Oesophageal Carcinoma is a severe form of gastrointestinal (GI) cancer and has a poor prognosis. It usually manifests as Oesophageal Squamous Cell Carcinoma or Oesophageal Adenocarcinoma. The p21 activated kinase (PAK) family of proteins have been implicated in a few forms of cancer, especially those that are GI. In this study, the levels of expression of total PAK1 and PAK4 as well as their phosphorylated forms were assessed via Western Blot and Immunophorescence analysis in (a) oesophageal squamous cell carcinoma, (b) oesophageal adenocarcinoma and (c) oesophageal metastatic cell lines. It was found that both PAK1 and PAK4 were over-expressed in all three types of cell lines. Phosphorylation of PAK4 was however insignificant or non-existent in the above mentioned cell lines. The recognition of both PAK1 and PAK4’s role in Oesophageal Carcinoma may allow for future applications in diagnostics and to a greater extent, therapeutics of the malignancy.
INTRODUCTION:
Oesophageal Carcinoma is one of the major types of GI cancers that has a wide geographical variation in incidence. Pathologically, it manifests in 2 forms i.e. squamous cell carcinoma (SCC) and adenocarcinoma. Approximately 90% of oesophageal cancers are of the former type whereas the remaining 10% thereof are adenocarcinomas. Usually both become detected in individuals over the mean age of 40 years.

SCC of the oesophagus frequently occurs in North China, where the mortality rate is known to exceed 100 per 100 000 males and 50 per 100 000 females (1). Other high risk areas include the regions of Brittany, France and Trieste, Italy. Adenocarcinoma of the oesophagus, unlike SCC affects 1-4 per 100 000 (during 1995-1997) in Western European countries and the US. A major risk factor that predisposes individuals to the disease is Barrett’s Oesophagus. The prognosis of oesophageal carcinoma is very poor, as only 5% of patients live for 5 years with the disease.

Countless mechanisms have been identified in the causation of oesophageal cancer. One such recent contributory mechanism uncovered has been the gain of chromosome 11q13. This was shown to have resulted in overexpression of the cortactin (CTTN) gene (2). Today, the scientific community acknowledges the role of the p21 activated kinase (PAK) protein family in an assortment of cancers. This group of mammalian Rac/Cdc42-associated serine/threonine protein kinases has diverse structural regulatory elements which can allow them to act as effectors in signalling pathways involving GTPases. They also participate in signalling events that are mediated by Src3 homology domains or caspase-mediated proteolytic cleavage (3).

The p21 activated kinase proteins have a variety of functions. They regulate actin cytoskeletal development e.g. neurite extension in neuronal cells. Apoptotic modulation as well as MAP kinase signalling are a few of the other important roles. Overall, they cater for cell morphogenesis, motility, survival, mitosis and angiogenesis (4).

Detailed studies have revealed that PAK1 has the ability to phosphorylate Bad (intrinsic death agonist) on the Ser112 and Ser136 residues of the apoptotic protein. Phosphorylation of this protein by PAK1 leads to inactivation and thus nullification of apoptosis, uncontrolled proliferation of cells, and thus tumorigenesis (5). PAK1 has been associated with invasiveness and significantly increased levels have been detected in malignant progressing Colorectal Carcinoma (6). Over-expression of PAK1 has been associated with the progression and metastasis of GI Carcinoma to the liver (7). PAK1 is phosphorylated on two residues – one is a serine site (Ser139), and the other being a threonine site (Thr423) (8). The activation of PAK1 is thought to be mediated by an assortment of molecules such as sphingosine, phosphatidic acid and 3-phosphoinositide-dependent kinase1 (PDK1) (9).

PAK4 on the other hand was actually the first member of the family to have been identified as a possible contributing factor to cell transformation. In a study by Callow et al, it was found to have been expressed in 78% of tumour cell lines which were assayed (10). The site of phosphorylation of PAK4 is the Ser474 residue leading to its activation (11).

The main objective in this study was to detect the presence of PAK1 and PAK4 both in their total, as well as phosphorylated forms in oesophageal cancer cell lines. The method used additionally allowed for comparison of levels of expression of these proteins. It should be appreciated that the quantification of the levels of expression of the phosphorylated forms of PAK1 and PAK4 provides us with possible insight into the role of such activated proteins in oesophageal carcinoma.

MATERIALS AND METHODS

This study involved the use of frozen Oesophageal Squamous Cell Carcinoma (SCC), Oesophageal Adenocarcinoma and Oesophageal Metastatic cell lines. The basic principles employed when attempting to detect over-expression of PAK1
and PAK4 (and their phosphorylated forms) in the abovementioned cell lines was that of Western Blotting followed by Immunophorescence. However, a number of important steps preceded these as discussed below.

To begin with, the following cell lines were obtained- KE3, KE6, KE8, KE10, OE19, OE33, OC1 and OC3. For clarification KE cell lines were Squamous Cell Carcinoma, OEs were Adenocarcinoma and OCs were Metastatic Oesophageal Carcinoma cell lines.

**Cell Culturing and Seeding:** KE3, KE6, KE8, KE10, OE19, OE33, OC1 and OC3 frozen cell lines were removed from liquid nitrogen and placed in a water bath at 37°C. 1 ml of 1 x 10⁶ cell suspension of each cell line was added to RPMI-1640 medium in incubating flasks. The flasks were then incubated at 37°C in the presence of 5% CO₂ for 36 hours. Following this, the incubated medium was extracted from the flasks. The flasks were then washed with PBS. The cell lines were then trypsinised by addition of 0.25% trypsin to the flasks. These flasks were then further incubated for 10 minutes at 37°C in the presence of 5% CO₂.

Each trypsinised cell solution (KE3, KE6, KE8, KE10, OE19, OE33, OC1 and OC3) was centrifuged at 1200rpmi for 5 minutes. Residual cellular pellets were obtained and resuspended in RPMI-1640 solution. The seeded cells were then incubated for 4 days at 37°C in the presence of 5% CO₂.

**Cell Lysate Preparation:** The incubation medium was removed and cells were treated with PBS. A lysis buffer consisting of 50mM sodium pyrophosphate, 200mM sodium fluoride, 1mg/ml Apr, 1mg/ml Leupeptin, 5mg/ml AEBSF and Tris-EDTA was prepared. All chemicals were of highest quality standard. Each group of cells were treated with this lysis buffer solution over ice. The residual cell lysate solutions were collected in eppendorfs and were sonicated thrice for 8 seconds each time. Centrifugation followed at 1200 rpm for 2 minutes at 4°C.

The supernatant obtained was used for a protein assay and Western Blot.

**Protein Assay and Laemmli Buffer Addition:** In order to quantify the amounts of protein in each sample of cell lysate, a protein assay was conducted. BSA standards were made up to 500µl – these standards ranged from 1.0µl – 20.0µl of BSA in a 2mg/ml concentration. Additionally a “blank” sample comprising of 500µl distilled water was used. A PIERCE® Micro BCA™ Working Reagent (assay) was then made up. 150µl of each standard (including blank) along with unknown samples of cell lysate were individually delivered to microplate wells. 150µl of Working Reagent were added to each well. All aliquots were incubated at 37°C for 2 hours. The absorbance was then measured at 562nm on a plate reader and a best fit protein curve was plotted. Fig 1 below illustrates this

![Fig 1. Protein Curve for Cell line sample](image_url)
For each sample, the volume of lysate that contained 30µg of protein was obtained. A Laemmli Buffer Solution (comprising of 0.5M Tris-HCl at pH 6.8, glycerol, 10% SDS, B-mercaptoethanol and 0.05% bromophenol and distilled water) was added cell line samples in a 1:2 ratio. This step was essential as it allowed for 30µg of protein to be present in each “track” of the gel electrophoresis unit. The combined cell line and Laemmli Buffer samples were centrifuged at 1200rpm for 5 seconds and stored at 4°C for 24 hours.

**SDS Polyacrylamide Gel Electrophoresis:** A Resolving and Stacking Gel were produced to load the abovementioned samples into the electrophoresis chamber. The Resolving Gel consisted of 30% acrylamide mix, distilled water, 1.5M Tris-HCl (at pH 8.8), 10% SDS, 10% ammonium persulphate and TEMED. The Stacking Gel was comprised of 30% acrylamide mix, distilled water, 1M Tris-HCl (at pH 6.8), 10% SDS, 10% ammonium persulphate and TEMED. The Resolving Gel was poured first between the electrophoretic plates and allowed to precipitate. The Stacking Gel was placed above the Resolving Gel and both were placed into an electrophoresis case which in turn was filled with Running Buffer. The Running Buffer was composed of Tris Base, glycine, SDS and distilled water. The combined cell line-Laemmli Buffer samples were loaded into wells within the Stacking Gel and underwent electrophoresis for 3 hours at 70V.

**Western Blotting:** Blotting paper, nitrocellulose membranes and cellophane sheets were immersed in a Transfer Buffer (consisting of Tris-Base, glycine, 10% SDS, methanol and distilled water) for 20 minutes. A semi-dry transfer unit was constructed from these materials and the Resolving Gel from the electrophoresis was excised. This excised Resolving Gel was placed within the semi–dry unit, covered with cellophane and subjected to a current of 80mA for 1 hour.

**Ponceau Red Test:** The nitrocellulose membrane post Western Blot was subjected to a Ponceau Red test to verify that transfer of the blot to the sad nitrocellulose membrane was successful.

**Blocking and Addition of Primary Antibody:** A blocking technique was employed to ensure that sites on the membrane not bound with protein were blocked. The nitrocellulose membrane was soaked in a Blocking Buffer (which consisted of TBS, dissolved milk in a 5% solution and 0.1% Tween washed in a Washing Buffer (0.1% TBS and Tween) for 1 hour. The said membrane was later washed 4 (four) times in a Washing Buffer (0.1% solution of TBS and Tween). Primary antibodies were diluted in a ratio of 1:1000 in a solution comprising of Bovine Serum Albumin (BSA) and PBS. Respective antibody solutions were then added to the nitrocellulose membrane blot. The primary PAK1 antibodies used was obtained from Cell Signaling Technology® and were derived from rabbit species, being polyclonal in origin and specific to human PAK1. Likewise PAK4 antibodies used were obtained from Cell Signaling Technology® being derived from rabbit species, polyclonal in origin but specific to human PAK4.

Antibodies specific to the phosphorylated PAK1s (phosphoPAK1-thr423 and phosphoPAK1-ser139) were obtained from Biosource™, polyclonal in origin, derived from rabbit species. The former however was specific to phosphor-peptides surrounding threonine 423 while the latter was specific to phosphor-peptides surrounding serine139.

The primary antibodies used for detection of phosphoPAK4 were obtained from Cell Signaling Technology®. Like the above, these were polyclonal and or rabbit origin however they specifically targeted phosphor-peptides around serine474 residues of human PAK4.

Treatment of the nitrocellulose membrane with primary antibodies lasted for 24hrs.

**Addition of Secondary Antibody:** After treatment with the primary antibodies as discussed above, the nitrocellulose membrane was washed and then further treated with a secondary antibody for 1 hour. This anti-rabbit antibody was biotinylated and was known to react with rabbit immunoglobulins of all classes.
Before being added to the nitrocellulose membrane, the secondary antibody was diluted with BSA in a 1:2000 ratio. The secondary antibody was obtained from DakoCytomation®.

Chemiluminescent Reaction and Imaging: Upon treatment of the nitrocellulose membrane with secondary antibody, a chemiluminescent substrate (which constituted of luminal enhancer solution, peroxide buffer and distilled water) was poured over the blot and left for 5 minutes. After this period, the said membrane blot was placed in an X-ray Photographic Chamber (dark box) and images were developed.

A Biotinylated Molecular Weight Marker (MWM) from Cell Signaling Technology®, within the range of 10-200kDa as a standard, allowed for identification of the protein bands responsible for PAK1, PAK4 and their phosphorylated counterparts’ presence.

Stripping of Antibodies from Nitrocellulose Membrane: This was an important step done each time, prior to the addition of the different antibodies.

Beta actin Detection: Beta actin was detected by using monoclonal mouse antibodies which were known to bind to an epitope located on the N-terminal end of the β-isoform of human actin. Such antibodies used were obtained from Sigma-Aldrich®.

RESULTS

The results from the chemiluminescent reaction and imaging are illustrated on a constructed figure as shown in Fig 2. This figure showed the expression of PAK1, PAK4, phosphoPAK1 and phosphoPAK4 as well as that of β-actin.

Fig 2. Figure confirming expression of total PAK1, total PAK4, phosphorylated PAK1 and phosphorylated PAK4.

Please see legend on separate page in appendix.

Legend for Fig 2.
1- PAK1 (total) expression
2- phospho-PAK1 (thr423) expression
3- phospho-PAK1 (ser139) expression
4- PAK4 (total) expression
5- phospho-PAK4 (ser474) expression
6- β-actin expression
A- KE-3 cell line sample  
B- KE-6 cell line sample  
C- KE-8 cell line sample  
D- KE-10 cell line sample  
E- OE-19 cell line sample  
F- OE-33 cell line sample  
G- OC-3 cell line sample  
H- OC-1 cell line sample.

PAK1 and PAK4 proteins were expressed significantly in the majority of cell lines. All squamous and adenocarcinoma cell lines in the experiment depicted increased expression of total PAK1. Likewise levels of expression of phosphoPAK1 (Thr423) were detected in all squamous and adenocarcinaoma lines. However metastatic cell lines i.e. OC-1 and OC-3 did not show significant levels of expression of this phosphorylated protein. The Ser139 phosphorylated PAK1 was absent in all cells.

PAK 4 was expressed in all cell lines except OE-19. Results also indicate absence of phosphorylated PAK4 in all of the cell lines.

**DISCUSSION**

As one can see from Fig 2, PAK1 and PAK4 proteins were generally expressed significantly in the majority of the cell lines.

All squamous and adenocarcinoma cell lines used in The Western Blot Analysis showed increased expression of total PAK1. However metastatic cell lines indicated little or no presence of the said protein. A similar pattern was noted with the detection of PAK1 phosphorylated on the Thr423 site. Nevertheless, phosphorylated PAK1 on the serine site was absent in all of the cell lines.  
This evidence may suggest that activated PAK1 (phosphorylated on the Thr423 site) may play a role in oesophageal carcinoma.

Despite the negative results portrayed with the metastatic cell lines, it would be unjust to say that activated PAK1 doesn’t contribute to metastasis. A possibility for poor expression could be the result of subsequent deactivation in the cell lines from generation to generation. Furthermore, it should make sense that phosphorylated PAK1 (PAK1 in its activated state) would most likely be expressed in a tumour cell and a normal cell (preceding transformation) before metastasis. . This would be the case in order for there to be cell migration, anchorage independent growth, and angiogenesis – all characteristics of metastases.

With the exception of 1 Oesophageal Adenocarcinoma cell line i.e. OE-19; PAK4 seemed to have been expressed significantly in all of the others. Interestingly, there was no phosphorylation of PAK4 in any of the cell line samples. This finding can direct us to think that perhaps PAK4 activation does not occur in the oesophageal cancer process. Whether or not inactive PAK4 plays a role in oesophageal carcinoma is still a question that remains unanswered, as more research is warranted in this field. Further work such as immunoperoxidase staining for the detection of PAK4 and phosphoPAK4 in tumour sections is recommended.

It is known that PAK is associated with the actin cytoskeleton in many different cells. Marler et al. found that endogenous PAK isoforms co-localised with actin in specific sites of neuroblastoma cells (12). In addition PAK proteins act through a guanidine nucleoside exchange factor (βPIX) to mediate Golgi/centrosome polarity and restrict actin polymerisation (13). PAK4 has been implicated in actin regulation which was responsible for podosome presence in macrophages (14). Gringel et al have also acknowledged that PAK1 and PAK4 link RhoGTPase signalling to actin cytoskeletal regulation.

Probing for beta actin was a useful control to ensure that all electrophoresis tracks had equal amounts of cell protein. A portion of Fig 2 shows beta actin expression. Unfortunately however, OE-19 and OC-3 cell line samples expressed less β-actin in comparison with the remainder. This means that such cell line samples could have had less protein and this...
should be taken into account when assessing levels of PAK proteins. For example, this could be a contributing factor for failure of detection of PAK4 in OE-19 cell line samples. Likewise OC-3 samples may not have illustrated expression of PAK1.

The concept of over-expression of PAK1 and PAK4 can provide us with a basis for further research such as the signalling mechanism whereby the activated protein triggers downstream events that cause oesophageal tumorigenesis. Knowing that PAK1 and PAK4 are over-expressed in oesophageal carcinoma can also be useful both therapeutically and diagnostically. There is possible scope for experimentation on compounds that have the potential to target PAK1 and PAK4 proteins in disease conditions. Laboratory and histopathological tests can also be based on the presence of phosphorylated PAK1 and PAK4 being over-expressed in biopsied tissue.

Nonetheless, these possibilities mentioned would have to be thoroughly tested for feasibility, accuracy, specificity and other characteristics over a long period of time before they become a reality and are developed. In the long term much more research is required in this field.

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