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Putting oesophageal cancer on the RACK
A study of the RACK1 scaffolding protein in oesophageal adenocarcinoma

by
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Abstract

Purpose

There is a need to identify new therapeutic targets for oesophageal adenocarcinoma. Deoxycholic acid is a component of gastro-oesophageal refluxate and is believed to promote oesophageal adenocarcinoma. RACK1 is an intracellular scaffolding protein whose expression is altered in many cancers. The role of RACK1 in oesophageal adenocarcinoma has not been investigated. The purpose of this study is to examine the expression of RACK1 in oesophageal adenocarcinoma and to assess its potential as a contributor to this disease. This study also aims to investigate if deoxycholic acid modulates RACK1 in oesophageal adenocarcinoma cells.

Methods

Normal oesophageal epithelial cell lines, Barrett's metaplastic and dysplastic cell lines and oesophageal adenocarcinoma cell lines were used to construct a model of oesophageal adenocarcinoma disease progression.
RACK1 expression levels were assessed by western blotting.
RACK1 subcellular localisation was examined by immunofluorescent confocal microscopy.

Results

RACK1 protein levels generally increase with increasing disease progression across a panel of cell lines representing the multistep sequence from normal oesophagus to oesophageal adenocarcinoma.

Deoxycholic acid downregulates RACK1 protein levels in SKGT-4 (31% reduction; P=0.01) and FLO-1 (26% reduction; P=0.03) oesophageal adenocarcinoma cells.

RACK1 has a diffuse, cytoplasmic localisation in SKGT-4 and FLO-1 cells and is most concentrated in the perinuclear area. This pattern of RACK1 subcellular localisation is not altered by deoxycholic acid.

Conclusion

RACK1 may be upregulated during the development of oesophageal adenocarcinoma. Deoxycholic acid can modulate RACK1 protein levels in oesophageal adenocarcinoma cells. RACK1 may contribute to the progression of oesophageal adenocarcinoma and further investigation into its role in this disease is warranted.

Keywords

Oesophageal adenocarcinoma, RACK1, deoxycholic acid.

Introduction

The incidence of oesophageal adenocarcinoma (OAC) is currently increasing at a rapid rate in western countries[1]. Furthermore, despite recent advances in treatment, prognosis for this malignancy is still poor with overall 5 year survival ranging from 15% to 25%[2].

The development of OAC is a multistep process and this cancer usually arises from a precursor lesion called Barrett's oesophagus; a metaplastic condition whereby the normal squamous epithelium of the oesophagus is replaced by an intestinal-type columnar epithelium[3]. The development of Barrett's oesophagus and OAC is strongly associated with chronic gastro-oesophageal reflux disease[4]. Gastro-oesophageal refluxate contains a mixture of acid, bile acids and other harmful chemicals which damage the oesophagus and promote inflammation[5]. Chronic exposure to such

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1 Pennathur, Arjun, Michael K. Gibson, Blair A. Jobe, and James D. Luketich. 2013. Oesophageal carcinoma. *The Lancet* 381 (9864):400-412.

2 Ibid.

3 de Almeida, J. C. Mendes, Paula Chaves, A. Dias Pereira, and Nasser K. Altorki. 1997. Is Barrett's esophagus the precursor of most adenocarcinomas of the esophagus and cardia? A biochemical study. Annals of surgery 226 (6):725.

4 Pennathur et al 2013.

5 Duggan, Shane P., Fiona M. Behan, Murat Kirca, Sinead Smith, John V. Reynolds, Aideen Long, and Dermot Kelleher. 2010. An integrative genomic approach in oesophageal cells identifies TRB3 as a bile acid responsive gene, downregulated in Barrett's oesophagus, which regulates NF-κB activation and cytokine levels. *Carcinogenesis* 31 (5):936-945.

Jenkins, G. J. S., K. Harries, S. H. Doak, A. Wilmes, A. P. Griffiths, J. N. Baxter, and J. M. Parry. 2004. The bile acid deoxycholic acid (DCA) at neutral pH activates NF-κB and induces IL-8 expression in oesophageal cells in vitro. *Carcinogenesis* 25 (3):317-323.

Jenkins, Gareth J. S., Francis R. D'Souza, Sinan H. Suzen, Zak S. Eltahir, Sally A. James, James M. Parry, Paul A. Griffiths, and John N. Baxter. 2006. Deoxycholic acid at neutral and acid pH, is genotoxic to oesophageal cells through the induction of ROS: the potential role of anti-oxidants in Barrett's oesophagus. *Carcinogenesis* 28 (1):136-142.

refluxate can promote the development of Barrett's oesophagus, which, in some cases, progresses further into OAC[6].

Evidence is accumulating to suggest that the bile acid component of the refluxate plays an important role in initiating and promoting cancer in Barrett's tissues[7]. Deoxycholic acid (DCA) is an unconjugated, bile acid found in the refluxate which has been particularly implicated in carcinogenesis[8]. It is a secondary bile acid which is produced from primary bile acids by microbial action in the gut[9]. DCA has been shown to alter the expression of many different genes in oesophageal cells and it may promote tumour progression through the activation of pro-tumourigenic signaling pathways and its selection for apoptotic-resistant cells[10]. However, the precise mechanisms through which DCA promotes OAC are still poorly understood.

RACK1 is an intracellular scaffolding protein with significant homology to the β subunit of G-proteins[11]. RACK1 interacts with a diverse range of binding partners and exerts its influence on them by shuttling them to particular subcellular locations, promoting or inhibiting their activity or modulating their stability or interaction with other molecules. Thus RACK1 participates in multiple signaling pathways and is implicated in a wide range of cellular functions and processes including transcription and protein synthesis, migration, adhesion and proliferation[12].

A number of studies have implicated RACK1 in the development and progression of cancer[13]. Its expression is altered in angiogenesis and in a

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6 McQuaid, K. R., L. Laine, M. B. Fennerty, R. Souza, and S. J. Spechler. 2011. Systematic review: the role of bile acids in the pathogenesis of gastro-oesophageal reflux disease and related neoplasia. *Alimentary pharmacology & therapeutics* 34 (2):146-165.
    Pennathur et al. *The Lancet*
7 Ibid.
    Jenkins et al. 2004.
8 Duggan et al. 2010
9 McQuaid et al. 2011.
10 Duggan et al. 2010.
    Jenkins et al. 2006.
    Huo, Xiaofang, Stefanie Juergens, Xi Zhang, Davood Rezaei, Chunhua Yu, Eric D. Strauch, Jian-Ying Wang, Edaire Cheng, Frank Meyer, and David H. Wang. 2011. Deoxycholic acid causes DNA damage while inducing apoptotic resistance through NF-κB activation in benign Barrett's epithelial cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 301 (2):G278-G286.
11 Adams, David R., Dorit Ron, and Patrick A. Kiely. 2011. RACK1, A multifaceted scaffolding protein: Structure and function. *Cell Commun Signal* 9 (1):22.
12 Ibid.
13 Li, J. J., and D. Xie. 2014. RACK1, a versatile hub in cancer. *Oncogene*.

wide variety of cancers[14]. For example, increased expression of RACK1 has been observed in oesophageal squamous cell carcinoma, glioma and in cancers of the lung and liver, while decreased RACK1 expression had been observed in gastric cancer[15]. RACK1 appears to exert its pro- or anti-tumourigenic effects through a number of different mechanisms which vary depending on the cell and cancer type and subtype. For example, RACK1 interacts with MKK7 in hepatocellular carcinoma cells and promotes proliferation by enhancing MKK7/JNK activity[16]. In oesophageal squamous cell carcinoma cells, RACK1 appears to drive cancer cell growth and migration through activation of the hedgehog signaling pathway[17]. RACK1 interacts with the IGF-IR in a variety of cell types and facilitates migration of breast and prostate tumour cells through a mutually exclusive interaction with β1 integrin or PP2a[18]. Conversely, RACK1 appears to have a tumour suppressive function in colon cancer cells where its inhibition of src delays cell cycle progression, suppresses colonic growth and induces apoptosis[19]. The vast number of proteins with which RACK1 interacts is likely to account for its contrasting roles in different cancer settings. It is likely that the particular cohort of binding partners with which RACK1 interacts at any particular moment, and in any particular disease or cell setting, will determine whether RACK1 acts as a tumour promoter or suppressor. Thus it is important that we consider cell and cancer type

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14 Ibid.

Adams, Ron, and Kiely 2011.

15 Ibid.

Hu, Fengqing, Zhen Tao, Mingsong Wang, Guoqing Li, Yunjiao Zhang, Hong Zhong, Haibo Xiao, Xiao Xie, and Mei Ju. 2013. RACK1 promoted the growth and migration of the cancer cells in the progression of esophageal squamous cell carcinoma. *Tumor Biology* 34 (6):3893-3899.

Peng, Renjun, Bing Jiang, Jianrong Ma, Zhiming Ma, Xin Wan, Hongwei Liu, Zigui Chen, Quan Cheng, and Rui Chen. 2013. Forced downregulation of RACK1 inhibits glioma development by suppressing Src/Akt signaling activity. *Oncology reports* 30 (5):2195-2202.

Deng, Yue–Zhen, Fan Yao, Jing–Jing Li, Zheng–Fa Mao, Ping–Ting Hu, Ling–Yun Long, Guo Li, Xiao–Dan Ji, Shuo Shi, and Dong–Xian Guan. 2012. RACK1 suppresses gastric tumorigenesis by stabilizing the β-catenin destruction complex. *Gastroenterology* 142 (4):812-823.

 Guo, Yuanyuan, Wendie Wang, Jing Wang, Jiannan Feng, Qingyang Wang, Jianfeng Jin, Ming Lv, Xinying Li, Yan Li, and Yuanfang Ma. 2013. Receptor for activated C kinase 1 promotes hepatocellular carcinoma growth by enhancing mitogen-activated protein kinase kinase 7 activity. *Hepatology* 57 (1):140-151.

16 Guo et al. 2013.

17 Hu et al. 2013.

18 Kiely, Patrick A., George S. Baillie, Martin J. Lynch, Miles D. Houslay, and Rosemary O'Connor. 2008. Tyrosine 302 in RACK1 is essential for insulin-like growth factor-I-mediated competitive binding of PP2A and β1 integrin and for tumor cell proliferation and migration. *Journal of Biological Chemistry* 283 (34):22952-22961.

19 Mamidipudi, V., and C. A. Cartwright. 2009. A novel pro-apoptotic function of RACK1: suppression of Src activity in the intrinsic and Akt pathways. *Oncogene* 28 (50):4421-4433.

when examining RACK1 function.

The subcellular localisation of RACK1 is also an important determinant of its cellular function as it interacts with different proteins at different locations within the cell[20]. Aberrant signaling, cellular stimuli or protein expression in cancer may alter RACK1 localisation which in turn may influence the function which RACK1 plays in cancer cells. For example, RACK1 normally has a diffuse cytoplasmic localisation in prostate cancer cells but it accumulates in the membrane compartment when expression of Trop-2 is upregulated[21]. This shift in RACK1 localisation increases the association between RACK1 and β1 integrin which results in increased β1 integrin-RACK1-FAK-src signaling and loss of cell adhesion[22]. Thus alterations in RACK1 subcellular localisation can have important impacts on its ability to promote or suppress carcinogenic processes and should thus be considered when examining the role of RACK1 in disease.

Thorough examination of RACK1 in a variety of cancer settings should enable us to increase our understanding of this multi-functional protein and may ultimately lead to the development of therapeutic strategies which hinder its pro-tumourigenic functions or promote its tumour suppressive functions.

RACK1 has not been previously studied in the context of OAC and it is not known if RACK1 expression is altered in this malignancy. Furthermore, DCA has been shown to modulate the expression and function of a range of proteins but the influence of this tumour promoting bile acid on RACK1 had not been investigated to date.

Therefore, in this study we examine RACK1 expression in OAC for the first time. We also examine the effects of DCA on RACK1 expression and localisation in OAC cell lines. We report that RACK1 protein levels generally increase with increasing disease progression across a panel of cell lines representing the multistep sequence from normal oesophagus to OAC. We also demonstrate a DCA-mediated decrease in RACK1 expression levels in two OAC cell lines.

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20 Adams, Ron, and Kiely 2011.
21 Trerotola, Marco, Jing Li, Saverio Alberti, and Lucia R. Languino. 2012. Trop-2 inhibits prostate cancer cell adhesion to fibronectin through the β1 integrin-RACK1 axis. *Journal of cellular physiology* 227 (11):3670-3677.
22 Ibid.

Methods

Cell lines and treatments

The non-tumourigenic squamous oesophageal cell line, HET-1A, was obtained from the American Type Culture Collection (LGC-standards, Middlesex, UK). Human oesophageal epithelial cells (HEECs) were obtained from 3H Biomedical (Uppsala, Sweden). HEECs and HET-1A cells were cultured in antibiotic free, BEBM media with SingleQuots™ growth supplements (Lonza, Berkshire, England). The Barrett's cell lines, QhTERT and GohTERT, were a kind gift from Dr Rabinovitch (University of Washington, WA, USA) and were cultured in BEBM media supplemented with 5% fetal bovine serum and SingleQuots™ growth supplements (Lonza, Berkshire, England). FLO-1 OAC cell lines were a kind gift from Dr Van Schaeybroeck (Queen's University Belfast) and were cultured in DMEM media supplemented with 10% fetal bovine serum. SKGT-4 cells (a kind gift from Dr Gisella Vaas, Trinity College Dublin) and OE33 cell lines (obtained from the European Collection of Cell Cultures) were both cultured in RPMI media supplemented with 10% fetal bovine serum.

DCA was obtained from Sigma and was solubilised in DMSO (Sigma). Exposure of SKGT-4 and FLO-1 cells to DCA was performed in media with 10% fetal bovine serum supplementation throughout the time course under study as this produced a more physiologically relevant setting than that which could be achieved under conditions of serum starvation. The concentrations and time periods used when treating cells with DCA are indicated in figure legends. Matched time point controls treated with vehicle (DMSO) were also conducted to normalise data for time or vehicle effects.

Preparation of cellular protein extracts

Cellular protein extracts were prepared by washing cells with ice cold phosphate buffered saline followed by incubation at 4 °C for 20 minutes in cell lysis buffer composed of 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40 and Na3VO4 (1 mM) supplemented with protease inhibitor cocktail (1/100, from Sigma). Following incubation, nuclear and cellular debris were removed by microcentrifugation at 10,000 rpm for 5 minutes at 4 °C.
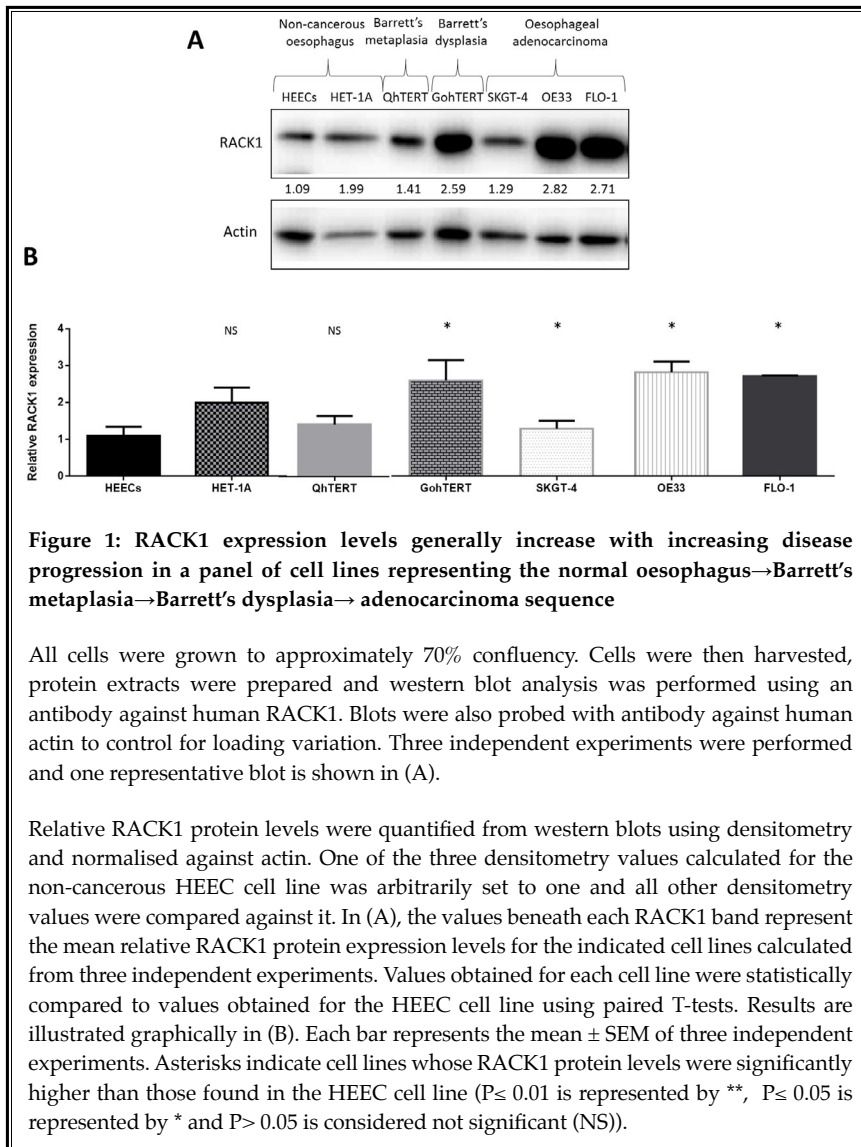
Western Blotting

Protein concentrations were determined by BCA assay (Pierce) and protein samples were prepared for SDS gel electrophoresis by boiling for 5 minutes in laemmli buffer. Proteins were resolved by SDS-PAGE on 10% polyacrylamide gels and then transferred to PVDF membranes, which were blocked for 1 h at room temperature in Tris-buffered saline containing 0.05% Tween20 and 5% milk (w/v). Primary antibody incubations were overnight at 4 °C. Primary antibodies employed were mouse anti-RACK1 mAb (BD transduction) and rabbit anti-actin (Sigma) and were used at dilutions of 1/3000 and 1/4500 respectively. Primary antibodies were detected with HRP conjugated anti-mouse or anti-rabbit secondary antibodies (Cell Signaling) which were both used at 1/5000 dilution. Secondary antibody incubations were at room temperature for two hours. Following antibody incubation, membranes were exposed to enhanced chemiluminescence developing solution (100mM Tris-HCl, pH 8.5, 0.009% H_2O_2, 1.2mM luminol, 1.9mM IBPA) and were then imaged using the FUSION gel imaging system (Vilber Lourmat). Densitometry was performed using ImageJ image analysis software (NIH).

Immunofluorescence

For immunofluorescence confocal microscopy, SKGT-4 and FLO-1 cells were seeded on coverslips, cultured for 36 hours in their appropriate media as detailed above and then treated with the indicated amounts of DCA or vehicle control. Cells were then fixed for 15 minutes with 4% paraformaldehyde in phosphate buffered saline, permeabilised for 10 minutes with 0.3% Triton X-100 in PBS, blocked for 30 minutes with 3% BSA in PBS before incubation with 1:200 mouse anti-RACK1 mAb (BD transduction laboratories) in 3% BSA for two hours at room temperature.

Antibody detection was with an Alexa-Flour 488 fluorescently labelled anti-mouse secondary antibody for 1 hour at room temperature. Controls with secondary antibody only were also employed. Cells were also stained with Hoechst nuclear stain (1/5000). The cells were visualised using a Zeiss LSM confocal microscope and all images were recorded using a 63X oil immersion objective.

Figure 1: RACK1 expression levels generally increase with increasing disease progression in a panel of cell lines representing the normal oesophagus→Barrett's metaplasia→Barrett's dysplasia→ adenocarcinoma sequence

All cells were grown to approximately 70% confluency. Cells were then harvested, protein extracts were prepared and western blot analysis was performed using an antibody against human RACK1. Blots were also probed with antibody against human actin to control for loading variation. Three independent experiments were performed and one representative blot is shown in (A).

Relative RACK1 protein levels were quantified from western blots using densitometry and normalised against actin. One of the three densitometry values calculated for the non-cancerous HEEC cell line was arbitrarily set to one and all other densitometry values were compared against it. In (A), the values beneath each RACK1 band represent the mean relative RACK1 protein expression levels for the indicated cell lines calculated from three independent experiments. Values obtained for each cell line were statistically compared to values obtained for the HEEC cell line using paired T-tests. Results are illustrated graphically in (B). Each bar represents the mean ± SEM of three independent experiments. Asterisks indicate cell lines whose RACK1 protein levels were significantly higher than those found in the HEEC cell line ($P \le 0.01$ is represented by **, $P \le 0.05$ is represented by * and $P > 0.05$ is considered not significant (NS)).

Statistics

Graphed data are presented as the mean and the standard error of the mean. Paired t-tests were used to identify significant differences in RACK1 protein levels between non-cancerous control cells (HEECs) and other cell lines or between untreated and DCA untreated cells. Analysis was carried out using Prism v6.0 (GraphPad Software, CA, USA). A P-value ≤ 0.05 was considered significant: *P≤ 0.05 and **P≤ 0.01.

Results

RACK1 expression levels generally increase with increasing disease progression in a panel of cell lines representing the normal oesophagus→Barrett's metaplasia→Barrett's dysplasia→ adenocarcinoma sequence.

OAC typically develops by a multi-step process recognised histologically as the normal-Barrett's metaplasia-Barrett's dysplasia-adenocarcinoma sequence[23]. We examined if RACK1 protein expression was altered during this sequence of cancer development by examining its protein levels by western blot analysis in cell lines derived from each stage of the sequence (Figure 1). The HEEC and HET-1A cell lines were used to represent non-cancerous oesophageal cells, the QhTERT and GohTERT represent Barrett's metaplasia and dysplasia respectively while three cell lines, SKGT-4, OE33 and FLO-1 represent oesophageal adenocarcinoma.

Three independent experiments were performed and relative RACK1 protein levels for each cell line was determined by densitometry as described in the legend of Figure 1. RACK1 protein levels between non-cancerous HEECs cells and each other cell line were statistically compared as is also described in the legend of Figure 1.

Of the seven cell lines examined, the non-cancerous cell line, HEECs, displayed the lowest protein levels of RACK1. The cell line representing Barrett's metaplasia (QhTERT) also had relatively low levels of RACK1. RACK1 expression was significantly increased by over 100% in the

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23 de Almeida, J. C. Mendes, Paula Chaves, A. Dias Pereira, and Nasser K. Altorki. 1997. Is Barrett's esophagus the precursor of most adenocarcinomas of the esophagus and cardia? A biochemical study. *Annals of surgery* 226 (6):725.

Barrett's dysplastic cell line (GohTERT) and in two of the three OAC cell lines examined. Thus, in general, levels of RACK1 increased across the sequence from normal to cancer. However there were some exceptions to this trend. Firstly, the SKGT-4 cell line had much lower levels of RACK1 than those observed in the other OAC cell lines and, of all the cell lines examined, it actually had the second lowest level of RACK1.

Secondly, the HET-1A cell line had much higher levels of RACK1 than those found in the HEECs cell line and actually had higher levels of RACK1 than those found in the cell line representing the next stage of the sequence, namely the QhTERT cell line representing Barrett's metaplasia. Nevertheless, the HET-1A cell line still had considerably lower levels of RACK1 expression than those found in the Barrett's dysplastic cell line (GohTERT) and in the OE33 and FLO-1 OAC cell lines. Furthermore, the ability of the HET-1A cell line to represent the 'normal', non-cancerous oesophageal epithelium has been questioned as it has been reported to have dysplastic properties and to lack some typical epithelial cell characteristics (Underwood et al. 2010). Thus, the HEECs cell line may provide a better representation of RACK1 protein levels in the non-cancerous oesophagus.

DCA suppresses cellular levels of RACK1 protein in two oesophageal adenocarcinoma cell lines (SKGT-4 and FLO-1).

DCA is present at micromolar concentrations (0-300 μM) in oesophageal aspirates[24]. 300 μM DCA is capable of inducing NF-κB expression and activation in OAC cell lines[25]. NF-κB can activate transcription of the murine RACK1 gene and may also regulate expression of the human RACK1 gene[26]. We therefore hypothesised that DCA may upregulate

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24 Nehra, D., P. Howell, C. P. Williams, J. K. Pye, and J. Beynon. 1999. Toxic bile acids in gastro-oesophageal reflux disease: influence of gastric acidity. *Gut* 44 (5):598-602.
25 Jenkins et al. 2004
    Abdel-Latif, Mohamed M. M., James O'Riordan, Henry J. Windle, Eleanor Carton, Nagunivan Ravi, Dermot Kelleher, and John V. Reynolds. 2004. NF-κB activation in esophageal adenocarcinoma: relationship to Barrett's metaplasia, survival, and response to neoadjuvant chemoradiotherapy. *Annals of surgery* 239 (4):491.
26 Choi, Doo-Sup, Hannah Young, Thomas McMahon, Dan Wang, and Robert O. Messing. 2003. The mouse RACK1 gene is regulated by nuclear factor-κB and contributes to cell survival. *Molecular pharmacology* 64 (6):1541-1548.
    Del Vecchio, Igor, Annalisa Zuccotti, Federica Pisano, Fabio Canneva, Silvia C. Lenzken, Francoise Rousset, Emanuela Corsini, Stefano Govoni, and Marco Racchi. 2009. Functional mapping of the promoter region of the GNB2L1 human gene coding for RACK1 scaffold protein. *Gene* 430 (1):17-29.

RACK1 protein levels through an NF-κB mediated increase in RACK1 gene expression. To investigate this we treated SKGT-4 OAC and FLO-1 cells with 300 μM DCA or vehicle control (DMSO) for 24 hours. RACK1 protein levels were then examined by western blot analysis. Blots were also probed with an antibody against actin to control for variations in loading. Three independent experiments were performed for each cell line and results show that treatment with 300 μM DCA actually reduced RACK1 protein levels in both cell lines (Figure 2). RACK1 protein levels were significantly reduced by an average of 31% in DCA treated SKGT-4 cells (P=0.01) and by an average of 26% in DCA treated FLO-1 OAC cells (P=0.03).

RACK1 has a cytoplasmic localisation in SKGT-4 cells and FLO-1 cells which is not altered by DCA.

We examined the subcellular localisation of RACK1 by immunofluorescent staining using a monoclonal antibody against human RACK1. We found that RACK1 has a predominately cytoplasmic distribution in both SKGT-4 and FLO-1 cells which is relatively diffuse and is slightly more concentrated in perinuclear areas (Figure 3A and 3B).
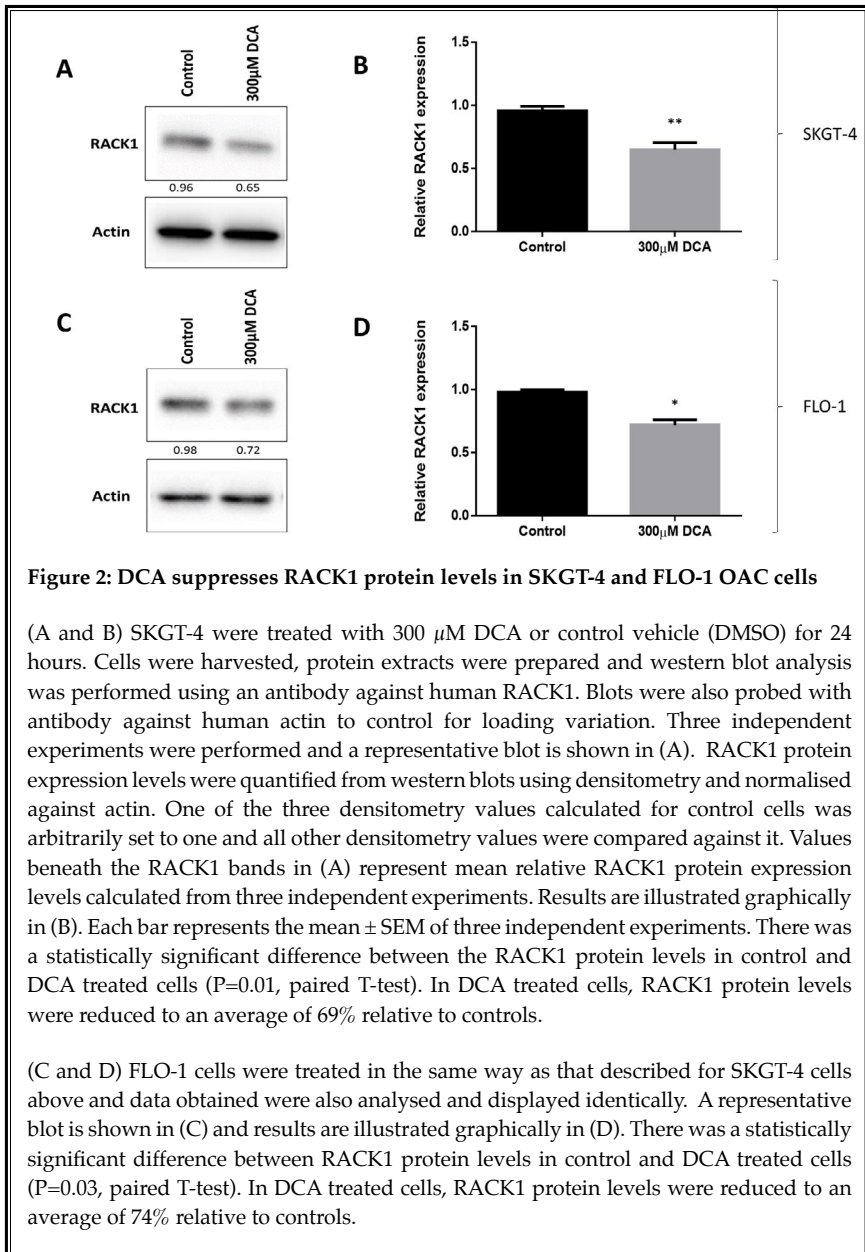
RACK1 plays an important role in shuttling its binding partners around the cell in response to certain stimuli and in targeting or anchoring its partners at particular cellular locations[27]. DCA can alter the subcellular localisation of a number of known RACK1 binding partners including IGF-IR, src and a number of PKC isoforms[28]. Thus, it is possible that RACK1 subcellular localisation might also be altered by DCA. To investigate this, we treated SKGT-4 and FLO-1 cells with 300 μM DCA for 90 minutes or 24 hours. RACK1 localisation was then examined in control and DCA treated cells using immunofluorescence. However, in both cell lines, neither 90 minute nor 24 hour treatment with DCA altered RACK1 subcellular localisation which remained predominately cytoplasmic in all cases (Figure 3A and 3B). SKGT-4 and FLO-1 cells were also treated with lower concentrations

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27 Adams, Ron, and Kiely 2011.

28 Ibid.

Morgan, Sherif, and Emmanuelle Meuillet. 2009. Abstract# 5048: Deoxycholic acid induces endocytosis of the insulin-like growth factor-I receptor in colorectal cancer cells. *Cancer Research* 69 (9 Supplement):5048-5048.

Looby, Eileen, Aideen Long, Dermot Kelleher, and Yuri Volkov. 2005. Bile acid deoxycholate induces differential subcellular localisation of the PKC isoenzymes β1, ϵ and δ in colonic epithelial cells in a sodium butyrate insensitive manner. *International journal of cancer* 114 (6):887-895.
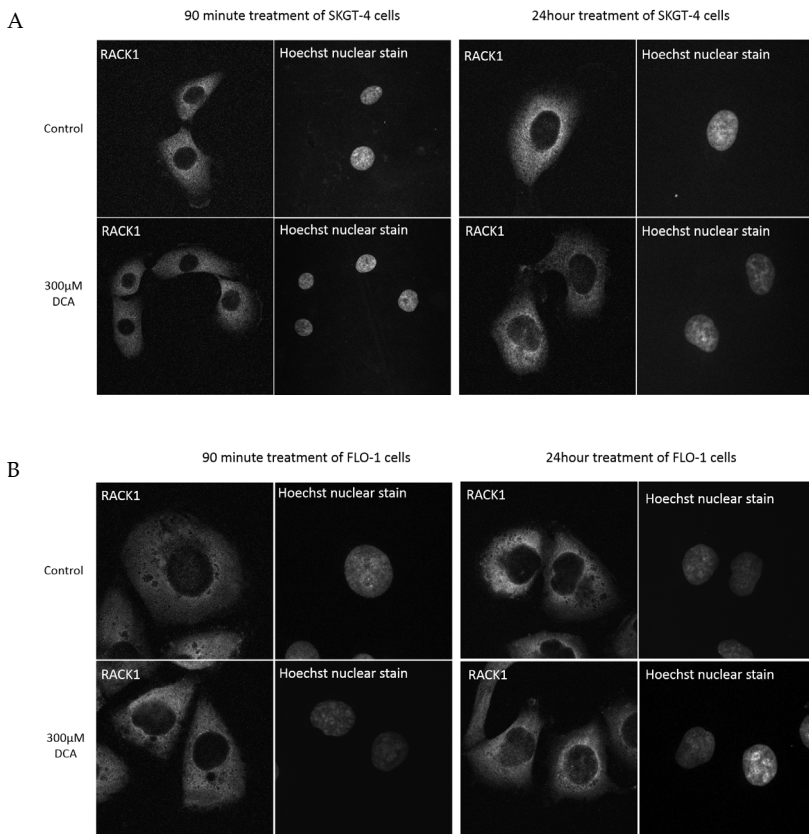
Figure 2: DCA suppresses RACK1 protein levels in SKGT-4 and FLO-1 OAC cells

(A and B) SKGT-4 were treated with 300 μM DCA or control vehicle (DMSO) for 24 hours. Cells were harvested, protein extracts were prepared and western blot analysis was performed using an antibody against human RACK1. Blots were also probed with antibody against human actin to control for loading variation. Three independent experiments were performed and a representative blot is shown in (A). RACK1 protein expression levels were quantified from western blots using densitometry and normalised against actin. One of the three densitometry values calculated for control cells was arbitrarily set to one and all other densitometry values were compared against it. Values beneath the RACK1 bands in (A) represent mean relative RACK1 protein expression levels calculated from three independent experiments. Results are illustrated graphically in (B). Each bar represents the mean ± SEM of three independent experiments. There was a statistically significant difference between the RACK1 protein levels in control and DCA treated cells (P=0.01, paired T-test). In DCA treated cells, RACK1 protein levels were reduced to an average of 69% relative to controls.

(C and D) FLO-1 cells were treated in the same way as that described for SKGT-4 cells above and data obtained were also analysed and displayed identically. A representative blot is shown in (C) and results are illustrated graphically in (D). There was a statistically significant difference between RACK1 protein levels in control and DCA treated cells (P=0.03, paired T-test). In DCA treated cells, RACK1 protein levels were reduced to an average of 74% relative to controls.

Figure 3: RACK1 has a cytoplasmic subcellular localisation in SKGT-4 and FLO-1 cells which is not altered by DCA

The subcellular localisation of RACK1 was examined using immunofluorescent and confocal microscopy. SKGT-4 cells (A) and FLO-1 cells (B) in the above images were treated with control vehicle or 300 μM DCA for 90 minutes or 24 hours prior to fixation, staining with an anti-RACK1 antibody and imaging by confocal microscopy. RACK1 subcellular localisation is similar in both control and DCA treated cells and is predominately cytoplasmic, relatively diffuse in nature and is most concentrated in perinuclear areas.

of DCA (100 μM) for 90 minutes and 24 hours and this also had no effect on RACK1 subcellular localisation (data not shown).

Discussion

OAC develops through a multi-step sequence whereby Barrett's metaplasia gives rise to Barrett's dysplasia and finally to fully malignant adenocarcinoma[29]. Bile acids, such as the secondary bile acid DCA, are believed to play an important role in promoting progression through this sequence and have been shown to modulate the expression of a variety of genes in oesophageal cells[30]. However, our understanding of the precise mechanisms through which bile acids promote tumourigenesis is still poor. Furthermore, while Barrett's oesophagus is a significant risk factor for OAC, only a small percent of patients with Barrett's go on to develop OAC[31]. At present, our ability to predict prognosis and to differentiate between Barrett's oesophagus patients who will and will not progress to OAC is poor[32]. Therefore, it is crucial that we further our understanding of the mechanisms and gene alterations which drive OAC as this should aid us in developing novel strategies to predict, diagnose and treat this serious malignancy.

A number of studies have reported that RACK1 expression is altered (usually upregulated) in a variety of cancers including oesophageal squamous cell carcinoma, glioma and cancers of the lung and liver[33]. Furthermore, correlations between RACK1 expression and tumour stage and prognosis have also been observed in a number of studies; for example, a recent study in non-small-cell lung cancer reported that upregulation of RACK1 was inversely correlated with tumour differentiation and positively correlated

29 de Almeida et al. 1997.

30 Duggan et al. 2010

Looby, Eileen, Mohamed M. M. Abdel-Latif, Veronica Athié-Morales, Shane Duggan, Aideen Long, and Dermot Kelleher. 2009. Deoxycholate induces COX-2 expression via Erk1/2-, p38-MAPK and AP-1-dependent mechanisms in esophageal cancer cells. *BMC cancer* 9 (1):190.

Byrne, Anne-Marie, Ruchika Sharma, Gina Duggan, Dermot Kelleher, and Aideen Long. 2012. Deoxycholic acid impairs glycosylation and fucosylation processes in esophageal epithelial cells. *Glycobiology* 22 (5):638-648.

31 Pennathur et al. 2013.

32 Ibid.

33 Li and Xie 2014.

Guo et al. 2013.

Hu et al. 2013.

Deng et al. 2012.

Peng et al. 2013.

with both tumour stage and metastasis[34].

However, prior to this study, the expression and role of RACK1 in OAC remained unknown. Furthermore, to our knowledge, the influence of DCA on RACK1 expression and function has not been investigated. Therefore, this is the first study to investigate RACK1 expression in OAC and to examine the influence which DCA has on RACK1.

We examined the expression of RACK1 in a panel of seven oesophageal cells representing each stage of the OAC progression sequence from normal oesophagus→Barrett's metaplasia→Barrett's dysplasia→OAC. In general, we found that RACK1 protein levels increased as the sequence progressed from normal oesophagus to OAC. The lowest level of RACK1 expression was found in a cell line (HEECs) derived from non-cancerous oesophageal tissue while the first, second and third highest levels of RACK1 expression were found in the OE33 OAC, FLO-1 OAC and GohTERT Barrett's dysplastic cell lines respectively (Figure 1). However two of the cell lines examined did make exceptions to this general trend. Firstly, the SKGT-4 OAC cell line displayed low RACK1 levels which were closer to those observed in non-cancerous or Barrett's metaplastic cells than to those found in the other OAC cell lines. Thus, our findings indicate that RACK1 may only be upregulated in some, but not all, OAC tumours. This is not all together surprising as cancer is a heterogeneous disease with many different subtypes occurring within the one cancer type which vary in their genetic alterations and their mechanisms of carcinogenesis.

Secondly, the HET-1A cell line, which is a non-cancerous oesophageal epithelial cell line, had much higher levels of RACK1 than those found in the other non-cancerous oesophageal cell line examined (HEECs) and also had higher levels of RACK1 than those found in the QhTERT Barrett's metaplastic cell line.

The HET-1A cell line was derived from normal oesophageal tissue, has remained non-tumourigenic in athymic, nude mice for over 12 months[35]

34 Shi, Shuo, Yue-Zhen Deng, Jiang-Sha Zhao, Xiao-Dan Ji, Jun Shi, Yu-Xiong Feng, Guo Li, Jing-Jing Li, Di Zhu, and H. Phillip Koeffler. 2012. RACK1 promotes non-small-cell lung cancer tumorigenicity through activating sonic hedgehog signaling pathway. *Journal of Biological Chemistry* 287 (11):7845-7858.

35 Stoner, Gary D., M. Edward Kaighn, Roger R. Reddel, James H. Resau, Douglas Bowman, Zenya Naito, Norio Matsukura, Ming You, Anthony J. Galati, and Curtis C. Harris. 1991. Establishment

and is widely used as a model of normal oesophageal epithelium cells. However, it has been reported that this cell line appears dysplastic when grown in organotypic culture, does not display evidence of squamous differentiation and is hyperproliferative in comparison to primary oesophageal epithelial cells[36]. Furthermore, HET-1A cells do not express the epithelial marker E-cadherin while they do express the mesenchymal marker vimentin[37]. A recent study in oesophageal squamous cell carcinoma has reported a positive correlation between RACK1 expression and vimentin, and an inverse correlation between RACK1 expression and E-cadherin[38]. If such findings are considered, it is plausible to suggest that HET-1A cells might have higher RACK1 expression levels than those normally found in non-cancerous oesophageal epithelial cells. Thus, the HEECs cell line may provide a better representation of RACK1 protein levels in the non-cancerous oesophagus.

Our study of RACK1 expression levels in this panel of OAC cell lines provides an insight into the potential modulation of RACK1 expression levels which may occur as OAC progresses. However, it is important to note that the cell lines used in this study were derived from different patients and are therefore not isogenic. Therefore, some of the differences in RACK1 levels which we observed between cells is likely to be due to the non-isogenic nature of these cells, rather than due purely to their particular stage in the cancer progression sequence. Thus, this study simply gives us an indication that RACK1 expression is upregulated in OAC but does not give us conclusive proof of same. Nevertheless, by providing an indication that RACK1 may be upregulated in OAC, we have shown that further examination of RACK1 expression levels in matched patient OAC and disease free control tissue is warranted, and that further investigation into the role of RACK1 in OAC is worthwhile.

and characterization of SV40 T-antigen immortalized human esophageal epithelial cells. *Cancer Research* 51 (1):365-371.

36 Underwood, Timothy J., Mathieu F. Derouet, Michael J. White, Fergus Noble, Karwan A. Moutasim, Eric Smith, Paul A. Drew, Gareth J. Thomas, John N. Primrose, and Jeremy P. Blaydes. 2010. A comparison of primary oesophageal squamous epithelial cells with HET-1A in organotypic culture. *Biology of the Cell* 102 (12):635-644.

37 Ibid.

38 Wang, Nana, Fang Liu, Fangli Cao, Yibin Jia, Jianbo Wang, Wei Ma, Bingxu Tan, Kai Wang, Qingxu Song, and Yufeng Cheng. 2015. RACK1 predicts poor prognosis and regulates progression of esophageal squamous cell carcinoma through its epithelial-mesenchymal transition. *Cancer Biology & Therapy* 16(4):528-540.

DCA has been identified as an important contributor to OAC progression and physiologically relevant concentrations of DCA can alter (both upregulate and downregulate) the expression of a variety of genes in OAC cells[39]. However, to our knowledge, the effect of DCA on RACK1 expression has not been previously investigated. 300 μM DCA induces both expression and activation of NF-κB expression in OAC cell lines (SKGT-4, OE33 and OE21)[40]. Interestingly, NF-κB binds to the murine RACK1 promoter and promotes RACK1 gene expression[41]. Bioinformatic analysis indicates that the human RACK1 promoter also contains binding sites for the NF-κB transcription factor[42]. Therefore, we hypothesised that DCA may induce an increase in RACK1 protein expression through a NF-κB mediated induction of RACK1 gene expression. However, our results show that 300 μM DCA actually decreases RACK1 protein levels in SKGT-4 and FLO-1 OAC cells (Figure 2). Use of both SKGT-4 and FLO-1 cell lines in this study enabled us to examine the effect of DCA on OAC cell lines with relatively low (SKGT-4) and high (FLO-1) basal levels of RACK1. Western blotting was used in this study in preference to rtPCR as it enabled us to examine DCA's effects on actual RACK1 protein levels as opposed to its effects on mere transcription of the RACK1 gene. This is particularly important in the context of DCA as published literature indicates that DCA may sometimes have opposing effects on gene expression and protein levels. For example, various studies in oesophageal cells have indicated that DCA can increase EGFR gene expression[43] but decrease EGFR protein expression[44]. Similarly, in this   present study we also report that DCA significantly reduces RACK1 protein levels in OAC cells, in spite of the literature discussed above which suggested that DCA could potentially increase RACK1 gene expression through its upregulation of NF-κB. DCA is a well-known inducer of oxidative stress[45] which in turn has negative effects on protein translation and can also influence protein turnover[46].

39 Jenkins et al. 2004.
   Looby et al. 2009.
40 Jenkins et al. 2004.
   Abdel-Latif et al. 2004.
41 Choi et al. 2003.
42 Del Vecchio et al. 2009.
43 Jenkins et al. 2004.
44 Byrne et al. 2012.
45 Duggan et al. 2010.
   Huo et al. 2011.
46 Vogel, Christine, Gustavo Monteiro Silva, and Edward M. Marcotte. 2011. Protein expression regulation under oxidative stress. *Molecular & Cellular Proteomics* 10 (12):M111-009217.

Thus it is possible that DCA does increase RACK1 gene expression through the NF-κB dependent mechanism proposed above but that this increase in expression is not seen at the protein level due to an oxidative stress mediated reduction in protein translation.

The DCA mediated decrease in RACK1 protein levels which we have observed is likely to have serious implications for the cell. RACK1 expression is believed to be tightly regulated in healthy cells and changes in its expression are likely to have dramatic consequences for the stability, activity and localisation of its multiple binding partners and could thus impact on a wide range of signaling pathways and cellular processes[47]. A decrease in RACK1 expression is likely to result in increased competition for RACK1 among its interaction partners. The consequences of decreased RACK1 expression may depend on the outcome of such competition and on the particular proteins which retain or lose their interactions with RACK1 in this situation.

For example, RACK1 interacts with and inhibits src in colon cancer cells which inhibits cell cycle progression and thus reduces proliferation[48]. In contrast, the interaction of RACK1 with other interaction partners can promote cell cycle progression; for example, RACK1's interaction with the DNA replication licensing factor, MCM7, promotes its activity and promotes cell cycle progression[49].

Thus, alterations in the RACK1 associated proteome as a result of a DCA mediated reduction in RACK1 expression could potentially have pro- or anti-tumourigenic effects. However, our finding that RACK1 expression levels generally increase across the sequence from non-cancerous cells to adenocarcinoma indicates that RACK1 may promote OAC and that increased RACK1 protein levels may be selected for as cancer develops. It could be that DCA-mediated down regulation of RACK1 protein levels may actually select for cells with increased RACK1 expression. DCA simultaneously induces apoptosis and activates survival signaling in oesophageal cells. Interestingly, a study in Barrett's cells has shown that DCA induces apoptotic resistance through NF-kB activation[50]. Thus DCA

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47 Adams, Ron, and Kiely 2011.
48 Mamidipudi and Cartwright 2009.
49 Li and Xie 2014.
50 Huo et al. 2011.

may select for cells with higher levels of NF-kB activation and it is possible that such cells may also have greater levels of RACK1 gene expression. It has been proposed that DCA mediated selection of apoptotic resistant cells plays an important role in neoplastic transformation[51]. RACK1 can protect against apoptosis in a variety of cellular settings, for example it can integrate survival signaling or can promote the ubiquitination-mediated degradation of pro-apoptotic proteins such as Fem1b and BimEL[52]. Thus, it is possible that the apoptotic resistant cells which DCA selects for may also be cells which have retained greater levels of RACK1 protein than their out-selected counterparts. To test this hypothesis, a DCA hyper-resistant OAC cell line could be generated by repeated passaging of an OAC cell line in the presence of DCA. RACK1 levels could then be compared between parental and hyper-resistant cell lines.

The diversity in RACK1 functions and in RACK1 interaction partners is reflected in its subcellular localisation patterns which are cell-type specific and are influenced by its binding partners and by cellular stimuli. Indeed, analysis of RACK1 in a variety of different cell contexts has resulted in the detection of RACK1 localisation in a wide range of subcellular compartments including the cytosol, nucleus, endoplasmic reticulum and stress granules[53]. No known organelle localisation motifs or nuclear entry or export signals are contained within the RACK1 sequence. Furthermore, there is no conclusive evidence to suggest that post-translational modification of RACK1 controls its subcellular localisation[54]. Thus, the particular set of interaction partners with which RACK1 associates at any one time, and in any one cell type, probably have the greatest influence on its subcellular localisation. Therefore, the subcellular localisation of RACK1 in a particular cell type, and under particular cellular conditions, can provide important clues regarding its current interaction partners and its function in that particular situation.

Therefore, we examined the subcellular localisation of RACK1 in SKGT-4 and FLO-1 cells. We report that RACK1 has a predominately cytoplasmic distribution which is relatively diffuse and is slightly more concentrated in perinuclear areas.

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51 Duggan et al. 2010.
52 Li and Xie 2014.
53 Adams, Ron, and Kiely 2011.
54 Ibid.

We also investigated if DCA induces changes in the subcellular localisation of RACK1 in SKGT-4 cells. RACK1 plays an important role in shuttling its binding partners around the cell and in targeting or anchoring its partners at particular cellular locations[55]. DCA can alter the localisation of a number of known RACK1 binding partners including IGF-IR, src and a number of PKC isoforms[56]. Thus, it is possible that RACK1 localisation might also be altered by DCA. Furthermore, it is possible that RACK1 may play a role in DCA mediated relocalisation of RACK1 binding partners.

However, we observed no alterations in RACK1 subcellular localisation following treatment of SKGT-4 and FLO-1 cells with 300 μM DCA for 90 minutes or 24 hours (Figure 3). Thus DCA does not appear to have any obvious effects on RACK1 localisation in SKGT-4 or FLO-1 cells. However, it is possible that DCA may modulate the localisation of particular subsets of RACK1 (e.g. a subset of RACK1 interacting with a particular binding partner) but that such effects are 'masked' by an opposing effect which DCA has on a different subset of RACK1, or simply by the diffuse distribution of RACK1 throughout the cytoplasm of both cell types.

For example, DCA has been shown to induce endocytosis of the IGF-IR receptor and thus mediate its removal from the cell membrane[57]. RACK1 interacts with the IGF-IR in a variety of cells and plays an important role in IGF-IR mediated signaling[58]. Thus it is plausible to suggest that DCA mediated endocytosis of the IGF-IR could alter the localisation of an IGF-IR-associated subset of RACK1 and possibly stimulate its translocation away from the membrane. In contrast, DCA could potentially induce the translocation of a PKC-associated subset of RACK1 to the membrane. A report in colorectal cancer cells has shown that DCA can stimulate translocation of PKC β1 and PKCε from the cytosol to the plasma membrane[59]. RACK1 is a well-known regulator of PKC function and it is known to interact with a number of PKC isoforms and to shuttle them to sites of action. In glioma, upon PMA mediated PKC activation, RACK1 translocates from the cytosol to the membrane and targets activated PKCε

55 Ibid.
56 Morgan and Meuillet 2009.
   Looby et al. 2005.
57 Morgan and Meuillet 2009.
58 Kiely et al. 2008.
59 Looby et al. 2005.

to integrin β chains[60].

Thus, it is possible that a subset of PKC-associated RACK1 may translocate to the membrane in response to DCA. However, if translocation of another subset of RACK1 (for example IGF-IR associated RACK1) away from the membrane also occurs, both translocations could 'mask' each other and preclude visual, immunofluorescence-mediated detection of such RACK1 translocations. Future studies which analyse the effects of RACK1 depletion (using short interfering RNA approaches) may be more successful in determining if RACK1 is involved in DCA mediated translocation of its binding partners. Such studies could focus on examining the location of RACK1 binding partners by immunofluorescence and examining if DCA mediated translocation of such partners is altered when RACK1 expression is suppressed.

In conclusion, we have found that RACK1 protein levels generally increase with increasing disease progression across a panel of cell lines representing the multistep sequence from normal oesophagus to OAC. We have also found that DCA can modulate RACK1 protein levels in OAC cells. RACK1 may play a role in promoting OAC and may have potential as a therapeutic target for this disease.

Acknowledgement/ Declaration of conflicts of interest

The author has no conflicts of interest to declare.

Abbreviations
Oesophageal adenocarcinoma: OAC
Deoxycholic acid: DCA
Dimethyl sulfoxide: DMSO
Dulbecco's modified eagle's medium: DMEM
Roswell Park Memorial Institute medium: RPMI
Bronchial epithelial basal medium: BEBM

60 Besson, Arnaud, Tammy L. Wilson, and V. Wee Yong. 2002. The Anchoring Protein RACK1 Links Protein Kinase Cε to Integrin β Chains REQUIREMENT FOR ADHESION AND MOTILITY. *Journal of Biological Chemistry* 277 (24):22073-22084.

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