Identification and Characterization of Rab25 as a Novel Tumor Suppressor Gene with Anti-Angiogenic and Anti-Invasive Activities in Esophageal Squamous Cell Carcinoma

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Abstract
Esophageal squamous cell carcinoma (ESCC), the major histologic subtype of esophageal cancer, is a devastating disease characterized by distinctly high incidences and mortality rates. Better understanding of the molecular events leading to development and progression of the disease is of paramount importance in identifying putative biomarkers for diagnosis, prognosis and personalized treatment. By high-throughput transcriptome sequencing (RNA-Seq) profiling of twelve non-tumor and ESCC clinical samples, we identified a subset of significantly differentially expressed genes involved in integrin signaling. Of these, Rab25 was found to be the only gene significantly down-regulated and was subsequently characterized as a novel tumor suppressor critical in ESCC development. In addition to the samples used for the original RNA-Seq screening, frequent down-regulation of Rab25 expression was also confirmed in a larger cohort of ESCC tumor specimens using qPCR and immunohistochemistry analyses. Reduced expression of Rab25 was also found to be correlated with decreased overall survival. Absent or weaker expression of Rab25 was detected in a panel of ESCC cell lines as compared to a pooled normal tissue control. Demethylation treatment and bisulfite genomic sequencing analyses found down-regulation of Rab25 in both ESCC cell lines and clinical samples to be associated with promoter hypermethylation. Functional studies using lentiviral-based overexpression and suppression systems lent direct support of Rab25 to function as an important tumor suppressor with both anti-invasive and anti-angiogenic abilities, through a deregulated FAK-Raf-MEK1/2-ERK signaling pathway. Further characterization of Rab25 may provide a prognostic biomarker for ESCC outcome prediction and a novel therapeutic target in ESCC treatment.
Introduction

Esophageal cancer ranks as the sixth leading cause of cancer-related deaths worldwide, with distinctly high incidences and mortality rates particularly in East Asia, Africa and North America (1). Esophageal squamous cell carcinoma (ESCC) is the most common form of esophageal cancer. The disease is characterized by regional variation in incidences. More than 50% of all ESCC cases in the world occur in China. In Linzhou and nearby cities in Henan Province of Northern China, ESCC constitutes over 90% of all esophageal cancer cases in the area, and has the highest incidences and mortality rates of esophageal cancer reported in the world (1-2). Despite advances in diagnostic techniques and therapeutic modalities, ESCC remains a devastating malignancy due to late diagnoses and the aggressive nature of the disease. A better understanding of the recurrent genetic alterations and underlying molecular mechanisms involved in ESCC development and progression will facilitate the identification of novel targets, allowing for more sensitive methods of detection, facilitating earlier diagnosis and prolonging patient survival.

With the advent of next-generation sequencing technologies in recent years, a new sequencing platform, called transcriptome sequencing (RNA-Seq), has been applied to delineate changes at the transcriptomic level. The development of ESCC, like many other cancers, is believed to be driven by the accumulation of genetic alterations, causing the transformation of normal cells to malignant cells. Thus, studying recurrent changes at the levels of functional transcripts in malignant cells compared to non-tumor cells may aid in the identification of deregulated molecular events and pathways involved in driving ESCC. In the present study, we performed RNA-Seq analysis on twelve patient-derived non-tumor and ESCC clinical samples and identified a number of commonly and significantly differentially expressed genes. Pathway enrichment analysis (DAVID databases) found the deregulated genes to be commonly associated with a number of cancer-related pathways. Of these, two of the most significantly enriched pathways are related to integrin signaling, which is commonly known to influence important cellular processes critical to tumor development and progression, including cell proliferation, cell survival, angiogenesis and cell motility and invasiveness (3-6). Among the differentially expressed genes involved in integrin signaling, Ras-related protein Rab25 was found to be the only significantly down-regulated gene in ESCC compared to non-tumor tissue; and was thus chosen for further studies. Rab25 belongs to the Rab family of small GTPases and plays a critical role in the maintenance of normal epithelial lining (7-9). Past studies have shown Rab25 to play very contrasting roles in cancer, depending on the tissue in which it is expressed. It has previously been implicated in the progression of ovarian and breast cancer (10-12), while in contrast, more recent studies have identified a tumor suppressive role of Rab25 in colon cancer and triple-negative
breast cancer (13-14). To date, the role of Rab25 in ESCC has not been explored. In the present study, frequent Rab25 down-regulation was identified in ESCC clinical specimens compared to its non-tumor counterparts, while reduced Rab25 expression significantly correlated with worst overall survival. Absent or significantly weaker expression of Rab25 was also likewise detected in a panel of ESCC cell lines as compared to a pooled normal tissue control. Down-regulation of Rab25 expression in both ESCC cell lines and clinical samples was found to be significantly associated with promoter hypermethylation, as evidenced by our results obtained from 5-aza-2’-deoxycytidine (5-aza-dC) demethylation treatment and bisulfite genomic sequencing (BGS). Finally, functional studies found Rab25 to possess both anti-invasive and anti-angiogenic abilities through a dysregulated MAPK/ERK signaling pathway. Taken together, our results suggest Rab25 to function as a novel tumor suppressor in ESCC by repressing invasion, angiogenesis and tumorigenicity.
Materials and methods

Collection of esophageal tissue samples

All clinical specimens used for RNA-Seq and Rab25 expression studies by qPCR, Western blot and immunohistochemistry were collected from ESCC patients who underwent surgical resection of tumor tissues at Linzhou Cancer Hospital (Henan, China). When available, paired adjacent non-tumor tissues from the proximal resection margins (over 5 cm away from the ESCC sample) were also collected. The patients had received no previous local or systemic treatment prior to operation. All clinical samples used in this study were approved by the committee for ethical review of research involving human subjects at Zhengzhou University and The University of Hong Kong. For RNA-Seq, twelve fresh frozen clinical specimens (including 3 paired tumor and non-tumor, 4 unpaired tumor and 2 unpaired non-tumor samples) were randomly selected for analysis. Supplemental Table 1 provides a summary of the clinico-pathological parameters (i.e. patient age, gender, TNM grade) of each of the patients collected for this purpose. Immunohistochemistry for Rab25 was performed on a tissue microarray (TMA) consisting of 270 pairs of formalin-fixed, paraffin-embedded ESCC tumor and non-tumor specimens (15).

RNA-Seq and differential expression analysis

RNA-Seq was performed on the Illumina Cluster Station and GAIIx using the Standard Cluster Generation kit v4 and the 36-Cycle Sequencing kit v3. cDNA libraries from 7 ESCC samples (1T, 2T, 3T, 6T, 7T, 8T, 9T) and 5 non-tumor samples (4N, 5N, 6N, 8N, 9N) were sequenced with 38-base single reads. Sequencing reads were filtered for polymers, primer adaptors, and ribosomal RNAs and then mapped against the human genome assembly (NCBI Build 37.1) using CLC Genomic Workbench. The expression abundance for each gene was measured by RPKM (number of exon reads mapped per kilobase per million mapped reads) (16). The differential expression between tumor and non-tumor samples was evaluated using the \( t \)-test and Baggerley’s test (17) by treating the same type of individuals as one group. The genes with a consistent type of regulation for both the \( t \)-test and Baggerley’s test were kept, and the genes with a Bonferroni corrected \( p \)-value less than 0.001 were considered to be significant.

ESCC cell lines and culture conditions

ESCC cell lines EC18 and EC109 were kindly provided by Professor George Tsao (Department of Anatomy, The University of Hong Kong). ESCC cell lines HKESC1 and KYSE520 were provided by Professor Gopesh Srivastava (Department of Pathology, The University of Hong Kong). The other five ESCC cell lines, KYSE30, KYSE140, KYSE180, KYSE410 and KYSE510 were obtained from DSMZ.
(Braunschweig, Germany), the German Resource Centre for Biological Material (18). KYSE30 and KYSE180 cell lines were cultured in DMEM. All other ESCC cell lines were maintained in RPMI. Both media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cell lines used in this study were regularly authenticated by morphological observation and tested for absence of Mycoplasma contamination (MycoAlert, Lonza Rockland, Rockland, ME).

**RNA extraction, cDNA synthesis and quantitative real-time PCR (qPCR)**

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). First-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the Advantage RT-for-PCR kit (Clontech Laboratories, Mountain View, CA) and was then used for qPCR analysis. qPCR was performed using SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA) on an ABI Prism 7900HT System (Applied Biosystems). β-actin was amplified as an internal control. Supplemental Table 2 provides a list of the primer sequence used to amplify Rab25 in the qPCR assay.

**DNA extraction, bisulfite modification and promoter methylation analysis**

Genomic DNA was extracted from normal and tumor esophageal tissues and cell lines by phenol-chloroform method followed by bisulfite modification using the EpiTECT Bisulfite Kit (Qiagen, Valencia, CA). Methylation-specific PCR (MSP) and bisulfite genomic sequencing (BGS) were performed as previously described (19) using primers listed in Supplemental Table 2.

**Immunohistochemistry (IHC)**

Paraffin sections were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. Slides were heated for antigen retrieval in 10mM citrate (pH 6.0). Sections were incubated with polyclonal rabbit anti-human Rab25 (1:400, gift from Dr. Kwai Wa Cheng, University of Texas MD Anderson Cancer Center), rat anti-human CD34 (Biogenex, San Ramon, CA), mouse anti-human PCNA (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-human cytokeratin 5/6 (Millipore, Billerica, MA) or rabbit anti-human phospho-ERK1/2 (Cell Signaling, Danvers, MA) antibodies overnight at 4°C. EnVision Plus System-HRP (DAB) (Dako, Denmark) was used according to manufacturer’s instruction. Staining was revealed by counter-staining with hematoxylin. Stained slides were scanned by Aperio Scanscope® CS system (Vista, CA). Evaluation of immunohistochemical staining for Rab25 was performed by pathologist Chan KW (Department of Pathology, The University of Hong Kong) who had no prior knowledge of patient data. Staining intensity was divided into three scores: no (0% staining), low
(less than 50% staining) or high (greater than 50% staining) Rab25 expression. Both relative intensity and number of cells stained was taken into consideration during the scoring process. The specificity of polyclonal rabbit anti-human Rab25 antibody has been verified and reported (10). Staining intensities of PCNA and phospho-ERK1/2 were quantified using Aperio Spectrum® positive pixel count algorithm. Microvessel density was assessed as previously described (20).
Results

Differential gene expression profiling by RNA-Seq

We sequenced twelve RNA libraries from 7 ESCC tumor and 5 non-tumor samples, generating a total of 91 million 38-bp single reads and an average of 7.6 million reads per sample (Supplemental Table 3). A total of 72% of the reads aligned with the human genome, of which 57% aligned with unique locations in the genome. Across the twelve samples, a total of 14,351 distant genes (42% of the total number of genes in the reference database) were expressed with at least 10 mappable exon reads. For each gene, the expression value was measured based on RPKM (16). The Euclidean distance between samples was calculated, and a hierarchical clustering tree based on this distance showed a two-branch (tumor vs. non-tumor) partition (Fig. 1A). The 5 non-tumor (N) samples and 7 tumor (T) samples clustered into two distinct groups, indicating that the gene expression between tumor and non-tumor samples differs significantly. Among the 7 tumor samples, samples 1T, 6T, 7T, 8T and 9T were more closely clustered than when compared with 2T and 3T (Fig. 1A). This observation further suggests that RNA-Seq is a powerful technique in delineating changes at the transcriptomic level. To identify differentially expressed genes of functional importance, the t-test and Baggerley’s test were performed to address the significance of the deregulated genes in tumor samples. Of the 14,351 represented genes, 1,598 genes were up-regulated and 132 were down-regulated with a Bonferroni-corrected p-value of less than 0.001 (red dots in Fig. 1B). The global profile of the differential expression profiling for the 1,730 deregulated genes is demonstrated as a heatmap (Fig. 1C), where the genes are listed in descending order according to the Baggerley’s test analysis. Subsequent pathway enrichment analysis by DAVID found the 1,730 differentially expressed genes to be commonly associated with a number of cancer-related pathways. Of these, two of the most significantly enriched pathways are related to integrin signaling (Fig. 1D). Supplemental Table 4 provides a full list of the significantly differentially expressed genes involved in integrin signaling pathway (p < 0.001). Among these, Ras-related protein Rab25 was found to be the only significantly down-regulated gene (p < 1E-20) in ESCC compared to non-tumor tissue (Fig. 1E and Supplemental Fig. 1); and was thus chosen for further studies. RPKM values obtained from RNA-Seq were reproducible upon qPCR analysis, showing higher Rab25 expression in normal samples versus tumor samples (Fig. 1F). The result indicates a high level of concordance of the differential expression measurements between both platforms.

Rab25 as a novel tumor suppressor gene in human ESCC
To determine whether down-regulation of Rab25 was a common event in ESCC, we extended our qPCR analysis to an additional 43 paired non-tumor/primary ESCC samples. Rab25 was significantly down-regulated in tumor tissues when compared with adjacent non-tumor tissues (paired t-test, $p < 0.0001$, Fig. 2A). We also examined the expression of Rab25 at the protein level by Western blot and IHC analyses. Of the 6 esophageal tissue samples examined by Western blot analysis, Rab25 expression was consistently lower in the 3 ESCC samples as compared with the 3 non-tumor esophageal samples (Fig. 2B). To investigate the clinical significance of Rab25 expression in ESCC, a TMA comprising 270 paired non-tumor/ESCC samples, with more detailed patient clinical data, was used for an immunohistochemical study. A strong Rab25 staining was observed in the non-tumor samples (Fig. 2C). More than half of all informative non-tumor samples displayed a high Rab25 expression level (89/171, 52.0%), whereas only a quarter of the informative tumor samples showed high Rab25 expression (42/164, 25.6%) (Fig. 2D, chi square test $p < 0.001$). In a Kaplan-Meier survival analysis comparing patients with different Rab25 expression levels, higher Rab25 expression was significantly associated with a longer survival time (log-rank test, $p = 0.009$). Among three groups of patients with different Rab25 expression levels, increased survival was observed with enhanced Rab25 expression (Fig. 2E). Patients with negative Rab25 expression had the worst prognosis, with a mean overall survival of 27.7 months, while patients with low Rab25 expression displayed a relatively improved survival of 34.3 months. Patients with high Rab25 expression had the longest mean survival time of 45.2 months.

The Rab25 promoter region is frequently hypermethylated in ESCC

In addition to expression analysis in clinical samples, Rab25 expression was also examined at both genomic and proteomic levels in 8 esophageal cell lines, by qPCR and Western blot analyses. Absent or significantly down-regulated Rab25 expression was observed in a panel of ESCC cell lines when compared with a pooled normal tissue control (5 cases of non-tumor esophageal clinical tissue samples pooled together). In particular, Rab25 was found to be completely absent in the ESCC cell lines EC109 and KYSE520 (Fig. 3A and B). Down-regulation of tumor suppressor genes in cancers is often associated with hypermethylation and histone deacetylation. To determine whether Rab25 down-regulation was associated with epigenetic regulation in ESCC, EC109 and KYSE520 cells that lacked Rab25 expression were treated with varying concentrations of the DNA methyltransferase inhibitor (5-aza-dC) and/or the histone acetylation agents (TSA or VPA) to investigate the effects of DNA demethylation and histone acetylation on Rab25 expression. As shown in Fig. 3C, qPCR analysis showed a dose-dependent restoration of Rab25 expression after demethylation treatment with 5-aza-dC. Treatment with the histone
acetylation agent TSA or VPA did not significantly alter Rab25 expression, and combined treatment with 10 μM 5-aza-dC and 0.2 μM TSA did not further enhance Rab25 expression. These studies support the idea that DNA methylation, but not histone modification, is involved in Rab25 inactivation in ESCC.

To substantiate the role of aberrant promoter hypermethylation in Rab25 silencing, we performed MSP or BGS to investigate the methylation status of the Rab25 promoter region. The 10 kb sequence directly upstream of the Rab25 gene was analyzed by two publicly available databases for potential CpG islands: CpG Island Searcher (http://www.cpgislands.com) and EMBOSS-CpGPlot (http://www.ebi.ac.uk/Tools/emboss/cpgplot). Three common CpG islands were predicted by both programs, including the regions at -8217 to -6690 (CpG I1), -6689 to -5717 (CpG I2) and -1287 to -453 (CpG I3) 5’ upstream of Rab25 (Fig. 4A). In addition, because a recent study also found the sequence from -173 to +17 (CpG I4) to contain the core promoter region of the Rab25 gene (21), we also investigated the methylation status in this region (Fig. 3D). We performed MSP at CpG I1 and BGS at CpG I2 and CpG I3, however, no difference in methylation status between Rab25 expressing or absent ESCC cell lines could be detected in these predicted CG dinucleotide-rich regions (data not shown). But BGS at CpG I4, which contains the promoter region of Rab25 (21), showed a high density of methylation in Rab25 absent EC109 and KYSE520 cell lines. In contrast, methylation was rarely detected in the same CpG sites in the Rab25 expressing KYSE30 cell line (Fig. 3D and E). As compared with untreated EC109, methylation was significantly reduced in EC109 cells treated with 5-aza-dC (Fig. 3E). In addition, we also investigated the methylation status in a pair of matched ESCC and non-tumor clinical sample. Rab25 expression was found to be significantly down-regulated in ESCC as compared to non-tumor in this case as confirmed by qPCR analysis. ESCC sample was found heavily methylated (75-100% methylation) in 18 of 21 CG dinucleotides examined, while the non-tumor samples showed significantly lower levels of methylation (0-25% methylation) in 17 of 21 CG dinucleotides examined (Fig. 3E, representative shown). Taken together, these observations provide strong evidence in support of the notion that DNA promoter hypermethylation is implicated in Rab25 inactivation in human ESCC.

**Rab25 suppresses in vitro migration, invasion and angiogenesis in ESCC**

Frequent epigenetic silencing of Rab25 in ESCC cell lines and human ESCC samples prompted us to further investigate the function of Rab25 in ESCC. To assess whether Rab25 might possess a tumor-suppressive function, EC109 cells without Rab25 expression, were stably transduced with lentivirus packaged with either a Rab25 expressing vector or an empty vector (EV) control to generate Rab25
overexpressing (EC109 Rab25) or control cells (EC109 EV). Similarly, KYSE30 cells, exhibiting high levels of Rab25 expression, was lentivirally transduced with either Rab25 shRNA (clones 849 and 852) or a non-target control (NTC) sequence to generate cells with Rab25 stably repressed (KYSE30 shRNA 849 or 852) or control cells (KYSE30 NTC). Stable Rab25 overexpression and knockdown was confirmed at both mRNA and protein levels by qPCR and Western blot, respectively (Fig. 4A and B). Stable Rab25 overexpression significantly reduced the ability of the cells to migrate and invade through transwell chambers. Conversely, suppressing Rab25 expression resulted in an opposing effect (Fig. 4C). Next, we also investigated whether overexpression of Rab25 has a suppressive effect on angiogenesis. HUVECs were treated with conditioned media collected from Rab25 overexpressed or suppressed cells and their respective controls. Morphological changes and differential tube-forming abilities of HUVECs following treatment with conditioned media is shown in Fig. 4D. HUVECs displayed elongated tube-like structures following treatment with conditioned media from Rab25 suppressed clones 849 and 852, as compared with control cell medium treatment. Conversely, treatment with conditioned media from Rab25 overexpressed cells displayed a contrasting effect. These observations suggest a role for Rab25 in the regulation of invasion, migration and angiogenesis in ESCC.

**Rab25 suppresses in vivo tumor formation and angiogenesis in ESCC**

An *in vivo* mouse model was further utilized to support our findings *in vitro*. Tumor formation ability of Rab25 overexpressed clone was significantly subdued as compared with EV control cells (Fig. 5A, left panel). On the contrary, mice injected with Rab25 repressed clone formed larger and more tumors than compared with NTC cells (Fig. 5A, right panel). Serial sections from the xenograft tumors were then subjected to H&E staining as well as IHC analysis. Histological analysis and CK5/6 IHC staining found tumors formed with EC109 Rab25, EC109 EV and KYSE30 shRNA 852 cells resembled an ESCC phenotype, while xenografts formed from KYSE30 NTC cells were composed mostly of necrotic cells and fibroblasts. Xenograft tissue sections were also examined for PCNA and CD34 expression by IHC. As compared with EC109 Rab25 overexpressing xenografts, xenografts generated with EV cells displayed an enhanced PCNA and CD34 expression, indicative of increased cell proliferation and microvessel density, respectively. Conversely, reduced PCNA and CD34 expression were observed in KYSE30 NTC xenografts compared to Rab25 repressed tumors (Fig. 5B).

**Rab25 drives ESCC through a deregulated MAPK/ERK signaling pathway**
Rab25 has previously been reported to control recycling of β1 integrin (11-13). FAK, which localizes with β subunit of integrins, is the major and most extensively studied downstream player activated by integrins. Activation and auto-phosphorylation of FAK has previously been shown to be critical in driving a number of cancer processes including promoting cell survival, cell proliferation and cell motility; through regulating downstream pathways including the MAPK/ERK pathway (4, 22-24). In view of the importance of MAPK/ERK pathway in regulating cell invasiveness and angiogenesis, we therefore examined if Rab25 could regulate the expression of activated kinases in this particular signaling cascade. As compared with EV control, Rab25 overexpression resulted in a reduction of phosphorylated FAK and c-Raf with a concomitant decrease in the downstream phosphorylation of MEK1/2 and ERK (Fig. 6A). Conversely, increased phosphorylation of FAK, c-Raf, MEK1/2 and ERK was detected in Rab25 suppressed cells, as compared with its respective NTC (Fig. 6A). Interestingly, immunohistochemical staining in the resected xenografts likewise showed a reduced expression of phospho-ERK in Rab25 overexpressing xenografts. Conversely, tumors formed with Rab25 repressed cells displayed an enhanced expression of phosphor-ERK (Fig. 6B). Taken together, our results suggested that Rab25 exerts its tumor suppressive function in ESCC through regulating the MAPK/ERK signaling pathway (Fig. 6C).
Discussion

Past studies on the genetic alterations in ESCC by our group and other research laboratories have mainly been focused on investigating differential gene expression, chromosomal aberrations and loss of heterozygosity using microarrays, comparative genomic hybridization and microsatellite DNA marker analysis techniques (25-28). In recent years, the advent of next-generation sequencing technologies has provided a new platform for generating vast amounts of data at genomic, epigenomic, transcriptomic and proteomic levels by means of a variety of high-throughput technologies (29). In particular, the recent development of RNA sequencing has provided a new approach for mapping and quantifying transcriptomes (30). Direct tabulation of the transcriptome by RNA-Seq has advantages over existing technologies in several ways. First, unlike hybridization-based approaches, RNA-Seq is not limited to detecting known genes but can also include the detection of novel transcripts and alternative splice forms (provided sequencing is performed to a sufficient depth). A second advantage of RNA-Seq relative to DNA microarrays is that RNA-Seq has high sensitivity for detecting transcripts of low expressed genes and that many sequencing reads can be unambiguously assigned to the genome. Finally, it is also shown to have high levels of reproducibility. RNA-Seq has now been successfully applied in the study of various disease models including Alzheimer’s disease (31), leukemia (32), prostate (33-34) and breast (35) cancers, but yet ESCC. In this present study, RNA-Seq was employed to investigate differential gene expression in twelve non-tumor and ESCC clinical samples. Using a stringent statistical cut-off of \( p < 0.001 \), 1,730 commonly differentially expressed genes were identified. Pathway and Gene Ontogeny analysis by DAVID in the 1,730 genes found 2 of the top 7 most enriched pathways to be associated with integrin signaling (i.e. integrin cell surface interactions and integrin signaling pathway), suggesting that this pathway should play a critical role in driving ESCC tumorigenesis. Integrin signaling has been extensively shown in the past to control initiation and progression of various cancer types. And this is also true in the context of ESCC, as a number of the identified differentially expressed genes involved in integrin signaling in this study (listed in Supplemental Table 4) have already been previously implicated in ESCC pathogenesis. These include CAV1, ROCK1, ITGB1, ICAM1, VCL, PXN and SHC1 (36-44).

Here, we report the identification and characterization of a tumor-suppressor gene Rab25 in ESCC, which was found to be significantly down-regulated in the integrin signaling associated pathway.

Rab25, with an epithelial distribution, was first discovered with its expression enriched in the normal gastrointestinal mucosa, kidney and lung, but absent in the brain, liver and skeletal muscle (7). It is a complex molecule that has been described as an intracellular transport protein (45-46). More recent
studies have also shown that it is a tumor modulator as it can regulate tumor growth and angiogenesis. A hand-full of studies have now been conducted to investigate the deregulated expression of Rab25 in different cancer types including ovarian (10, 47), breast (14, 48) and colon cancers (13). Based on these studies, Rab25 seems to display very contradicting roles dependent on cellular context. Concordant with findings in triple-negative breast cancer and colon cancer (13-14, 48), we also found Rab25 to function as a tumor suppressor in ESCC. And as Nam et al. reported for colon cancer (13), Rab25 was also found to have prognostic value in ESCC. Cheng et al. has previously reported that Rab25 can act through multiple pathways to enhance apoptosis and to suppress angiogenesis and invasion in triple-negative breast cancer by modulating VEGF-A and VEGFR-1 expression (14). Contrary to their findings, our preliminary findings by qPCR show unaltered VEGF-A and VEGFR-1 expression in Rab25 overexpressed or repressed ESCC cells (data not shown). However, we did identify another pathway by which Rab25 mediates tumorigenicity, metastasis and angiogenesis in ESCC – through a deregulated FAK-Raf-MEK1/2-ERK signaling. This study is also the first comprehensive study to identify a role of promoter hypermethylation in the inactivation of Rab25 expression in ESCC. It is interesting to note that Rab25 has been suggested, along with a number of other genes (CDCA8, ATAD2, AURKA, etc.) to have a strong correlation for methylation dependent expression changes in ovarian cancer; although data is yet to be validated (49). On the contrary, loss of Rab25 expression as a result of chromosome 1q22-23 mutation has previously been suggested for breast cancer cell lines (48) while up-regulated Rab25 expression has been suggested to be associated with chromosome amplification in ovarian cancer (10) and PKA-dependent regulation of the proximal promoter in gastric cancer cell line model (21). Our preliminary studies of DNA copy number in two ESCC cell lines (KYSE520 and EC109) and two ESCC clinical samples with no or low Rab25 expression suggested that down-regulation of Rab25 is not associated with deletion of the Rab25 gene (Supplemental Fig. 2). Although there is no solid explanation to conclusively explain the different outcomes observed by different groups with regard to Rab25 expression, its regulation, its functional role and its mediated pathways, several important factors should be considered. Most of the data that indicates Rab25 as an oncogene is derived from studies in ovarian and breast cancers, which is in a completely different cellular context from ESCC. The stage of the disease can also determine whether a certain gene acts as a tumor promoter or suppressor. For instance, TGF-beta has been shown to act as a tumor suppressor in early stages of pancreatic cancer, but then switches to become an oncogene in metastatic pancreatic cancer (50). Further, the cellular / mechanistic pathway most impacted may also be vital in determining the nature of Rab25’s action in different cellular models. For instance, Cheng et al. found Rab25 to exert tumor suppressive properties in MDA-MB-231 breast cancer cells in
part through effects on VEGF-A secretion and VEGFR-1 expression (14) while our present study found Rab25 to exert tumor suppressive effects in ESCC through a deregulated MAPK/ERK pathway. In ovarian and breast cancers, however, Rab25 has been shown to possess oncogenic functions through suppressing Bak/Bax pro-apoptotic molecules and activation of PI3K/Akt pathway (10). These factors may potentially contribute to determine whether Rab25 promotes or impedes tumor growth. Regardless, all these data do suggest a multi-functional role of Rab25 in different cellular contexts and either a gain or loss of Rab25 expression could potentially lead to tumorigenesis in different organ models.
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References


Figure Legends

Figure 1. RNA-Seq expression profiling data. A, cladogram clustering diagram based on the gene expression values for twelve ESCC and non-tumor samples. B, volcano plot of the \( p \)-value as a function of weighted fold-change for the 5 non-tumor vs. 7 tumor samples. Blue dots represent genes not significantly differentially expressed (Bonferroni-corrected \( p \)-value > 0.001), and red dots represent genes significantly differentially expressed (Bonferroni-corrected \( p \)-value \( \leq \) 0.001). C, heatmap of expression profiles for the 1,730 genes that showed significant expression changes (1,598 up-regulated and 132 down-regulated). D, pathway enrichment analysis of significantly differentially expressed genes. E, expression tiling of Rab25 in 3 pairs of matched ESCC and corresponding non-tumor clinical samples. F, qPCR analysis of the relative expression of Rab25 in the original twelve ESCC and non-tumor clinical samples used for RNA-Seq. Table shows the RPKM expression values of the twelve samples analyzed by RNA-Seq. Validation study to determine the correlation between real-time qPCR and RNA-Seq data.

Figure 2. Down-regulation of Rab25 in ESCC. A, Rab25 expression in matched non-tumor (NT) and ESCC tumor samples (\( n = 43 \)) as detected by qPCR. \( \beta \)-actin was used as an internal control. Values displayed as average delta Ct values. Boxes in the box plot contain the values between the 25\(^{th}\) and 75\(^{th}\) percentiles. The lines across the boxes indicate the median. The whiskers extend to the highest values, excluding outliers and extremes. B, Rab25 protein expression in ESCC tumor (T) and non-tumor (N) tissues as detected by Western blot. C, representative IHC staining of Rab25 in ESCC tumor and paired non-tumor squamous epithelium tissue from one patient sample. D, bar chart summary of the distribution of different Rab25 expression levels in non-tumor versus tumor for all informative cases on the TMA. (E) Kaplan-Meier survival analysis comparing the overall survival time of ESCC patients with different Rab25 expression levels.

Figure 3. Rab25 promoter region is frequently hypermethylated in ESCC. A and B, measurement of genomic and proteomic Rab25 expression levels in a panel of ESCC cell lines compared with a pooled normal tissue control by qPCR and Western blot. C, real-time qPCR analysis of Rab25 expression after demethylation and/or histone acetylation treatment with 5-aza-dC (blue bars), TSA (pink bars) and/or VPA (green bars) in ESCC cell lines without Rab25 expression. Double (grey bars) – combined 10\( \mu \)M 5-aza-dC and 200nM TSA treatment. D, a schematic diagram showing the distribution of four predicted CpG islands 5’ upstream of the Rab25 gene. The CpG islands cover the regions -8217 to -6690 (CpG I1), -6689 to -5717 (CpG I2), -1287 to -453 (CpG I3) and -173 to +17 (CpG I4). MSP was performed at CpG
I1 and BGS was performed at CpG I2, CpG I3 and CpG I4. Specifically, 23 CpG dinucleotides are present at CpG I4. BGS primers (4BGS-F and 4BGS-R) were designed to amplify the sequence in this region. Transcription factor binding sites for Sp1 and CRE are predicted within this core promoter region. E, mapping of the methylation status of CpG dinucleotides within CpG I4 in a case of matched ESCC and non-tumor samples, Rab25 expressing KYSE30 cells, Rab25 negative KYSE520 and EC109 cells and EC109 cells treated with 5-aza-dC by BGS. The percentage of methylation at each CpG dinucleotide is displayed in the pie charts.

**Figure 4.** Rab25 from ESCC cell lines suppresses migration, invasion and angiogenesis *in vitro*. Stable repression or overexpression of Rab25 in KYSE30 and EC109 cells, respectively, by lentiviral transduction was confirmed by A, qPCR and B, Western blot. C, representative migration and invasion assays in Rab25 overexpressed or repressed clones as compared to their controls. Bar chart summary of the number of migrated or invaded cells per field (*p < 0.05). D, representative images showing morphological changes and differential tube-forming abilities of HUVECs following treatment with conditioned media from Rab25 overexpressed or repressed clones as compared to their controls. Bar chart summary showing the number of tubes formed in each assay (**p ≤ 0.01 and ***p ≤ 0.001).

**Figure 5.** Rab25 suppresses *in vivo* tumor initiation and angiogenesis in ESCC. A, representative images of the subcutaneous tumors formed in nude mice following injection of Rab25 overexpressed or repressed clones and their respective controls. Tumor incidence denoted in parentheses. B, H&E and IHC staining for CK5/6, PCNA and CD34 expression in the resected xenografts. Bar chart summary of average staining intensity of PCNA and microvessel density in five randomly selected ‘hot spot’ regions (*p < 0.05, **p ≤ 0.01).

**Figure 6.** Rab25 drives ESCC tumorigenesis via a deregulated MAPK/ERK signaling pathway. A, detection of expression of phospho-FAK, total FAK, phospho-c-Raf (Ser259), total c-Raf, phospho-MEK1/2, phospho-ERK and total ERK in Rab25 overexpressed or repressed clones and their respective controls by Western Blot. β-actin was used as a loading control. B, phospho-ERK IHC staining in the resected xenografts generated from Rab25 overexpressed (EC109 Rab25) or suppressed (KYSE30 shRNA 852) cells and their respective control cells. Bar chart summary of average staining intensity of phospho-ERK in five randomly selected ‘hot spot’ regions (**p ≤ 0.01). C, a schematic diagram illustrating the proposed Rab25 regulated tumor suppressive mechanism in ESCC tumorigenesis.
Rab25 is a tumor suppressor gene with anti-angiogenic and anti-invasive activities in esophageal squamous cell carcinoma

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