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SHORT COMMUNICATION

# Potential Role of TRAIL in Metastasis of Mutant KRAS Expressing Lung Adenocarcinoma

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**Abstract** Apo2L/tumor necrosis factor (TNF)-α-related apoptosis-inducing ligand (TRAIL, TNFSF10) is an important cytokine in the tumor microenvironment and plays a major role in the balance of cell survival/death pathways. Bioinformatic analyses of 839 adenocarcinoma (AC) and 356 squamous cell lung carcinoma patient data (SCC) by cBioPortal (genomic analyses) shows that TRAIL expression leads to differential outcomes of disease free survival in AC and SCC. Oncomine datamining (transcript analyses) reveal that TRAIL is upregulated in 167 SCC as compared to 350 AC patients from six data sets. Genomic analyses using cBioPortal revealed high rates of KRAS mutation in AC accompanied by higher incidence of metastasis and increased amplifications of TRAIL gene in SCC. Bioinformatic analyses of an additional lung cancer patient database also showed that risk of disease progression was significantly increased with high TRAIL expression in AC (461 samples). In vitro studies demonstrated that TRAIL increased phosphorylation of ERK only in adenocarcinoma cell lines with mutant KRAS. This was associated with increased migration that was abrogated by MEK inhibitor PD98059. Effects of increased migration induced by TRAIL persisted even after exposure to ionizing radiation with suppression of DNA damage response. These results help understand the role of TRAIL signaling in metastasis which is essential to develop strategies to revert these signals into pro-apoptotic pathways.

**Keywords** TRAIL · cBioPortal · Oncomine · Adenocarcinoma · Squamous cell carcinoma · Mutant KRAS · ERK signaling

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## Introduction

Non-small cell lung cancer (NSCLC) represents 75–80 % of all types of lung cancer, and includes different histological subtypes viz., squamous cell carcinomas, adenocarcinomas, and large-cell carcinomas. The histologic subtypes of lung adenocarcinoma and squamous cell carcinoma display different molecular characteristics and gene expression signatures and hence, there is a compelling need to consider them separately for treatment purposes [1–3]. The Apo2L/tumor necrosis factor (TNF)-α-related apoptosis-inducing ligand (TRAIL, TNFSF10), a member of the TNF family, induces apoptosis and TRAIL receptor targeting agents have shown great promise in NSCLC models and early clinical studies (reviewed in [4]). However, resistance is often encountered due to multiple mechanisms [4–6]. These include the inhibition of death inducing signaling complex (DISC) assembly and increase in several microRNAs that down regulate critical molecules like caspase 8 [7] and interferon stimulated gene 12a [8].

Though 90 % of NSCLC express TRAIL and its receptors [9], its expression in different subtypes of lung cancer and its role in metastasis, if any, are not known. The rationale of the present study is that altered TRAIL signaling in the microenvironment may have a role in promoting tumor growth and metastasis. The potential role of TRAIL in lung cancer was investigated by extensive bioinformatic analyses of the

genomic and transcript level changes of TRAIL, its receptors and KRAS in adenocarcinoma (AC) and squamous cell lung carcinoma (SCC) and its association with metastasis. In vitro studies were carried out in AC cell lines to assess the effect of TRAIL on migration and its possible mechanism.

## Methods

### Bioinformatic Analyses

Genomic analysis was carried out in 839 AC and 356 SCC patient datasets through cBioPortal for Cancer Genomics, a platform to analyze genetic alterations of TRAIL, its receptors and KRAS, as well as their correlation to metastasis and disease free survival [10]. Oncomine, a cancer microarray database and web-based data-mining platform comprising of transcriptome data from patient derived cancer samples was used to analyze the expression of TRAIL [11]. Comparisons were made between microarray data of these two types of lung cancer across multiple datasets. The median intensity of TRAIL along with 10th and 90th percentile data from Oncomine was plotted using Graphpad Prism software. The transcript analyses were carried out in 350 AC and 167 SCC samples.

The probability of disease progression was calculated using Kaplan Meier (KM) estimate from 982 patients' data available in KM plotter database. It is an online tool that allows analysis of published cancer gene expression and clinical datasets to identify biomarkers related to metastasis and survival [12].

### Cell Culture and Treatments

Human lung adenocarcinoma cell lines A549, NCI-H23 and NCI-H522 were obtained from National Center for Cell Sciences, Pune, India and maintained in DMEM containing 10 % foetal bovine serum (FBS) (HiMedia, Mumbai, India) at 37 °C in a 5 % $CO_2$ atmosphere. Cells were serum starved overnight prior to treatment with TRAIL (Miltenyi Biotec, Gladbach, Germany) and further maintained in 1 % serum containing medium (LSM) in humidified incubators with 5 % $CO_2$ at 37 °C till completion of the experiment. Cells were exposed to ionizing radiation (IR) using Bhabhatron, a $Co^{60}$ source (Panacea Biotech Ltd., New Delhi, India) with a dose rate of 1 Gy/min.

### Wound Healing and Migration Assays

Changes in the mobility of cells were assessed by wound healing and migration assay. Wounds were created in a confluent monolayer of cells using a sterile pipette tip. It was followed by treatment of the cells with TRAIL (100 ng/ml). The cells were maintained in LSM for 24 h and stained with acetomethoxy derivative of calcein. Fluorescence images of the wound were acquired at 0 and 24 h and the number of migrated cells was enumerated using CellProfiler software [13].

Adherent cells in transwell inserts (with 8 μm pore membrane) (BD Biosciences, Franklin Lakes, NJ, USA) were treated with TRAIL (50–200 ng/ml). All cells that migrated to the bottom of the membrane were stained with crystal violet, photographed in a light microscope and counted using CellProfiler software.

### Flow Cytometry

Cells were trypsinized and fixed in ice-cold 70 % alcohol) at −20 °C overnight. The cells were blocked for 30 min in 5 % FBS in phosphate buffered saline (PBS) and then incubated with phospho ERK1/2 followed by anti-goat alexafluor 488 antibody. Cells labeled only with anti-goat alexafluor 488 served as negative controls. Twenty thousand cells were acquired in Cyflow space™ flow cytometer (Partec, Germany) using Flowmax software and analysed using FCS express software.

### RT- PCR

The expression of integrins and matrix metalloproteinases (MMPs) in cells treated with TRAIL (24 h) was assessed using Real-time PCR. Total RNA was extracted using RNA isolation kit (5 Prime GmbH, Deutschland, Germany). One microgram of total RNA was reverse transcribed to cDNA using first strand cDNA synthesis kit (Roche Applied Science, Penzberg, Upper Bavaria, Germany). Equal amount of cDNA (0.5 μl) was used for PCR amplification of GAPDH, ITGA2 and MMP genes using specific primers (Integrated DNA technologies, Coralville, Iowa, USA). Primer sets used were as follows. GAPDH-F′: ATGACATCAAGAAGGTGGTG, GAPDH-R′: CATACCAGGAAATGAGCTTG, ITGA2-F′: GCAGATGGACCACACTTTGA, ITGA2-R′: TGTCTGTGCCCTTTTCCTCT, MMP1-F′: GGGAGATCATCGGGACAACTC and MMP1-R′: GGGCCTGGTTGAAAAGCAT. qRT-PCR was carried out on a LightCycler® 480 System (Roche Applied Science, Penzberg, Upper Bavaria, Germany). All reactions were performed with SYBR green in triplicates. Relative mRNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method ($\Delta Ct = Ct_{Target} - Ct_{Ref}$, $\Delta\Delta Ct = \Delta Ct_{TRAIL} - \Delta Ct_{untreated}$), using GAPDH as the reference gene.

### Western Blotting

Cells were scraped in lysis buffer (50 mM Tris–HCl pH 7.2, 100 mM DTT, 2 % SDS, 0.1 % bromophenol blue, 10 % glycerol, phosphatase and protease inhibitor cocktail). The cells were incubated on ice for 30 min and lysates were clarified by centrifugation at 14,000 rpmfor 15 min at 4 °C. The lysates were boiled for 10 min for denaturing the proteins after which equal amounts were separated by 12 % SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred to polyvinylidenedifluoride membranes. Membranes were blocked with 5 % non-fat milk in PBS with 0.05 % Tween-20 (PBST) at room temperature for 1 h and incubated overnight at 4 °C with primary antibodies diluted in 4 % BSA. Bound antibodies were visualized with HRP-conjugated secondary antibodies with the use of enhanced chemiluminescence.

### Statistical Analysis

All results are expressed as mean ± standard error. Statistical difference between means was assessed using Student's t test, and a $P$ value less than 0.05 was considered significant.

## Results

### Alterations in TRAIL Expression Correlate with Poor Disease Free Survival in AC

cBioPortal was used to assess association of TRAIL with disease free survival (DFS), in lung AC and SCC. A total of 839 patients from five datasets of adenocarcinoma and 356 patients from two datasets of squamous cell carcinoma were analyzed (Fig. 1a). Alterations in TRAIL expression were associated with increased relapse and decrease in DFS in AC ($P = 0.005$) whereas it was the reverse in SCC ($P = 0.508$). Bioinformatic analyses using Oncomine database was carried out to assess transcript levels of TRAIL in 350 AC and 167 SCC samples from six datasets (Fig. 1b–g). Interestingly, Oncomine microarray analysis revealed that TRAIL was among the top 10 % of the differentially regulated genes in lung cancer patients. Expression of TRAIL was upregulated 1.7 fold in SCC as compared to AC in Garber (53 samples) and Bittner (100 samples) lung cancer microarray data analyses respectively ($P < 0.01$; Fig. 1b, c respectively). Analysis of Bild (101 samples) and Hou (72 samples) lung cancer data revealed a 2.2 fold increase in SCC as compared to AC ($P < 0.01$; Fig. 1d, e respectively) Su multi cancer data (28 samples) and Bhattarcharjee lung data (153 samples) too demonstrated 3.6 and 6.4 fold increase in expression of TRAIL in SCC ($P < 0.01$; Fig. 1f, g respectively). These analyses, therefore, indicated that TRAIL was overexpressed in SCC as compared to lung AC.

### Increased KRAS Mutations in AC and TRAIL Amplification in SCC

Since there was an increase in transcript levels of TRAIL, its genomic alterations along with the death receptors DR4 and DR5 were studied. Recently, it has been reported that oncogenic Kirsten rat sarcoma viral oncogene homolog (K-RAS) and its effectors can convert death receptors into invasion-inducing receptors in colorectal cancer [14]. Therefore cBioPortal was used to examine the same AC and SCC datasets studied for DFS for multiple gene alterations, mutations and amplifications of TRAIL, its receptors and KRAS. In lung AC, 2.7–36.8 % patients had mutations or other alterations and 4.1–6.4 % had amplifications (Fig. 2a) for all four genes. On the other hand, 36–50 % of patients with SCC had amplifications with 1.1–2.2 % showing mutations in these genes. The split up of these gene alterations was visualized in a representative AC (Fig. 2b) and SCC (Fig. 2c). As can be seen, there was no change in expression of DR4 and DR5 (6–7 %) between AC and SCC. However, with respect to KRAS mutation and TRAIL amplification, there was a completely opposite pattern in the two cancer types. While AC harbors significantly higher KRAS mutations with fewer TRAIL alterations, SCC has significantly higher proportion of TRAIL amplifications. From these figures, it can also be seen that the proportion of patients with metastasis are higher in AC as compared to SCC. This can be seen with TRAIL alterations as well as in KRAS mutant patients. These results were confirmed with bioinformatics analyses of an additional dataset. The Kaplan Meier plotter, a meta-analysis based biomarker assessment tool can assess the effect of 22,277–54,675 genes on progression and survival using 2437 lung cancer patients. When we assessed the effect of TRAIL expression in lung AC patients (461 samples), the probability of cancer progression was found to be statistically significant with high TRAIL expression as compared to that of low TRAIL expression (Fig. 2d; $P = 0.0054$). However, in SCC patients (141 samples), the effect on cancer progression was not statistically significant (Fig. 2e; $P = 0.32$).

### TRAIL Increases ERK Mediated Migration of AC Cell Lines with Mutant KRAS

A549 cells (mutant KRAS) are resistant to TRAIL induced apoptosis (Fig. 3a). However, there was a dose dependent increase in migration as seen by transwell assay. TRAIL at concentrations of 10 and 20 ng/ml did not increase migration (data not shown) whereas there was a significant increase at >50 ng/ml (Fig. 3b). This increased migration was also

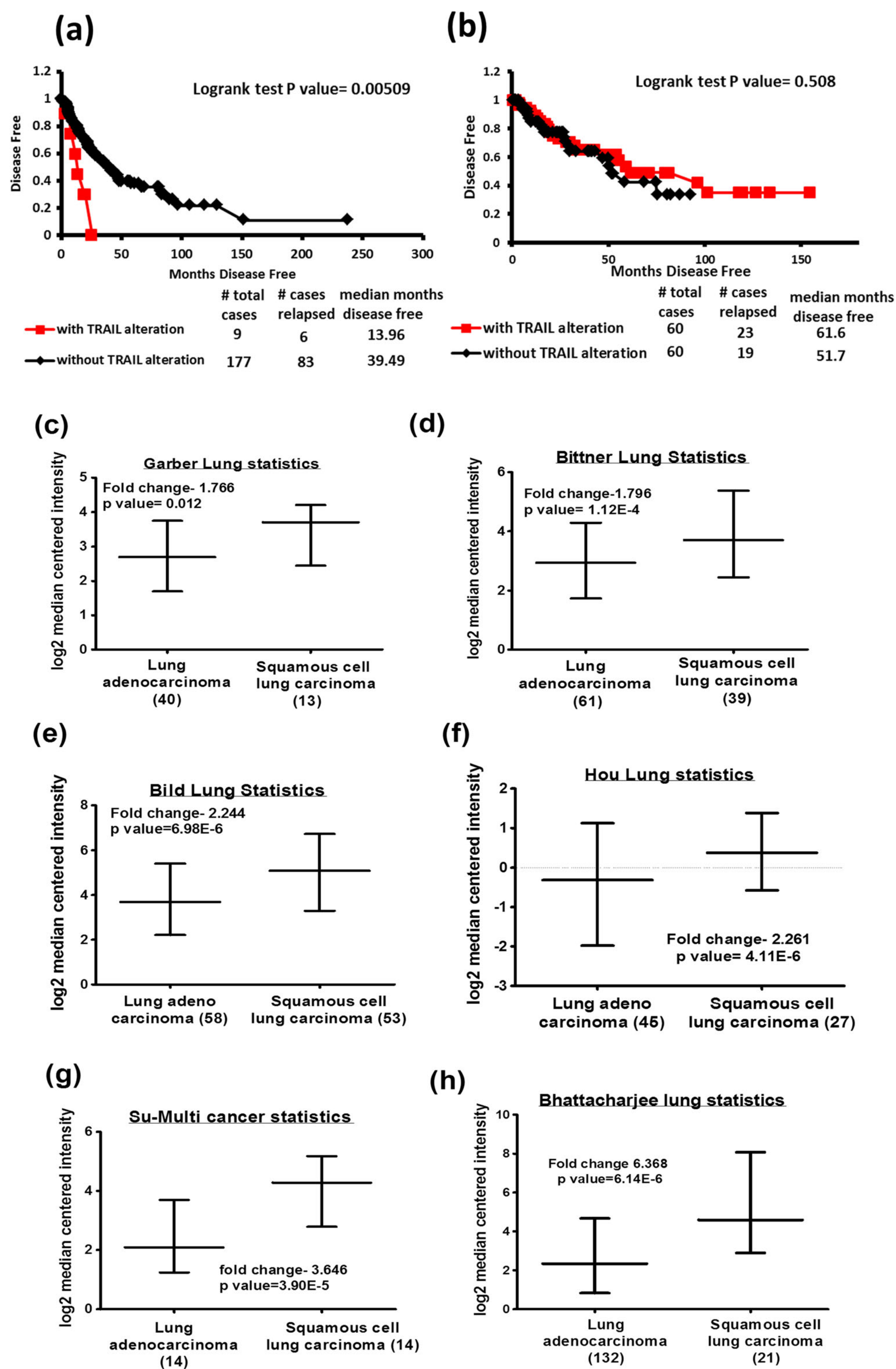


**Fig. 1** *Disease free survival in lung AC and SCC with TRAIL alteration and transcript analysis of TRAIL.* cBioPortal analysis for disease free survival in patients with TRAIL alterations in **a** AC and **b** SCC. TRAIL (TNFSF10) transcript analysis in AC and SCC in **c** Garber **d** Bittner **e** Bild, **f** Hou, **g** Su Multi-cancer and **h** Bhattacharjee datasets using Oncomine datamining platform. Y-axis represents median intensity of TRAIL expression with 10th and 90th percentile values. Numbers in parentheses indicate the number of samples

confirmed by wound healing assay (Fig. 3c). TRAIL increased migration of another mutant KRAS lung AC cell line, NCI-H23 (Fig. 3d) whereas this effect was not observed in NCI H522 harboring wild type KRAS (Fig. 3e). To find out the mediators, we studied the activation of some signaling pathways. There was no appreciable activation of AKT (data not shown). However, TRAIL increased phosphorylation of ERK in A549 and NCI-H23 cell lines with mutant KRAS and no change was observed in NCI-H522 with wild type KRAS (Fig. 4a–d). This was also associated with an increased

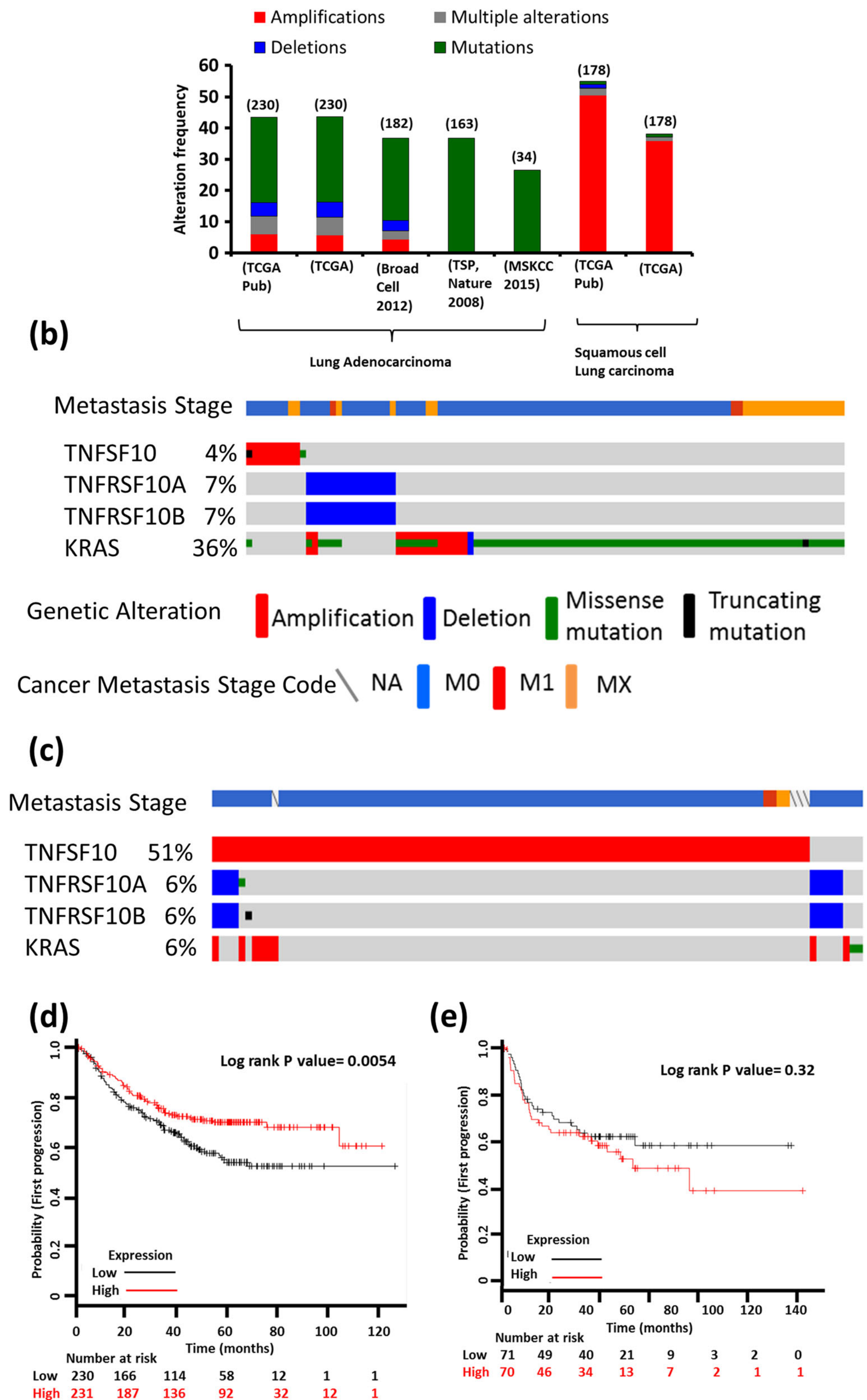


**Fig. 2** *Genetic alterations of TRAIL, its receptors and KRAS and probability of progression to metastasis in AC and SCC.* **a** cBioPortal analysis for genetic alterations of TRAIL (TNFSF10), its receptors (TNFRSF10A and B) and KRAS (multiple alterations, mutations, amplifications and deletions) in lung cancer. Numbers in parentheses indicate the number of samples. Status of TRAIL, its receptors and KRAS in individual samples along with their clinical stage of metastasis from a representative **b** AC **c** SCC TCGA study shown in Fig. 2a. Per cent genetic alterations of individual genes are indicated. **d** Kaplan-Meier plot for first progression with TRAIL gene in lung AC datasets. The probability of cancer progression has been plotted over a period of 120 months for patients with high or low TRAIL expressions. Numbers at risk indicate the number of patients at risk at corresponding time points **e** K-M plot in SCC

expression of MMP1 and ITGA2 (Fig. 4e) in A549 cells. MEK inhibitor PD98059 was able to completely inhibit TRAIL induced migration in A549 cells indicating the involvement of ERK signaling in TRAIL induced migration (Fig. 4f). Though ERK activity is widely regarded to provide proliferation and migration signals to cancerous cells, it can

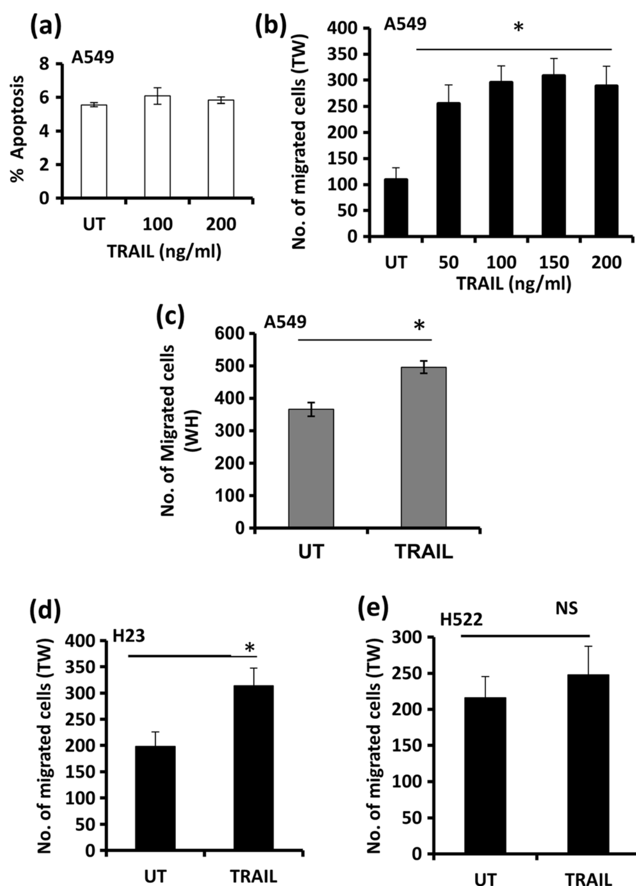


**Fig. 3** *TRAIL increases migration of lung adenocarcinoma cell lines with mutant KRAS.* **a** Per cent apoptosis in A549 cells treated with TRAIL. Cell migration was assessed using 8 μm trans-well inserts. The cells on the top of the insert were removed using a cotton swab and the migrated cells on the bottom of the membrane were fixed and stained with crystal violet and 10× photomicrographs of the same were acquired using NIS elements software in Nikon Eclipse microscope. **b** TRAIL induced migration of A549 cells in transwell assay (TW). **c** To assess migration, wound healing (WH) assay was also performed by capturing photomicrographs of the wound area before and 24 h after treatment. Cells that migrated to the wound area in 24 h were enumerated using CellProfiler software. TRAIL induced migration assessed by transwell assay in **d** NCI-H23 cells and **e** NCI-H522 cells. $^{*}P < 0.05$; NS- not significant

also contribute to altering the radio sensitivity of cells [15]. When we assessed the effects of TRAIL pre-treatment on migration following exposure to IR, it was found to be increased as compared to IR alone (Fig. 4g and h). A suppression of DNA damage response genes, p53 and γ- H2AX following exposure to ionizing radiation in TRAIL pre-treated cells could contribute to this effect (Fig. 4i).

## Discussion

The unique capacity of TRAIL to induce apoptosis selectively in cancer cells in vitro and in vivo has provided the basis for the use of recombinant TRAIL and development of TRAIL-receptor agonists (TRAs), which have demonstrated robust anticancer activity in a number of pre-clinical studies. However, these have not translated very successfully into anti-cancer activity in patients (reviewed in [16]). The primary function of TRAIL is to induce apoptosis which can be type-I with robust caspase 8 activation or type- II which requires the involvement of mitochondrial pathway. In cancers, several intrinsic mechanisms have evolved to prevent aberrant activation of TRAIL [17–20] as well as TRAIL induced non canonical pro-survival signaling (reviewed in [21]) particularly in the context of KRAS mutations [14]. Hence an attempt was made in the present study, to utilize bioinformatics analysis to evaluate the expression of TRAIL, its receptors and KRAS in AC vs SCC patients. The interesting information that emerged was that KRAS mutations were highest in AC and TRAIL amplifications in SCC. Although the frequency of TRAIL alterations was small in AC, it was associated with poor disease free survival. On the other hand, in SCC, the amplification of TRAIL gene as well as increased expression of mRNA, did not lead to metastasis and resulted in better disease free survival. Similar TRAIL signaling in conjunction with mutant K-RAS or increased epidermal growth factor receptor mediated tissue transglutaminase leading to increased migration and metastasis have been reported in non-small lung cancer and pancreatic ductal adenocarcinoma [22, 23].

Cells resistant to TRAIL induced apoptosis activate a pro-tumorigenic [24] or pro-survival signaling, [25] particularly in the context of mutant KRAS leading to cancer progression, invasion, and metastasis [22]. Our in vitro studies confirmed that rhTRAIL induced ERK phosphorylation which resulted in migration of cell lines with mutant KRAS (A549 and NCI-H23) and not in those with wild type gene (NCI-H522). Abrogation of TRAIL induced migration in mutant KRAS AC by MEK inhibitor PD98059 further confirms the involvement of this signalling pathway. Though involvement of ERK in TRAIL induced migration has been demonstrated earlier, it was not associated with mutant KRAS [24]. A critical step in the progression of cancer is the process of degradation of extracellular matrix proteins by MMP and epithelial mesenchymal transition regulated by integrins [26] which was increased by TRAIL in A549 cells. Modulation of ERK signaling by TRAIL also affected the response to IR in these cells.

Though our studies have given insight into the potential role of TRAIL and possible mechanism of mediating mutant KRAS AC metastasis, a key limitation is that the genomic and transcript level changes as well as the KM plot have not been studied in the same set of samples. However, since similar trend of increased TRAIL expression was observed in microarray analyses of several datasets, along with differences in disease progression, further data can

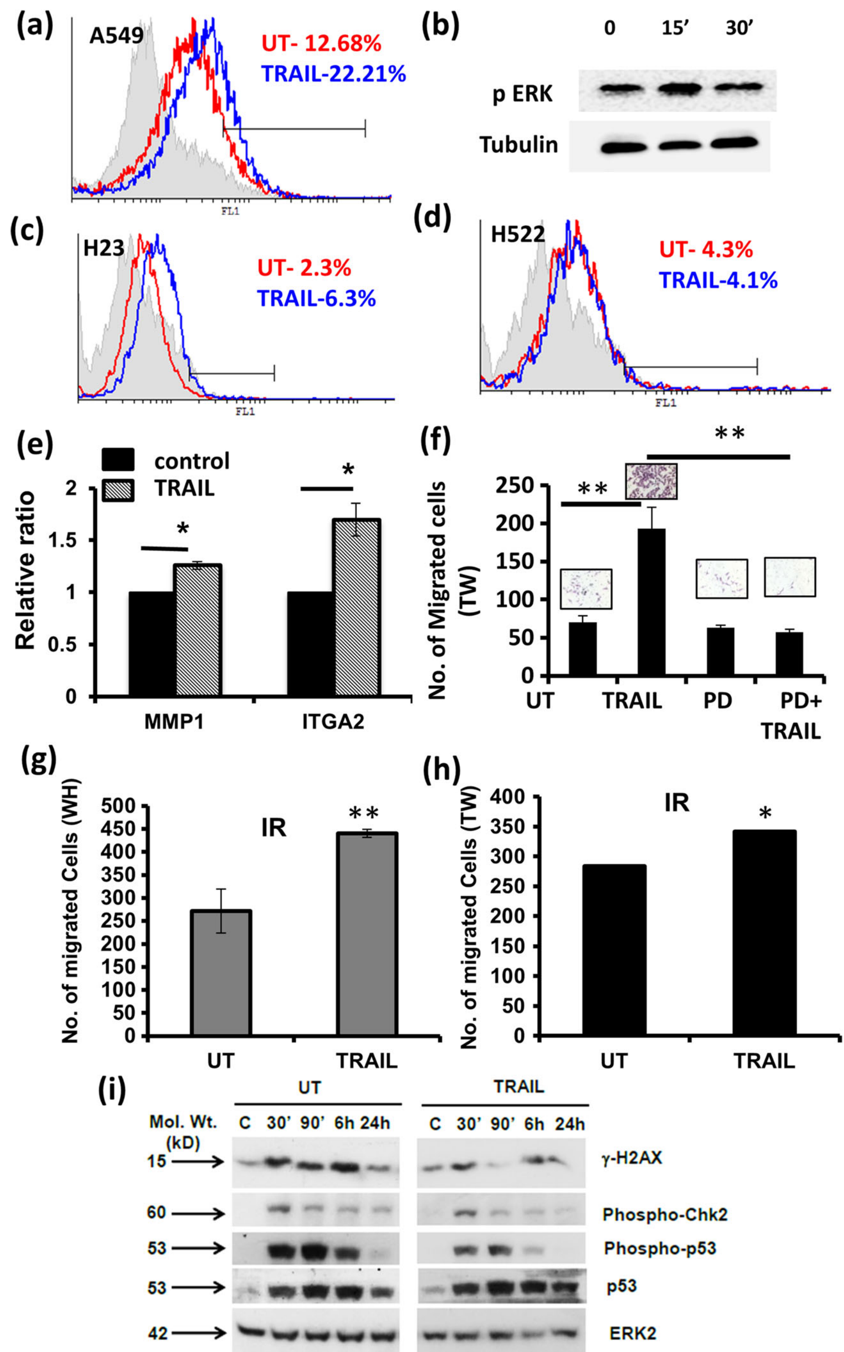


**Fig. 4** *Involvement of ERK signaling in TRAIL induced migration in mutant KRAS AC.* Phosphorylation of ERK following TRAIL treatment in **a** A549 cells - flow cytometry **b** A549 cells - western blotting (15 and 30 min after TRAIL) **c** NCI-H23 cells **d** NCI-H522 cells. **e** mRNA levels of MMP1 and ITGA2 in A549 cells (relative to GAPDH) **f** MEK inhibitor PD98059 abrogates TRAIL induced migration. Inserts are representative images of the migrated cells. Migration in TRAIL pre-treated A549 cells exposed to IR **g** wound healing assay **h** transwell assay **i** Western blot of DNA damage response associated proteins in TRAIL pre-treated cells following treatment with IR. **P* < 0.05; ***P* < 0.01

be generated to strengthen the observations on the association between TRAIL and AC metastasis with mutant KRAS. These results highlight the importance of knowledge of KRAS status prior to decision of TRAIL therapy, particularly in lung adenocarcinoma.

## Conclusion

The current findings underscore the importance of TRAIL in the metastasis of mutant KRAS expressing lung adenocarcinoma.

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**Compliance with Ethical Standards**

**Conflicts of Interest** The authors declare no conflicts of interest.

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