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A QUANTITATIVE MULTIPARAMETRIC CYTOCHEMICAL ANALYSIS OF SMALL CELL CARCINOMA OF THE LUNG

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ABSTRACT

In general lung cancer has become the leading cause of cancer mortality in the Western World. However, geographical variations in lung cancer incidence can be seen worldwide as well as at a regional level within the UK, with Merseyside having the highest incidence and mortality. Many previous studies have subjectively assessed two-dimensional histological sections of SCLC with different immunocytochemical markers. Therefore, this study was initially designed to extract single whole tumour cells from routinely processed small cell lung cancer (SCLC) samples and to use immunocytochemical techniques on the cell monolayer produced. However, immunostaining was found to be not possible on these single cells. Therefore, serial histological sections were studied from 172 SCLC cases using immunocytochemical markers to cell proliferation antigens, oncoproteins and tumour suppressor proteins, as well as several cytochemical techniques. The immunostaining results were then assessed subjectively and fifty one cases were then selected on the basis of sample size and morphological preservation to be assessed objectively using a computerised digital image analysis system. However clinical data was only available on thirty one of these cases.

After detailed statistical analysis of the results a panel of five markers (P53, Mib1, PCNA, NM23, CMYC) was shown to be of possible significance in predicting patient survival. Also several markers (NM23, BCL2, P53, Mib1) showed a difference in expression between the different histological sub-types of SCLC. For markers such as NM23 this is the first study in SCLC, whereas for markers such as BCL2 it is the first *quantitative* study in SCLC.

The results generated and the conclusions reached by this study provide many directions for further work to take, in terms of studying different marker combinations, further quantitation and studying genes directly rather than gene products.

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1. AIMS AND OBJECTIVES

Human small cell lung cancer (SCLC) is an extremely aggressive disease which accounts for approximately 25% of all lung cancer cases and is often extensively metastasized at the time of diagnosis. Due to the aggressive nature of the tumour any information that may contribute towards a greater understanding of the tumour biology of this form of neoplasia would be useful, as a better understanding of the biological nature of a tumour may lead to improvements in treatment and management.

Initially, this study looked at developing a reliable and reproducible method for obtaining single whole cells from formalin-fixed, paraffin-embedded SCLC tissue. Once this method had been developed it was further intended to immunostain the whole cells obtained for various different oncoproteins and cellular proliferation markers. The results obtained were also to be compared to haematoxylin and eosin stained histological sections for comparison, so that the extra-cellular matrix could be seen and the cell monolayer results put into context in relation to the actual tumour specimen used.

Eventually a reproducible technique was developed to extract single whole cells from routinely processed tissue, as well as a method of removing background debris for accurate quantitation. However, it was not found possible to reliably immunostain the cells thus obtained and this remained the case regardless of the permutation of modifications applied to the original technique.

The study then reverted to a more conventional histological format, whereby a large series of SCLC serial histological sections were immunostained for a range of immunocytochemical markers. Following subjective analysis a proportion of these cases were then selected, on the basis of morphological preservation, for objective analysis using a digital image analysis system. This data was then subjected to statistical analysis. As the results were analysed it became apparent that a panel of immunocytochemical

markers was emerging that could possibly provide useful information regarding survival prediction.

To summarize, this study was originally aiming to develop a novel technique for isolating single whole tumour cells from routinely processed human SCLC tissue (with minimal background contamination) and to immunostain the cells obtained. However, after exhaustive technical modifications it was found to be not possible to reliably immunostain the single cell monolayers obtained. A serial section histological approach was then taken, using the same range of immunocytochemical markers proposed for use on the cell monolayers. After initial subjective analysis, followed by objective quantitation a panel of markers emerged that may provide useful survival information. This was a useful initial study combining immunocytochemical staining with digital image analysis for the objective quantitation of results.

2. INTRODUCTION

2.1 GENERAL

The existence of cancer has been recognized for over 200 years and is now even thought to have been known to the early Egyptians. The actual term "cancer" refers to a collection of different diseases that generally at least have the common feature of uncontrolled growth. Cancers are generated by a multi step process known as carcinogenesis, which results from the accumulation of errors in vital regulatory pathways. Of all the types of human neoplasia lung cancer has the highest mortality rate, with 1992 USA statistics quoting less than 15% of patients surviving for five years after diagnosis (Boring, Squires and Tong, 1993). However, although lung cancer is the most fatal form of neoplasia it does not necessarily have the highest incidence, as breast cancer and prostate cancer are currently the most prevalent in women and men respectively in the Western world. However, there is considerable regional variation of lung cancer incidence even within the UK with areas such as East Anglia having a lower than average incidence and Merseyside having a much higher than usual incidence. In fact, the Merseyside lung cancer incidence figures are 30% above the average for men and 40% above the average for women (personal communication from Mr. R. Donnelly, Head of the Roy Castle Cause For Hope Foundation, Liverpool).

Generally lung cancers are categorized into two main groups, reflecting their biology and management; namely non-small cell lung cancers (NSCLC) and small cell lung cancers (SCLC), with further sub-classification according to 1981 World Health Organization (WHO) guidelines. Although due to the heterogeneous nature of lung cancers any classification system must be less than absolute. NSCLC account for approximately 75% of all lung cancer cases and the major subtypes are squamous cell carcinomas and adenocarcinomas. These tumours have a five year survival rate of roughly 10 to 20%. SCLC account for approximately 25% of lung cancer cases and have a five year survival rate of just 2 to 5%, with the average patient surviving for approximately one year after diagnosis (Richardson and Johnson, 1993). The high mortality rate is mainly due to the disease being at a late stage at the time of diagnosis, and as a result the treatment required is usually radical and rarely effective. If the preferred chemotherapy/radiotherapy combination is effective, the tumour is still likely to recur and it will then resist any further therapy.

One of the most important clinical features of SCLC is it's tendency for early and widespread dissemination via metastasis, resulting in the formation of secondary neoplasms at distant sites within the body such as the brain. Due to the highly aggressive nature of SCLC and the comparatively few studies carried out exclusively on this type of tumour (usually due to lack of material) only SCLC were chosen for inclusion within this study. In order to metastasize, tumour cells must have the ability to degrade extracellular matrix structures including the basement membrane (which is characteristic of SCLC). They must also be able to evade host immunosurveillance and to eventually form an autonomous lesion at a secondary site (Egan, Wright and Greenberg, 1991). Several proteases have been implicated in the degradation of the extracellular matrix, including plasminogen activators, cathepsins and certain metalloproteinases (MacDonald and Steeg, 1993). However, if a primary or metastatic tumour is to exceed a few millimetres in size it must develop it's own vascular network via angiogenesis (formation of new blood vessels), so this process is critical to metastatic progression. Although aggressive primary tumours such as SCLC shed many cells, less than 0.01% of these will ultimately initiate successful metastatic colonies (Liotta and Stetler-Stevenson, 1990).

Histologically, SCLC are characteristically composed of uniform, small, round cells classically expressing neuroendocrine tumour antigens, and often having electron dense neurosecretory granules (Russell, O'Mara and Raghaven, 1990). Due to their neuroendocrine properties SCLC are often referred to as tumours of the diffuse neuroendocrine system (DNES), and often produce ectopic peptide hormones including adrenocorticotrophic hormone (ACTH), antidiuretic hormone (ADH) and calcitonin. However, since the identification of the DNES the term "ectopic" is no longer appropriate, though still commonly used, as cells of the DNES normally produce peptide hormones.

SCLC are often also staged by pathologists according to the extent of the tumour as either limited stage or extensive (Quoix, Charloux, Pepin and Pauli, 1993; Fraire, Johnson, Yesner, Zhang, Spjut and Greenberg, 1992; Souhami and Law, 1990; Rawson and Peto, 1990). Also, tumours may be sub-classified on the basis of histological subtype as either oat cell (more aggressive) or intermediate cell type (less aggressive), although this classification is

somewhat controversial with studies both supporting it (Korkolopoulou, Oates, Crocker and Edwards, 1993; Deb, Jackson, Subler and Martin, 1992; World Health Organization, 1982) and opposing it (Fraire et al, 1992; Bepler, Neumann, Holle, Havemann and Kalbfleisch, 1989; Hirsch, Matthews, Aisner, Campobasso, Elema, Gazdar, Mackay, Nasiell, Shimosato, Steele, Yesner and Zettergren, 1988). However, as far as this particular study was concerned staging information was absent but data on histological subtype was available, so this was used for further sub-classification.

The vast majority of human SCLC specimens are available only as archival material. So, in order to evaluate the potential value of immunocytochemical markers in SCLC it was essential that a large number of cases be initially studied, and therefore the main material used for this research was formalin-fixed and paraffin wax-embedded (i.e. routinely processed). Thus, this survey is a retrospective study of biopsy material generously supplied by Dr. Colin Clelland of Nottingham City Hospital. The majority of histological and immunocytochemical studies performed generally have their results assessed subjectively using a semi-quantitative scoring system. Part of the aim of this study was to carry out extensive quantitative work on routinely processed tissue using a digital image analysis system. The major limitation of using histological sections for quantitative evaluation is that invariably whole, complete cells cannot be studied due to the very nature of the sectioning process, and this has to be taken into account when interpreting results. Therefore, part of this work involved the development of a procedure to obtain single whole cells from formalin-fixed, paraffin wax-embedded biopsy material. Also, these cells were to be clearly morphologically recognizable tumour cells of direct relationship to those observed in the histological material. However, the cell monolayers obtained proved unsuitable for immunocytochemical staining, so a conventional histological serial sectioning approach was taken.

As well as assessing the value of quantitative multiparametric analysis, it was also envisaged that the results would provide useful information regarding the fundamental cell biology of human SCLC. The majority of the work carried out was a retrospective study of routinely processed SCLC material obtained from patients at Nottingham City Hospital between 1988

and 1995 (inclusive).

Markers were selected based on results obtained from tumours of different human tissues, or as a follow up to preliminary work already carried out on lung cancers. The selected markers were investigated using immunocytochemical staining (as outlined in section 2.2) which allowed a broad range of markers to be assessed within a standardised system, and can also be used routinely. It was essential to develop reliable and consistent immunocytochemical procedures for the demonstration of candidate SCLC markers, and to ensure that these techniques were optimized for both subjective and objective assessment. The candidate SCLC markers selected fell into one of three general categories - (i) Cell proliferation markers, (ii) Oncogenic proteins, or (iii) Tumour suppressor gene proteins. These are discussed in more detail at a later stage. The results obtained were first assessed subjectively and then selected cases were quantitated objectively using a microscope-based, computer-assisted image analysis system. The quantitative results obtained were then subjected to critical statistical analysis in order to determine the most useful marker(s) in the diagnostic and prognostic assessment of SCLC.

More information about the immunocytochemical procedures, candidate markers and the image analysis system used are given below.

2.2 PRINCIPLES OF IMMUNOCYTOCHEMISTRY

The definition of immunocytochemistry is :

> *"A technique for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions, the site of antibody binding being identified either by direct labelling of the antibody or by use of a secondary labelling method."*
> (Bancroft and Stevens, 1990).

There are numerous immunocytochemical techniques which may be used to localize antigens, with varying degrees of sensitivity and specificity. These include the direct method, the two-step indirect method, the protein A method, the peroxidase-anti-peroxidase method, the

avidin-biotin method and the immunogold method. The streptavidin-biotin complex technique (Hsu, Raine and Fanger, 1981) was chosen for this study as it is a highly sensitive, reliable and commonly used technique and it does not have a black end-reaction product, so a positive result will not be confused with carbon deposits which are prevalent in human lung tissue. The principles of the procedure are outlined in detail within this section.

Avidin is a basic glycoprotein of molecular weight 68 kiloDaltons (kD) which has an affinity for the small (244D), water-soluble vitamin biotin (vitamin H). Various methods have been devised to exploit the high efficiency and specificity of the avidin-biotin reaction. It is possible to conjugate up to 150 biotin molecules to one antibody molecule, so that the biotinylated antibody can bind to more than one avidin molecule (Robinson, Ellis and Maclennan, 1990) thereby enhancing the sensitivity of the reaction. However, avidin has two distinct disadvantages for use in immunocytochemistry. It has a high isoelectric point of approximately 10 and therefore is positively charged at neutral pH, so it may bind non-specifically to negatively charged structures such as the nucleus. The other problem is that as avidin is a glycoprotein it reacts with molecules such as lectins via the carbohydrate moiety, both of these difficulties lead to a reduced specificity of the reaction. These two problems are overcome with the substitution of streptavidin for avidin. Streptavidin is a protein of molecular weight 60kD isolated from the bacterium *Streptomyces avidinii* and, like avidin, has four high affinity binding sites for biotin. Also, streptavidin has an isoelectric point close to neutral pH, and as it is not a glycoprotein it does not bind lectins (Beesley, 1993).

Various labels are available for the visualisation of the antigen-antibody reaction and these include fluorescent labels, colloidal metals and enzymes. Immunofluorescent techniques were the original labelling methods used in immunocytochemistry, with fluorescein being currently the most widely used fluorochrome. However, for retrospective studies the disadvantages definitely outweigh any advantages of this technique, a major problem being fixation-induced background staining, which is why this technique is usually only used on fresh-frozen tissue. Other problems include the non-permanence of the fluorescein label due to it's instability and limitations when counterstaining. Colloidal gold is the most popular metal label in use and is enhanced using silver in the immunogold-silver staining procedure,

which produces a black end-reaction product. However, any technique that produces a black reaction is unsuitable for work in human lung, this is due to the presence of carbon deposits within lung biopsies. The carbon is due to industrial pollution and is invariably present to some degree as a black deposit across the section, making the distinction between the carbon and a black positive reaction unreliable and highly subjective.

Enzymes are the most widely used labels in immunocytochemistry because they produce a stable coloured reaction product suitable for light microscopy. Horseradish peroxidase (HRP) is the most commonly used immunocytochemical enzyme label in combination with the chromogen DAB (3,3'-diaminobenzidine tetrahydrochloride) which produces an insoluble, dark brown reaction end-product. However, this colour can still prove difficult to distinguish from carbon deposits in the lung. Also, the removal of endogenous peroxidase can be problematic and requires the use of a methanol-hydrogen peroxide mixture for removal. Alkaline phosphatase is the most widely used alternative to HRP and can be used with a variety of chromogens, usually giving a red or blue colour which provides a good contrast to any carbon deposits. Also, the removal of endogenous enzyme is easily achieved by adding levamisole to the developing solution (although this will not block the small intestine isoform of alkaline phosphatase). As alkaline phosphatase can be used to produce various chromogenic reactions the four most frequently used, non-commercial systems were evaluated for use within this study. The chromogens evaluated were - fast red, hexazotised new fuchsin, fast blue and tetrazolium, and these are discussed in detail in the methods section (3.1). As some of these chromogens are reported to be soluble in xylene, various mounting media were also evaluated, namely D.P.X., PVA, Farrant's medium and "Aquaperm".

Once the histological sections have been taken to water (i.e. the wax has been removed) then the first step for immunocytochemistry is to flood the sections with diluted normal animal serum (generally rabbit for monoclonal antibodies, and non-rabbit for polyclonal antibodies) which effectively binds to any free immunoglobulins within the tissue and blocks protein binding groups, so preventing them from interfering with the immunocytochemical reaction and causing non-specific background staining. Then a primary antibody specific for the

antigen under investigation is applied to the tissue section (mouse for monoclonal antibodies, non-mouse for polyclonal antibodies). This is followed by the addition of a biotinylated secondary antibody that is specific for the primary antibody. The avidin-biotin complex that has been allowed to pre-form for 30 minutes at room temperature is then applied. Careful stoichiometric control ensures that binding sites remain free to bind with the biotinylated antibody, which allows the pre-formed complex to bind and results in a very high signal at the antigen binding site. The enzyme bound to the avidin-biotin complex is then developed so that the label can be visualized and quantitated. Generally, a nuclear counterstain (e.g. haematoxylin) is also applied so that the location of the antigen within a cell can be determined. It is very important that extensive washes in buffer are performed between each stage to ensure that the previous reactant is totally removed before the next stage is begun. Endogenous enzyme activity must also be blocked at some stage if an enzyme label is being used. For peroxidase labels the sections are treated with a hydrogen peroxide solution at the beginning of the procedure, whereas for alkaline phosphatase systems the levamisole block is added to the substrate solution used to visualize the label towards the end of the technique.

However, the immunocytochemical detection of an antigen can be influenced by many variables, including the absolute level of antigen, affinity of the antibody for the antigen, duration of the incubation, sensitivity of the detection system and the consequences of fixation. Also, the signal given in an immunohistological assay is not linear with the antibody concentration (or with any other variable), and with a given set of conditions there will be a specific threshold below which no signal is obtained (Hall and Lane, 1994).

The major artefact induced by fixation, as far as immunocytochemistry is concerned, is the masking of tissue antigens. Time, temperature, nature of the fixative used, fixative concentration and the availability of nearby proteins to be cross-linked are the variables related to the extent of masking. Proteolytic enzymes such as trypsin, pepsin and pronase are well known agents that can restore the accessibility of antibodies to epitopes previously masked by fixation (Huang, Minassian and More, 1976). However, enzymes have a limited range of effectiveness in paraffin sections and have been reported to possibly only break surface loops (Cattoretti, Pileri, Parravicini, Becker, Poggi, Bifulco, Key, D'Amato, Sabattini,

Feudale, Reynolds, Gerdes and Rilke, 1993). As a result heat-mediated antigen retrieval is now often the method of choice, either using microwaves or autoclaving ("pressure cooking").

Microwave oven heating of sections in heavy metal salt solutions has been found to increase the immunocytochemical detection of antigens (Shi, Key and Kalra, 1991). However, as pH is now thought to be the most important property of the buffer used (Shi, Imam, Young and Cote, 1995), then buffers such as citrate can be used instead of heavy metal salts so reducing the problem of toxicity. Protein denaturation is thought to be induced by the heating effect of the microwave oven. However, although microwave oven heating is an improvement in antigen retrieval compared to enzymes, the most appropriate buffer (and it's pH) and the optimum irradiation time have to be determined for each antibody under investigation (Evers and Uylings, 1994).

Another novel technique that is reported to be possibly more effective than microwave oven heating is wet autoclave pretreatment ("pressure cooking"), which involves the immersion of dewaxed slides into superheated citrate buffer. It has been proposed that the presence of citrate ions at the temperature of superheated steam (120°C to 130°C) is necessary for the antigen unmasking (Bankfalvi, Navabi, Bier, Bocker, Jasani and Schmid, 1994). Another author reports that further protein denaturation together with the rupture of some of the aldehyde cross-links is the mechanism of action (Norton, Jordan and Yeomans, 1994). However, an important point to bear in mind concerning antigen retrieval is that because it markedly alters antigen detection thresholds, it may lead to situations where many cells become falsely positive (McKee, Hobbs and Hall, 1993), so affecting the results obtained.

In order for the immunocytochemical technique to be as sensitive as possible, all the assay parameters need to be optimized. This means that for each monoclonal and/or polyclonal antibody being used the optimum antibody concentration, optimum antibody incubation time and incubation temperature must all be determined. Also, the most appropriate form of antigen retrieval should be determined along with exposure times, concentrations and buffers to be used. Steps should also be taken to ensure that reagents do not evaporate off the

sections during incubation (this is likely with the small quantities being used) and that the washing steps are extensive enough to remove all unbound reagent before the next step.

A problem at any stage in the immunocytochemical procedure (especially the early stages) can lead to false negative or positive results or high background staining levels, making analysis impossible. In order to ensure that the technique is running correctly each time it is performed control sections should always be included. These should consist of two histological sections of tissue that is known to be positive for the target antigen, one of which is taken through the whole technique normally (positive control) and one of which has no primary antibody applied (negative control) but is otherwise treated as the positive control and the test sections. The use of these controls each time ensures that the results obtained are valid and reproducible, and goes some way towards providing an element of inter-assay quality control.

2.3 CELL PROLIFERATION

Cell proliferation is one of the most fundamental of biological processes (Hall and Levinson, 1990). The control of proliferation, like other cellular responses, depends upon interlinked systems of intracellular communication. The multiple elements that complete the regulatory pathways collectively allow fine control of cell proliferation and provide a system of checks and balances that is a barrier to disordered growth. The concept of the cell cycle and it's division into several phases was introduced by Howard and Pelc in the 1950's. The diagram below outlines the various stages. After M (mitosis) phase the daughter cells enter G_1 (first gap) phase, the length of which varies according to the tissue type. They then enter S (synthetic) phase where DNA replication occurs, and this is followed by a second gap (G_2) phase before cells divide again. G_0 is effectively a phase where cells can rest before re-entering the cell cycle.

The time between the two mitoses is referred to as the cell cycle time and varies depending on the length of the G_1 phase. G_1 is also where the most regulation occurs and where growth factors and proliferation inhibitors act (Quinn and Wright, 1990).

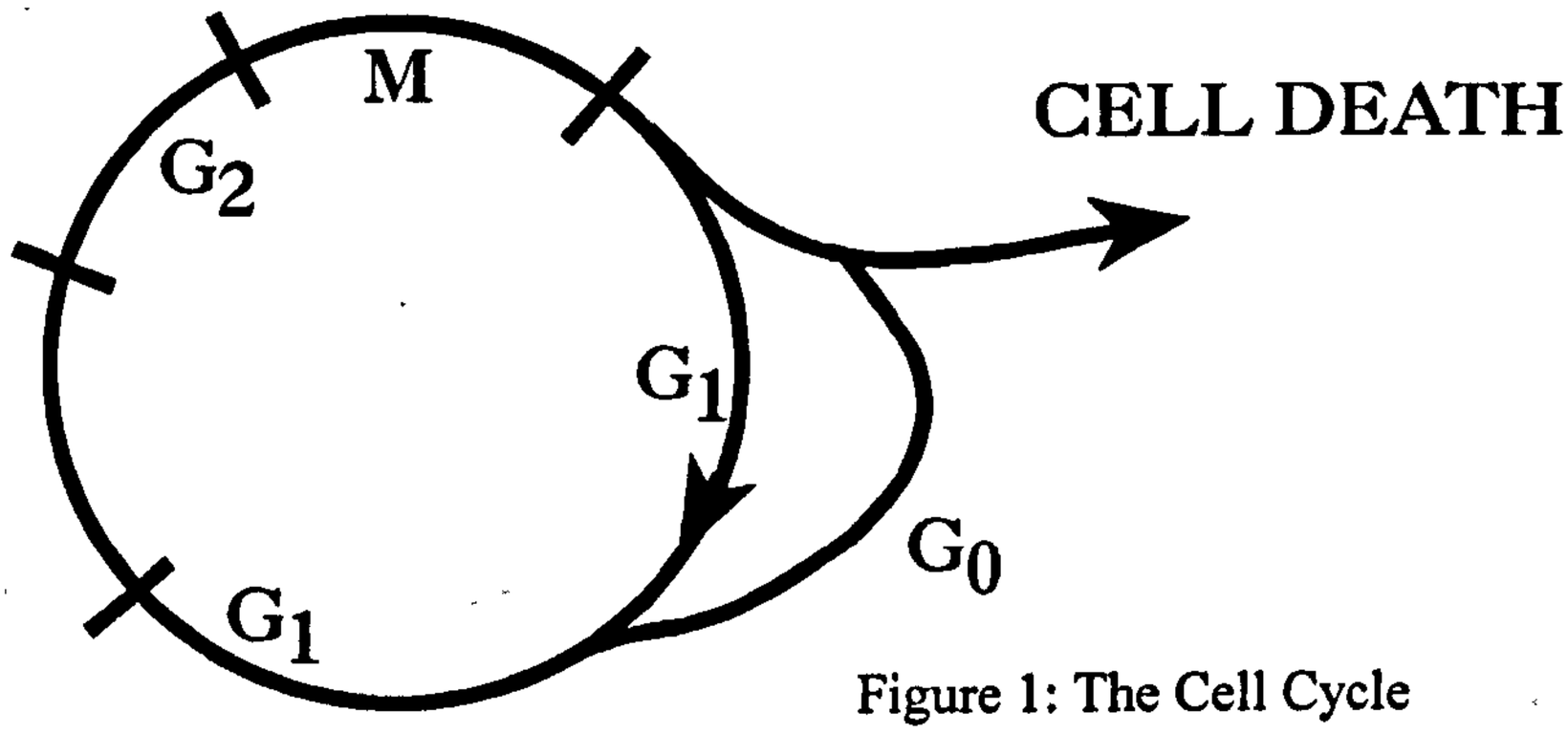


Figure 1: The Cell Cycle

Many studies of tumour cell kinetics have indicated a relationship between high cell proliferation rates and tumour aggressiveness but this also obviously depends on the tumour cell death rate (see section 2.6). There are a variety of different methods available for measuring cell proliferation, including mitotic counts, thymidine labelling index and bromodeoxyuridine incorporation, flow cytometry, and immunocytochemistry using cell cycle specific antibodies.

The mitotic count is a subjective technique, the validity of which remains controversial, and is defined as the number of mitoses per ten high powered fields. It is subject to many errors, not least of which is inter-observer variation, plus it takes no account of cell size and is generally non-reproducible (Quinn and Wright, 1990). In addition, it is not an independent parameter of cell proliferation but requires further pathological and clinical information for interpretation of the results. Another drawback is that this technique is only a measure of the proliferating cells that are actually in M phase. The thymidine labelling index (TLI) and bromodeoxyuridine (BrdU) incorporation both measure the percentage of tumour cells in S phase of the cell cycle, but they do not measure the duration of that S phase. Therefore, it is possible for a tumour to have a slow proliferation rate but high TLI and BrdU indices. Other disadvantages of these techniques include the requirement of fresh tissue and specialized equipment that would not be routinely available, plus the techniques are time consuming. Also, for use in humans the practice of in vivo administration is not justifiable. Flow cytometry, although widely used, does not take into account the heterogeneity of tumour cell

populations or the presence of normal cells, and the spatial relationships of cells to each other is lost. It can deliver faster results than immunocytochemical evaluation, but does not provide as much morphological information (Crocker, 1994), as the tissue architecture is destroyed when the cell suspension that is required as a sample is prepared. Therefore immunocytochemistry was the method of choice for this study as on balance it was the best method available.

2.3.1 Proliferating Cell Nuclear Antigen (PCNA)

There are several cell cycle specific antigens that when detected immunohistologically are known to provide important information about cell proliferation state, the most well known of which are Proliferating Cell Nuclear Antigen (PCNA) and Ki67 or Mib1 (see section 2.3.2). PCNA, which is also known as cyclin, is an auxiliary protein for DNA polymerase δ and is an absolute requirement for DNA synthesis. PCNA represents a 36kD acidic, non-histone protein which can be demonstrated in routinely processed tissues (Garcia, Coltrera and Gown, 1989) and so can be used in retrospective studies. It has been found to be maximally elevated in late G_1 and S phase of the cell cycle and is also expressed in DNA synthesis associated with DNA repair. PCNA results require careful interpretation as it has now been found to be expressed in some non-cycling cells (McCormick, Chong, Hobbs, Datta and Hall, 1993). This may be due to the long half-life of the detectable protein, which is over 20 hours, or the presence of malignant cells inducing PCNA expression in adjacent normal cells. Also, PCNA is often present at very low levels in non-cycling cells so that upregulation can occur upon entry into the cell cycle, so PCNA is generally only considered as being totally down-regulated in long term quiescent cells (Wolf and Dittrich, 1992). There are several different antibodies to PCNA which recognize different epitopes and there are differences in the effects of fixation and processing on the detectability of these epitopes. PC10 is the antibody that is most commonly used as this has been found to recognize the epitope most resistant to formalin fixation (Wolf and Dittrich, 1992).

2.3.2 Mib1

The Ki67 (or Mib1) antigen was first identified in 1983 by Gerdes, Schwab and Stein and has been confirmed as a nuclear antigen expressed only on proliferating cells. The actual Ki67

antibody has been shown to recognize a nuclear non-histone protein doublet of molecular weights 395 and 345kD. The Ki67 antigen has been shown to be expressed at all stages of the cell cycle except G_0 and early G_1, but the actual level has been reported as varying throughout the cell cycle with a maximum during G_2 and M phases (Crocker, 1994). However, there is evidence that in nutritionally deprived cells (which are common in tumours) there can be a discrepancy between Ki67 immunoreactivity and other parameters of cell proliferation (Brown and Gatter, 1990). Prior to the latest antigen retrieval developments (see section 2.2) Ki67 monoclonal antibodies were only suitable for work on frozen sections, although they have now also been found to work on fixed tissue using microwave antigen retrieval (Cuevas, Jones and Wright, 1993). An antibody designated as Mib1 was raised to recognize the Ki67 epitope on routinely processed material (Cattoretti, Becker, Key, Duchrow, Schluter, Galle and Gerdes, 1992) before it was found that Ki67 could be used, however, the results obtained with Mib1 are still reported to be much stronger (Cuevas *et al*, 1993). Other antibodies to different cell cycle related antigens are also available, but these are generally less well characterized and less reliable (Crocker, 1994). There are several points to bear in mind when assessing cell proliferation, such as that the results will give an indication of cell proliferation state but not rate and as most tumours consist of heterogeneous cell populations there will be different proliferation rates within a tumour.

2.3.3 AgNORs

Nucleolar organizer regions (NORs) are chromosomal segments in which rRNA is encoded, and in the human karyotype they are located on each of the short arms of the chromosomes 13, 14, 15, 21 and 22. The acidic non-histone proteins associated with these areas were found to be argyrophilic and are referred to as AgNORs, although it is important to bear in mind that only transcriptionally active NORs bind silver (Crocker, 1996). The AgNOR silver staining reaction was first described in 1975 (Goodpasture and Bloom) as a three-step method, and has since been modified to a one-step method (Howell and Black, 1980) and the reaction temperature has been lowered to 20°C from 60°C (Ploton, Menager, Jeanneson, Himber, Pigeon and Adnett, 1986). By means of the AgNOR procedure interphase NORs appear as well defined black dots (1-2μm diameter), distributed within the nucleolar body, which can be easily quantified either subjectively (using x100 oil immersion) or objectively

using image analysis (Ruschoff, Plate, Contractor, Kern, Zimmermann and Thomas, 1990 ; Derenzini and Trere, 1991 ; Botticelli, 1996).

They have often been described as being more numerous in malignant cells in comparison to normal cells and there is still some argument about whether this reflects a rise in cell proliferation (Janmohamed, Murray, Crocker and Leyland, 1990 ; Smith, Skilbeck, Harrison and Crocker, 1993) or merely an increase in cell protein production (Underwood and Giri, 1988), but either way AgNOR status gives an indication of malignancy. However, AgNORs have been quoted as increasing from early G_1 phase and reaching a maximum in S phase (as proliferation antigens do) (Derenzini and Ploton, 1994).

AgNOR counts have previously been found useful for differentiating between low grade and high grade non-Hodgkin's lymphomas (Crocker and Nar, 1987), as well as for differentiating between benign and melanocytic lesions of the skin (Egan and Crocker, 1988). Also, a study of AgNORs in NSCLC found interphase AgNOR quantitation to be of prognostic value (Kaneko, Ishida, Sugio, Yokoyama and Sugimachi, 1991), whereas Boldy, Ayres, Crocker, Waterhouse and Gilthorpe (1991) reported AgNOR counts as being of little value in squamous cell carcinoma of the bronchus. However, AgNOR counts have been shown to be of no use in distinguishing between SCLC and bronchial carcinoids (Benbow and Cromie, 1989), although they can help in identifying lymphocyte infiltrates within SCLC (Crocker, Ayres and McGovern, 1987).

The AgNOR technique has some associated problems, such as precipitates across the section, which have limited it's routine use and interpretation, despite various proposed chemical modifications and physical barrier techniques. However, it has been found possible to stain a section for both AgNORs and immunocytochemical markers and so study two parameters simultaneously (Janmohamed *et al*, 1990).

2.4 ONCOGENES

Protooncogenes are normal cellular genes that become "activated" to oncogenes through particular mutations so that either the normal protein is overexpressed or a mutant protein is produced. Activation of oncogenes may occur by mutation, chromosomal translocation, gene amplification or deregulation of protein expression. There are two main classes of oncogenes, these being (a) dominant oncogenes - require a change in a single allele to become oncogenic, and (b) recessive oncogenes or tumour suppressor genes (see section 2.5) - require a change in both alleles. Examples of oncogenes activated in lung cancer include ras, raf, jun and abl, however, the ras family is rarely overexpressed in SCLC and the rest have not been widely studied. For these reasons the oncogene proteins studied were C-MYC, C-ERBB-2, MDM-2, BCL-2, NM23, P53 and RB.

2.4.1 C-MYC

The v-myc oncogene was initially identified in the MC29 avian retrovirus and belongs to a family of oncogenes with high evolutionary conservation. In humans the family is known to consist of five members - C-myc, N-myc, L-myc, R-myc and B-myc (LeGouy, 1987) that code for nuclear phosphoproteins which bind to DNA and are presumed to have transcriptional regulatory functions. Activation of these genes usually occurs by mechanisms other than mutation e.g. gene amplification, rearrangements and point mutations have all been reported (Gazdar, 1992). Approximately 80-90% of SCLC overexpress a myc gene, however, only one member of the family may be amplified in a given tumour, with c-myc being the usual amplification noted in SCLC cases (Kern and Filderman, 1993). Whereas amplification of the myc genes in NSCLC is much less common (Gazdar, 1992). The c-myc gene has been mapped to chromosome 8q24 and codes for a 64-67kD protein. Anderson and Spandidos (1993) found an apparent correlation between the degree of c-myc gene amplification in the primary tumour and the patient survival time. Cmyc has also been implicated to participate in DNA replication by influencing the initiation of DNA synthesis (Prins, DeVries and Mulder, 1993). Expression of C-MYC appears to be associated with a cellular state in which DNA replication occurs provided suitable extrinsic factors are present, but from which cells die by the process of apoptosis (see 2.6) should these factors be withdrawn (Evan, Wyllie, Gilbert, Littlewood, Land, Brooks, Waters, Pann and Hancock, 1992).

2.4.2 MDM2

The human mdm2 (mouse double minute 2) gene is located on chromosome 12q13-14 and is often found to be amplified in sarcomas. The 90 kD MDM2 protein is capable of forming a complex with mutant or wild-type P53 (see section 2.5.1) and suppressing its transactivation function (Bueso-Ramos, Yang, DeLeon, McCown, Stass and Albitar, 1993). When MDM2 protein is expressed in a cell where P53 is active it blocks further P53 function, which results in less MDM2 being made, so the activity of P53 and levels of MDM2 in a cell are kept in balance by this autoregulatory feedback loop (Wu, Bayle, Olson and Levine, 1993). Factors that act to increase MDM2 levels or increase MDM2 activity will promote cell proliferation, whereas factors that alter the ability of P53 protein to stimulate MDM2 or inactivate MDM2 should lead to growth arrest. MDM2 has been shown to be able to overcome the G_1 arrest capability of both P53 and RB (see section 2.5.2) as well as enhancing the activity of the growth promoting transcriptional activator E2F.

2.4.3 C-ERBB-2

The neu oncogene was first identified in a chemically induced rat neuroblastoma by Schechter, Hung, Vaidyanathan, Weinberg, Yang-Feng, Francke, Ullrich and Coussens in 1984. The human equivalent of the rat neu gene was independently cloned from a cDNA library and was called HER-2 (Coussens, Yang-Feng, Liao, Chen, Gray, McGrath, Seeburg, Libermann, Schlessinger, Francke, Levinson and Ullrich, 1985), however, after cloning from genomic DNA it was designated as c-erbB-2 (Semba, Kamata, Toyoshima and Yamamoto, 1985) and has been mapped to chromosome 17q21. The c-erbB-2 gene (erythroblastoma-2) encodes a transmembrane, tyrosine-specific protein kinase called $p185^{neu}$, which is a putative growth factor receptor, related in sequence and structure to the epidermal growth factor receptor (EGFR). EGFR and $p185^{neu}$ plus the proteins encoded by the genes c-erbB-3 and c-erbB-4 form the EGFR superfamily (Pastan, 1993). Amplification or overexpression of the protein has been indicated as a negative prognostic factor in breast cancer. $p185^{neu}$ has been found to be overexpressed in NSCLC, and to indicate shortened survival times in lung adenocarcinomas (Kern, 1990). A lack of c-erbB-2 mRNA and protein expression has been reported as a unique feature of SCLC, with possible diagnostic relevance (Schneider, Stohr, Spies, Bohm, Fink, Manning, Nekarda, Prauer and Roth, 1992).

2.4.4 BCL-2

The bcl-2 protooncogene (B cell leukaemia and lymphoma gene 2) was originally discovered at the chromosomal breakpoint of the translocation 14;18 found in human follicular lymphoma. The translocation juxtaposes the bcl-2 gene from chromosome 18 with the immunoglobulin heavy chain locus on chromosome 14. As a result abnormally high levels of BCL-2 protein are produced, although expression of the protein is not specific to this translocation as it is also seen in normal tissue and other malignancies. The bcl-2 gene is believed to be localized to the inner mitochondrial membrane (Hockenbery, Nunez, Milliman, Schreiber and Korsmeyer, 1990) and codes for a 26kD protein. The bcl-2 gene family contains several members including bcl-2, bax, bcl-x and bak, all of which show structural homology (Lu, Abel, Foster and Lalani, 1996).

High levels of BCL-2 expression prevent cell death from a wide variety of cell stresses and cytotoxic chemicals, including growth factor depletion, heat shock, ionising radiation and a range of chemotherapeutic drugs (Fanidi, Harrington and Evan, 1992 ; Bissonnette, Echeverri, Mahboubi and Green, 1992). In oncogenesis, the deregulation of BCL-2 expression may contribute to the accumulation of mutations by suppressing the apoptotic deletion of cells that normally follows DNA damage. BCL-2 has the ability to bypass P53-dependent and independent apoptotic pathways (Chiou, Rao and White, 1994). BCL-2 may act in concert with C-MYC in cell transformation, by suppressing the cell death pathway promoting function of c-myc. Other studies have shown that CMYC and BCL-2 can co-operate to overcome P53-induced growth inhibition by the exclusion of P53 from the nucleus during a critical period in G_1 phase (Ryan, Prochownik, Gottleib, Apel, Merino, Nunez and Clarke, 1994). Whilst BCL-2 expression may inhibit cell death it does not enhance proliferation, and although the rescue of cells from apoptosis by BCL-2 can occur at any stage of the cell cycle the actual mechanism of apoptotic inhibition is unclear (Lu *et al*, 1996).

BCL-2 protein has been reported as being absent in differentiated cells of normal bronchial epithelium, however, abnormal expression has been found in bronchial dysplasias of all histological grades, suggesting that changes in the expression of this molecule arise early in the transformation of normal to dysplastic epithelium (Walker, Robertson, Myskow and

Dixon, 1995a). In NSCLC, BCL-2 overexpression has been found to be related to better overall survival and a less aggressive tumour phenotype (Pezzella, Turley, Kuzu, Tungekar, Dunnill, Pierce, Harris, Gatter and Mason, 1993 ; Fontanini, Vignati, Bigini, Mussi, Lucchi, Angeletti, Basolo and Bevilacqua, 1995). SCLC cell lines have been found to express high levels of BCL-2 protein (Ikegaki, Katsumata, Minna and Tsujimoto, 1994), and other studies have found the majority of SCLC tumour samples to be positive for BCL-2 expression (Jiang, Sato, Kuwao and Kameya, 1995 ; Yan, Chen, Tsai and Jin, 1996). However, the expression of BCL-2 was not found to be of any prognostic significance in this tumour type (Yan *et al*, 1996). Due to it's expression in neuroendocrine tumour types (such as SCLC) and it's close correlation with neuroendocrine markers, BCL-2 has been suggested as a possible marker of neuroendocrine differentiation (Jiang, Kameya, Sato, Yanase, Yoshimura and Kodama, 1996).

2.5 TUMOUR SUPPRESSOR GENES

2.5.1 P53

The P53 protein was discovered in the late 1970's as a cellular 53kD nuclear phosphoprotein (hence the name p53) bound to the large transforming antigen of the SV40 DNA virus (Lane and Crawford, 1979). The actual p53 gene itself has now been mapped to chromosome 17. p53 mutations are generally regarded as being the commonest abnormality in human malignancies. The wild type (normal) P53 protein is usually found in the cell nucleus, but it has a very short half-life and is present in such small quantities that it cannot be detected by immunohistochemistry. However, mutant P53 accumulates to levels that allow detectability by immunohistochemical systems. Approximately 90% of P53 mutations are missense mutations, causing one amino acid to be substituted for another, usually altering protein conformation and causing nuclear accumulation (Harris, 1993). Different carcinogens have been found to cause different characteristic mutations, for example, the predominant base change found in lung cancer is a G to T transversion believed to be caused by the carcinogen benzo(a)pyrene present in tobacco smoke (Mazur and Gluckmann, 1988).

Kastan, Onyekwere, Sidransky, Vogelstein and Craig (1991) have shown that accumulation

of P53 mediates arrest of the cell cycle at G_1 phase. Normal P53 monitors the integrity of the genome. If DNA is damaged, P53 accumulates and switches off replication to allow extra time for repair, and if repair fails P53 may trigger apoptosis. Tumour cells where P53 is inactivated by mutation cannot carry out this cell cycle arrest, therefore they are generally less stable and will accumulate mutations and chromosomal rearrangements at an increased rate, leading to rapid selection of malignant clones (Lane, 1992). The halting of the cell cycle is believed to be due to the protein product of the WAF1/Cip1 gene which is under p53 transcriptional control. Recent studies investigating the mechanisms underlying the biological activity of P53 indicate that the protein is involved in gene transcription, DNA synthesis and repair, genomic plasticity and apoptosis (Greenblatt, Bennett, Hollstein and Harris, 1994). P53 also has the ability to form complexes with other cellular proteins as well as viral oncoproteins which bind to P53 and alter it's ability to interact with cellular proteins. MDM2 (see section 2.4.2) is one of the cellular proteins that can bind to P53 and inhibit it's transcriptional activity.

Various studies agree that P53 mutations occur at a higher frequency in SCLC (approx 70%) compared to NSCLC (approx 20 - 30%) (Miller, Simon, Aslo, Kok, Yokota, Buys, Terada and Koeffler, 1992 ; Fontanini, Bigini, Vignati, Macchiani, Pepe, Angeletti, Pingitore and Squartini, 1993). However, studies have shown no increase in P53 protein levels in pulmonary carcinoids (Iggo, Gatter, Bartek, Lane and Harris, 1990). There are, however, several controversial aspects of P53 assessment, such as the time when the P53 mutations appear. In 1994 Fontanini, Vignati, Bigini, Merlo, Ribecchini, Angeletti, Basolo, Pingitore and Bevilacqua found no P53 immunoreactivity in histologically normal epithelium, hyperplastic epithelium or squamous metaplastic lesions. However, Walker, Dixon and Myskow (1995b) found the abnormal expression of P53 protein to be an early though not obligatory event in lung cancer development that may precede visible histological change. The use of antigen retrieval in P53 immunohistochemistry is also a controversial area.

Hall and Lane (1994) recommended that care is needed when interpreting P53 results, as a positive P53 reaction may be due to the accumulation of wild type protein caused by DNA damage. Baas, Van den Berg, Mulder, Clement, Slebos, Hamilton and Offerhaus (1996) have

also recommended a limit to the extent of which antigen retrieval should be used for P53 immunohistochemistry.

2.5.2 Retinoblastoma (RB)

The rb gene was first identified in retinoblastoma, a rare form of paediatric eye tumour (Knudson, 1971) and encodes a nuclear phosphoprotein ($p110^{RB}$) that is associated with DNA binding activity. The rb gene has been mapped to chromosome 13q14. The RB protein is phosphorylated in an undulating fashion throughout the cell cycle. It becomes hyperphosphorylated in late G_1 and remains so in S, G_2 and M phases. Cells in G_1 and G_0 express RB in an underphosphorylated form. This regulation of phosphorylation during the cell cycle suggests that the RB protein acts as a cell cycle regulator, the inactivation of which may lead to unbridled cell growth (Hensel, Hseih, Gazdar, Johnson, Sakaguchi, Naylor, Lee and Lee, 1990). The cell cycle-dependent phosphorylation of the RB protein is mediated by activated cyclin D-cyclin dependent kinase (CDK) complexes during G_1 (Sakaguchi, Fujii, Hirabayashi, Yoon, Komoto, Oue, Kusafuka, Okada and Maksuda, 1996). Several studies have shown that many, if not all, of the phosphorylations of RB protein are carried out by cyclins and CDKs (Hinds and Weinberg, 1994). Xiao, Chen, Levine, Modjtahedi, Xing, Sellers and Livingston (1995) showed that MDM-2 interacts with the RB protein inhibiting it's growth regulatory function. RB protein can protect cells from P53-induced apoptosis as well as non P53-induced apoptosis, although the mechanism of protection remains obscure (Kouzarides, 1995).

Inactivation of RB is a frequent event in SCLC. Harbour, Lai, Whang-Peng, Gazdar, Minna and Kaye (1988) found RB abnormalities to be a common event in both SCLC and pulmonary carcinoids but rare in other lung tumour types. Another study in 1994 by Higashiyama, Doi, Kodama, Yokouchi and Tateishi also found complete lack of RB protein expression in the majority of SCLC patients (88%) and regarded it as possibly a minor event in NSCLC. It has been suggested that inactivation of the rb gene in SCLC may be correlated not only with the initiation of the tumours but also with tumour development in terms of aggressiveness of biological and clinical behaviour.

2.5.3 NM23

nm23 (non-metastasizing gene 23) is a candidate metastasis suppressor gene first identified in 1988 by Steeg, Bevilacqua, Kopper, Thorgeirsson, Talmadge, Liotta and Sobel. In 1991 Stahl, Leone, Rosengard, Porter, King and Steeg found a second nm23 gene (nm23-H2) that was 81% identical to the first nm23 gene (nm23-H1), both of which produce 17kD proteins. Both nm23 genes have been localized to chromosome 17q21 (Backer, Yendola, Kovesdi, Fairhurst, O'Hara, Eddy Jr, Shaws, Mathew, Murty and Chaganti, 1993). Reduced expression of the nm23-H1 mRNA content has been correlated with reduced survival in breast cancer (Hennessey, Henry, May, Westley, Angus and Lennard, 1991). Reduced expression of NM23-H1 has also been associated with distant metastases of human hepatocellular carcinoma (Nakayama, Ohtsuni, Nakao, Shima, Nakata, Watanabe, Ishii, Kimura and Nagataki, 1992) and with the early appearance of metastases after diagnosis of malignant melanoma (Florenes, Aamdal, Myklebost, Maelandsmo, Bruland and Fadstad, 1992). However, in squamous cell carcinoma of the lung high levels of nm23 mRNA have been correlated with advanced stages of the disease, thereby not exhibiting a metastasis suppressor function (Engel, Theisinger, Seib, Seitz, Huwer, Zang, Welter and Dooley, 1993). To date there have been no published studies investigating the expression of nm23 gene or its protein product in SCLC.

2.6 APOPTOSIS

There are two major categories of cell death - necrosis and apoptosis. Necrosis is a pathologic form of cell death that often occurs as a result of massive tissue damage and results in the deletion of large numbers of cells. Apoptosis (also called programmed cell death) describes a process where single cells may be deleted in the midst of living tissue. The two processes also exhibit distinctly different morphological characteristics. In necrosis the cell loses it's homeostatic control and becomes swollen with fluid, eventually leading to cell lysis and release of the intracellular contents, which in turn stimulates the inflammatory process (Arends and Harrison, 1994). In apoptosis the cell shrinks and loses contact with neighbouring cells, followed by a loss of specialized cell surface structures such as microvilli and cell-cell junctions. Apoptotic bodies (membrane-bound vesicles) are then produced, and

these are rapidly recognized and removed by macrophages and neighbouring cells before the inflammatory process can be stimulated (Wyllie, 1992).

The actual process of apoptosis was first recognized by Kerr, Wyllie and Currie in 1972. Apoptosis is a normal physiological process that is important in embryogenesis, immune development and other normal body processes. Many cancer treatments also rely on the induction of apoptosis and may be one mechanism responsible for the development of drug resistance (Arends and Harrison, 1994). The role of oncogenes and tumour suppressor genes in the prevention and induction of apoptosis is now becoming widely recognized (see sections 2.4 and 2.5). Also enzymes such as interleukin-1β converting enzyme (ICE) and ICE-like cysteine proteases are capable of inducing apoptosis in susceptible cells. These oncogenes and enzymes provide a promising area of interest for the development of new methods for the detection of apoptosis. The problem with quantitating apoptosis is that the morphological characteristics exist for only 1-2 hours, so the window of detection is relatively small, leading to under-estimation of the apoptotic population. The latest detection method to be proposed is the *in situ*-end labelling (ISEL) technique, which is also referred to as the TUNEL technique, for use on histological sections (Gavrieli, Sherman and Ben-Sasson, 1992 ; Wijsman, Janker and Keijzer, 1993). Gaffney, O'Neill and Staunton (1995) found that the ISEL technique was able to identify cells in the early stages of apoptosis in SCLC, however, false positives due to exogenous/endogenous mutations are a possibility with this technique. Moser (1995) has also described a modified silver stain that detects the argyrophilia exhibited by clumped chromatin in apoptotic cells.

2.7 QUANTITATION

The classical evaluation of a microscopic sample in pathology is based on the visual analysis of the image with the aim of detecting and classifying it's diagnostic features. These characteristics are compared with commonly approved schemes (e.g. WHO classification of tumours). The interpretation of the microscope image, which provides valuable information for individual therapy, depends a great deal on a subjective view and subjective decision making capability, based on the individual's experience. The subjective element in the

diagnosis can result in divergence of opinions between individuals (Julis, University and Mikes, 1992). However, a conventional tissue section represents only a 2 dimensional image of 3 dimensional structures, but this can be overcome using techniques such as stereology or confocal microscopy, however, they require specialized equipment which was unavailable for this study and are also unlikely to be used routinely in hospital laboratories. Computerized image analysis is the term used to describe that area of work that utilizes computers in the morphometric measurements of histological or cytological features. The ability of computers to display complex graphics and digitally stored images has greatly enhanced the analysis of visual data. Machine vision cannot replace classical visual diagnosis but enables new and reproducible criteria to be introduced into the subjective decision making process, and a more precise assessment to be made of some biological features important for the estimation of prognosis and diagnosis.

Computerized image analysis systems simulate, in a limited way, the function of the human brain. Characteristic features of the colour signal captured by the camera are sent to the image processor and analysed. These features include saturation which corresponds with stain purity, hue which represents the principal wavelength and intensity (or luminance) which is the sum of the intensities of received light in each of it's 3 red, green and blue channels. The analysis of a reaction product in real colours used to represent a major obstacle for many image analysis systems. To circumvent the problem of making densitometric measurements in colour, only one colour channel is selected at any one time. From the red, green and blue components the one should be chosen which undergoes the maximum absorption by a coloured reaction product (Julis *et al*, 1992), in a similar way to conventional spectrophotometers.

In conventional histological sections the whole cell is never seen, due to the very nature of the sectioning process, therefore a technique was developed from that proposed by Van Driel Kulker, Mesker, Tanke, Feichtinger and Ploem (1985) to obtain whole cells from formalin-fixed, paraffin-embedded tissue. The theory was to produce a monolayer of single, whole tumour cells on a slide which were capable of being stained both by routine histological methods and by immunocytochemical techniques (see section 3.18). Used in conjunction

with histological sections from the same material it was hoped to obtain quantitative data (as in flow cytometry) with the maximum morphological information (as in histological immunostaining).

The only chromogenic stain that has gathered worldwide acceptance for DNA cytophotometry is the Feulgen reaction (1924), as it is currently the only histological staining technique which is stoichiometric for DNA i.e. the staining of DNA is both quantitative and specific (McCormick and Hall, 1994). The Feulgen reaction starts with acid hydrolysis where the purine bases are removed from the DNA and the apurinic acid molecule produced is degraded. These two reactions have their own kinetics and produce a reaction curve known as the hydrolysis profile which has 4 phases with the plateau (phase III) being the point of optimum hydrolysis. The use of hot acid produces a short profile and it may be difficult to stop the reaction at the plateau, whereas in cold hydrolysis (using a higher concentration of acid at room temperature) the plateau phase lasts several minutes. After acid hydrolysis the sections are placed in Schiff's reagent which binds covalently to the exposed aldehyde groups, producing a bright magenta colour.

Various studies have been performed on tumour nuclei stained by the Feulgen technique and quantitated by image analysis systems. Kayser, Shite and Tocke (1993) showed that the measurement of nuclear DNA content was not associated with tumour size or lymph node infiltration. DNA content has been shown to have a tendency to increase through the sequence from typical carcinoids to atypical carcinoids to SCLC (Larsimont, Kiss, DeLaunoit and Melamed, 1990), and may have a role in the distinction of premalignant and non-premalignant squamous dysplasias (Aufferman and Bocking, 1985). In SCLC the cell dimensions are partly dependent upon the size of the biopsy and the difficulty of studying these lesions objectively was emphasized by Vollmer (1982), but Thunnisson and Diegenbach (1986) disagreed with these findings. In order to ensure any degree of reproducibility within the image analysis system controls must be used, and these can be provided by the use of lymphocytes within sections as these are known diploid cells and so provide a reference against which malignant nuclei can be measured.

A large portion of this work involved the development or evaluation of methods (see section 3) for use with the image analyser. As immunohistochemistry was so important for this study it was imperative that the alkaline phosphatase detection system produced the optimum chromogenic result for the image analysis system. Four detection methods were evaluated for this purpose (see section 3.1). Also, each antibody required complete optimization. The technique for demonstrating AgNORs was also modified to produce the best overall result, as the high optical density of AgNOR dots is ideal for measurement using image analysis (Ruschoff, Plate, Contractor, Kern, Zimmermann and Thomas, 1990).

3. MATERIALS AND METHODS

For a list of abbreviations see Appendix I.

For a full list of materials and ordering information see Appendix II.

All materials and the methods in which they were used were evaluated under Control Of Substances Hazardous to Health (C.O.S.H.H.) guidelines at the outset of this study and reviewed, checked and approved by Health and Safety officers at regular intervals throughout.

3.1 DEVELOPMENT OF ALKALINE PHOSPHATASE DETECTION SYSTEM

In order to obtain the maximum information from the image analysis system, the optimum method of visualization of the alkaline phosphatase label needed to be determined. The four most widely used chromogens were evaluated for this reason. The methods used were - fast red method, hexazotised new fuchsin method, tetrazolium method and fast blue method, and these are outlined in detail below. These methods were evaluated on frozen rat small intestine as this contains very high levels of alkaline phosphatase. However, in order to preserve the tissue morphology the material required fixation prior to freezing (see below).

Tissue fixation for alkaline phosphatase techniques

- Take piece of tissue less than 5mm in size and place into paraformaldehyde solution at 4°C
- Leave at 4°C for 24 hours, then transfer to 5% sucrose at 4°C
- Leave overnight at 4°C, changing solution once
- Cool tissue on pelcool or in cryostat until frozen
- Store in liquid nitrogen for sectioning in cryostat at -20°C

3.1.1 Method 1 (Fast red method)

STOCK SOLUTION

- Take 0.1g Naphthol AS-MX phosphate (Sigma) and add 5ml of dimethylformamide (DMF)
- *Dissolve well.* Add 500ml of distilled water
- Add 500ml of 0.2M Tris buffer at pH 8.4, then check solution pH is 8.4

SUBSTRATE SOLUTION

- Take 25ml of the stock solution
- Add 15mg (0.015g) of Fast red violet LB salt (Sigma)
- Add 25µl of 1M levamisole solution (Sigma)
- Filter and use immediately

3.1.2 Method 2 (Hexazotised new fuchsin method)

HEXAZOTISED NEW FUCHSIN

A - Take 0.5g of new fuchsin (BDH) and add 10ml of 20% aqueous hydrochloric acid (HCl)

Use mild heat with constant agitation (stirrer/hotplate) to dissolve

Cool and filter the solution

Store at 4°C (stable for months)

B - Take 1g of sodium nitrite (BDH), add 25ml of distilled water, and dissolve

Store at 4°C (stable for 1 week only)

- Immediately before use mix together equal parts A and B
- Leave to stand for approx 2 mins, and use within 30 min of synthesis

SUBSTRATE SOLUTION

- Take 25mg (0.025g) Naphthol AS-TR phosphate, sodium salt (Sigma)
- Add 0.5ml of DMF, and *dissolve well*
- Add 50ml of 0.2M Tris-HCl buffer at pH 9.2
- Add 0.5ml of freshly hexazotised new fuchsin (see above)
- Check pH is 9.2 (can adjust with 1M NaOH)
- Filter before use

3.1.3 Method 3 (Tetrazolium method)

- Take 2.5mg of 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (Sigma)
- Add 10mg of tetranitro blue tetrazolium (Sigma)
- Dissolve these in 0.7ml of DMF
- Add 10ml of 0.2M Tris-HCl buffer pH9.2
- Mix and filter before use

For this particular method these quantities only apply for small intestine

3.1.4 Method 4 (Fast blue method)

STOCK SOLUTION

- Add 10mg of Naphthol AS-MX phosphate (Sigma) to 0.25 ml DMF, *dissolve well*
- Add 25ml of Tris buffer pH 8.6 and 25ml of distilled water
- Mix together well and add 50mg of PVP (Sigma)

SUBSTRATE SOLUTION

- Add 25mg of fast blue RR salt (Sigma) to 50ml of stock solution
- Mix well
- Check pH is 8.3, filter and use immediately

The optimum incubation times were found to be 10-20 min for the fast red method, 20 min for the hexazotised new fuchsin method, 10 min for the tetrazolium method and fast blue method. Also, the optimum mounting media for these methods was investigated. Farrant's medium and D.P.X. caused the colour to fade quite quickly and D.P.X. actually affected the appearance of the chromogen, making it appear "grainy". PVA or Aquaperm (from Immunon) proved to be the optimum mounting media for all four methods.

3.1.5 Method Selected

After considering the colour produced and the ease of the technique to perform, the best overall method was found to be the fast red method. This technique produced a bright red colour (see figure 2), and the solutions required are relatively simple to prepare and can be stored for long periods of time.

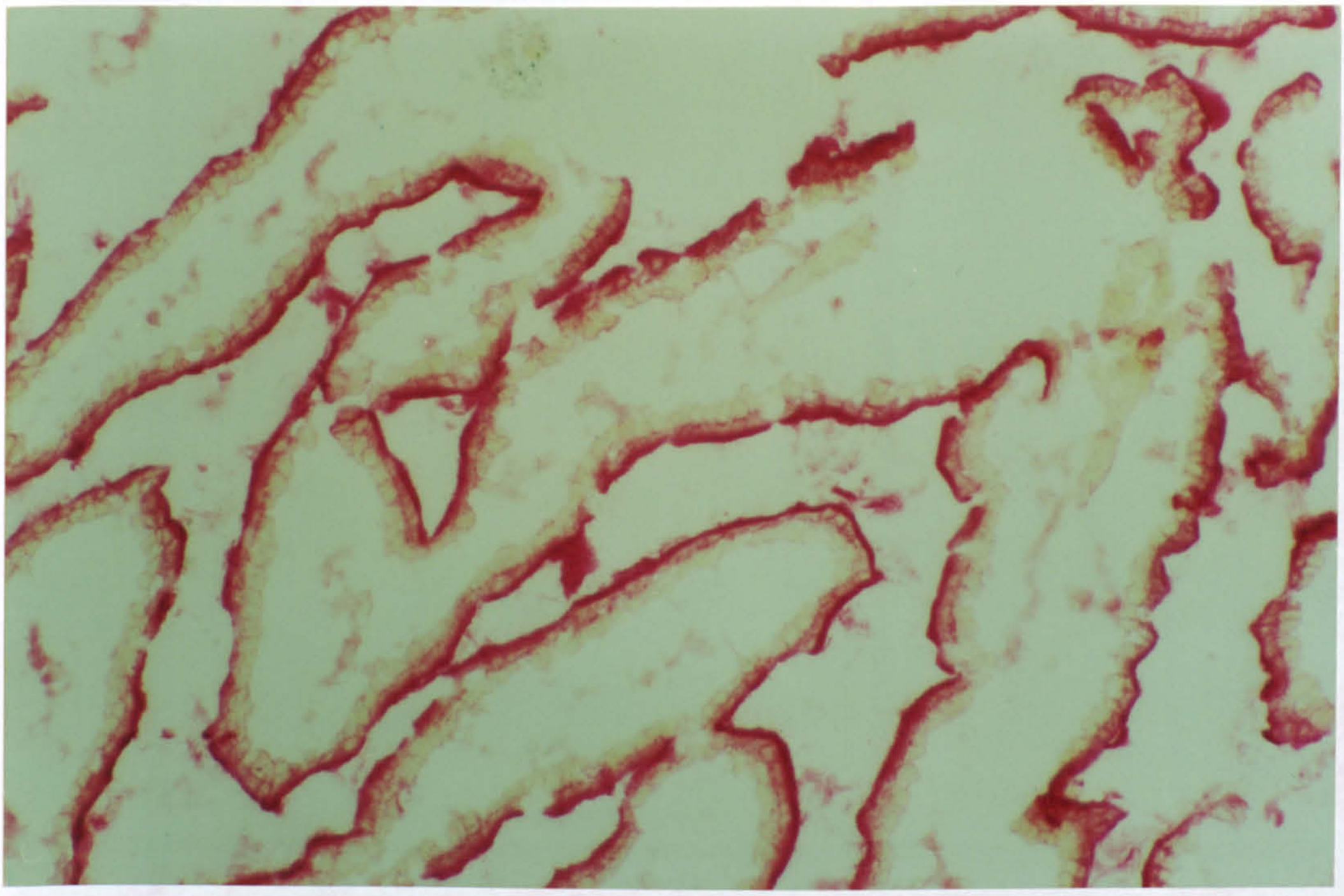


Figure 2 : Chromogenic reaction produced using the alkaline phosphatase fast red detection system (method 1). The red colour indicates the site of alkaline phosphatase activity within rat small intestine. Magnification x250.

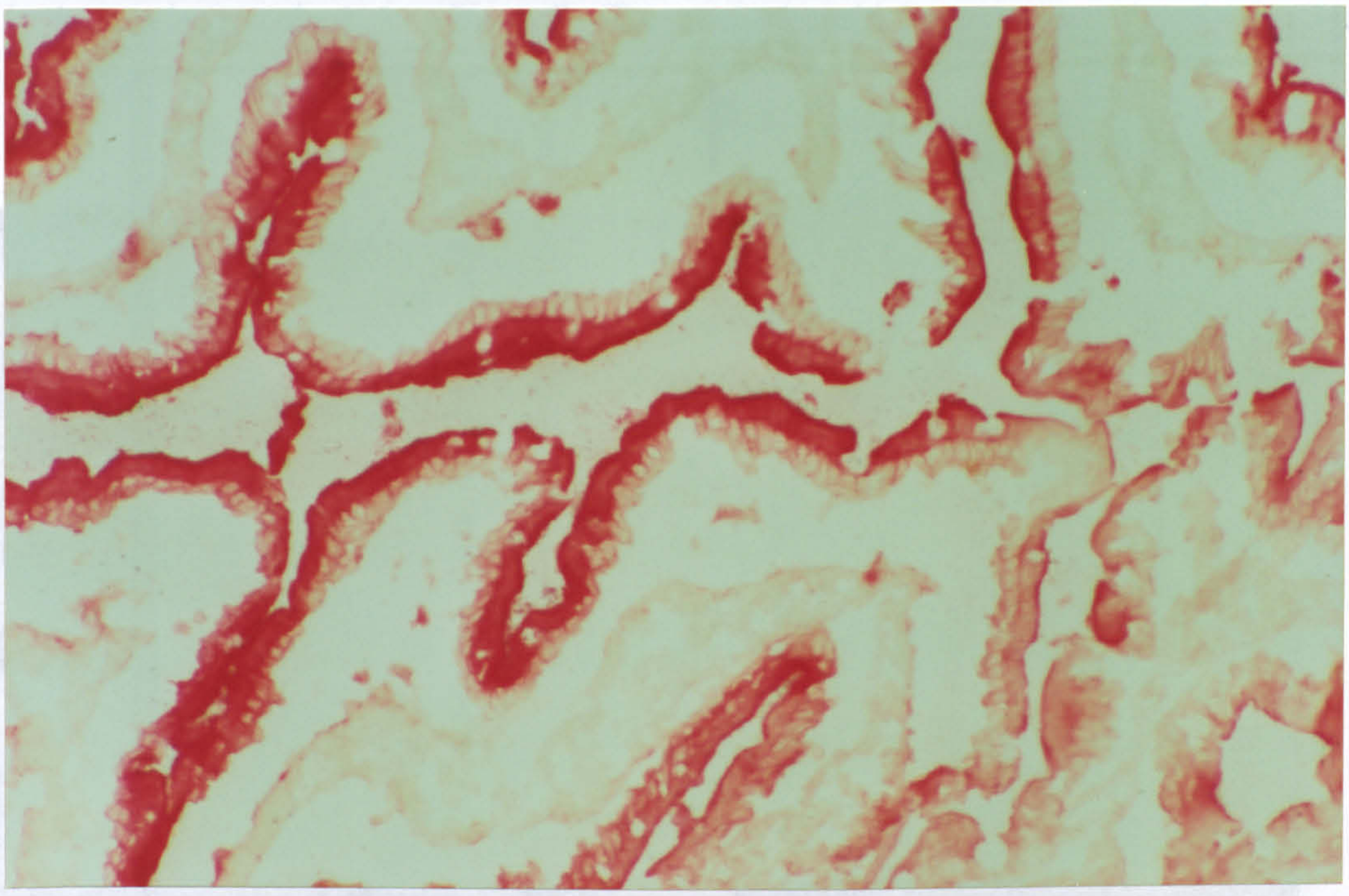


Figure 3 : Chromogenic reaction produced using the alkaline phosphatase hexazotised new fuchsin detection system (method 2). The red colour represents the site of alkaline phosphatase activity within rat small intestine. Magnification x250.

The hexazotised new fuchsin method (figure 3) actually gave an even brighter red colour than the fast red method (figure 2) but the solutions used required more preparation time. So, this method would only really be useful in certain situations, such as for slides that are to be photographed.

The tetrazolium method produced a very strong black colour at the site of alkaline phosphatase activity (see figure 4). The colour was very strong and developed very quickly, however, it could not be used on lung samples as it would be too easily confused with carbon deposits, which are prevalent in lung tissue, causing false positive results for immunocytochemistry.

The fast blue method produced a blue colour that was of a weaker intensity than the colours in the previous methods (see figure 5). This method could, however, prove useful as a second chromogen in a double immuno-staining method.

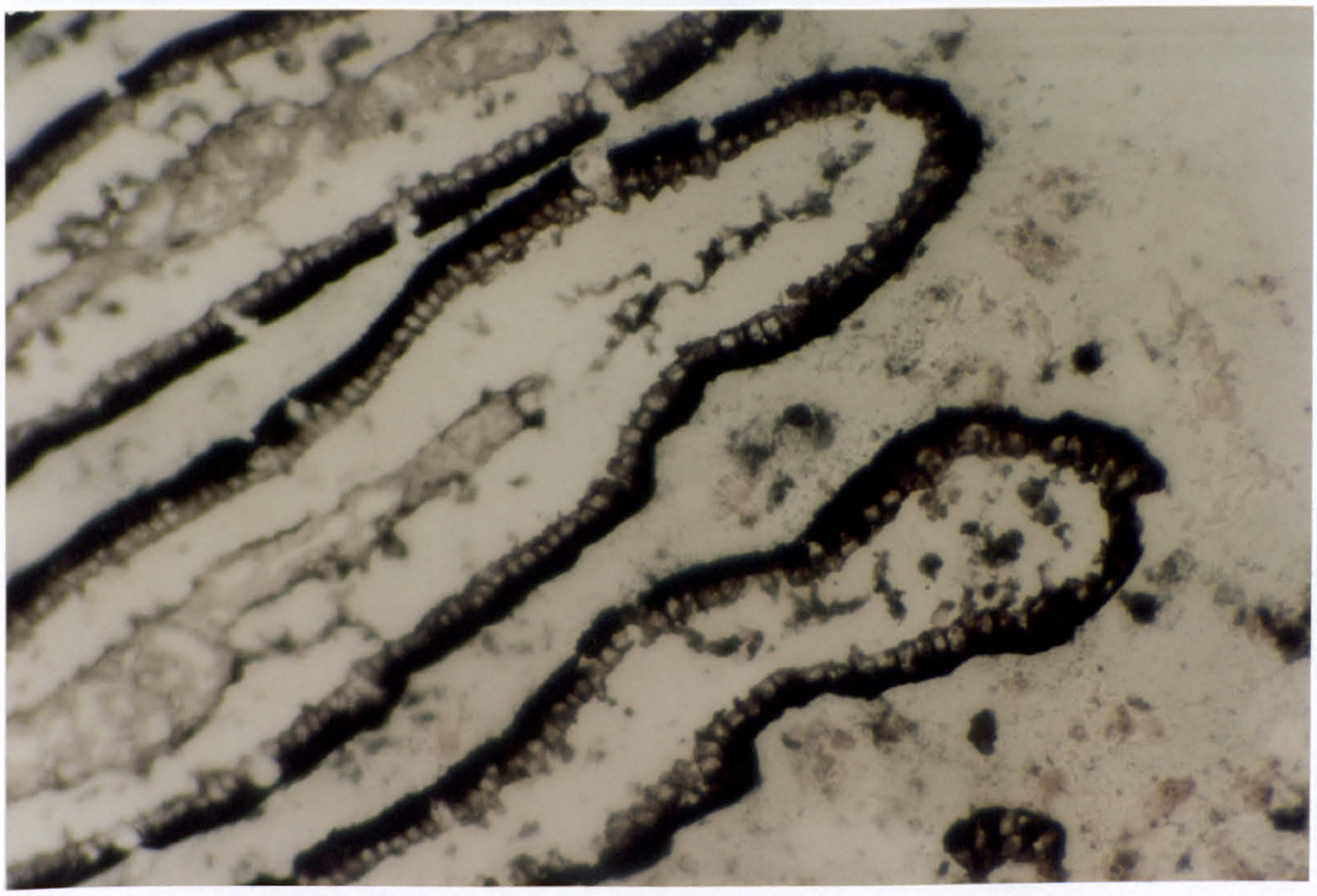


Figure 4 : Chromogenic reaction produced using the alkaline phosphatase tetrazolium detection system (method 3). The black colour represents the site of alkaline phosphatase activity within rat small intestine. Magnification x250.

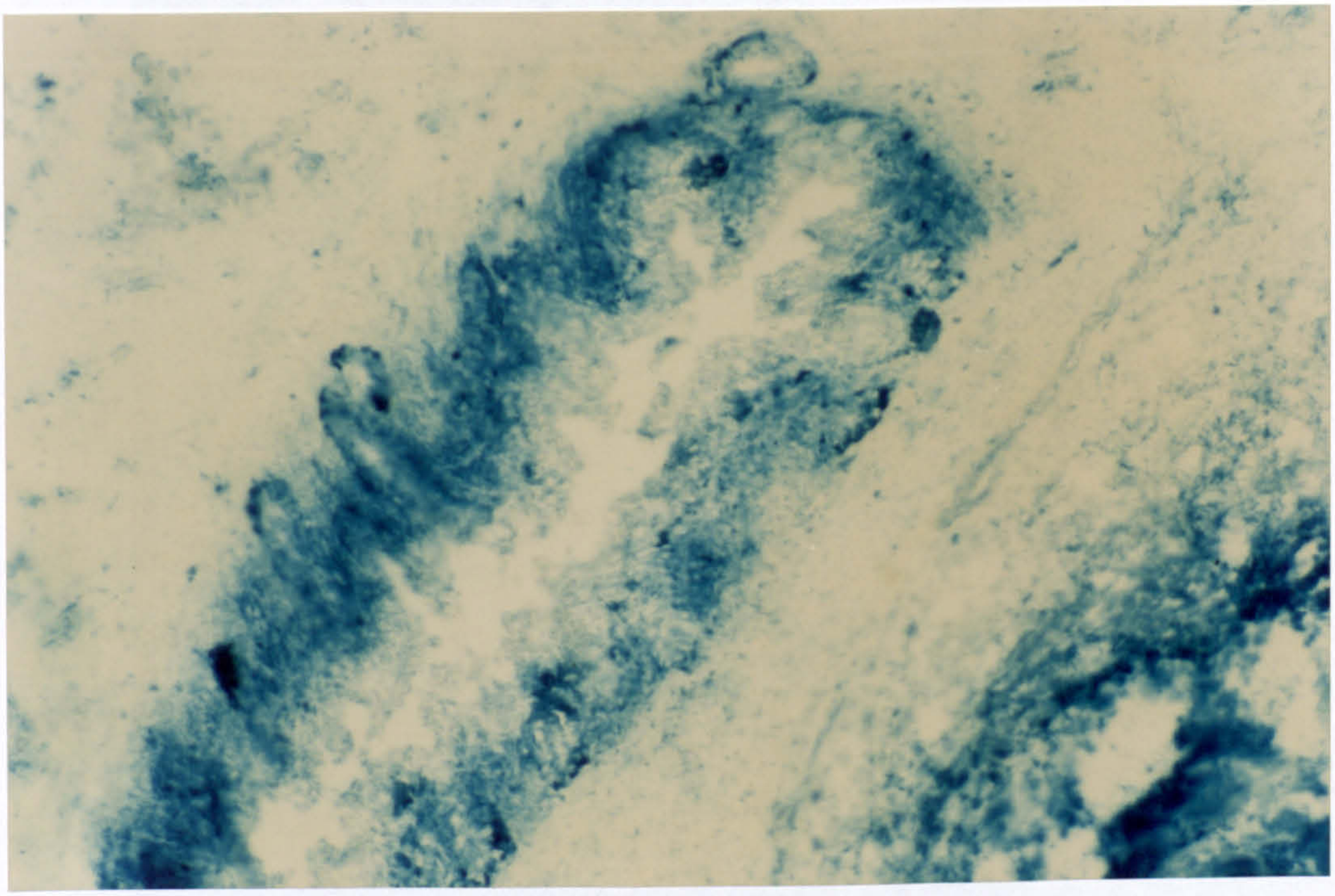


Figure 5 : Chromogenic reaction produced using the alkaline phosphatase fast blue detection system (method 4). The blue colour represents the site of alkaline phosphatase activity within rat small intestine. Magnification x250.

3.2 DEVELOPMENT OF CELL MONOLAYER PREPARATION

In histological sections the majority of cells are not whole, and this can reduce the accuracy of results obtained from the image analysis system. So, in order to maximize the quantitative information obtained a technique needed to be developed that would provide whole cells with minimal background contamination. The starting point for this work was a technique proposed by Van Driel Kulker *et al* in 1985, which was then modified for use with routinely processed tissue, as outlined below.

3.2.1 Preparation from unembedded tissue

The tissue used for this part of the work was formalin fixed, but unembedded. The technique was developed first on normal rabbit lung and then on a human lung tumour.

- Take 0.22g of tissue and cut into 4 pieces
- Wash tissue in wash buffer (6.8% sucrose on 0.05M PBS) at least 3 times (once overnight)
- Cut 1 piece of tissue and place in 0.05% trypsin solution (Difco), containing glass beads (Orme)
- Incubate for **90 min**, vortex mixing intermittently
- Syringe suspension through capillary pipette
- Filter solution through 100μm nylon gauze
- Centrifuge suspension (2500 rpm for 5 min) and decant supernatant
- Add 1 ml of carbowax (2% polyethylene glycol 1500 in 50% ethanol)
- Break up cell clumps by adding glass beads and vortexing
- Dilute solution 1:2 with phosphate buffered saline (PBS)
- Cytocentrifuge onto APES slides (1000 rpm for 15 min, using 500μl volume)
- Fix in 95% ethanol for at least **30 min**
- Stain sections and mount in DPX

Parameters Altered

Enzyme used - trypsin, proteinase K, collagenase IV, protease XXIV (all from Sigma)

Incubation times - 30 min, 60 min, 90 min, 120 min (for each enzyme)

3.2.2 Preparation from paraffin embedded tissue

Used human formalin-fixed, paraffin-embedded lung tumour tissue to develop the technique.

- Cut 30μm section off block
- Place in 2 changes of 3ml of xylene for **10 min**
- Place in 3ml of 100% alcohol for **10 min**
- Place in 3ml of 95% alcohol for **10 min**
- Place in 3ml of 70% alcohol for **10 min**
- Place in 3ml of 50% alcohol for **10 min**
- Place in 3ml PBS for **10 min**

- Centrifuge and decant supernatants to change solutions
- Add 2ml of 0.05% trypsin solution at 37°C
- Incubate for **90 min** with glass beads, vortexing the solution intermittently
- Syringe suspension through capillary pipette
- Filter the solution through 100μm nylon gauze (to remove beads and tissue)
- Centrifuge suspension (2500 rpm for 5 min) and decant supernatant
- Add 3ml of PBS and syringe through capillary pipette. Repeat
- Remove debris from cell solution (see 3.2.3)

Parameters Altered

Enzymes used - trypsin, proteinase K, protease XXIV, collagenase IV, trypsin-EDTA

Enzyme concentrations - 0.05%, 0.1%

Incubation time - 30 min, 60 min, 90 min, 120 min (for each enzyme and concentration)

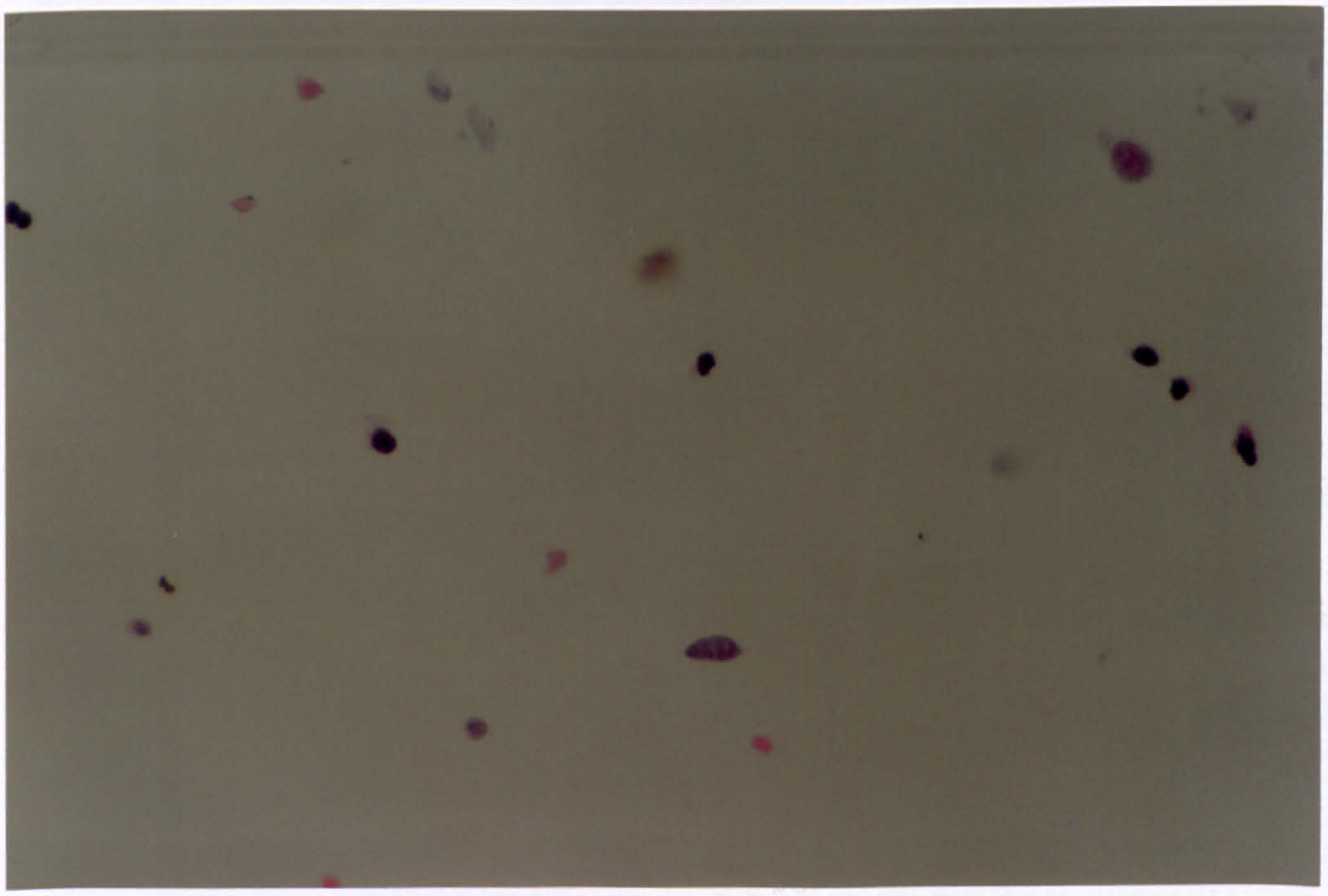


Figure 6 : Cell monolayer preparation before the removal of debris which can clearly be seen in the background, as can the SCLC cells (case no - 21/95). Magnification x400

3.2.3 Removal of debris

After finding the best overall technique for producing the monolayer there was still too much background debris for the image analyser to be effective, as the background "noise" would be too high. Three methods of debris removal were assessed (outlined below)

A) Differential Centrifugation

The first method tested was differential centrifugation using Optiprep (Nycomed) in a non-continuous gradient. This did not work. Next, was the use of a continuous gradient made from two very dense layers mixed in a gradient mixer (1.163 and 1.32 g/ml in saline). This was also unsuccessful. Eventually a gradient was found that worked which consisted of just very minimally diluted optiprep (1 layer), however, this was found to give unreproducible results.

B) Membrane Filtration

With equipment available at the Royal Liverpool University Hospital Trust cytology department tested the usefulness of a polycarbonate membrane of 5μm pore size (see below). All membranes were handled using tweezers to minimize contamination.

- Filter sample through a membrane under negative pressure
- Fix membrane in 95% ethanol for approximately **10 min**
- Clip membrane onto the slide (cell-side upward)
- Stain with H&E and take slide to xylene
- Invert membrane onto the slide and blot
- Seal membrane edges using a stick dipped in chloroform and dry at 60°C
- Dissolve membrane in chloroform (approximately 45 min)
- Dip in xylene and mount using DPX

Then tried using 12μm pore size polycarbonate membrane (CoStar UK Ltd) with a positive pressure system (syringe) and a pressure-fixation method of transferring the cells to a glass slide (Oud *et al*, 1984), which is outlined below:

- Place membrane onto filter holder with shiny side toward inlet
- Draw 5ml PBS into syringe, attach to holder
- Expel PBS through holder to wet the membrane
- Draw sample into syringe and expel through holder
- Draw up 5ml of PBS and rinse membrane
- Take membrane out of filter holder and place cell side down onto glass slide
- Place sponge soaked in ethanol over membrane and apply pressure for **60 secs**
- Remove pressure and sponge and fix slide for further **30 min** in ethanol
- Stain, clear and mount as required

The "cytoshuttle" from Cell-Path which employed the same principle as the above membrane filtration technique was also investigated, but to remove the cells from the filter it was claimed that only blotting was required. This method was found to give an extremely low cell yield and was therefore ruled out.

C) Gauze Filtration

Samples of 20µm and 30µm gauze mesh were obtained from Cell-Path for use in 2 different filtration methods outlined below.

METHOD 1

- Fix gauze over top of filter funnel and apply sample
- Invert gauze and elute cells by adding PBS
- Take 500µl of solution and spin onto slide using cytospin
- Fix, stain and mount as required

This method gave a low cell yield and was unreproducible

METHOD 2

- Cut 25mm circles from the gauze and place in filter holder
- Draw PBS into syringe and run through holder to wet the gauze
- Draw sample into syringe and expel through holder
- Invert gauze in the holder (using tweezers)

- Draw up PBS and expel through holder and collect cells
- As before, spin cells onto slide using cytospin
- Fix, stain and mount as required

Both the second filtration method and the polycarbonate membranes were found to remove the debris and leave an adequate number of cells for investigation.

Figure 7 : Cell monolayer preparation after the removal of debris by 20μm gauze filtration (case no -21/95). The difference after debris removal is quite clear as the tumour cells are now obviously isolated. Magnification x400

3.2.4 Immunocytochemistry on cell monolayers

For all immunocytochemistry on monolayers used PCNA, as this is a reliable, well known antibody that should have been positive on both the runt pig small intestine and lung tumour used. The monolayers were prepared as in section 3.2.2 and cleaned up as described in 3.2.3. For all the procedures the primary antibody was applied overnight at 4°C, the secondary antibody and the streptABC/AP were both applied for 2 hours each at RT.

Parameters Altered

Antigen retrieval - Microwaving 20 min / stand 20 min, microwaving 10 min / stand 20 min

Pressure cooking 1 min ; No antigen retrieval

Concentration - 1:50, 1:100, 1:150

The antigen retrieval procedures removed the cells so when antigen retrieval was required the slides needed to be coated in LVN (as in section 3.13). This procedure was repeated a further 4 times but no reaction was observed.

There was the possibility that the long exposure to trypsin during the tissue digestion stage was destroying or altering the antigen, so an alternative was investigated. An ultrasonic cleaning bath was used to disaggregate the cells for the monolayer which was then used for PCNA immunostaining throughout.

Parameters Altered

Ultrasonic bath - 60 min, 90 min, 120 min, 180 min

The sample was placed in PBS in water in the sonic bath and was mixed every 30 min.
Removed after 120 min and solution filtered through 100μm gauze to remove tissue left.
Immunostaining was still negative.

Permeabilization solution was also tried on the slides before immunostaining.
Permeabilization solution = 0.1% triton x-100 in 0.1% sodium citrate for 2 mins, 4°C
Also tried 5 min and 10 min exposure, with microwaving and pressure cooking and without.
Immunostaining still negative.

The next alternative was to carry out all immunostaining and antigen retrieval in test tubes and only at the end put them onto slides.

Parameters Altered

Tried different forms of antigen retrieval on cells in test tube:
Pressure cooking 1 min, pressure cooking 3 min
microwaving 20 min / stand 20 min

trypsinization for 15 min at 37°C

protease treatment for 5 min at 37°C

Immunostaining results were still negative.

TRYPSINIZATION

Solution consists of 0.1% trypsin in TBS with 0.1% calcium chloride

- Add calcium chloride to TBS and warm to *exactly* 37°C
- Add trypsin when solution is 37°C
- Treat sections (cells) for 5-15 min and wash

PROTEASE TREATMENT

Solution consists of 0.03g protease XXIV in 300ml TBS

- Prewarm TBS to 37°C and add enzyme just prior to use
- Treat sections (cells) for 1-10 min and wash

As a final test to show once and for all that these cells just could not be immunostained a combination of enzymes with and without permeabilization solution was tried.

Parameters Altered

Trypsin for 20 min at 37°C

Permeabilization solution for 2 min at 4°C, trypsin for 15 min at 37°C

Protease for 15min 37°C

Permeabilization solution for 2 min 4°C, protease for 10 min 37°C

ALL RESULTS NEGATIVE FOR IMMUNOSTAINING

Therefore, the study reverted back to using conventional histological serial sections in conjunction with image analysis.

3.3 PROCEDURE FOR HAEMATOXYLIN AND EOSIN (H&E) STAINING

All sections used were stained by H&E (Bancroft and Stevens, 1990) as this provides a picture of the general tissue morphology.

- Take sections to water
- Place in Gadsdons haemalum for **5 min**
- Rinse in running tap water for **1 min**
- Place in 1% acid-alcohol solution for **10 secs** (check under microscope)
- Rinse in running tap water for **2 min**
- Rinse in *warm* running tap water for **1 min**
- Place in Eosin solution for 5 min
- Rinse briefly in running tap water
- Dehydrate, clear and mount in DPX.

(1% acid-alcohol = 1% conc HCl in 70% ethanol)

3.4 PREPARATION FOR IMMUNOCYTOCHEMICAL METHODS

Optimization of the immunocytochemical procedure is essential for both quantitative and subjective assessment of the antigen under investigation. This requires determining the optimum incubation time and temperature and the most appropriate form of antigen retrieval, using as a basis the recommendations provided by the supplier. One parameter is altered at a time and so the procedure has to be repeated several times before it is optimized. Due to the aggressive nature of the antigen retrieval techniques and the numerous washing steps required sections are often lost from slides, so now *all slides* used in immunocytochemistry are coated with 3- aminopropyltriethoxysilane (APES) solution (see below) which causes stronger adherence of the sections to the slide preventing them from floating off. The general procedure for the microwave oven and wet autoclave pretreatment (pressure cooking) forms of antigen retrieval are also given below. Trypsinisation as a form of antigen retrieval was tried but the results obtained from the other procedures were better and more reliable. When the sections used are very small (e.g. the needle biopsies used in this study) it is possible to draw around the sections using an immunocytochemistry pen (Dako) which forms a hydrophobic barrier, ensuring that the reagents applied remain on the sample.

3.4.1 Preparation of APES slides

- Take clean slides to tap water
- Transfer to hot, soapy water
- Rinse very well in running tap water
- Rinse in distilled water
- Dry in slide dryer (approximately 30 min)
- Transfer to pot of acetone, then drain
- Dip in 2% APES solution (Sigma) in acetone
- Allow to drain, but not fully dry (slide goes grainy)
- Rinse in running tap water
- Rinse in distilled water
- Dry and return to clean slide box

3.4.2 Antigen retrieval by microwave irradiation

- Take sections to water
- Place 500ml of 0.01M citrate buffer pH 6.0 at room temperature into microwavable container
- Put slides in plastic rack into container
- Microwave on high power for **5.5 min**
- Place on simmer power for optimum time
- Leave sections to stand in the cooling buffer for optimum time (if appropriate)
- Put sections into room temperature tap water
- Gently wash in running tap water for 3-5min
- Put sections into tris-buffered saline (TBS) for immunocytochemistry

3.4.3 Antigen retrieval by wet autoclave pre-treatment procedure (pressure cooking)

- Place 1.5l of 0.01M citrate buffer pH 6.0 in a pressure cooker
- Switch hotplate onto highest temperature
- While buffer is boiling take sections to water
- When the buffer is boiling add the sections in metal slide racks
- Seal lid on correctly and put weight onto 1 dot position
- When timer behind weight lifts start timing for **1 min** (time may differ)

- After 1 min switch weight to 2 dot position
- Take off the heat and place in running cold water
- Place sections in *warm* water (take care handling hot racks!)
- Transfer sections to TBS for immunocytochemistry

FOR ALL IMMUNOCYTOCHEMICAL PROCEDURES

StreptABC/AP solution = 1ml TBS + 10µl solution A + 10µl solution B

Diluted Gadsdons Haemalum = Gadsdons haemalum diluted 1:2 with distilled water

All other dilutions required were made in TBS

Each time the procedure is carried out control sections are included to check that all stages of the reaction are working correctly. The positive control is a section of known positive material, whereas the negative control is a known positive section that has had no primary antibody applied, just TBS.

3.5 IMMUNOCYTOCHEMICAL PROCEDURE FOR MIB-1 ANTIGEN

- Take sections to water
- Carry out antigen retrieval for **1 min** using 0.01M citrate buffer pH 6.0 in a pressure cooker
- Rinse sections in TBS
- Apply normal rabbit serum (NRS) diluted 1:3 for **10-15 min**, room temperature (RT)
- Drain slides and wipe around section -DO NOT WASH
- Apply anti-Mib-1 (Immunotech cat-0505) at 1:100 for **60 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply biotinylated rabbit anti-mouse (BRAM) secondary antibody at 1:400 for **30 min**, RT
- Make up streptABC/AP solution and leave to stand for **30 min**, RT
- Drain slides and wash in TBS for 5-10 mins
- Apply pre-mixed streptABC/AP solution for **30 min**, RT
- Drain slides and wash in TBS for 5-10 mins
- Make up and apply fast red substrate solution for **20 min**, RT
- Wash off using TBS
- Apply diluted Gadsdons haemalum for **10-20 secs**

- Rinse slides in running tap water for approximately 2mins
- Dip slides *briefly* into 1% HCl solution and return to tap water
- Dehydrate in 80% ethanol and mount in PVA

CONTROL TISSUE - Runt pig small intestine

Suppliers Recommendations

1:50 to 1:100 1hour RT ; Microwave 15min / stand 20 min

Parameters Altered

Antigen retrieval - Microwaving 20 min/stand 20 min, microwaving 10 min/standing 20 min

Pressure cooking 1 min, pressure cooking 3min

No antigen retrieval

Concentration - 1:75, 1:100, 1:150, 1:200, 1:250

Temperature/Time - 1hour RT, overnight 4°C

3.6 IMMUNOCYTOCHEMICAL PROCEDURE FOR NM23 ANTIGEN

- Take sections to water
- Rinse slides in TBS
- Drain slides and apply NRS diluted 1:3 for **10-15 min**, RT
- Drain slides and wipe around section - DO NOT WASH
- Apply anti-NM23 (Novocastra cat-NCL-NM23) at 1:300, incubate **overnight** at 4°C
- Drain slides and wash in TBS for 5-10 min
- Apply BRAM secondary antibody at 1:400 for minimum **30 min**, RT
- Make up streptABC/AP solution and leave to stand for **30 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply pre-mixed streptABC/AP solution for **30 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply fast red substrate solution for **20 min**, RT
- Wash off using TBS

- Apply diluted Gadsdons haemalum for **10-20 secs**
- Rinse slides in running tap water for approximately 2 mins
- Dip slides *briefly* into 1% HCl solution and return to tap water
- Dehydrate in 80% ethanol and mount in PVA

CONTROL TISSUE - Human breast cancer

Suppliers recommendations

1:100 to 1:250 for 30 min RT ; No antigen retrieval

Parameters Altered

Antigen retrieval - Microwaving 20 min / stand 20 min, Microwaving 10 min / stand 20 min

Pressure cooking 1 min, pressure cooking 3min

No antigen retrieval

Concentration - 1:150, 1:200, 1:250, 1:300

Temperature / Time - 30 min RT, 60 min RT, overnight 4°C

3.7 IMMUNOCYTOCHEMICAL PROCEDURE FOR C-MYC ANTIGEN

- Take sections to water
- Carry out antigen retrieval for **1 min** using 0.01M citrate buffer pH 6.0 in a pressure cooker
- Rinse sections in TBS
- Drain slides and apply NRS diluted 1:3 for **10-15 min**, RT
- Drain slides - DO NOT WASH
- Apply anti-C-MYC (Novocastra cat - NCL-CMYC) at 1:150 for **60 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply BRAM secondary antibody at 1:400 for minimum **30 min**, RT
- Make up streptABC/AP solution and leave to stand for **30 min**, RT
- Drain and wash in TBS for 5-10 min
- Apply pre-mixed streptABC/AP solution for **30 min**, RT
- Drain slides and wash in TBS for 5-10 min

- Apply fast red substrate solution for **20 min,** RT
- Wash off using TBS
- Apply diluted Gadsdons haemalum for **10-20 secs**
- Rinse slides in running tap water for approximately 2 min
- Dip slides *briefly* into 1% HCl solution and return to tap water
- Dehydrate in 80% ethanol and mount in PVA

CONTROL TISSUE - Human colon cancer

Suppliers recommendations

1:150 to 1:250 1 hour RT ; May need antigen retrieval

Parameters Altered

Antigen retrieval - Microwaving 20 min / stand 20 min, microwaving 10 min / stand 20 min
Pressure cooking 1 min, pressure cooking 3 min

Concentration - 1:150, 1:200, 1:250, 1:300, 1:400

3.8 IMMUNOCYTOCHEMICAL PROCEDURE FOR MDM-2 ANTIGEN

- Take sections to water
- Carry out antigen retrieval for **1 min** using 0.01M citrate buffer pH 6.0 in a pressure cooker
- Rinse sections in TBS
- Drain slides and apply NRS diluted 1:3 for **10-15 min,** RT
- Drain slides and wipe around section - DO NOT WASH
- Apply anti-MDM2 (Novocastra cat - NCLMDM2) at 1:50 and incubate **overnight** at 4°C
- Drain slides and wash in TBS for 5-10 min
- Apply BRAM secondary antibody at 1:400 for minimum **30 min,** RT
- Make up streptABC/AP solution and leave to stand for **30 min,** RT
- Drain slides and wash in TBS for 5-10 min
- Apply pre-mixed streptABC/AP solution and leave for **30 min,** RT
- Drain slides and wash in TBS for 5-10 min

- Apply fast red substrate solution for **20 min,** RT
- Wash off using TBS
- Apply Gadsdons haemalum solution for **10-20 secs**
- Rinse slides in running tap water for approximately 2 min
- Dip slides *briefly* into 1% HCl and return to tap water
- Dehydrate in 80% ethanol and mount using PVA

CONTROL TISSUE - Human sarcoma

Suppliers Recommendations

1:50 to 1:100 overnight 4°C ; Pressure cook antigen retrieval for 1 min 0.01M citrate buffer

Parameters Altered

Antigen retrieval - Microwaving 20 min / stand 20 min, microwaving 10 min / stand 20 min

Pressure cooking 1 min, pressure cooking 3min

Concentration - 1:40, 1:50, 1:75, 1:100, 1:150

Temperature / Time - 1 hour RT, overnight 4°C

3.9 IMMUNOCYTOCHEMICAL PROCEDURE FOR BCL-2 ANTIGEN

- Take sections to water
- Carry out antigen retrieval for **1 min** using 0.01M citrate buffer pH 6.0 in a pressure cooker
- Rinse slides in TBS
- Drain slides and apply NRS diluted 1:3 for **10-15 min,** RT
- Drain slides and wipe around section - DO NOT WASH
- Apply anti-BCL-2 (Dako cat - M887) at 1:75 for **60 min,** RT
- Drain slides and wash in TBS for 5-10 min
- Apply BRAM secondary antibody at 1:400 for minimum **30 min,** RT
- Make up streptABC/AP solution and leave to stand for **30 min,** RT
- Drain slides and wash in TBS for 5-10 min
- Apply pre-mixed streptABC/AP solution for **30 min,** RT

- Drain slides and wash in TBS for 5-10 min
- Apply fast red substrate solution for **20 min**, RT
- Wash off using TBS
- Apply diluted Gadsdons haemalum for **10-20 secs**
- Rinse slides in running tap water for approximately 2 min
- Dip slides *briefly* into 1% HCl solution and return to tap water
- Dehydrate in 80% ethanol and mount in PVA

CONTROL TISSUE - Normal human tonsil

Suppliers Recommendations

1:40 to 1:80 1hour RT ; Microwaving 10 min / stand 20 min

Parameters Altered

Antigen retrieval - Microwaving 10 min / stand 20 min, Microwaving 20 min / stand 20 min
Pressure cooking 1 min, pressure cooking 3min
Concentration - 1:50, 1:75, 1:100, 1:150

3.10 IMMUNOCYTOCHEMICAL PROCEDURE FOR P53 ANTIGEN (D07 clone)

- Take sections to water
- Carry out antigen retrieval for **1 min** using 0.01M citrate buffer pH 6.0 in a pressure cooker
- Rinse slides in TBS
- Drain slides and apply NRS diluted 1:3 for **10-15 min**, RT
- Drain slides - DO NOT WASH
- Apply anti-P53 (Dako cat - M7001) at 1:100 for **60 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply BRAM at 1:400 for minimum **30 min**, RT
- Make up ABC/AP solution and leave to stand for **30 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply ABC/AP solution for **30 min**, RT

- Drain slides and wash in TBS for 5-10 min
- Apply fast red substrate solution for **20 min**, RT
- Wash off using TBS
- Apply diluted Gadsdons haemalum for **10-20 secs**
- Rinse slides in running tap water for approximately 2 mins
- Dip slides *briefly* into 1% HCl solution and return to tap water
- Dehydrate in 80% ethanol and mount in PVA

CONTROL TISSUE - Human non-small cell lung cancer

Suppliers Recommendations

1:50 to 1:100 1hour RT ; Microwaving 10 min / stand 20 min

Parameters Altered

Antigen retrieval - Microwaving 20 min / stand 20 min, microwaving 10 min / stand 20 min
Pressure cooking 1 min, pressure cooking 3min
Concentration - 1:50, 1:100, 1:150, 1:200, 1:300

3.11 IMMUNOCYTOCHEMICAL PROCEDURE FOR C-ERBB-2 MONOCLONAL

- Take sections to water
- Rinse sections in TBS
- Drain slides and apply NRS diluted 1:3 for **10-15 min**, RT
- Drain slides and wipe around sections - DO NOT WASH
- Apply C-ERBB-2 monoclonal antibody (Novocastra cat - NCLCB11) at 1:40 for **60 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply BRAM secondary antibody at 1:400 for minimum **30 min**, RT
- Make up streptABC/AP solution and leave to stand for **30 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply pre-mixed streptABC/AP solution for **30 min**, RT
- Drain slides and wash in TBS for 5-10 min

- Apply fast red substrate solution for **20 min**, RT
- Wash off using TBS
- Apply diluted Gadsdons haemalum for **10-20 secs**
- Rinse slides in running tap water for approximately 2 mins
- Dip slides *briefly* into 1% HCl solution and return to tap water
- Dehydrate in 80% ethanol and mount in PVA

CONTROL TISSUE - Human breast cancer

Suppliers Recommendations

1:40 1hour RT ; No antigen retrieval

Parameters Altered

Antigen retrieval - Microwaving 20 min/ stand 20 min, microwaving 10 min/ stand 20 min

Pressure cooking 1 min, pressure cooking 3min

No antigen retrieval

Concentration - 1:40, 1:50, 1:100

3.12 IMMUNOCYTOCHEMICAL PROCEDURE FOR PCNA ANTIGEN (PC-10 epitope)

- Take sections to water
- Carry out antigen retrieval using 0.01M citrate buffer pH 6.0 in microwave 20 min /stand 20 min
- Rinse slides in TBS
- Drain slides and apply NRS diluted 1:3 for **10-15 min**, RT
- Drain slides and wipe around section - DO NOT WASH
- Apply anti-PCNA (Dako cat - M879) at 1:700 for **60 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply BRAM secondary antibody at 1:400 for minimum **30 min**, RT
- Make up streptABC/AP solution and leave to stand for **30 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply pre-mixed streptABC/AP solution for **30 min**, RT

- Drain slides and wash in TBS for 5-10 min
- Apply fast red substrate solution for **20 min**, RT
- Wash off using TBS
- Apply diluted Gadsdons haemalum for **10-20 secs**
- Rinse slides in running tap water for approximately 2 mins
- Dip slides *briefly* into 1% HCl and return to tap water
- Dehydrate in 80% ethanol and mount using PVA

CONTROL TISSUE - Runt pig small intestine

Suppliers Recommendations

1:50 to 1:100 1hour RT ; No antigen retrieval

Parameters Altered

Antigen retrieval - Microwaving 20 min / stand 20 min, microwaving 10 min / stand 20 min
Pressure cooking 1 min, pressure cooking 3min
No antigen retrieval

Concentration - 1:100, 1:200, 1:400, 1:500, 1:600, 1:700

3.13 IMMUNOCYTOCHEMICAL PROCEDURE FOR C-ERBB-2 POLYCLONAL

A polyclonal antibody is generally raised in a rabbit (unlike monoclonal antibodies which are raised in mice) so rabbit serum and rabbit secondary antibodies cannot be used due to cross-reactivity. For this particular polyclonal antibody swine serum and a swine secondary antibody were employed.

- Take sections to water
- Carry out antigen retrieval for **1 min** using 0.01M citrate buffer pH 6.0 in a pressure cooker
- Rinse slides in TBS and then apply normal swine serum diluted 1:3 for **10-15 min**, RT
- Drain slides and wipe around section - DO NOT WASH
- Apply C-ERBB-2 polyclonal antibody (Dako cat - A485) at 1:300 for **60 min**, RT
- Drain slides and wash in TBS for 5-10 min

- Apply secondary antibody (biotinylated swine anti-rabbit) at 1:400 for minimum **30 min**, RT
- Make up streptABC/AP solution and leave to stand for **30 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply pre-mixed streptABC/AP solution for **30 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply fast red substrate solution for **20 min**, RT
- Wash off using TBS
- Apply diluted Gadsdons haemalum for 10-20 secs
- Rinse slides in running tap water for approximately 2 mins
- Dip slides *briefly* into 1% HCl solution and return to tap water
- Dehydrate in 80% ethanol and mount using PVA

CONTROL TISSUE - Human breast cancer

Suppliers Recommendations

1:100 to 1:200 1 hour RT ; Microwaving 10 min / stand 20 min

Parameters Altered

Antigen retrieval - Microwaving 20 min / stand 20 min, microwaving 10 min / stand 20 min

Pressure cooking 1 min, pressure cooking 3min

Concentration - 1:100, 1:200, 1:300, 1:400

3.14 IMMUNOCYTOCHEMICAL PROCEDURE FOR Retinoblastoma (RB) ANTIGEN

Several problems were encountered in obtaining a reliable and reproducible protocol for this particular antigen. Different commercial antibodies were used with varying degrees of success, as well as different immunohistochemical detection systems and other technical modifications, as outlined below.

NOVOCASTRA RB1

The first RB antibody tried was from Novocastra (cat - NCL RB1).

Suppliers Recommendations

1:50 overnight 4°C ; No antigen retrieval

Parameters Altered

Antigen retrieval - Microwaving 20 min/ stand 20 min, microwaving 10 min / stand 20 min
Pressure cooking 1 min, pressure cooking 3min
No antigen retrieval

Temperature / Time - 1 hour RT, overnight 4°C

Concentration - All 1:50 (as should definitely work according to supplier)

As all results obtained were negative Novocastra supplied a sample of a different batch of the same antibody, which was tested as listed below

Parameters Altered

Tried 1:50, overnight 4°C, no antigen retrieval

This procedure was used with the ABC/AP method (as used for the previous monoclonal antibodies) and the ABC/HRP method (given below)

Also an NM23 control section was stained at the same time (see section 3.4) to control the other stages of the procedure.

3.14.1 ABC/HRP Immunocytochemical Procedure

- Take sections to absolute alcohol
- Place sections in 3% hydrogen peroxide in 100% methanol for **12-15 min**
- Wash well in tap water
- Drain slides and apply NRS diluted 1:5 for **15-30 min**
- Drain slides and wipe around section - DO NOT WASH
- Apply anti-RB at 1:50 and leave **overnight** at 4°C
- Drain slides and wash in TBS for 5-10 min
- Apply BRAM secondary antibody at 1:400 for minimum **30 min**, RT
- Make up streptABC/HRP solution (in same way as ABC/AP) and leave to stand for **30min**, RT

- Drain slides and wash in TBS for 5-10 min
- Apply pre-mixed streptABC/HRP solution for **30 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply diaminobenzidine (DAB) solution for **10-15 min**, RT
- Wash off using TBS
- Apply Gadsdons haemalum diluted 1:2 for **1-2min**
- Rinse slides in running tap water for approximately 2 mins
- Dehydrate, clear and mount in DPX

DAB solution - Take a 0.01g/ml frozen aliquot of 3-3'diaminobenzidine tetrachloride
Add to 0.2ml of 30% hydrogen peroxide when slightly defrosted
Make up to 20ml with TBS and filter onto sections

CONTROL TISSUE - Human breast cancer

Both the ABC/AP and the ABC/HRP methods failed to work for the RB antigen, but the actual procedure was working, as a positive reaction was obtained for the NM23 section.
Another modification was using 3% bovine serum albumin (BSA) in place of the NRS
The antibody was used at 1:50 overnight 4°C, no antigen retrieval - *No reaction*

A different secondary antibody (BRAM) was obtained from Vector Labs
The antibody was used at 1:50 overnight 4°C, no antigen retrieval - *No reaction*

NOVOCASTRA RB

Novocastra then sent a sample of their new rb clone to try (cat - NCL RB)

Suppliers Recommendations

1:20 - 1:50 30 min RT ; High temperature antigen retrieval

It was decided to try the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique (described below) using both the RB1 antibodies and the new RB clone, because for an unknown reason the results of the streptABC/AP technique were unreliable and unreproducible.

RB1 - 1:50 overnight 4°C, pressure cooking 1 min in 0.01M citrate buffer pH 6.0

1:50 overnight 4°C, pressure cooking 1 m in 0.01M citrate solution pH 6.0

This procedure was repeated for both the original RB1 and the newer sample of RB1.

RB - 1:40 1 hr RT, pressure cooking 1 min 0.01M citrate buffer pH 6.0

1:40 1 hr RT, pressure cooking 1 m in 0.01M citrate solution pH 6.0

The APAAP procedure was found to work for the RB clone as described below.

3.14.2 APAAP Immunocytochemical Procedure

- Take sections to water
- Place slides in 0.01M citrate solution pH 6.0 for **1 min** in a pressure cooker
- Rinse slides in TBS and then apply NRS diluted 1:3 for **10-15 min**
- Drain slides and wipe around section - DO NOT WASH
- Apply anti-RB (Novocastra cat - NCL RB) at 1:40 for **60 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply non-BRAM secondary antibody at 1:50 for **60 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply APAAP solution at 1:10 for **60 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply fast red substrate solution for **20 min**, RT
- Wash off using TBS
- Apply diluted Gadsdons haemalum for 10-20 secs
- Rinse in running tap water for approximately 2 mins
- Dip *briefly* into 1% HCl solution and return to tap water
- Dehydrate in 80% ethanol and mount in PVA

However, the result obtained was not very strong, so the RB antibody was used in the strept ABC/AP procedure.

Parameters Altered

Antigen retrieval - Pressure cooking 1 min, pressure cooking 3 min

Microwaving 20 min / stand 20 min, microwaving 10 min / stand 20 min

Both citrate solution and citrate buffer were tried as antigen retrieval solutions

Concentration - 1:20, 1:40, 1:75

Temperature / Time - 30 min at RT, 60 min at RT

CONCLUSION

The results obtained were inconclusive even after repeating 3 times, as well as using the APAAP method with the same altered parameters as listed above. The outcome was that the antibody gave unreliable and inconsistent results, so another commercial clone was ordered from a different supplier.

BIOGENEX RB

An rb antibody sample was obtained from Biogenex that came prediluted to 1:40, and was recommended to be microwaved for 15 min and to stand for 15 min.

This was found to work using the streptABC/AP method and, after repeating several times, was found to give reliable and consistent results therefore this antibody was used for the study.

3.14.3 Immunocytochemical Procedure Used For RB

- Take sections to water
- Carry out antigen retrieval using 0.01M citrate solution pH 6.0 for **1 min** in a pressure cooker
- Rinse slides in TBS
- Drain slides and apply NRS diluted to 1:3 for **10-15 min**, RT
- Drain slides and wipe around section - DO NOT WASH
- Apply anti-RB (Biogenex cat - MU290-UC) at 1:40 for **60 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply BRAM secondary antibody at 1:400 for minimum **30 min**, RT
- Make up streptABC/AP solution and leave to stand for **30 min**, RT
- Drain slides and wash in TBS for 5-10 min
- Apply pre-mixed streptABC/AP solution for **30 min**, RT

- Drain slides and wash in TBS for 5-10 min
- Apply fast red substrate solution for **20 min**, RT
- Wash off using TBS
- Apply diluted Gadsdons haemalum for **10-20 secs**
- Rinse in running tap water for approximately 2 mins
- Dip *briefly* into 1% HCl solution and return to tap water
- Dehydrate in 80% ethanol and mount in PVA

CONTROL TISSUE - Human breast cancer

Suppliers Recommendations

1:40, 30 min, RT ; Antigen retrieval required

Parameters Altered

Antigen retrieval - Microwaving 20 min / stand 20 min, microwaving 15min / stand 15min

Pressure cooking 1 min, pressure cooking 3min

Concentration - 1:40, 1:50, 1:75

3.15 PROCEDURE FOR AgNOR STAINING

All water used for this procedure was ultra pure water (to minimize contamination)

- Take sections to water
- Prepare 2% gelatine in 1% formic acid (AgNOR solution)
- Prepare 10% aqueous silver nitrate solution
- Mix AgNOR solution 1:2 with silver nitrate solution
- Apply to sections and incubate in the dark for **6 hrs**, RT
- Wash well in ultra-pure water
- Dehydrate, clear and mount in DPX.

CONTROL TISSUE - Pancreas

The actual AgNOR technique used requires determination by each individual laboratory, as different individuals tend to find that their own particular method works best for them, although the one-step method proposed by Ploton, Menager, Jeanneson, Himber, Pigeon and Adnet (1986) is the general method used. Various modifications were investigated to produce the optimal AgNOR technique for this study. These included changing the colloidal developer from gelatine to polyvinylpyrollidine (PVP) or polyethylene glycol (PEG) 20 000 (Rowlands, Crocker and Ayres, 1990), altering the concentrations of silver nitrate as well as the incubation temperature used, coating the slides and treating the slides in various ways after AgNOR staining. The optimum staining method decided upon after looking at these modifications is the one described above.

Parameters Altered

Silver nitrate concentration - 50%, 20%, 10%, 5%

Incubation times - 30 min, 45 min, 60 min, 3 hrs, 6 hrs, overnight

Incubation temperatures - RT, 45°C, 4°C

Colloidal developers - gelatin, PVP, PEG 2000

Post-AgNOR stain treatments - 1%gold chloride, 5% sodium thiosulphate, blue toning

BLUE TONING AgNOR STAINING

(Yekeler, Erel, Yumbul, Doymaz, Dogan, Ozercan and Iplikci, 1995)

- Take sections to water
- Carry out AgNOR staining as described above
- Apply blue toning solution for maximum **10 secs**
- Wash in water
- Dehydrate, clear and mount in DPX

BLUE TONING SOLUTION

30mmol/l iron chloride plus 11mmol/l potassium hexacyanoferrate (III) plus 33mmol/l oxalic acid

This modification still requires more work to ensure reproducibility and consistency, so it was not included as part of the AgNOR staining performed for this study.

APES slides were found to attract the deposition of silver grains, possibly due to the slight charge they have, in order to overcome this celloidin or low viscosity nitrocellulcose (LVN) were used to coat APES slides (see below).

LVN COATING OF SLIDES

- Dissolve approximately 25% LVN in a mixture of 50% diethyl ether - 50% absolute alcohol
- Add slides to solution and then remove slowly and steadily
- Leave slides to dry
- When slides dry dip into 70% ethanol to set the coating
- Stain slides (using the AgNOR technique)
- When stained dissolve coating in the diethyl ether - alcohol mixture
- When coating dissolved wash the slides in water

Parameters Altered

The concentration of the LVN solution required altering as no silver stain was able to get through the coating, as it was too thick.
Tried - 75%, 50%, 25% of original strength.
50% gave the best result but was not very reproducible.

It was decided to just cut fresh sections onto chrome alum slides which showed no increase in silver deposit during the AgNOR reaction.

CHROME ALUM COATING OF SLIDES

- Clean slides in soapy water, as for APES slides
- Dip slide into chrome alum solution

(Dissolve 2g gelatin in 400ml distilled water, when cool add 0.2g of chrome alum)

- Dry slides and store at 4°C

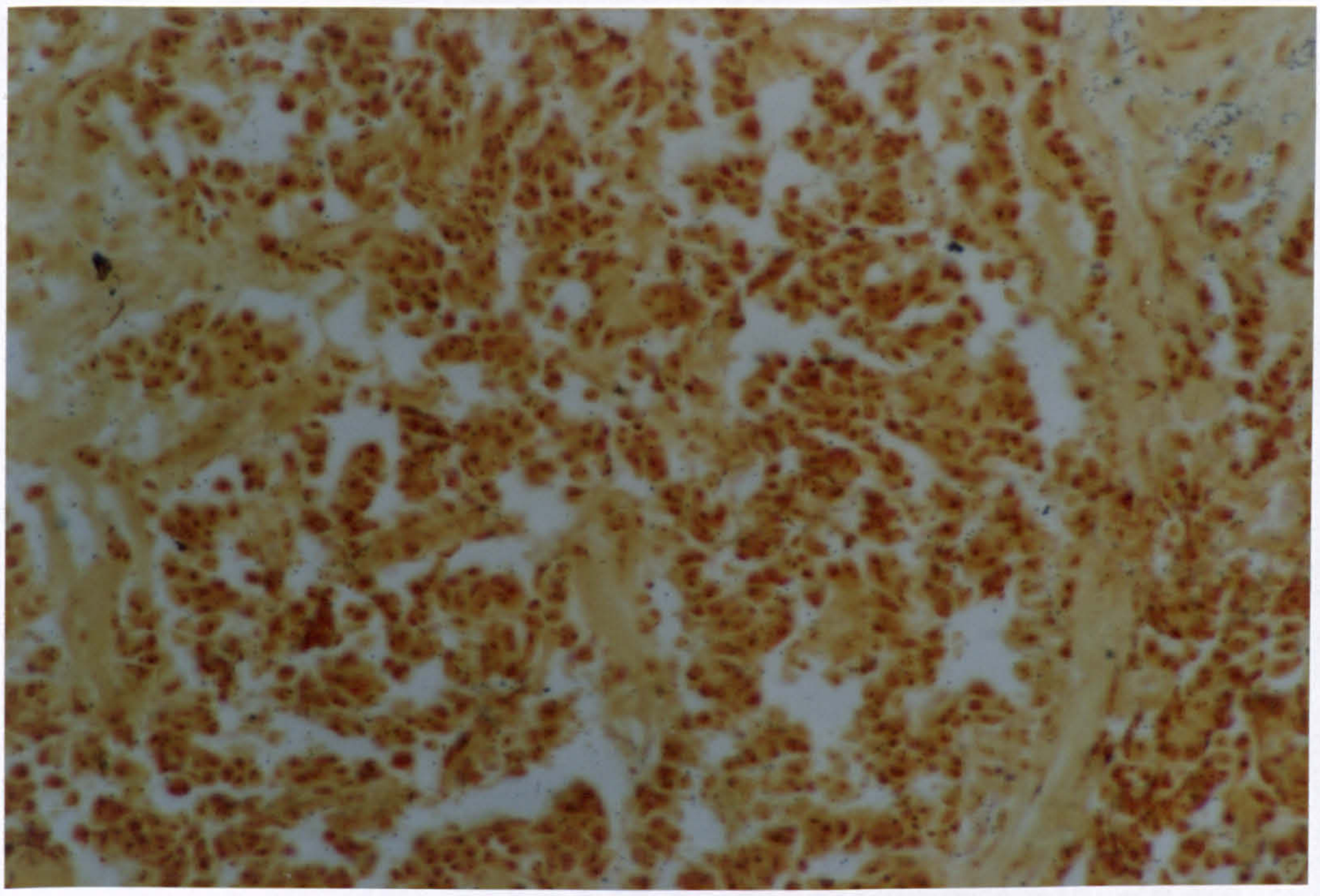


Figure 8 : Optimum AgNOR staining method on rat pancreas. The black dots represent the actual AgNORs which are easily distinguishable from the brown background. Magnification x400

3.16 PROCEDURE FOR FEULGEN REACTION (Cold)

(The cold Feulgen technique was developed by Elias JM, Conkling K and Makar M in 1972)

- Take sections to water
- Place sections in 5M HCl at RT for **45-60 min**
- Rinse in tap water
- Rinse very well in distilled water
- Place in Schiff's reagent at RT for **30 min**
- Rinse very well in distilled water
- Counterstain using 0.1% aqueous light green solution for **2 mins**, RT
- Wash in water
- Dehydrate, clear and mount in DPX.

CONTROL TISSUE - Nasal polyp or spinal cord

Negative control (TCA removal of nucleic acids)

- Take sections to water
- Treat with 4% trichloroacetic acid (TCA) at *exactly* 90°C for **15min**
- Wash in water, carry out Feulgen reaction

3.17 IMAGE ANALYSIS PROCEDURES

All clinical material used within this study was supplied by Nottingham City Hospital and was identified only by case number (no names etc. were given) so that awareness of the clinical data could not influence the results, even subconsciously. The cases for subjective study were selected on the basis of being diagnosed as SCLC and having enough tissue within the block to cut 15 serial sections (3μm each). For objective analysis the cases were selected on H&E for having reasonable morphological preservation for quantitation purposes.

3.17.1 Image Analysis System

There are various types of image analysis systems. The system used within this study acquired images from a Nikon Labophot-2 light microscope using a single chip JVC camera (TK-1270) whose signal was captured by a DT 2871 frame grabber board. The image analysis software package was Foster-Findlay PC Image for Windows (version 1.64) used on a Dan 486DX2 50 PC. The system used RGB (red, green, blue) colour representation as this was found to be a more reliable system than HSI (hue, saturation, intensity) for the software and hardware used. For image analysis the signal received from the camera is digitized and stored in the digitizing board memory as a grey value. The digitization assigns a grey scale value between 0 and 255 where the value 0 represents no light and 255 represents maximum light, indicating that no absorbing structure is situated within the light path. Thresholding (or segmentation) is then used to discriminate between the object and the background, by setting a range of grey level values to select the pixels to be measured, which transposes the grey value image into a binary one, where 1 represents the area occupied by cells and 0 represents the background.

3.17.2 Quantitation On Feulgen Slides

Each time the image analysis system is used the light microscope should be set up for optimal Kohler illumination. Firstly, a field was arbitrarily selected that contained a tumour nucleus, an image of this was then captured by the camera. The system was then calibrated for the magnification being used on the microscope which for all slides in this study was the x40 objective. By a system of trial and error it was found that the most effective colour channel to work on for this stain was green. A threshold level was then set for the nucleus being investigated using the green channel only, after first sampling the image to give an approximation of the range of values to be used. This results in the formation of a binary image, which may be represented in either black or white (as required). The required measurements were then selected, which in this case were optical weight, detected area, and circularity. These measurements were made on individual objects (nuclei) by selecting them with the mouse pointer and "clicking" the left mouse button. The results were stored on the hard disk, with a floppy disk back-up (as a Foster Findlay internal format) and also saved as a text file for later use in spreadsheet packages. This procedure was repeated for at least 20 nuclei in each of the tumour samples and for a population of lymphocyte infiltration within a sample. It was necessary to select the magnification and parameters measured only at the beginning of each image analysis session.

3.17.3 Quantitation For Nuclear Antigens (PCNA, Mib1, RB, P53)

As before, a field was containing a nucleus showing a positive reaction (red colour) was randomly selected and the image was captured, again on x40 magnification. Once again, the green channel was found to be the most effective, and the image was thresholded. The binary image colour was changed to black in order to enhance visualisation against the very pale background. Detected area (DA) and optical weight (OD) only were measured on at least 20 nuclei and saved as above, which allows the calculation of OD/DA per nucleus. This was all that was required for Rb and p53, although measurements were divided into central and edge of tumour results for Mib1, Mibc and Mibe respectively, although the central results appeared to represent the true reaction. As there is a problem with false positive reactions when using PCNA, it was decided to try setting a cut-off threshold point. This was set at 0-127on the green channel with cells above this value regarded as positive and those below classified as negative.

3.17.4 Quantitation For Cytoplasmic Antigens (CMYC, MDM2, BCL2, NM23,C-ERBB2)

The microscope was set up for Kohler illumination, the x40 magnification objective was selected and a random image containing positive nuclei was captured. The threshold was set using the green channel so that all positive cells within the field were selected as a binary image, and the optical weight for the field was measured and stored. The threshold was then increased so that all cells within the field were selected, the total object area was then measured. This procedure was performed on a total of 8 fields for each tumour and the average optical weight and object area calculated.

For each antigen either nuclear or cytoplasmic only those sections that were subjectively positive (weak, moderate or strong) were measured objectively. This is because the image analysis system still always has a degree of bias i.e. the operator sets the thresholding levels, therefore it was decided that only those cases that had a red positive reaction detectable by the naked eye upon light microscopy would be considered as measurable. An example of a typical negative reaction is provided photographically in the results section.

3.17.5 Quantitation Of AgNORs

Generally AgNORs are counted using x100 oil immersion on a light microscope. With this method the numbers of AgNOR dots are counted within a cell nucleus for an arbitrary number of fields, and the mean number of AgNORs per cell can be calculated. However, this does not take into account the actual size of the AgNORs, except as subjective observations that may be included. Several studies have previously advocated the use of image analysis systems to optimise and standardise AgNOR quantitation (Ruschoff *et al*, 1990 ; Derenzini and Trere, 1991). Within this study the image analysis system was used to calculate the AgNOR area per nucleus for 100 nuclei in each tumour, a reference lymphocyte population was also measured. The results of 9 cases plus the lymphocytes are given below (SD = standard deviation, CV = co-efficient of variation).

CASE NO.	MEAN AgNOR AREA(μ^2)	SD	CV
10084/90	1.736	0.792	46%
9136/89	2.443	0.881	36%
15041/89	4.847	3.202	66%
11928/90	1.632	0.834	51%
15230/92	1.785	0.901	50%
1826/95	1.892	0.860	45%
2896/91	3.431	2.518	73%
4024/89	4.430	1.580	36%
4580/92	1.589	0.847	53%
LYMPHS	0.398	0.175	44%

Table 1 : Results of AgNOR image analysis

From these results it can be seen that the data obtained from image analysis of AgNORs was, in this case at least, virtually meaningless because of the huge variations within the tumours demonstrated by the high CV values. The large CV value in the lymphocyte population may be due to the fact that these were an infiltrate in one of the tumours, so some could have been proliferating while others were not giving rise to a variation in AgNOR values. Due to the large variations recorded in these randomly selected tumours it was decided to discontinue further AgNOR quantitation within this study.

3.18 STATISTICAL ANALYSES

Statistical investigations were carried out using the software Minitab (version 10.1)within Windows on a networked PC.

3.18.1 Statistics on Subjective Data (172 cases)

The only statistical test used on the subjective data was a simple correlation to check for significant correlations between pairs of biological markers.

3.18.2 Statistics on Objective Data

Firstly, the objective data was considered using the biological markers only, for 51 cases. Again, a simple correlation was used to check for relationships between pairs of markers. Then only the 31 cases that had survival data also available were considered, still in terms of the biological markers only. However, upon plotting a histogram of the survival data it was found to be very skewed so $\log_{10}$ of the survival data was used to overcome this problem. A best subset regression analysis (multiple regression) was carried out on these cases to identify the best combination of markers able to predict survival, up to a maximum of 5 parameters as too many would only cause further confusion.

Then the results were tested using the biological markers in combination with data on the SCLC histological subtype (intermediate and oat cell), using a simple correlation analysis. A one-way analysis of variance (ANOVA) was carried out to determine if the 2 subtypes had different survival patterns. This was followed by a general multiple analysis of variance (MANOVA) to identify which of the biological markers have a statistically significant expression in either of the subtypes.

The rest of clinical data was then brought into the analysis (treatment, age, sex) and again a simple correlation was carried out. A best subset regression was then performed once again to see if the clinical parameters had any influence upon predicting survival. FITS data was obtained from this, which is achieved by applying the regression equation generated to each case, thereby giving a value for predicted survival. A graph was then generated of predicted survival (FITS) against actual survival and any outliers were checked to see if they were patients whom no death date was available for (therefore they had to be considered as possibly still surviving).

3.18.3 Comparative Statistics On Objective Plus Subjective Results (51 cases)

The 51 cases that had been quantitated objectively for the biological markers were then compared against the same data available for these cases on subjective assessment. The subjective cases were scored on a scale of 0 - 3, with 0 being negative, 1 being weakly positive, 2 being moderately positive and 3 being strongly positive. This data was sorted into categories of 0-3 for each marker, the objective data was then placed alongside this and where there was enough data

available (i.e. more than 2 results available) the mean, SD and standard error (SE) was calculated for all the categories for each marker. The purpose of this was to illustrate the concordance between the two different methods of analysis.

4. RESULTS

4.1 CELL MONOLAYER RESULTS

After many different modifications (described in detail in section 3.2) a method was developed which isolated single whole SCLC cells from formalin-fixed, paraffin embedded material. This technique was then further modified to allow for the removal of background debris to facilitate quantitation. It was possible to stain the cells obtained with a H and E, but it was initially impossible to immunostain the cells obtained via the original technique developed. Therefore, the technique was further modified to increase the permeability of the cells to allow the dyes and antibodies to enter. However, no matter how many different modifications were attempted immunostaining was found to be impossible and so a serial histological sectioning approach was used.

4.2 IMMUNOCYTOCHEMICAL RESULTS

The optimised methods used for immunocytochemical staining and how they were determined are outlined in section 3. An example of a typical negative immunocytochemical reaction (blue) and a typical positive immunocytochemical reaction (red) for each of the markers used are shown photographically below.

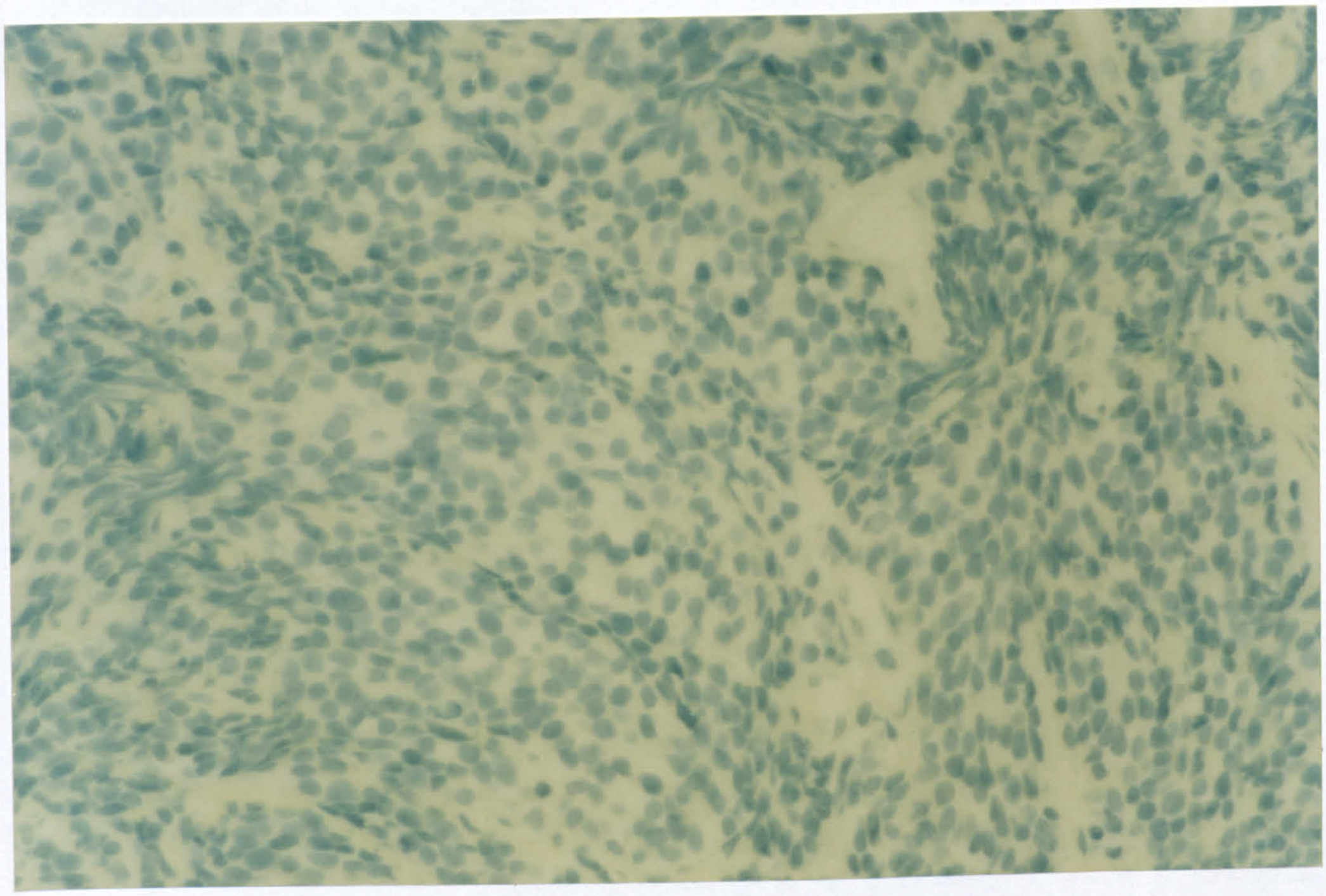


Figure 9 : A typical negative immunocytochemical reaction on a SCLC (case no - 9628/95). As this is a negative control section no primary antibody has been applied, so no red colour should be seen as there will be no reaction apart from non-specific background staining. The only colour visible is the blue of the haematoxylin counterstain. Magnification x400.

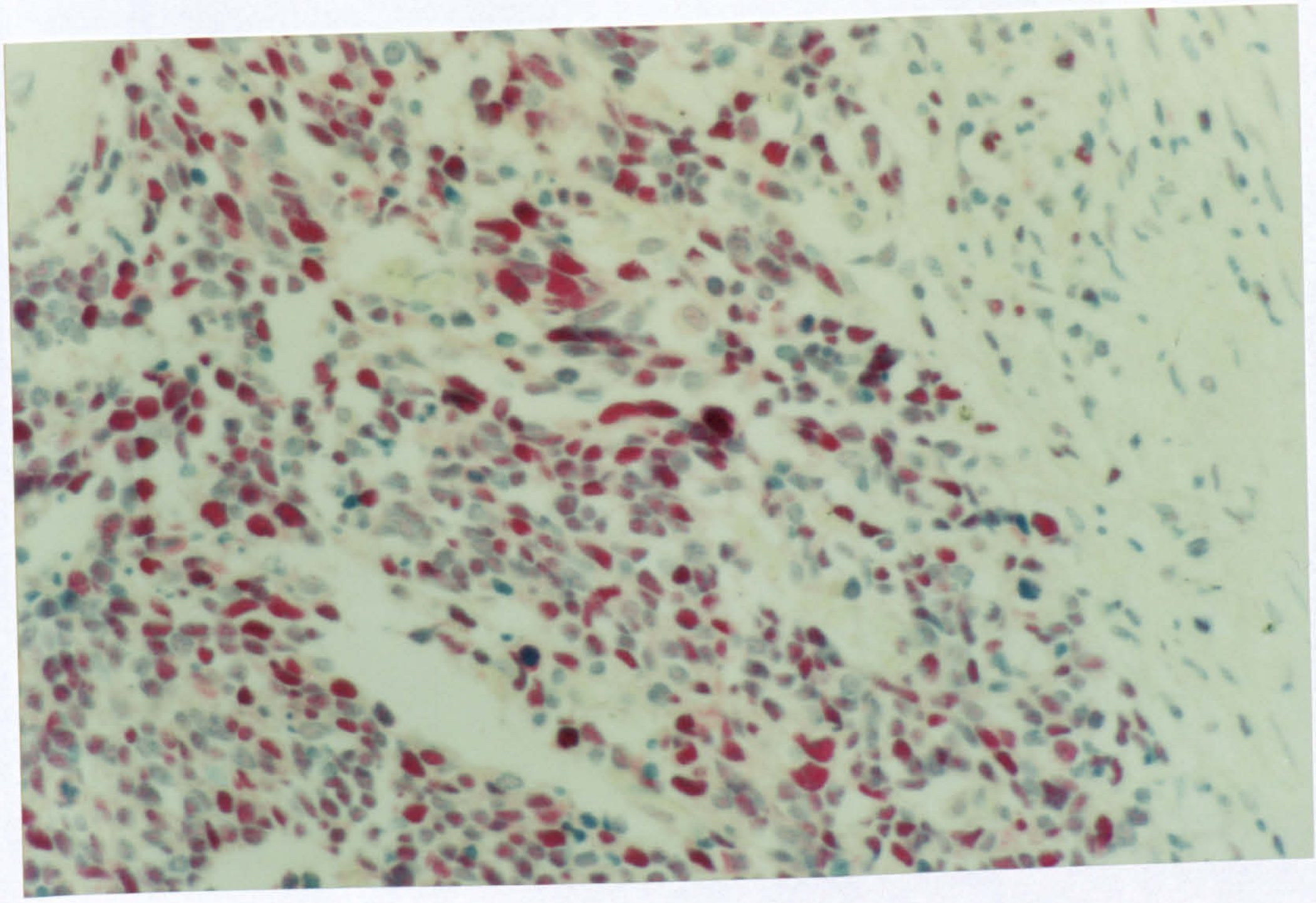


Figure 10 : A tumour section showing a positive immunocytochemical reaction with the PCNA antibody (case no - 10669/93). The red colour is localized to the nucleus for this particular antigen. The red-stained positive cells are expressing this antigen and, according to the data on PCNA, are therefore proliferating. Magnification x400.

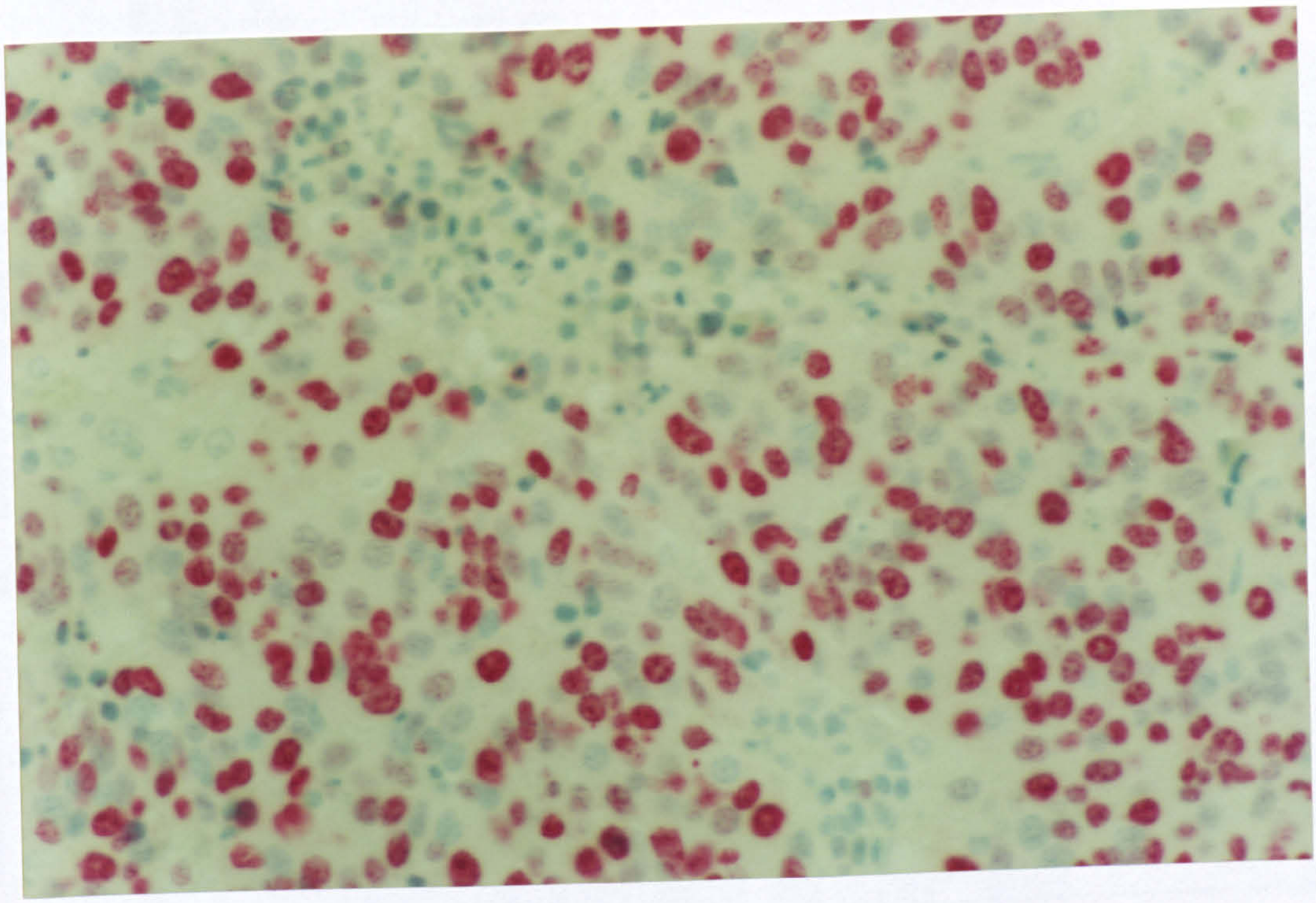


Figure 11 : Tumour section showing immunocytochemical positivity with the Mib-1 antibody (case no - 5133/95). The Mib-1 antigen is localized to the nucleus which corresponds to the red nuclear staining seen. The positive cells are presumably proliferating as they express Mib-1 which is a marker of cell proliferation. Magnification x400.

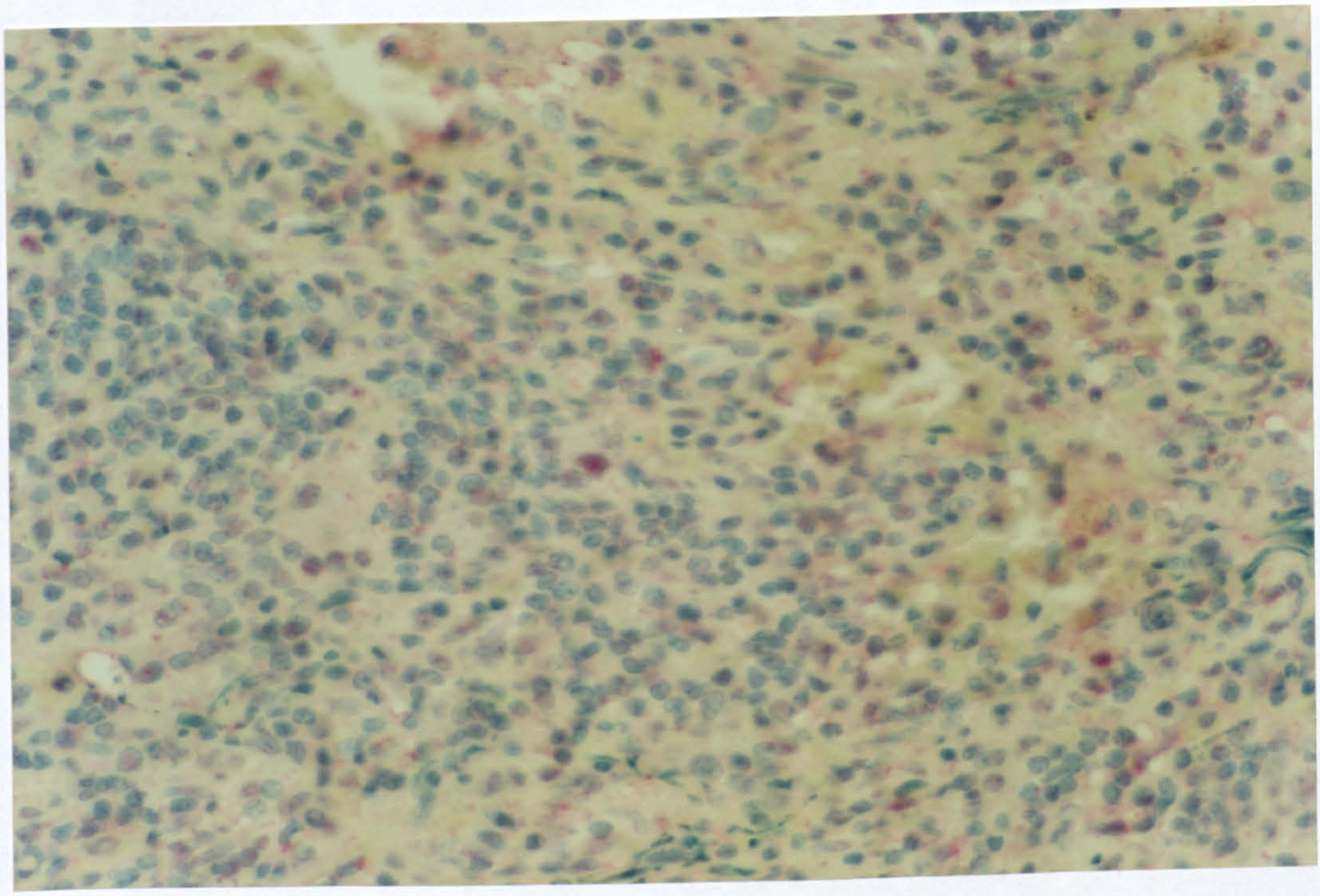


Figure 12 : A tumour section showing a comparatively rare positive immunocytochemical reaction with the RB antibody (case no - 5133/95). The red positive reaction seen with this antibody is much weaker compared to that seen with the other antibodies used within this study. There were very few positive reactions as SCLC rarely expresses the RB protein. Magnification x400.

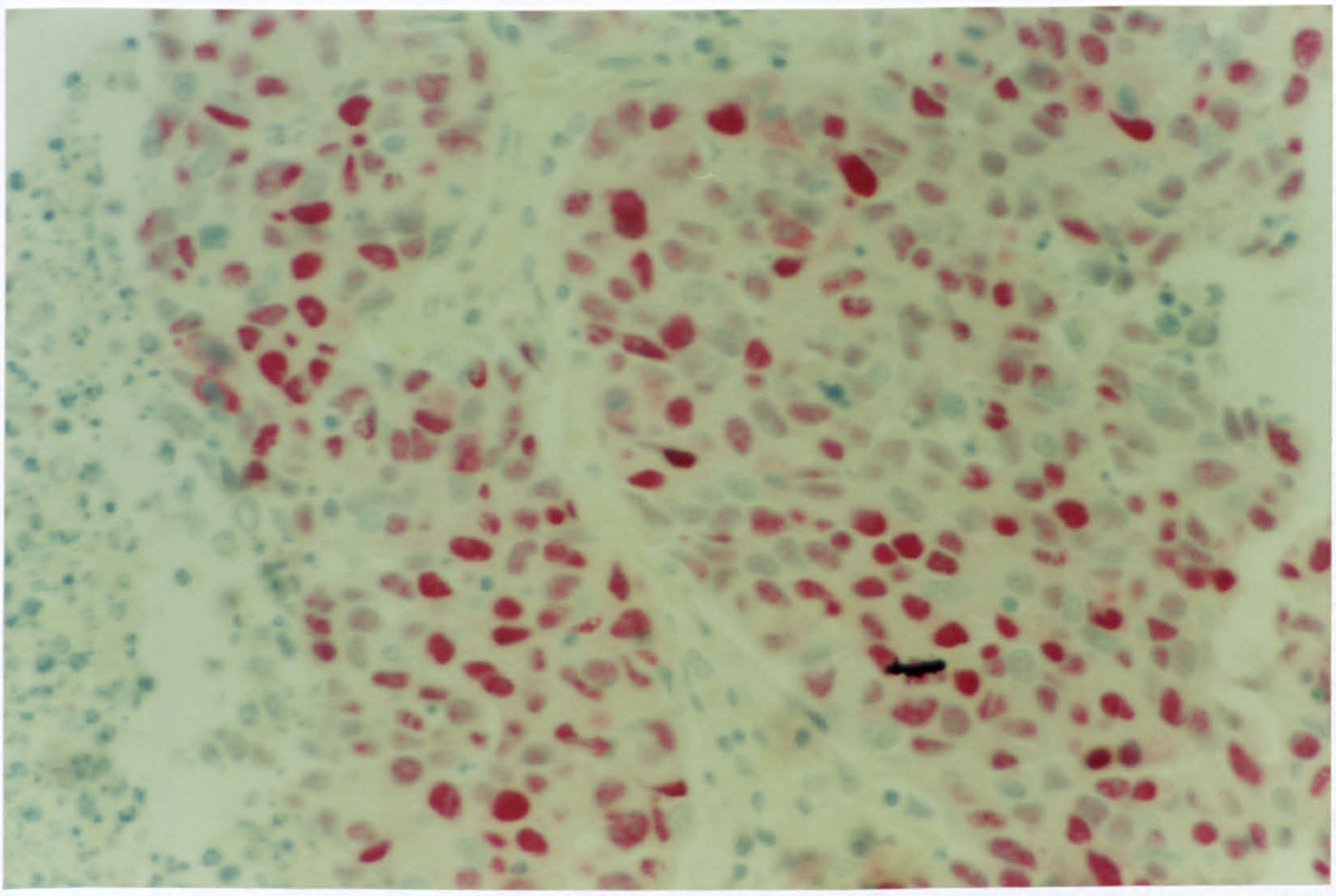


Figure 13 : Tumour section showing a positive nuclear immunocytochemical reaction with the P53 antibody DO-7, which identifies both wild type and mutant forms of the protein (case no - 5361/94). However the positive reaction seen will only represent mutant P53 as this the form that reaches levels detectable by immunocytochemistry within the nucleus. Magnification x400.

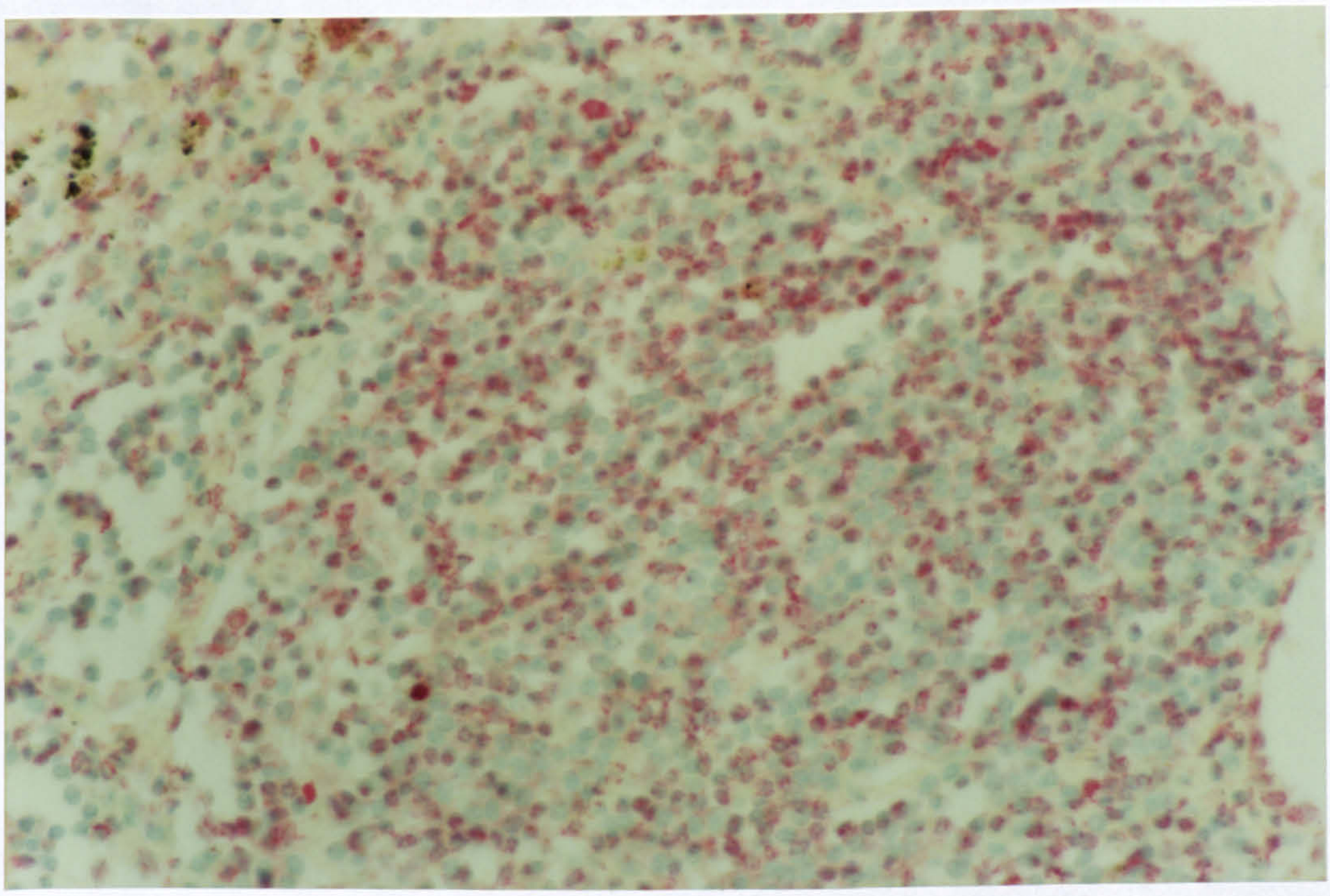


Figure 14 : Tumour section showing the positive cytoplasmic staining reaction produced with the MDM-2 antibody (case no - 1826/95). The red reaction indicates the site of the 90kD protein produced by the mdm-2 gene, which is localized to the cytoplasm. Magnification x400.

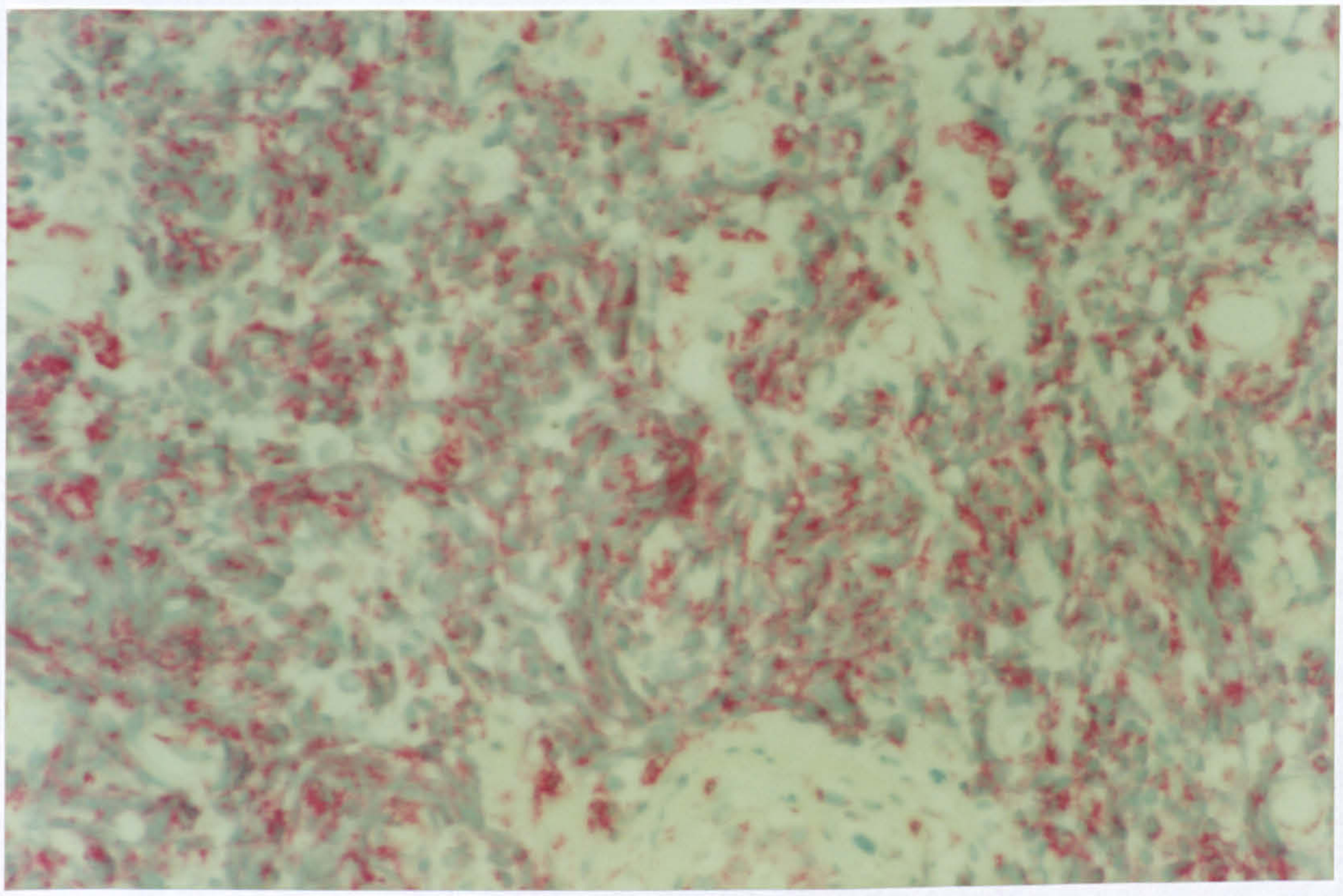


Figure 15 : Tumour section showing a positive immunocytochemical reaction with the C-MYC antibody (case no - 11221/91). The positive reaction can be seen to be cytoplasmic and variable in intensity. The actual site of the red reaction represents the site of the C-MYC antigen which is perinuclear or cytoplasmic following formalin fixation of tissue. Magnification x400.

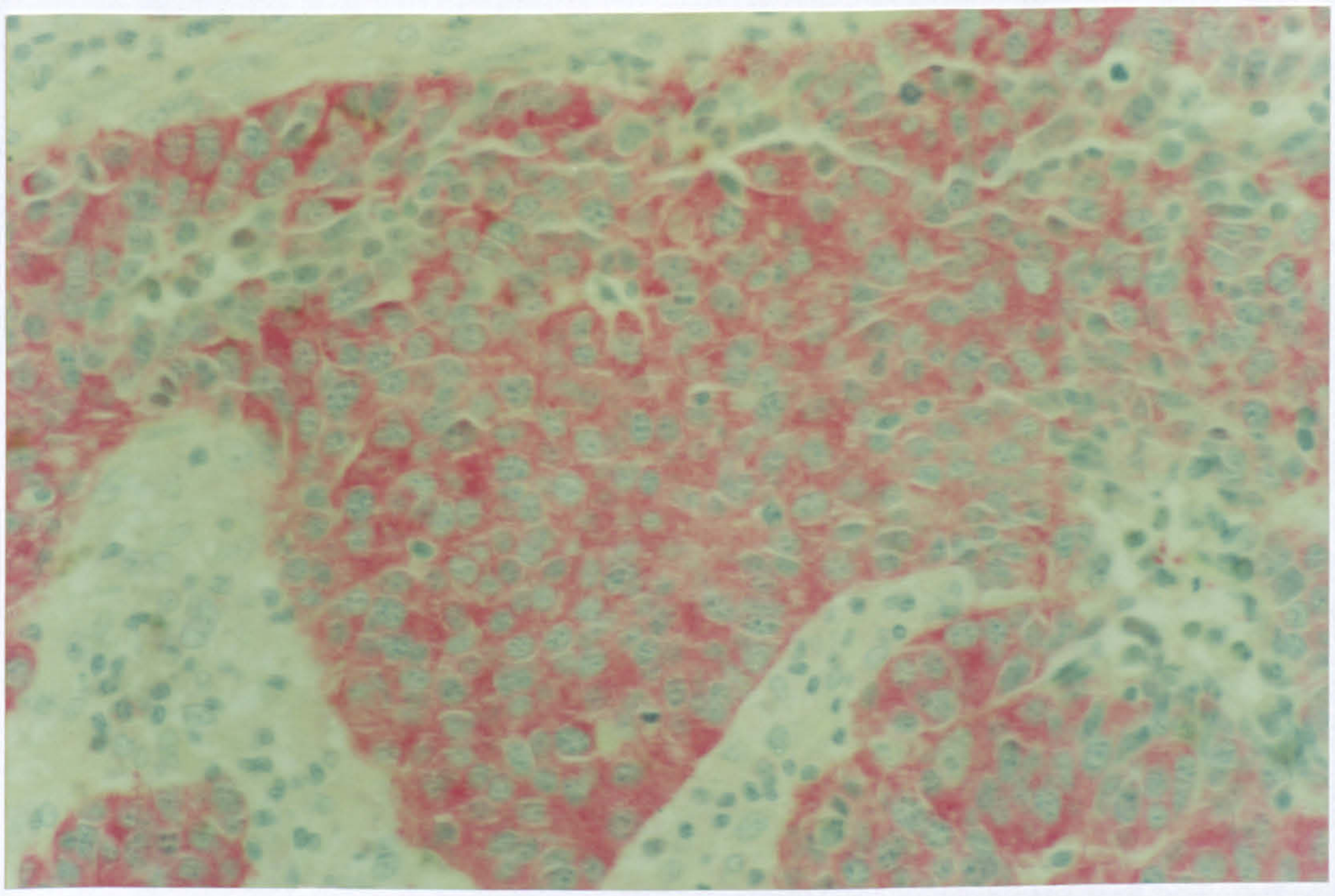


Figure 16 : Tumour section showing a positive immunocytochemical reaction with the NM23 antibody (case no - 11241/92). The red positive reaction produced by the NM23 antibody is strong in comparison to the reaction products of other antibodies within this study. A definite pattern of protein expression is apparent with areas of red positivity seen very obviously in comparison with the blue negative areas. Magnification x400.

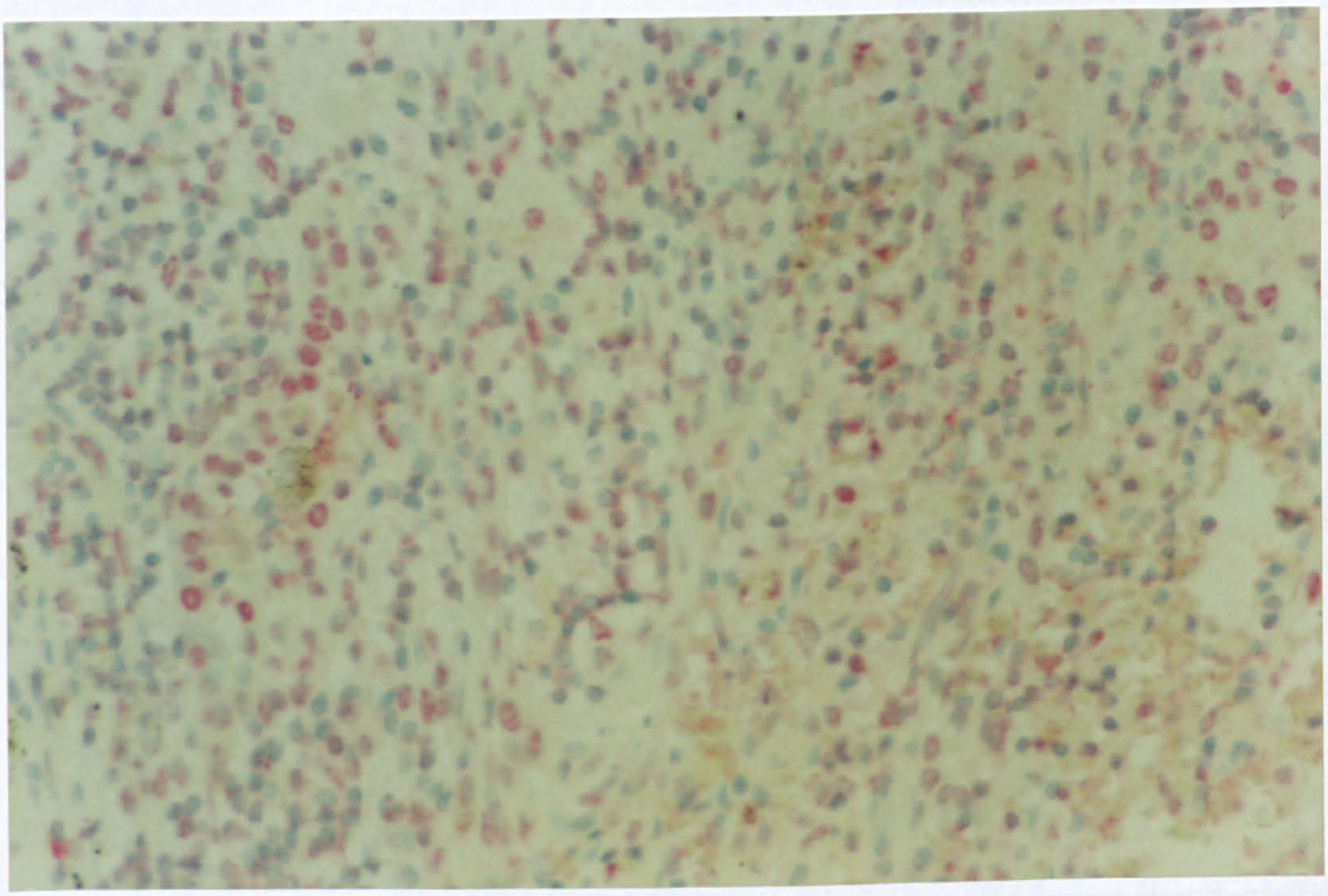


Figure 17 : Tumour section showing a positive immunocytochemical reaction with the BCL2 antibody (case no - 6958/95). This red reaction is once again cytoplasmic and is relatively weak, with some cells staining stronger than others. Magnification x400.

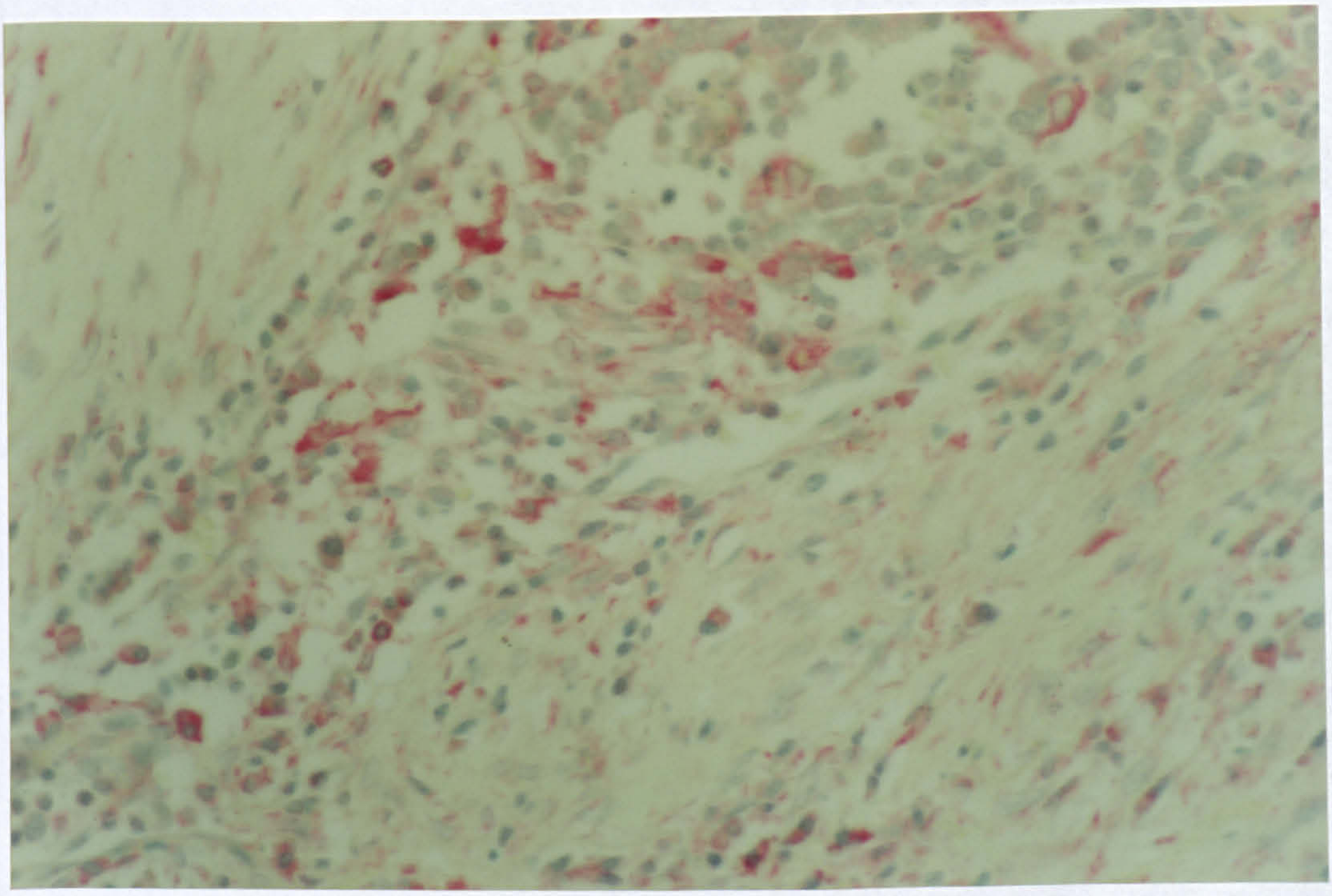


Figure 18 : Tumour section showing a positive immunocytochemical staining reaction with the C-ERBB-2 monoclonal antibody (case no - 10669/93). The red positive staining can be seen to be variable with some areas being much more positive than others. Magnification x400.

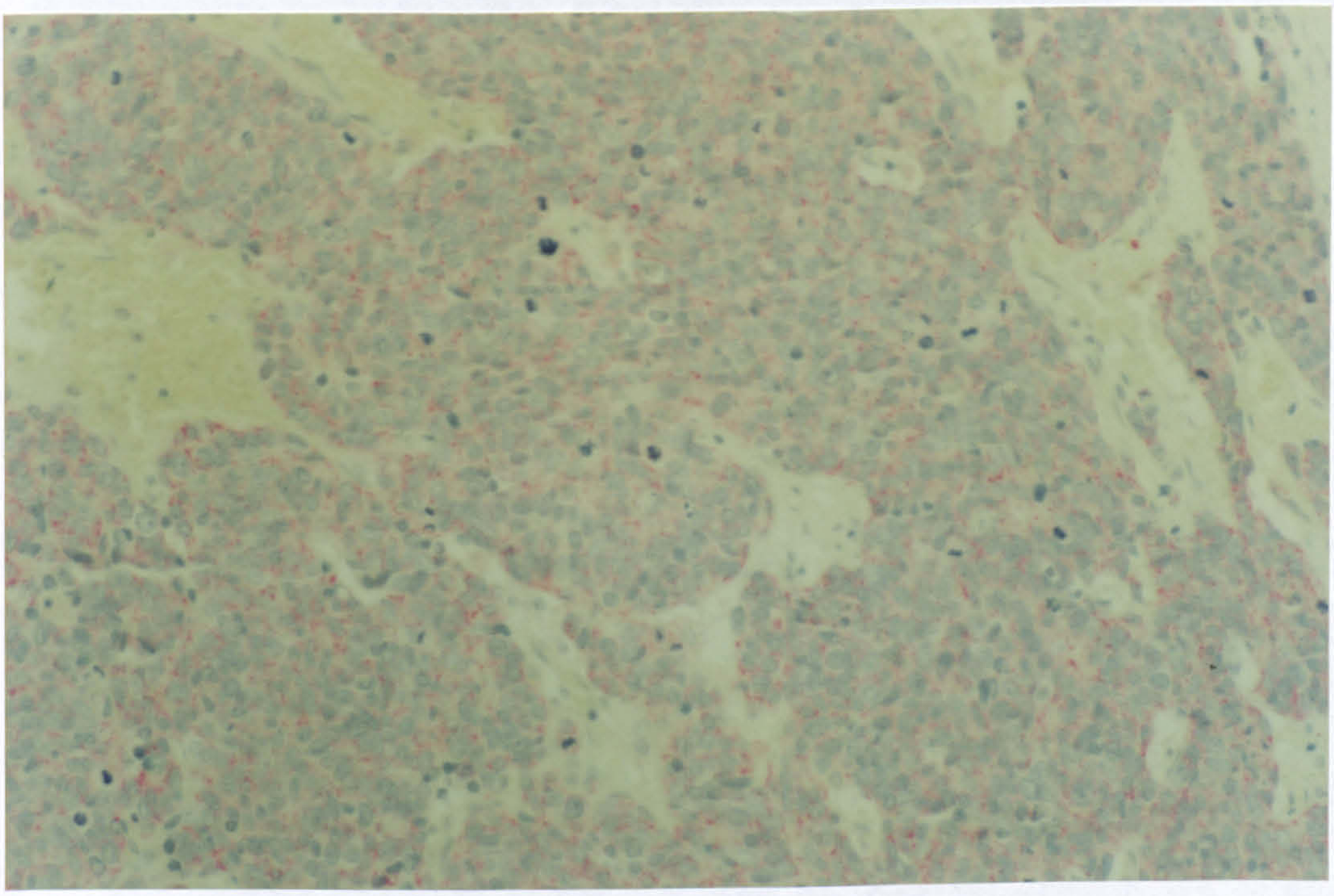


Figure 19 : Tumour section showing a positive immunocytochemical staining reaction with the C-ERBB-2 polyclonal antibody (case no - 6583/92). The red positive cytochemical reaction can be seen to be weak, as were all reactions with this antibody. However, it does appear less variable than the reaction seen with the monoclonal antibody to this antigen (see . Magnification x400.

4.3 SUBJECTIVE RESULTS

All 172 cases were initially assessed subjectively by light microscopy (magnification x400). The cases were assessed as negative if no red reaction product was observed.. If a red reaction was noted cases were then graded on the strength of that reaction either as weakly, moderately or strongly positive. The photographic examples of positive immunocytochemical reactions in SCLC that are shown in section 4.2 are moderate or strong reactions (where possible). In order to compare the results obtained with those produced by other studies, a summary of the results obtained for each strength of reaction and for each

antigen was compiled from the somewhat lengthy table of full results which is provided in appendix IV. These numerical results were also converted into percentages to make comparisons easier, and these may be seen in table 2 below.

ANTIGEN	-VE		WK +VE		MOD +VE		ST +VE	
MIB 1	89/172	52%	38/172	22%	26/172	15%	19/172	11%
PCNA	85/172	49%	30/172	17%	35/172	20%	22/172	13%
P53	130/172	76%	17/172	10%	14/172	8%	11/172	6%
RB	163/172	95%	5/172	3%	4/172	2%	-	-
BCL2	132/172	77%	18/172	10%	13/172	8%	9/172	5%
NM23	124/172	72%	30/172	17%	12/172	8%	6/172	3%
MDM2	16/172	9%	26/172	15%	89/172	52%	41/172	24%
C-ERBB-2p	159/172	92%	11/172	6%	2/172	2%	-	-
CMYC	94/172	55%	45/172	26%	28/172	16%	5/172	3%
C-ERBB-2m	142/172	82%	22/172	13%	7/172	4%	1/172	1%

Table 2 : A summary of the graded subjective results

In order to compare the subjective results obtained with those from studies that did not grade strength of reaction, a more simplified table was compiled that merely gave percentages of positive and negative immunocytochemical reactions for each antigen studied. These overall subjective results in their simplest form are given in table 3 below.

ANTIGEN	NEGATIVE	POSITIVE
MIB 1	52%	48%
PCNA	49%	51%
P53	76%	24%
RB	95%	5%
BCL2	77%	23%
NM23	72%	28%
MDM2	9%	91%
cerbB2 p	92%	8%
CMYC	55%	45%
cerbB2 m	82%	18%

Table 3 : A summary of the overall subjective results (full details in appendix V).

4.4 OBJECTIVE RESULTS

Of the 172 SCLC cases studied 51 cases were selected on the basis of morphological preservation for objective quantitation using digital image analysis. These results were compiled to illustrate the percentages of positive and negative results for each antigen studied objectively, rather than subjectively, and are given in table 4. For the objective results Mib1 was assessed at the edge of the tumour section (Mibe) and at the centre (Mibc). Analysis deemed Mibc to be the true Mib1result with Mibe appearing to be artefactual, possibly due to trauma incurred whilst removing the tissue sample.

ANTIGEN	- VE		+ VE	
PCNA	19/51	37%	32/51	63%
MIB e	39/51	76%	12/51	24%
MIB c	24/51	47%	27/51	53%
CMYC	25/51	49%	26/51	51%
MDM2	4/51	8%	47/51	92%
C-ERBB-2p	43/51	84%	8/51	16%
BCL2	35/51	69%	16/51	31%
NM23	26/51	51%	25/51	49%
C-ERBB-2m	43/51	84%	8/51	16%
P53	35/51	69%	16/51	31%
RB	48/51	94%	3/51	6%

Table 4 : A summary of objective results for the biological markers (full details available in appendix IV)

4.5 STATISTICAL ANALYSIS RESULTS

4.5.1 Subjective Data

All 172 cases available were assessed subjectively for each of the antigens studied and scored according to the reaction seen as either 0 (negative), 1 (weak positive), 2 (moderate positive), or 3 (strong positive). The results of a simple correlation test showed only 3 weakly negative correlations, the rest all being positive. The only statistically significant correlation seen ($p<0.00$) was between CMYC and C-ERBB-2 polyclonal, this was however a misleading result as graphically very little relationship was obvious. Also, it is possible that the polyclonal antibody is picking up something apart from the C-ERBB-2 antigen. Basically, the correlation results illustrate a high degree of inter-relationship between the markers i.e. when one marker is expressed others are also to a greater or lesser degree.

4.5.2 Objective Data

4.5.2.1 BIOLOGICAL DATA

A correlation test on all 51 results for the biological markers showed 15 weakly negative correlations, with the rest being positive. As before this shows all the markers to be correlated to some degree, with high inter-relationship between pairs of markers. The data used was then restricted to the 31 cases where survival data was available and multiple regression analysis was performed (best subset regression). This generated the best combination of markers (up to 5) that could be used to predict survival.

For the available biological marker data the most statistically significant predictor was NM23, with a p value of 0.009 (same p value for the equation) and the regression equation being - log survival = 0.665 + 0.369nm23. The positive value preceding NM23 in the equation implies a possible positive prognostic role i.e. presence of NM23 is beneficial for patient survival.

The best marker combination was for 5 markers, these being PCNA, Mibc (Mib1), CMYC, NM23 and P53. The p values were - PCNA $p<0.014$, Mib1 $p<0.023$, CMYC $p<0.081$, NM23 $p<0.009$, P53 $p<0.201$, and regression equation p value <0.007. The regression equation is given below -

Log survival=0.625 + 0.102 PCNA - 0.120 Mib1 - 0.394 CMYC +0.406 NM23 + 0.0887 P53

From the low p value of the equation (less than 0.05 is statistically significant) it can be seen that in combination these markers are highly statistically significant. The p values for the markers show NM23, Mib1 and PCNA to be statistically significant, and although strictly speaking CMYC is not significant alone it is only just outside significance, whereas P53 is well outside of this range. However, if P53 is removed from the combination the other markers lose significance, as does the combination of markers. It therefore seems likely that there is high inter-relationship between the markers in combination, so although P53 is not statistically significant alone it is important and necessary in combination. The contradicting signs mathematical signs on the regression equation for Mib and PCNA were investigated by

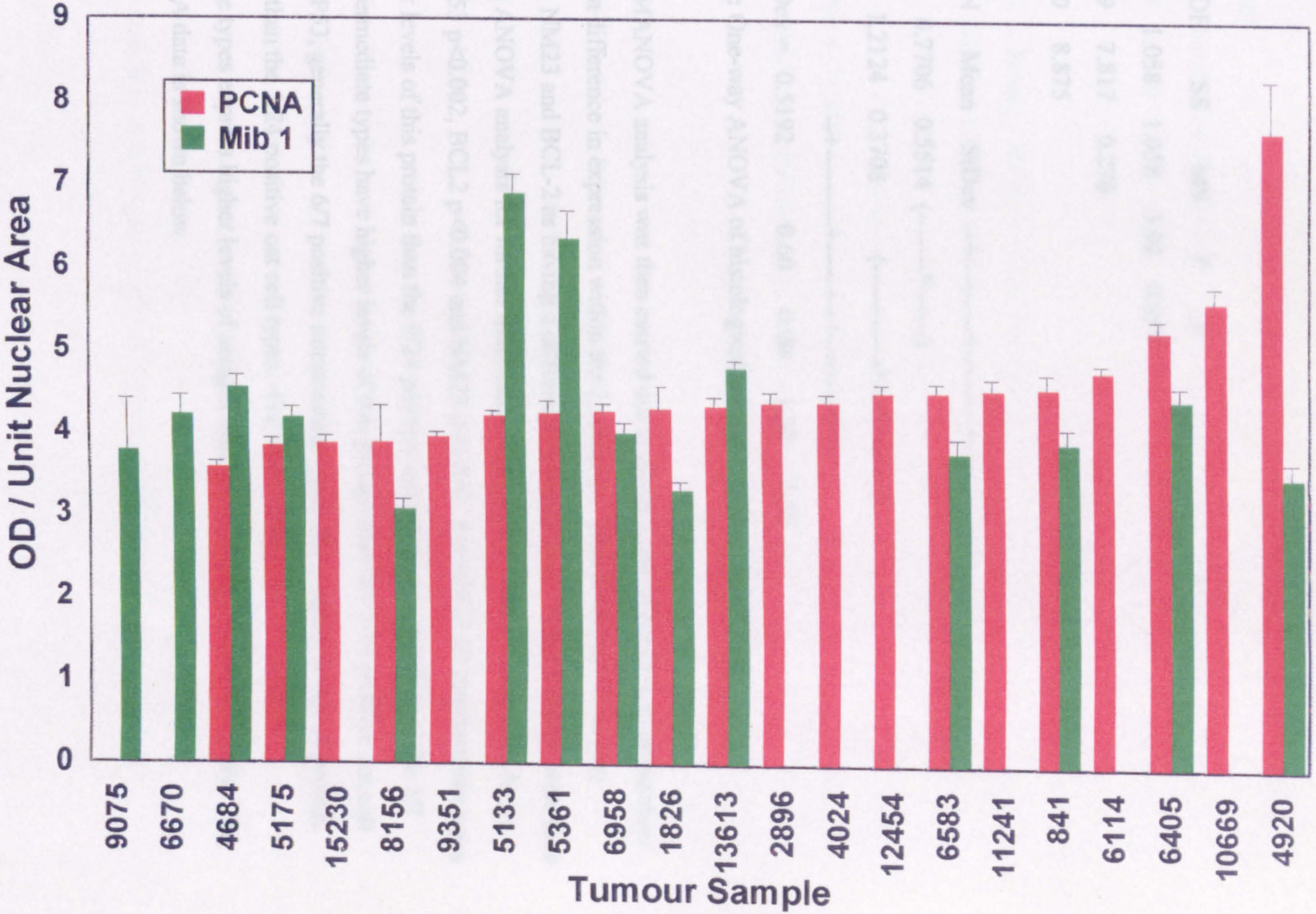
carrying out a separate regression analysis for each of the 2 markers. The actual values in the equation should not be taken as absolute as it may be the contrast between the markers that is the important factor. A comparison of the levels of these two markers in the 31 cases studied is given graphically in Figure 20, where it can be seen that 2 case were negative for PCNA but positive for Mib1 and 6 cases were positive for PCNA and negative for Mib1. As these antigens both represent markers of cellular proliferation the significance of this result is unclear, although the cases in question were immunostained several times in order to ensure that these results were genuine.

The 51 cases used for objective analysis were selected randomly on the basis of morphological preservation and this was further reduced to 31 cases based on eliminating those on which survival and clinical information was unavailable, thereby eliminating bias. However, this presented a problem for the analysis of MDM2 protein as only 2 negative cases were included in the 31 objective cases studied. Therefore, the data on this marker is skewed and it will never achieve statistical significance in this study.

4.5.2.2 BIOLOGICAL MARKERS IN HISTOLOGICAL SUBTYPES

The same 31 cases were then looked at in combination with data on the tumour histological subtype (either intermediate or oat cell). A one-way ANOVA was carried out to illustrate that the two different subtypes had definitely different survival patterns, with oat cell having a generally lower survival period than intermediate cell.

Figure 20 : Comparison of Mib1 and PCNA levels



Source	DF	SS	MS	F	p
hist	1	1.058	1.058	3.92	0.057
Error	29	7.817	0.270		
Total	30	8.875			

Level	N	Mean	StDev	--+---------+---------+---------+----
0	24	0.7706	0.5514	(-------*------)
1	7	1.2124	0.3708	(-----------*-----------)
				--+---------+---------+---------+----
Pooled StDev =		0.5192		0.60 0.90 1.20 1.50

Figure 21 : One-way ANOVA of histological subtypes against log survival

A general MANOVA analysis was then carried out to identify any of the biological markers which had a difference in expression within the 2 subtypes. This identified 4 markers - Mib1, P53, NM23 and BCL-2 as having a difference in expression. These markers were then included in ANOVA analysis for further illustration (see below). The p values were Mib1 $p<0.017$, P53 $p<0.002$, BCL2 $p<0.004$ and NM23 $p<0.000$. For NM23 all intermediate types have higher levels of this protein than the 9/24 positive oat cell types. For BCL-2 the 5/7 positive intermediate types have higher levels of this protein than the 6/24 positive oat cell types. For P53, generally the 6/7 positive intermediate types show higher levels of protein expression than the 5/24 positive oat cell types. For Mib1 (Mibc) the 5/7 positive intermediate types express higher levels of antigen than the 9/24 positive oat cell types. The full ANOVA data is shown below.

Source	DF	SS	MS	F	p
hist	1	24.42	24.42	11.62	0.002
Error	29	60.96	2.10		
Total	30	85.38			

Level	N	Mean	StDev	---------+---------+---------+------
0	24	0.687	1.389	(----*----)
1	7	2.810	1.664	(--------*--------)

Pooled StDev = 1.450 1.2 2.4 3.6

Figure 22 : One-Way ANOVA for P53 in Histological Subtypes

Source	DF	SS	MS	F	p
hist	1	8.066	8.066	41.69	0.000
Error	29	5.611	0.193		
Total	30	13.678			

Level	N	Mean	StDev	---------+---------+---------+-------
0	24	0.2821	0.4286	(---*--)
1	7	1.5021	0.4807	(------*------)

Pooled StDev = 0.4399 0.50 1.00 1.50

Figure 23 : One-Way ANOVA for NM23 in Histological Subtypes

Source	DF	SS	MS	F	p
hist	1	2.106	2.106	9.65	0.004
Error	29	6.329	0.218		
Total	30	8.435			

Level	N	Mean	StDev	---------+---------+---------+------
0	24	0.2087	0.3896	(-----*-----)
1	7	0.8322	0.6877	(---------*---------)
				---------+---------+---------+------
Pooled StDev =		0.4672		0.35 0.70 1.05

Figure 24 : One-Way ANOVA for BCL-2 in Histological Subtypes

Source	DF	SS	MS	F	p
hist	1	29.01	29.01	6.41	0.017
Error	29	131.28	4.53		
Total	30	160.29			

Level	N	Mean	StDev	-------+---------+---------+---------
0	24	1.441	1.923	(-----*-----)
1	7	3.755	2.777	(---------*---------)
				-------+---------+---------+---------
Pooled StDev =		2.128		1.5 3.0 4.5

Figure 25 : One-Way ANOVA for Mib1 in Histological Subtypes

Simple correlation analysis showed a high correlation (0.768) between NM23 and histological subtype ($p<0.000$), which just backs up the information already shown by ANOVA and MANOVA analyses. A high correlation was seen between C-ERBB-2 monoclonal and Mibe (0.641), but this was purely coincidence as there were only 4 positive

C-ERBB-2 results and 6 positive Mibe results. A similar correlation (0.649) was seen with RB and Mibe, but as there were only 2 positive RB results this was not considered significant. Another correlation was shown between P53 and Mibc (Mib1) (0.632) with a p value of 0.000, and this was reflected by the inter-relationship apparent in the best subset regression, as when P53 is removed from the combination Mib1 loses statistical significance.

4.5.2.3 BIOLOGICAL DATA PLUS CLINICAL DATA

A simple correlation test provided no new information, only that there is more positive than negative correlations and everything is related to some degree. When predicting survival histology was not statistically significant as a combination of biological markers had a higher significance i.e. there was a stronger correlation between biological markers and survival than between histological subtype and survival. As before multiple regression analysis was performed as best subset regression (up to a combination of 5 markers). NM23 was still the most statistically significant single predictor (same equation and p values as previously). However, once the number of factors increases above one NM23 loses significance and only appears in the second best combinations. The best combination of was found to be PCNA, Mibc (Mib1), P53, chemotherapy, and radiotherapy. The p values were - PCNA $p<0.002$, Mib1 $p<0.003$, P53 $p<0.01$, chemo $p<0.002$, radio $p<0.009$ and equation $p<0.000$.
The regression equation generated is shown below :

Log survival = 0.151 + 0.115 PCNA + 0.699 chemo + 0.403 radio - 0.135 Mib1 + 0.154 P53

As before it is likely to be relative levels that are important rather than the actual values given in the equation. The only possible negative prognostic factor is Mib1, which is still showing a contradictory prognostic role to PCNA. This time P53 is significant, although still to a lesser degree than the other factors in the combination. The results of the 2 treatment factors are as expected i.e. patient has a good prognosis if they have received chemotherapy or radiotherapy. In view of this the preferred combination to quote would be the biological markers only, as this can provide an prediction of survival based on the biology of the tumour at diagnosis before treatment has commenced. The relative levels of the markers in the best combination are demonstrated graphically in figure 26. As it can be seen not all markers are

expressed in each case which may reflect the inherent heterogeneous nature of all human tumours.

FITS data was also collected during multiple regression analysis, this is generated by applying the regression equation to each tumour case and getting a value for predicted survival. This FITS data was then plotted against actual survival (Figure 27) to see if the relationship between the two was as close as expected. The outlying values were examined to see if they were caused by the "censored" data i.e. the survival data used where a death date was not available for the patient so the survival data used was the number of months that they were recorded to have survived, or where the patient is still alive.

4.5.3 Comparison of Subjective and Objective Data

The subjective results were grouped as 0, 1, 2, 3 along with the objective data. The objective mean, standard deviation and standard error were then calculated for each group per antigen (table of these results in appendix ...). Basically this showed that there was good agreement between the two types of result in that the objective results showed an increase going from 0 to 3. CMYC was the only antigen that showed a decrease from 1 to 2. Although for RB and the two C-ERBB-2 antibodies the results were inconclusive due to lack of data.

Figure 26 : Comparison of Marker Levels

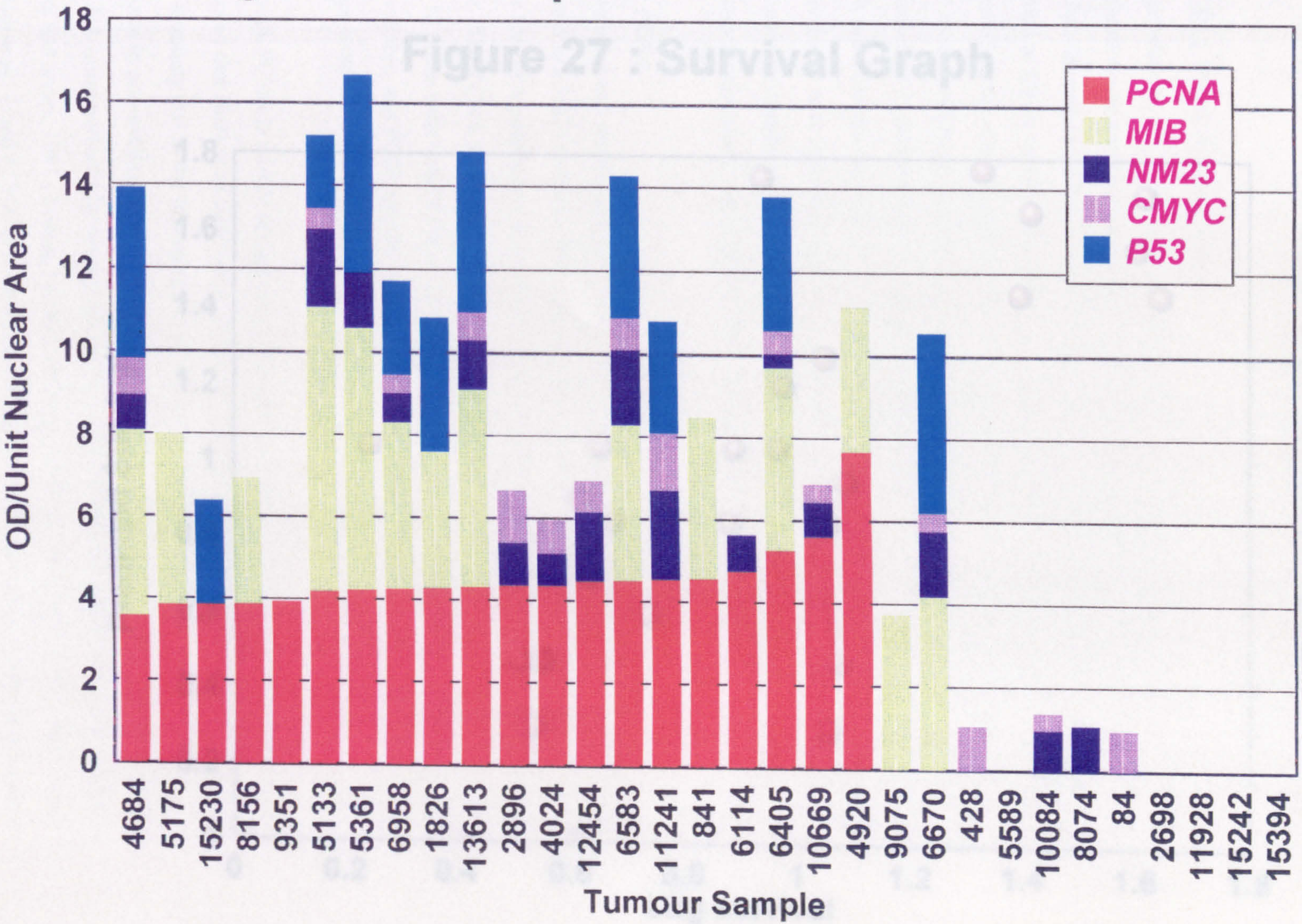
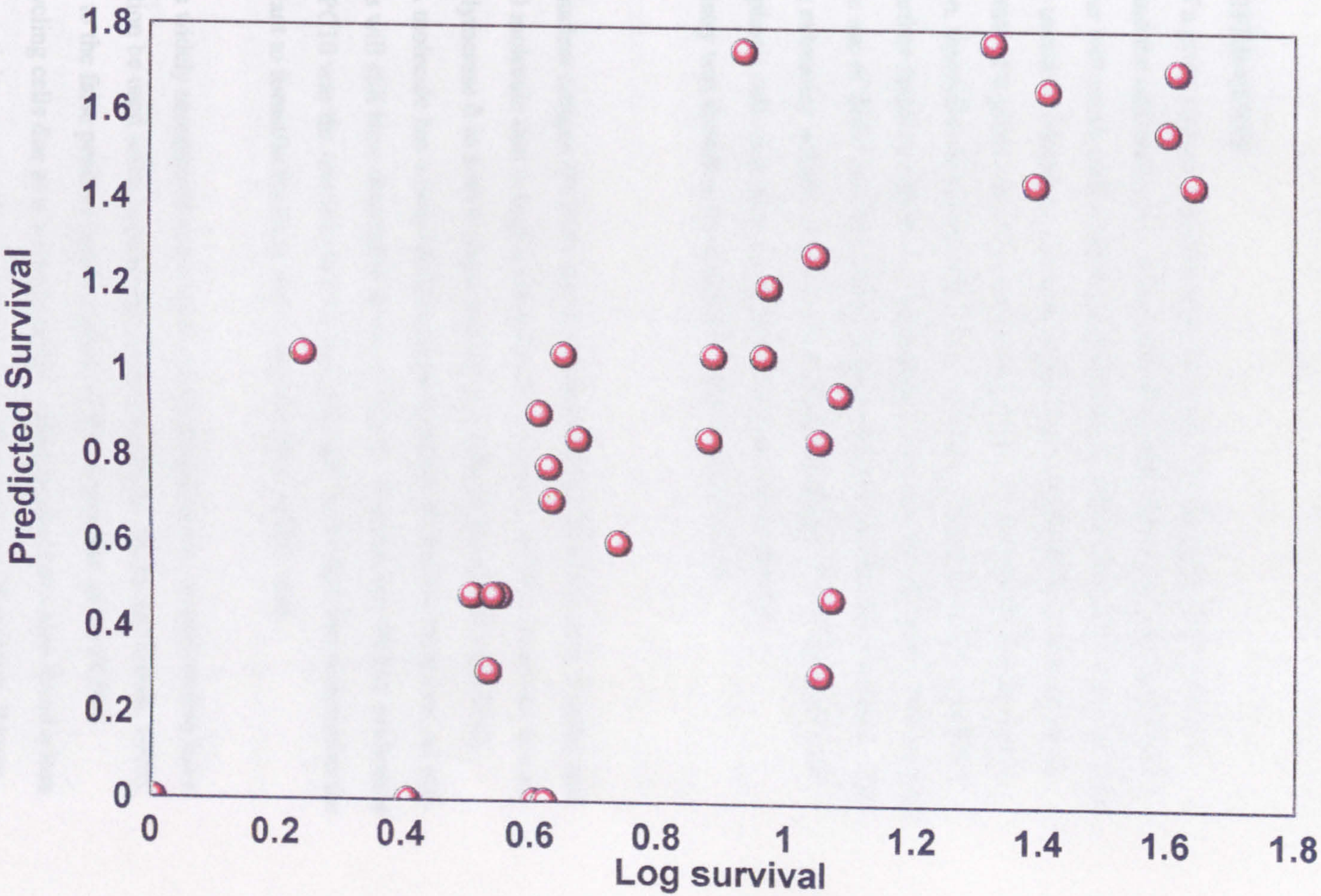


Figure 27 : Survival Graph



5. DISCUSSION

5.1 CELL PROLIFERATION

The growth rate of a given tumour depends upon two main factors, these being the cell proliferation rate and the cell death rate. If the cell proliferation rate exceeds the cell death rate then the tumour will continually expand, so information on this parameter has long been regarded as highly useful by clinicians. Several methods are available for determining the cell proliferation state of a given tumour at a particular time. These include mitotic index, BrdU incorporation, thymidine labelling index, flow cytometry and immunocytochemistry (all described in further detail in chapter 1). The material available for this study was archival which ruled out the use of BrdU and thymidine, as these require the use of fresh tissue. The mitotic index is an extremely subjective and controversial technique. Flow cytometry also measures non-neoplastic cells and takes no account of tumour heterogeneity. Immunocytochemistry was therefore the method of choice for this study.

5.1.1 PCNA

Proliferating cell nuclear antigen (PCNA) was first identified in 1978 (Miyachi, Fritzler and Tan) and is a 36kD molecule that is highly conserved in evolution. PCNA functions as a co-factor for DNA polymerase δ in both S phase and DNA synthesis associated with DNA repair. The PCNA molecule has a long half-life (approximately 20 hours), therefore, on exit from M phase cells will still have detectable levels of PCNA. Various anti-PCNA antibodies are available, and PC10 was the one selected for this study due to the fact that it identifies the epitope most resistant to formalin fixation, and is also the most widely used.

Although PCNA is widely recognized as a marker of cell proliferation, several studies have suggested that caution be used when interpreting results (Coltrera, Skelly and Gown, 1993). This is mainly due to the false positive results caused by the expression of the PCNA molecule in non-cycling cells due to it's long half-life. Other studies have also found a loss of PCNA immunoreactivity upon prolonged exposure to formaldehyde (Rowlands, Brown, Barber and Jones, 1991 ; Leong, Milios and Tang, 1993). In 1993 Fujii, Motoi, Saeki, Aoe and Moriwaki found PCNA to be of prognostic significance in NSCLC, although they also

noted a great heterogeneity of PCNA expression. Ishida, Kaneko, Akazawa, Tateishi, Sugio and Sugimachi (1993) also found that NSCLC with a high proliferation index, as assessed by PCNA to have a poor prognosis.

When the results generated by this study were analysed the role of PCNA as a possible indicator of survival was found to be closely linked to the expression of four other antigens.

5.1.2 Mib-1 (Ki-67)

Ki67 is an IgG_1 class murine monoclonal antibody raised against a crude nuclear fraction of the Hodgkin's disease derived cell line L428 (Brown and Gatter, 1990), and was first identified in 1983 by Gerdes *et al.* The Ki67 antibody reacts with a nuclear non-histone protein doublet of molecular weight 395 and 345 kD, present in all parts of the cell cycle (G_1, S, G_2 and M) except G_0 (Sawhney and Hall, 1992). The Mib1 antibody was produced to identify the Ki67 antigen in routinely processed tissue, as the Ki67 antibody was originally only intended for use on frozen sections. Several studies have subsequently shown Mib-1 to be true Ki67 equivalent (Cattoretti *et al*, 1992 ; Cuevas *et al*, 1993). It has also been shown that Mib-1 staining correlates with semi-conservative DNA synthesis, rather than excision-repair DNA synthesis (McCormick *et al*, 1993).

SCLC has been found to have a high Ki67/Mib-1 labelling index (Tungekar, Gatter, Dunnill and Mason, 1991). A study on neuroendocrine lung cancers using image analysis with Mib-1 immunostaining, found that Mib-1 may be able to identify subgroups of lung cancer (Bohm, Koch, Gais, Jutting, Prauer and Hofler, 1996). Mib-1 has been verified as a reliable marker for the assessment of cell proliferation (Querzoli, Albonico, Ferretti, Rinaldi, Magri, Indelli and Nenci, 1996). There is also evidence that Mib-1 staining may be useful prognostically (Gee, Douglas-Jones, Hepburn, Sharma, McClelland, Ellis and Nicholson, 1995).

The results of this study indicate that Mib1could be possibly useful in predicting survival in combination with other markers. There was also a difference in expression between SCLC histological subtypes, in that intermediate cell type expressed relatively higher levels of Mib1 than the oat cell types.

5.1.3 Ploidy

For this study ploidy was determined on the basis of the cold Feulgen stain for DNA, which was measured using digital image analysis. This showed the majority of tumours to be diploid and when it was employed as a parameter within the statistical analysis it was found to have very little significance. Full results are given in Appendix V.

5.2 ONCOGENES

5.2.1 CMYC

The myc family of protooncogenes encode nuclear proteins that have DNA binding properties and are believed to be involved in transcriptional regulation. The three best characterized members of this family are C-myc, N-myc and L-myc. C-myc is the cellular homologue of the avian retrovirus oncogene v-myc. Amplification and overexpression of the N and L-myc genes have been described in SCLC but were not consistent with any morphologic, biological or clinical features, unlike c-myc (Gazdar, 1992). Only one member of the myc gene family is amplified in any given tumour. Amplification of myc genes is much less common in NSCLC compared to SCLC (Kern and Filderman, 1993). C-myc has been implicated to participate in DNA replication by influencing the initiation of DNA synthesis. As well as it's role in cell transformation, differentiation and cell cycle progression, c-myc also is also involved in some forms of apoptosis. Several studies have also shown that the oncogene bcl-2 (see section 5.2.4) can prevent c-myc induced apoptosis (Evan *et al*, 1992 ; Fanidi *et al*, 1992). One study found there to be no correlation between the degree of myc gene amplification in primary tumours and survival time, and found myc amplification more frequent in patients who have undergone chemotherapy (Anderson and Spandidos, 1993).

The results of this study did not highlight any significant association between C-MYC and BCL-2 protein expression in SCLC. Although the relative levels of these two proteins, as assessed quantitatively was similar, and much lower than the majority of the other markers studied. However, CMYC was found to be able to predict survival time in patients in combination with several other markers.

5.2.2 MDM-2

Mdm-2 is found on chromosome 12 and produces a 90kD protein that forms a complex with mutant and wild type p53, suppressing it's trans-activation function. The mdm-2 gene has been reported to be amplified in sarcomas. MDM-2 has also been shown to interact with the RB protein , and to inhibit the Rb growth regulatory function (Xiao *et al*, 1995). Wild type P53 induces MDM-2 expression in response to DNA damage while over-expression of MDM-2 inhibits the ability of wild type P53 to stimulate the expression of target genes. Regulation of MDM-2 expression by P53 represents a potential feedback control of P53 function. Analysis of MDM-2 and P53 in sarcomas indicates one or other to be mutated in 70% of tumours.

Analysis of the results of this study showed no significant association between MDM-2 and P53 protein expression. However, for both the subjective and objective data less than 10% of cases were negative for MDM-2, and of the 31 cases used for statistical analysis only 2 MDM-2 negative results were included. The imbalance in this data means that, in this study at least, MDM-2 will never be of any statistical significance.

5.2.3 C-ERBB-2

The neu oncogene was first identified in rats (Schechter *et al*, 1985), whilst the human equivalent was independently cloned from a cDNA library and called HER-2 (Coussens *et al*, 1985). However, after the gene was cloned from genomic DNA it was designated c-erbB-2 (Semba *et al*, 1985). c-erbB-2 gene sequence and protein product (p185) are closely related to the epidermal growth factor receptor (EGFR), and show an overall homology of 50%. The gene has been mapped to chromosome 17q21, also, the p185 protein belongs to class I of the superfamily of tyrosine kinase receptor molecules.

Amplification or overexpression of the c-erbB-2 gene has been found to be a negative prognostic factor in breast cancers (Gazdar, 1992 ; DePotter, 1994). Yokota, Yamamoto, Tayashima, Terada, Sugimura, Battifora and Cline (1986) carried out a study of c-erbb-2 amplification in a range of tumour types, and found amplification to be specific to adenocarcinomas, inclusive of lung tumours. Amplification of c-erbB-2 gene has been

observed in 15-40% of breast cancers and 30% of ovarian cancers, with high levels of protein expression linked to shortened survival and disease free interval, as well as lymph node metastasis. Also, C-ERBB-2 protein expression has been identified as a negative prognostic factor in NSCLC (Kern, Schwartz, Nordberg, Weiner, Greene, Torney and Robinson, 1990). Further data was provided to back up this study, and reported the expression of the C-ERBB-2 protein to carry important negative prognostic information in lung adenocarcinomas (Kern and Filderman, 1993). A study in 1992 found approximately 60% of NSCLC to express C-ERBB-2 protein but noted an absence of expression in SCLC (Shi, Imam, Young and Cote, 1992). These results were verified by Schneider *et al* (1992) who found absence of c-erbB-2 mRNA and protein in SCLC, and suggested this as possibly being unique and maybe useful in a diagnostic sense. A direct concordance between c-erbB-2 gene amplification and increased protein expression was demonstrated by Venter, Tuzi, Kumar and Gullick in 1987.

Statistical analyses of the results generated by this study found no correlations between the C-ERBB-2 protein and any of the other markers used with either the polyclonal or monoclonal antibody. As a comparison of the two antibodies the results were as expected in that the monoclonal antibody was more sensitive than the polyclonal antibody i.e. showed a higher level of positivity. Although the number of cases used for the objective study were approximately equal.

5.2.4 BCL-2

The bcl-2 gene was first identified at the chromosomal breakpoint of the t (14;18) translocation in follicular lymphomas. In this translocation it is brought under the control of the promoter of the Ig heavy chain gene, leading to dysregulation of the gene product. High levels and aberrant patterns of BCL-2 protein expression have been reported in a wide variety of human cancers including colorectal, renal, neuroblastoma, non-Hodgkin's lymphomas and chronic and acute leukaemias (Reed, 1995). Immunolocalization studies have shown BCL-2 to be an inner mitochondrial membrane protein of molecular mass 25 kD (Hockenbery *et al*, 1990). BCL-2 expression inhibits cell death but does not enhance cell proliferation. BCL-2 has also been implicated in the regulation of intracellular calcium distribution, as well as an antioxidant role. Some studies *in vivo* have shown that levels of P53 in breast cancer are

inversely related to expression of bcl-2 (Lu *et al*, 1996). Haldar, Negrini, Manne, Sabbioni and Croce (1994) also found an inverse correlation between the expression of BCL-2 and mutant P53 protein in breast cancer cells, suggesting that P53-induced apoptosis may be due to the down-regulation of the BCL-2 protein. Vaux, Cory and Adams (1988) showed that BCL-2 co-operates with C-MYC in cell transformation.

Pezzella *et al* (1993) showed 20% of NSCLC to be positive for BCL-2 protein (on immunocytochemistry) and that these tumours had a better clinical outcome. They also demonstrated absence of BCL-2 protein expression in undifferentiated cells of the normal bronchial epithelium. Fontanini *et al* (1995) found a high level of BCL-2 positivity in NSCLC (mainly squamous cell carcinomas) at 67%, but agreed with Pezzella *et al* that BCL-2 overexpression seems able to induce a less aggressive phenotype. They demonstrated an inverse relationship between P53 and BCL-2 protein expression, and found no relationship to cell proliferation. Also, loss of BCL-2 expression was shown to be associated with shorter overall survival and metastatic development in this tumour type. High expression of BCL-2 gene has been shown in SCLC cell lines, and the same study also demonstrated that BCL-2 and C-MYC expression does not always occur together (Ikegaki *et al*, 1994). A study by Ben-Ezra, Kornstein, Grimes and Krystal (1994) showed a high level of BCL-2 protein expression in SCLC (65%). Yan *et al* (1996) suggested that BCL-2 expression is involved in the relatively early phases of SCLC tumorigenesis, due to the high level of positivity recorded (93%). They also suggest that expression of the BCL-2 protein in SCLC is not due to dysregulation of the protein but that it is a succeeding property directly derived from the SCLC progenitor cells. They found no correlation between BCL-2 and P53 expression as well as reporting BCL-2 expression to be of no use as a prognostic marker. No correlation between P53 and BCL-2 protein expression was reported by Walker *et al* (1995a) when they examined a series of lung cancers (only included 1 SCLC). A large study (60 cases) of BCL-2 protein expression in SCLC by immunocytochemistry found 90% to be BCL-2 positive, and suggested that this could be related to the theory that BCL-2 protein expression correlates with neuroendocrine differentiation (Jiang *et al*, 1995). A further study concluded that BCL-2 protein expression is differentially regulated in lung cancer histological types and may be closely related to neuroendocrine differentiation. The study involved carried out BCL-2

immunocytochemistry on 111 SCLC and found 104 to be positive for BCL-2 protein (Jiang *et al,* 1996). An inverse relationship between P53 and BCL-2 protein expression has also been demonstrated in colon cancers (Watson, Merritt, Jones, Askew, Anderson, Becciolini, Balzi, Potten and Hickman, 1996).

The results of this study did not indicate any relationship between BCL-2 and any other marker, including P53 and CMYC. However, the levels of BCL-2 and P53 protein expression were almost identical, but CMYC was positive in many more cases. It was shown that BCL-2 protein was expressed at higher levels in intermediate type, than in oat cell type. This agrees with the findings of Pezzella *et al* and Fontanini *et al* who found BCL-2 overexpression to induce a less aggressive phenotype in NSCLC, as this study found a higher level of BCL-2 protein positivity in the less aggressive intermediate cell type compared to the more aggressive oat cell type. Previous studies which reported high levels of BCL-2 protein in SCLC did not comment on any differences in expression between histological subtypes of SCLC.

5.3 TUMOUR SUPPRESSOR GENES

5.3.1 P53

The p53 gene was first identified in 1979 (Lane and Crawford) by it's ability to bind the SV40 large tumour (T) antigen, and was subsequently reported in non-virally transformed cells. The gene is located on chromosome 17p13 and encodes a 53 kD nuclear phosphoprotein. Normal (wild type) P53 protein is always nuclear and has a short half-life so it doesn't accumulate, whereas mutant P53 protein may be found in both the nucleus and cytoplasm and has a longer half-life making it detectable by immunocytochemistry. Over 90% of p53 mutations are missense mutations that change the identity of an amino acid. This can alter the conformation and increase the stability of the p53 protein and can indirectly alter the sequence-specific binding and transcription factor activity (Harris, 1993). P53 protein alterations due to missense mutations and loss of P53 protein by nonsense or frameshift mutations provide a selective advantage for clonal expansion of preneoplastic and neoplastic cells. Studies indicate that P53 protein is involved in gene transcription, DNA synthesis and

repair, genomic plasticity and programmed cell death. Abnormalities of the p53 gene or protein can be detected by fluorescent *in situ* hybridisation (FISH), restriction fragment length polymorphism (RFLP) analysis, sequencing of mutated exons, single strand conformational polymorphism (SSCP) analysis and immunocytochemistry. However, immunocytochemistry is of no use for detecting mutations which cause deletion or truncation of the protein, rather than accumulation. The predominant mutation seen in lung cancers is a G:C to T:A transversion (Mazur and Gluckman, 1988).

BCL-2 has been shown to block P53-dependent apoptosis, but as it doesn't alter P53 levels or localization it is likely that it acts downstream of p53 by suppressing p53 dependent and independent pathways (Chiou, Rao and White, 1994). In fact, a reciprocal expression of BCL-2 and P53 protein expression has been shown in breast cancer (Haldar *et al*, 1994). Cells that co-express BCL-2 and C-MYC overcome P53-induced growth inhibition, possibly by exclusion of P53 from the nucleus during a critical period in G_1 (Ryan *et al*, 1994). p53 mutations have been shown to be involved in 40-70% of colon cancers, 50% of lung cancers, 10-65% of liver cancers, 30-60% of stomach cancers, 40-60% of ovarian cancers and 90% of cervical cancers, as well as bladder cancer, breast cancer, brain cancer, leukaemias, lymphomas and melanomas (Hager and Rogers, 1997).

An increase in P53 protein has been demonstrated in SCLC, but shown not to occur in carcinoids (Iggo *et al*, 1990). In 1992 Sameshima, Matsuno, Hirohashi, Shimosato, Mizoguchi, Sugimura, Terada and Yokota demonstrated p53 gene alterations in over 80% of SCLC and found these to occur early in SCLC development, and these results were echoed by D'Amico, Carbone, Mitsudomi, Nau, Fedorko, Russell, Johnson, Buchhagen, Bodner, Phelps, Gazdar and Minna (1992). Whereas P53 protein mutations were shown in 70% of SCLC (Miller *et al*, 1992). Several studies have shown a general level of P53 protein positivity in lung cancers to be approximately 50% (McLaren et al 1992 ; Brambilla *et al*, 1993). In 1992 Quinlan, Davidson, Summers, Warden and Doshi showed a strong correlation between P53 accumulation and poor survival in NSCLC. Several studies have shown that P53 immunostaining predicts for a favourable clinical prognosis in NSCLC, but that it is also independent of stage and histology (Passlick, Izbicki, Reithmuller and Panteller, 1994 ; Volm

and Mattern, 1994 ; Lee, 1995). P53 is also quoted as an independent prognostic factor in prostate cancer (Shurbaji, Kalbfleisch and Thurmond, 1995). Fontanini *et al* (1994) further found p53 alterations able to confer a more aggressive phenotype in NSCLC.

Deb *et al* (1992) demonstrated that P53 protein expression is higher in non-oat cell SCLC than in other lung cancer histological types. They also noted a correlation between PCNA and P53 protein expression, which could be due to the theory that mutant P53 may directly activate the PCNA promoter. Mutant P53 and PCNA expression was also found to be highest in non-oat cell SCLC by Korkolopoulou *et al* (1993). These results were further supported by a study showing abnormal P53 protein accumulation in 45% of intermediate cell and 11% of oat cell SCLC. A distinct inverse relationship between P53 immunocytochemistry and apoptosis in SCLC was also noted (Eerda *et al*, 1997). Data concerning the reliability of immunocytochemistry in detection of P53 mutations remains conflicting. In 1994 Gazzeri, Brambilla, Caron de Fromental, Gouyer, Moro, Perron, Berger and Brambilla demonstrated a strong correlation between P53 gene mutations and abnormal P53 protein expression as detected by immunocytochemistry. Wiethege, Voss and Miller (1995) also recommended the use of immunocytochemistry for detecting P53 mutations. However, Casson, McCuaig, Craig, Ayed, Inculet, Kerkvliet and O'Malley (1994) found immunocytochemistry to correlate poorly with genetic analysis when examining P53 mutations. One of the major problems in this argument is that very rarely has the immunocytochemical threshold been set using an objective basis (Dowell and Hall, 1995). A cautionary note on the excessive use of antigen retrieval techniques in P53 immunocytochemistry was given by Baas *et al* (1996).

Statistical analysis of the results of this study showed P53 to be able to predict patient survival in combination with some other markers, although not being statistically significant itself it is related to the other markers to such an extent that they lose significance if P53 is removed from the combination. P53 was also found to be expressed at a higher level in intermediate cell type rather than oat cell type SCLC. A reciprocal expression of BCL-2 and P53 expression was not noted in this study.

5.3.2 Retinoblastoma

The rb gene was the first tumour suppressor gene isolated (Knudson, 1971). It has now been mapped to chromosome 13q14 and codes for a 105 kD nuclear phosphoprotein. The phosphorylation state of the RB protein varies throughout the cell cycle, with a maximum in S phase and a reduced phosphorylation after M phase. This indicates that the protein acts in a cell cycle specific way to regulate cell proliferation, which function may be carried out by the underphosphorylated form, while the phosphorylated form may participate in the control of growth inhibitory activities. RB protein can also protect cells from p53-dependent apoptosis (Kouzarides, 1995).

A study on SCLC tumours and cell lines using a rb cDNA probe showed that abnormalities in structure and expression of the rb gene are common in SCLC and atypical pulmonary carcinoids, but rare in other lung cancer cell types (Harbour *et al*, 1988). In fact, Hensel *et al* (1990) reported inactivation of rb to be a frequent, if not universal, event in SCLC. In SCLC mutations of the rb gene are almost always present, but are seen in only about 20% of NSCLC. The protein may be expressed in SCLC but it usually lacks phosphorylation and is of an abnormal size (Gazdar, 1992). Abnormalities of both rb alleles, abnormal gene transcription products or absence of RB protein is almost always seen in SCLC, but in only 10-20% of NSCLC (Kern and Filderman, 1993). A large study by Reissmann *et al* in 1993 found rb gene inactivation in approximately one third of the NSCLC examined. They also reported immunocytochemistry to be very sensitive in detecting RB inactivation. Further evidence was provided in a study that found 88% of SCLC to be negative for the RB protein and only 23% of the NSCLC examined to be positive for abnormal RB protein expression, indicating that abnormalities of RB protein may be a minor event in NSCLC (Higashiyama *et al*, 1994).

The RB protein was not found to be of any statistical significance or to have any associations with any other markers used in this study. However, the results do confirm the previously published data by demonstrating a low incidence of RB protein expression in SCLC (5%). This is also the first time a quantitative study has been performed on this antigen, and it showed that even when the protein is expressed it is not at particularly high levels. RB was

never likely to achieve any statistical significance as of the 31 results used for statistical analysis there were only two RB positive results.

5.3.3 NM23

nm23 is a putative metastasis suppressor gene which was isolated on the basis of it's reduced expression in murine melanoma cell lines of high metastatic potential (Steeg *et al*, 1988). Two human nm23 genes - nm23-H1 and nm23-H2 - which are 88% homologous have been described (Stahl *et al*, 1991). The genes are on chromosome 17q22, 18kb apart, and encode for the two polypeptide subunits of a nucleoside diphosphate kinase (NDPK) (Chandrasekharappa, Gross, King and Collins, 1993; Backer *et al*, 1993). nm23 has been shown at both the level of the protein and mRNA to have a higher expression in breast tissues of low metastatic potential than in corresponding high metastatic variants. However, in other tumours e.g. lung cancers no association between nm23 expression and metastasis has been reported.

Low tumour nm23 mRNA content has been found to correlate with significant disease-free and overall survival in breast cancer patients (Hennessey *et al*, 1991). NM23 protein expression has been reported in colon adenocarcinomas (Royds, Cross, Silcocks, Scholefield, Ross and Stephenson, 1994).

Reduced expression of the nm23 gene is closely associated with distant metastases in human hepatocellular carcinomas (Nakayama *et al*, 1992), and with the early appearance of metastases after diagnosis of malignant melanoma (Florenes *et al*, 1992). In contrast, an increased NM23 protein level in neuroblastoma has been reported to be associated with the aggressive stages of the disease. A study in 1993 (Backer *et al*) showed NM23 expression to increase with the grade of malignancy in all NSCLC, except adenocarcinomas. A study by Higashiyama *et al* in 1992 looked at NM23 expression, using an antibody to NDPK, in pulmonary adenocarcinomas. They found no relationship between NDPK expression and grade of malignancy in this type of lung tumour.

The results of this study implicated NM23 to be by far the most significant of all the markers

studied. Upon multiple regression analysis the best single predictor of survival was NM23, and it was also included in the best combination of markers. NM23 protein was found to be expressed at a much higher level in intermediate type than in the positive cases of oat cell type SCLC. This appears to be possibly indicative of the role of NM23 protein expression as a possible positive prognostic indicator in SCLC, as it is expressed at higher levels in the less aggressive intermediate cell type in comparison with the few cases in which it is expressed in the oat ell type SCLC. As no published work on NM23 protein expression, quantitative or subjective, has been found a comparison with other authors work is not possible at this time, but it does provide an interesting area for possible future studies.

5.4 OVERALL CONCLUSIONS

The original aim of this study was to develop a method for isolating single whole SCLC cells from formalin-fixed, paraffin-embedded tissue and immunostaining the cell monolayer produced. However, although it was technically possible to produce a single cell monolayer of tumour cells from archival material they were unable to be reliably immunostained despite various attempts to improve the cells permeability. The study then reverted to a serial histological section approach using a panel of 10 immunocytochemical markers and quantitating a proportion of the cases objectively using digital image analysis. As the results were analysed they were found to be possibly relevant to predicting survival by means of a possible panel of immunocytochemical markers. The markers studied showed differences in relevance for survival prediction, indeed some of the markers selected (such as NM23) had not previously been studied in SCLC and so these results are preliminary as there are no other studies for comparison.

This study itself is novel in several ways, the major way being the extensive quantitative digital image analysis carried out. The value of objective numerical techniques cannot be stressed enough, as they remove observer bias and allow for direct comparison of results between different laboratories, even using different techniques due to the use of both internal and external controls. Also, the number of cases included within the study lend more validity to the statistical data derived, especially when the objective data can be used to compare the

general trends observed in the much larger series of subjective data obtained.

All the markers used in this study were correlated to some degree, the majority being positively correlated. This reflects the essentially heterogeneous nature of lung cancers and the fact that there is no "magic marker", thereby leading to the conclusion that a panel of markers is to be the only likely way of getting useful information from tumours. Which is logical when the range of mutations produced by tobacco smoke is considered, because cigarette smoke is known to be the major cause of lung cancer generally and is linked to SCLC particularly, and it produces a large range of mutations therefore a large panel of markers would be needed to detect these changes either to reflect diagnosis or survival prediction. Although NM23 was found to be the best single predictor of survival it was even better when placed in combination with four other markers - PCNA, P53, Mib1 and C-MYC. Even after taking into account the clinical data this remained the most useful marker combination feasible from the range of markers used within this study.

Also, for the vast majority of the markers used within this study it was the first time that a quantitative image analysis system had been used to assess the expression of the markers. Although the image analysis system is quoted as being objective there is still an element of subjectivity as the operator sets the threshold limits, and is inevitably responsible for selecting the field to be measured. There was a definite difference in expression between some markers in the histological subtypes, although only three of the markers from the selected panel showed this difference in staining intensity, and it was the only point where BCL-2 stood out. One problem was the high numbers of MDM-2 positive cases included in the study which precluded any statistically significant results from this marker, but that is just an unfortunate result of using "blind" data (i.e. had no preconceived notions of what the results should or would be). The important thing to remember is that the oncogenic markers used within this study looked at protein products of genes, not the actual genes themselves.

5.5 FUTURE WORK

The main purpose of any future work needs to be to duplicate the study already carried out on a different series of patients. The regression equation that was generated from this study to predict survival should then be applied to the data obtained from each patient to verify the use of the panel of markers suggested. Another avenue would be to carry out a similar study using different markers, either immunocytochemical or biochemical, to study in more detail how information on oncoproteins, proliferation and apoptosis can improve the diagnosis and predict prognosis and survival times in SCLC. As well as using different markers, a range of techniques could be evaluated for the same reasons. A larger quantitative study would be useful in terms of improving the statistical value of the analysis of the results, and this may become more likely as computer technology is constantly improving and image analysis systems are upgraded, so the element of subjectivity inherent in these systems presently where the operator has to set the threshold limits could be eliminated.

In terms of individual markers NM23 definitely requires further investigation in all types of lung cancer quantitatively. A more detailed study of the dynamics of the relationship of expression between P53, BCL-2 and CMYC as well as between P53 and MDM2 would also be of great value and much interest. Also further investigations on the differences of expression of markers such as P53, NM23, BCL-2 and Mib1 within histological subtypes to provide more information that could lead to an improvement in prognostic assessment. An interesting study would be to look at lung cancers as a whole to see if there are any major discrepancies that form a cut-off point between different histological types and sub-types, as there are often mixed tumours reported.

It would also be useful to attempt a prospective study of SCLC patients to see when markers become expressed and how they interact through the course of the disease. This would be possible as advances in histological and cytological techniques are producing less and less invasive methods for sampling tumours. A possible avenue available for exploration on prospective material is to study how or if the levels of selected markers for predicting survival alter if a patient stops smoking tobacco, both in terms of genes and gene products. The majority of work to be carried out at the Cause For Hope Lung Cancer Research Foundation,

Liverpool is to be based upon genetic research which could provide a useful comparison to the work on gene products carried out in this study. Indeed as areas such as *in situ* hybridisation continue to advance and easy to use kits are developed it should become as relatively quick and simple to detect genetic alterations directly as it is currently to detect protein products using immunocytochemical techniques.

The main advantage of computerised numerical techniques becomes apparent on cytological samples such as bronchial brushings and washings and sputum samples, which is the type of material most often collected in prospective studies. These samples contain relatively few malignant cells and positive reactions may be easily missed subjectively, which is unlikely objectively. It would also be useful to have multi-centre trials carried out nationally and internationally on both prospective and a retrospective basis. These sort of studies would highlight regional and geographical differences in lung cancer incidence and survival generally, but most especially in the highly aggressive SCLC. Organisations such as the Cause For Hope Foundation with offices in other areas of the country, as well as having its headquarters in Merseyside which is quoted as the lung cancer capital of England, would be ideally suited to initiate this type of study at a national level.

In summary the large series of cases studied in a unique study using a wide range of markers assessed in the most objective way possible have provided a great deal of useful data and proposed many more avenues of possible future research. As computer and medical technology continue to advance then more and more can be understood about the nature of the human body and how it becomes disrupted in conditions such as cancer. Each novel piece of work contributes to this understanding in some way, which will ultimately benefit the patients involved. It is hoped that this study will go some way towards providing a shift in the direction of research in the area of SCLC towards the computerised, standardised objective interpretation of results especially in large studies such as this.

This work is currently being prepared for submission as a series of publications in reputable international journals.

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APPENDIX I - Abbreviations

SCLC - small cell lung cancer

NSCLC - non-small cell lung cancer

DNES - diffuse neuro-endocrine system

ACTH - adrenocorticotrophic hormone

ADH - anti-diuretic hormone

PCNA - proliferating cell nuclear antigen

DNA - deoxyribose nucleic acid

RNA - ribose nucleic acid

NOR - nucleolar organizer region

AgNOR - silver staining nucleolar organizer region

WHO - World Health Organization

DMF - dimethylformadide

PVP - polyvinylpyrollidine

PEG - polyethylene glycol

TBS - tris-buffered saline

PBS - phosphate-buffered saline

PVA - polvinyl acetate

ABC/AP - avidin-biotin complex/ alkaline phosphatase

BRAM - biotinylated rabbit anti-mouse antibody

NRS - normal rabbit serum

RLUHT - Royal Liverpool University Hospital Trust

5-HT - 5-Hydroxytryptamine

Rb - retinoblastoma

H&E - haemalum and eosin stain

HCl - hydrochloric acid

Tris - Tris(hydroxymethyl)methylamine

NaOH - sodium hydroxide

BSA - bovine serum albumin

cDNA - complementary DNA

M phase - mitotic phase

S phase - synthetic phase

G phase - gap phase

TLI - thymidine labelling index

BrdU - bromodeoxyuridine

CDK - cyclin dependent kinase

ICE - interleukin-1β-converting enzyme

ISEL - *in situ* end labelling

APES - 3-aminopropyltriethoxysilane

RT - room temperature

ABC/HRP - avidin biotin complex / horesradish peroxidase

APAAP - alkaline phosphatase anti-alkaline phosphatase

LVN - low viscosity nitrocellulose

TCA - trichloroacetic acid

Mdm-2 gene - mouse double minute 2 gene

Bcl-2 gene - B cell leukaemia and lymphoma 2 gene

mRNA - messenger RNA

DAB - 3-3'diaminobenzidine

nm23 gene - non-metastasizing gene

nm23-H1 - nm23 homologue 1

C.O.S.H.H - Control Of Substances Hazardous to Health

OD - optical density

DA - detected area

CV - co-efficient of variation

ANOVA - analysis of variance

MANOVA - multiple analysis of variance

SD - standard deviation

SE - standard error

RGB - red green blue

HSI - hue saturation intensity

Mibc - Mib1 centre

Mib e - Mib 1 edge

EGFR - epidermal growth factor receptor

FISH - fluorescent *in situ* hybridisation

RFLP - restriction fragment length polymorphism

SSCP - single strand conformational polymorphism

NDPK - nucleoside diphosphate kinase

rRNA - ribosomal RNA

kD - kiloDalton

D.P.X. - dibutylthalate in xylene

APPENDIX II - Materials

Supplied by Sigma chemical company :

Naphthol AS-MX phosphate (cat-N4875) ; Fast red violet LB salt (cat-F-3381) ; Levamisole (cat-L9756) ; Naphthol AS-TR phosphate (cat-N6125) ; 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (cat-B8503) ; Tetranitro BT (cat-T4000) ; Fast blue RR salt (cat-F0500) ; 3-aminopropyltriethylsilane (APES)(cat-A3468) ; Collagenase IV (cat-C1538) ; Protease XXIV (cat-D8001) ; Polyvinylpyrrolidine (PVP) (cat-PVP40) ; Proteinase K (cat-P6556) ; 0.05% Trypsin-EDTA solution (cat-T3924) ; 3-3' diaminobenzidine (cat-D8001) ; Schiffs reagent (cat-395-2-016) ; Dimethylformamide (cat-28425)

Supplied by BDH :

New Fuchsin (cat-34184) ; Fast red B salt (cat-34119); Silver nitrate (cat-10233 3J) ; Gelatine powder (cat-44045 4B) ; Tris(hydroxymethyl)methylamine (cat-27119) ; Sodium chloride (cat-30123) ; Trisodium citrate (cat-10242) ; Sodium nitrite (cat-30188) ; Paraformaldehyde (cat-29447); Hydrochloric acid (cat-28507) ; Sodium hydroxide (cat-30617) ; Bovine serum albumin, fraction V (cat-44155) ; Polyethylene glycol 20 000 (cat-29864) ; Hydrogen peroxide solution, 30% (cat-10128) ; Cloroauric acid (gold chloride) (cat-19049) ; Sucrose (cat-30299) ; Polyethylene glycol 1500 (cat-29575 4U) ; Iron (III) chloride (cat-10110) ; Oxalic acid (cat-29423) ; Chloral hydrate (cat-27668) ; Eosin Y (cat-34027) ; Light green (cat-34043) ; Aluminium potassium sulphate (potassium alum) (cat-10009) ; Glycerol (cat-284546F) ; Sodium sulphite (cat-30223) ; Formic acid (cat-28429) ; DPX mountant (cat-36029 4H) ; Lithium carbonate (cat-29065) ; Sodium iodate (cat-30171) ; Glacial acetic acid (cat-27013) ; Trichloroacetic acid (cat-30489) ; Low viscosity nitrocellulose wool (cat-36013) ; Triton X-100 (cat-30632) ; Diethyl ether (cat-28132) ; Industrial methylated spirits (absolute alcohol) (cat-30244) ; Chromium (III) potassium sulphate (chrome alum) (cat-27758) ; Calcium chloride (cat-10069) ; Sodium thiosulphate (cat-30236) ; Acetone (cat-27023) ; Methanol (cat-26129) ; Disodium hydrogen phosphate (cat - 10249) ; Sodium dihydrogen phosphate (cat-10245) ; Calcium nitrate (cat-27607)

Supplied by DAKO :

Rabbit anti-mouse biotinylated immunoglobulins (cat-E354) ; Streptavidin-biotin complex/alkaline phosphatase (cat-K391); Bcl-2 oncoprotein, mouse anti-human (cat-M887) ; Proliferating cell nuclear antigen monoclonal antibody PC10 (cat-M879) ; c-erbB-2 oncoprotein polyclonal, rabbit anti-human (A485) ; Swine anti-rabbit biotinylated immunoglobulins (E353) ; Strept ABC/AP kit (cat-K377) ; APAAP mouse monoclonal (cat-D651) ; Rabbit anti-mouse immunoglobulins, absorbed with rat immunoglobulin (cat-Z456) ; Monoclonal mouse anti-human p53 protein (cat-M7001) ; Dako pen for immunocytochemistry (cat-S2002)

Supplied by Novocastra Laboratories :

C-myc oncoprotein monoclonal antibody (cat-NCL-CMYC) ; Mdm-2 monoclonal antibody (NCL-MDM2) ; Nm23 protein monoclonal antibody (cat-NCL-NM23) ; Retinoblastoma gene protein, mouse monoclonal (cat-NCL-RB) ; Retinoblastoma gene protein, mouse monoclonal (cat-NCL-RB1) ; C-erbB-2 oncoprotein, mouse monoclonal (cat-NCL-CB11)

Other Reagents :

Aquaperm mountant (Immunon cat-484975) ; Mib-1 monoclonal antibody (Immunotech cat-0505) ; Polycarbonate membranes (25mm, 12μm pore size) (CoStar UK cat-110616) ; Polyvinyl acetate (PVA) (Aldrich cat-18949.9) ; Glass beads 1.01-1.27 mm diameter (ORME cat-G50-100) ; Normal swine serum (SeraLab cat-S-0005) ; Normal rabbit serum (SeraLab cat-S-0009) ; Biotinylated anti-mouse IgG (Vector cat-BA-9200) ; Retinoblastoma protein, monoclonal (Biogenex cat-MU-290-UC) ; Apoptag *in situ* hybridisation kit (Oncor cat-S1700-kit) ; Optiprep (Nycomed cat-1030061) ; Citric acid (Hopkin and Williams cat-3224) ; Potassium hexacyanoferrate (Hopkin and Williams cat-698000) ; Haematoxylin (Raymond A Lamb cat-75290) ; Trypsin (Difco cat-0152-13-1) ; Nylon gauze, 100μm (Lockertex)

APPENDIX III - Preparation of Solutions

0.2M TRIS BUFFER

- Dissolve 24.2g of tris in 500ml of distilled water
- Add 70ml 1M hydrochloric acid
- Mix well and make up to 1l with distilled water
- Check pH is as desired

40% SODIUM HYDROXIDE SOLUTION

- Take 200g of sodium hydroxide
- Add ***slowly*** to 300ml of distilled water, with stirring
- Make up to 500ml when cool (CAUTION! - Exothermic reaction)

40% FORMALDEHYDE SOLUTION (From Paraformaldehyde)

- Take 80g of paraformaldehyde
- Add ***slowly*** to 200ml of distilled water at 65°C
- Use constant stirring to dissolve (hotplate/stirrer)
- Mix well for 1 minute
- Add 1ml of 40% sodium hydroxide solution
- Mix for 5 min, with constant stirring
- The solution should become clear and be of pH 9.3

This must all be carried out at 65°C, in a fume cupboard wearing gloves and goggles

- Seal the flask and cool to room temperature
- Check pH is **7.2-7.4**
- Store at 4°C
- DILUTE 1:10 WITH DISTILLED WATER FOR USE

CONCENTRATED 0.05M TBS pH7.6

- Take 60.55g of Tris and add 85.2g of sodium chloride
- Add 500ml of distilled water and mix until dissolved

- Add approximately 185ml of 2M hydrochloric acid to get pH7.6
- Take final volume up to 1litre with distilled water
- Dilute 1:10 with distilled water for use

0.01M CITRATE BUFFER pH6.0

SOLUTION A - 0.1M citric acid (21.01g in 1 litre of water)

SOLUTION B - 0.1M sodium citrate (29.4g in 1 litre of water)

- Take 9ml of solution A and 41ml of solution B
- Make up to 500ml with distilled water and adjust pH to 6.0

GADSDON'S HAEMALUM

- Mix well together - 5.5g haemalum powder
  - 100ml absolute ethanol
  - 20ml glacial acetic acid
  - 600ml filtered saturated aqueous potassium alum
- Dissolve 0.5g of sodium iodate in 10ml of distilled water and add to above mixture
- Mix well and leave for 1 hour
- Add 300ml of glycerol and filter before use

SCHOFIELD'S EOSIN

- Prepare 1% eosin Y in o.1% aqueous calcium nitrate
- Filter before use

CONCENTRATED PBS (0.5M pH7.2)

- Prepare 0.5M disodium hydrogen phosphate
- Add conc sodium dihydrogen phosphate to get pH 7.2
- Add 9% sodium chloride
- Dilute 1:10 with distilled water for use to give 0.05M pH7.2, 0.9% sodium chloride

PVA MOUNTANT

- Dissolve 5g of PVA (polyvinyl acetate) in 20ml of 80% ethanol
- Leave for 24 hours to dissolve before use

MAYERS HAEMALUM

- Take 1 litre of distilled water
- Add 1g haematoxylin, 0.2g sodium iodate and 50g potassium or ammonium alum
- Dissolve by warming and stirring or leave at room temperature overnight
- Add 50g chloral hydrate and 1g citric acid and boil for 5 min
- Cool and filter (can use immediately)

APPENDIX IV - OBJECTIVE DATA

CASE	PCNA	Mib e	Mib c	Cmyc	Mdm2	cerbB2p	Bcl-2	nm23	p53	cerbB2m
4684/94	3.58	0.0	4.54	0.89	1.12	0.0	0.47	0.84	4.11	0.0
5175/92	3.85	0.0	4.18	0.0	1.91	0.0	0.0	0.0	0.0	0.0
15230/92	3.88	3.7	0.0	0.0	2.54	0.0	0.0	0.0	2.54	0.81
8156/92	3.89	0.0	3.08	0.0	1.73	0.0	0.69	0.0	0.0	0.0
9351/91	3.96	2.72	0.0	0.0	2.44	0.0	0.0	0.0	0.0	0.0
5133/95	4.21	8.21	6.9	0.51	1.56	0.0	1.18	1.87	1.76	0.57
5361/94	4.25	0.0	6.36	0.0	1.27	0.0	1.4	1.33	4.77	0.0
6958/95	4.27	0.0	4.07	0.45	1.57	0.0	1.82	0.7	2.25	0.0
1826/95	4.31	0.0	3.33	0.0	3.12	0.0	0.0	0.0	3.23	0.0
13613/92	4.34	0.0	4.81	0.67	0.075	0.0	0.0	1.19	3.86	0.0
2896/91	4.39	0.0	0.0	1.3	1.74	0.0	0.0	1.03	0.0	0.0
4024/89	4.39	0.0	0.0	0.82	1.87	0.0	0.0	0.77	0.0	0.0
12454/91	4.51	3.25	0.0	0.77	1.76	0.0	0.0	1.67	0.0	0.0
6583/92	4.52	0.0	3.8	0.78	1.13	0.71	1.04	1.8	3.41	0.43
11241/92	4.57	0.0	0.0	1.39	1.64	0.0	0.0	2.17	2.7	0.0
841/95	4.59	0.0	3.92	0.0	0.0	0.0	0.0	0.0	0.0	0.0
6114/91	4.79	0.0	0.0	0.0	1.47	0.0	0.0	0.88	0.0	0.0
6405/92	5.29	5.13	4.45	0.56	0.57	0.57	0.71	0.33	3.21	0.0
10669/93	5.64	4.24	0.0	0.45	1.47	0.31	0.0	0.83	0.0	0.62
4920/89	7.7	0.0	3.53	0.0	1.94	0.0	0.0	0.0	0.0	0.0
9075/90	0.0	0.0	3.77	0.0	1.82	0.0	0.0	0.0	0.0	0.0
6670/94	0.0	0.0	4.21	0.47	1.02	0.0	0.72	1.57	4.34	0.0
428/93	0.0	0.0	0.0	1.1	0.0	0.0	0.88	0.0	0.0	0.0
5589/89	0.0	0.0	0.0	0.0	2.72	0.0	1.21	0.0	0.0	0.0
10084/90	0.0	0.0	0.0	0.43	1.72	0.0	0.0	0.99	0.0	0.0
8074/92	0.0	0.0	0.0	0.0	2.26	0.0	0.0	1.11	0.0	0.0
84/89	0.0	0.0	0.0	0.99	1.56	0.0	0.0	0.0	0.0	0.0
2698/93	0.0	0.0	0.0	0.0	2.04	0.0	0.0	0.0	0.0	0.0
11928/90	0.0	0.0	0.0	0.0	1.97	0.0	0.0	0.0	0.0	0.0
15242/90	0.0	0.0	0.0	0.0	1.93	0.0	0.0	0.0	0.0	0.0
15394/88	0.0	0.0	0.0	0.0	1.74	0.0	0.0	0.0	0.0	0.0

CASE	Rb	PLOIDY	HIST	SEX	AGE	CH	RA	SU	SURV	Log_{10} SURV	FITS
4684/94	0.0	2	0	2	67	1	0	1	7	0.845	0.881
5175/92	0.0	2	0	2	69	1	0	0	1	0.00	0.605
15230/92	0.0	2	0	1	69	1	0	0	51+	1.708	1.619
8156/92	0.0	1	0	2	72	1	0	0	3	0.477	0.507
9351/91	2.51	2	0	1	77	0	1	0	7	0.845	0.676
5133/95	3.12	1	1	1	45	1	1	1	4	0.602	0.738
5361/94	0.0	2	1	1	67	1	1	1	28+	1.447	1.396
6958/95	0.0	2	1	1	61	1	1	1	16	1.204	0.977
1826/95	0.0	1	0	2	74	0	1	0	3	0.477	0.549
13613/92	0.0	1	1	1	73	1	1	1	7+	0.845	1.054
2896/91	0.0	2	0	2	44	1	1	0	56	1.748	0.939
4024/89	0.0	2	0	1	65	1	0	1	3	0.477	1.071
12454/91	0.0	2	1	1	68	1	1	1	46	1.663	1.415
6583/92	0.0	2	0	2	67	0	1	0	5	0.699	0.633
11241/92	0.0	2	1	2	56	1	0	1	28+	1.447	1.645
841/95	0.0	2	0	2	69	1	0	0	11	1.771	1.329
6114/91	0.0	1	0	2	71	1	0	1	59+	0.477	0.539
6405/92	0.0	2	0	2	69	0	0	0	3	1.568	1.605
10669/93	0.0	2	0	1	54	1	1	1	37+	0.954	1.086
4920/89	0.0	2	0	2	44	1	1	0	9	1.041	0.244
9075/90	0.0	2	0	1	69	1	1	0	11	1.279	1.060
6670/94	0.0	2	1	1	57	1	0	1	19	0.00	0.409
428/93	0.0	2	0	2	51	0	0	0	1	0.301	0.533
5589/89	0.0	2	0	2	69	1	0	0	2+	1.041	0.889
10084/90	0.0	2	0	1	57	1	1	0	11	0.301	1.054
8074/92	0.0	2	0	1	52	1	0	0	2	0.00	0.00
84/89	0.0	1	0	1	66	1	0	0	1	0.903	0.614
2698/93	0.0	2	0	2	66	1	1	0	8	0.00	0.622
11928/90	0.0	2	0	2	75	1	0	0	1	0.778	0.628
15242/90	0.0	2	0	2	63	1	1	0	6	1.041	0.653
15394/88	0.0	2	0	1	65	1	1	0	11	1.041	0.967

Table 5 : Full objective data on 31 cases used

KEY FOR TABLE 5

HIST = Histological subtype - 0 = oat cell, 1 = non-oat cell

CH = chemotherapy - 0 = not treated, 1 = treated

RA = radiotherapy - 0 = not treated, 1 = treated

SU = surgery - 0 = not treated, 1 = treated

SURV = survival - + indicates still alive when records ceased on patient

SEX - 1 = male, 2 = female

FITS = predicted survival

CASE	PCNA	Mib e	Mib c	C-myc	Mdm2	C-erbB2 p
12859/94	3.78	0.0	4.61	0.82	1.27	0.44
9132/90	5.25	3.43	3.42	0.615	1.69	.052
12828/88	0.0	0.0	0.0	0.0	0.0	0.0
4580/92	4.64	0.0	4.4	0.7	1.97	0.0
15131/88	4.08	0.0	0.0	0.0	1.5	0.0
10151/94	3.65	5.35	4.64	0.61	0.92	0.0
9628/95	0.0	0.0	0.0	0.0	1.19	0.0
13861/92	0.0	2.38	0.0	0.0	1.75	0.0
4155/92	4.6	3.3	3.79	0.6	1.43	0.29
3939/89	0.0	0.0	0.0	0.0	2.64	0.0
248/89	0.0	0.0	2.67	0.0	1.34	0.0
310C/89	0.0	0.0	0.0	0.99	1.23	0.0
310D/89	0.0	0.0	4.23	0.0	0.0	0.0
2953/88	0.0	0.0	3.42	0.106	1.77	0.0
3007/90	4.05	2.56	2.64	0.0	1.08	0.0
15041/89	4.54	0.0	3.74	1.16	2.26	0.0
7761/89	5.9	2.33	3.3	0.86	1.92	0.0
15470/92	4.71	0.0	0.0	0.67	1.41	0.587
9136/89	4.9	0.0	3.85	0.0	2.02	0.0
1759/87	5.19	0.0	4.72	1.33	2.00	0.065

CASE	Bcl-2	nm23	cerbB2 m	p53	Rb	PLOIDY
12859/94	0.0	0.0	0.0	0.0	3.08	2
9132/90	0.0	0.99	0.0	0.0	0.0	2
12828/88	0.0	0.0	0.0	0.0	0.0	2
4580/92	0.0	0.0	1.14	0.0	0.0	2
15131/88	0.0	0.96	0.0	0.0	0.0	1
10151/94	0.0	0.96	0.0	0.0	0.0	1
9628/95	0.0	0.0	0.0	3.64	0.0	1
13861/92	0.0	0.0	0.72	1.95	0.0	1
4155/92	0.0	1.47	0.65	0.0	0.0	2
3939/89	0.0	0.0	0.0	0.0	0.0	2
248/89	0.0	0.0	0.0	0.0	0.0	1
310C/89	0.72	0.0	0.0	0.0	0.0	1
310D/89	0.45	0.0	0.0	0.0	0.0	2
2953/88	1.06	0.0	0.0	0.0	0.0	2
3007/90	1.36	1.17	0.0	0.0	0.0	2
15041/89	0.0	0.0	0.0	0.0	0.0	1
7761/89	0.0	1.21	0.0	3.52	0.0	3
15470/92	0.5	1.72	0.91	3.46	0.0	2
9136/89	0.0	0.91	0.0	0.0	0.0	1
1759/87	0.0	0.62	0.0	2.99	0.0	2

Table 6 : Objective data on further 20 cases where clinical data unavailable

APPENDIX V - FULL SUBJECTIVE DATA

CASE	Mib1	PCNA	p53	Rb	Bcl-2	nm23	Mdm2	Cmyc	cerbB2m	cerbB2p
3369/89	-	++nr	-	-	+	++	+++	+	-	-
1759/87	+++n	+++nr	+++n	-	-	++	+++	+++	-	+
9132/90	++r	+++nr	-	-	-	+	++	++	-	++
10669/93	+n	+++nr	-	-	-	++	+++	++	-	+
733/88	-	++n	-	-	-	-	++	++	-	-
15470/92	-	+++nr	+++nr	-	+	+	+++	+++	+++	++
7761/89	+n	+++nr	+++nr	-	-	++	++	++	-	-
12454/91	++r	+++nr	-	-	+	++	++	++	-	-
6405/92	+++nr	+++nr	++n	-	+	+	+	++	-	+
11449/94	+++r	+++r	+++nr	-	-	+	++	++	+	+
2457/87	+n	+r	++r	-	-	+	++	+	-	-
14632/94	++r	+nr	++nr	-	-	-	++	++	+	+
12859/94	++r	++n	-	++nr	-	-	+++	+++	-	++
4155/92	++nr	+++nr	-	-	-	++	+++	++	+	+
6958/95	+++nr	+++nr	++n	-	+++	+	+++	++	-	-
10226/88	++r	+r	+r	-	+++	+	++	++	+	-
4623/88	+n	-	-	-	+++	+	++	++	+	-
11974/89	+n	+r	++r	-	+++	+	++	++	+	-
10123/88	-	+r	+r	-	-	+	++	+	+	-
10146/87	-	-	-	-	-	-	+	+++	+	+
10297/91	++r	+++nr	++r	-	-	-	-	++	+	-
3569/87	+n	+n	+nr	-	+++	-	+++	+	+	-
3691/87	-	+nr	-	-	++	-	+++	++	++	+
6583/92	+++r	+++nr	+++nr	-	++	+++	++	++	++	+
15473/87	-	-	++r	-	++	-	++	-	+	-
5076/88	+nr	-	+nr	-	-	+++	-	-	-	-
14186/90	-	+nr	-	-	+	++	-	++	-	-
13501/87	+n	+nr	+n	-	++	-	-	+	+	-
1392/87	-	-	-	-	-	-	+++	++	+	-

CASE	Mib1	PCNA	p53	Rb	Bcl-2	nm23	Mdm2	Cmyc	cerbB2m	cerbB2p
6923/87	+n	++r	+n	-	++	-	++	+	+	-
10048/88	+n	-	++n	-	-	-	++	-	-	-
15310/88	+nr	-	++r	-	+	-	+	+	+	-
9652/88	+n	-	-	-	+	-	++	+	-	-
482/87	+n	-	+++r	-	+++	-	++	+	-	-
8581/87	-	+nr	-	-	-	-	+	+	-	-
11177/88	+n	+nr	++r	-	-	-	++	++	-	-
15159/87	-	-	+nr	-	-	-	+	-	-	-
1491/87	-	-	++r	-	-	-	+	+	-	-
13974/87	-	-	-	-	-	-	+	-	-	-
2186/95	-	++nr	-	-	-	+	+++	-	-	-
558/93 I	++r	-	-	++nr	-	-	+++	+	-	-
558/93 E	++r	-	-	-	-	-	++	++	-	-
3007/90	++n	+++n	-	-	+++	+++	+++	+	-	-
546/90 A	++r	-	-	+nr	-	-	++	++	-	-
546/90 B	+++nr	-	-	-	-	-	++	++	-	-
788/87	-	-	-	-	-	-	++	-	-	-
6399/87	+n	+r	++nr	-	++	+	+++	+	-	-
441/90	++r	++nr	+nr	++nr	-	+	+++	+	-	-
248/89	+n	-	-	-	-	-	++	-	-	-
4684/94	+++nr	+r	+++nr	-	++	+++	++	+	-	-
13613/92	+++nr	++nr	+++nr	-	-	++	+	++	-	-
10151/94	+++nr	+r	-	-	-	++	++	++	-	-
428/93	-	-	-	-	+++	-	-	+	-	-
21/95	+++r	+r	-	-	-	++	++	-	-	-
5133/95	+++nr	+++nr	++n	++nr	++	+++	+++	+	+	-
319/89	+n	-	-	+nr	-	-	++	+	-	-
5361/94	+++nr	++nr	+++nr	-	+++	+++	+	-	-	-
84/89	-	-	-	-	-	-	+++	++	-	-
6670/94	+++nr	-	+++nr	-	++	+++	+++	+	-	-
310/89 C	-	-	-	-	+	-	+	+	-	-

CASE	Mib1	PCNA	p53	Rb	Bcl-2	nm23	Mdm2	Cmyc	cerbB2m	cerbB2p
310/89 D	+n	-	-	-	+	-	-	-	-	-
6449/94	+++nr	++nr	+++nr	-	++	-	++	++	-	-
6462/87	+nr	-	+nr	-	-	-	++	+	-	-
4142/88	+n	+n	-	-	++	-	-	+	-	-
13977/87	-	-	-	-	-	-	++	-	-	-
2953/88	+n	-	-	-	+	-	+++	+	-	-
6224/91	++r	-	-	-	-	-	-	-	-	-
10106/90	-	-	-	-	-	-	+	-	-	-
2768/90	+n	-	-	-	++	-	++	-	-	-
1018/91	++r	-	-	-	-	-	+++	-	-	-
5194/90	-	-	-	-	-	-	++	-	-	-
2492/89	+nr	-	-	-	-	-	++	-	-	-
2698/93	-	-	-	-	-	-	+	-	-	-
13861/92	+nr	-	+nr	-	-	-	+++	-	+	-
11225/90	-	-	-	-	-	-	-	-	-	-
13396/94	+nr	+++nr	-	-	-	-	++	-	-	-
13152/91	-	-	-	-	-	-	++	+	+	+
841/95	++nr	+r	-	-	-	-	-	-	-	-
780/92	-	-	-	-	-	-	++	+	-	-
9075/90	++nr	-	-	-	-	-	++	-	-	-
14003/90	+nr	-	-	-	-	-	++	-	++	-
6352/91	-	-	-	-	-	-	++	-	-	-
7920/95	+++nr	-	-	-	-	-	+++	-	-	-
7037/95	+++nr	-	-	-	-	-	+	-	-	-
5544/93	++r	-	-	-	-	-	+++	+	++	+
15216/88	++r	-	-	-	-	-	++	-	-	-
11928/90	-	+nr	-	-	-	-	+++	-	-	-
731/90	-	-	-	-	-	-	++	-	-	-
9351/91	++r	++nr	-	+n	-	-	++	-	-	-
1826/95	++nr	+++nr	+n	-	-	-	+++	-	-	-
16786/92	+nr	++r	+nr	-	-	-	++	-	-	-

CASE	Mib1	PCNA	p53	Rb	Bcl-2	nm23	Mdm2	Cmyc	cerbB2m	cerbB2p
3558/94	+++r	++nr	-	-	-	-	++	-	-	-
8156/92	++r	+++r	-	-	++	-	++	-	-	-
4920/89	++r	++r	-	-	-	-	++	-	-	-
5175/92	+++nr	++nr	-	-	-	-	+	-	-	-
8086/91	++r	+++nr	-	+r	-	-	++	-	-	-
3276/91	+nr	++r	-	-	-	-	++	-	+	-
4580/92	+++nr	++nr	-	-	-	-	++	+	+	-
307/91	+nr	-	-	-	-	-	++	-	+	-
14818/93	-	-	-	-	-	-	+	-	-	-
2071/89	-	-	-	-	-	-	++	-	-	-
14424/94	-	-	-	-	-	-	+	-	-	-
10149/93	-	+nr	-	-	-	-	++	-	-	-
6786/91	+nr	-	-	-	-	-	++	-	-	-
9628/95	-	-	+r	-	-	-	++	-	-	-
15242/90	-	-	-	-	-	-	++	-	-	-
4970/92	-	-	-	-	-	-	-	-	-	-
13691/92	-	+nr	-	-	+	+	++	-	-	-
1230/92	-	-	-	-	-	-	++	-	-	-
1976/92	-	+n	-	-	-	-	++	-	-	-
7883/90	-	-	-	-	-	-	++	+	-	-
12393/92	-	++r	-	-	-	+	-	-	-	-
8890/90	-	-	-	-	-	-	++	+	-	-
10447/89	-	-	-	-	-	-	++	-	++	-
5595/90	-	-	-	-	-	+	-	-	-	-
17147/92	-	-	-	-	-	-	++	-	-	-
3191/92	-	-	-	-	-	+	+++	+	-	-
14494/89	-	-	-	-	-	+	++	-	-	-
6195/92	-	-	-	-	-	-	++	-	-	-
6652/90	-	-	-	-	-	-	++	-	-	-
5896/91	-	-	-	-	-	-	++	-	-	-
11419/93	-	-	-	-	-	-	++	-	-	-

CASE	Mib1	PCNA	p53	Rb	Bcl-2	nm23	Mdm2	Cmyc	cerbB2m	cerbB2p
706/89	++r	-	-	-	-	-	++	-	-	-
2699/89	-	-	-	-	-	-	++	-	-	-
4922/89	-	-	-	--	-	-	++	-	-	-
12589/92	-	++r	-	-	+	-	+++	-	-	-
3168/95	-	++nr	-	-	+	+	++	+	-	-
16220/90	-	-	-	-	-	+	++	-	-	-
12828/88	-	-	-	-	-	-	-	-	-	-
7304/91	-	+n	-	-	-	++	+++	++	++	-
10084/90	-	-	-	-	-	+	++	+	-	-
14815/90	-	-	-	-	-	+	+	+	-	-
3369/95	+nr	+nr	-	-	-	-	++	-	-	-
2410/91	-	-	-	-	-	-	++	-	-	-
13749/88	-	-	-	-	-	-	++	+	-	-
3939/89	-	-	-	-	-	-	+	-	-	-
11221/91	++r	++nr	-	-	-	-	-	+++	+	-
10268/91	-	+++nr	-	-	+	-	+++	-	-	-
712/89	+n	++r	-	-	-	-	++	-	-	-
7124/92	+n	++nr	-	-	-	+	++	-	-	-
15394/88	-	-	-	-	-	-	++	-	-	-
15070/94	-	++nr	-	-	-	-	+	-	-	-
9684/91	-	++nr	-	-	+	-	+	+	-	-
7132/90	-	+nr	-	-	-	-	+	-	-	-
5410/92	+n	+++nr	-	-	-	-	++	-	-	-
10088/90	-	+nr	-	-	-	-	++	+	-	-
3583/95	-	++nr	-	-	-	-	+	-	-	-
12259/90	-	-	-	-	-	-	+	-	-	-
5073/90	-	-	-	-	-	-	-	+	-	-
5589/89	-	-	-	-	+	-	++	-	-	-
13084/90	-	-	-	-	-	-	+	-	-	-
8074/92	-	-	+n	-	-	+	+++	-	-	-
2896/91	-	+n	-	-	-	+	+++	++	-	-

CASE	Mib1	PCNA	p53	Rb	Bcl-2	nm23	Mdm2	Cmyc	cerbB2m	cerbB2p
816/91	-	+nr	-	-	-	-	++	-	-	-
15230/92	++r	+nr	+r	-	-	-	+++	-	+	-
12825/89	-	-	-	-	-	-	+	+	-	-
6827/93	+n	++r	-	+r	-	-	+++	-	-	-
12260/88	-	-	-	-	-	-	++	-	-	-
16088/94	-	+nr	-	-	-	-	++	-	-	-
15131/88	-	++nr	-	-	-	+	+++	-	-	-
1909/89	-	+n	-	-	-	-	++	+	++	-
4024/89	-	++nr	+nr	-	-	+	+++	+	-	-
9136/89	+n	+++nr	-	-	-	++	+++	-	-	-
15041/89	+n	+++nr	-	-	-	-	+++	+	-	-
1505/90	-	-	-	-	+	-	++	-	-	-
3887/90	-	++nr	-	-	++	+	+++	+	-	-
6114/91	-	++nr	-	-	-	+	++	-	-	-
7684/91	+n	++r	-	-	-	+	++	-	-	-
8111/91	-	++nr	-	-	-	-	+++	+	-	-
15087/91	-	+++nr	++nr	-	-	-	+++	-	-	-
497/92	-	++r	-	-	-	-	+	-	-	-
11241/92	-	++nr	+n	-	-	+++	+++	+	-	-

Table 7 : Full subjective results assessed semi-quantitatively

KEY FOR SUBJECTIVE RESULTS TABLE

- = negative
+ = weak positive
++ = moderate positive
+++ = strong positive
n = result based on number of positive cells
r = result based on strength of reaction

APPENDIX VI - DETAILS OF STATISTICAL ANALYSIS

One-Way Analysis of Variance

Analysis of Variance on P53

Source	DF	SS	MS	F	p
hist	1	24.42	24.42	11.62	0.002
Error	29	60.96	2.10		
Total	30	85.38			

Individual 95% CIs For Mean
Based on Pooled StDev

Level	N	Mean	StDev	----------+---------+---------+------
0	24	0.687	1.389	(----*----)
1	7	2.810	1.664	(--------*--------)

Pooled StDev = 1.450 1.2 2.4 3.6

Analysis of Variance on NM23

Source	DF	SS	MS	F	p
hist	1	8.066	8.066	41.69	0.000
Error	29	5.611	0.193		
Total	30	13.678			

Individual 95% CIs For Mean
Based on Pooled StDev

Level	N	Mean	StDev	---------+---------+---------+-------
0	24	0.2821	0.4286	(---*--)
1	7	1.5021	0.4807	(------*------)

Pooled StDev = 0.4399 0.50 1.00 1.50

Analysis of Variance on BCL2

Source	DF	SS	MS	F	p
hist	1	2.106	2.106	9.65	0.004
Error	29	6.329	0.218		
Total	30	8.435			

Individual 95% CIs For Mean
Based on Pooled StDev

Level	N	Mean	StDev	----------+---------+---------+------
0	24	0.2087	0.3896	(-----*-----)
1	7	0.8322	0.6877	(----------*---------)

Pooled StDev = 0.4672 0.35 0.70 1.05

Analysis of Variance on MIBc

Source	DF	SS	MS	F	p
hist	1	29.01	29.01	6.41	0.017
Error	29	131.28	4.53		
Total	30	160.29			

Level	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
0	24	1.441	1.923	(-----*-----)
1	7	3.755	2.777	(----------*----------)

Pooled StDev = 2.128 1.5 3.0 4.5

Correlations (Pearson)(Subjective data)

	mib-1	pcna	p53	rb	bcl-2	nm23	mdm-2	c-myc
pcna	0.364							
p53	0.402	0.284						
rb	0.243	0.129	0.002					
bcl-2	0.214	0.149	0.383	-0.030				
nm23	0.316	0.388	0.413	0.044	0.293			
mdm-2	0.100	0.245	0.088	0.202	0.041	0.178		
c-myc	0.302	0.289	0.378	0.128	0.253	0.311	0.122	
cerbb2m	0.079	0.079	0.221	-0.030	0.158	0.070	0.147	0.344
cerbb2p	0.197	0.291	0.209	0.131	-0.004	0.147	0.165	0.522

	cerbb2m
cerbb2p	0.402

Correlations (Pearson)(Objective data)

	PCNA	MIBe	MIBc	CMYC	MDM2	CERBB2p	BCL2	NM23
MIBe	0.328							
MIBc	0.373	0.188						
CMYC	0.141	0.050	-0.087					
MDM2	-0.120	-0.000	-0.366	-0.406				
CERBB2p	0.284	0.284	0.182	0.188	-0.265			
BCL2	0.080	0.193	0.477	0.121	-0.200	0.222		
NM23	0.212	0.267	0.168	0.481	-0.185	-0.131	0.209	
CERBB2m	0.261	0.641	0.055	0.001	0.095	0.348	0.082	0.053
P53	0.265	0.084	0.632	0.216	-0.263	0.283	0.415	0.375
RB	0.134	0.649	0.214	-0.054	0.127	-0.081	0.153	0.188
PLOIDY	-0.141	-0.122	-0.227	0.014	0.012	0.152	0.035	-0.073

	CERBB2m	P53	RB
P53	0.157		
RB	0.296	-0.030	
PLOIDY	-0.039	-0.091	-0.238

Correlations (Pearson)(objective and clinical data)

	PCNA	MIBe	MIBc	CMYC	MDM2	CERBB2p	BCL2	NM23
MIBe	0.328							
MIBc	0.373	0.188						
CMYC	0.141	0.050	-0.087					
MDM2	-0.120	-0.000	-0.366	-0.406				
CERBB2p	0.284	0.284	0.182	0.188	-0.265			
BCL2	0.080	0.193	0.477	0.121	-0.200	0.222		
NM23	0.212	0.267	0.168	0.481	-0.185	-0.131	0.209	
CERBB2m	0.261	0.641	0.055	0.001	0.095	0.348	0.082	0.053
P53	0.265	0.084	0.632	0.216	-0.263	0.283	0.415	0.375
RB	0.134	0.649	0.214	-0.054	0.127	-0.081	0.153	0.188
PLOIDY	-0.141	-0.122	-0.227	0.014	0.012	0.152	0.035	-0.073
SEX	0.046	-0.324	-0.073	-0.048	-0.044	0.161	-0.116	-0.414
AGE	-0.068	-0.172	0.014	-0.396	0.113	0.056	-0.090	-0.406
chemo	-0.132	-0.155	-0.068	-0.115	0.093	-0.545	-0.149	0.325
radio	0.096	0.202	0.156	0.019	0.109	0.117	0.156	0.177
surg	0.495	0.174	0.314	0.292	-0.229	-0.123	0.267	0.712
hist	0.190	0.209	0.425	0.288	-0.251	-0.168	0.500	0.768

	CERBB2m	P53	RB	PLOIDY	SEX	AGE	chemo	radio
P53	0.157							
RB	0.296	-0.030						
PLOIDY	-0.039	-0.091	-0.238					
SEX	-0.279	-0.124	-0.288	0.048				
AGE	-0.182	0.120	-0.125	-0.176	0.073			
chemo	-0.017	-0.212	-0.202	0.007	-0.222	-0.195		
radio	0.163	0.115	0.288	-0.048	-0.349	-0.037	-0.131	
surg	0.079	0.390	0.089	-0.114	-0.343	-0.256	0.349	0.077
hist	0.008	0.535	0.205	-0.126	-0.440	-0.154	0.237	0.285

	surg
hist	0.680

Regression Analysis

Regression Analysis on nm23
The regression equation is
log surv = 0.665 + 0.369 NM23

Predictor	Coef	Stdev	t-ratio	p
Constant	0.6645	0.1153	5.76	0.000
NM23	0.3693	0.1329	2.78	0.009

s = 0.4916 R-sq = 21.0% R-sq(adj) = 18.3%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	1	1.8651	1.8651	7.72	0.009
Error	29	7.0096	0.2417		
Total	30	8.8747			

Unusual Observations

Obs.	NM23	log surv	Fit	Stdev.Fit	Residual	St.Resid
3	0.00	1.7076	0.6645	0.1153	1.0431	2.18R
15	2.17	1.4472	1.4660	0.2319	-0.0188	-0.04 X

R denotes an obs. with a large st. resid.
X denotes an obs. whose X value gives it large influence.

Regression Analysis on PCNA and nm23
The regression equation is
log surv = 0.491 + 0.319 NM23 + 0.0688 PCNA

Predictor	Coef	Stdev	t-ratio	p
Constant	0.4907	0.1470	3.34	0.002
NM23	0.3191	0.1310	2.44	0.022
PCNA	0.06881	0.03815	1.80	0.082

s = 0.4736 R-sq = 29.2% R-sq(adj) = 24.2%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	2	2.5947	1.2974	5.78	0.008
Error	28	6.2799	0.2243		
Total	30	8.8747			

SOURCE	DF	SEQ SS
NM23	1	1.8651
PCNA	1	0.7297

Unusual Observations

Obs.	NM23	log surv	Fit	Stdev.Fit	Residual	St.Resid
3	0.00	1.7076	0.7574	0.1224	0.9502	2.08R

R denotes an obs. with a large st. resid.

Regression Analysis on PCNA, nm23 and Mibc

The regression equation is

log surv = 0.540 + 0.340 NM23 + 0.0919 PCNA - 0.0655 MIBc

Predictor	Coef	Stdev	t-ratio	p
Constant	0.5401	0.1456	3.71	0.001
NM23	0.3400	0.1277	2.66	0.013
PCNA	0.09190	0.03949	2.33	0.028
MIBc	-0.06555	0.03929	-1.67	0.107

s = 0.4592 R-sq = 35.9% R-sq(adj) = 28.7%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	3	3.1817	1.0606	5.03	0.007
Error	27	5.6930	0.2109		
Total	30	8.8747			

SOURCE	DF	SEQ SS
NM23	1	1.8651
PCNA	1	0.7297
MIBc	1	0.5870

Regression Analysis on PCNA, nm23, Mibc and cmyc

The regression equation is

log surv = 0.609 + 0.448 NM23 + 0.0994 PCNA - 0.0792 MIBc - 0.332 CMYC

Predictor	Coef	Stdev	t-ratio	p
Constant	0.6088	0.1488	4.09	0.000
NM23	0.4479	0.1427	3.14	0.004
PCNA	0.09936	0.03881	2.56	0.017
MIBc	-0.07923	0.03932	-2.02	0.054
CMYC	-0.3319	0.2145	-1.55	0.134

s = 0.4478 R-sq = 41.3% R-sq(adj) = 32.2%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	4	3.6618	0.9154	4.57	0.006
Error	26	5.2129	0.2005		
Total	30	8.8747			

SOURCE	DF	SEQ SS
NM23	1	1.8651
PCNA	1	0.7297
MIBc	1	0.5870
CMYC	1	0.4801

Unusual Observations

Obs.	NM23	log surv	Fit	Stdev.Fit	Residual	St.Resid
25	1.11	0.3010	1.1055	0.2049	-0.8045	-2.02R

R denotes an obs. with a large st. resid.

Regression Analysis on PCNA, nm23, Mibc, cmyc and p53

The regression equation is

log surv = 0.625 + 0.406 NM23 + 0.102 PCNA - 0.120 MIBc - 0.394 CMYC
+ 0.0887 P53

Predictor	Coef	Stdev	t-ratio	p
Constant	0.6251	0.1472	4.25	0.000
NM23	0.4063	0.1443	2.82	0.009
PCNA	0.10168	0.03832	2.65	0.014
MIBc	-0.12002	0.04967	-2.42	0.023
CMYC	-0.3942	0.2168	-1.82	0.081
P53	0.08873	0.06753	1.31	0.201

s = 0.4416 R-sq = 45.1% R-sq(adj) = 34.1%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	5	3.9985	0.7997	4.10	0.007
Error	25	4.8762	0.1950		
Total	30	8.8747			

SOURCE	DF	SEQ SS
NM23	1	1.8651
PCNA	1	0.7297
MIBc	1	0.5870
CMYC	1	0.4801
P53	1	0.3367

Unusual Observations

Obs.	NM23	log surv	Fit	Stdev.Fit	Residual	St.Resid
20	0.00	1.0414	0.1722	0.2072	0.8692	2.23R

R denotes an obs. with a large st. resid.

Regression Analysis on PCNA

The regression equation is

log surv = 0.611 + 0.0885 PCNA

Predictor	Coef	Stdev	t-ratio	p
Constant	0.6109	0.1498	4.08	0.000
PCNA	0.08852	0.04032	2.20	0.036

s = 0.5123 R-sq = 14.2% R-sq(adj) = 11.3%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	1	1.2645	1.2645	4.82	0.036
Error	29	7.6101	0.2624		
Total	30	8.8747			

Regression Analysis on Mibc

The regression equation is

log surv = 0.899 - 0.0144 MIBc

Predictor	Coef	Stdev	t-ratio	p
Constant	0.8987	0.1310	6.86	0.000
MIBc	-0.01442	0.04361	-0.33	0.743

s = 0.5522 R-sq = 0.4% R-sq(adj) = 0.0%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	1	0.0333	0.0333	0.11	0.743
Error	29	8.8413	0.3049		
Total	30	8.8747			

Regression Analysis on chemo, radio and PCNA

The regression equation is

log surv = - 0.056 + 0.583 chemo + 0.364 radio + 0.0933 PCNA

Predictor	Coef	Stdev	t-ratio	p
Constant	-0.0563	0.2554	-0.22	0.827
chemo	0.5827	0.2237	2.60	0.015
radio	0.3641	0.1646	2.21	0.036
PCNA	0.09329	0.03591	2.60	0.015

s = 0.4508 R-sq = 38.2% R-sq(adj) = 31.3%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	3	3.3877	1.1292	5.56	0.004
Error	27	5.4870	0.2032		
Total	30	8.8747			

SOURCE	DF	SEQ SS
chemo	1	0.8194
radio	1	1.1967
PCNA	1	1.3716

Unusual Observations

Obs.	chemo	log surv	Fit	Stdev.Fit	Residual	St.Resid
2	1.00	0.0000	0.8852	0.1204	-0.8852	-2.04R

R denotes an obs. with a large st. resid.

Regression Analysis on chemo, radio, PCNA and Mibc

The regression equation is

log surv = - 0.012 + 0.581 chemo + 0.400 radio + 0.117 PCNA - 0.0655 MIBc

Predictor	Coef	Stdev	t-ratio	p
Constant	-0.0117	0.2474	-0.05	0.963
chemo	0.5810	0.2155	2.70	0.012
radio	0.4004	0.1599	2.50	0.019
PCNA	0.11689	0.03711	3.15	0.004
MIBc	-0.06554	0.03730	-1.76	0.091

s = 0.4343 R-sq = 44.7% R-sq(adj) = 36.2%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	4	3.9701	0.9925	5.26	0.003
Error	26	4.9045	0.1886		
Total	30	8.8747			

SOURCE	DF	SEQ SS
chemo	1	0.8194
radio	1	1.1967
PCNA	1	1.3716
MIBc	1	0.5824

Unusual Observations

Obs.	chemo	log surv	Fit	Stdev.Fit	Residual	St.Resid
21	1.00	1.2788	0.2932	0.1942	0.9855	2.54R

R denotes an obs. with a large st. resid.

Regression Analysis on chemo, radio, PCNA, Mibc and p53

The regression equation is

log surv = - 0.151 + 0.699 chemo + 0.403 radio + 0.115 PCNA - 0.135 MIBc
+ 0.154 P53

Predictor	Coef	Stdev	t-ratio	p
Constant	-0.1509	0.2261	-0.67	0.511
chemo	0.6988	0.1967	3.55	0.002
radio	0.4028	0.1426	2.82	0.009
PCNA	0.11537	0.03308	3.49	0.002
MIBc	-0.13479	0.04155	-3.24	0.003
P53	0.15402	0.05541	2.78	0.010

s = 0.3871 R-sq = 57.8% R-sq(adj) = 49.3%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	5	5.1280	1.0256	6.84	0.000
Error	25	3.7467	0.1499		
Total	30	8.8747			

SOURCE	DF	SEQ SS
chemo	1	0.8194
radio	1	1.1967
PCNA	1	1.3716
MIBc	1	0.5824
P53	1	1.1578

Best Subsets Regression on biological data

Response is log surv

Vars	R-sq	Adj. R-sq	C-p	s	PCNA	MIBc	CMYC	CERBB2p	NM23	CERBB2m	P53	RB
1	21.0	18.3	8.3	0.49164					X			
1	14.2	11.3	11.3	0.51227	X							
1	6.4	3.2	14.8	0.53507						X		
1	3.5	0.2	16.1	0.54339							X	
1	0.7	0.0	17.4	0.55124			X					
2	29.2	24.2	6.6	0.47359	X				X			
2	26.3	21.0	7.9	0.48328					X	X		
2	23.8	18.4	9.0	0.49138					X			X
2	23.4	18.0	9.2	0.49263			X		X			
2	23.0	17.5	9.4	0.49406		X			X			
3	35.9	28.7	5.7	0.45918	X	X			X			
3	33.1	25.7	6.9	0.46896	X				X			X
3	32.3	24.8	7.2	0.47164					X	X		X
3	32.1	24.5	7.4	0.47247	X		X		X			
3	31.9	24.3	7.4	0.47323	X				X	X		
4	41.3	32.2	5.3	0.44777	X	X	X		X			
4	38.3	28.8	6.6	0.45896	X	X			X			X
4	38.2	28.7	6.6	0.45938	X				X	X		X
4	38.1	28.6	6.7	0.45964	X	X			X	X		
4	37.8	28.2	6.8	0.46081	X	X			X		X	
5	45.1	34.1	5.6	0.44164	X	X	X		X		X	
5	44.9	33.9	5.6	0.44225	X	X	X		X			X
5	43.2	31.8	6.4	0.44919	X	X	X		X	X		
5	42.5	31.0	6.7	0.45187	X		X		X	X		X
5	42.4	30.9	6.7	0.45200	X	X			X	X		X

Best Subsets Regression on biological and clinical data

Response is log surv

Vars	R-sq	Adj. R-sq	C-p	s	PCN	MIB	NM2	CERB2	P5m3	chedm3	rasiro3	sug
1	21.0	18.3	14.8	0.49164				X				
1	20.5	17.8	15.0	0.49325								X
1	14.2	11.3	18.3	0.51227		X						
1	10.5	7.4	20.3	0.52329						X		
1	9.2	6.1	21.0	0.52704							X	
2	29.2	24.2	12.4	0.47359		X		X				
2	28.9	23.8	12.6	0.47465						X	X	
2	27.1	21.9	13.5	0.48063				X		X		
2	27.0	21.8	13.6	0.48111		X					X	
2	26.3	21.0	14.0	0.48328				X	X			
3	38.2	31.3	9.7	0.45080		X				X	X	
3	35.9	28.7	10.9	0.45918		X	X	X				
3	35.5	28.4	11.1	0.46030			X				X	X
3	34.6	27.3	11.6	0.46367		X		X			X	
3	34.5	27.3	11.6	0.46388		X		X				X
4	44.7	36.2	8.2	0.43432		X	X				X	X
4	44.3	35.7	8.5	0.43601		X	X			X	X	
4	42.7	33.9	9.3	0.44220		X	X	X				X
4	42.5	33.6	9.4	0.44318		X		X			X	X
4	40.9	31.8	10.2	0.44905		X	X					X
5	57.8	49.3	3.3	0.38713		X	X			X	X	X
5	50.0	40.0	7.4	0.42113		X	X	X			X	X
5	47.6	37.1	8.7	0.43137		X	X				X	X
5	46.5	35.9	9.3	0.43562		X	X	X		X	X	
5	45.5	34.6	9.8	0.43969		X	X		X		X	X