***c-myc* Oncogene product P62^{c-myc} in ovarian mucinous neoplasms: immunohistochemical study correlated with malignancy**

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SUMMARY The monoclonal antibody Myc 1-6E10 was used to determine the cellular distribution of the *c-myc* oncogene product p62^{c-myc} in 60 mucinous ovarian tumours. Three patterns of immunostaining were apparent: (i) nuclear staining alone; (ii) staining of the nucleus and basal cytoplasm; and (iii) staining of the entire cell. Of the 21 cases of mucinous cystadenoma, 11 showed nuclear staining alone, and a further case showed additional weak staining of the basal cytoplasm. Nuclear staining alone was not present in any of the 17 borderline mucinous tumours examined. Strong staining of the nucleus and basal cytoplasm was seen in 16 of these borderline cases, six of which also showed focal staining of the apical cytoplasm. All 22 cases of mucinous cystadenocarcinoma showed staining of the cell nucleus and entire cell cytoplasm. Focal staining of the apical cytoplasm in six of 17 borderline mucinous tumours produced a pattern of *c-myc* immunostaining similar to that of cystadenocarcinoma. Retrospective analysis of the clinical data showed that no significant differences between patients with borderline tumours of these two categories could be defined.

Although immunostaining with Myc 1-6E10 can be used in the categorisation of mucinous ovarian tumours, it is concluded that standard histological criteria are more accurate indicators of tumour behaviour than is an assessment of *c-myc* expression.

Oncogenes are highly conserved regions of the normal genome. A change in the coding or controlling regions of these genes has been implicated in the pathogenesis of neoplasia. The human *c-myc* oncogene is a cellular homologue of the avian *v-myc* gene found in some leukaemogenic retroviruses. The gene has been shown to have an important role in early embryogenesis,¹ the control of cell growth,² cellular differentiation^{3,4} and in tissue repair processes.⁵ Expression of the gene is therefore considered to be a major component in the regulatory processes associated with normal cell proliferation and differentiation. This view is reinforced by the demonstration of gene amplification or an increase in the level of gene product p62^{c-myc} in a variety of pathological states in which there is a perturbation of cell proliferation and differentiation, and these include several malignant⁶⁻⁸ and premalignant conditions.⁹

Ovarian neoplasia is an important cause of

mortality and morbidity worldwide and considerable effort has been expended in attempts to define useful prognostic indices, particularly in advanced ovarian cancer.¹⁰ Among these, clinical staging, the presence of ascites, and the volume of residual tumour following surgery have all been identified as important determinants of survival.¹¹ More recently, attempts have been made to examine the role of oncogene activation in tumours of the ovary^{12,13} and possible prognostic implications of such expression have been considered.¹⁴

Tumours derived from ovarian surface epithelium comprise nearly three quarters of all ovarian tumours and in excess of 90% of all ovarian malignancies. Of these some 20% show mucinous differentiation, most of which are benign tumours. Mucinous carcinomas of the ovary are relatively uncommon, accounting for only 5%–10% of ovarian malignancy.¹⁵ Borderline mucinous tumours are an additional tumour category characterised by architectural and cytological atypia in the absence of stromal invasion. Most of these tumours behave benignly but metastases undoubtedly

do occur, often a considerable time after definitive treatment has been performed.¹⁵

Whilst the role of *c-myc* has been studied in ovarian tumour cell lines¹² and in a single unspecified *in vivo* ovarian cancer,¹³ no attempt has previously been made to examine its role in this important category of ovarian tumour. This present study therefore investigates the expression of the gene in such tumours using the monoclonal antibody Mycl-6E10 in an immunohistochemical demonstration of the *c-myc* oncogene product $p62^{c-myc}$.

Material and methods

Tumour tissue blocks were obtained from 60 patients with mucinous ovarian tumours presenting between 1979 and 1985. Using standard histological criteria these tumours were classified into benign mucinous cystadenoma (n = 21), malignant mucinous cystadenocarcinoma (n = 22), and borderline mucinous tumours (n = 17). Borderline tumours were characterised by epithelial stratification and atypia, the presence of epithelial tufting, and evidence of mitotic activity in the absence of stromal invasion.

Case notes from 58 patients were available for review. The mean age at presentation of those patients with benign, borderline, and malignant tumours was 45.7, 47.0, and 61.7 years, respectively. Analysis of these data using the unpaired *t* test showed a significant difference between those with malignant disease and those with benign or borderline lesions (*p* < 0.01). Most patients presented with abdominal distension or pain, with no difference between those in the various disease categories.

In each case outcome of disease was determined by retrospective review of the case notes. The follow up interval was defined as the time interval between original tumour diagnosis and the last entry recorded in the case notes. The mean follow up interval was 51 months, and during this time 52% of those with malignant tumour had died of their disease. Most of these had stage III and IV disease as defined by FIGO (Fédération Internationale des Gynécologues et Obstétristes) staging. A single death occurred in the benign tumour cohort; this was due to non-gynaecological disease. No deaths occurred in the group of patients with borderline mucinous tumour nor was there evidence of tumour recurrence during the follow up interval.

Sections were cut at 4 μ m and placed on glass microscope slides and oven-dried at 50°C overnight. Sections were dewaxed and rehydrated using xylene and alcohols, and endogenous peroxidase was blocked by a 10 minute incubation in 0.5% hydrogen peroxide in methanol. The sections were washed briefly and incubated for two hours in Myc 1-6E10, diluted

1/10 000 in Tris-buffered saline with 1% bovine serum albumin and 0.25% Triton X-100 (pH 7.3). After further washing biotinylated sheep anti-mouse immunoglobulin was applied to the sections at a dilution of 1/100 and incubated for 60 minutes. After additional washes an incubation in Vectorstain ABC reagent (Vector Labs, England) was performed for one hour, followed by a final incubation in diaminobenzidine (100 mg in 200 ml 0.5% hydrogen peroxide solution) for 10 minutes. The sections were washed in tap water and counterstained in Harris's haematoxylin for 60 seconds.

All washes were performed using Tris-buffered saline containing 1% bovine serum albumin, and incubations were at room temperature.

Specificity of Mycl-6E10 has previously been investigated and its immunoreactivity found to be annulled by addition of the peptide used as immunogen in the production of the antibody.¹⁶

Results

Essentially three patterns of immunostaining with Myc 1-6E10 were shown. These were classified as nuclear staining alone, staining of the nucleus and basal cytoplasm, and staining of the entire cell. For the purpose of this study staining was classified as basal cytoplasmic if $p62^{c-myc}$ staining was restricted to the basal one third of the cell cytoplasm, and staining of the entire cell was $p62^{c-myc}$ positivity of nucleus, basal, and apical cytoplasm. Analysis of these results have shown a close correlation between the pattern of immunostaining observed and the tumour classification (table 1).

Of the 21 cases of benign mucinous cystadenoma examined, 19 showed positive staining of the nucleus (fig 1). Of these, 11 cases showed nuclear staining alone, while a further eight cases showed additional, although weak, staining of the basal cytoplasm. Basal cytoplasmic staining alone was seen in a single case and an additional case seemed to be entirely negative.

Nuclear staining alone was not seen in any of the 17 borderline mucinous tumours examined. Strong staining of the nucleus and basal cytoplasm was seen in 16 cases (fig 2), six of which also showed focal staining of the apical cytoplasm (fig 3). A further case failed to stain with the antibody.

Table 1 Immunostaining pattern

	Nucleus alone	Nucleus and basal cytoplasm	Entire cell
Benign	11	8	0
Borderline	0	10	6
Malignant	0	0	22

Fig 1 Benign mucinous cystadenoma showing positive nuclear staining of epithelium with Myc1-6E10.

Fig 2 Borderline mucinous tumour showing positive staining of epithelial nucleus and basal cell cytoplasm.

All 22 cases of mucinous cystadenocarcinoma showed weak staining of the nucleus and moderate to strong staining of basal and apical cytoplasm (fig 4). Staining intensity was noted to be generally reduced in poorly differentiated tumours.

All tumour categories showed focal expression of the *c-myc* gene in stromal fibroblasts, inflammatory cells (particularly lymphocytes and plasma cells), and vascular endothelial cells.

Focal staining of the apical cell cytoplasm in six of 17 borderline mucinous tumours produced a pattern of *c-myc* immunostaining similar to that of

cystadenocarcinoma, although the intensity of apical staining in the borderline tumours tended to be less. These cases were separately analysed with respect to patient's age, mode of presentation, and subsequent progress (table 2). Using these variables no difference between the two subgroups of borderline tumour could be defined.

Discussion

The role of cellular oncogenes in the development of ovarian neoplasia has received relatively scant attention. We have shown both expression of the *c-myc* gene in mucinous ovarian neoplasms and a correlation between the cellular distribution of the gene product p62^{c-myc} and the tumour category.

In non-neoplastic fibroblasts the induction of *c-myc* expression seems to accompany the release of such cells from Go and their re-entry into the cell cycle. This view has been confirmed in serum stimulated BT3 cells and in mitogen stimulated lymphocytes.¹⁷ In neoplastic states these high levels of gene expression persist throughout the cell cycle and this is reflected in raised *c-myc* mRNA transcript copy numbers in many tumours.¹⁸ In some instances evidence of gene

Table 2 Borderline tumours

	Entire cell staining (n = 6)	Nuclear and basal cytoplasmic staining alone (n = 10)
Mean (years) age at presentation (SEM)	41.7 (8.9)	49.9 (3.4)
Presenting complaint:		
Abdominal pain	3	4
Abdominal distension	2	5
Other	1	1
Survival (%)	100	100
Follow up (months)	49	53

Fig 3 Borderline mucinous tumour showing focus of epithelium in which the nucleus and entire cell cytoplasm stain positively with Myc1-6E10.

Fig 4 Malignant mucinous cystadenocarcinoma showing positive staining of epithelial nucleus and entire cell cytoplasm.

amplification is present, although this is not invariable, and indeed *c-myc* deregulation within neoplastic cells may also occur by gene mutation, gene rearrangement, or promotor insertion.¹⁹

Amplification of the *c-myc* oncogene has been shown in ovarian cancer,¹³ where DNA extracted from tumour cells showed gene amplification in three of 11 cases. DNA from tumour metastases and circulating blood leucocytes showed similar amplification in two of 11 cases. Amplification of the *c-myc* gene has also recently been shown in the ovarian tumour cell lines OS-4 and OvCa-1.¹² Where specifically examined, a close correlation has been shown between *c-myc* mRNA copy number and $p62^{c-myc}$ abundance as determined by immunocytochemistry.¹⁸ This correlation fully supports the use of Myc 1-6E10 in the assessment of *c-myc* gene activity in clinical material.

In the present study an assessment of $p62^{c-myc}$ abundance based on the cellular distribution of the gene product or staining intensity would be unjustified; within any one tumour category, however, such an assessment might be valid. Staining intensity seemed to vary most in the malignant mucinous tumours, where the least differentiated neoplasms

stained most weakly. Similar observations have been made in colonic^{18,20} and testicular cancer²¹ and would be consistent with a role for the gene product in cellular differentiation. The clinical importance of these observations is supported by the reported correlation between low concentrations of $p62^{c-myc}$ and poor disease outcome.²²

The overall distribution of $p62^{c-myc}$ shown in this study is similar to that reported in glandular neoplasms elsewhere in the body. Malignant neoplasms show both an increased volume of distribution of the gene product and an overall change in the cellular compartments containing the product, with a much greater emphasis on the cell cytoplasm.^{18,22} This may in part be related to mucin depletion in malignant tumours with a consequent loss of mucin vacuoles and thus a potentially greater volume for distribution of the gene product in the malignant cell. The cellular distribution of the gene product may also represent a genuine change in the biology of the malignant cell.

The *c-myc* gene product is normally associated with the cell nucleus, a feature related to the known DNA binding of the protein product.²³ Evidence from the present study shows that this nuclear association of

p62^{c-myc} is strongly maintained in benign mucinous tumours of the ovary but it becomes more tenuous in borderline tumours, until ultimately in malignant tumours most gene product appears in the cytoplasm of the cell. Predominantly cytoplasmic staining has also been reported in colonic adenomas,²⁰ while colonic carcinoma¹⁸ and untreated coeliac disease²⁴ show both a nuclear and cytoplasmic distribution of the gene product. The cytoplasmic distribution of p62^{c-myc} has provoked concern in previous studies, with a suggestion that this distribution may be artefactual. This concern has been countered, however, by the observation in familial polyposis that the staining pattern of normal colonic epithelium is quite different from that of the epithelium of adjacent adenomatous polyps in the same immunocytochemical preparation.²⁵ In normal mucosa nuclear staining is seen, but the adenomatous polyps show cytoplasmic staining. Identical patterns of immunostaining were also seen using the alternative antibodies Myc-CT14 and *pan-myc*. These antibodies recognise different epitopes on the *c-myc* gene product from that recognised by Myc 1-6E10. It seems likely, therefore, that the differing patterns of gene product distribution do indeed reflect a modification of the biology of the neoplastic cell.

This study has shown that borderline tumours of the ovary display a pattern of immunostaining which in most cases seems to be distinct from either benign or malignant tumour categories. Six of the 17 cases of borderline tumour, however, showed a pattern of staining focally identical with that seen in malignant tumours. Subsequent analysis of these cases has not shown any difference in biological behaviour of these tumours when compared with the remaining borderline cases. In view of the occasional late recurrence of borderline mucinous tumours these cases are the subject of a further prospective study. As the pattern of immunostaining alone might suggest a more aggressive clinical course than was in fact the case we conclude that standard histological criteria are more accurate indicators of tumour behaviour than is an assessment of *c-myc* expression. Recent work on the *ras* gene in advanced ovarian cancer has shown a similar poor correlation, not only between gene expression and clinical outcome, but also between expression and histological grade and ploidy.^{14,26}

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