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RhoA and RhoC proteins promote both cell proliferation and cell invasion of human oesophageal squamous cell carcinoma cell lines *in vitro* and *in vivo*

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ABSTRACT

There is growing evidence that Rho proteins are deregulated by overexpression in tumours; and according to some reports, this correlates with disease progression. Our previous clinical study had demonstrated a correlation between RhoA expression and tumour progression in oesophageal squamous cell carcinoma (ESCC). These findings prompted us to study, using nude mice, pathological roles of Rho proteins in human ESCC cells. Western blot analysis in ESCC cell lines, in addition to cell proliferation and *in vitro* migration assays, were performed to observe the malignant potential of RhoA and RhoC in untransfected and transfected cells. Constitutively active RhoA, RhoC and dominant negative RhoA (dn-RhoA) proteins were transfected to ESCC (TE-1 and TE-2) cells. The stably transfected cells were injected into nude mice, and the growth and metastasis of these cells to the lungs were analysed. Tumour tissues were then examined using immunohistochemical methods for proteins Ki-67 (MIB-1), FAK, MMP-1, MMP-9 and TIMP-3. Protein levels of RhoA and RhoC in ESCC cell lines were visualised by Western blotting, and showed highest expression in TE-2 cells. Results from the migration assay illustrated that both RhoA and RhoC play a role in migration of ESCC cells. In TE-2 transfected cells, RhoC showed greater migration compared to RhoA. By using an experimental metastasis model in nude mice, RhoA was found to promote more tumour growth than RhoC, whereas RhoC induced lung metastasis in comparison to RhoA. Ki-67 labelling index was used to evaluate the proliferation potential of tumour tissue inoculated from nude mice. In TE-2 cells RhoA gave a proliferation capacity of 24.8 ± 0.5, which was significantly higher than those of TE-2 RhoC 10 ± 0.4 ($P < 0.01$). Strong immunoreactivity for FAK, MMP-1 and MMP-9 proteins was present in all tumour cells. By contrast, loss of TIMP-3 expression was observed in all tumour cells. In conclusion, our results indicate that pro-oncogenic Rho proteins are involved in promoting tumour growth, cell migration and metastasis in human ESCC cells in nude mice. The results from this study suggest that active Rho proteins may induce a transforming effect that leads to a malignant phenotype.

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1. Introduction

Ras homolog (Rho)-family of small guanosine triphosphatase (GTPases) comprise a large branch within the Ras family of low-molecular-weight guanine nucleotide-binding proteins.[1–4] Rho GTPases have been implicated in the control of diverse physiological responses such as cell proliferation and cell motility as well as in pathological processes such as transformation and metastasis.[5,6] There is growing evidence that Rho proteins become deregulated by overexpression in tumours; and in some cases, this deregulation correlates with disease progression.[7] Interestingly, despite the high homology of the different Rho isoforms (RhoA, RhoB and RhoC),[8,9] their physiological roles are distinct.[4] Whereas RhoA and RhoC proteins have been shown to have a positive role in proliferation and malignant transformation,[10–12] the role of RhoB in these processes appears to be more divergent.[13,14] Our previous clinical study demonstrated the correlation between RhoA and tumour progression in human oesophageal squamous cell carcinoma (ESCC).[15] The importance of other closely related proteins such as FAK, MMPs and TIMP in human ESCC[16–18] prompted us to use nude mice to study the pathological role of Rho in human ESCC cells.

2. Materials and methods

2.1. Cell lines

Six human ESCC cell lines (TE-1, TE-2, TE-8, TE-13, TE-14, TE-15) and one human keratinocytes cell (HaCaT) were used in this study. TE cell lines were derived from human ESCC with varying degrees of differentiation.[19] All cells were cultured in RPMI-1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 Uml^{-1} penicillin and 100 μgml^{-1} streptomycin).

2.2. Protein extraction and Western blot analysis

All cells were harvested at approximate 80% confluence. The protein concentration was determined using BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Protein (40 μg) was resolved on 16.5% Tris-Tricine ReadyGel (Bio-Rad, Tokyo, Japan) and transferred to hybond-enhanced nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). RhoA protein levels were determined using monoclonal anti-RhoA and polyclonal anti-RhoC specific antibodies (1:1000, Santa Cruz Labs, CA, US). Presence of protein was detected using an enhanced chemiluminescence detection system according to the manufacturer's instruction (Amersham). An anti-β-actin antibody (Sigma) served as control. Horizontal scanning densitometry was performed by using acquisition into Adobe Photoshop (Apple, Inc., Cupertino, CA) and analysis by the Quantity One (BioRad).

2.3. Cell proliferation assay

TE-1 and TE-2 cells (5×10^3/well) were seeded into 96-well microtiter plates, in a total volume of 100 μl in each well. Briefly, WST-8 (Dojindo Lab, Tokyo, Japan), 10 μl of the cell counting solution was added and incubated in a humidified 5% CO_2 atmosphere at 37 °C for 3 h. The medium was removed and formazan was dissolved in 100 μl/well 1N HCl. The absorbance of the solution was read at 450 nm using a microtiter plate reader (Becton Dickinson, Franklin Lakes, N.J). All experiments were performed in triplicate.

2.4. In vitro cell migration assay

Cell migration assay was performed using a Transwell apparatus (Corning) as described previously.[20] In brief, the cell suspension (~2.5×10^5 cells in 100 μl of serum-free medium) was transferred to a polycarbonate filter (pore size, 8 μm) in the upper compartment of the apparatus, and 300 μl of culture medium was placed in the lower compartment. The apparatus was then placed for 24 h at 37 °C in the humidified incubator containing 5% CO_2. Cells that had migrated through the membrane were exposed to staining solution of WST-8 assay kit (Dojindo) and 30 μl of the cell counting solution was added to each well of the plates, and incubated in a humidified 5% CO_2 atmosphere at 37 °C for 3 h prior to colourimetric analysis. The absorbance at 450 nm was measured using a microtiter plate reader (Becton Dickinson, Franklin Lakes, N.J). Each experiment was performed in triplicate.

2.5. Cell lines and transfection

Two ESCC cells (TE-1 and TE-2) were used in these experiments. Cells (80% confluent in 96-well plate) were transfected with 1 μg of vector plasmid pMIG-RhoA, pMIG-RhoC or pMIG-N19RhoA (gift from Dr. R.O. Hynes, Massachusetts Institute of Technology, USA). The empty vector plasmid, the retroviral bi-cistronic expression vector pMX-IRES-GFP, contained the coding sequence for enhanced green fluorescent protein (GFP), which became the marker for protein expression.[21–23] The vector plasmids were used to transform competent TE-1 and TE-2 cells, and grown on LB Broth agar plates containing 50 μg/ml ampicillin. A single positive clone (with each vector plasmid) was checked by restriction enzyme digestion (*Bam*HI and *Eco*RI) and resolved on 2% (w/v) agarose gel with etidium bromide. In brief, the TE cells were co-transfected with 1 μg of each vector and pcDNA3.1Zeo (+) (Invitrogen). Transfections were performed according to the manufacturer protocol (FuGENE 6, Roche). Reagents were used in amounts of 6 μl/well in antibiotic-free medium. After 24 h, the medium was removed. The transfected cell was transfered to a new plate with medium containing no antibiotic. The following day the medium was replaced with fresh medium containing 200 μg/ml Zeocin selection marker. The stable transfectants were incubating for 2 week and protein expression was verified by Western blotting using GFP antibody (BD Bioscience).

2.6. Detection of GFP transgene expression

TE-1 and TE-2 cells were plated in 6-well culture plates (Falcon, Oxnard, CA) at a density ~2.5×10^5 cells/well and cultured overnight. Cells were transfected with the pMIG-Rho vector plasmids coding for GFP. After 24 h, cells were fixed

in 4% paraformaldehyde. Subsequently, GFP expression was visualised under a Leica PVCAM Image fluorescence stereo microscope (Leica Imaging Systems Ltd, Cambridge, England).

2.7. Tumourigenicity and metastatic potential experiment in nude mice

Specific pathogen-free BALB/c nude (*nu/nu*) mice, 6–8 week old males were obtained from CLEA Inc. Tokyo, Japan. All mice were housed in the Institute of Experimental Animal Research, Gunma University, under conditions with a laminar air-flow. They were maintained on standard laboratory feed and in 12 h light/dark cycles. This study was approved by the Animal Research Ethics Board of Gunma University. TE-2 transfected cell lines were injected subcutaneously (s.c.) (0.1 ml) into the dorsal flank or intravenously (i.v.) (0.2 ml) into the lateral tail vein. All mice were sacrificed at 42 days after injection. The sizes of the primary tumours in the s.c. were measured. The volume of the primary tumour was calculated using the formula: $0.5 \times (\text{long diameter}) \times (\text{short diameter})^2$. The results are shown as mean size of tumours (mm^3) from each group of mice ± standard deviation (SD).

2.8. Histopathology and immunohistochemistry

The samples of primary tumours and lungs were fixed in 10% (v/v) formalin, embedded in paraffin, and 4 μm sections were processed with hematoxylin eosin (H&E). Lung metastasis was evaluated by histological examination under light microscopy. Tissue sections were treated with monoclonal anti-Ki67 (MIB-1), FAK, MMP-1, MMP-9, and TIMP-3 antibodies for immunohistochemical analysis. The sections were examined using light microscopy to establish the presence or absence of immunostaining and its distribution. Proliferating cells were detected by Ki-67, the proliferation index (PI) was calculated as the percentage of nuclear staining of tumour cells among more than 500 cells counted in three consecutive fields. A tumour was regarded as (+) if any tumour cells showed immunostaining and classified as (–) if there was a complete absence of immunostaining.

2.9. Statistical analysis

The significances between TE-1 and TE-2 transfected cell groups were determined using the student *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. RhoA protein expression in ESCC cell lines

Six ESCC cells were used to determine levels of RhoA and RhoC protein expression. HaCaT cells were used as a positive control. Levels of RhoA expression in different cell lines are shown in Fig. 1, upper panel; RhoC expression is shown in Fig. 1, middle panel; and β-actin served as the loading control (Fig. 1, lower panel). A high level of RhoA expression was observed in TE-2 cell line; moderate in TE-8, TE-13, TE-14, and TE-15 cell lines; and low in TE-1 cells. RhoC expression was observed only in TE-2 cells.

3.2. Cell proliferation assay

Based on the Western blotting results (Fig. 1), cells expressing the highest (TE-2) and the lowest (TE-1) level of Rho protein were selected for cell proliferation assays. The results of the cell proliferation assay (Fig. 2) showed that TE-2 untransfected cells exhibited higher proliferation ability than TE-1 untransfected cell (after 24 h, $P < 0.01$).

3.3. In vitro cell migration assay

Cell migration assay showed that the number of cells which had migrated to the lower chamber was greater for TE-2 untransfected cell (35%) than TE-1 untransfected cell (12%). The migration assay showed that TE-2 untransfected cell

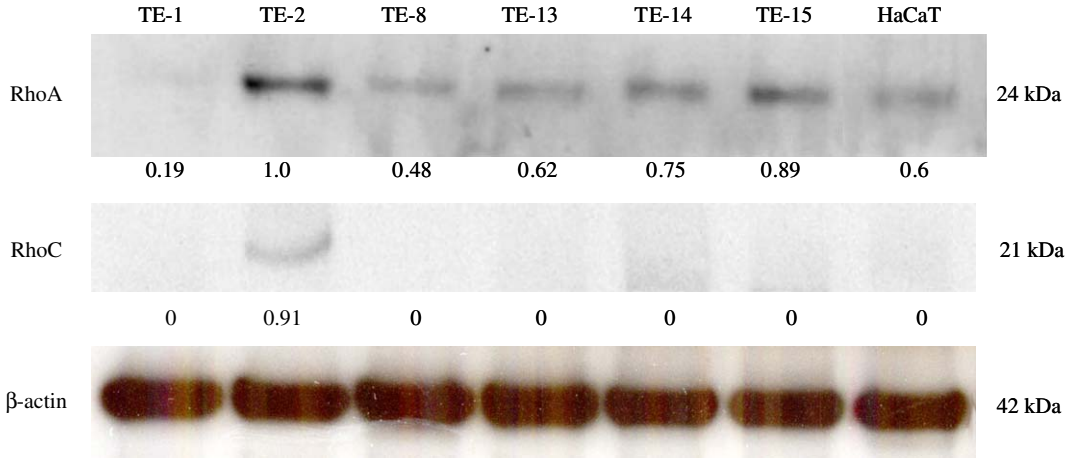

Fig. 1 – Western blotting of cell extract from six human ESCC cell lines and one human keratinocytes cell. It shows the expression of RhoA (24 kDa); RhoC (21 kDa); β-actin (42 kDa). The values underneath the bands represent the densitometric estimation of the density of the bands. The level of β-actin served as the loading control.

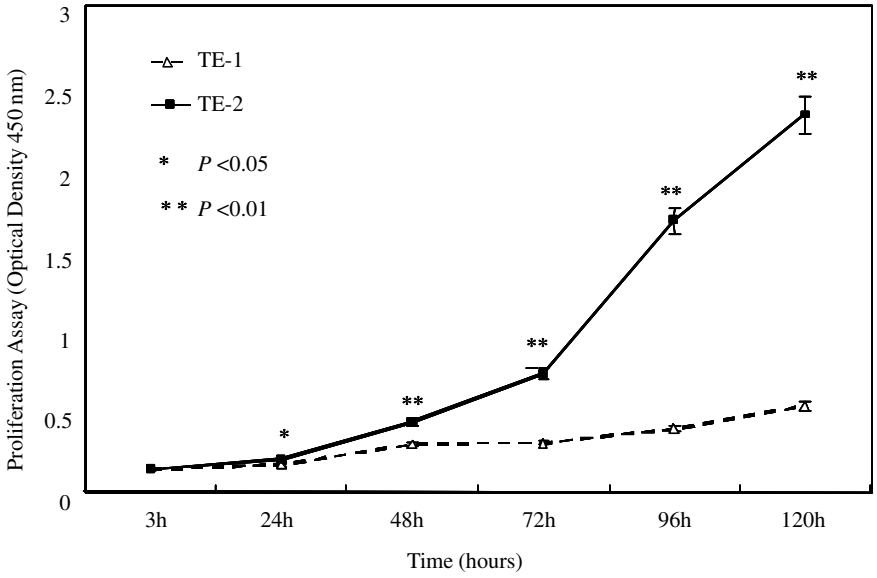

Fig. 2 – Growth properties of ESCC cells, TE-2 cells exhibited the higher proliferation ability than TE-1 cells. Each bar represents the mean of triplicate experiment.

had migratory ability 3 times higher compared to TE-1 untransfected cell ($P = 0.032$) (see supplementary Fig. 1). TE-1 and TE-2 transfected cell migration was measured using a polycarbonate filter (pore size 8 μm). Significantly, 2.5 times more TE-2 transfected cells than TE-1 transfected cells migrated through the pores ($P < 0.05$). Two out of three transfected cells (constitutive active RhoA and RhoC) induced significantly more migration than untransfected TE-1 and TE-2 cells ($P < 0.05$). Furthermore, in TE-2 transfected cells, RhoC had significantly greater migration ability than RhoA and dnRhoA (Fig. 3; $P < 0.05$ and $P < 0.005$). No significant differences were observed between TE-2 RhoA and TE-2 dnRhoA. For each transfected cell, TE-2 cells had significantly greater migration ability than TE-1 cells. Collectively, these data illustrate that both RhoA and RhoC play a role in the migration of ESCC cells.

3.4. *Ectopic expression of Rho in ESCC cells*

Cells transfected with pMIG-RhoA, pMIG-RhoC and pMIG-dnRhoA expressed GFP as the pMIG plasmid also comprised GFP cDNA downstream of the encephalomyocarditis virus

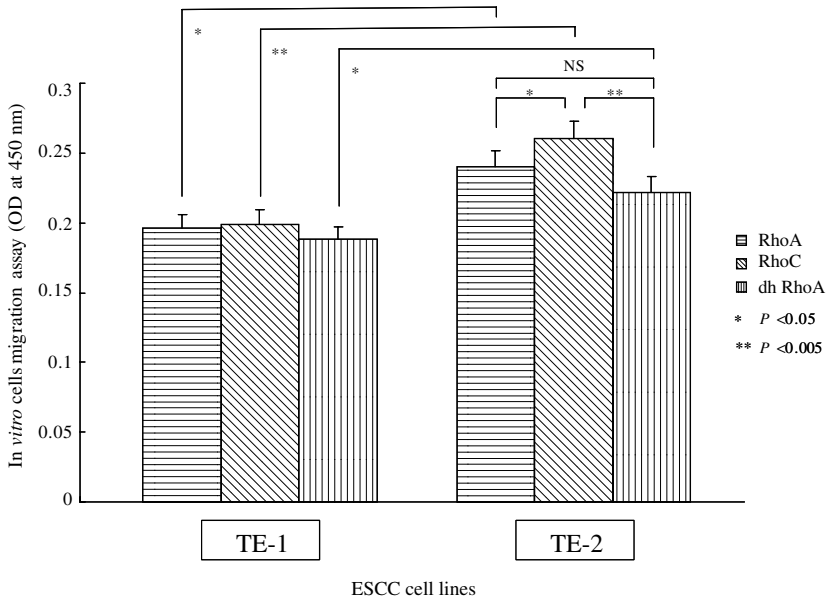

Fig. 3 – Migration ability of TE-1 and TE-2 transfected cells, TE-2 RhoC had significantly greater migration ability than TE-2 RhoA and TE-2 dnRhoA. No significant difference was observed between TE-2 RhoA and TE-2 dnRhoA (NS: not significant). For each transfected cell, TE-2 cells had significantly greater migration ability than TE-1 ($^{*}P < 0.05$, $^{**}P < 0.005$).

internal ribosomal entry sequence.[23] Using GFP-specific antibody, the expression levels of pMIG-RhoA, pMIG-RhoC and pMIG-dnRhoA were evaluated by Western blotting of transfected TE-1 and TE-2 cell lysates. Transfected cells showed levels of GFP expression (Fig. 4, 2 weeks post-transfection); expression of each Rho target protein was also significantly enhanced (Fig. 5); while the 'mock' served as negative control. Increases in the intensities of Western blotting bands corresponding to RhoA after pMIG-dnRhoA transfections is to be expected since the antibody for RhoA also recognises dnRhoA, as the only change between RhoA and dnRhoA protein is a the substitution *Thr* for *Asn* at codon 19 of *RhoA*.[24] Expression of GFP-tagged Rho proteins, as visualized through a fluorescence microscope, is shown in Fig. 6.

3.5. *Tumourigenicity and metastatic potential experiment in nude mice*

TE-2 stably transfected cells were implanted s.c. in nude mice, and tumour sizes were measured over a period of 42 days. As shown in Table 1, none of the mock cells or TE-2 dnRhoA formed tumours in the nude mice, and only adipocyte tissue was observed (Fig. 7C). By contrast, the stably transfected cells expressing TE-2 RhoA grew to an average tumour size of 164.5 ± 35.5 mm^3; and those expressing TE-2 RhoC 97 ± 19 mm^3. To measure experimental metastatic potential, TE-2 stable transfected cells were injected into the lateral tail vein of nude mice. The occurrence of lung metastasis was examined on the day of sacrifice. Briefly, the lung was fixed with 10% (v/v) formalin, and metastasis foci were counted. Because the metastasis foci on the lung surface could hardly be observed, the very small nodules were counted by microscopy. As expected, lung metastasis was found in 80% (4 of 5, n = 5) of TE-2 RhoC groups, whereas there was no lung metastasis found in the other groups (Fig. 7D-H).

3.6. *Histopathology and immunohistochemistry*

Primary tumours were excised and analysed using histological methods. Mice inoculated s.c. with the TE-2 RhoA had larger tumours than those with TE-2 RhoC, whereas there was no tumour development in other groups. Tumour cells were poorly differentiated, with a small cytoplasm and large nuclei with abundant mitosis (Fig. 7A-B). On the other hand in TE-2 cells expressing dnRhoA, only adipocyte tissue was observed (Fig. 7C). Tumour cells grew in a solid, capsu-

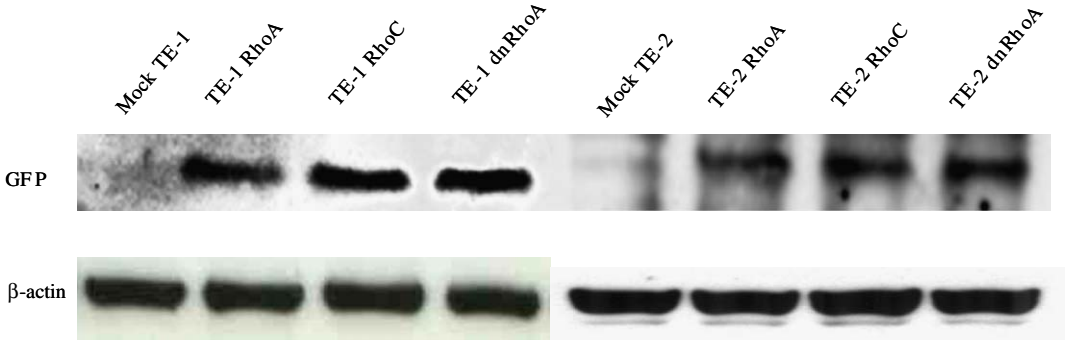

Fig. 4 – Stably transfected TE-1 or TE-2 cells with pMIG-RhoA, pMIG-RhoC or pMIG-dnRhoA were subjected to 16.5% SDS-PAGE (2 weeks post-transfection). Using the GFP antibody, the expression of transfected cells (upper) were evaluated by Western blotting. Anti β–actin served as control (lower).

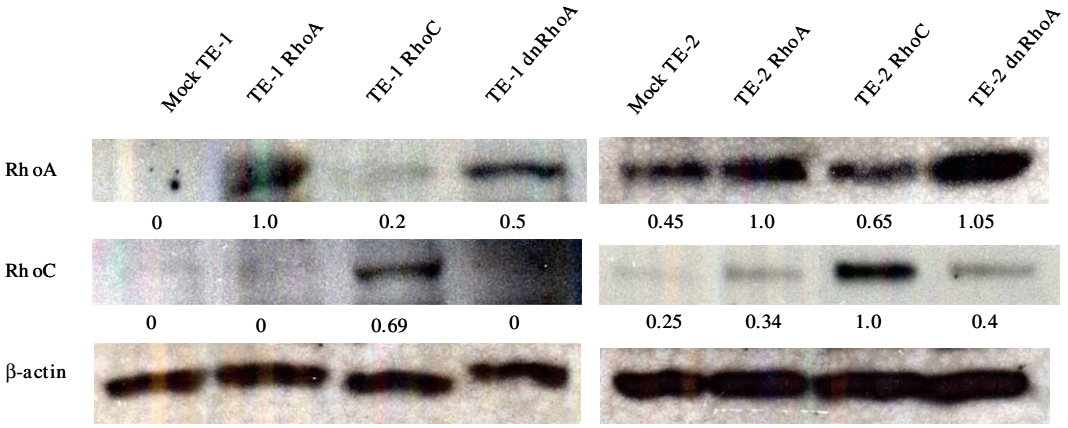

Fig. 5 – Expression of RhoA and RhoC in ESCC transfected cells. Up regulation of RhoA and RhoC was observed in transfected cells compare with mock transfected cells. Equal loading was confirmed by immunoblotting the membrane with an antibody to β–actin.

lated, diffuse pattern. All experiments are summarised in Table 1.

Number of Ki-67-positive cancer cells (see supplementary Fig. 2) was 24.8 ± 0.5 in TE-2 RhoA groups, and was significantly higher than those of TE-2 RhoC 10 ± 0.4 ($P < 0.01$). Using immunohistochemical methods with anti-FAK, MMP-1 and MMP-9 antibodies, cytoplasmic staining of tumour cells was observed in sections of tumour tissues in nude mice. The staining for each protein expression appeared uniform in all tumour cells, with no apparent intra-tumour heterogeneity. Strong immunoreactivity for FAK, MMP-1 and MMP-9 was present in all tumour cells (Fig. 8A-F). By contrast, loss of TIMP-3 expression was observed in all tumour cells (Fig. 8G-H).

4. Discussion

The activation or overexpression of RhoA proteins seems to promote tumour progression of ESCC.[15] To gain a clearer understanding of the roles of Rho proteins in tumour progression and metastasis in ESCC, the constitutively active form of RhoA, RhoC, and dominant negative RhoA was stably transfected into ESCC cells. Tumour growth, migration and metastatic potential of these stably transfected cells were then analysed.

The variability of RhoA expression at the protein level in ESCC cell lines was observed. The highest expression was observed in TE-2 and the lowest in TE-1 cells. On the other hand, only TE-2 cells showed expression of RhoC. Based on

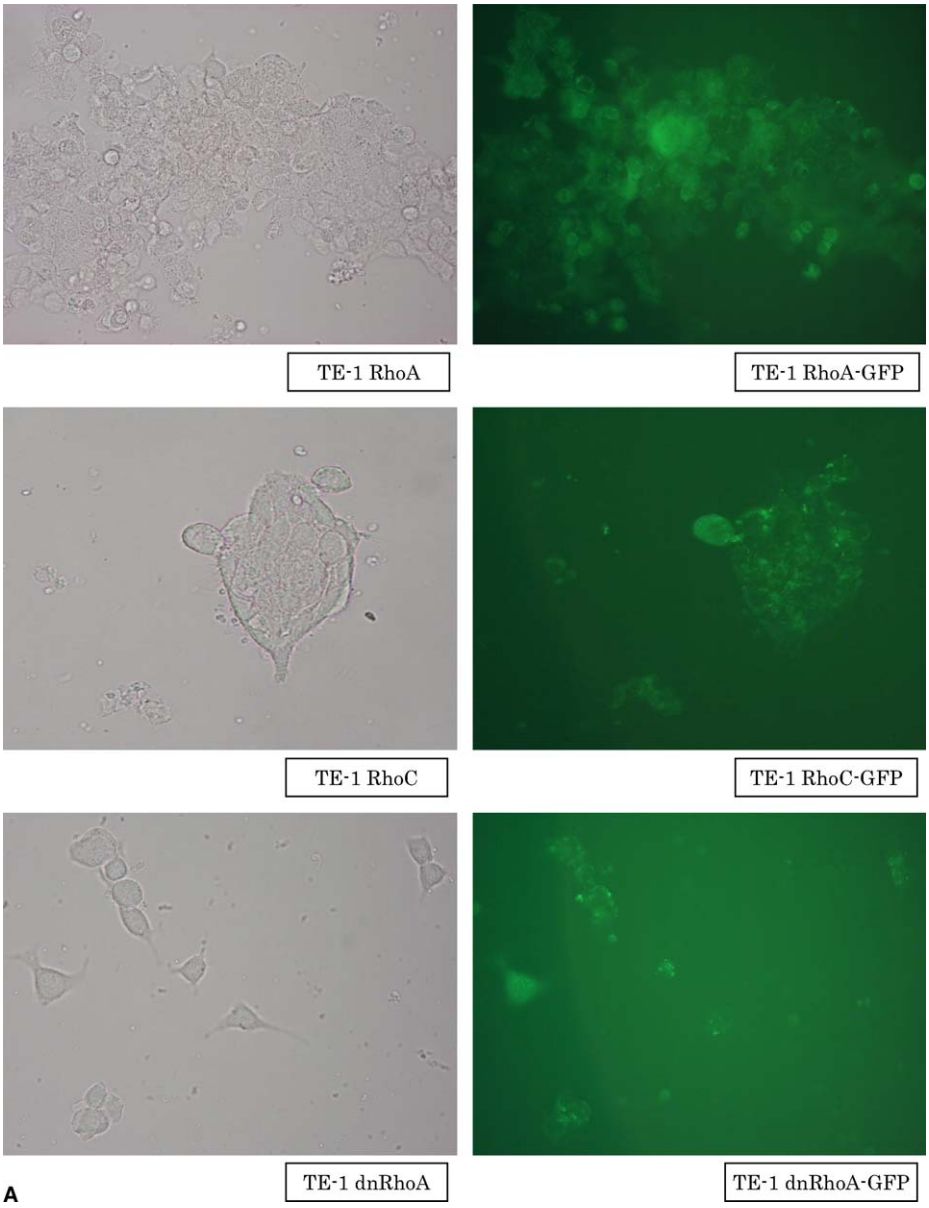

Fig. 6 – TE-1 and TE-2 cells were plated in six-well culture plates at a density ~2.5×10^5 cell/well and transfected with the Rho vector plasmids containing Green Fluorescence Proteins (GFP). GFP expression was visualised under a Leica PVCAM Image fluorescence stereo microscope. (A) TE-1 GFP transgene expression Upper: RhoA; middle: RhoC; lower: dnRhoA. (B) TE-2 GFP transgene expression Upper: RhoA; middle: RhoC; lower: dnRhoA.

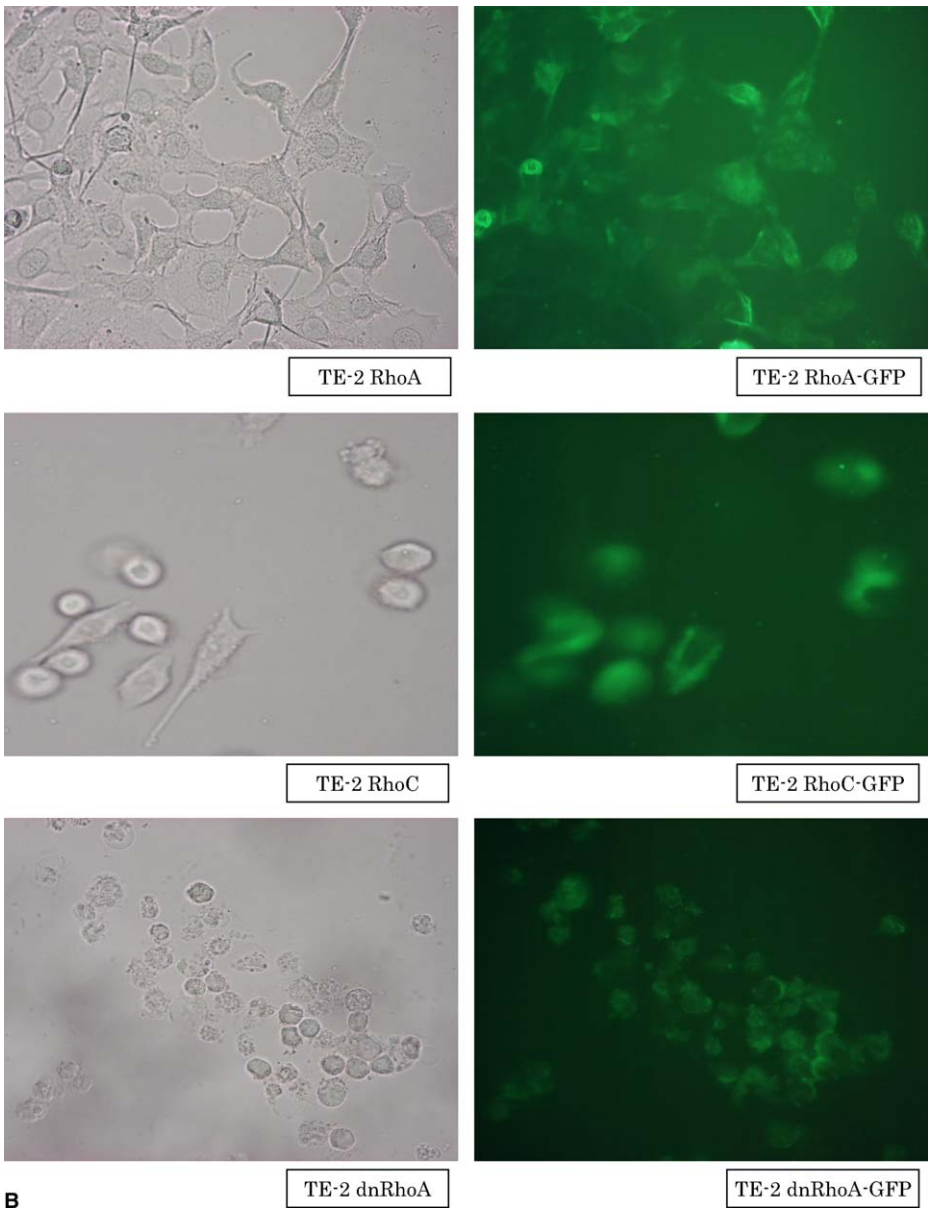

Fig. 6 –*continued*

Table 1 – The experimental metastatic results and primary tumour volume

Cell line	No. Tumour	No. lung metastasis	No. Mice	Mean ± SD tumour volume	P-Value
Mock	0/5	0/5	5	0	
TE-2 RhoA	5/5	0/5	5	164.5 ± 35.5 mm^3	A/C : 0.03*
TE-2 RhoC	5/5	4/5	5	97 ± 19 mm^3	
TE-2 dnRhoA	0/5	0/5	5	0	

SD: Standard Deviation; *: Significant; A: RhoA; C: RhoC; dn: dominant negative.

the Western blot analysis, we compared the proliferation and migration abilities between TE-1 and TE-2 untransfected cell lines. The cell proliferation assay showed that TE-2 had higher proliferation capability than TE-1 cell. As expected, the migration assay showed that TE-2 untransfected cells migrated 3-fold higher than TE-1 untransfected cell and indicated the malignant potential of Rho proteins in ESCC cell lines. However, the role played by Rho GTPases in the progression of malignant potential in ESCC cells has not been determined yet. Further, we performed transfection and

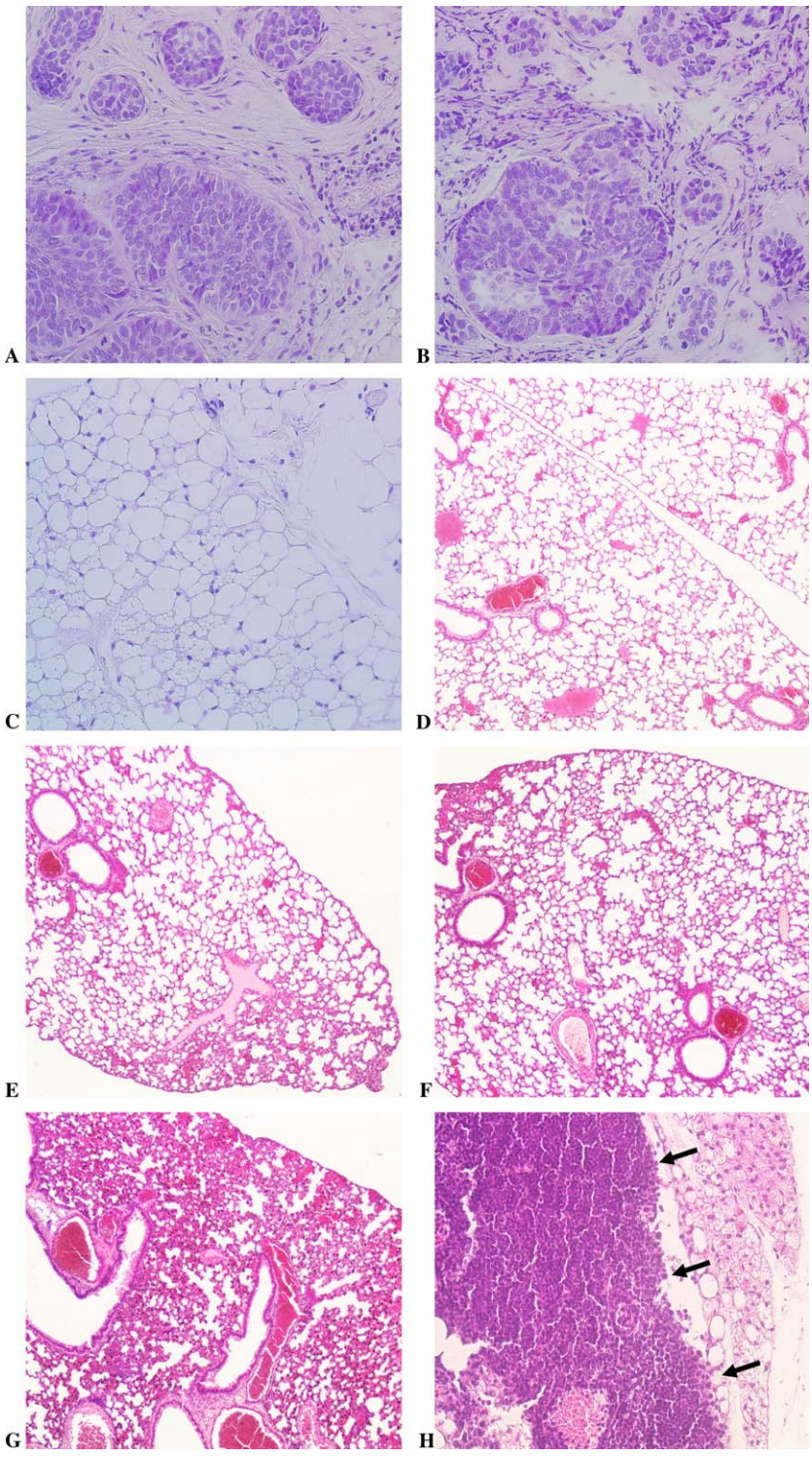

Fig. 7 – Histopathological analysis of solid tumour of TE-2 RhoA and -RhoC inoculated mice. (A) General view of H&E stained from tumour derived from TE-2 RhoA inoculated mice (×100). (B) TE-2 RhoC inoculated mice (×100). These tumour cells presented a poorly differentiated, small cytoplasm and large nuclei with abundant mitosis. (C) TE-2 dnRhoA inoculated cells replaced by adipocyte tissue, which is remained normal. This altered cell morphology was followed by a decreased in its cell proliferation (×100). (D-F) Histological findings of lung tissue from mice injected with mock transfected cells (i.v.), TE-2 RhoA (i.v.), and TE-2 dnRhoA (i.v.). (G) H &E section of TE-2 RhoC lung metastases. The boundary of the lesion was well demarcated, and the alveolar architecture is distorted. (H) The lung tissue from mice injected with RhoC (i.v.) shows the metastasis tumour growth. The arrow mark indicates the lung tumour (×200).

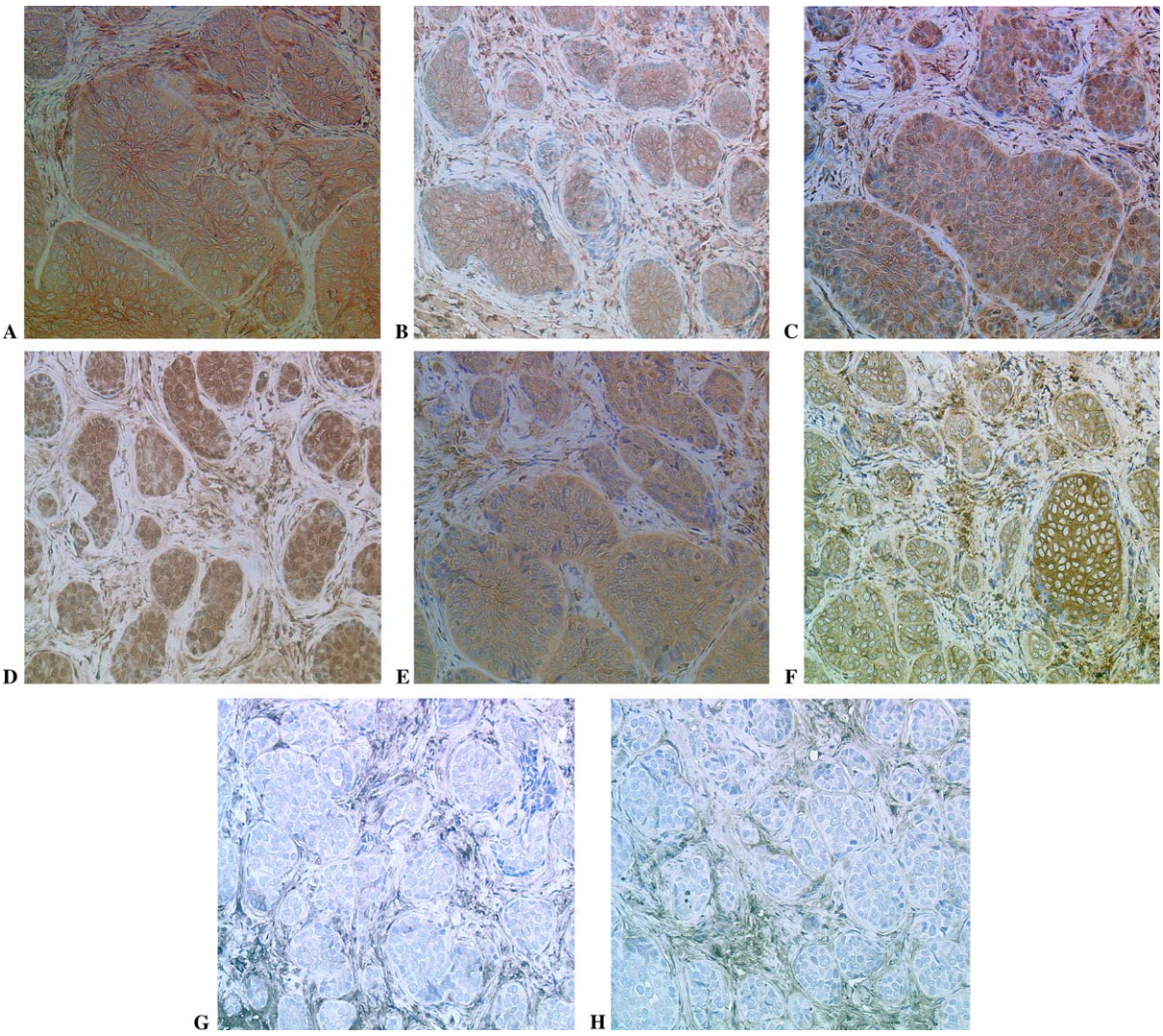

Fig. 8 – Immunohistochemical findings of tumour tissue growth in nude mice, FAK immunoreactivity was found to be diffuse and strong in (A) TE-2 RhoA and (B) RhoC. MMP-1 immunoreactivity was found to be diffuse and strong in (C) TE-2 RhoA and (D) RhoC. MMP-9 immunoreactivity was found to be diffuse and strong in (E) TE-2 RhoA and (F) RhoC. Loss of TIMP-3 immunostaining in (G) TE-2 RhoA and (H) RhoC was observed in all tumour tissue.

experimental metastasis assays to explore the role of Rho-family in ESCC cells.

To investigate the malignant potential of Rho proteins in ESCC cells, TE-2 cells were stably transfected with vector plasmids coding for an active form of RhoA (pMIG-RhoA), RhoC (pMIG-RhoC) or a dominant negative RhoA (pMIG-N19RhoA). Transfected cells with each vector, was injected s.c. into nude mice to induce tumour growth or i.v. to induce lung metastasis. Mock transfectant cells were also injected s.c. or i.v. and served as negative controls. All mice in TE-2 RhoA and RhoC group developed s.c. solid tumours. As RhoA and RhoC are 94% identical (i.e. only 11 amino acids are different),[25] they may influence the relative interaction with other effectors protein. Here we present evidence that the stable expression of constitutively active RhoA and RhoC induces a proliferation advantage in TE-2 ESCC cells. Conversely, expression of dominant negative RhoA decreases TE-2 ESCC cell proliferation (Fig. 7A-C). The cell proliferation of TE-2 RhoA injected s.c. was greater than the proliferation of TE-2 RhoC stably transfected cells (Table 1). In this study, we found that the distant metastasis observed in the lungs of tumour-bearing mice was caused by TE-2 RhoC (Fig. 7G-H). In contrast, the constitutively active TE-2 RhoA, dominat negative RhoA and mock transfectant cells did not induce lung metastasis (Fig. 7D-F). These results indicate that constitutively active RhoA and RhoC induce TE-2 ESCC cell transformation and tumourigenesis. Ki-67 labelling index was used to evaluate the proliferation potential of tumour tissue inoculated from nude mice, in TE-2 RhoA it was 24.8 ± 0.5 and was significantly higher than those of TE-2 RhoC 10 ± 0.4 ($P < 0.01$). These results suggest that RhoA promotes tumour growth more than RhoC, whereas RhoC induces distant metastasis in comparison to RhoA. Our findings are in agreement with those of Clark and colleagues, who showed that RhoC had better motogen than RhoA when expressed in melanoma and that RhoC overexpression can promote the ability of melanoma cells to exit

the blood and colonise lungs.[21] Recently, a study by Ikoma and colleagues showed a crucial role of RhoC in lung cancer metastasis.[26] All these observation suggest different functions of the two proteins in tumour progression.

Palazzo and colleagues showed that the integrin-FAK signalling pathway enables Rho to activate its effectors.[27] The activities of both Rho and ROCK are required for the assembly of integrins into focal adhesion.[28] Rho proteins can modulate the degradation and remodelling of the extracellular matrix (ECM) either by regulating the levels of matrix metalloproteinase (MMP) that degrade the ECM or by regulating the levels of their antagonists, tissue inhibitors of metalloproteinase (TIMPs).[29] An imbalance in the activities of MMPs and TIMPs, resulting in excessive degradation of the matrix, is specific to tumour invasion and metastasis.[30,31] The presence of MMP-1 and MMP-9 was examined in oesophageal cancer and was found to be related to poor prognosis and to be an independent prognostic factor.[17] Tumours isolated from these experiments were found to cause the uniform overexpression of FAK, MMP-1 and MMP-9 observed by immunohistochemistry. On the other hand, in the same tumour tissue, TIMP-3 expression was lost. Increased expression of TIMP-3 suppresses the invasion of colon carcinoma cells and *in vivo* and *in vitro* tumour growth.[32] Miyazaki and colleagues found that in ESCC, tumour tissues having metastasis ability did not express TIMP-3.[18] All these observation support our results.

By experimental metastasis in nude mice, we generated solid, subcutaneous, capsulated tumours and lung metastasis from constitutively active Rho proteins. The results of our study are in agreement with those of recent reports showing the malignancy potential of Rho proteins *in vivo*.[33,34] Overexpression of constitutively active RhoA and RhoC in certain advanced tumours indicates that they might promote tumour invasiveness and metastasis.

In conclusion, our study supports the notion that prooncogenic Rho proteins are involved in promoting tumour growth, cell migration and metastasis in human ESCC cells in nude mice. Thus, an active Rho protein may induce a transforming effect that leads to a malignant phenotype.

Conflict of interest statement

None declared.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2006.02.012.

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