Oesophageal adenocarcinoma is associated with a deregulation in the MYC/MAX/MAD network

JKR Boul\textsuperscript{1}, P Tanière\textsuperscript{1}, MT Hallissey\textsuperscript{2}, MJ Campbell\textsuperscript{2} and C Tselepis\textsuperscript{*,1}

\textsuperscript{1}CRUK Institute for Cancer Studies, University of Birmingham, Vincent Drive, Birmingham B15 2TH, UK; \textsuperscript{2}University Hospital Birmingham NHS Foundation Trust, Birmingham B15 2TH, UK; \textsuperscript{*}Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

The incidence of oesophageal adenocarcinoma has increased more rapidly over the past three decades than any other cancer in the Western world. Elevated expression of c-MYC has been demonstrated in oesophageal adenocarcinoma; however, the expression of other members of the MYC/MAX/MAD network has not been addressed. The aims of this work were to characterise the expression of c-MYC, MAX and the MAD family in adenocarcinoma development and assess the effects of overexpression on cellular behaviour. mRNA expression in samples of Barrett’s metaplasia and oesophageal adenocarcinoma were examined by qRT–PCR. Semi-quantitative immunohistochemistry and western blotting were used to examine cellular localisation and protein levels. Cellular proliferation and mRNA expression were determined in SEG1 cells overexpressing c-MYCER or MAD1 using a bromodeoxyuridine assay and qRT–PCR, respectively. Consistent with previous work expression of c-MYC was deregulated in oesophageal adenocarcinoma. Paradoxically, increased expression of putative c-MYC antagonists MAD1 and MXI1 was observed in tumour specimens. Overexpression of c-MYC and MAD proteins in SEG1 cells resulted in differential expression of MYC/MAX/MAD network members and reciprocal changes in proliferation. In conclusion, the expression patterns of c-MYC, MAX and the MAD family were shown to be deregulated in the oesophageal cancer model.

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**Keywords:** c-MYC; MAD; oesophageal adenocarcinoma; Barrett’s metaplasia

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**Correspondence:** Dr C Tselepis; E-mail: c.tselepis@bham.ac.uk

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is lost or mutated in human cancers (Gray et al, 1995; Bartsch et al, 1996; Kuczyk et al, 1998).

There have been a number of isoforms of MXI1 isolated from glioblastoma and haematological cells that suggest dominant negative activity; antagonising the normal activity of MXI1 and demonstrating differential expression (Engstrom et al, 2004; Kawamata et al, 2005). Of particular interest is MXI1-0, an isoform with an alternative first exon, which demonstrates elevated expression in glioblastoma, aberrant cellular localisation and fails to repress c-MYC dependent transcription (Engstrom et al, 2004).

While c-MYC expression has been widely characterised in oesophageal adenocarcinoma, expression of members of the MAD family of putative c-MYC antagonists is yet to be studied in any detail in gastrointestinal carcinogenesis. The gene-encoding MAD1 (MADX1) has previously been identified as one of six genes downregulated at the transcriptional level in oesophageal adenocarcinoma (Hourihan et al, 2003), however, this observation has not been confirmed and other members of the MAD family have not been addressed. We hypothesised that MAD expression would be repressed in the progression of oesophageal adenocarcinoma in a reciprocal pattern to c-MYC. Overexpression of MAD family proteins in other cell systems has demonstrated decreased cell proliferation and reduced apoptosis; therefore, we postulate that modulating the expression of MAD1 would have a similar effect in SEG1 cells and may represent a mechanism by which tumour growth could be retarded.

### MATERIALS AND METHODS

#### Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. Ethical approval for this study was approved by University Hospital Birmingham Trust (LREC 2003/331). All patients provided informed written consent.

#### Patient tissue

(i) Oesophageal adenocarcinoma resection specimens. Samples of oesophageal adenocarcinoma (n = 37), some of which were matched with Barrett’s metaplasia (BM) from the same resection specimen (n = 11), were collected during surgery and each tissue specimen was divided for RNA and protein extraction and pathological confirmation.

(ii) Endoscopic specimens. Samples of long segment (≥3 cm) Barrett’s metaplasia (n = 14), defined as columnar mucosa with intestinal type goblet cells, with matched normal oesophageal squamous mucosa (S) and gastric fundal mucosa (F) from the same patient were collected during routine endoscopy. Any patients with Barrett’s metaplasia with evidence of dysplasia or adenocarcinoma were excluded from this study.

(iii) Archived tissue. Paraffin sections of normal oesophagus (n = 10), normal gastric fundus (n = 10), Barrett’s metaplasia (n = 25), Barrett’s with dysplasia (BD, n = 20) and oesophageal adenocarcinoma (OAC, n = 25) were identified within an archived tissue bank, Department of Pathology, Queen Elizabeth Hospital Birmingham, and processed for immunohistochemistry.

#### Quantitative real-time RT–PCR

Quantitative real-time RT–PCR (qRT–PCR) was performed as described previously (Brookes et al, 2006) using 18S ribosomal RNA as an internal standard (Applied Biosystems, Warrington, UK) and sets of primers and 5’FAM 3’TAMRA probes listed in Table 1.

#### Table 1  Taqman probe and primer sequences used for qRT–PCR

| Gene     | Sequence                                                                 |
|----------|--------------------------------------------------------------------------|
| MYC      | Forward: TCAAGAGGTTGCCAGCTCTCC, Reverse: TCTTGCGACGAGTAGTCCT          |
| MXD1     | Forward: CACTTAAACCGGAGAAACAAATCC, Reverse: AGCGAAGATGAGCCGCTCTA       |
| MXI1 (Exon 1/2 boundary) | Forward: GGAGGAGGAGGATGTGACATG, Reverse: TCTGTGCTCCGGCTCAAC        |
| MXI1-0 (Exon 0/2 boundary) | Forward: CTACCTGGAGCAGATCGGAAAG, Reverse: TGGCCGATGGCGGGAAT        |
| MAX      | Forward: AGGTGAGGAGCGGAGAAGAG, Reverse: GTGCAATTTGATGAGGCGGTGTT |

#### Western blotting

Western blotting was performed as described previously (Brookes et al, 2006) with a mouse monoclonal antibody to c-MYC (1 μg ml⁻¹, clone 9E10, Applied Biosystems) or a rabbit polyclonal antibody to MAD1 (1 μg ml⁻¹, clone C-19, Autogen Bioclear, Calne, Wiltshire, UK) or MXI1 (1 μg ml⁻¹, clone G-16, Autogen Bioclear). A mouse monoclonal antibody to cytokeratin 19 (CK19) (0.5 μg ml⁻¹, clone A53-B/A2.26; Merck Chemicals Ltd, Nottingham, UK) was employed for normalisation of epithelial protein loading. Immunoreactive bands were subject to densitometry using a BioRad GS800 calibrated densitometer and Quantity One software. Where available a blocking peptide was used to confirm specific immunoreactive bands (MAD1 5 μg ml⁻¹; MXI1 5 μg ml⁻¹).

#### Immunohistochemistry

Immunohistochemistry was performed as previously described (Brookes et al, 2006) using microwave antigen retrieval and antibodies to c-MYC (2 μg ml⁻¹), MAD1 (2 μg ml⁻¹, clone FL–221, Autogen Bioclear), MXI1 (2.7 μg ml⁻¹) or MAX (50 ng ml⁻¹, clone C-17, Autogen Bioclear). Positive control tissue was included, and omission of primary antibody and, where available, blocking peptides (MXI1 13.5 μg ml⁻¹; MAX 250 ng ml⁻¹) were used as negative controls. The slides were scored by a previously described method for (i) intensity of staining (0 = negative, 1 = weak, 2 = moderate, 3 = intense) and (ii) percentage of epithelial cells staining (0 = 0–5%; 1 = 6–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–100%). These two values were multiplied to yield a final staining score of between 0 and 12. In addition, cellular localisation was assessed (Di Martino et al, 2006). All sections were scored independently by two observers.

#### Cell culture

The cell line SEG1 (Hughes et al, 1997; a kind gift of Dr David Beer, University of Michigan, Ann Arbour, MI) was routinely cultured in DMEM with 10% FCS supplemented with 100 U ml⁻¹ penicillin and 0.1 mg ml⁻¹ streptomycin (Invitrogen, Paisley, Renfrewshire, UK).
Cells were transfected with pcDNA3.1-MYCER, encoding a chimeric protein that consists of human c-MYC fused at its carboxyl terminus to the hormone-binding domain of a mutant mouse oestrogen receptor (Littlewood et al., 1995), pcDNA3-MAD1 or the corresponding empty vector using Lipofectamine and Plus reagent according to manufacturer’s instructions (Invitrogen). Twenty-four hours after transfection with c-MYCER, the fusion protein was activated by replacing the medium with 100 nM 4-hydroxytamoxifen (4-OHT) (or control medium). Cells were cultured for a further 24 h before cells processing for RNA or protein or up to 96 h for a proliferation assay.

**Bromodeoxyuridine incorporation assay**
A colorimetric cell proliferation ELISA was performed according to the manufacturer’s instructions (Roche Applied Biosystems, Burgess Hill, Sussex, UK). Briefly, cells were labelled with bromodeoxyuridine (BrdU) followed by fixation and incubation with anti-BrdU peroxidase, the immune complex was then detected using a 3,3,5,5-tetramethylbenzidine substrate reaction with the reaction product assessed at 370 nm.

**Statistics**
All data are presented as means ± 1 s.e.m. Statistical significance was calculated using paired t-test for mRNA analysis, Mann-Whitney test for analysis of immunohistochemical staining and unpaired Student’s t-test for in vitro data. Significance was accepted at *P* ≤ 0.05. All analyses were performed using SPSS version 10.0 (SPSS Inc., Chicago, Illinois, USA).

**RESULTS**

**mRNA expression of the MYC/MAX/MAD network in Barrett’s metaplasia and oesophageal adenocarcinoma**
Quantitative real-time RT-PCR was utilised to assess the expression of the mRNAs encoding c-MYC, MAD1, MXI1, MXI1-0 and MAX in normal epithelia, Barrett’s metaplasia and oesophageal adenocarcinoma specimens. This revealed that all the transcripts analysed were expressed at a higher level in oesophageal adenocarcinoma tissue than in matched normal gastric (Figure 1) and oesophageal (data not shown) controls. The only gene to be significantly altered in Barrett’s metaplasia in comparison with normal gastric mucosa was MXI1-0, the alternatively transcribed isoform of MXI1. When expression in adenocarcinoma was evaluated in comparison to matched Barrett’s metaplasia it was apparent that expression of MYC, MXI1 and MAX were significantly elevated in the malignant transformation of Barrett’s metaplasia.

**Expression of MYC and MAD family proteins in Barrett’s metaplasia and oesophageal adenocarcinoma**
Western blotting analysis was employed to confirm the alteration in expression at the protein level. In comparison to matched normal gastric controls the expression of c-MYC and MXI1 was significantly upregulated in Barrett’s metaplasia; conversely, the expression of MAD1 was significantly lower in the metaplastic lesion than in the normal mucosa (Figure 2). In accordance with the mRNA data c-MYC, MAD1 and MXI1 expression was significantly higher in oesophageal adenocarcinoma than in matched normal gastric controls. While a difference in MYC and MXI1 was demonstrated between Barrett’s metaplasia and adenocarcinoma at the level of mRNA, there was no significant alteration in protein expression in malignancy. However, while MXD1 expression was not altered at the transcript level, MAD1...
Protein was expressed more highly in adenocarcinoma than Barrett's metaplasia.

**Immunolocalisation of MYC/MAX/MAD network proteins in the progression to oesophageal adenocarcinoma**

Immunohistochemical staining was utilised to establish MYC/MAX/MAD network protein localisation in normal oesophageal and gastric epithelia, Barrett's metaplasia, dysplastic Barrett's epithelium and oesophageal adenocarcinoma (Figure 3). To allow semi-quantitative evaluation of protein expression, the epithelial component of each section was scored as described in the methods for intensity of immunoreactivity and percentage of epithelial cells stained (Di Martino et al, 2006) (Table 2).

**c-MYC** staining in native squamous oesophageal epithelium was confined to the nuclei of cells in the proliferative basal layer. No immunoreactivity was observed on sections of normal gastric fundus. Barrett's metaplasia exhibited weak to moderate nuclear staining; however, heterogeneity is suggested since staining was not evident in all glands within a single specimen. Dysplastic Barrett's glands demonstrated evidence of nuclear and cytoplasmic c-MYC expression. Immunoreactivity was widespread and intense in the majority of adenocarcinoma sections, indicating both nuclear and cytoplasmic expression in most cases (Figure 3). Semi-quantitative analysis suggested that c-MYC expression in dysplastic Barrett's mucosa and adenocarcinoma tissue was significantly higher than in normal oesophageal and gastric mucosae. Expression in Barrett's metaplasia was lower than in both dysplasia and adenocarcinoma but significantly higher than in normal stomach (Table 2).

Weak diffuse cytoplasmic MAD1 immunoreactivity was observed in normal oesophageal and gastric mucosae and Barrett's metaplasia. In squamous epithelial staining was localised to the suprabasal differentiated compartment of the epithelium, gastric mucosa also demonstrated evidence of nuclear immunoreactivity in some of the positively stained glands. Only 25% of Barrett's metaplasia displayed MAD1 immunoreactivity, similarly half of the examples of dysplastic Barrett's epithelium remained negative; immunoreactivity on the positive sections was indicative of increased cytoplasmic expression in both cases. Staining in adenocarcinoma was more evident, with sections demonstrating cytoplasmatic staining with intensity ranging from weak to strong (Figure 3). Semi-quantitative analysis indicated that expression in adenocarcinoma was greater than normal oesophageal and gastric epithelia and non-dysplastic Barrett's metaplasia (Table 2).

The majority of normal oesophageal epithelium sections did not display any MX11 immunoreactivity, in those that did staining was weak and localised to the nuclei and cytoplasm of the epibasal cells. In gastric fundus expression of MX11 was limited, with a small amount of weak cytoplasmic staining in some sections; the nuclei of the adjacent lymphoid cells stained positively for MX11. Immunoreactivity was evident in approximately one-third of Barrett's metaplasia specimens and demonstrated cytoplasmic expression of weak to moderate intensity. A pattern of expression was also evident in dysplastic tissue with staining consistently moderate in intensity. MX11 immunoreactivity in oesophageal adenocarcinomas was largely moderate in intensity and cytoplasmic in localisation (Figure 3). Epithelial immunoreactivity in tumours was significantly more intense and widespread than in normal oesophagus, stomach and benign and dysplastic Barrett's metaplasia (Table 2).

In normal oesophageal epithelium MAX immunoreactivity was moderately intense in the nuclei and weak in the cytoplasm of the suprabasal layers of the stratified epithelium. In normal fundal mucosa immunoreactivity was moderately intense and largely nuclear; Barrett's metaplasia however, displayed weak immuno-
reactivity that was predominantly cytoplasmic. Staining in Barrett’s dysplasia was also cytoplasmic but of moderate intensity. Adenocarcinoma sections displayed MAX immunoreactivity localised to both nuclei and cytoplasm (Figure 3). Semi-quantitative analysis suggested that MAX expression was significantly higher in adenocarcinoma than in normal mucosae and Barrett’s metaplasia (Table 2).

**Table 2** Semi-quantitative analysis of c-MYC network proteins in the oesophagus

| Protein | S       | F       | BM      | BD      | OAC     |
|---------|---------|---------|---------|---------|---------|
| c-MYC   | 1.50 ± 0.2 | 0.50 ± 0.3 | 2.42 ± 0.5* | 4.00 ± 0.7** | 7.70 ± 0.8** |
| MAD1    | 1.00 ± 0.4 | 1.25 ± 0.3 | 0.54 ± 0.3 | 1.81 ± 0.5 | 3.97 ± 0.6** |
| MXI1    | 0.50 ± 0.3 | 1.50 ± 0.4 | 0.78 ± 0.2 | 1.42 ± 0.6 | 4.06 ± 0.5** |
| MAX     | 4.33 ± 1.3 | 2.00 ± 1.2 | 1.90 ± 0.3 | 3.7 ± 0.7** | 8.00 ± 0.6** |

Immunohistochemistry for c-MYC, MAD1, MXI1 and MAX was performed on paraffin sections of normal squamous oesophageal mucosa (S), normal gastric fundus (F), Barrett’s metaplasia (BM), dysplastic Barrett’s (BD) and oesophageal adenocarcinoma (OAC). Immunoreactivity was scored as described in Materials and methods. The mean score is displayed ± 1 s.e.m. * denotes significant change in comparison to S; † denotes statistical significance compared to F; ‡ denotes significance relative to BM, (P<0.05).

**Figure 3** Immunolocalisation of MYC/MAX/MAD network proteins in Barrett’s metaplasia and oesophageal adenocarcinoma. Paraffin sections of normal oesophagus, normal gastric fundus, Barrett’s metaplasia and oesophageal adenocarcinoma were subjected to immunohistochemistry using antibodies designed against c-MYC, MAD1, MXI1 and MAX. Magnification × 40.

**Overexpression of c-MYCR and MAD1 in SEG1 cells**

An oesophageal adenocarcinoma cell line, SEG1, was transiently transfected with pcDNA3.1-MYCR, pcDNA3-MAD1 or the corresponding empty vectors. To activate c-MYC 4-OHT was applied to the cells 24 h after transfection. Significant overexpression of c-MYC and MAD1 was demonstrated at mRNA and protein level by qRT-PCR and western blotting, respectively 48 h following transfection (Figure 4A and B).

The expression of other members of the MYC/MAX/MAD network was assessed in transfected cells. MYCER expressing cells demonstrated elevated levels of the mRNA encoding MXI1-0 (P<0.001) (Figure 5A), whereas MAD1-overexpressing SEG1 cells demonstrated elevated MYC, MXII and repressed MAX expression (P=0.002, P=0.014, P=0.03, respectively) (Figure 5B). Activated SEG1-MYCR cells demonstrated significantly higher BrdU incorporation over 72 h than the untreated controls (107 ± 2%, P=0.024), conversely SEG1-MAD1 proliferated at a significantly slower rate than mock transfected controls (87 ± 2%, P=0.006). These overexpression studies were similarly reproduced in other
In light of the observations of reduced MAD1 expression in Barrett’s metaplasia and previous evidence of MXD1 repression in oesophageal adenocarcinoma (Hourihan et al., 2003), the trend towards upregulation of MAD1 in adenocarcinomas was not anticipated and would appear to be in conflict with the suggestion that MAD1 may have a role as a tumour suppressor (Ayer et al., 2004) and therefore may represent the overexpressed isoform MXI1. Similarly, MAD1 expression has been identified in benign and malignant murine skin tumours where the differentiation capacity was retained (Lymboussa-Kaki et al., 1996). In support of the observations made here, MAD1 expression has been identified in benign and malignant murine skin tumours where the differentiation capacity was retained (Lymboussa-Kaki et al., 1996).

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**DISCUSSION**

Progressive overexpression of MYC in the oesophageal metaplasia-dysplasia-adenocarcinoma sequence has been observed previously (Tselepis et al., 2003; Schmidt et al., 2007); prior to this study however, the expression of other members of the MYC/MAX/MAD network had not been studied in any detail in the oesophagus. In the only study in this area the gene encoding MAD1 was identified as one of six genes downregulated at the mRNA level in oesophageal adenocarcinoma when compared with normal squamous oesophageal mucosa (Hourihan et al., 2003). Expression of c-MYC and MAD in normal oesophageal epithelium was consistent with previous studies in tissues compartmentalised with respect to proliferation and differentiation (Larsson et al., 1994; Chin et al., 1995; Hurlin et al., 1995a). c-MYC was confined to the proliferative compartment, the basal cell layer, a pattern previously observed in epidermis and colonic epithelium (Osterland et al., 1990; Tselepis et al., 2003). MAD1 expression was evident in the suprabasal layers, in differentiating postmitotic cells consistent with the role of MAD1 in terminal differentiation (Vastrik et al., 1995; Lymboussa-Kaki et al., 1996; Queva et al., 1998).

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**Figure 4** Confirmation of expression in transfected SEG1 cells. SEG1 cells were transfected with pcDNA3.1/Zeolite-MYC or the corresponding empty vector (VO); in the case of MYCER the chimeric product was then activated by the addition of 4-OHT. (A) qRT–PCR was utilised to evaluate the expression of MXD1, MXI1, MXI1-0 and MAX mRNA in SEG1 cells transiently overexpressing MYCER. Relative gene expression is expressed as a ratio of SEG1-MYCER not stimulated using 4-OHT normalised to one. (B) Western blotting demonstrated the expression of the chimeric protein in SEG1-MYCER. A representative blot is also shown. Values represent the mean of two independent experiments each performed in triplicate ± 1 s.e.m. * denotes statistical significance (P<0.05).

**Figure 5** c-MYC network expression in SEG1 cells expressing exogenous MYC/MAX/MAD network proteins. (A) qRT–PCR was utilised to evaluate the expression of MXD1, MXI1, MXI1-0 and MAX mRNA in SEG1 cells transiently overexpressing MYCER. Relative gene expression is expressed as a ratio of SEG1-MYCER not stimulated using 4-OHT normalised to one. (B) Expression of MYC, MXD1, MXI1-0 and MAX mRNA was assessed in SEG1 cells transiently overexpressing MAD1. Relative gene expression is expressed as a ratio of mock transfected cells normalised to one. Data represent the mean of two independent experiments each performed in triplicate ± 1 s.e.m. * denotes statistical significance (P<0.05).

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putatively dominant negative isoform may be involved in the evolution of Barrett's metaplasia. Haematological tissue expresses three additional MX1 isoforms, lacking either the SID-encoding first exon, the DNA-binding domain encoded by exon 3 or both these domains, which could also be considered in the oesophagus. Like MX1I-0, these isoforms may potentially exert a dominant negative effect (Kawamata et al, 2005).

MAX expression is widely acknowledged to be ubiquitous and in excess of other network members, whose expression is more tightly regulated (Blackwood and Eisenman, 1991; Berberich et al, 1992). Therefore, it was interesting to observe an increase in its expression in oesophageal adenocarcinoma. It has been suggested that although MAX is likely to be in excess of c-MYC and other binding partners in most circumstances, it may be limiting during the period when c-MYC levels are sharply elevated during cell cycle entry (Walker et al, 2005). This raises the possibility that MAX may also be limiting in tumours where c-MYC levels are very high. The observed overexpression demonstrated here may act to overcome this limitation, and may limit the antagonistic relationship between c-MYC and the MAD proteins that are evidently coexpressed. Increased MAX expression has been linked to increased proliferation (Martel et al, 1995), but the mechanism by which MAX overexpression may occur in tumours is elusive.

Cytoplasmic expression of MYC/MAX/MAD network proteins may be an indication that, although overexpression occurs, these proteins may not be completely functional. It has been suggested that cytoplasmic localisation of c-MYC in colon cancer may be due to alterations in the C terminus of the protein, reducing the efficiency of nuclear targeting (Royds et al, 1992). While this may also be the case for other network members, evidence of murine mmp2-mediated cytoplasmic translocation of bad proteins (Yin et al, 1999) and cytoplasmic localisation of MXI-0 (Engstrom et al, 2004) lend other potential mechanisms to cytoplasmic expression. Consistent with in vitro and transgenic models of MYC amplification (Pelenargas et al, 2002), an increase in cellular proliferation was demonstrated following c-MYCER activation in SEG1 cells. c-MYC overexpression resulted in an increase in the expression of MXI-0 but had no effect on MXI1 suggesting alternative factors involved in their expression. Indeed Engstrom et al (2004) suggest that regulation of MXI1-0 may differ from the AP2-mediated repression of the MXI1 promoter (Benson et al, 1999). As MXI1-0 is thought to lack the antagonistic effects of MXI1, one may suggest that increased expression may facilitate the activity of c-MYC.

MAD1 overexpression in SEG1 cells resulted in a reduction in cellular proliferation at 72h in concordance with earlier studies associating MAD1 with reduced cell cycling and compromised tumourigenicity and colony formation (Chen et al, 1995; Wechsler et al, 1997). MAD1 overexpression has previously been associated with accumulation of cells in G0/G1 mediated in part by limited G1 phase cyclin/CDK complex kinase activity and moderate increases in the expression of CDK inhibitors p27KIP1 and p21CIP1. Although the implications made by these studies may be limited during tumourigenesis, they oppose the observation that MAD1 is overexpressed in oesophageal adenocarcinoma.

To conclude, the overexpression of c-MYC in Barrett’s metaplasia and oesophageal adenocarcinoma has been confirmed. Interestingly, this was accompanied by an overexpression of c-MYC antagonists MAD1 and MXI1 in many tumours. These observations demonstrate that the expression patterns and regulation of this network of proteins may be more complex than initially predicted. This may, in part, be due to the natural heterogeneity of tumour tissue, indeed localisation by immunohistochemistry demonstrated heterogeneous staining. Multiple isoforms of MXI1 have been identified in a variety of tissues, which raises the possibility that alternative isoforms of other network members might exist that interfere with their previously known functions. Therefore, it is worth considering that any MYC-targeted therapy approach may also need to take into account the effect of the MAD family proteins.

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Competing interests
None declared.
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