INTERNATIONAL JOURNAL OF Experimental Pathology

Int. J. Exp. Path. (2018), 99, 10–14

ORIGINAL ARTICLE

Immunohistochemical assessment of Survivin and Bcl3 expression as potential biomarkers for NF-κB activation in the Barrett metaplasia–dysplasia–adenocarcinoma sequence

Ignazio Puccio*, Saif Khan*, Adil Butt*, David Graham*, Vinay Sehgal*, Dominic Patel*, Marco Novelli†, Laurence B. Lovat*, Manuel Rodriguez-Justo† and Rifat A. Hamoudi*,‡

**Research Department of Tissue and Energy, Division of Surgery & Interventional Science, University College London, London, UK, †Research Department of Pathology, University College London, London, UK and ‡Sharjah Institute for Medical Research, College of Medicine, University of Sharjah, Sharjah, United Arab Emirates*

INTERNATIONAL JOURNAL OF EXPERIMENTAL PATHOLOGY

doi: 10.1111/iep.12260

Received for publication: 8 October 2017
Accepted for publication: 12 December 2017

Correspondence
Rifat A. Hamoudi
Wing 2.4
The Cruciform Building
Division of Surgery & Interventional Science
University College London
Gower Street
London WC1E 6BT
UK
Email: r.hamoudi@ucl.ac.uk

SUMMARY

Non-dysplastic Barrett's oesophagus (NDBE) occurs as a consequence of an inflammatory response triggered through prolonged gastro-oesophageal reflux and it may precede the development of oesophageal adenocarcinoma. NF-κB activation as a result of the inflammatory response has been shown in NDBE, but the possible mechanism involved in the process is unknown. The aim of this study was to assess, using immunohistochemistry, Survivin and Bcl3 expression as potential biomarkers for NF-κB activation along the oesophageal metaplasia–dysplasia–adenocarcinoma sequence. Survivin is an NF-κB-inducible anti-apoptotic protein, and Bcl3 is a negative regulator of NF-κB. There was progressive upregulation of Survivin expression along the oesophageal metaplasia–dysplasia–adenocarcinoma sequence. Bcl3 expression was upregulated in non-dysplastic Barrett's oesophagus, low-grade, high-grade dysplasia and oesophageal adenocarcinoma when compared to squamous group. The study shows the differential expression of Bcl3 between the squamous and Barrett's stage, suggesting that Bcl3 could be a surrogate marker for early event involving constitutive NF-κB activation. In addition, the study suggests that NF-κB activation may infer resistance to apoptosis through the expression of anti-apoptotic genes such as Survivin, which showed progressive increase in expression throughout the oesophageal metaplasia–dysplasia–adenocarcinoma sequence. This ability to avoid apoptosis may underlie the persistence and malignant predisposition of Barrett's metaplasia.

Keywords
Barrett's oesophagus, Bcl3, immunohistochemistry, inflammatory response, nuclear factor kappa B, Survivin

Introduction

Non-dysplastic Barrett's oesophagus (NDBE) is defined as the replacement of the normal squamous epithelium of the lower oesophagus by metaplastic columnar epithelium (Garud *et al.* 2010). It occurs as a consequence of the inflammatory response triggered through prolonged gastro-oesophageal reflux and precedes the development of oesophageal adenocarcinoma, which is a malignancy that has shown a rapidly rising incidence over the last two decades (Pohl & Welch 2005) and currently has the third worst survival rate amongst cancers in men in the United Kingdom (CRUK Oesophageal Cancer Statistics). Most cases of NDBE will not progress to oesophageal adenocarcinoma (OAC); however, its presence is an indication for further endoscopic biopsy surveillance (Pohl & Welch 2005).

Inappropriate activation of nuclear factor kappa B (NF-κB) has been linked to a variety of inflammatory and neoplastic conditions including colorectal, breast (Buckley *et al.* 2016) and prostate cancers (Mak *et al.* 2015). NF-κB

activation occurs in oesophageal cancer cell lines in response to acid and bile, and a marked overexpression of NF-κB in patients with NDBE or OAC has been reported (Abdel-Latif *et al.* 2004; O'Riordan *et al.* 2005). NF-κB is a ubiquitous transcription factor that regulates the activation of many NF-κB-inducible genes involved in pro-inflammatory responses, differentiation and growth (Baeuerle & Baltimore 1996; Chen *et al.* 1999). NF-κB resides in the cytoplasm of most cells in an inactive form as a heterodimer consisting of p65, p50 and RelA subunits complexed to inhibitory molecules Bcl3, IκBα, IκBβ, IκBγ, IκBε and IκBζ, which prevent the migration of the heterodimer to the nucleus (Yamamoto & Gaynor 2004).

Bcl3 is a key negative feedback regulator of the NF-κB pathway and is known to be overexpressed in different tumours such as colorectal (Saamarthy *et al.* 2015), breast (Cogswell *et al.* 2000), nasopharyngeal (Thornburg *et al.* 2003), endometrial (Pallares *et al.* 2004), prostate cancer (Ahlqvist *et al.* 2013) and cervical carcinoma (Zhao *et al.* 2016). In addition, Bcl3 was shown to be a prognostic biomarker for inflammatory diseases such as ulcerative colitis (Reissig *et al.* 2017). Survivin is an anti-apoptotic, NF-κB-inducible gene (Kawakami *et al.* 2005; Wang *et al.* 2010). It is involved in the control of proliferation and apoptosis in T-cell development (Xing *et al.* 2004) in various cancers such as myeloma (Locke *et al.* 2015), lung cancer (Li & Ding 2015) and ovarian cancer (Berinstein *et al.* 2015).

The aim of this study is to assess Bcl3 and Survivin expression in a series of biopsies spanning the oesophageal Barrett metaplasia–dysplasia–adenocarcinoma sequence highlighting their roles in the initiation and progression to oesophageal adenocarcinoma. Bcl3 expression would suggest a possible molecular mechanism of NF-κB activation involved in the initiation of the inflammatory process. Survivin expression would suggest a possible role in the progression to oesophageal adenocarcinoma.

Materials and methods

Patients and samples

A panel of formalin fixed paraffin-embedded (FFPE) oesophageal specimens (biopsies and endoscopic mucosal resections) of normal and pathological oesophagus was identified from the University College London Hospital upper gastrointestinal clinical database. Ethical approval was obtained from the UK Research Ethics Committee (15/YH/0311; 08/H808/8; 08/H0714/27).

Samples were selected from 74 independent patients containing, in order of disease severity; normal squamous tissue ($n = 12$; Sq), non-dysplastic Barrett's epithelium ($n = 16$; NDBE), low-grade dysplasia ($n = 10$; LGD), high-grade dysplasia ($n = 22$; HGD) and invasive oesophageal adenocarcinoma ($n = 14$; OAC). The samples chosen were of the highest pathological grade the patient had at the time of sampling. Sections were stained with haematoxylin and eosin (H&E) and the reported pathological grade confirmed by two expert GI pathologists (MN, MRJ). All cases in each group were homogenous for disease grade. Biopsies predominantly represented mucosal tissue, although some adenocarcinoma samples included submucosal tissue (e.g. endoscopic resection specimen).

Immunohistochemistry

Immunohistochemistry (IHC) for Survivin and Bcl3 proteins was carried out using the automated Bond-Max system (Leica Biosystems Ltd., Newcastle) using 4-μm FFPE sections. Antigen retrieval used Bond epitope retrieval solution 2 (EDTA based on pH 9.0) for 20 min prior to incubation with either Survivin [clone D-8 sc-17779 (Santa Cruz Biotechnology)] and Bcl3 (clone 1E8 Leica Biosystems Ltd) (1:150). Slides were counterstained with haematoxylin and mounted using DPX. IHC for Bcl3 demonstrated nuclear and cytoplasmic localization whilst Survivin demonstrated nuclear localization on examination of tonsil and diffuse large B-cell lymphoma sections as positive control.

Interpretation

Prior to inclusion in this study, all H&E-stained sections were reviewed by gastrointestinal histopathologists (MRJ and MN) to confirm the classification. All slides were scored by MN, MRJ using the Allred scoring system (Allred *et al.* 1998) to take into account heterogeneous staining. The Allred system scores IHC intensity positivity as negative (0), weak (1), moderate (2) and strong (3+). Separately scores the extent of biomarker positivity according to the proportion of tissues positively stained as negative (0), <1% (1), 1–10% (2), 11–33% (3), 34–66% (4) and 67–100% (5), and the final Allred score is the addition of these two scores. For Bcl3 staining, the nuclear and cytoplasmic intensity staining was added to give a final intensity score. Positive cases were defined as those staining 2+/3+ intensity in >10% of the pathology examined.

Statistical analysis

Multivariate analysis was carried out using the Kruskal–Wallis test across the five histological groups: squamous (Sq), non-dysplastic Barrett's oesophagus (NDBE), low-grade dysplasia (LGD), high-grade dysplasia (HGD) and oesophageal adenocarcinoma (OAC). Two-way comparisons between the groups were carried out using the Mann–Whitney *U*-test. $P < 0.05$ was taken to be statistically significant. All statistical analysis was carried out using SPSS (IBM, UK).

Results

Clinicopathological features of the patients

Tissue samples from 74 patients were analysed to provide an overview of the oesophageal Barrett metaplasia–dysplasia–adenocarcinoma sequence. The main clinical and

histological features of the 74 patients at diagnosis are detailed in Materials and Methods section and summarized in Table 1.

Bcl3 staining along the oesophageal metaplasia–dysplasia–adenocarcinoma sequence

Sections from patients along the oesophageal Barrett metaplasia–dysplasia–adenocarcinoma sequence were stained for Bcl3 using immunohistochemistry (Figure 1, a–e). Bcl3 was noted in both the cytoplasm and nucleus in all samples. Staining was scored using the semiquantitative Allred method to take into account the heterogeneity (Figure 2). Bcl3 staining did not change along the sequence, however when compared to Sq; NDBE, LGD, HGD and OAC demonstrated Allred mean fold change of 2.68, 1.82, 2.48 and 2.79 respectively. In addition, compared to Sq, there is evidence of upregulation of Bcl3 in NDBE ($P = 0.0006$), HGD ($P = 0.0022$) and OAC ($P = 0.0012$).

Table 1 Main clinical and histopathological features of the patients selected for this study to give an overview of the oesophageal Barrett metaplasia–dysplasia–adenocarcinoma sequence

Characteristic	Number	Percentage (%)
Age at diagnosis mean (range)	66 (18–84)	NA
Gender		
Male	16	20
Female	62	80
Histological grade		
Sq	12	15
NDBE	16	20
LGD	14	18
HGD	22	29
OAC	14	18

NA, not applicable; Sq, squamous; NDBE, non-dysplastic Barrett's oesophagus; LGD, low-grade dysplasia; HGD, high-grade dysplasia; OAC, oesophageal adenocarcinoma.

Survivin staining along the oesophageal metaplasia–dysplasia–adenocarcinoma sequence

After staining for Bcl3, sequential sections were stained with Survivin, which showed a nuclear distribution consistent

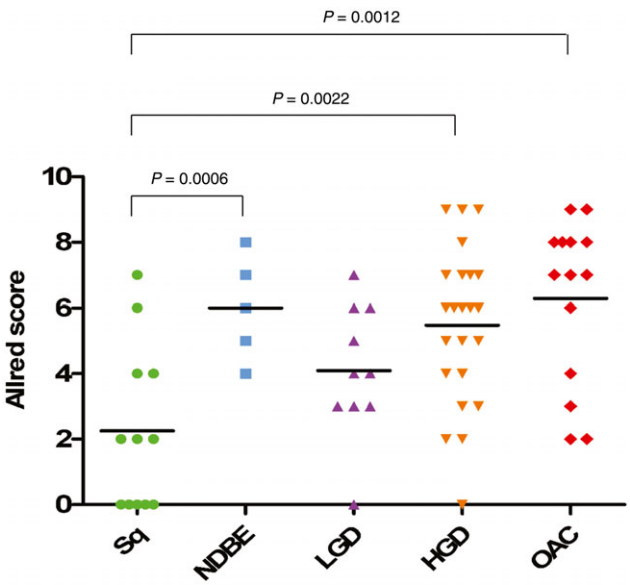

Figure 2 Allred Bcl3 staining scores for squamous (Sq), non-dysplastic Barrett's oesophagus (NDBE), low-grade dysplasia (LGD), high-grade dysplasia (HGD) and oesophageal adenocarcinoma (OAC). Mann–Whitney *U*-test was used for comparison between the groups. [Colour figure can be viewed at wileyonlinelibrary.com]

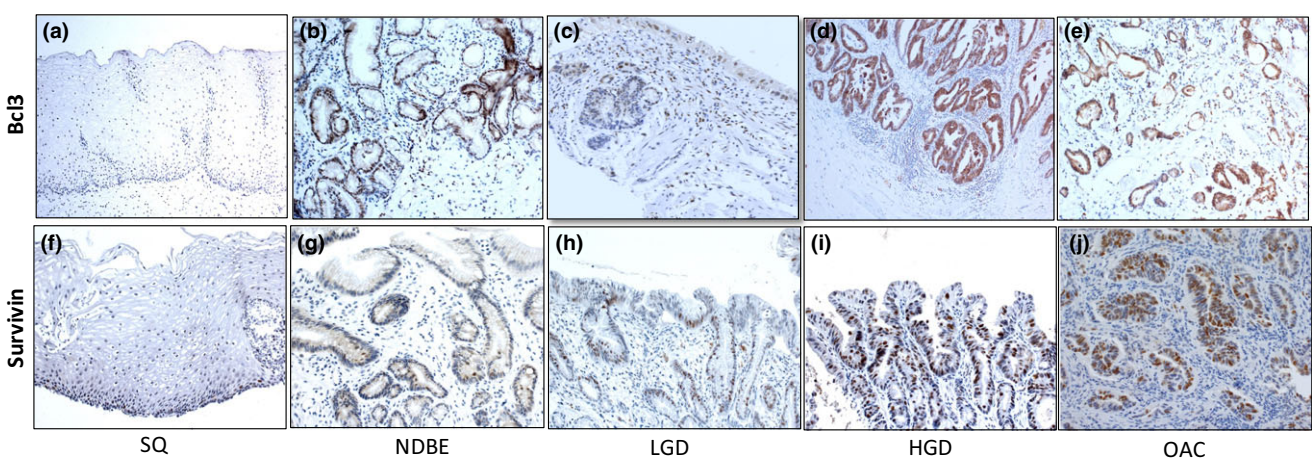

Figure 1 Oesophageal sections and immunohistochemistry for Survivin & Bcl3 protein expression (X20) using monoclonal antibody clones D-8 sc-17779 and 1E8 respectively. Sections were stained for either Bcl3 (a–e) or Survivin (f–j) (brown) and counterstained with haematoxylin (blue). A range of pathology is shown; SQ, squamous; NDBE, non-dysplastic Barrett's oesophagus; LGD, low-grade dysplasia; HGD, high-grade dysplasia; OAC, adenocarcinoma. [Colour figure can be viewed at wileyonlinelibrary.com]

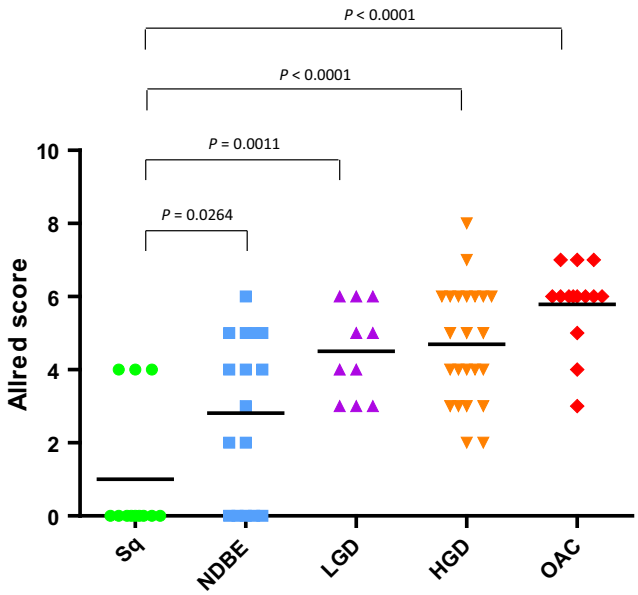

Figure 3 Allred Survivin staining scores for squamous (Sq), non-dysplastic Barrett's oesophagus (NDBE), low-grade dysplasia (LGD), high-grade dysplasia (HGD) and oesophageal adenocarcinoma (OAC). Mann–Whitney *U*-test was used for comparison between the groups. Mann–Whitney *U*-test was used for comparison between the groups, and Kruskal–Wallis test was used to carry out multivariate analysis across the groups. [Colour figure can be viewed at wileyonlinelibrary.com]

with the literature (Figure 1, f–i). Compared to Sq, all neoplastic tissues showed increased staining with Allred mean scores of NDBE 2.81 ($P = 0.0264$), LGD 4.50 ($P = 0.0011$), HGD 4.64 ($P < 0.0001$) and OAC 5.79 ($P < 0.0001$) respectively (Figure 3). All diagnostic categories showed a wide range of scores; however, there was clear evidence of a progressive increase in Survivin in NDBE through LGD, HGD to OAC ($P < 0.0001$, using the Kruskal–Wallis test).

Discussion

In this study, we have demonstrated for the first time the differential expression of Bcl3 between squamous and Barrett's mucosa and progressive increase in Survivin expression along the Barrett's metaplasia–dysplasia–adenocarcinoma sequence in the oesophagus.

The role of Bcl3 in the NF-κB pathway has previously been described (Cogswell *et al.* 2000), and our results show, for what we believe is the first time, the differential expression of Bcl3 in squamous and higher grade oesophageal neoplasia samples implying that Bcl3 may be involved in the constitutive activation of the NF-κB pathway, which is an early event in the metaplasia–dysplasia–carcinoma sequence (Abdel-Latif *et al.* 2004). Given that the NF-κB pathway is known to initiate the inflammatory response, we hypothesize that Bcl3 may be responsible for the inflammatory response that is known to occur in Barrett's oesophagus (Abdel-Latif *et al.* 2004; O'Riordan *et al.* 2005; Hormi-Carver *et al.* 2009). Interestingly, Bcl3 expression seems to suggest constitutive NF-κB activation, which occurs in NDBE and remains high in LGD, HGD and OAC compared to Sq. The results suggest that the cohort of normal squamous is likely to exhibit some abnormal phenotype suggesting that inflammation has already occurred probably due to acid or bile reflux. Also, the high Bcl3 expression in the latter stages of the metaplasia–carcinoma sequence compared to Sq suggests continuation in inflammatory signatures, probably due to the fact that chronic inflammation has developed at those stages.

Few studies have used immunohistochemistry of the NF-κB subunits such as p50 and p65 to show constitutive NF-κB activation; however, this has been shown to be unreliable because NF-κB subunits shuttle between the nucleus and cytoplasm in constitutively active NF-κB cells (Herkenham *et al.* 2011). In this study, we show that measuring the NF-κB inhibitors such as Bcl3 may be a more reliable measure of constitutive NF-κB activation.

Survivin expression progressively increases along the Barrett's metaplasia–dysplasia–adenocarcinoma sequence indicating that it could potentially be a useful biomarker for the progression to OAC. Survivin is an anti-apoptotic gene that is a member of the IAP family (Zhao *et al.* 2016). It is also NF-κB-inducible gene (Kawakami *et al.* 2005; Wang *et al.* 2010) and has been shown to be involved in proliferation and apoptosis during T-cell development (Song *et al.* 2008), both of which are important in the development of oesophageal adenocarcinoma.

Taken together, Bcl3 overexpression could be a surrogate biomarker for NF-κB activation in Barrett's epithelial cells leading to deregulation of NF-κB-inducible gene expression, inferring resistance to apoptosis through the activation of anti-apoptotic genes such as Survivin. This ability to avoid apoptosis may underlie the persistence and malignant predisposition of Barrett's metaplasia.

Conclusions

This study demonstrated for the first time the differential expression of Bcl3 between normal squamous mucosa and NDBE, indicating an early mechanism of NF-κB activation that might play a role in the initiation of inflammation in response to acid or bile reflux. In addition, the study showed that NF-κB activation may infer resistance to apoptosis through the activation of anti-apoptotic NF-κB-inducible genes such as Survivin, which is known to be involved in proliferation and apoptosis during T-cell development. Survivin showed progressive upregulation through the oesophageal metaplasia–dysplasia–adenocarcinoma sequence showing its importance in the progression to OAC. This ability to avoid apoptosis may underlie the persistence and malignant predisposition of Barrett's metaplasia. Therefore, Bcl3 can be used as early biomarker for of NDBE and Survivin can be used as biomarker for the progression of NDBE to OAC.

Contributors

RAH conceived the project. RAH, IP and MRJ collected the data, analysed the results and drafted the manuscript. All authors contributed to the final draft of the manuscript.

Funding

MR-J and MN are supported by the UCLH/UCL NIHR Biomedical Research Centre. We acknowledge Dangoor Education for their support towards this work.

Competing interests

None declared.

References

Abdel-Latif M.M., O'Riordan J., Windle H.J. *et al.* (2004) NF-kappaB activation in esophageal adenocarcinoma: relationship to Barrett's metaplasia, survival, and response to neoadjuvant chemoradiotherapy. *Ann. Surg.* **239**(4), 491–500.

Ahlqvist K., Saamarthy K., Syed Khaja A.S., Bjartell A. & Massoumi R. (2013) Expression of Id proteins is regulated by the Bcl-3 proto-oncogene in prostate cancer. *Oncogene* **32**(12), 1601–1608.

Allred D.C., Harvey J.M., Berardo M. & Clark G.M. (1998) Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod. Pathol.* **11**(2), 155–168.

Baeuerle P.A. & Baltimore D. (1996) NF-kappa B: ten years after. *Cell* **87**(1), 13–20.

Berinstein N.L., Karkada M., Oza A.M. *et al.* (2015) Survivin-targeted immunotherapy drives robust polyfunctional T cell generation and differentiation in advanced ovarian cancer patients. *Oncoimmunology* **4**(8), e1026529.

Buckley N.E., Haddock P., De Matos S.R. *et al.* (2016) A BRCA1 deficient, NFkappaB driven immune signal predicts good outcome in triple negative breast cancer. *Oncotarget* **7**(15), 19884–19896.

Chen F., Castranova V., Shi X. & Demers L.M. (1999) New insights into the role of nuclear factor-kappaB, a ubiquitous transcription factor in the initiation of diseases. *Clin. Chem.* **45**(1), 7–17.

Cogswell P.C., Guttridge D.C., Funkhouser W.K. & Baldwin Jr A.S. (2000) Selective activation of NF-kappa B subunits in human breast cancer: potential roles for NF-kappa B2/p52 and for Bcl-3. *Oncogene* **19**(9), 1123–1131.

CRUK oesophageal cancer statistics (http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/oesophageal-cancer) [http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/oesophageal-cancer]

Garud S.S., Keilin S., Cai Q. & Willingham F.F. (2010) Diagnosis and management of Barrett's esophagus for the endoscopist. *Therap. Adv. Gastroenterol.* **3**(4), 227–238.

Herkenham M., Rathore P., Brown P. & Listwak S.J. (2011) Cautionary notes on the use of NF-kappaB p65 and p50 antibodies for CNS studies. *J. Neuroinflammation* **8**, 141–148.

Hormi-Carver K., Zhang X., Zhang H.Y. *et al.* (2009) Unlike esophageal squamous cells, Barrett's epithelial cells resist apoptosis by activating the nuclear factor-kappaB pathway. *Can. Res.* **69**(2), 672–677.

Kawakami H., Tomita M., Matsuda T. *et al.* (2005) Transcriptional activation of survivin through the NF-kappaB pathway by human T-cell leukemia virus type I tax. *Int. J. Cancer* **115**(6), 967–974.

Li Y. & Ding J. (2015) Optimized generation of survivin-specific cytotoxic T lymphocytes against lung cancer. *Mol. Med. Rep.* **12**(2), 2169–2174.

Locke F.L., Menges M., Veerapathran A., Coppola D., Gabrilovich D. & Anasetti C. (2015) Survivin-specific CD4 + T cells are decreased in patients with survivin-positive myeloma. *J. Immunother. Cancer* **3**(20), 1–11.

Mak P., Li J., Samanta S. & Mercurio A.M. (2015) ERbeta regulation of NF-kB activation in prostate cancer is mediated by HIF-1. *Oncotarget* **6**(37), 40247–40254.

O'Riordan J.M., Abdel-Latif M.M., Ravi N. *et al.* (2005) Proinflammatory cytokine and nuclear factor kappa-B expression along the inflammation-metaplasia-dysplasia-adenocarcinoma sequence in the esophagus. *Am. J. Gastroenterol.* **100**(6), 1257–1264.

Pallares J., Martinez-Guitarte J.L., Dolcet X. *et al.* (2004) Abnormalities in the NF-kappaB family and related proteins in endometrial carcinoma. *J. Pathol.* **204**(5), 569–577.

Pohl H. & Welch H.G. (2005) The role of overdiagnosis and reclassification in the marked increase of esophageal adenocarcinoma incidence. *J. Natl Cancer Inst.* **97**(2), 142–146.

Reissig S., Tang Y., Nikolaev A. *et al.* (2017) Elevated levels of Bcl-3 inhibits Treg development and function resulting in spontaneous colitis. *Nat. Commun.* **8**, 15069. https://doi.org/10.1038/ncomms15069.

Saamarthy K., Bjorner S., Johansson M. *et al.* (2015) Early diagnostic value of Bcl-3 localization in colorectal cancer. *BMC Cancer* **15**(341), 1–9.

Song J., Lei F.T., Xiong X. & Haque R. (2008) Intracellular signals of T cell costimulation. *Cell. Mol. Immunol.* **5**(4), 239–247.

Thornburg N.J., Pathmanathan R. & Raab-Traub N. (2003) Activation of nuclear factor-kappaB p50 homodimer/Bcl-3 complexes in nasopharyngeal carcinoma. *Cancer Res.* **63**(23), 8293–8301.

Wang K., Brems J.J., Gamelli R.L. & Holterman A.X. (2010) Survivin signaling is regulated through nuclear factor-kappa B pathway during glycochenodeoxycholate-induced hepatocyte apoptosis. *Biochim. Biophys. Acta* **1803**(12), 1368–1375.

Xing Z., Conway E.M., Kang C. & Winoto A. (2004) Essential role of survivin, an inhibitor of apoptosis protein, in T cell development, maturation, and homeostasis. *J. Exp. Med.* **199**(1), 69–80.

Yamamoto Y. & Gaynor R.B. (2004) IkappaB kinases: key regulators of the NF-kappaB pathway. *Trends Biochem. Sci.* **29**(2), 72–79.

Zhao H., Wang W., Zhao Q., Hu G., Deng K. & Liu Y. (2016) BCL3 exerts an oncogenic function by regulating STAT3 in human cervical cancer. *Onco. Targets Ther.* **9**, 6619–6629.