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Significant up-regulation of 1-ACBP, B-ACBP and PBR genes in immune cells within the oesophageal malignant tissue and a possible link in carcinogenic angiogenesis

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Summary. Oesophageal cancer ranks as the sixth most common malignancy in the world, and recent evidence has shown that its incidence is increasing. ACBPs (Acyl-coA binding proteins) act as intracellular carrier-proteins for medium to long chain acyl-coA, mediating fatty acid transport to the mitochondrion for β -oxidation. ACBPs are also believed to be putative ligands of PBR (peripheral benzodiazepine receptor), and once they bind to this receptor they facilitate mitochondrial membrane permeabilization, presumably favouring apoptosis. The main aim of the study was to establish the expression patterns of 1- Acyl-coA binding proteins (1-ACBP), B- Acyl-coA binding proteins (B-ACBP), and peripheral benzodiazepine receptor (PBR) in oesophageal cancer, and to link their roles with the disease. *In situ* hybridization and quantitative real-time PCR methods were performed to determine localization and the expression levels of the three genes in oesophageal cancer. All three genes products illustrated substantial up-regulation within the malignant tissue sections as compared to normal oesophageal sections. All three transcripts localized specifically to mast cells, plasma cells and lymphocytes in diseased and normal tissue section. In the diseased tissue, B-ACBP and 1-ACBP mRNA localized to endothelial cells of blood vessels in the submucosa. B-ACBP also localized to the nucleus of squamous epithelial cells. PBR localization was found in tumour islands of invasive tissue sections. Quantitative

RT-PCR also indicated that the expression levels of PBR were higher as compared to the ACBP genes expression in tumours. These results show that 1-ACBP, B-ACBP and PBR play a role in the pathogenesis of oesophageal tumours and possibly in carcinogenic angiogenesis.

Key words: Oesophagus cancer, 1-ACBP, B-ACBP, PBR

Introduction

Oesophageal cancer is the sixth leading cause of cancer related mortalities world-wide and its pathogenesis is still poorly understood (Ferlay et al., 2010). Oesophageal cancer is a disease with a high and ever increasing incidence worldwide (Munoz, 1993). It is caused by both effects of environmental factors and lifestyle factors; and these lifestyle factors are leading influencing factors of the disease (Wu et al., 2011; Chen et al., 2013). To improve the diagnosis of the disease, there is ongoing research where animal models are used in order to show how oesophageal carcinogenesis develops or progresses (Fang et al., 2013; Kaz and Grady, 2014). There are many genes that have been shown by numerous studies that are speculated to play an important role in the carcinogenesis of oesophageal tumours, but there is presently no evidence showing how the tumour develops. Many researchers believe that human papilloma virus (HPV) is one of the most important factors contributing to the development of squamous cell carcinomas. However, more research is required to confirm the link between HPV and the

occurrence of oesophageal carcinoma in more than a small percentage of cases, as has been shown (Matsha et al., 2002). Others believe that mutations of apoptosis regulating genes, tumour suppresser genes and oncogenes are involved in the initiation and development of oesophageal cancer. A gene directly related to oesophageal cancer has yet to be identified, for an effective therapeutic strategy to be determined (Cui et al., 2003). Oesophageal cancer develops in a sequential form from normal to malignant tissue in four stages; metaplasia, dysplasia (low and high grade), carcinoma *in situ*, and then finally an invasive carcinoma stage. This study was carried out to determine the expression patterns of three genes, 1-ACBP, B-ACBP and PBR in oesophageal carcinoma, and determine their roles in the development of oesophageal carcinogenesis. Acyl-coenzyme A binding protein (ACBP) has five isoforms that are very similar. ACBPs bind to peripheral-type

benzodiazepine receptor (PBR) and play a role in steroidogenesis, this interaction is also believed to play a vital role in apoptosis and any disturbance in the expression of these genes may lead to cancer.

ACBPs are primary intracellular proteins which consist of 86-103 amino acids and were originally isolated from human brain tissue as putative ligands of PBR (Yang et al., 2012). They have been characterised and found to be conserved among different species ranging from yeast to mammals (Swinnen et al., 1998; Yang et al., 2012). The vital function of ACBPs is to act as intracellular carrier-proteins for medium to long chain acyl-coA, mediating fatty acid transport to the mitochondrion for β -oxidation. (Swinnen et al., 1998). ACBPs are grouped into at least four groups, 1-ACBP, B-ACBP, T-ACBP, and M-ACBP. The two ACBP variants being focussed on in this study are the 1-ACBP

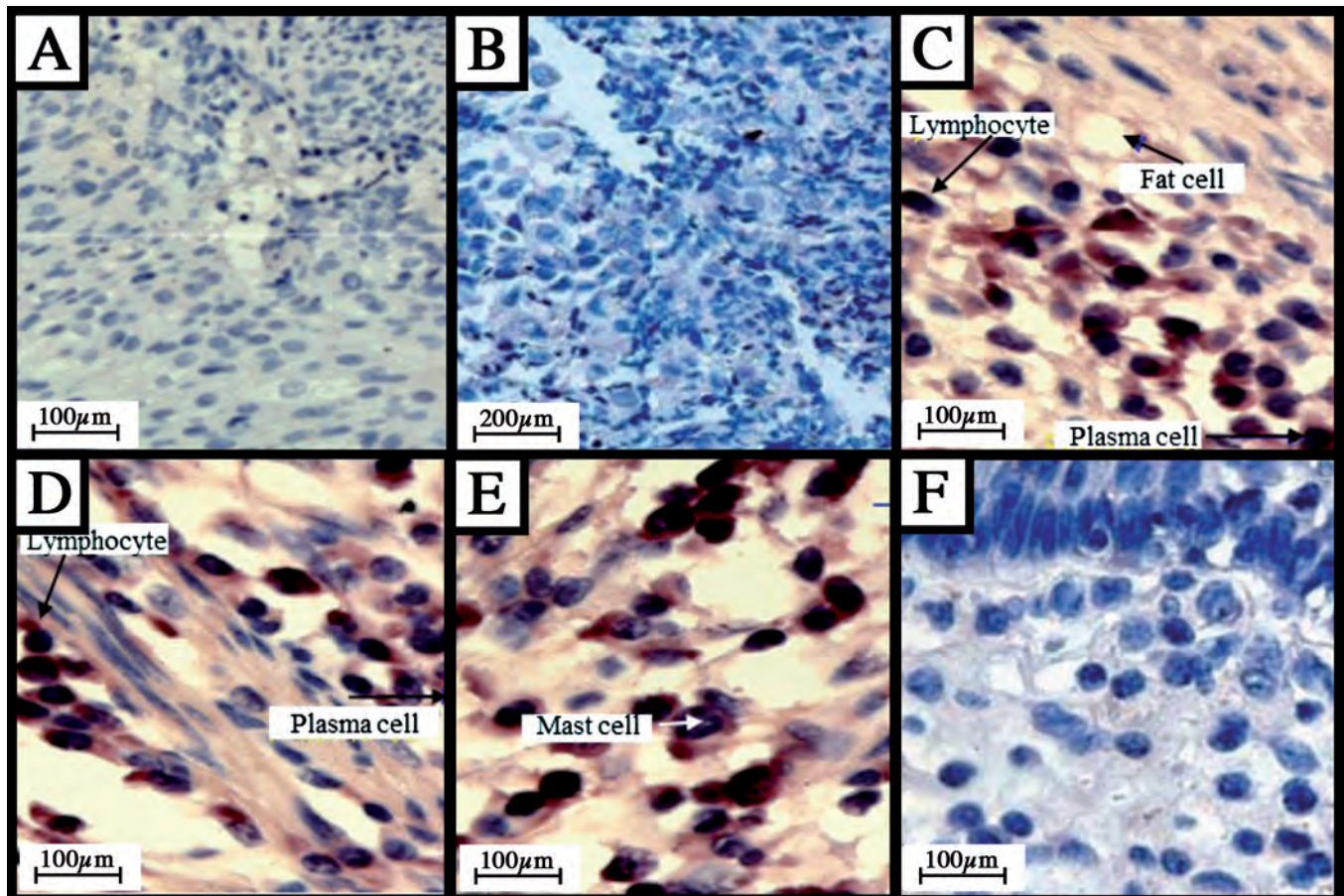


Fig. 1. Localization of 3' 1-ACBP. **A, B.** Negative controls. **C.** Localization of 3' 1-ACBP probe to the cytoplasm of plasma cells and limited localization in lymphocytes. **D.** Indicates plasma cells in between skeletal muscle layer of an infiltrating moderately differentiated squamous cell carcinoma, the 3' 1-ACBP probe localizes to plasma cells, and little lymphocytes, but no localization occurs in fibroblasts of skeletal muscle. **E.** 3' 1-ACBP was highly expressed in plasma cells and mast cells in the lamina propria of a well-differentiated, high-grade squamous cell carcinoma of the oesophagus. **F.** 3' 1-ACBP showed no localization within the lamina propria of normal oesophageal tissue sections.

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and B-ACBP isoforms. PBRs are relatively small proteins (18kDa) of 169 amino acids, which have been identified in peripheral tissues and are localised on the outer membrane of the mitochondria (Imaizumi et al., 2008). Despite the name, PBRs are not only located in peripheral organs but also present in the CNS. PBR forms part of a trimeric complex with voltage-dependent anion channel (VDAC) and adenine nucleotide carrier (ANC) (Anholt et al., 1986). PBRs are primarily located on the outer mitochondrial membrane. ACBPs and PBR are of great interest due to their involvement in numerous biological functions, including the regulation of cellular proliferation, immunomodulation, regulation of steroidogenesis and apoptosis. *In situ* hybridisation studies were utilized to elucidate the expression patterns of these genes at the mRNA level in oesophageal carcinoma. *In situ* hybridisation required the generation of probes for the three genes, 1-ACBP, B-ACBP and

PBR.

Materials and methods

Ethics clearance

Ethics clearance was obtained from the Human Ethics Committee, University of the Witwatersrand.

Sample collection

Oesophageal carcinoma tissue sections were obtained from Prof Stewart Goetsch at University of the Witwatersrand in the Department of Anatomical Pathology. The normal tissue sections were obtained from Prof M Scholtz at the University of the Witwatersrand Medical School, Department of Anatomical Pathology.

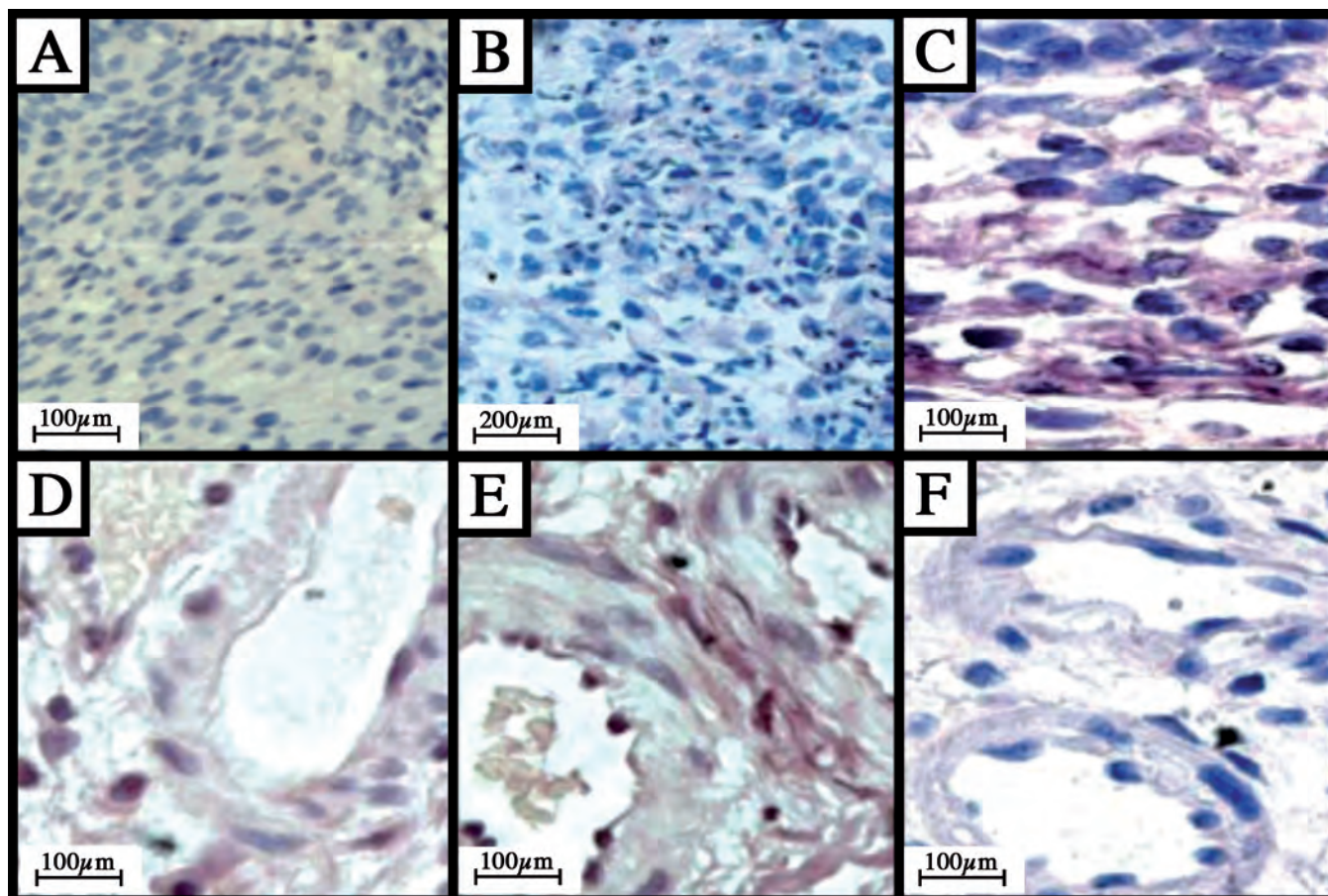


Fig. 2. Localization of 5' 1-ACBP. **A, B.** Negative controls. **C.** Well differentiated low grade squamous carcinoma localization of 5' 1-ACBP to the lamina propria, indicating an up-regulation of the gene in plasma cells and lymphocytes. **D.** 5' 1-ACBP localizes in the submucosa with nuclear localization in plasma cells, mast cells, macrophages, and low expression in small lymphocytes. **E.** 5' 1-ACBP localizes in the connective tissue of well differentiated low-grade dysplasia in the endothelium of arteries, but does not localize in the fibroblasts. **F.** The ACBP probe does not localize to endothelial cells in normal oesophagus, except to the cytoplasm of a mononuclear mast cell.

Histopathology

Haematoxylin and eosin staining of wax embedded tissue sections, approximately 4 μm thick, of the paraffin wax embedded tissue were cut and adhered on to poly-L-lysine coated slides. The sections were stained with haematoxylin and eosin to determine the histological diagnosis of the resection and biopsy specimens.

In situ hybridization

RNA Probes that are specific to the genes encoding for 1-ACBR, B-ACBR and PBR were PCR-amplified and cloned into pGEM-T easy vectors. The cloned products were sequenced and screened against the GENBANK database (NCBI) to determine the identity and orientation of the 1-ACBR, B-ACBR and PBR genes within the vector. Antisense and sense probes

were then generated using T7 or Sp6 polymerase (Roche Diagnostics, Mannheim, Germany). 4 μm cut tissue sections from normal and oesophageal cancer samples were dewaxed, rehydrated, and then permeabilized using 20 $\mu\text{g}/\text{ml}$ of proteinase K (Promega) for *In situ* hybridization. Hybridization was performed with DIG labeled cRNA probes overnight at 55°C. After hybridization, sections were washed in 2 x saline sodium citrate (SSC) and 1 x SSC at 55°C, then 0.5 x SSC and 0.1 x SSC at room temperature. Sections were blocked in 10% (w/v) blocking reagent (Roche Diagnostics), and then incubated for one hour with anti-DIG alkaline phosphatase conjugated antibody (Roche diagnostics, Germany) for 1 h at room temperature. Localization was visualized with 5-bromo-4-chloro-indolyl phosphate/nitroblue tetrazolium (Roche diagnostics, USA), counterstained with Mayer's haematoxylin (Sigma, Germany) and mounted with aqueous mounting medium

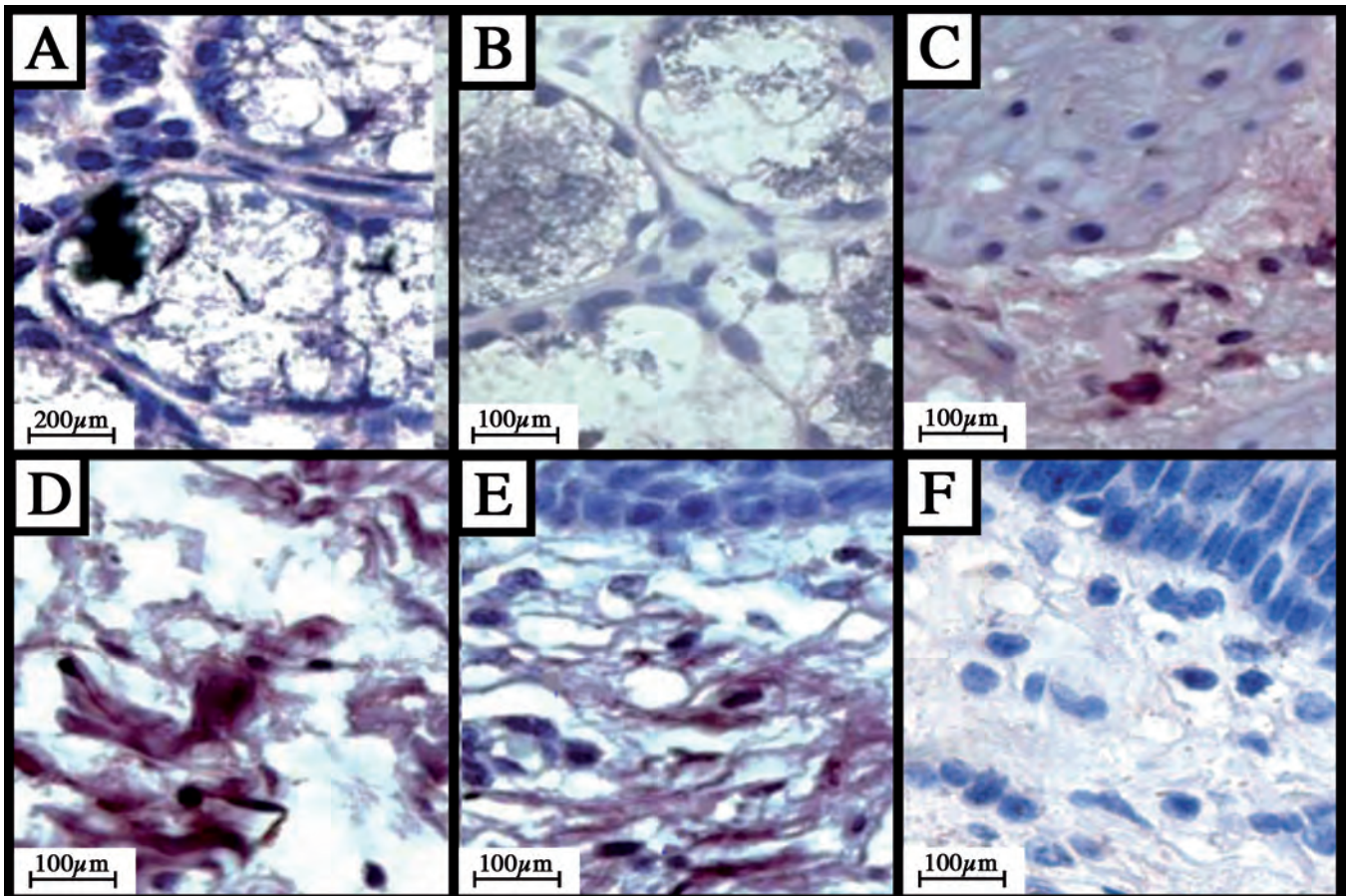


Fig. 3. Localization of 3' B-ACBP. **A.** Localization of 3' B-ACBP in plasma cells in low-grade dysplastic squamous cell carcinoma and intermediate expression level of the gene is detected. **B.** Normal oesophageal tissue section shows no localization. **C.** Localization within plasma cells, mast cells and lymphocytes in the longitudinal muscle layer of a moderately well differentiated high-grade squamous carcinoma and is up-regulated as compared to normal oesophageal tissue. **D.** Up-regulation of 3' B-ACBP in the connective tissue of a high-grade dysplasia squamous carcinoma. **E.** 3' 1-ACBP was highly expressed in mast cells, plasma cells in the lamina propria of a well-differentiated, high-grade squamous cell carcinoma of the oesophagus. **F.** No localization of 3' 1-ACBP within the lamina of normal oesophageal tissue sections.

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(Serotec, United Kingdom).

Quantitative real time PCR

mRNA expression levels were determined using 1X iQ™ SYBR Green Mix I (Bio-Rad, USA) to determine if the expression of the genes was correlated in anyway. Gene expression PCR was performed using 1 µg of cDNA in 18 µl reaction volume. The reaction mixture consisted of 1X iQ™ SYBR Green Mix I (Bio-Rad Laboratories, Hercules, CA, USA), 5 mM MgCl₂, 10 µmol/ µl of each PCR primer, and PCR-grade H₂O. Instead of adding cDNA as in the case of the genes of interest, cDNA from β-globin was used as a positive control while H₂O was added as negative control. Pre-incubation and denaturation of the cDNA was performed at 95 degrees Celsius for a period of 10 minutes for 1 cycle. Amplification of the target sequence was performed for 45 cycles at temperatures of 95, 58 and 72

degrees Celsius, at incubation times of 10, 5 and 10 minutes respectively. Melting curve analysis for product identification was performed for 1 cycle at three different temperatures of 95, 65 and 95 degrees Celsius, at incubation times of 0, 15 and 0 minutes respectively.

Results

In situ hybridization localization detected a substantial up-regulation of all three genes within the malignant tissue sections when they were compared with normal oesophageal tissue sections, within the lamina propria, muscularis mucosa, submucosa, connective tissue and longitudinal muscle layers. Table 1 represents gene expression and localization of the three genes. The two transcripts of ACBP, 1-ACBP and B-ACBP were localized to the mast cells, plasma cells and lymphocytes in diseased and normal tissue sections. These tissue

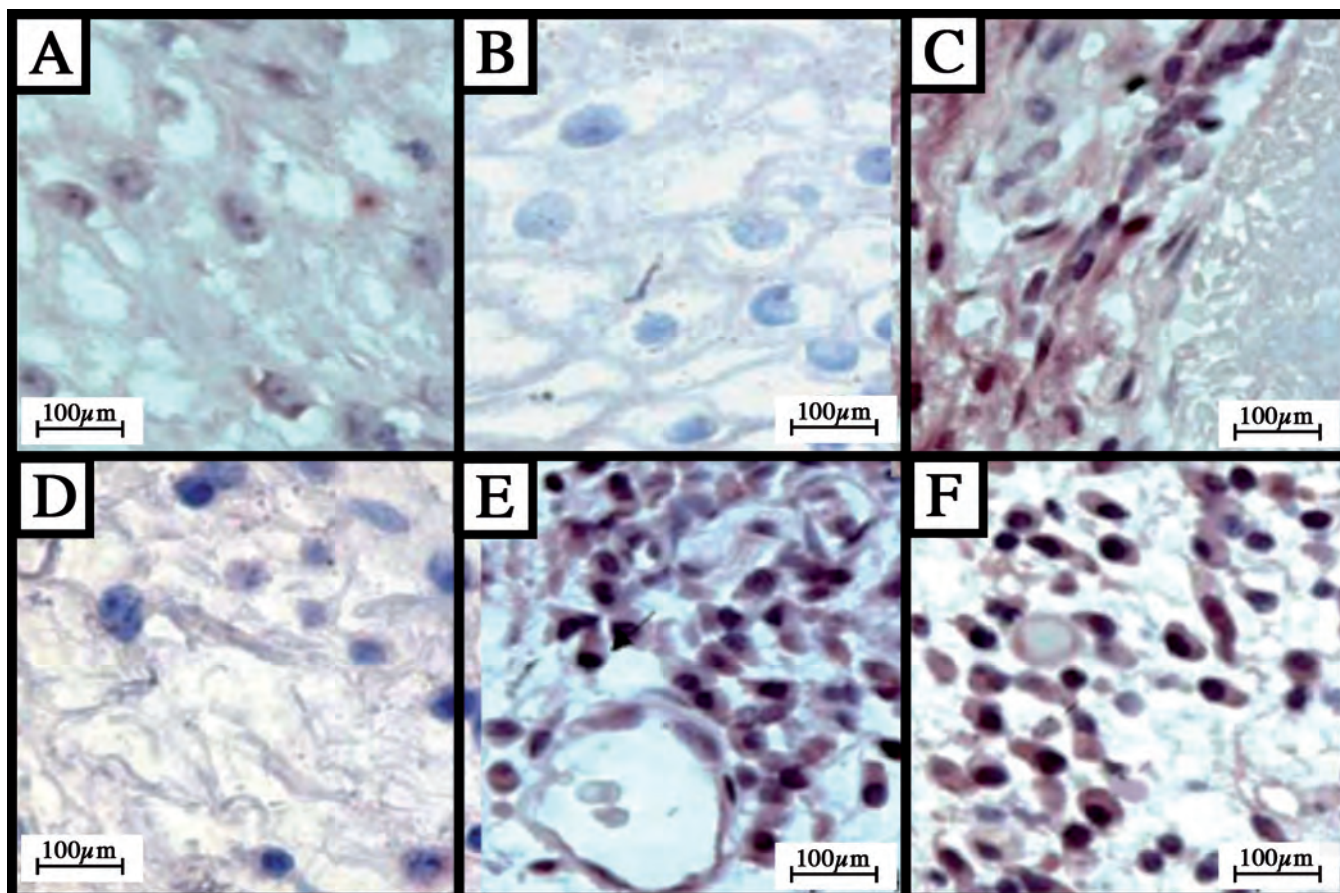


Fig. 4. Localization of 5' B-ACBP. **A.** Localization of 5' B-ACBP within the nucleus of epithelial cells of a low-grade dysplastic tissue section and Negative control **(B)**. **C.** Localization of 5' B-ACBP in the cytoplasm of the lymphocytes and plasma cells and **(D)**, indicates no localization of the 5' B-ACBP in normal oesophageal tissue. **E.** The 5' B-ACBP mRNA localizes mainly to mast cells and plasma cells in the connective tissue section of the submucosa of a moderately well differentiated high-grade dysplastic tissue sample of the oesophagus. **F.** An upregulation of 5' B-ACBP occurs within the lamina propria in mast cells of a well-differentiated high-grade dysplastic tissue sample of the oesophagus.

sections indicated an increase in expression as the disease progressed. 3' and 5' Probes of 1-ACBP and B-ACBP illustrated similar results in the degree and localization of expression. The ACBP transcripts localized to endothelial cells in arteries within the connective tissue of diseased submucosa, but no localization was found in these cells in the normal oesophageal submucosa. The results of 3' and 5' 1-ACBP indicated its localization of PBR and up-regulation of the gene in the mast cells, plasma cells and lymphocytes, as indicated in Figures 1 and 2. The results for 3' and 5' B-ACBP also showed localization of PBR and up-regulation of the gene in the mast cells, plasma cells and lymphocytes and no expression of either gene was detected in normal tissue controls (Figs. 3, 4). B-ACBP localized to the nucleus in epithelial cells of the transformed epithelial tissue, but no localization of this gene was found when compared with normal epithelial cells. PBR was localized to tumour islands in invasive

squamous carcinomas (Fig. 5). Quantitative RT-PCR illustrated the comparable expression results, with B-ACBP having the highest expression level, followed by PBR and 1-ACBP with the lowest expression in tumours as indicated in (Fig. 6).

Discussion

Since all three genes localize to mast cells, plasma cells and some lymphocytes, there is a possibility that these genes may be interacting with each other in some way to play a role in the immunological response to cancer cells. These roles could be pro- or antiapoptotic and therefore it would be useful to determine the exact role of these genes in immunology, so that potential therapeutic strategies can be determined to combat this disease. Since 1-ACBP, B-ACBP and PBR are all localised to mainly plasma cells in response to cancer cells invading the body, they are believed to play a role

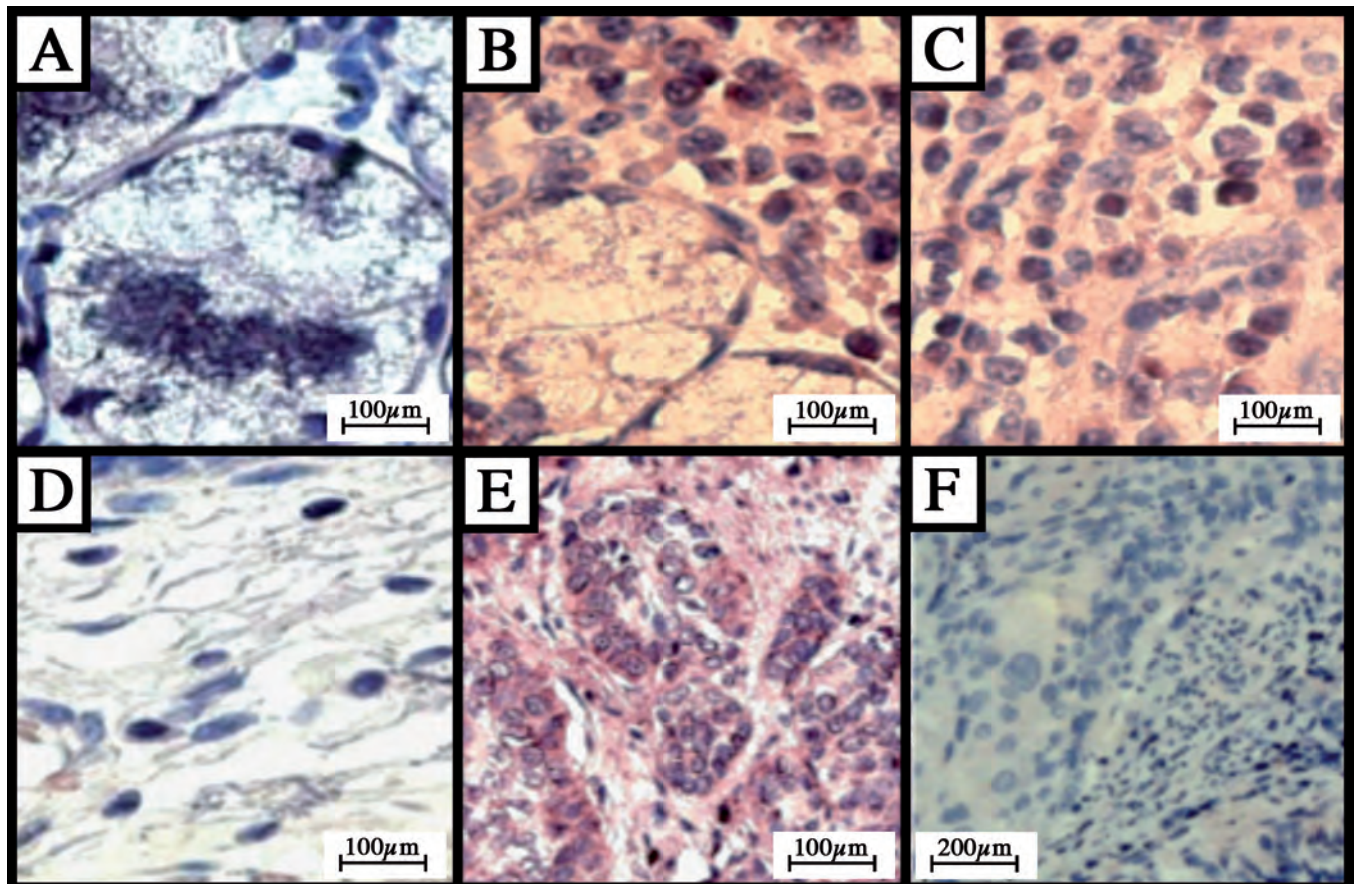


Fig. 5. Localization of PBR. **A.** Up-regulation of PBR mRNA around mucous acini glands in the connective tissue of the submucosa, in a high-grade invasive squamous carcinoma and is localized in the plasma membrane cells and lymphocytes. **B.** Indicates mucous acini glands of normal oesophagus tissue that shows no PBR localization. **C.** PBR localization in the lamina propria of a high-grade invasive squamous carcinoma in plasma cells, mast cells and a few lymphocytes. **D.** Localization of PBR in lamina propria section of the normal oesophagus. **E.** PBR localization shows its expression in the tumour islands but not in the nucleus of the tumour cells. **F.** Indicates no localization of the sense strand (negative control) of PBR in experimental control.

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in the production of antibodies needed to combat the tumour cells.

The main functions of ACBPs are to stimulate transport of cholesterol from the outer to inner mitochondrial membrane, by binding to PBR, playing a role in steroidogenesis. Plasma cells are mature B-lymphocytes that are specialised for antibody production. These cells consist of a nucleus that is eccentric and oval in shape, cytoplasm that has plentiful studded or rough ribosomes- for a highly metabolically active cell. Mature plasma cells are often oval or fan shaped. Plasma cells are rarely found in the peripheral blood. They comprise from 0.2% to 2.8% of the bone marrow white cell count. The bone marrow (BM) is well known to be the major site of Ig production in secondary immune responses; thus, the microenvironment of BM is considered to be essential for final differentiation of plasma cells. The unique finding of upregulation of these genes in activated mast cells is the first indication of a possible role in carcinogenic angiogenesis. Mast cell infiltration is thought to cause prominent proliferation of

capillaries (angiogenesis) and venules, probably initiated by release of the angiogenic actor, vascular endothelial growth factor, which enhances the extensive progression of tumours (Elpek et al., 2001; Tomita et al., 2001; Hiromatsu and Toda, 2003). Mast cells play a dual role, one of which is to secrete mediators during degranulation that are mitogenic and lead to emigration of cancer cells (Theoharides and Conti, 2004). The expression of these genes in mast cells in oesophageal tumour cells may be important in the infiltration and metastatic progression of oesophageal cancer. Evidence exists that angiogenesis is related to mast cell function. Several mast cell mediators are angiogenic and regulate endothelial cell proliferation and function. Mast cell products such as the proteolytic enzyme tryptase also degrade connective tissue matrix to provide space for neovascular sprouts (Hiromatsu and Toda, 2003). Mast cell infiltration is thought to correlate with poor prognosis in certain cancers. However, recent evidence suggests that mast cells may also participate in immune surveillance against cancer cells by triggering caspase

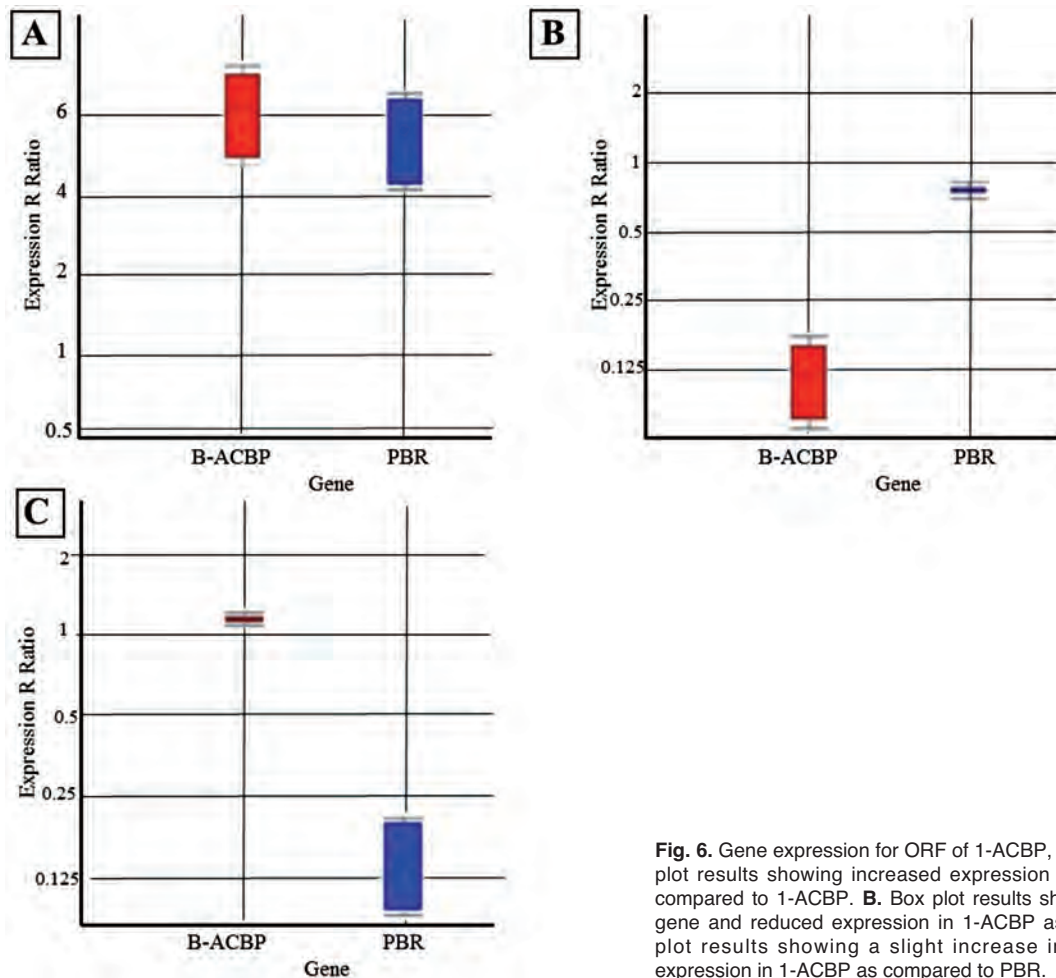


Fig. 6. Gene expression for ORF of 1-ACBP, B-ACBP and PBR genes. **A.** Box plot results showing increased expression of B-ACBP and PBR genes as compared to 1-ACBP. **B.** Box plot results showing a slight reduction in PBR gene and reduced expression in 1-ACBP as compared to B-ACBP. **C.** Box plot results showing a slight increase in B-ACBP gene and reduced expression in 1-ACBP as compared to PBR.

independent apoptosis via a cell surface molecule, consistent with a role for mast cells as antitumour effector cells (Gallagher et al., 2003).

1-ACBP and B-ACBP localised to endothelial cells of arteries in high-grade squamous carcinomas. Endothelial cells are involved in the inhibition or promotion of angiogenesis, which is the development of new blood vessels for the blood and nutrient supply for tumour survival. Endothelial cell proliferation and migration promote angiogenesis, whereas apoptosis of endothelial cells leads to vessel regression. It has been found that stimulation of endothelial cell apoptosis results in tumour degeneration, due to the tumour blood supply being withdrawn. A study has shown that inhibition of VEGF (Vascular endothelial growth factor) in tumours has led to endothelial cell detachment, apoptosis, vascular collapse, haemorrhage, and tumour necrosis (Dimmeler and Zeiher, 2000). ACBP functions in endothelial cells are believed to be that of steroidogenesis, due to the high-energy requirement of angiogenesis for tumour survival in high-grade squamous carcinomas. B-ACBP has been found expressed in the nuclei of epithelial cells in high-grade stratified epithelium of well-differentiated squamous cell carcinoma tissue sections. In an aggressive human breast cancer cell line, PBR along with 1-ACBP was found to be highly expressed, and PBR was found to be responsible for the increased cholesterol transport into the nuclei of the cell line (Hardwick et al., 1999). Increased expression in the nuclei of the epithelial cells could be due to the increase of cholesterol transport into the nuclei of the epithelial cells, mediated by some other factor other than PBR. PBR was also found to be overexpressed in the islands of tumours in invasive squamous cell carcinoma tissue sections. Tumour islands consist of compressed tumour cells and these cells therefore, have high levels of PBR and high levels of localisation are observed in these islands. No localisation for ACBP molecules occurs in these islands due to these molecules having short half life spans. It has been found that discrepancies between micromolar concentrations of PBR ligands inhibit cell growth, whereas nanomolar concentrations promote cell growth. ACBPs have a high affinity for PBR and displace benzodiazepines with an inhibitory constant of 1-2 μM (Papadopoulos and Brown, 1995). High concentrations of PBR and correlating ACBP concentrations have been found in many tumours and therefore are believed to play a role in the pathogenesis of malignancy. In this study however, high expression of these genes was found in plasma cells and an interaction between these genes is believed to take place for high-energy production, for the generation of antibodies. These findings indicate that PBR, 1-ACBP and B-ACBP play an immunological role in plasma cells, in response to oesophageal cancer. Localization of the ACBP genes to endothelial cells and nuclei of epithelial cells was detected, but no PBR expression was found in these locations. These findings therefore, suggest that the ACBP genes could have a

potentially pathogenic role in oesophageal carcinoma, without the help of PBR, suggesting that other factors may be involved in tumour development of the oesophagus.

Relative quantification was also used to compare the expression levels of the three genes' open reading frames in oesophageal cancer cell line SNO 1. The expression levels of 3' and 5' 1-ACBP indicated a variation in concentration of the amplicons generated, which was not expected and believed to be due to the primer annealing to the template cDNA. The 5' 1-ACBP was also compared to the open reading frame of 1-ACBP, and showed a comparable PCR product amplification, which was expected, as the same gene was amplified and a similar expression level should be seen. 3' 1-ACBP primer annealing may therefore be much more efficient in binding to the cDNA template, increasing the amount of product generated. Another consideration with regards to the alteration in the expression level of 3' 1-ACBP could be the higher concentration of an altered mRNA transcript. It has been found that PBR has two transcripts in diseased tissue, one that lacks exon 2 and one that contains exon 2. The one that lacks exon 2 has been found to be expressed at a much higher level in diseased tissue and this transcript is not translated into protein (Gavish et al., 1999). The transcript that contains exon 2 is translated into a normal protein even in diseased tissue. It has recently been shown that a decrease of the mitochondrial membrane potential is a prerequisite for PBR-ligand induced apoptosis (Sutter et al., 2003), and induction of apoptosis is dependent on the activation of the p38 mitogen-activated kinase (MAPK) pathway (Sutter et al., 2003). A drastic decrease in energy supply for neoplastic cells to proliferate combined with the PBR-ligand-induced apoptosis pathway could be a potential strategy to combat the development and progression of cancer.

It could therefore be a possibility that the same result occurs in the case of the 3' 1-ACBP, where an alteration in the 3' end of this gene, resulting in a much higher transcript concentration, is not translated into protein. Looking at quantitative RT-PCR results, the open reading frames of 1-ACBP, B-ACBP and PBR concentrations were then compared and the results showed comparable expression levels, with B-ACBP having the highest concentration, PBR was the second highest and 1-ACBP the lowest as indicated in (Fig 6). Since B-ACBP is expressed in squamous epithelial cells, endothelial cells and plasma cells, it is expected that a higher concentration of this gene product and transcript should occur. And since B-ACBP and PBR have the highest concentrations compared to 1-ACBP, it is most likely that B-ACBP binds more often to PBR having cell proliferation effects on the cell, or it could have apoptotic effects on the cell. Further analysis will have to be carried out to determine the exact roles of these genes in the development of cancer, and also mutation analysis would determine whether these genes are mutated in the diseased oesophagus.

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Conclusion

1-ACBP, B-ACBP and PBR have all been localised mainly to mast cells, plasma cells and neutrophils in the tissue sections of the diseased oesophagus. This data supports other findings that PBR and ACBP ligands might have an immunomodulation role. The ACBP genes were found to be expressed in endothelial cells of the diseased oesophagus and are believed to play a role in the development of new blood vessels for tumour survival. PBR localised to tumour islands indicates that a high level of the gene localises to tumour cells, and supports other findings, where PBR is upregulated in other cancers. Though ACBP and PBR have been documented to interact with each other in diseased tissue, no interaction seemed to have occurred between these genes in the development of the disease. Further analysis would have to be carried out to confirm whether there are any interactions between the ACBP molecules and PBR in the development of oesophageal cancer.

From this study the ACBP genes are implicated in playing a role in the development and progression of cancers, due to endothelial cell localisation, suggesting a potential role in assisting in the process of angiogenesis, by steroidogenesis. A suggested therapeutic strategy would be to cut off the energy supply in this process and therefore block tumour development. But since all cells need ACBP to function, these ACBP inhibiting molecules would have to be specifically targeted to tumour cells. Once the tumour cell ACBPs are targeted, the effect on the cell could be observed to determine whether direct ACBP inhibition results in energy starvation, and hopefully cell death. These results show that 1-ACBP, B-ACBP and PBR play a vital role in the pathogenesis of oesophageal cancer as well as immunology. In conclusion, our results are the first to demonstrate the upregulation of 1-ACBP, B-ACBP and PBR in oesophageal carcinoma. The identification of PBR receptors in oesophageal cancer may have therapeutic implications leading to the possible use of PBR receptor antagonists.

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Conflict of interest. The authors declare that there are no conflicts of interest.

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