ARTICLE
Journal of Cellular Biochemistry 9999:1–6 (2016)

Journal of Cellular Biochemistry

A Crosstalk Between *K ras* (Kirsten Rat Sarcoma Viral Oncogene Homologue) and Adherence Molecular Complex Leads to Disassociation of Cells—A Possible Contribution Towards Metastasis in Colorectal Cancer

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ABSTRACT

Constitutive activation of mutant *K ras* (*Kirsten rat sarcoma viral oncogene homologue*) and disassembly of E-cadherin–catenin complex (E-cadherin, α-catenin, β-catenin, and γ-catenin) play an important role in apoptosis, differentiation, and cell proliferation. In this study, the expression pattern of K ras and E-cadherin–catenin complex has been evaluated in normal and mutant colorectal cancer cell lines with an object to determine its impact on disassociation of cells from one another. We addressed the expression analysis of *K ras* with reference to its association with adherence molecules in two colorectal cancer cell lines, that is, Caco-2 (wild type *K ras* served as a control) and DLD1 (heterozygous mutation at codon 13) at message level by qRT-PCR and translational level by western blotting. Compared to the control Caco-2 cell lines, the *K ras* in DLD1 cell lines showed slightly higher values while α-catenin showed a slight lower (1.3-folds), β-catenin and E-cadherin showed significantly lower expression (4.2-fold decrease). It can be inferred that a possible cross talk exists between *K ras* and adherent junction mediated signalling. Mutation at codon 13 (G to D) leads to the overexpression of K ras and reduced expression of adherent junction complex resulting in metastasis. J. Cell. Biochem. 9999: 1–6, 2016.

KEY WORDS: COLORECTAL CANCER; METASTASIS; GENE EXPRESSION; KIRSTEIN RAS SARCOMA VIRAL ONCOGENE HOMOLOGUE; E-CADHERIN (*CDH1*); α-CATENIN (*CTNNA1*); β-CATENIN

Colorectal cancer (CRC) is a multifactorial disease constituting a series of genetic events which convert normal mucosa to a benign adenoma and then invasive carcinoma. Metastasis in CRC has been associated with the reduction of cell–cell adhesion and invasion into surrounding tissue [Waldner and Neurath, 2010]. Adherent molecules or E-cadherin–catenin complex (E-cadherin, α-catenin, β-catenin, and γ-catenin) play a vital role in cell proliferation, apoptosis, differentiation, and tumor metastasis by transducing signals from cellular receptors to signalling cascades within the cell. Reduced expression of cadherin-mediated adhesion may act as a promoter of tumour cell detachment from the primary site, stimulation for invasion of adjacent normal tissue, and metastasis and this loss of expression has been associated with an aggressive tumour phenotype and highly invasive neoplasms [Bukholm et al., 1998]. In CRC, loss of E-cadherin (*CDH1*) has been associated with a more malignant phenotype and poor differentiation by stimulation of invasion [Tsanou et al., 2008]. In cancerous cells, β-catenin (*CTNNB1*) is oftenly found in the

Conflicts of interest: The authors have no conflict of interest to declare.

Grant sponsor: Higher Education Commission of Pakistan; Grant sponsor: School of Medicine Swansea University, United Kingdom; Grant sponsor: King Saud University, Riyadh, Kingdom of Saudi Arabia: Prolific Research; Grant number: 1436-011.

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Manuscript Received: 29 February 2016; Manuscript Accepted: 1 March 2016

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 00 Month 2016

DOI 10.1002/jcb.25531 • © 2016 Wiley Periodicals, Inc.

cytoplasm and nucleus where it associates with TCF family members to form a complex and activate transcriptionally number of oncogenes, including *c-Myc, cyclinD*1 [Goss and Groden, 2000]. It plays an important role in tissue morphogenesis, organogenesis, regulation of cadherin mediated cell recognition, and adhesion and activation of transducing oncogenic signaling cascades [Miller et al., 1999; Taipale and Beachy, 2001]. Tyrosine phosphorylation of *CTNNB*1 in response to growth factor stimulation induces disassembly of the E-cadherin–catenin complex, disrupting normal cell adhesion processes [Shibamoto et al., 1994].

Ras proteins (H-, N- and K-ras) function as molecular switches in cell proliferation and transiently activate in response to the extracellular signals. Constitutive activation of *Ras* mutations is considered as an early event found in progression of approximately 30% of colorectal cancers [Waldner and Neurath, 2010]. The activated GTP bound Ras interacts with other effector proteins (Raf kinases, etc.) [O'Neill et al., 2004]. Activation of K ras-mediated ERK signalling activates MMP-9, leading to the disruption of cell–cell adhesion by the cleavage of E-cadherin [Wang et al., 2009]. *K ras* and *β-catenin* signalling synergize to stimulate tumor formation in colon and prostate [Janssen et al., 2006]. Activation of either of these two pathways results in tumor development and progression [Miller et al., 1999; Taipale and Beachy, 2001]. Zhang et al. [2003] have reported that mutational activation of *K* ras and oncogenic activation of *β-catenin* has an inverse correlation. Synchronous activation of both these genes may exert synergistic or syngeneic effects in invasion and metastasis of colorectal cancers. A possible link proposed for this behavior was tyrosine phosphorylation of β-catenin (disruption of cell adhesion molecules complex) by activated Ras [Kinch et al., 1995]. By altering the expression of integrin proteins and cell migration through activation of the PI3-K/Akt pathway, activated Ras pathway has also implications in cytoskeletal rearrangements [Okudela et al., 2004; Fleming et al., 2005]. To understand the molecular mechanism of CRC, mutational spectrum and presence of interaction or cross-talk between different proteins is very necessary. Comparative and combined analysis of these genes is promising in molecular diagnosis and determining the malignant potential of colorectal cancers. Mutations at codon 12, 13, and 61 of *K ras* are considered to be responsible to account for most Ras-mediated cancers but some of the recent evidences have suggested that non canonical mutations (outside of codons 12, 13, and 61) may also contribute to the genetic aberrations leading to the Ras-associated oncogenesis [Murtaza et al., 2012; Stites et al., 2015]. An association between codon 13 mutations and lymph node metastasis with advanced clinical stages of CRC has previously been reported in some studies [Moerkerk et al., 1994; Bazan et al., 2002]. Present study was aimed at understanding the possible role or association of K ras to tumor metastasis with the reference of expression of other important proteins like adherent complex. Two colorectal cancer cell lines, that is, Caco-2 and DLD1 were selected as model for this analysis. Caco-2 cell lines contains a normal *K ras* [Delag et al., 1993], while DLD1 has heterozygous mutation at codon 13 of *K ras* which is responsible for its malignant properties [Trainer et al., 1988]. DLD1 also possesses a *p53* mutation, which has not been found responsible for malignant properties in this particular cell line [Shirasawa et al., 1993].

MATERIALS AND METHODS

All cell culture manipulations were carried out using aseptic technique in class II cytomat pharmaceutical safety cabinets and cells were incubated in LEEC MK11 proportional temperature controller incubators at 37°C with an atmosphere of 5% CO_2. Cell culture medium was prepared freshly and sterility tests were performed for the presence of Mycoplasma by following the standard optimized protocols. For comparison of expression of *K ras* and adherent molecule complex such as *E-cadherin*, α-*catenin*, and *β-catenin* (at message and translational level), two human colorectal cancer cell lines were selected, that is, Caco-2 and DLD1 provided by American Type Culture Collection (ATCC) were used. DLD1 was grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco-BRL, Paisley, UK) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (50 in 500 ml of medium) (Gibco-BRL, Paisley, UK), 2 mM Glutamine (Invitrogen, Paisley, UK), and 1% Non-essential Amino acids (NEAA) (Invitrogen) while Caco-2 was grown in Rosswell Park Memorial Institute (RPMI) 1640 (Gibco) supplemented with 10% (v/v) Fetal Bovine serum (FBS) (Gibco-BRL, Paisley, UK), and 2 mM Glutamine (Invitrogen, Paisley, UK).

Sequence of codon 13 was analyzed in both of the cell lines. PCR product of 576 bp was amplified and sequenced by using K ras-S forward and K ras-S reverse primers with annealing at 52°C for 60 s [Claire et al., 2009].

EXPRESSION OF *K-RAS*, *E-CADHERIN*, *α-CATENIN*, AND *β-CATENIN* IN CACO-2 AND DLD1 CELL LINES

The expression of *K ras*, *E-cadherin*, α-*catenin*, and *β-catenin* was compared in Caco-2 and DLD1 cells at transcriptional level by measuring mRNA through Real time PCR (*MY IQ-2*, Bio-Rad, Hertfordshire, UK). Total RNA was extracted from the cells using RNeasy kit (Qiagen, UK) and cDNA was prepared by using "High Capacity cDNA reverse transcription Kit" (Applied Biosystems). *K ras*, *E-cadherin*, α-*catenin*, *β-catenin*, and *β-actin* were amplified by the designed primers used for

K ras	
Forward	5′-GCAAGAGTGCCTTGACGATA-3′
Reverse	5′-TCCAAGAGACAGGTTTCTCCA-3′
E-cadherin	
Forward	5′-CTGTCGAAGCAGGATTGCAAA-3′
Reverse	5′-GAAGAAACAGCAAGAGCAGCA-3′
α-*catenin*	
Forward	5′-CTGGGAGGAGAGCTCATCA-3′
Reverse	5′-TTTCACTGTTTGCACTACAGCATTC-3′
β-catenin	
Forward	5′-TGTTCTCAGATTTCTGGTTGTT-3′
Reverse	5′-CACTTTCTGAGATACCAGCC-3′

β-actin was used as housekeeping gene as an internal control for normalization [Doak et al., 2004]. The assay was performed in triplicate and each experiment was repeated for the confirmation of results. The reaction mixture comprised of 2 μl cDNA, 12.5 μl of Syber green Master Mixture (containing polymerase, PCR buffer, and $MgCl_2$ by Bio-Rad, UK), and 0.4 μl of (25 mM) dNTPs range (0.5–1.5).

The expression pattern of *K ras*, *E-cadherin*, α-*catenin*, and *β-catenin* in Caco-2 and DLD1 on translational level was compared by Western blotting [Hongbao, 2006]. *β-actin* was used as a control. Briefly, protein was extracted, quantified, and detected by blotting

on membranes using Immun-Star WesternC chemiluminescence detection assay (Bio-Rad) and visualized by ChemiDoc XRS (Bio-Rad). Primary antibodies used were Mouse monoclonal anti-K-ras (Santa Cruz, Biotechnology; 1:2,000), Rabbit anti-α-catenin (1:5000; Sigma–Aldrich), Mouse anti-β-catenin (1:1000; BD Biosciences, San Jose, CA), Rabbit anti-E-cadherin (1:1000; Cell Signaling Technology Inc.), and Mouse anti- β-actin (1:1000; Cell Signaling Technology Inc.) while HRP-conjugated antibodies were used as secondary antibody.

DATA ANALYSIS

Real time expression data was analyzed using the iCycler-iQ software. Comparison of expression of K ras and adherent molecules in Caco-2 (control and wild type for *K ras*) and DLD1 (mutant for *K ras*) cell lines was calculated by the iCycler-iQ software automaticaly (in ΔΔCT units). The difference in expression was finally calculated in fold change and significant range was 0.5–1.5 (for down and upregulation, respectively).

RESULTS

RELATIVE AVERAGE EXPRESSION OF *K RAS*, *α-CATENIN*, *β-CATENIN*, AND *E-CADHERIN*

The specific amplified region containing the codon 13 of *K ras* was sequenced and it was confirmed that Caco-2 used in our experiments contains a wild type *K ras* (GGC at codon 13) and DLD1 contains heterozygous mutation at codon 13 (GAC). When compared on message level, a slightly higher expression of *K ras* was observed in DLD1 cell lines but the calculated fold increase was not significant (1.83), while *β-catenin* and *E-cadherin* showed significant decrease in expression in DLD1 (as compared to Caco-2) (Fig. 1). A change of 4.2-fold was observed in the expression of both of these genes while in case of α-*catenin*, a slight reduction in expression was noticed. The observed change was 1.3-folds (non significant). When expression of *K ras* was compared on translation level by western blotting, results correlated with the real time data (Fig. 2). As compared to Caco-2, a slight increase in expression of K ras, slight down regulation in α-catenin and significant down regulation in β-catenin and E-cadherin was observed in DLD1.

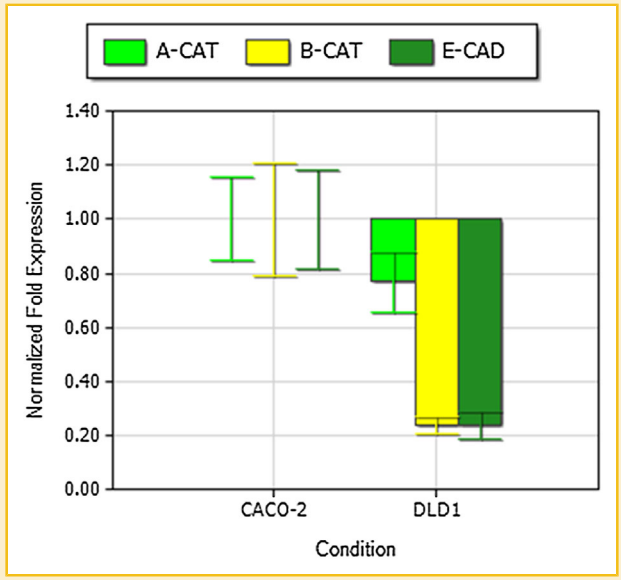

Fig. 1. Comparison of expression of *E-cadherin* (E-CAD), *β-catenin* (B-CAT), and *α-catenin* (A-CAT) in Caco-2 and DLD1 lines by qRT-PCR. *E-cadherin* and *β-catenin* showed slightly reduced while *α-catenin* showed significantly reduced expression in DLD1 as compared to Caco-2 cell lines.

DISCUSSION

K ras is one of the important member in Ras signaling or MAP kinase pathway and formation of adherens junction seems to activate the MAP kinase pathway indirectly by ligand independent activation of the EGFR [Pece and Gutkind, 2000]. Stimulation of growth factor receptor stimulates the adherens junctions assembly and activation of Ras which results in activation of Raf kinases at the cell membrane [Orton et al., 2005]. On activation, Raf kinases phosphorylate Mitogen activated protein kinase kinase (MEK) to be phosphorylated, which in turn activates Mitogen activated protein (MAP) signal transduction cascade [Kolch, 2000]. Active MAP kinase effects the cell survival by alteration to downstream targets, which control the activities of transcription of genes involved in cell survival and inhibition of the pro-apoptotic protein [Bonni et al., 1999]. The adherent molecules play a crucial role in epithelial cell–cell adhesion and in the maintenance of tissue architecture. Distraction of this particular complex can lead to scattering of cells [Keller et al., 2007]. Loss of E-cadherin-mediated adhesion has been reported to be involved in the neoplastic process, allowing cells to escape normal growth control signals, resulting in loss of differentiation and increased cell proliferation associated with invasive behavior [Lochner and Birchmeier, 1991; Tsanou et al., 2008]. As compared to Caco-2, a slightly higher expression of *K ras* was observed in DLD1 cell lines, but the calculated increase was not significant. When expression of adherent junction proteins was compared in these two cell lines, α-catenin showed a slight decrease (1.3-folds) in expression level, while β-catenin and E-cadherin were significantly down regulated (4.2-fold decrease) at transcriptional level by qRT-PCR. The results of real time analysis were found to be concordant with western blotting.

Down regulation in expression of adherent molecules has also been reported previously in different cancers. Reduced expression in E-cadherin, β-catenin, or α-catenin has been observed in CRC with frequent lymph node metastasis [Takayama et al., 1998]. Joo et al. [2002] have demonstrated that reduced expression of β-catenin and α-catenin is associated with tumor differentiation. It has been observed that α-catenin acts as a tumor suppressor in DLD1 cell lines and it′s over expression can mitigate the effect of mutant *K ras* in these cell lines.

Down regulation in the expression of E-cadherin has been reported to be associated to advanced stage of CRC [Zheng et al., 2010], tumour size, histopathology, and differentiation [Tsanou et al., 2008]. Herzig et al. [2007] have reported that loss of E-cadherin expression results in β-catenin degradation and blocking of Tcf/β-catenin-mediated transcriptional activity in mammalian

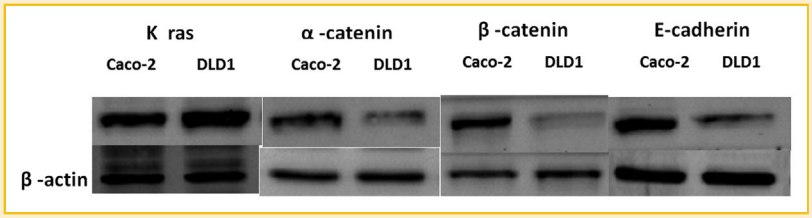

Fig. 2. Expression of *K ras*, *α-catenin*, *β-catenin*, and *E-cadherin* in Caco-2 and DLD1 cell lines as determined by estimation of translational level through Western blotting technique. β-actin is the expression of house keeping gene.

cell, but the forced expression of constitutive-active β-catenin or genetic ablation of Tcf/β-catenin transcriptional activity in these cells does not affect tumor progression. So this is an indication that signals other than β-catenin/Tcf-mediated Wnt signaling is induced by the loss of E-cadherin during this tumor progression.

Loss of α-catenin has been found to increase the aggressiveness of tumor cells (with mutation in *K ras*) but on reintroduction cell adhesion and proliferation was significantly reduced. So it is being considered as a potent suppressor of colorectal tumors (having mutations in *K ras*) [Claire et al., 2009]. Ahmad et al. [2011] have reported that ras pathway activation strongly cooperates with Wnt signaling to drive bladder cancer. Oncogenic *K ras* has been observed to effect the expression of E-cadherin in human prostate and pancreatic ductal cells, also Kwon et al. [2010] have reported that over expression of K ras reduces the expression of E-cadherin in pancreatic cells by hypermethylation of promoter which decreases aggregation of cells leading to increase in metastasis in human prostate cells. Agbunag and Bar-Sagi [2004] have reported that its oncogenic expression trigger the mitogenic activity of pancreatic ductal cells by reducing the expression of E-cadherin. On the basis of accumulating evidences by present study and some of the previous studies [Shah et al., 1994; Downward, 2003; Tang et al., 2000; Taipale and Beachy, 2001; Li et al., 2005; Shahrzad et al., 2005], we propose a possible model in Figure 3A and B which represent the presence of a possible cross talk between *K ras* and adherent junction mediated signaling cascades. We suggest that heterogenous mutation (G to D) in codon 13 is responsible for overexpression of *K ras* and down regulation of expression of adherent complex in DLD1 cell lines compared to Caco-2. It can be suggested that K ras-mediated decrease in the adherent junction complex (specially E-cadherin) expression may contribute to the metastatic nature of the DLD1 cells. The down

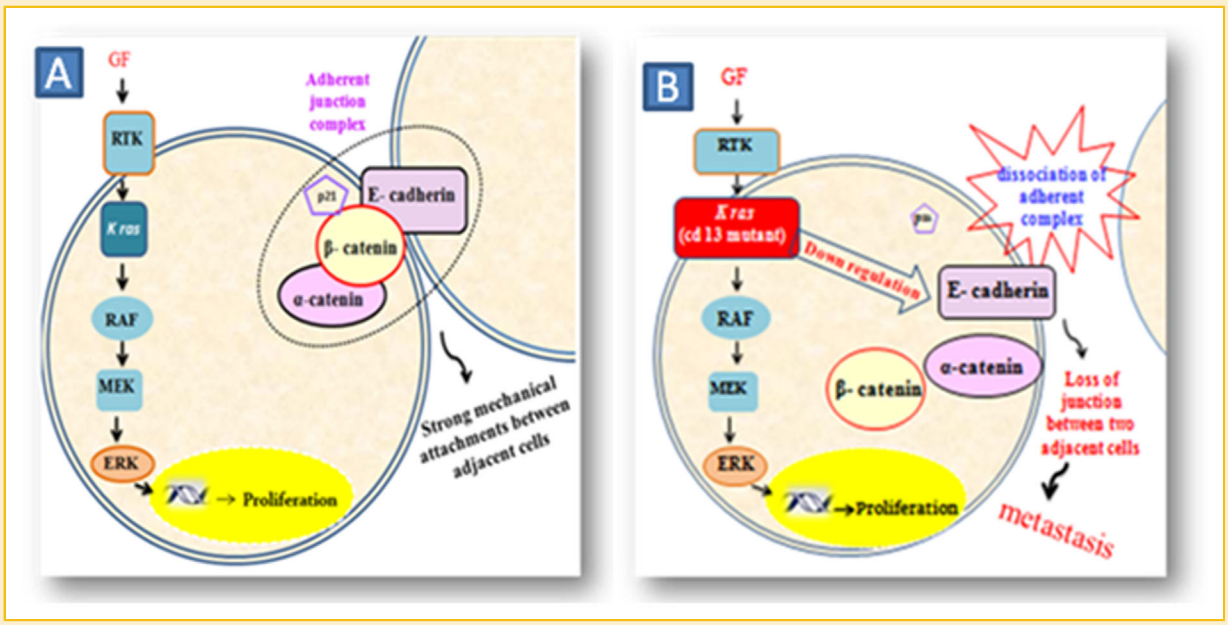

Fig. 3. A: Ras signaling in CRC cell having wild type codon 13 in *K ras*, adherent junction complex is providing strong mechanical attachment to hold the two adjacent cells together. B: Ras signaling in cell having mutation in codon 13 in *K ras* leading the downregulation in expression of adherent junction complex partner proteins, that is, E-cadherin, α-catenin, and β-catenin which can cause the dissociation of adherent complex resulting in intravasation of malignant cells from the primary site causing metastasis.

regulation of expression of E-cadherin, α-catenin, and β-catenin leads to dissociation of the adherent junction complex causing loosening of the junction between the tumor cells and hence leading to metastasis. Some of the previous studies also support our suggested model as DLD1 cell lines show cell migration, proliferation, and highly aggressive tumorigenicity while Caco-2 is non metastatic cell line [Shah et al., 1994; Shahrzad et al., 2005]. Experimentally induced mutations in *K ras* stimulate the migration of human CRC cells (Caco-2) cells by decreasing the level of E-cadherin/beta-catenin/p120 protein (adherent junction) complex formation [Li et al., 2005].

Results presented in this study are based on cultured cells and hence in vitro analysis and thus need to be confirmed in vivo. To demonstrate the effect of *K ras* mutations on expression of these proteins and their role in signaling and tumorigenicity, the mechanism should be studied in normal and cancerous CRC cell lines by disruption of *K ras*, *α-catenin*, *β-catenin*, *and E-cadherin*. Re-introduction of these protein and effect on their carcinogenesis ability can better explain their exact role.

CONCLUSIONS

K ras showed slightly higher level in DLD1 cell lines, which has a heterozygous mutation at codon 13, compared to control Caco-2 cell line, along with 1.3-fold decrease in α-catenin and 4.2-fold decrease in β-catenin and E-cadherin. It can be inferred that a possible cross talk exists between *K ras* and adherent junction mediated signaling. Mutation at codon 13 (G to D) leads to the overexpression of K ras and reduced expression of adherent junction complex resulting in metastasis.

ACKNOWLEDGMENTS

We would like to thank Drs. Helen Whiteland and Mathew Turner, and Mr. Mark Roberts and Ms. Margaret Clatworthy for their valuable suggestions, guidance, and cooperation. This project was funded by Higher Education Commission of Pakistan and School of Medicine Swansea University, United Kingdom. The authors would also like to extend their sincere appreciation to the Deanship of Scientific Research at King Saud University, Riyadh, Kingdom of Saudi Arabia for funding this research through the Prolific Research Group (project number–1436-011).

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