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Aberrantly methylated *PKP1* in the progression of Barrett's esophagus to esophageal adenocarcinoma

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Abstract

The aberrant DNA methylation of tumor suppressor genes occurs frequently in Barrett's esophagus (BE) and esophageal adenocarcinoma (EAC) and likely affects the initiation and progression of BE to EAC. In the present study we discovered *PKP1* as a novel methylated gene in EAC and then investigated the role of loss of *PKP1*, a constituent of the desmosome complex found in stratified epithelial layers, on the behavior of Barrett's esophagus and esophageal adenocarcinoma cells. Using primary esophageal tissue samples, we determined that *PKP1* was rarely methylated in normal squamous esophagus (5/55; 9.1%) and BE (5/39; 12.8%) and more frequently methylated in Barrett's esophagus with high-grade dysplasia (HGD) or EAC (20/60; 33.3%; $p<0.05$). Furthermore, PKP1 levels were decreased in BE and HGD/EAC cases compared to normal squamous esophagus cases. Knockdown of PKP1 in the BE cell lines CP-A and CP-D (both normally express PKP1) resulted in increased cell motility. Thus, *PKP1* loss secondary to promoter methylation, as well as other mechanisms, may promote the progression of BE to EAC in a subset of patients via decreased desmosome assembly and increased cell motility.

Keywords

Barrett's esophagus; esophageal adenocarcinoma; DNA methylation; desmosome; *PKP1*

Introduction

The incidence of esophageal adenocarcinoma (EAC) has increased dramatically in the U.S. in recent years, making it the most rapidly increasing solid cancer in the Western world

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(Blot and McLaughlin 1999). Most esophageal adenocarcinoma originates in Barrett's esophagus (BE), a pre-malignant condition in which normal squamous epithelium is replaced by metaplastic, specialized intestinal-type columnar epithelium (Spechler 2002). EAC appears to arise via a metaplasia-dysplasia-carcinoma sequence whereby Barrett's metaplasia progresses through low-grade dysplasia, high-grade dysplasia, intramucosal carcinoma, and finally becomes invasive carcinoma (Spechler 2002). Although the frequency and timing of these events has not been clearly elucidated, several predictable histologic and concurrent genetic changes have been described (Flejou 2005; Maley, et al. 2006; Reid, et al. 2000; Werner, et al. 1999). In addition, epigenetic modifications of numerous genes, in the form of DNA hypermethylation with subsequent gene silencing, have been demonstrated to occur frequently in BE and EAC (Clement, et al. 2006; Hamilton, et al. 2006; Jin, et al. 2009; Tischoff, et al. 2007). A subset of these aberrantly methylated tumor suppressor genes are predicted to play an important role in the pathogenesis of BE and/or EAC. Furthermore, some of these methylated genes might be useful prognostic markers as they appear to precede and thus predict the progression of BE to EAC (Clement, et al. 2006).

Despite the fact that numerous genes appear to be methylated in Barrett's esophagus and esophageal adenocarcinoma, it remains to be determined which methylated genes play a functional role in the malignant transformation of BE ("driver alterations") versus those that are methylated coincidentally during the BE-EAC progression sequence but have no effect on the pathogenesis of EAC ("passenger alterations"). Previous studies of CpG island methylation in human esophageal tissue have determined that groups of genes demonstrating an intermediate degree of methylation are the most useful in discriminating normal tissue (squamous esophageal and gastric) from BE and EAC (Eads, et al. 2001). Others have shown that in BE specimens obtained from patients who progressed to cancer or high grade dysplasia, *APC*, *TIMP3*, and *TERT* were frequently methylated, whereas in BE tissue obtained from patients who did not progress, methylation of these genes was uncommon (Clement, et al. 2006). More recently, a panel of eight methylated biomarkers has been indentified that distinguishes individuals with BE who later progressed to HGD or EAC ("BE progressors") versus individuals who did not show this type of progression ("BE nonprogressors"), highlighting the feasibility of a methylation-based biomarker panel to predict neoplastic progression in BE (Jin, et al. 2009). While these studies underscore the potential value of methylated genes to predict which individuals with BE will develop cancer, the studies were not designed to determine whether a particular methylated gene is simply a high-risk marker or whether it is driving the biological process of carcinogenesis.

In light of recently published studies demonstrating a role of junctional complexes in the formation of BE and in the development of several types of epithelial cancers (Jovov, et al. 2007; Moll, et al. 1997; Papagerakis, et al. 2003; Schmitt-Graeff, et al. 2007) we assessed the potential role of genes encoding the proteins in these complexes in BE and EAC formation. We performed a review of the genes involved in tight junctions and desmosomal complexes that had CpG islands in their promoters, and consequently chose to evaluate plakophilin-1 (*PKP1*) in more detail. *PKP1* is a gene normally expressed in the squamous epithelium-lined esophagus that we felt merited further study in BE and EAC based upon its role in the maintenance of normal epithelial tissue architecture and its description as a tumor-suppressor gene in other cancers. *PKP1* encodes a protein that is a component of the desmosome complex, a cell-cell adhesive junction found in various stratified epithelial tissue layers including skin, oropharyngeal, and esophageal mucosa (Cheng, et al. 2005). *PKP1*, along with the desmosomal plaque proteins desmoplakin and plakoglobin, forms a complex of proteins at the desmosome that links the cadherins to the intermediate filaments of the cell (Sobolik-Delmaire, et al. 2007). Defects in desmosome-mediated cell-cell adhesion can lead to tissue fragility syndromes, affecting the skin and the heart, as well as to

defects in embryological development (Cheng, et al. 2005; Kottke, et al. 2006). Keratinocytes lacking normal plakophilin demonstrate reduced desmosomal stability and increased motility, highlighting the critical role of plakophilin in desmosomal integrity and tissue maintenance (South, et al. 2003). *PKP1* can also be found residing in the nucleus and in the cytoplasmic pool, and therefore may be involved with intracellular signaling, much like other armadillo proteins such as beta-catenin (Hatzfeld 2007).

The plakophilin proteins 1, 2, and 3 have also been associated with the neoplastic phenotype. In basal and squamous cell skin carcinomas, reduced levels of *PKP1* have been associated with poorly differentiated and invasive cancers (Moll, et al. 1997). Similarly, *PKP1* expression was inversely correlated with tumor grade and local recurrence or metastasis in a recent evaluation of head and neck squamous cell cancers (Papagerakis, et al. 2003). PKP1 expression was also shown to be reduced or altogether lost in adenocarcinomas with high Gleason scores (Breuninger, et al. 2010). Others have examined the expression of *PKP1* in a group of cervical carcinomas and determined that its expression is reduced early in the process of cervical carcinogenesis (Schmitt-Graeff, et al. 2007). These studies provide evidence that *PKP1* can act as a tumor suppressor gene in several cancers derived from squamous epithelia.

We have now identified hypermethylated *PKP1* in a subset of cases of EAC and provide evidence that reduced levels of PKP1 seen in BE result in increased cell motility, which would be predicted to favor the progression of BE to EAC. Thus, *PKP1* appears to be a tumor suppressor gene in EAC and a potential prognostic marker for BE progression.

Materials and Methods

Tissue samples and cell lines

Human esophageal biopsy specimens were obtained from the pathology archives at Vanderbilt University, Case Western Reserve University, the University of Washington, and the VA Puget Sound Health Care System. IRB approval was obtained from each of the parent institutions. The EPC2 normal human esophageal cells (obtained in June 2006 as a generous gift from Dr. Anil Rustgi, University of Pennsylvania) were grown in KSFM media (Invitrogen, Carlsbad, CA) containing 50mg/L bovine pituitary extract and 1 μg/L epidermal growth factor (EGF). The CP-A and CP-D human Barrett's esophageal cells (obtained in July 2004 as a generous gift from Dr. Peter Rabinovitch, University of Washington) were grown in MCDB-153 media (Sigma, St. Louis, MO) containing 0.4 μg/ml hydrocortisone, 20 ηg/ml EGF, 10^{-10} M cholera toxin, 20 mg/L adenine, 140 μg/ml bovine pituitary extract, 100 units/ml Pen/Strep, 0.25 μg/ml amphotericin B, 5 μg/ml insulin-transferrin-selenium, and 4 mM L-glutamine. The OE33 human esophageal carcinoma cells (obtained in April 2008 from the European Collection of Cell Cultures) were grown in RPMI 1640 (Gibco/BRL, Gaithersburg, MD) containing 2 mM glutamine, 10% fetal bovine serum, and 1X Pen/Strep. All cells were grown at 37°C in 5% CO_2.

DNA extraction and sodium bisulfite treatment

An experienced gastrointestinal pathologist (MPU or JEW) reviewed H&E-stained sections of esophageal tissues and then marked formalin-fixed, paraffin-embedded sections on glass slides in order to clearly segregate histological subtypes from each other. In those sections with mixed histology (e.g. Barrett's and squamous esophagus), care was taken to keep subtypes separated during microdissection, which was carried out using a sterile razor blade (Supplemental Figure 1). Sections where histological subtypes could not be easily segregated were not used. For EAC cases that contained a mixture of tumor and stromal cells only samples containing ≥ 75% tumor cells were used. DNA was extracted using

InstaGene Matrix (Bio-Rad, Hercules, CA). Sodium bisulfite treatment of the DNA was performed as previously described (Grady, et al. 2000). Positive and negative controls, consisting of methylated and unmethylated DNA obtained from colon cancer cell lines and normal human lymphocytes, were included with each round of bisulfite treatment and methylation-specific PCR (MSP) (Lind, et al. 2004).

RNA extraction from cell lines and primary tissues

RNA was extracted from esophageal cell monolayers using Trizol reagent (Sigma) and then purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturers' instructions. RNA was purified from sections of formalin-fixed, paraffin-embedded human esophageal tissue using the RNeasy FFPE kit (Qiagen) following the manufacturer's instructions.

Methylation-specific PCR (MSP) and sodium bisulfite sequencing

Following extraction and sodium bisulfite conversion, DNA was subjected to MSP using primer pairs designed to discriminate between methylated and unmethylated alleles of *PKP1* as previously described (Kaz, et al. 2007; Petko, et al. 2005). The optimal annealing temperature for the *PKP1* methyl- and unmethyl-specific primers (64°C) was determined using DNA samples that were known to carry unmethylated or methylated *PKP1*. The primer sequences used were as follows: methyl forward = 5′-GTTTAGCGTTTTATATAGGGGATTC-3′, methyl reverse = 5′-AACTCCCTACAACTACTCCTAACG-3′, unmethyl forward = 5′-TTAGTGTTTTATATAGGGGATTTGT-3′, unmethyl reverse = 5′-ACTCCCTACAACTACTCCTAACACT-3′. 20 μl MSP reactions were carried out for 40–45 cycles in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). For bisulfite sequencing, 20 μl PCR reactions using bisulfite-treated DNA were carried out at an annealing temperature of 60°C using 0.5 μM of forward and reverse primers (forward primer = 5′ – TTTTGGGGTTTTAAGGTTTG-3′, reverse primer = 5′-AAAACTCCCTACAACTACTCCTAAC-3′). PCR products were cloned into One Shot TOP10 competent cells (Invitrogen) using the TOPO TA cloning kit (Invitrogen) following the manufacturer's instructions. Colonies were selected for DNA extraction and sequencing with M13R primers. Generally, 10 clones from each sample were sequenced and analyzed using FinchTV (Geospiza, Seattle, WA) and BiQ analyzer (Bock, et al. 2005).

5-aza-deoxycytidine treatment and RNA extraction from cell lines

OE33 esophageal adenocarcinoma cells were treated with 5 μM 5-aza-deoxycytidine (Sigma) in DMSO or mock-treated with DMSO only for four consecutive days, with the 5-aza-containing media (or mock solution) changed daily. RNA was then extracted from the cells on day seven using Trizol reagent (Sigma) and purified using an RNeasy Mini Kit (Qiagen) following the manufacturers' protocols.

Quantitative reverse transcription-PCR

TaqMan gene expression assays (Assays-on-Demand from Applied Biosystems) for *PKP1* and *GUSB* were used for quantitative RT-PCR. The assays were run on an Opticon 2 DNA Engine real-time PCR system (Bio-Rad) and *GUSB* expression values were used for normalization of cDNA loading between samples.

siRNA transfection of cell lines

PKP1 On-Target Plus siRNA or siGENOME non-targeting control siRNA #3 (a negative control siRNA with at least four mismatches to any human, rat, or mouse gene; both from Thermo Scientific-Dharmacon, Waltham, MA) were prepared for transfection according to

the manufacturer's instructions. 600 μl of either siRNA transfection mix was added to both CP-A and CP-D Barrett's esophagus cells seeded in wells of a 24-well plate. The cells were incubated for 48 hours at 37°C in 5% CO_2 prior to being harvested for Western blots or cell motility assays.

Western blotting of PKP1 from Barrett's esophagus cell lysates

CP-A and CP-D cell lysates were collected using RIPA lysis buffer following transfection with PKP1 siRNA. Proteins were resolved by SDS-PAGE using a 12% separating gel, and then electrophoretically transferred to PVDF membranes. The membranes were then blocked in 5% non-fat dry milk in TBS + 0.1% Tween 20. PVDF membranes were incubated overnight at 4°C with rabbit anti-PKP1 primary antibody (Sigma) diluted 1:300 and then incubated for one hour with goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:5000. β-actin was used as a protein loading control.

Cell motility assays

Twenty-eight microliters of degassed media were added to the bottom portion of a 48-well chemotaxis chamber (NeuroProbe, Gaithersburg, MD) and then covered with a polycarbonate filter membrane with 8 μM pores (NeuroProbe). For CP-A and CP-D cells transfected with siRNA, 15,000 cells were added to top portion of the chemotaxis chamber. Cells were allowed to migrate for 20 hours, after which non-migrating cells were removed from the original (top) side of the membrane by scraping the membrane with a glass microscope slide. Migrated cells were visualized by staining the underside of the membrane with the Diff-Quik Stain Set (Dade Behring, Eschborn, Germany), and the number of migrated cells per filter was counted for two to three replicate wells per cell type.

Immunohistochemistry

Immunohistochemistry was performed using 4 μM sections of formalin-fixed, paraffin-embedded specimens. Following paraffin removal and rehydration of tissues, sections were steam treated for antigen retrieval using the Trilogy method (Cell Marque, Rocklin, CA; #920P-10). After preheating the Trilogy buffer in a steamer, slides were added for 20 minutes, followed by a 20 minute cool down. Samples were then blocked in TCT buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.25% Casein, 0.1% Tween20). Specimens were then incubated with anti-PKP1 antibody (Sigma #HPA027221, lot R12607) diluted in TBS +1%BSA at 1:50 (3 μg/ml) for 60 minutes. Detection was performed using biotin-free anti-rabbit Mach 2 polymer (Biocare Medical, Concord, CA) for 30 minutes. Finally, sections were treated with DAB (2 × 4 minutes) and hematoxylin (2 minutes) before being alcohol dehydrated and coverslipped.

Statistical analyses

Data were analyzed using a two-sided *t* test, assuming equal variances. Methylation versus expression data in the EAC cases were compared using a two-way table (contingency table) and Pearson's Chi-square test. Values of $P < 0.05$ were considered significant.

Results

PKP1, a gene involved with cell-cell adhesion in stratified epithelia, is methylated in a subset of esophageal adenocarcinoma cases

We used methylation-specific PCR (MSP) to assess for methylated *PKP1* in BE and EAC cases (Figure 1). *PKP1* was more frequently methylated in cases of Barrett's esophagus with high-grade dysplasia (HGD) and esophageal adenocarcinoma (33.3%, N=20/60) when

compared to Barrett's esophagus (12.8%, N=5/39) and to normal squamous esophagus (9.1%, N=5/55) cases ($p<0.05$ for both normal and BE versus HGD/EAC) (Table 1). Sodium bisulfite sequencing of the same region amplified by the MSP primers was used in order to validate the MSP results for two squamous and two adenocarcinoma cases. Sequencing of SSS1-treated DNA was included to demonstrate the completeness of bisulfite treatment (Supplemental Figure 2). The sequencing results confirmed the accuracy of the MSP assays for determining the methylation status of *PKP1* and also demonstrated that the methylation of PKP1 is heterogeneous in the EAC samples, likely reflecting a mixture of stromal and tumor DNA in the sample as well as possible clonal heterogeneity.

The aberrant methylation of *PKP1* induces its loss of expression in EAC

The esophageal adenocarcinoma cell line OE33, which carries methylated *PKP1* (Table 1) has a low level of *PKP1* expression (Figure 2). Following treatment with 5μM 5-aza-2′ deoxycytidine, *PKP1* expression increased ~5.4 fold, consistent with the effect of methylation of *PKP1* leading to its transcriptional repression.

PKP1 is often repressed in esophageal adenocarcinoma secondary to methylation dependent- and methylation- independent mechanisms

The level of *PKP1* expression in squamous esophagus (N=10), Barrett's esophagus (N=12), and high-grade dysplasia/esophageal adenocarcinoma (N=11) specimens was assessed using qRT-PCR. Overall, the average *PKP1* mRNA expression was highest in the squamous esophagus samples (relative expression = 27.2) compared to the BE samples (relative expression = 4.4) and HGD/EAC samples (relative expression = 2.0) ($p<0.001$, squamous esophagus vs. BE and vs. HGD/EAC) (Figure 3).

We then compared PKP1 mRNA expression between the EAC cases with methylated and unmethylated *PKP1.* In this set of experiments, we chose an arbitrary value of 100 relative expression units (REUs) as a positive expression threshold based on the expression in our positive control, and considered EAC cases where PKP1 expression exceeded 100 REUs to be 'expressors' and the remaining cases to be 'nonexpressors'. Using this cutoff, 2/8 (25%) of EAC cases with methylated *PKP1* were expressors, and 8/11 (73%) of EAC cases with unmethylated *PKP1* were expressors. This trend toward reduced PKP1 expression in methylated cases was not statistically significant ($p=0.07$), and is consistent with PKP1 expression being regulated by both methylation dependent and independent mechanisms.

The PKP1 protein relocates from the cell-cell interfaces in squamous esophagus cells to the cytoplasm in esophageal adenocarcinoma cells

Immunohistochemical analysis demonstrated an overall reduction in PKP1 in BE and EAC cells compared to SQ esophageal cells, confirming the decrease in PKP1 mRNA expression noted above (Figure 4). Additionally, the subcellular location of PKP1 varied among these cells types. There was a strong PKP1 signal located predominantly at the cell-cell junctions in the squamous esophagus, little or no signal in the BE cells, and a moderately intense cytoplasmic signal in the EAC cells (Figure 4).

Decreased PKP1 expression enhances cell motility

CP-A and CP-D Barrett's esophagus cells, which carry unmethylated *PKP1* and express relatively high levels of PKP1, were treated with either siRNA against *PKP1* or a control non-targeting siRNA with at least four mismatches to any human, rat, or mouse gene. Following siRNA knockdown we noted an adequate reduction in *PKP1* mRNA expression, in the range of 33–69% versus non-targeting siRNA control treatment (Supplemental Figure

3). Western blots of total CP-A and CP-D cell lysates confirmed reduced levels of PKP1 protein following siRNA treatment against PKP1 versus the control siRNA (Figure 5).

We then assessed the effect of alterations of PKP1 levels on cell motility in the CP-A and CP-D cell lines. Knockdown of PKP1 in CP-A cells increased cell motility 3.6-fold when measured using a Boyden chamber assay (11.3+/−2.3 control cells vs. 40.3 +/−7.1 PKP1 knock-down cells). Similarly, knockdown of PKP1 in CP-D cells resulted in a 16.3-fold increase in cell motility (1.5 +/−0.7 control cells vs. 24.5 +/−3.5 PKP1 knock-down cells). (Each treatment was done in triplicate and the number of migrating cells was averaged; $p = 0.01$ for both CP-A and CP-D) (Figure 6).

Discussion

In the present study, we evaluated plakophilin-1 (*PKP1*), a novel gene that is hypermethylated in a subset of EAC cases and infrequently methylated in BE cases, for the role it plays in the progression of BE to EAC. *PKP1* is a member of the plakophilin family of desmosomal plaque proteins and a constituent of desmosome complexes found in stratified epithelia. The plakophilin family (comprised of PKP1, 2, and 3) is a recently identified family of proteins that are central members of the desmosome complex. The plakophilins are associated with the ectodermal dysplasia/fragility syndrome and have been shown to be deregulated in a variety of cancers. With regards to cancer, there is evidence that they may act as tumor suppressor genes or oncogenes depending on the cellular context (Bass-Zubek, et al. 2009). In light of the role of PKP1 in cell adhesion and the regulation of the cytoskeleton, we chose to study this gene in more detail because of the biological plausibility that it may influence the progression of BE to EAC.

We first evaluated the methylation status of *PKP1* in a collection of human esophageal biopsy specimens using methylation-specific PCR (MSP) and found that *PKP1* was methylated in significantly more of the combined high-grade dysplasia (HGD) and esophageal adenocarcinoma (EAC) cases than normal esophagus and Barrett's esophagus cases. These results suggest that the aberrant methylation of *PKP1* occurs late in the BE-EAC progression sequence in a subset of esophageal cancer patients and may influence the malignant transformation of BE to EAC.

In order to demonstrate the effect of methylation on *PKP1* expression, we treated the OE33 esophageal adenocarcinoma cell line, which carries methylated *PKP1*, with the demethylating agent 5-aza-deoxycytidine and found a 5.4-fold increase in expression following treatment, providing evidence that methylation of *PKP1* can suppress its expression.

We have also shown that the expression of PKP1 is significantly repressed in Barrett's with high-grade dysplasia or adenocarcinoma tissues (HGD/EAC) as compared to normal squamous esophageal specimens. When we compared PKP1 expression in the EAC cases with methylated *PKP1* to cases with unmethylated *PKP1*, there was no statistically significant difference between these groups. We suspect this was due in part to the fact that PKP1 expression was generally very low in these cases. Also, only 19 EAC cases were available for both methylation and expression analyses; with additional cases the differences might demonstrate statistical significance. Additionally, it is becoming increasingly clear that the relationship between promoter methylation and expression is more complex than initially believed. In a recent publication by Hinoue et al., this group used the Illumina HumanMethylation27 array to evaluate methylation patterns in 125 colorectal samples and then correlated methylation with gene expression (Hinoue, et al. 2011). When examining the effect of DNA hypermethylation on gene expression, they found only 7.3% of the high

methylation gene markers they evaluated in these cancers were associated with a strong reduction in corresponding gene expression. It is also known that that the location of aberrant methylation within a gene promoter is an important feature of epigenetic regulation and it is likely that this phenomenon at least partly explains the low correlation between expression and methylation found by Hinoue et al. Peng et al. used pyrosequencing to analyze the methylation status of the entire *MT3* gene promoter region in esophageal cancer cases. They found that in particular regions of the promoter, the tumor and normal samples exhibited very similar methylation patterns, while in other regions they noted hypermethylation in tumor versus normal DNA (Peng, et al. 2011). They concluded that hypermethylation in only these distinct promoter sites accounted for the reduced MT3 expression they noted in the cancer cases.

We also found that PKP1 expression in the non-dysplastic Barrett's esophagus cases was reduced compared to normal squamous esophagus even though only approximately 13% of cases had methylated *PKP1*, suggesting there are additional mechanisms responsible for the reduction of PKP1 levels in the majority of the BE cases. Loss of heterozygosity (LOH) of *PKP1* is one mechanism that could account for reduced levels of PKP1 in BE, although when we compared ten cases of BE that had matched normal squamous esophagus tissue available, we found no evidence of LOH in any of our cases (data not shown). Additionally, translational control of mRNA, including regulation at the 3′ untranslated region (UTR), commonly occurs at the initiation stage of protein synthesis and could be another mechanism that is affecting PKP1 expression (de Moor, et al. 2005; Jackson, et al. 2010; Meyer, et al. 2004). Another possibility is that methylation of *PKP1* occurs during the transition from BE to EAC after the gene is silenced by other means. Hinshelwood *et al.* examined the temporal progression of gene silencing and methylation in $p16^{INK4A}$ and found that gene silencing was a precursor to epigenetic suppression and histone modifications (Hinshelwood, et al. 2009). In a similar fashion, it is possible that *PKP1* methylation, which occurs in a subset of EAC cases, serves to 'reinforce' the silencing of *PKP1* that occurs secondary to other mechanisms in BE. Although *PKP1* expression was low in most of our patients with BE, the great majority of individuals with BE do not develop EAC. We propose that the process of esophageal carcinogenesis is the cumulative effect of both reduced PKP1 expression plus additional genetic and/or epigenetic alterations.

In light of PKP1's role as a critical component of the desmosome complex and in cell-cell adhesion, we next assessed the effect of *PKP1* silencing on cell motility in BE. Impaired desmosome function has been previously reported in various squamous cell carcinomas, including esophageal, skin, and oropharyngeal cancers (Depondt, et al. 1999; Kurzen, et al. 2003; Neuber, et al. 2010; Sobolik-Delmaire, et al. 2007). Given PKP1's role in the organization and integrity of the desmosome complex, we hypothesized that altered levels of PKP1 might affect cell migration in Barrett's esophagus cell lines. We found that PKP1 knock-down in both the CP-A and CP-D Barrett's esophagus cells resulted in significantly increased cell migration. These studies highlight the importance of PKP1 in cell-cell interactions and cell movement in cell lines, and as such reduced levels of PKP1 could be a mechanism of BE progression through decreasing cell adhesion and enhancing cell motility.

Another interesting finding from this study was that the subcellular location of PKP1 varied between squamous esophagus cells (where PKP1 was located at cell-cell interfaces), Barrett's esophageal cells (where PKP1 was essentially absent), and esophageal adenocarcinoma cells (where PKP1 was absent or located in the cytoplasm). The fact that PKP1 was detectable in a subset of EAC cancers argues against the idea that PKP1 is lost in BE and EAC cells as a result of passive gene expression changes associated with metaplasia or neoplasia.

It is well-known that armadillo-related proteins, such as PKP1, APC, and β-catenin, have dual roles as cell-contact and cytoplasmic/nuclear signaling molecules that affect gene expression (Papagerakis, et al. 2003; Wijnhoven, et al. 2000). Previous studies have correlated the cytoplasmic localization of these armadillo-related molecules with the neoplastic phenotype. In oropharyngeal tumors, cytoplasmic localization of PKP1 has been associated with local recurrence (Papagerakis, et al. 2003), and alterations in expression of cadherin and catenin complexes have been described previously in Barrett's esophagus (Bailey, et al. 1998). The expression of the p120 catenin family of proteins, which are closely related to the plakophilins, have been found to be frequently upregulated in the cytoplasm and down regulated in cell-cell junctions in gastric, breast, and other cancers, suggesting a role for the p120 catenins in ligand-induced mitogenic signaling and cell transformation (Jawhari, et al. 1999; Shibata, et al. 2004).

Our results suggest that a similar mechanism occurs during the transformation from normal squamous esophagus to BE to EAC, where the loss of PKP1 at cell-cell junctions leads to disordered desmosome assembly and ensuing increased cell motility. Aberrant methylation of *PKP1* occurs in a subset of BE and EAC cases and there are presumably additional mechanisms responsible for reducing *PKP1* expression, and for modifying its subcellular location, in the remainder of cases. Later during the neoplastic process, analogous to the role of other armadillo-like proteins, an increase in the PKP1 protein in the cytoplasm appears to have a mitogenic effect, ultimately favoring the development of esophageal adenocarcinoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

PKP1	plakophilin-1
BE	Barrett's esophagus
EAC	esophageal adenocarcinoma
HGD	high-grade dysplasia
MSP	methylation-specific PCR

References

Bailey T, Biddlestone L, Shepherd N, Barr H, Warner P, Jankowski J. Altered cadherin and catenin complexes in the Barrett's esophagus-dysplasia-adenocarcinoma sequence: correlation with disease progression and dedifferentiation. Am J Pathol. 1998; 152(1):135–144. [PubMed: 9422531]

Bass-Zubek AE, Godsel LM, Delmar M, Green KJ. Plakophilins: multifunctional scaffolds for adhesion and signaling. Curr Opin Cell Biol. 2009; 21(5):708–716. [PubMed: 19674883]

Blot WJ, McLaughlin JK. The changing epidemiology of esophageal cancer. Semin Oncol. 1999; 26(5 Suppl 15):2–8. [PubMed: 10566604]

Bock C, Reither S, Mikeska T, Paulsen M, Walter J, Lengauer T. BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. Bioinformatics. 2005; 21(21): 4067–4068. [PubMed: 16141249]

Breuninger S, Reidenbach S, Sauer CG, Strobel P, Pfitzenmaier J, Trojan L, Hofmann I. Desmosomal plakophilins in the prostate and prostatic adenocarcinomas: implications for diagnosis and tumor progression. Am J Pathol. 2010; 176(5):2509–2519. [PubMed: 20348237]

Cheng X, Den Z, Koch PJ. Desmosomal cell adhesion in mammalian development. Eur J Cell Biol. 2005; 84(2–3):215–223. [PubMed: 15819402]

Clement G, Braunschweig R, Pasquier N, Bosman FT, Benhattar J. Methylation of APC, TIMP3, and TERT: a new predictive marker to distinguish Barrett's oesophagus patients at risk for malignant transformation. J Pathol. 2006; 208(1):100–107. [PubMed: 16278815]

de Moor CH, Meijer H, Lissenden S. Mechanisms of translational control by the 3′ UTR in development and differentiation. Semin Cell Dev Biol. 2005; 16(1):49–58. [PubMed: 15659339]

Depondt J, Shabana AH, Florescu-Zorila S, Gehanno P, Forest N. Down-regulation of desmosomal molecules in oral and pharyngeal squamous cell carcinomas as a marker for tumour growth and distant metastasis. Eur J Oral Sci. 1999; 107(3):183–193. [PubMed: 10424382]

Eads CA, Lord RV, Wickramasinghe K, Long TI, Kurumboor SK, Bernstein L, Peters JH, DeMeester SR, DeMeester TR, Skinner KA, Laird PW. Epigenetic patterns in the progression of esophageal adenocarcinoma. Cancer Res. 2001; 61(8):3410–3418. [PubMed: 11309301]

Flejou JF. Barrett's oesophagus: from metaplasia to dysplasia and cancer. Gut. 2005; 54(Suppl 1):i6–12. [PubMed: 15711008]

Grady WM, Willis J, Guilford PJ, Dunbier AK, Toro TT, Lynch H, Wiesner G, Ferguson K, Eng C, Park JG, Kim SJ, Markowitz S. Methylation of the CDH1 promoter as the second genetic hit in hereditary diffuse gastric cancer [In Process Citation]. Nat Genet. 2000; 26(1):16–17. [PubMed: 10973239]

Hamilton JP, Sato F, Greenwald BD, Suntharalingam M, Krasna MJ, Edelman MJ, Doyle A, Berki AT, Abraham JM, Mori Y, Kan T, Mantzur C, Paun B, Wang S, Ito T, Jin Z, Meltzer SJ. Promoter methylation and response to chemotherapy and radiation in esophageal cancer. Clin Gastroenterol Hepatol. 2006; 4(6):701–708. [PubMed: 16678495]

Hatzfeld M. Plakophilins: Multifunctional proteins or just regulators of desmosomal adhesion? Biochim Biophys Acta. 2007; 1773(1):69–77. [PubMed: 16765467]

Hinoue T, Weisenberger DJ, Lange CP, Shen H, Byun HM, Van Den Berg D, Malik S, Pan F, Noushmehr H, van Dijk CM, Tollenaar RA, Laird PW. Genome-scale analysis of aberrant DNA methylation in colorectal cancer. Genome Res. 2011

Hinshelwood RA, Melki JR, Huschtscha LI, Paul C, Song JZ, Stirzaker C, Reddel RR, Clark SJ. Aberrant de novo methylation of the p16INK4A CpG island is initiated post gene silencing in association with chromatin remodelling and mimics nucleosome positioning. Hum Mol Genet. 2009; 18(16):3098–3109. [PubMed: 19477956]

Jackson RJ, Hellen CU, Pestova TV. The mechanism of eukaryotic translation initiation and principles of its regulation. Nat Rev Mol Cell Biol. 2010; 11(2):113–127. [PubMed: 20094052]

Jawhari AU, Noda M, Pignatelli M, Farthing M. Up-regulated cytoplasmic expression, with reduced membranous distribution, of the src substrate p120(ctn) in gastric carcinoma. J Pathol. 1999; 189(2):180–185. [PubMed: 10547572]

Jin Z, Cheng Y, Gu W, Zheng Y, Sato F, Mori Y, Olaru AV, Paun BC, Yang J, Kan T, Ito T, Hamilton JP, Selaru FM, Agarwal R, David S, Abraham JM, Wolfsen HC, Wallace MB, Shaheen NJ, Washington K, Wang J, Canto MI, Bhattacharyya A, Nelson MA, Wagner PD, Romero Y, Wang KK, Feng Z, Sampliner RE, Meltzer SJ. A multicenter, double-blinded validation study of methylation biomarkers for progression prediction in Barrett's esophagus. Cancer Res. 2009; 69(10):4112–4115. [PubMed: 19435894]

Jovov B, Van Itallie CM, Shaheen NJ, Carson JL, Gambling TM, Anderson JM, Orlando RC. Claudin-18: a dominant tight junction protein in Barrett's esophagus and likely contributor to its acid resistance. Am J Physiol Gastrointest Liver Physiol. 2007; 293(6):G1106–1113. [PubMed: 17932229]

Kaz A, Kim YH, Dzieciatkowski S, Lynch H, Watson P, Kay Washington M, Lin L, Grady WM. Evidence for the role of aberrant DNA methylation in the pathogenesis of Lynch syndrome adenomas. Int J Cancer. 2007; 120(9):1922–1929. [PubMed: 17278092]

Kottke MD, Delva E, Kowalczyk AP. The desmosome: cell science lessons from human diseases. J Cell Sci. 2006; 119(Pt 5):797–806. [PubMed: 16495480]

Kurzen H, Munzing I, Hartschuh W. Expression of desmosomal proteins in squamous cell carcinomas of the skin. J Cutan Pathol. 2003; 30(10):621–630. [PubMed: 14744087]

Lind GE, Thorstensen L, Lovig T, Meling GI, Hamelin R, Rognum TO, Esteller M, Lothe RA. A CpG island hypermethylation profile of primary colorectal carcinomas and colon cancer cell lines. Mol Cancer. 2004; 3(1):28. [PubMed: 15476557]

Maley CC, Galipeau PC, Finley JC, Wongsurawat VJ, Li X, Sanchez CA, Paulson TG, Blount PL, Risques RA, Rabinovitch PS, Reid BJ. Genetic clonal diversity predicts progression to esophageal adenocarcinoma. Nat Genet. 2006; 38(4):468–473. [PubMed: 16565718]

Meyer S, Temme C, Wahle E. Messenger RNA turnover in eukaryotes: pathways and enzymes. Crit Rev Biochem Mol Biol. 2004; 39(4):197–216. [PubMed: 15596551]

Moll I, Kurzen H, Langbein L, Franke WW. The distribution of the desmosomal protein, plakophilin 1, in human skin and skin tumors. J Invest Dermatol. 1997; 108(2):139–146. [PubMed: 9008225]

Neuber S, Muhmer M, Wratten D, Koch PJ, Moll R, Schmidt A. The desmosomal plaque proteins of the plakophilin family. Dermatol Res Pract. 2010; 2010:101452. [PubMed: 20585595]

Papagerakis S, Shabana AH, Depondt J, Gehanno P, Forest N. Immunohistochemical localization of plakophilins (PKP1, PKP2, PKP3, and p0071) in primary oropharyngeal tumors: correlation with clinical parameters. Hum Pathol. 2003; 34(6):565–572. [PubMed: 12827610]

Peng D, Hu TL, Jiang A, Washington MK, Moskaluk CA, Schneider-Stock R, El-Rifai W. Location-specific epigenetic regulation of the metallothionein 3 gene in esophageal adenocarcinomas. PLoS One. 2011; 6(7):e22009. [PubMed: 21818286]

Petko Z, Ghiassi M, Shuber A, Gorham J, Smalley W, Washington MK, Schultenover S, Gautam S, Markowitz SD, Grady WM. Aberrantly methylated CDKN2A, MGMT, and MLH1 in colon polyps and in fecal DNA from patients with colorectal polyps. Clin Cancer Res. 2005; 11:1203–1209. [PubMed: 15709190]

Reid BJ, Levine DS, Longton G, Blount PL, Rabinovitch PS. Predictors of progression to cancer in Barrett's esophagus: baseline histology and flow cytometry identify low- and high-risk patient subsets. Am J Gastroenterol. 2000; 95(7):1669–1676. [PubMed: 10925966]

Schmitt-Graeff A, Koeninger A, Olschewski M, Haxelmans S, Nitschke R, Bochaton-Piallat ML, Lifschitz-Mercer B, Gabbiani G, Langbein L, Czernobilsky B. The Ki67+ proliferation index correlates with increased cellular retinol-binding protein-1 and the coordinated loss of plakophilin-1 and desmoplakin during progression of cervical squamous lesions. Histopathology. 2007; 51(1):87–97. [PubMed: 17593084]

Shibata T, Kokubu A, Sekine S, Kanai Y, Hirohashi S. Cytoplasmic p120ctn regulates the invasive phenotypes of E-cadherin-deficient breast cancer. Am J Pathol. 2004; 164(6):2269–2278. [PubMed: 15161659]

Sobolik-Delmaire T, Katafiasz D, Keim SA, Mahoney MG, Wahl JK 3rd. Decreased plakophilin-1 expression promotes increased motility in head and neck squamous cell carcinoma cells. Cell Commun Adhes. 2007; 14(2–3):99–109. [PubMed: 17668353]

South AP, Wan H, Stone MG, Dopping-Hepenstal PJ, Purkis PE, Marshall JF, Leigh IM, Eady RA, Hart IR, McGrath JA. Lack of plakophilin 1 increases keratinocyte migration and reduces desmosome stability. J Cell Sci. 2003; 116(Pt 16):3303–3314. [PubMed: 12840072]

Spechler SJ. Clinical practice. Barrett's Esophagus. N Engl J Med. 2002; 346(11):836–842. [PubMed: 11893796]

Tischoff I, Hengge UR, Vieth M, Ell C, Stolte M, Weber A, Schmidt WE, Tannapfel A. Methylation of SOCS-3 and SOCS-1 in the carcinogenesis of Barrett's adenocarcinoma. Gut. 2007; 56(8): 1047–1053. [PubMed: 17376806]

Werner M, Mueller J, Walch A, Hofler H. The molecular pathology of Barrett's esophagus. Histol Histopathol. 1999; 14(2):553–559. [PubMed: 10212817]

Wijnhoven BP, Dinjens WN, Pignatelli M. E-cadherin-catenin cell-cell adhesion complex and human cancer. Br J Surg. 2000; 87(8):992–1005. [PubMed: 10931041]

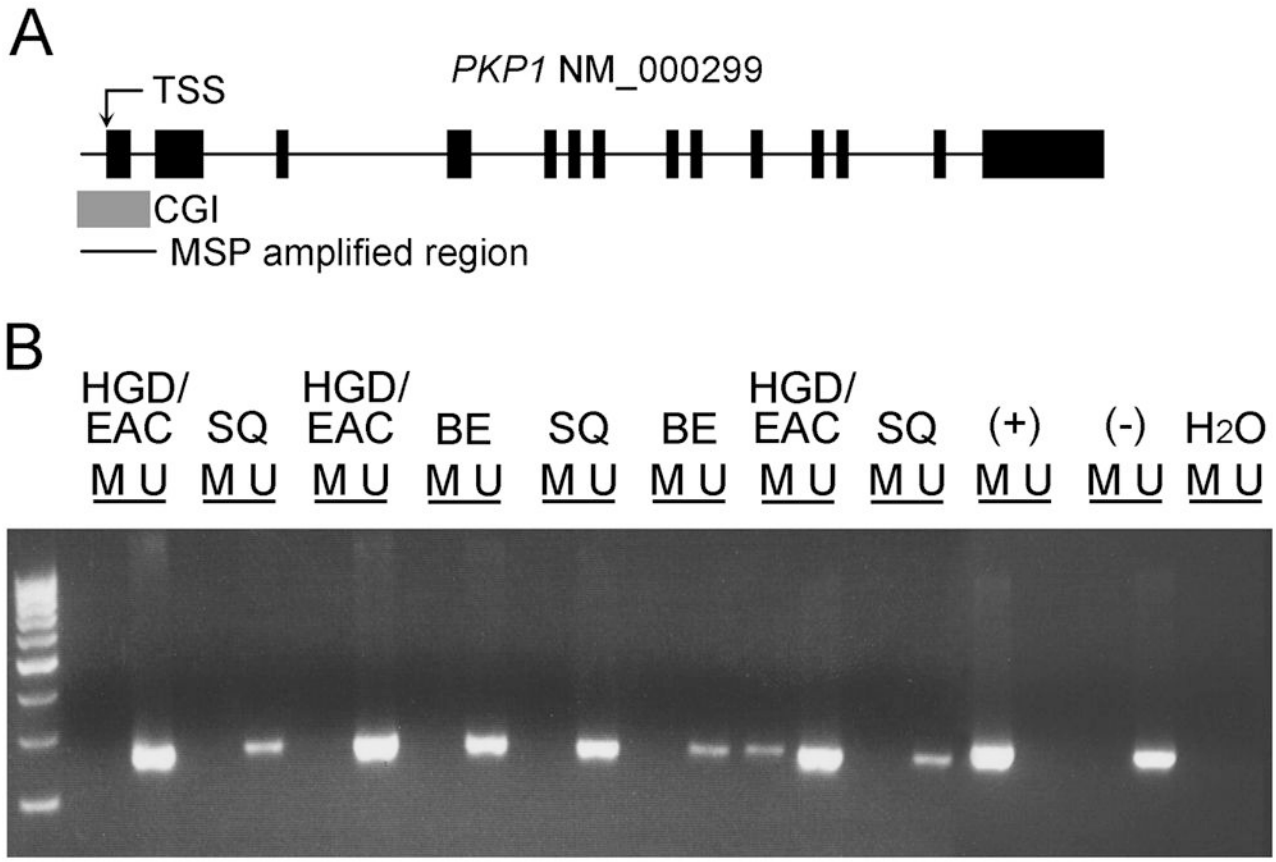

Figure 1.

Methylation-specific PCR (MSP) of *PKP1* in esophageal samples. Panel A depicts the *PKP1* gene, with exons shown as black rectangles, along with the location of the transcription start site (TSS), the promoter-associated CpG island (CGI; grey box), and the region amplified by the MSP reaction. Panel B demonstrates MSP results for a collection of esophageal DNAs. In these representative cases, *PKP1* was unmethylated in all cases except for one of the HGD/EAC samples, where it showed partial methylation. Positive (+), negative (−), and no-template (H_20) controls are included with each set of reactions. SQ= normal squamous esophagus, BE=Barrett's esophagus, HGD/EAC=combined high-grade dysplasia and esophageal adenocarcinoma.

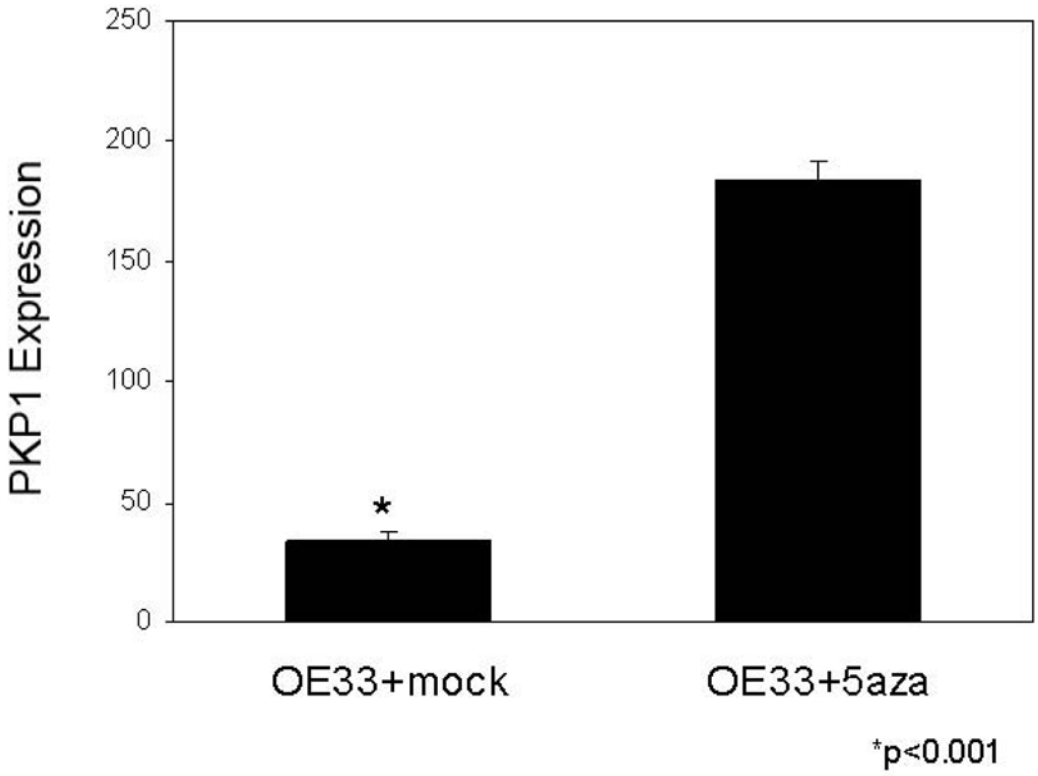

Figure 2.

OE33 esophageal adenocarcinoma cells, which carry methylated *PKP1*, demonstrate a low level of *PKP1* mRNA expression at baseline (mock treated; left bar). Following treatment with 5-aza-2′deoxycytidine, *PKP1* expression increased ~5.4 fold (right bar), consistent with the methylation of *PKP1* mediating its transcriptional repression. The Y-axis depicts PKP1 expression as 'Relative Expression Units' (REUs), which was calculated using *GUSB* mRNA levels to control for variable cDNA levels between samples.

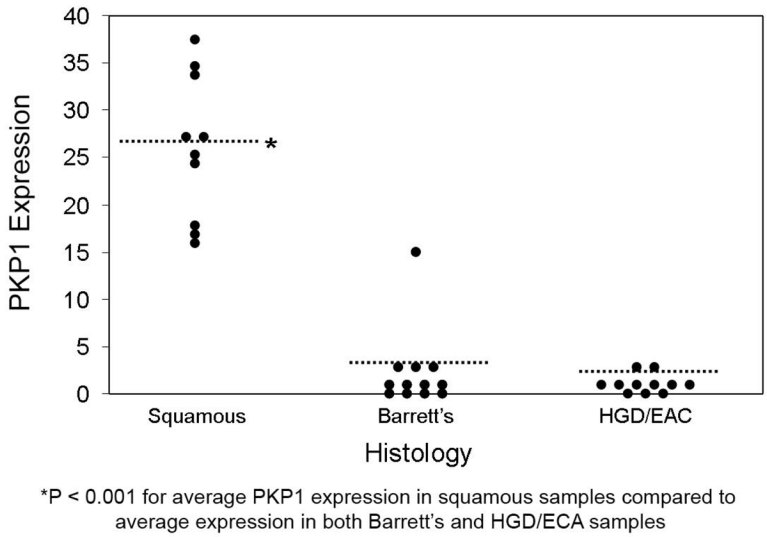

Figure 3.

Expression of *PKP1* in a collection of primary esophageal tissues, including squamous esophagus, Barrett's esophagus, and combined high-grade dysplasia/esophageal adenocarcinoma (HGD/EAC) using quantitative RT-PCR. Average relative expression, depicted as a dashed line, is highest in the squamous esophageal tissues (27.2) while average expression is repressed in BE (4.4) and HGD/EAC (2.0). The Y-axis depicts relative expression as 'Relative Expression Units' (REUs).

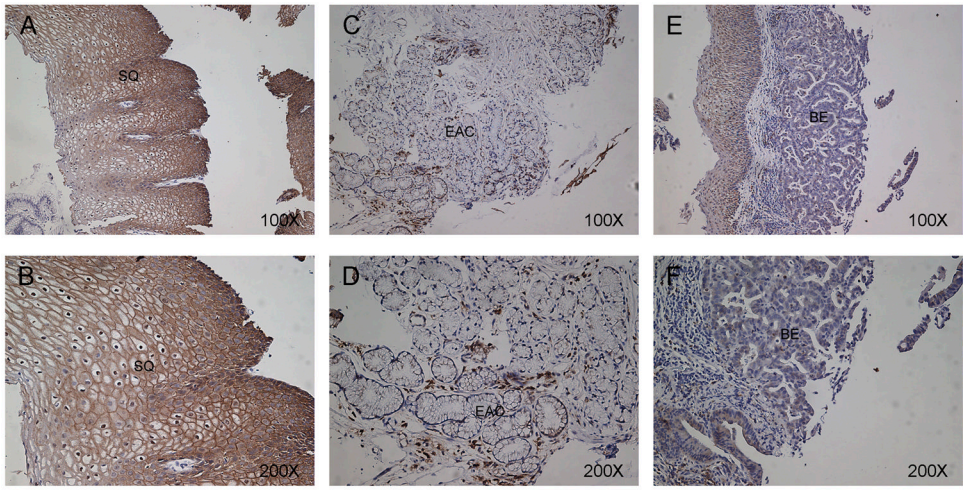

Figure 4.

Immunohistochemical analysis of PKP1 in esophageal tissues demonstrating the subcellular distribution of the PKP1 protein. In the squamous esophagus (SQ), PKP1 was primarily located at cell-cell interfaces, consistent with its role as a constituent of the desmosome (Panels A and B). In Barrett's esophagus cells (BE), there was little or no PKP1 protein expression (Panels C and D). In the esophageal adenocarcinoma (EAC) case shown, moderate levels of PKP1 were seen in the cytoplasm of the cancer cells (Panels E and F).

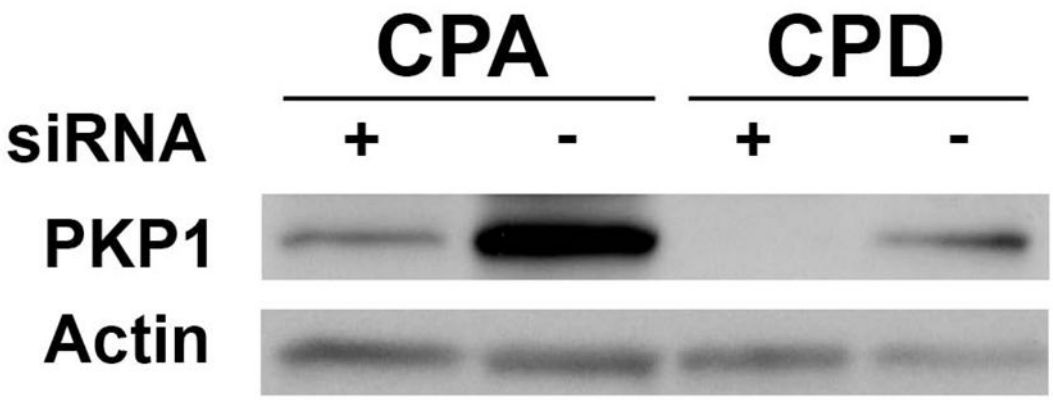

Figure 5.
Western blotting analysis demonstrating the PKP1 protein in CP-A and CP-D cell lysates following PKP1-targeted siRNA knockdown (+) versus control (−). In both cell lines, PKP1 levels were significant down regulated by PKP1-targeted siRNA transfection as compared to transfection with the non-targeting siRNA control. β-actin was used as a protein loading control.

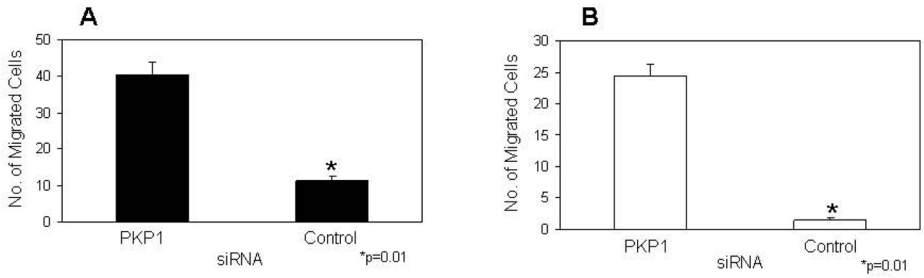

Figure 6.

Results of cell migration assays from the Barrett's esophagus cell lines CP-A (panel A) and CP-D (panel B). The left bar of each graph represents the number of cells that migrated across a polycarbonate membrane with eight micron pores 20 hours after siRNA knockdown of PKP1; the right bar of each graph depicts the same measurement after control non-targeting siRNA treatment. Both Barrett's cell lines contain unmethylated *PKP1* and express relatively high levels of PKP1. In both cell lines, cell motility is low at baseline (control) but increases significantly following siRNA knockdown of PKP1.

Table 1

PKP1 methylation in primary esophageal tissues and cell lines

Tissue Diagnosis	No. Methylated Samples/Total Samples (% methylation)
Squamous	5/55 (9.1)
Barrett's	5/39 (12.8)
BE+HGD	1/4 (25.0)
EAC	17/54 (31.5)
HGD/EAC combined	20/60 (33.3) *

Cell Line	*PKP1* Methylation Status
EPC2 (normal squamous esophagus)	U
CP-A (Barrett's esophagus)	U
CP-D (Barrett's esophagus)	U
OE33 (esophageal adenocarcinoma)	M

* $p<0.05$ for both SQ and BE versus HGD/EAC

U=unmethylated; M=methylated