatory processes that contribute to aberrant growth properties of the tumour cell.

Considerable evidence now implicates Ras proteins as critical relay switches that control signalling pathways that connect the cell surface with the nucleus. Among these pathways, the best characterised involves intracellular signalling events which are triggered by epidermal growth factor (EGF) [10, 11]. EGF binds to the EGF receptor (EGFR), causes stimulation of its tyrosine kinase activity, which then causes receptor autophosphorylation of specific tyrosine residues [12]. The activated receptor causes a recruitment of SOS, via the Grb2 adaptor protein, to the plasma membrane-associated receptor, where it causes a transient activation of Ras [13, 14]. Activated Ras then triggers the activation of a cascade of serine/threonine kinases, which includes the Raf-1 and mitogen-activated protein (MAP) kinases [15]. Activated MAP kinases then translocate to the nucleus where they phosphorylate and activate transcription factors (e.g. Elk, Jun) which cause changes in gene expression responsible for growth stimulation [16, 17]. The signalling events are terminated, in part, by a GAP-mediated conversion of Ras to its inactive, GDP-bound form. Analogous signalling events are also responsible for activation of Ras by other extracellular ligands.

Although proliferation of normal cells is tightly regulated by growth factor-stimulated events, there is considerable evidence that tumour cells have escaped growth factor control by a variety of mechanisms. First, a common feature of many tumour cells is induction of an autocrine mechanism to facilitate growth factor-independent growth. For example, the induction of transforming growth factor alpha (TGFα), which stimulates growth via interaction with EGFR, is observed with many tumour cells [18]. Second, a number of studies have established that Ras transformation can alter the response of various growth factor receptors to ligand stimulation. For example, Ras-transformed cells have been found to be refractory to platelet-derived growth factor (PDGF) or EGF stimulation [19]. In the present study, we have addressed the consequences of mutated Ras on EGF responsiveness of endometrial carcinoma cells.

## MATERIALS AND METHODS

*Plasmids*

pZIP-Neo SV(X)1 retrovirus vector and constructs containing cDNA sequences encoding either wild-type K-Ras4B or [$^{12}$Val]K-Ras4B were gifts from Dr Channing J Der (University of North Carolina). This [$^{12}$Val]K-Ras cDNA sequence encodes an additional N-terminal 10 residues from vector-derived sequences, and the resulting chimeric K-Ras protein migrates at approximately 24 kDa in a sodium dodecyl sulphate (SDS) polyacrylamide gel. This distinct mobility allowed us to distinguish clearly the chimeric K-Ras protein from the endogenous Ras protein.

*Cell culture*

Three human endometrial carcinoma cell lines (Ishikawa, HHUA and HOUA) were used for the present study. Ishikawa cells harbouring either wild-type or mutant (12V) versions of K-*ras*4B were established by transfecting Ishikawa cells with pZIP-NeoSV(x)1 retrovirus vector constructs containing cDNA sequences encoding either wild-type K-Ras4B or K-Ras4B(12V) using lipofectin (Gibco BRL, Life Technologies, Inc., Rockville, Maryland, U.S.A.). Stably transfected cells were selected and isolated in growth medium containing 400 μg/ml G418 (Geneticin; Gibco BRL, Life Technologies, Inc., Rockville, Maryland, U.S.A.) to establish cell lines expressing each Ras protein. Ras protein expression was confirmed by immunoprecipitation as described below. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Seika, Japan) supplemented with 10% fetal calf serum (FCS; Gibco BRL, Life Technologies, Inc., Rockville, Maryland, U.S.A.).

*Immunoprecipitation analysis of Ras*

Cells were labelled overnight in growth medium supplemented with [$^{35}$S]methionine/cysteine (Tran$^{35}$S-label, ICN; Costa Mesa, California, U.S.A., Met/Cys; 400 μCi/ml). Ras proteins were immunoprecipitated using the Y13-259 rat anti-Ras monoclonal antibody (Oncogene Science, Uniondale, New York, U.S.A.), resolved by SDS ph polyacrylamide gel electrophoresis (SDS/PAGE), and subjected to fluorographic analysis as described previously [20].

*EGFR expression*

EGFR expression was analysed by immunohistochemistry using an avidin-biotinylated immunoperoxidase technique (Vector Labs, Burlingame, California, U.S.A.). Cells were plated at approximately $1\times10^4$ cells per two-well chamber (Nunc, Roskilde, Denmark). After 2 days, cells were permeabilised and fixed by treatment with 10% formalin and incubated with mouse monoclonal anti-EGFR antibody (Sigma Chemical Co., St Louis, Missouri, U.S.A.) overnight at 4°C. Bound antibody was detected with a biotinylated antimouse IgG secondary antibody and avidin–biotin complex linked to horseradish peroxidase followed by incubation with diaminobenzidine tetrahydrochloride as the substrate.

*$^{125}$I-EGF binding assay*

Approximately $10^5$ cells per well were incubated with $^{125}$I-EGF of various concentrations (0.6–30.0 ng/ml) in MEM containing 20 mM and 0.5% bovine serum albumin (BSA) at 4°C for 2 h. A 200-fold excess of unlabelled EGF was added to determine specific binding. After the incubation, cells were washed four times with ice-cold phosphate buffered saline (PBS), and cell associated radioactivity was counted. Dissociation constants and EGFR number were determined from Scatchard analysis.

*Growth rates*

Cells were plated at $2.5\times10^4$ cells per 2.0 mm$^2$ 24-well plates (Falcon, Becton Dickinson Labware, Lincoln Park, New Jersey, U.S.A.) in DMEM supplemented with 10% FCS and incubated overnight. To determine the consequences of EGF stimulation on cell growth, the medium

was replaced with serum-free DMEM which was supplemented with 0.1% BSA and varying concentrations of EGF (0–10 ng/ml). After 3 days, the number of viable cells were counted. To determine if EGF similarly affects growth when incubated in low serum concentrations (0.1%), after 2 days of incubation in low serum (0.1%) medium, cells were counted to determine the number of cells at day 0, and medium was replaced in the presence or absence of 10 ng/ml EGF. Cells were cultured for a further 3 days and viable cells were counted. Cell counts were performed in triplicate using a haemocytometer. Each experiment was repeated three times.

In vivo *guanine nucleotide association*

Analysis of *in vivo* guanine nucleotide association was carried out essentially as described previously [21, 22]. Briefly, cells were metabolically labelled overnight with 1 mCi/ml [$^{32}$P] orthophosphate (Amersham, Buckinghamshire, U.K.). The labelled cells were washed in Tris buffered saline (TBS) with 1 mM sodium vanadate and lysed with 1% NP-40 detergent buffer, then subjected to immunoprecipitated Ras proteins with Y13-259. The bound guanine nucleotides were released from the immunoprecipitated Ras proteins, separated by thin layer chromatography on a PEI cellulose plate, and the percentage of GTP bound was quantitated by BAS 1000 imaging analyser (Fuji, Japan) and calculated as follows: GTP (cpm)/1.5×GDP (cpm) + GTP (cpm). To monitor the EGF effects on the GTP/GDP ratio of resting cells, cells were incubated with 5% FCS/DMEM for 7 days before labelling. EGF was added for 2 min at the end of labelling.

*Soft agar analysis*

Anchorage-dependent growth assays were performed as described above. Briefly, $2\times10^4$ cells were seeded per well and incubated overnight before addition of 0.2 μg/ml erbstatin (Biomol, Plymouth Meeting, Pennsylvania, U.S.A.) which inhibits tyrosine kinase activity of EGFRs [23]. After 4 days' incubation, viable cells were counted. Cell counts were performed in triplicate using a haemocytometer. For the anchorage-independent growth assay, $10^4$ cells were seeded with or without 1 μg/ml erbstatin in growth medium supplemented with 10% FCS and 0.3% Bactoagar over a hardened 0.5% agar base layer in 60-mm dishes. After 18 days, colonies were counted in triplicate.

*Quantitation of TGFα*

Cells were plated at $2.0\times10^5$ cells per 2.0 mm$^2$ 24-well plates (Falcon, Becton Dickinson Labware, Lincoln Park, New Jersey, U.S.A.) in DMEM supplemented with 10% FCS and incubated overnight. The medium was replaced with serum-free medium and TGFα production was investigated using the enzyme linked immunosorbant assay (ELISA) method (TGFα ELISA-Kit, Oncogene Research Products, Cambridge, Massachusetts, U.S.A.) according to the manufacturer's instructions.

*Detection of apoptosis*

Cells were plated at $2.0\times10^5$ cells per 35 mm$^2$ plates in DMEM supplemented with 10% FCS and incubated overnight. The medium was replaced with serum-free DMEM which was supplemented with 0.1% BSA, and 0 or 10 ng/ml EGF. After 3 days, cells were fixed in 4% formalin. *In situ* apoptosis detection was carried out according to the manufacturer's instructions. (ApopTag Plus, Oncor, Inc., Gaithersburg, Maryland, U.S.A.).

## RESULTS

*Effect of EGF on cell growth*

To evaluate a role of aberrant Ras function in endometrial carcinomas, we first evaluated the status of *ras* gene mutations in the HHUA, HOUA and Ishikawa endometrial carcinoma cell lines. Dot blot hybridisation with mutation-specific oligomers were used to screen for missense mutations at codons 12, 13 or 61 of the K-, H- and N-*ras* genes and further direct sequence analysis was performed on polymerase chain reaction (PCR)-amplified DNA isolated from each of the cell lines. We detected a single base substitution at codon 12 of K-*ras* in HHUA or HOUA cells that encoded a glycine to valine substitution. No mutations were detected at codons 12, 13 or 61 of the K-*ras*, H-*ras* or N-*ras* genes in Ishikawa cells (data not shown). These findings are in agreement with reported observations [4, 6].

We next determined whether the presence of mutated *ras* was associated with any differences in the levels of EGFR expression, determined in $^{125}$I-EGF binding assays. Compared with Ishikawa cells, HOUA cells expressed a slightly lower level of EGFR, whereas HHUA cells expressed the highest level of EGFR (Table 1). Our $^{125}$I-EGF binding assay showed that the EGFR number for both high and low affinity was not related to the presence or absence of mutated *ras* in the endometrial carcinoma cells.

To determine if the mutation status of *ras* was associated with any differences in the growth response of endometrial carcinoma cells to EGF stimulation, we compared the growth rates of HHUA and Ishikawa cells in response to exogenous EGF. Both Ishikawa and HHUA cells grew exponentially in the presence of 10% calf serum. In the absence of serum, the growth of Ishikawa cells was almost static. In contrast, HHUA cells grew slowly and showed a 2-fold increase in cell number after 3 days' incubation. When these cells were maintained in medium supplemented with 10 ng/ml EGF, we observed an approximately 10-fold increase in the number of Ishikawa cells after 3 days. In contrast, we saw no increase in HHUA cell numbers with EGF supplements (Figure 1a).

We also determined the growth proliferative response to EGF after the cells had been first maintained in growth medium supplemented with a low concentration of serum (0.1% FCS), then followed by 3 days' incubation in growth medium supplemented with 10 ng/ml EGF. In the medium containing 0.1% serum, control cultures of 3 days for each cell line resulted in the slow growth of cells. A 2-fold increase in cell number was observed in Ishikawa cells and HHUA cells and a 1.2-fold increase in HOUA cells. Whereas we observed a 1.5-fold increase in the number of Ishikawa cells, the addition of EGF did not result in promotion of growth in both HHUA and HOUA cells which also contain the

*Table* 1. *Dissociation constants and epidermal growth factor receptor (EGFR) numbers*

| | High | | Low | |
|---|---|---|---|---|
| Cell | $K_d$(M) | No./cell | $K_d$(M) | No./cell |
| IK | $4.21\times10^{-9}$ | $1.05\times10^4$ | $26.5\times10^{-9}$ | $2.38\times10^4$ |
| HHUA | $9.8\times10^{-9}$ | $2.90\times10^4$ | $29.5\times10^{-9}$ | $3.76\times10^4$ |
| HOUA | $1.4\times10^{-9}$ | $0.78\times10^4$ | $43.0\times10^{-9}$ | $2.2\times10^4$ |

[$^{12}$Val]K-*ras* mutation (Figure 1b). EGF inhibited cell growth. Thus, an impaired growth response to EGF was observed for cells harbouring mutated *ras*. The lesser growth stimulation in response to EGF observed in Ishikawa cells incubated in medium containing 0.1% calf serum might reflect the presence of minimal growth factors essential for their growth.

*Effect of EGF on Ras activation*

The mitogenic action of EGF is due, in part, to activation of the Ras signal transduction pathway. Thus, EGF has been shown to cause a rapid and transient elevation in active, Ras-GTP levels in stimulated cells [19]. Therefore, we determined if the responsiveness of the different endometrial carcinoma cell lines to EGF growth stimulation was associated with different responses to EGF-stimulated changes in Ras-GTP levels. First, we determined the levels of Ras-GTP in proliferating cells. For this analysis, cells were incubated with phosphate-free DMEM supplemented with 10% FCS and metabolically labelled with $^{32}$P-orthophosphate in phosphate-free DMEM. The cells were then lysed and Ras was immunoprecipitated with the Y13-259 anti-Ras antibody, and the nucleotides bound to Ras were determined by thin layer chromatography. Ras-GTP levels in Ishikawa and HHUA cells were found to be 13 and 27% (% GTP/GTP + GDP), respectively (Figure 2a). The higher levels of Ras-GTP in HHUA cells most likely reflect the presence of mutated K-*ras* in these cells. However, this level contrasts with higher levels that are typically observed for oncogenic Ras in transfected fibroblast cells (70–90%), presumably because of the presence of comparable levels of normal Ras protein in HHUA cells. Incubation of Ishikawa and HHUA cells in the presence of 5% calf serum for 7 days resulted in cells accumulating in the resting phase (data not shown). Thus, we determined the levels of Ras-GTP in quiescent Ishikawa and HHUA cells stimulated with 50 ng/ml EGF. Whereas serum-starved Ishikawa cells showed low levels of Ras-GTP (3.0%) prior to EGF stimulation, EGF stimulation caused a 3-fold increase in active Ras-GTP (10.6%) within 2 min. However, the significantly higher levels of Ras-GTP (13.1%) seen in serum-starved HHUA cells were not affected by EGF addition (13.0%) (Figure 2b). We determined the levels of Ras-GTP in HHUA cells at 10 and 20 min after EGF addition. No significant change in the level was detected (data not shown). These results suggest that EGF could cause activation of Ras in Ishikawa, but not in HHUA cells.

*Influence of the introduction of mutated* ras *on EGF responsiveness*

Our observation that HHUA cells were insensitive to EGF-mediated stimulation of growth or Ras-GTP formation suggests that expression of oncogenic K-Ras(12V) may influence

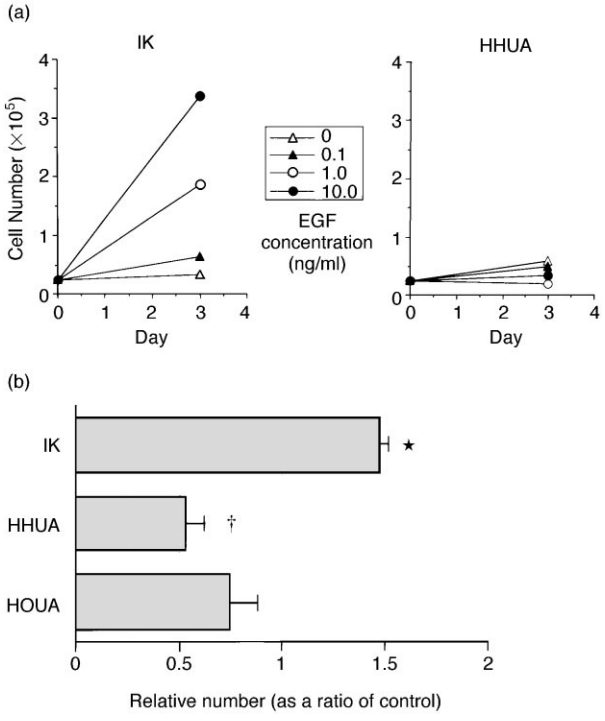


**Figure 1. * Ishikawa and HHUA cells show different growth responses to epidermal growth factor (EGF) stimulation. (a) Initial growth in 10% fetal calf serum (FCS). Results are representative of three independent experiments. (b) Initial growth in 0.1% FCS. Relative increases in cell numbers in response to EGF are shown as ratios of cell numbers after EGF treatment over cell numbers without EGF treatment. Columns, means; bars, standard error of the mean (SEM); $n = 3$. Significant differences between the EGF-treated and control culture are $P < 0.01$. *EGF > control †control > EGF.**

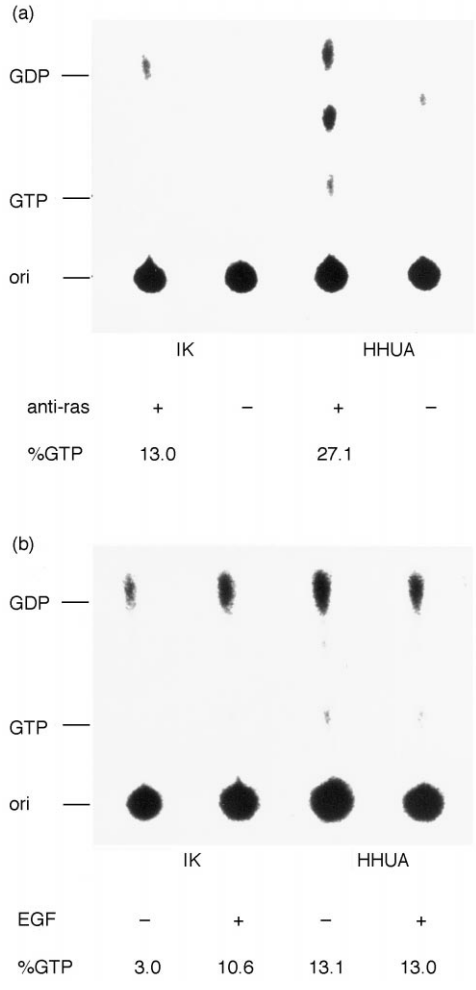


**Figure 2. Ras-GTP in Ishikawa and HHUA cells. (a) Ras-GTP levels after growth in 10% fetal calf serum (FCS). (b) Ras-GTP levels after growth in 5% FCS (i.e. resting cells) and following epidermal growth factor (EGF).**

EGF responsiveness in endometrial carcinoma cells. However, since these two independently-derived tumour cell lines are likely to possess genetic differences beyond those seen for *ras*, we cannot ascribe differences in EGF responsiveness solely to the mutation status of *ras*. For example, a single base substitution at nucleotide 756 of the *p53* gene has been documented in Ishikawa cells [24]. Therefore, we determined if the introduction of mutated *ras* alone could alter the EGF responsiveness of Ishikawa cells. Expression vectors encoding wild-type or mutated K-*ras*4B were transfected into Ishikawa cells and stably transfected cell lines expressing each Ras protein were established. The relative levels of wild-type versus mutant Ras were 3:2 in IK12V pools and IK12V c3, and 1:4 in IK12V c5 (Figure 3a). IK12V cells had a tendency to express a higher level of low affinity EGFR and a lower level of high affinity EGFR, compared with that in IK mock cells (Table 2). The difference in EGFR expression pattern did not correspond to the *ras* mutation, but rather reflected the variation between IK12V subclones. IK12V c5 clone expressed a similar level of low affinity EGFR to that in IK mock cells.

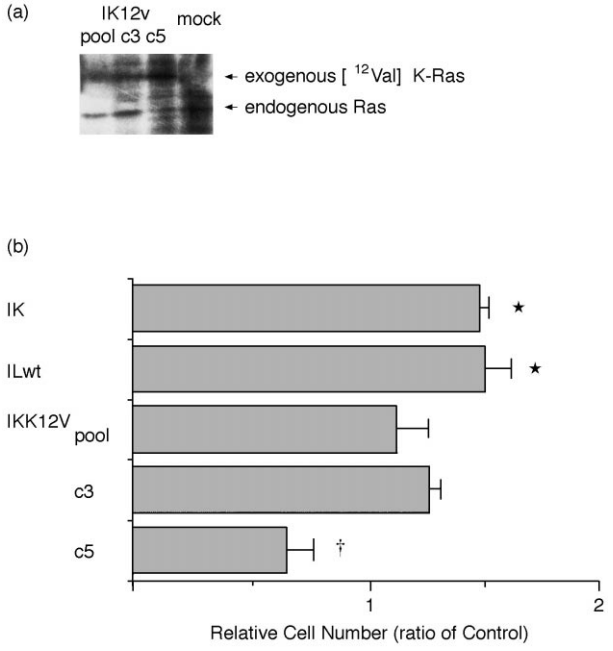


**Figure 3. The effect of mutated *ras* on epidermal growth factor (EGF) responsiveness of Ishikawa cells. (a) Wild-type and mutant protein expression. (b) EGF on parental and transfected Ishikawa cells. Data are expressed as ratios of cell numbers after EGF treatment over cell numbers without EGF treatment. Pool, pooled population of IK12V cells. c3 and c5 were individual clones derived from IK12V cells. Column, means; bars, standard error of the mean (SEM) as a ratio to the control cell number (=1); $n=3$. Significant differences between the EGF-treated and control cultured are $P<0.01$. *EGF > control †control > EGF.**

*Table 2. Receptor number in established Ishikawa cells*

| | No. of receptors/cell | |
|---|---|---|
| Cell | Low ($\times 10^3$) | High ($\times 10^3$) |
| IKmock | 1.0 | 8.2 |
| IKK12V pool | 12.0 | 7.2 |
| c3 | 6.7 | 1.9 |
| c5 | 1.5 | 1.3 |

We then compared their growth responses to EGF. The transfected cell lines were maintained for 2 days in growth medium supplemented with 0.1% FCS, followed by an additional 3 days in growth medium, either with or without 10 ng/ml EGF (Figure 3b). After 3 days' incubation, the control culture grew slowly and a 2.0-fold increase in cell number was observed in mock cells, a 2.7-fold increase in Ishikawa cells transfected with wild-type K-Ras cDNA (IKwt cells), and a 2.0-fold increase in IK12V cells. In response to EGF, mock and IKwt cells showed a 1.6- and a 1.5-fold increase in cell number compared with that of each control culture, respectively. Growth stimulation in response to EGF was less remarkable in IK12V cells; a 1.1-fold increase in pooled populations, a 1.2-fold increase in c3 and a 0.65-fold increase in c5. These observations provide strong support for the role of oncogenic Ras in abolishing the growth-promotive effect of EGF in endometrial carcinoma cells.

*Programmed cell death (PCD) induced by EGF in endometrial carcinoma cells carrying oncogenic K-*Ras

Many epithelial and endothelial cells responded to serum deprivation by increasing their rate of programmed cell death (PCD) while they continued to cycle, resulting in apparent stasis, a slow increase or a decrease in cell number. Thus, we compared the incidence of PCD in response to EGF in Ishikawa cells, HHUA cells and IK12V cells. Three-day incubation of these cells in the absence of serum resulted in the appearance of cells positively stained by *in situ* apoptosis detection (0.6% in Ishikawa cell, 6.0% in HHUA cells and 7.0% in IK12V cells). EGF (10 ng/ml) stimulated PCD in HHUA (22%) and IK12V cells (25%) whereas Ishikawa cells did not respond to EGF (0.5%) (Figure 4). Our results suggest that carcinoma cells carrying oncogenic [$^{12}$Val] K-*ras* are susceptible to EGF-mediated PCD. The oncogenic K-*ras* mutation would affect the EGF-mediated signal transduction and convert the EGF effect from promotion of cell growth to PCD induction. This assumption is compatible with the present result that EGF suppressed the growth of HHUA and HOUA cells containing the [$^{12}$Val] K-*ras* mutation. As constitutive c-myc expression resulted in PCD in serum-deprived Rat-1 fibroblasts [25], we investigated c-myc expression

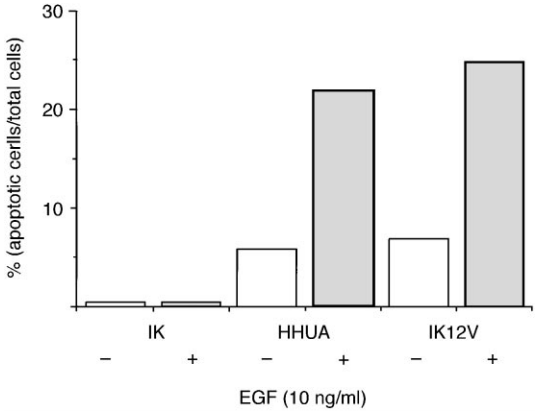


**Figure 4. Programmed cell death in cells harbouring mutated *ras* incubated in serum-free medium and epidermal growth factor (EGF). −, serum-free medium; +, serum-free medium in the presence of 10 ng/ml EGF.**

*Table 3. Effect of erbstatin on anchorage-independent cell growth*

| | Cell line | | | | |
|---|---|---|---|---|---|
| | IK | IK WT | IK 12V | HHUA | HOUA |
| (A) Control | 37.7±6.6* | 22.3 ± 2.4 | 68.7 ± 4.0 | 147 ± 2.9 | 138 ± 35.6 |
| (B) Erbstatin | 7.7 ± 2.0 | 6.7 ± 0.47 | 40.7 ± 8.0 | 160 ± 20.9 | 107 ± 29.8 |
| Ratio of B:A (fold inhibition) | 4.9 | 3.3 | 1.7 | 0.9 | 1.3 |

*Mean colony number/plate ± standard error of the mean (SEM), $n = 3$.

using Western blots. However, any increases of c-myc protein levels in response to EGF could not be observed in these three cells (data not shown).

*Effect of a tyrosine kinase inhibitor on anchorage-independent growth*

The EGF-insensitive phenotype of endometrial carcinoma cells harbouring mutated *ras* suggested that growth of these cells was no longer dependent on EGFR-mediated stimulation. To address this possibility, we determined the effects of an inhibitor of the EGFR tyrosine kinase activity, erbstatin [23], on the growth of cells expressing wild-type versus mutant Ras proteins. First, we observed that erbstatin inhibited the anchorage-dependent growth of Ishikawa, but not HHUA cells (Figure 5). Second, we observed that erbstatin caused a remarkable suppression of anchorage-independent growth in Ishikawa cells (approximately 5-fold) and IKwt cells (3.3-fold) (Table 3). However, a less remarkable suppression of colony formation in soft agar was noticeable in erbstatin-treated HHUA cells and IK12V cells. Erbstatin also did not notably suppress colony formation of HOUA cells. These results suggest that the carcinoma cells carrying the wild type K-*ras* gene require promoted EGF-mediated signal transduction for their proliferative activities.

TGFα binds EGFR and activates EGFR-mediated signal transduction. Thus, we investigated the levels of TGFα production in Ishikawa, HHUA, Ishikawa mock and IK12V cells by ELISA, in order to address whether the K-*ras* mutation would alter the response of cells to EGF by promoting TGFα production. Small amounts of TGFα were detectable in these cells. However, we could not obtain any evidence indicating a positive correlation between the presence of the K-*ras* mutation and enhanced production of TGFα (Table 4).

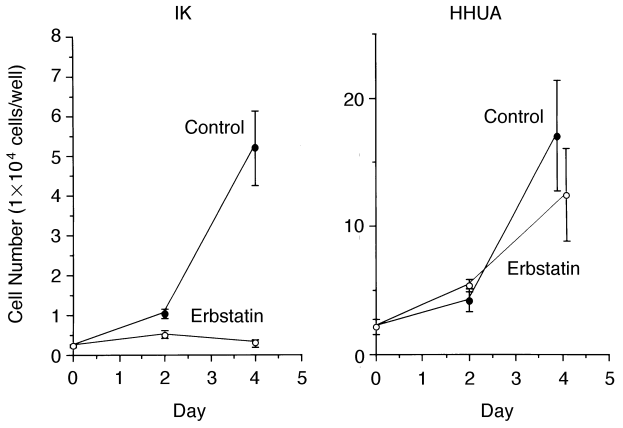


**Figure 5. The effect of erbstatin on the growth of endometrial carcinoma cells harbouring mutated *ras*.**

## DISCUSSION

Although precise genetic events involved in endometrial carcinoma development and the contribution of each genetic lesion remains to be defined, considerable evidence has identified a number of these putative genetic events. Microcell hybridisation experiments suggest that chromosome 1 carries a putative tumour suppressor gene for endometrial carcinoma [26]. K-*ras* mutations and 18q allelic losses have been detected in 20–30% of endometrial carcinomas [27]. Mutations in the *p53* tumour suppressor gene may correlate with the appearance of aggressive endometrial carcinoma cells [28]. Thus, the accumulation of at least four genetic events has been implicated in endometrial carcinoma development. Our results suggest that K-*ras* mutations alter the response of endometrial carcinoma cells to EGF stimulation. Aberrant Ras function may correlate with the transition process from hyperplasia to carcinoma stages, since K-*ras* mutations have been detected both in atypical hyperplasia, that was premalignant, and carcinomas [5]. This means that K-*ras* mutations may contribute to the critical steps in the carcinogenesis of endometrial cells.

Although both Ishikawa and HHUA cells expressed abundant cell surface EGFR, only Ishikawa cells exhibited responsiveness to EGF. EGFR number was not related to the presence or absence of mutated K-*ras* in these cells. Previous studies have observed the loss of growth factor responsiveness in Ras-transformed cells [19]. Similar to our observation, Ras-transformed cells display impaired responses to PDGF responsiveness, yet retain expression of the PDGF receptor [29]. Whether the presence of oncogenic Ras prevents EGF stimulation and activation of the EGFR in endometrial carcinoma cells is unknown. The exact basis for this defect in EGF responsiveness in endometrial carcinoma cells that harbour mutated *ras* remains to be clarified.

Although K-*ras* mutations have been documented in endometrial carcinomas, 80% of these tumours do not contain any detectable *ras* mutations. Thus, the aberrant function of Ras and the Ras signal transduction pathway may not contribute to the progression of the majority of endometrial carcinomas. In addition, the presence of *ras* mutations in endometrial carcinomas has not correlated with transformed phenotypes and the prognosis factors (grade, myometrial

*Table 4. Transforming growth factor alpha (TGFα) production of endometrial carcinoma cells*

| | Cell line | | | | |
|---|---|---|---|---|---|
| | IK | HHUA | IK mock | IK12V c3 | IK12V c5 |
| TGFα (pg/$10^5$ cells/24 h) | 2.86 | 2.59 | 2.07 | 1.54 | 6.35 |

invasion, lymph node metastasis, etc.) [7]. Other genetic events which cause chronic activation of the Ras signal transduction pathway may be important for the development of *ras* mutation negative tumours. For example, chronic upregulation or overexpression of tyrosine kinases, which mediate their transforming actions, in part, through the activation of Ras may trigger the same consequences as mutations in Ras itself. This may occur as a consequence of an autocrine mechanism involving TGFα and other EGF-related growth factors. Although the low levels of Ras–GTP in Ishikawa cells argue against this possibility, it is possible that the constitutive activation of components that function downstream of Ras can also lead to cellular transformation (e.g. Raf-1 and MEK). Thus, constitutive activation of the Ras signalling pathway, in the absence of chronic Ras activation, may still contribute to malignant growth properties of endometrial carcinoma cells that do not possess mutated *ras* genes.

In spite of the frequent occurrence of *ras* mutations in diverse human tumours, the precise contribution of aberrant Ras function to the malignant phenotype of most types of tumours remains to be established. Our observations that mutated *ras* perturbs the sensitivity of endometrial carcinoma cells to EGF stimulation and that a functional EGFR is dispensable for the growth of oncogenic Ras-containing tumour cells support an important contribution of mutant *ras* to the growth factor requirements of endometrial carcinoma cells.

While this paper was in preparation, two papers were published. In one, PANC-1 human pancreatic carcinoma cell line which harbours the K-*ras* gene mutation in codon 12 was examined. PANC-1 cells responded to neither EGF nor exogeneous TGF-α [30]. The result was consistent with our data. The other paper described several human colon carcinoma cell lines containing mutations in codon 12 or 13 of the K-*ras* gene. It was shown that Ras function in growth factor signalling was maintained in spite of the presence of the K-*ras* mutation in the cells [31]. The difference in the response to growth factor could not be explained by the position of the mutation. Although it is unclear, at this time, why there is a discrepancy among human endometrial, pancreatic and colon carcinoma cells, the implication would be that in endometrial carcinoma cells, EGF receptor signalling relies heavily or solely upon K-*ras*, rather than Ha- or N-*ras*, or direct effects upon protein kinase A or other Ras-independent pathways. However, signal transduction from the EGF receptor bypassing K-Ras would be important for colon carcinoma cells.

The suppression of *in vitro* cell growth by the EGF receptor inhibitor demonstrated in the present observations is important in understanding *ras* mutation negative tumours. Erbstatin was isolated as a potent inhibitor of EGFR associated tyrosine protein kinase and inhibits EGFR-autophosphorylation with an $IC_{50}$ of 0.55 μg/ml *in vitro*. Based on these data, we used 0.2 μg/ml erbstatin for cells plated on plastic, resulting in suppression of growth of carcinoma cells harbouring the wild-type K-*ras* gene. However, suppression of colony formation in soft agar required 1.0 μg/ml erbstatin. Degradation of erbstatin during the longer cultivation could explain the requirement for higher concentrations for suppressing colony formation in soft agar. Although it remains unknown whether a similar degree of inhibition is expected for *in vivo* tumours, dependence of cell growth on external stimuli through EGFR provides the possibility that the inhibitor is applicable to the diagnosis and treatment of endometrial carcinomas that carry the wild type K-*ras* gene. However, application of the inhibitor for clinical use will require verification of the intact regulation of signal transduction from the Ras-encoded protein to various downstream targets through which it transmits a signal for growth of carcinoma cells with the wild type K-*ras* gene.


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