

## **Microarray analysis of pathways involved in bladder cancer invasion and metastasis.**

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Dedicated to Claudia





**Microarray analysis of pathways involved in bladder cancer  
invasion and metastasis**

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## Microarray analysis of pathways involved in bladder cancer invasion and metastasis

Abstract (word count =300)

Hypoxia-inducible genes have been linked to the aggressive phenotype of cancer. However, nearly all work on hypoxia-regulated genes has been conducted in vitro on cell lines. Here the hypoxia transcriptome in primary human bladder cancer was investigated using cDNA microarrays to compare genes induced by hypoxia in vitro in bladder cancer cell line EJ28 with genes upregulated on an in vivo array of 39 bladder tumours (27 Ta/T1 12 T2-T4). mRNA array fold-changes were correlated with carbonic anhydrase IX (CA IX) staining and necrosis of tumours as surrogate markers of hypoxia. Of 6000 genes 32 were repeatedly hypoxia-inducible in vitro more than 2-fold, five of which were novel, including lactate transporter SLC16A3 and RNase 4. Eight of 32 hypoxia-inducible genes in vitro were also upregulated on the vivo array. Vascular endothelial growth factor (VEGF) mRNA was upregulated 2-fold by hypoxia and 2 to 18-fold in 31/39 tumours. Also up regulated on both arrays was GLUT 1 mRNA, and fold changes on the in vivo genearray significantly correlated with CA IX staining of tumours ( $p=0.008$ ). However Insulin-like growth factor binding protein 3 (IGFBP-3) mRNA was the most strongly differentially expressed gene in both arrays and its upregulation was confirmed in the urine of bladder cancer patients ( $n=157$ ,  $p<0.01$ ) and in cell line supernatants. Angiogenin was also upregulated in urine of bladder cancer patients. Selected genes upregulated by hypoxia (HIF  $1\alpha$ , HIF  $2\alpha$ , CA IX and NIP3) were studied by immunohistochemistry for their prognostic significance and association with necrosis in 98 cystectomy specimens. Normal human urothelial cells were also grown in culture and a hypoxia genearray profile compared with EJ28, peripheral blood monocytes and T-cells. This thesis studies the prevalence of hypoxia and necrosis in bladder cancer, its relationship with prognosis, genes associated with the hypoxic phenotype and hypoxia related molecular pathways.

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**Paper**                      **Ord JJ**, Agrawal S, Thamboo T, Roberts I, Campo L, Turley H, Han C, Fawcett D, Kulkarni RP, Cranston D, Harris AL An investigation into the prognostic significance of necrosis and hypoxia in high grade and invasive bladder cancer. **Journal of Urology** August 2007 (Chapter 6)

**Paper**                      **Ord JJ**, Streeter E Jones A Joel SP Le Monnier K Cranston D Crew J Rogers MA Banks RE Roberts ISD Harris AL Phase I Trial of intravesical suramin in superficial transitional cell bladder carcinoma. **British Journal of Cancer** 2005 Jun 20; 92(12): 2140-7

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# **Chapter One**

## **Introduction**



## 1.1 Bladder Cancer, epidemiology, screening, clinical presentation, risk of recurrence and progression to invasion of muscularis propria.

Bladder cancer is the fourth commonest cancer in men, with an incidence of 30/100,000 in England and Wales in 1998, 11,500 new cases and 4,500 deaths in the U.K. per annum. A quarter of all cases are in women in whom the incidence is 10/100,000. Non-invasive bladder cancer is managed by endoscopic resection, intravesical chemotherapy and surveillance cystoscopy for a period of at least 10 years. Up to a half of all non-invasive bladder cancers progress and a quarter of all bladder cancers present with disease invading into the muscularis propria of the bladder (muscle invasive bladder cancer). The 5-year survival rate of muscle invasive disease with radical treatment is 50%. 4% of all cancer deaths in England and Wales are due to bladder cancer. The incidence of bladder cancer increases directly with age, with median age at diagnosis 69 years in men and 71 in women (Lynch and Cohen 1995).

Metastases occur to lymph glands, liver, lung, bone, intestine and adrenals (in order of frequency). Patients who develop metastasis do so at a mean of 11 months from diagnosis (Babaian, Johnson et al. 1980) making it a fast growing cancer. Unlike prostate cancer, thyroid cancer or even lung cancer, bladder cancer is not reported to be found as a latent cancer at autopsy (Imaida, Hasegawa et al. 1997). Multifocal and recurrent tumours have been shown to have a monoclonal origin (Chern, Becich et al. 1996) but a recent review of the evidence suggests an oligoclonal origin to bladder cancer (Duggan, Gray et al.



2004). Clonality assays of muscle-invasive bladder cancer have repeatedly shown that there is evidence for subclonal evolution. That is, all tumours shared some genetic events but there were many accumulated differences, indicating a great overall genetic instability (Anderson 2005). Case reports of familial transitional cell carcinoma have been described with a variety of genetic defects described (Kiemenev and Schoenberg 1996). There have been few epidemiological studies, one suggesting an increased risk of 2-fold for first degree relatives, but another in the founder population of Iceland argued against a hereditary subtype of bladder transitional cell carcinoma, at least in that population (Kiemenev, Moret et al. 1997). The only known associated clinical syndrome associated with transitional cell carcinoma is hereditary nonpolyposis colon cancer, which increases the risk of upper tract tumours but not bladder tumours by 10-fold (Watson and Lynch 1993).

The most common presenting symptom of bladder cancer is painless haematuria, which occurs in about 85% of patients (Varkarakis, Gaeta et al. 1974). In reality nearly all patients with cystoscopically detectable bladder cancer have at least microscopic haematuria if enough urine samples are taken. Most commentators agree that there is no value in screening the general population because the incidence of bladder cancer in those under the age of 40 is so low and the incidence of microscopic haematuria in this group may be as high as 5.4% (Tomson and Porter 2002) . However a case has been made for screening selected high-risk groups such as older men. In one large study

(n=1340) with repeated urine testing in men over 50 years of age the prevalence of microscopic haematuria was 21%(Messing, Young et al. 1992). Urothelial cancer was found in 8% of this group (or 1.2% of those screened). Screening for bladder tumours in this way can pick them up at a significantly earlier stage with a benefit to prognosis (Messing, Young et al. 1995). However screening would have to be completed at least annually (Messing, Young et al. 1995), and there has never been a randomised trial of such a screening programme. Furthermore given the proportion of bladder cancer cases attributable to smoking is at least one third (Burch, Rohan et al. 1989) (Howe, Burch et al. 1980) any money spent on bladder cancer might be better spent on primary prevention rather than screening.

Sylvester has published tables that allow urologists to easily calculate a superficial bladder cancer patient's short- and long-term risks of recurrence and progression after transurethral resection. An analysis was carried out of individual patient data from 2596 superficial bladder cancer patients included in seven European Organization for Research and Treatment of Cancer trials. A simple scoring system was derived based on six clinical and pathological factors: number of tumors, tumor size, prior recurrence rate, T category, carcinoma in situ, and grade. The probabilities of recurrence and progression at one year ranged from 15% to 61% and from less than 1% to 17%, respectively. At five years, the probabilities of recurrence and progression ranged from 31% to 78% and from less than 1% to 45%. With these probability tables, the urologist can discuss the different options with the patient to determine the most

appropriate treatment and frequency of follow-up. (Sylvester, van der Meijden et al. 2006)

(see table below taken from Sylvester)

**Table 3: Weighting used to calculate recurrence and progression scores**

<b>Factor</b>	<b>Recurrence</b>	<b>Progression</b>
<b>Number of Tumours</b>		
Single	0	0
2 to 7	3	3
≥ 8	6	3
<b>Tumour Diameter</b>		
< 3 cm	0	0
≥ 3 cm	3	3
<b>Prior Recurrence Rate</b>		
Primary	0	0
≤ 1 recurrence/year	2	2
> 1 recurrence/year	4	2
<b>Category</b>		
Ta	0	0
T1	1	4
<b>CIS</b>		
No	0	0
Yes	1	6
<b>Grade (1973 WHO)</b>		
G1	0	0
G2	1	0
G3	2	5
<b>Total Score</b>	<b>0 - 17</b>	<b>0 - 23</b>

CIS = carcinoma in situ, rec/yr = recurrence per year.

**Table 4: Probability of recurrence and progression according to score**

<b>Recurrence score</b>	<b>Probability of recurrence at 1 year (95% CI)</b>	<b>Probability of recurrence at 5 years (95% CI)</b>
0	15% (10%, 19%)	31% (24%, 37%)
1-4	24% (21%, 26%)	46% (42%, 49%)
5-9	38% (35%, 41%)	62% (58%, 65%)
10-17	61% (55%, 67%)	78% (73%, 84%)
<b>Progression score</b>	<b>Probability of progression at 1 year (95% CI)</b>	<b>Probability of progression at 5 years (95% CI)</b>
0	0.2% (0%, 0.7%)	0.8% (0%, 1.7%)
2-6	1% (0.4%, 1.6%)	6% (5%, 8%)
7-13	5% (4%, 7%)	17% (14%, 20%)
14-23	17% (10%, 24%)	45% (35%, 55%)



## 1.2 Hypoxia is common in solid cancers and is a major cause of radio and chemoresistance

Hypoxia is a common feature of solid human tumours (Vaupel, Kallinowski et al. 1989). Tissue oxygen levels in human tumours have been measured with eppendorf electrodes since 1984. The tumours measured by this technique include sarcomas, cervix, prostate, and head and neck tumours (Brizel, Scully et al. 1996; Hockel, Schlenger et al. 1996; Becker, Hansgen et al. 1998; Movsas, Chapman et al. 1999; Movsas, Chapman et al. 2001). A significant proportion of these tumours have median oxygen tensions below 10mm Hg. In these same studies it was shown that neighbouring muscle (periprostatic (Movsas, Chapman et al. 1999) or sternocleidomastoid (Becker, Hansgen et al. 1998)) and normal tissue (prostate (Movsas, Chapman et al. 1999)) had comparatively higher tissue levels of oxygen. End-capillary (mixed venous) oxygen tension is 40mm Hg (75% saturation), and a reasonable estimate of mean resting tissue pO<sub>2</sub> is 10mmHg (Berne and Levy 1996). Hypoxic tumours are usually defined as having a median pO<sub>2</sub> of <10mmHg.

Xenograft experiments measuring oxygenation with eppendorf electrodes in tumours at different stages of growth suggest that although smaller tumours may vary in their proportion of hypoxic cells, larger tumours (above only 2g) are universally hypoxic (De Jaeger, Merlo et al. 1998). Seminal observations by L.H.Gray 50 years ago (Thomlinson and Gray 1955) on human lung cancer

suggested that cancer cells grow in cords around blood vessels. Gray analysed 160 tumours and with only one exception no intact tumour cell was seen more than 180 $\mu$ m from the stroma, and there was no tumour cord more than 200 $\mu$ m in radius that was not without central necrosis. Regions of necrosis are believed to demarcate regions of severe, 'chronic' hypoxia. Gray's observations and experiments suggest that very early on in tumour development, tumour cells receive inadequate perfusion for their needs and areas of hypoxia and necrosis can develop even in small tumours. Another type of hypoxia known as acute hypoxia occurs when aberrant blood vessels are shut down. Closed vessels can be reopened leading to reperfusion of hypoxic tissues with oxygenated blood, leading to reperfusion injury.

Eppendorf measurements have shown tumour hypoxia to be independently related to prognosis, being associated with aggressive local growth, metastasis, and treatment failure (Brizel, Scully et al. 1996; Hockel, Schlenger et al. 1996; Nordmark and Overgaard 2000). Pelvic recurrences of cervical cancer are significantly more hypoxic than the primary tumour (Hockel, Schlenger et al. 1998).

The impact of oxygen on radiosensitivity of mammalian tumours was demonstrated as early as the 1950s when Gray et al (Gray, Conger et al. 1953) published their seminal treatise on oxygen and radiation. The summary of the in vitro experiments is shown (Fig 1.1). As oxygen levels fall from 21%,

radiosensitivity falls in all in vitro experimental systems tested including; growth rate of *Vicia faba* root tips, lethal mutations in *Drosophila melanogaster*, and cytological damage to mouse ascites tumour cells. Gray's paper describes a large in vivo experiment on 124 mice with the Ehrlich mouse ascites tumour. The mouse tumours were exposed to 1000 rad x-rays in air, or 1000 rad in pure oxygen, or 1500 rad or 2000 rad in air. Breathing pure oxygen critically increased radiosensitivity of the tumours to the equivalent of an extra 500 rad. Interestingly Gray also observed in this paper that tumours that had been allowed to grow for 9 days or longer, the effectiveness of extra inhaled oxygen was only very slight. Gray hypothesised that in larger tumours the capillary structure may be such that when the animal breathes oxygen there are regions of tumour tissue in which the oxygen tension is not significantly raised. Gray also proposed the correct mechanism that the role of dissolved oxygen is to modify the nature of the chemical intermediates formed along the tracks of the ionising particles.

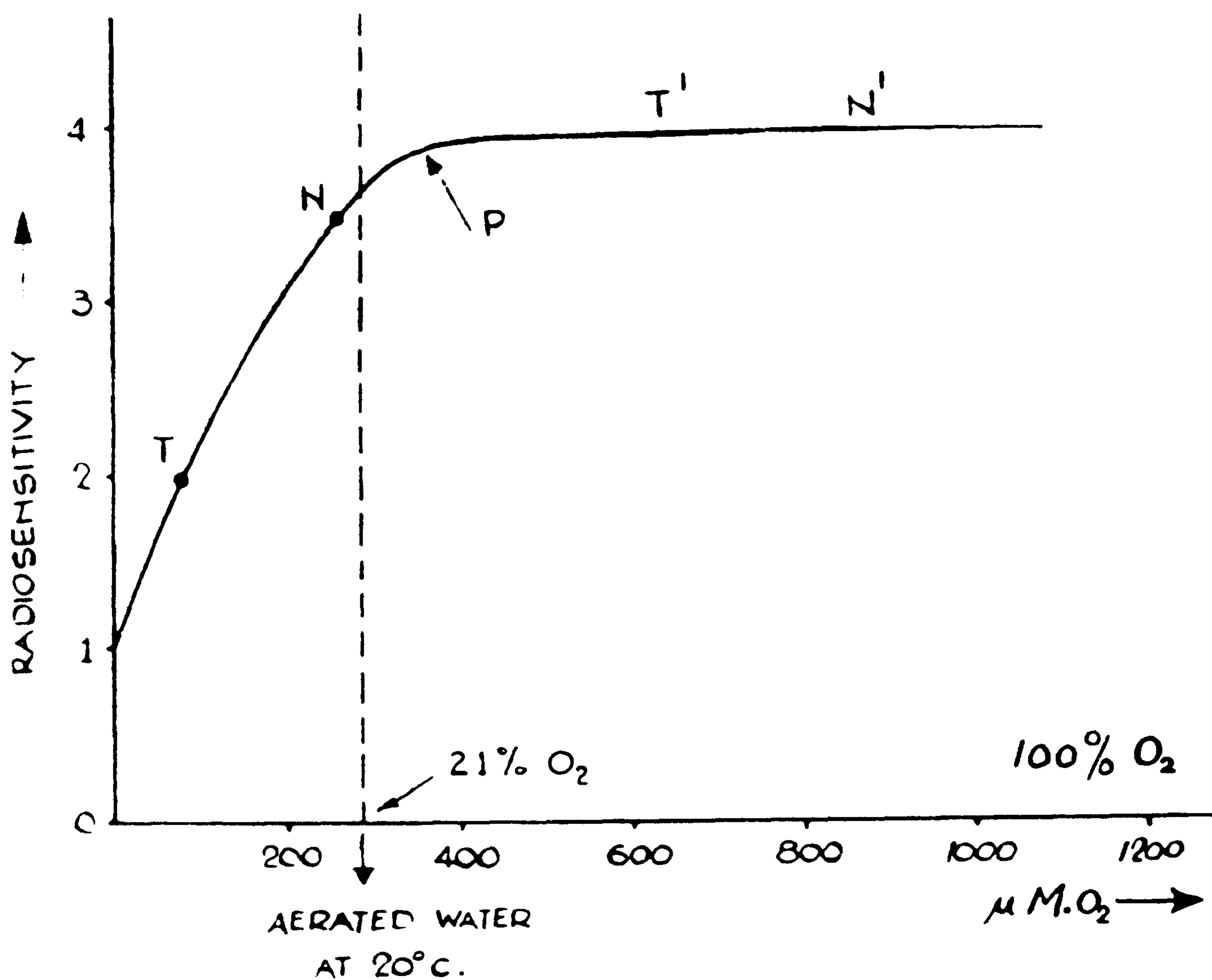


FIG. 1B.

Typical curve of radiosensitivity as a function of oxygen tension at the time of irradiation.

*Abscissae:*  $O_2$  tension at the time of irradiation.

*Ordinates:* Radiosensitivity.

Fig 1.1 Summary of the findings of the in vitro experiments of oxygen on radiosensitivity. (Gray, Conger et al. 1953)

Trials of hyperbaric oxygen as a sensitizer for radiotherapy in the 1970s were positive for head and neck and cervical carcinomas but not for bladder tumours (1978). In bladder cancer there may be areas where oxygen tension is not significantly raised. Encouragingly subsequent trials of the use of 95% oxygen in the form of inhaled carbogen have shown a positive radiosensitising effect in



bladder cancer (Hoskin, Saunders et al. 1999) and Phase III trials are now starting in the U.K.(Hoskin, Rojas et al. 2005).

Long-term follow-up of radical radiotherapy failures in the treatment of invasive bladder cancer has consistently shown that T stage (T2/T3a vs T3b/T4) and tumour bulk are the main factors affecting survival (Gospodarowicz, Rider et al. 1991). One of the aims of this thesis was to accurately measure the proportion of cancers with necrosis at each stage to see if this might be a likely factor in radioresistance..

Hypoxia also affects chemotherapeutic agents adversely by leading to growth arrest, which makes the cells less amenable to cytotoxic effects. Hypoxia also induces the multidrug resistance gene 1 (Comerford, Wallace et al. 2002) among other stress response genes. However hypoxia within cells can also be used to an advantage. The agent mitomycin, which is current standard of treatment given intravesically to all patients as a one off dose immediately after superficial bladder tumour resection is known to be activated preferentially in hypoxic cells. As will be seen later the luminal surface of superficial bladder cancers is hypoxic and so intravesical installation of this agent is the ideal route. Levels of two reductase enzymes upregulated in hypoxic cells are known to correlate with mitomycin activity (Gan, Mo et al. 2001). The corresponding down-regulation of these enzymes in more aggressive tumours contributes to mitomycin resistance (Baumann, Hodnick et al. 2001).



### 1.3 The HIF 1 pathway

One of the ways in which cells respond to reduced oxygen levels is through hypoxia-inducible transcription factor 1 (HIF-1)(Harris 2002; Semenza 2003). HIF-1 is a heterodimer that consists of the hypoxic response subunit HIF-1 $\alpha$  and the constitutively expressed protein HIF-1 $\beta$  subunit. As for any protein, the level of HIF-1 $\alpha$  expression is determined by the rates of protein synthesis and protein degradation. In the case of HIF-1 $\alpha$ , synthesis is regulated by O<sub>2</sub>-independent mechanisms (Fig 1.3), whereas degradation is regulated primarily via O<sub>2</sub>-dependent mechanisms (Fig 1.4). In the presence of oxygen, the prolyl hydroxylase enzymes (1-3) hydroxylate HIF-1 $\alpha$  at proline residues 402 and 564. This allows the Von Hippel-Lindau (VHL) protein to bind HIF-1 $\alpha$  labelling it for proteasome degradation. VHL binding is also promoted by acetylation of lysine (K) residue 532 by the ARD1 acetyltransferase. O<sub>2</sub>-dependent hydroxylation of asparagine (N) residue 803 by the enzyme FIH-1 (factor inhibiting HIF-1) blocks the binding of the transcriptional complex (p300 and CBP) to HIF-1 $\alpha$  and therefore also inhibits HIF-1 $\alpha$  mediated gene transcription (although more recent references show this is not confirmed.). In hypoxic conditions the prolyl and asparagines residues are not hydroxylated and HIF-1 $\alpha$  consequently is not degraded and is able to proceed with transcription.

An additional complicating factor is the existence of the related protein HIF-2 $\alpha$ , which can also dimerise with HIF-1 $\beta$ . Initially a specific role in endothelium was suggested for HIF-2 $\alpha$  however experiments in control and hypoxic rats showed

that HIF-2 $\alpha$  was not detectable under baseline conditions, and marked hypoxic induction occurred in all organs investigated. In some organs induction of HIF-2 $\alpha$  was exclusively nonparenchymal (kidney) but predominantly parenchymal in others (liver and intestine). Fitting with this result, experiments with small interfering RNAs have shown that hypoxia induction of three known hypoxia-inducible genes (CA IX, GLUT-1 and BNIP3) was found to be critically dependent on HIF-2 $\alpha$  in renal cancer cell lines but not in other cell lines tested which were HIF-1 $\alpha$  dependent (Sowter, Raval et al. 2003). Furthermore down regulation of HIF-2 $\alpha$  may be sufficient to suppress tumour formation by pVHL-defective renal carcinoma cells (Kondo, Kim et al. 2003).







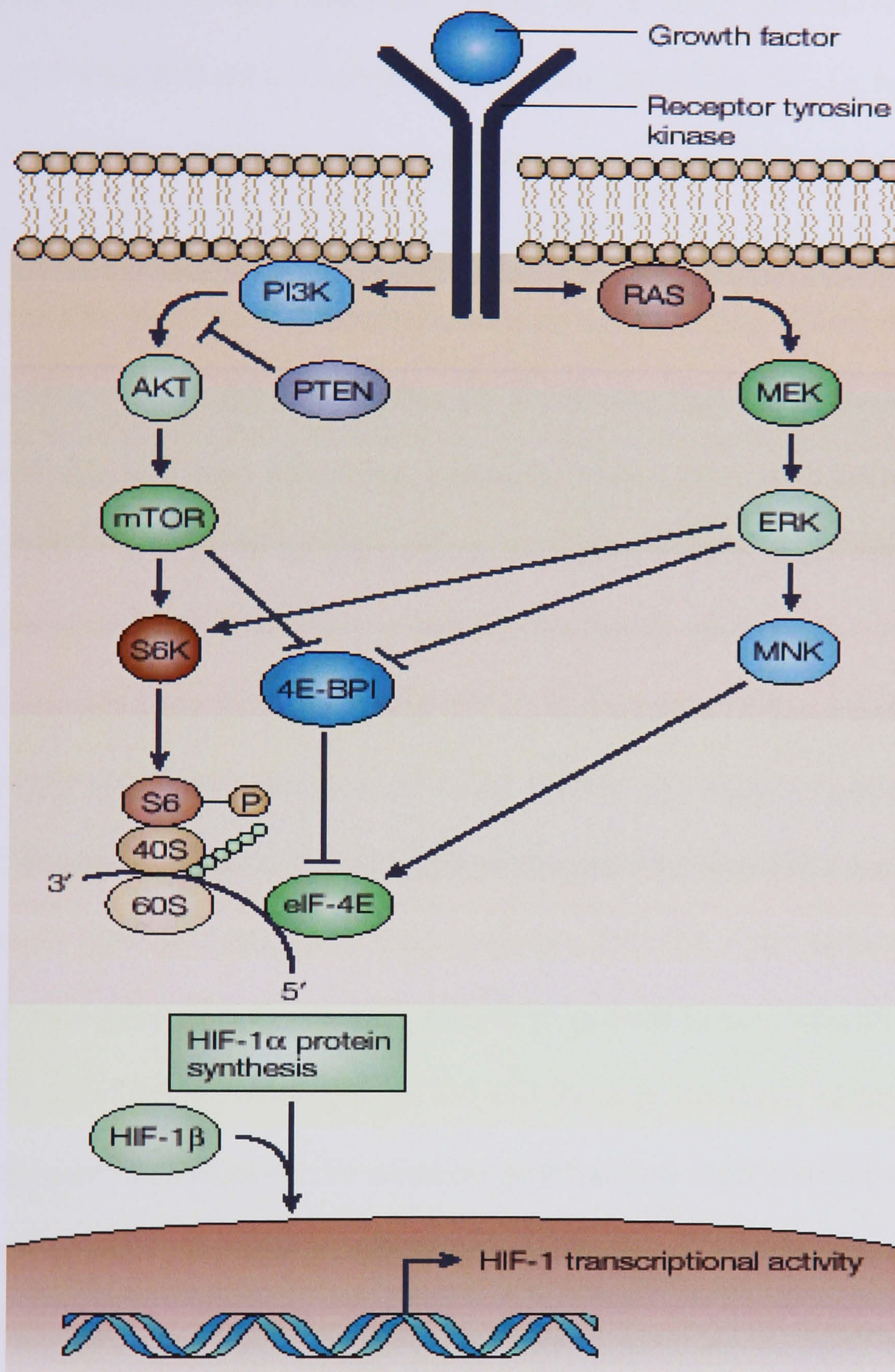


Fig 1.3 Oxygen-independent regulation of HIF-1 $\alpha$  synthesis. Growth factor binding to a cognate receptor tyrosine kinase activates the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. PI3-Kinase and RAS activate downstream pathways that increase the rate at which a subset of mRNAs within the cell are translated into protein. (Semenza 2003)



Oxygen-independent regulation of HIF-1 differs from oxygen-dependent regulation in two respects. First, as already mentioned, growth factors, cytokines and other signaling molecules stimulate HIF-1 $\alpha$  synthesis rather than degradation. Second, whereas hypoxia increases HIF-1 $\alpha$  levels in all cell types, growth-factor stimulation induces HIF-1 $\alpha$  expression in a cell-type-specific manner via activation of phosphatidylinositol kinase (PI3K) or mitogen activated protein kinase (MAPK) pathways. Of course these pathways are not specific to HIF-1 $\alpha$  and also affect the translation of dozens of different mRNAs. HIF-1 $\alpha$  protein is likely to be particularly sensitive to changes in the rate of synthesis because of its extremely short half-life under non-hypoxic conditions. This link between growth factors and HIF-1 is probably of functional and evolutionary importance. Proliferation of cells increases oxygen consumption in a given microenvironment. As one cell produces two daughter cells, O<sub>2</sub> consumption increases. It is not surprising therefore that the main pathways that transduce proliferative and survival signals from growth-factor receptors also induce HIF-1 $\alpha$  expression. This can be viewed as a preemptive strategy for maintaining oxygen homeostasis. In addition proliferating cells preferentially use glycolytic rather than oxidative metabolism to generate ATP. The induction of glycolysis in proliferating cells is mediated partly by activating HIF-1 (see Glycolysis). This is demonstrated by the coordinated upregulation of HIF-1 $\alpha$  with proliferation of migrating basal keratinocytes seen in normal wound healing in humans (Elson, Ryan et al. 2000).

#### 1.4 The function of HIF-1

HIF-1 $\alpha$  knockout mice die in utero on day E11 (Kotch, Iyer et al. 1999). Although vascular malformations are evident, the principle cause of cell death is thought to be mesenchymal because cell death precedes vascular malformation. This suggests a primary role of HIF-1 in the survival of cells. In these embryos VEGF (Vascular Endothelial Growth Factor) was surprisingly not deficient. HIF-1 $\alpha$  is not essential for VEGF production.

Xenografted HIF-1 $\alpha$  or HIF-1 $\beta$  knockout cancerous cells have a slower rate of tumour growth ((Jiang, Agani et al. 1997; Maxwell, Dachs et al. 1997; Ryan, Lo et al. 1998; Ryan, Poloni et al. 2000)) (except in (Carmeliet, Dor et al. 1998)) while cells with increased HIF-1 $\alpha$  activity have an increased rate of growth ((Ravi, Mookerjee et al. 2000; Akakura, Kobayashi et al. 2001; Maranchie, Vasselli et al. 2002)). One experiment suggested that HIF-1 $\alpha$  knockout cells become necrotic in poorly vascularised areas but grow well in vascularised areas (Blouw, Song et al. 2003), which would support the hypothesis that HIF-1 $\alpha$  acts as a survival factor in areas of hypoxia.

Further evidence supporting this hypothesis comes from tissue-specific knockout mice. The advantage of such mice is that they survive the early developmental death of the global knockout. Tissue-specific targeting to delete HIF-1 $\alpha$  in an avascular tissue; the cartilaginous growth plate of developing bone shows that cells that lack HIF-1 $\alpha$  in the hypoxic interior of the growth

plate die (Schipani, Ryan et al. 2001). Tissue specific knockouts of HIF-1 $\alpha$  in monocytes and macrophages of the myeloid lineage demonstrated dramatically that HIF-1 $\alpha$  is essential for regulation of glycolytic capacity: When HIF-1 $\alpha$  is absent, the cellular ATP pool is drastically reduced, the metabolic defect results in profound impairment of myeloid cell function resulting in experimental skin wounds having no inflammatory component to their healing compared to controls (Cramer, Yamanishi et al. 2003).

### 1.5 HIF-1 $\alpha$ and human cancers

Immunohistochemical analysis using monoclonal antibodies revealed that HIF-1 $\alpha$  is over expressed in many human cancers. Although immunohistochemical analysis can be useful in determining whether a specific protein is present at higher levels in cancer cells compared with surrounding normal tissue, it does not reveal whether the protein carries any alterations which could affect its function (for example with non-functional p53 protein overexpression).

Tumour type	Association	References
Cervical, early stage	Increased mortality	(Birner, Schindl et al. 2000)
Cervical, RXT	Increased mortality	(Burri, Djonov et al. 2003)
Lung, NSCLC	Decreased mortality	(Volm and Koomagi 2000)
Lung, NSCLC	Increased mortality (HIF-2a)	(Giatromanolaki, Koukourakis et al. 2001)
Breast, LN-positive	Increased mortality	(Schindl, Schoppmann et al. 2002)
Breast, LN-negative	Increased mortality	(Bos, van der Groep et al. 2003)
Oligodendroglioma	Increased mortality	(Birner, Gatterbauer et al. 2001)
Oropharyngeal SCC	Increased mortality, radiation resistance	(Aebersold, Burri et al. 2001)
Ovarian	Increased mortality (with p53)	(Birner, Schindl et al. 2001)
Oesophageal, early stage	Increased mortality resistance to PDT (with BCL2)	(Koukourakis, Giatromanolaki et al. 2001)
Endometrial	Increased mortality	(Sivridis, Giatromanolaki et al. 2002)
Head and Neck SCC	Increased mortality	(Koukourakis, Giatromanolaki et al. 2002)
GI stromal tumour of stomach	Increased mortality	(Takahashi, Tanaka et al. 2003)

Table 1.1 Increased HIF-1 $\alpha$  levels in human cancers by immunohistochemistry. GI, gastrointestinal; LN, lymph node; NSCLC, non-small-cell lung cancer; PDT, photodynamic therapy; RXT, radiation therapy; SCC, squamous-cell carcinoma (Taken from (Semenza 2003))



At the time of initiating work there were no studies investigating HIF-1 $\alpha$  in bladder cancer. However one group did publish two papers while work was in progress (see Chapter 6.3). Significant associations between HIF-1 $\alpha$  overexpression and patient mortality have been shown in cancers of the brain, breast, cervix, oropharynx, ovary and uterus (endometrial) (Table 1.1). In some cancer subtypes, such as oropharyngeal cancer, the association was observed among tumours from all patients, whereas in other cancer types the association was observed only in specific subgroups, such as in early-stage cervical cancer. By contrast, associations between HIF-1 $\alpha$  overexpression and decreased mortality were reported for patients with head and neck cancer and non-small-cell lung cancer although other studies failed to replicate these results. The mechanism for this upregulation varies in different cell types and tumours. In the VHL syndrome and over 90% of sporadic renal cell carcinomas this is characteristically due to VHL loss of function and reduced degradation of HIF-1 $\alpha$ . In other tumours increased synthesis from oncogenic and signaling changes plays the main part (Table 1.2).

Alteration in tumour	Mechanism of HIF-1 $\alpha$ induction	Reference
VHL loss of function	Decreased ubiquitylation	(Maxwell, Wiesener et al. 1999)
P53 loss of function	Decreased ubiquitylation	(Ravi, Mookerjee et al. 2000)
PTEN loss of function	Increased synthesis	(Zundel, Schindler et al. 2000)
PI3K-AKT-mTOR signalling	Increased synthesis	(Laughner, Taghavi et al. 2001)
MEK-ERK signalling	Increased synthesis	(Fukuda, Hirota et al. 2002)
ERBB2 gain of function	Increased synthesis	(Laughner, Taghavi et al. 2001)
EGFR signalling	Increased synthesis	(Zhong, Chiles et al. 2000)
IGF1R signalling	Increased synthesis	(Fukuda, Hirota et al. 2002)
PGE <sub>2</sub> signalling	Increased synthesis	(Liu, Kirschenbaum et al. 2002; Fukuda, Kelly et al. 2003)
SRC gain of function	Increased synthesis	(Jiang, Agani et al. 1997)
ARF loss of function	Decreased nucleolar sequestration	(Fatyol and Szalay 2001)

Table 1.2 Genetic alterations that increase HIF-1 activity. Where the alteration is due to increased signalling this may be due to genetic alteration in a component of the pathway or an upstream activator. For example AKT gain of function mutation or PTEN loss-of-function mutation induces PI3K-AKT-mTOR signalling; EGFR amplification or TGF- $\alpha$  overexpression induces EGFR (and PI3K-AKT-mTOR and MEK-ERK) signalling. EGFR, epidermal growth factor receptor; ERK, extracellular-signal-regulated kinase; IGFR1, insulin-like growth-factor-1 receptor; MEK, MAP/ERK kinase; mTOR, mammalian target of rapamycin; PGE<sub>2</sub>, prostaglandin E2; PI3K, phosphatidylinositol-3-kinase; VHL, von Hippel-Lindau protein. (Taken from (Semenza 2003))

### 1.6.1 HIF-1 target genes and biological responses to hypoxia

More than 60 putative direct HIF-1 targets have been identified. These can be grouped into a variety of biological responses. (Fig 1.5) One of the first targets of HIF-1 to be recognized was erythropoietin production in renal cells, which increases hemoglobin production. More universally, hypoxic cells undergo a shift from aerobic to anaerobic metabolism; they upregulate the enzyme carbonic anhydrase IX to regulate pH; they produce growth factors that produce angiogenesis (new blood vessel formation); and they produce factors that promote invasion and metastasis. All of these biological responses are potentially cancer promoting and are discussed below. However hypoxia also has been shown to cause cell cycle arrest in response to hypoxia in untransformed cells (embryonic murine fibroblasts and splenic B lymphocytes), and HIF-1 is a major regulator of cell cycle arrest in this circumstance, independent of p53 (Goda, Ryan et al. 2003). Upregulation of p21 and p27 cyclin-dependent kinase inhibitors is associated with this arrest. HIF-1 also upregulates apoptotic pathways such as BNIP3 and NIX (Sowter, Ratcliffe et al. 2001)). Therefore it can be said that presence of HIF-1 signals low oxygen, but the biological response of the cell may depend on the developmental, physiological and, in the case of cancer, oncogenic environment in which the cell finds itself.



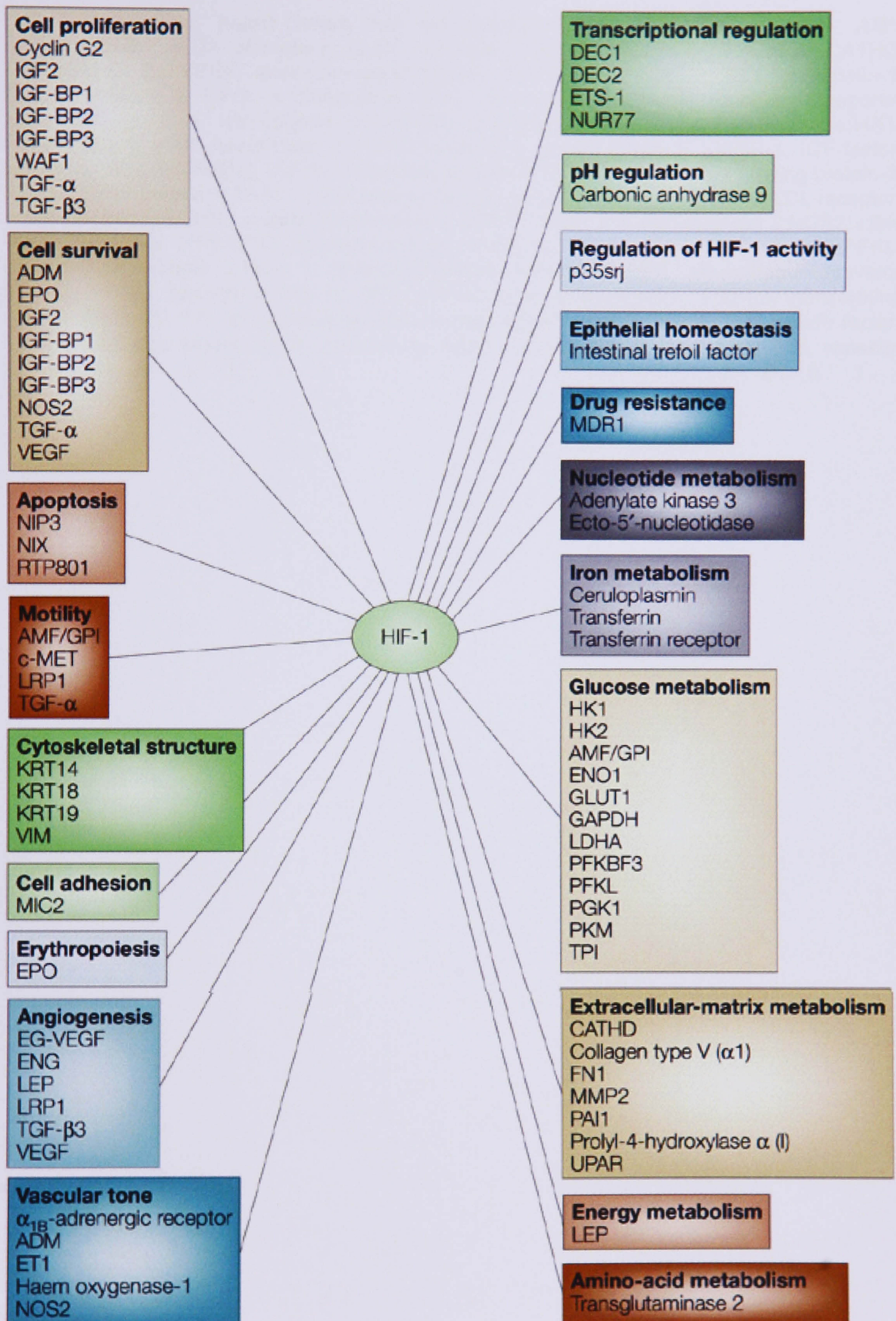




Fig 1.4 (previous page) Genes that are transcriptionally activated by HIF-1. ADM adrenomedullin; ALDA, aldolase A; ALDC, aldolase C; AMF, autocrine motility factor; CATHD, cathepsin D; EG-VEGF, endocrine-gland-derived VEGF; ENG, endoglin; ET1, endothelin-1; ENO1, enolase 1; EPO, erythropoietin; FN1, fibronectin 1; GLUT1, glucose transporter 1; GLUT3, glucose transporter 3; GAPDH, glyceraldehydes-3-P-dehydrogenase; HK1, hexokinase 1; HK2, hexokinase 2; IGF2, insulin-like growth factor 2; IGFBP-1, IGF-factor-binding-protein-1; IGFBP-2, IGF-factor binding protein-2; IGFBP-3, IGF-factor binding protein-3; KRT14,18,19 keratin14,18,19; LDHA, lactate dehydrogenase A; LEP, leptin; LRP1, LDL-receptor-related protein-1; MDR1, multidrug resistance 1; MMP2, matrix metalloproteinase 2; NOS2, nitric oxide synthase 2; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphate-3; PFKL, phosphofructokinase L; PGK 1, phosphoglycerate kinase 1; PAI1, plasminogen-activator inhibitor 1; PKM, pyruvate kinase M; TGF- $\alpha$ , transforming growth factor- $\alpha$ ; TGF- $\beta$ 3, transforming growth factor- $\beta$ 3; TPI, triosephosphate isomerase; VEGF, vascular endothelial growth factor, UPAR, urokinase plasminogen activator receptor; VEGFR2, VEGF receptor-2; VIM, vimentin  
( S e m e n z a 2 0 0 3 )



## 1.6.2 Glycolysis

Over seven decades ago classical biochemical studies showed that tumours have altered metabolic profiles and display high rates of glucose uptake and glycolysis (Dang and Semenza 1999). Normal cells utilize oxygen for efficient production of ATP by oxidative phosphorylation. Tumour cells rely on the less efficient glycolytic pathway to produce ATP, even in the presence of oxygen. Pyruvate at the end of glycolysis is converted to lactate via lactate dehydrogenase to replete NAD used in glycolysis (Fig 1.6).

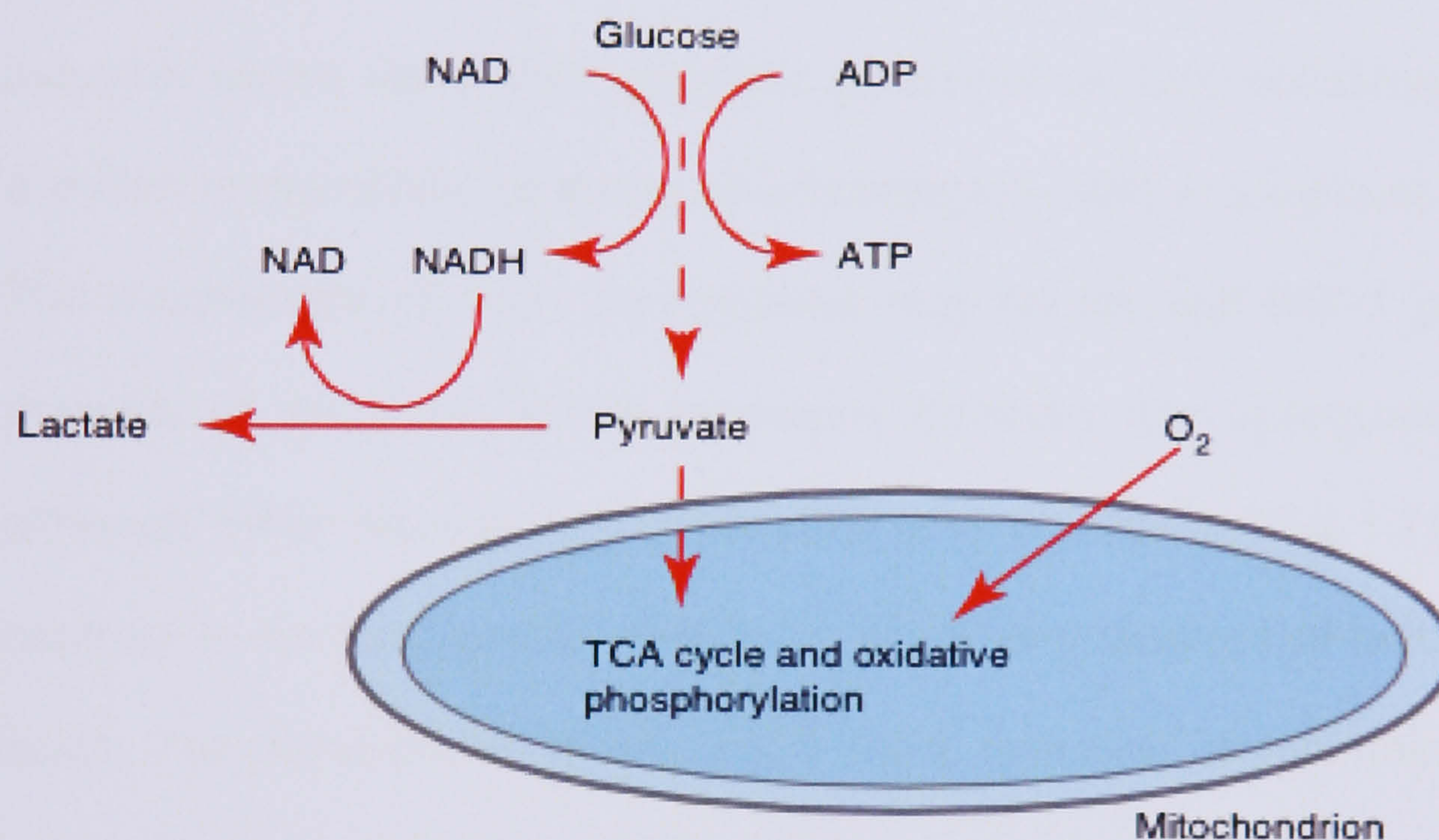


Fig 1.5 The production of lactate from pyruvate repletes NAD used in glycolysis. (Dang and Semenza 1999)

Many glycolytic enzymes are raised in the serum of cancer patients (Cameron, 1971). For example there is a close correlation of changes in glucose phosphate isomerase levels with progression of the disease. Phosphoglucomutase and ketose 1-phosphate aldolase is also often



raised in disseminated cancer. Increases of these enzymes also occur in cerebrospinal fluid in association with cerebral tumours but also in cerebrovascular accidents. The only glycolytic enzyme in routine use as a cancer marker in the serum however is lactate dehydrogenase (LDH). LDH is an independent factor for prognosis in all tumour types studied. LDH is raised in the serum independent of the nature of the tumour and the site of metastasis. There are 5 isozymes of LDH and only the hypoxia-inducible one is raised in cancer (Yoshimura, Takemori et al. 1988; Koukourakis, Giatromanolaki et al. 2003). This implies that tumour cells may be primarily up regulating hypoxia-inducible genes rather than glycolytic genes per se. One could hypothesize that a switch to glycolysis would be an advantage to cells in a hypoxic environment. The mechanism of such upregulation may be through HIF-1 given that the majority of enzymes in the glycolytic pathway are upregulated by HIF-1, although other oncogenic mechanisms also contribute (Fig 1.7). Tumours in contrast to normal tissues exist in an acidic environment of lactate and other acids. The intracellular pH of tumour cells, however, is maintained as it is in normal cells or even more alkaline which facilitates intracellular signaling.



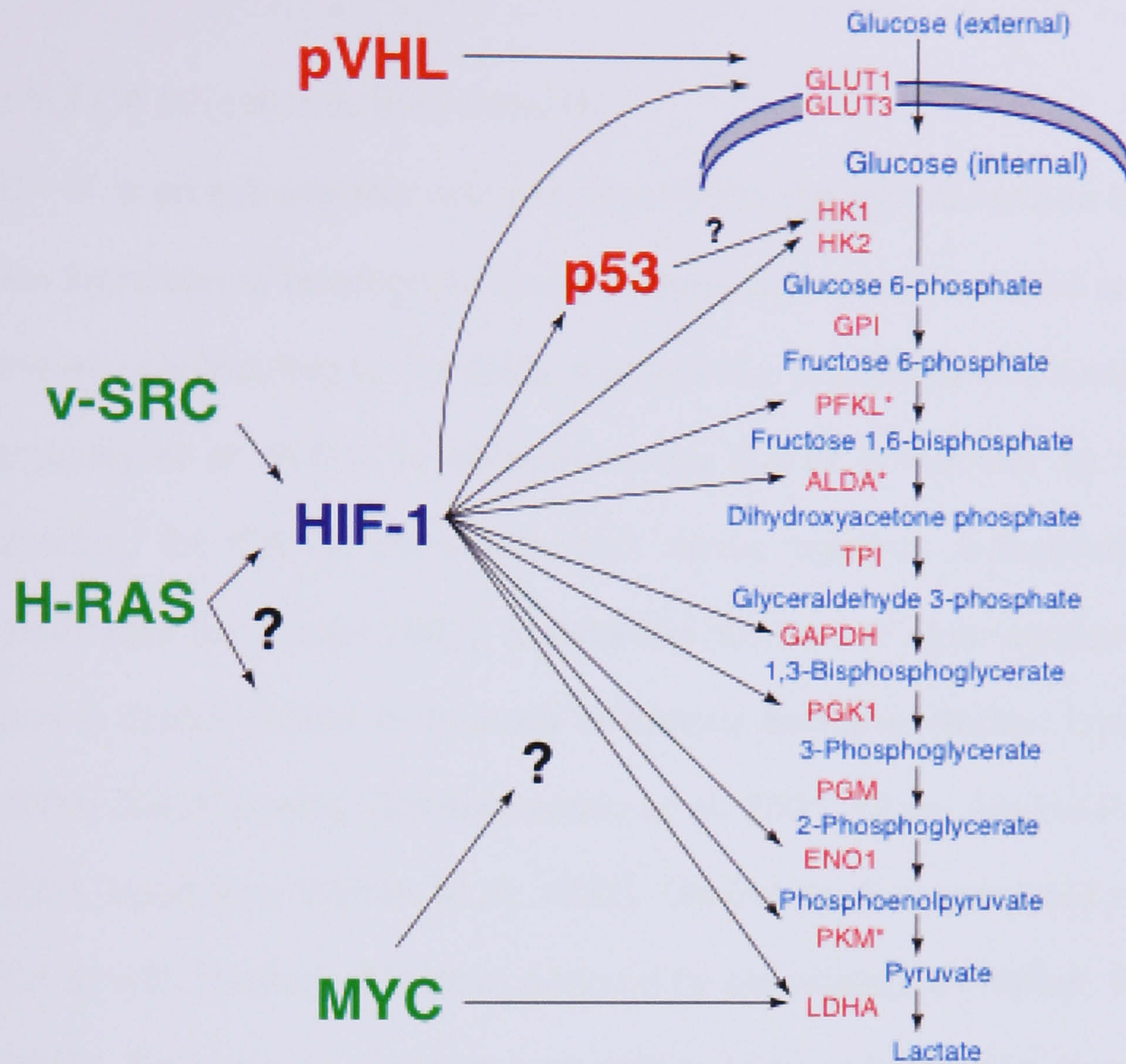


Fig 1.6 HIF-1 upregulates the majority of enzymes in glycolysis. Oncogenes also upregulated glycolysis either directly or indirectly via HIF-1 (from (Dang and Semenza 1999)). C-myc may be responsible for the upregulation of HIF-1 seen in normal healing skin wounds (see text), it is also turned on in the normal regeneration of liver tissue after resection (Robbins).



### 1.6.3 CA IX (carbonic anhydrase IX)

CA IX is an extracellular enzyme attached to the cell membrane that catalyses the formation of bicarbonate and hydrogen ions from water and carbon dioxide thereby contributing to the acidic extracellular environment of tumours (Wykoff, Beasley et al. 2000). In cervical cancer CA IX is induced by hypoxia, and staining for CA IX correlates with tissue hypoxia measured by oxygen electrodes (Loncaster 2001). Substantial numbers of other studies indicate that it is a useful marker of hypoxia in tumour sections. (Airley, Loncaster et al. 2003) (Koukourakis, Giatromanolaki et al. 2001; Olive, Aquino-Parsons et al. 2001; Kaanders, Wijffels et al. 2002). Clinical studies correlated expression of CA IX with staining of bladder tumours by pimonidazole (Wykoff, Beasley et al. 2000). The latter is a recognised marker of tumour hypoxia given to patients preoperatively and metabolised in hypoxic areas to an adduct that can be detected histochemically.

### 1.6.4 Invasion and metastasis

Proteins that are known to be upregulated by HIF-1 are known to play an established role in the pathophysiology of invasion. Urokinase plasminogen activator receptor (uPA), matrix metalloproteinase 2, cathepsin D, fibronectin 1, vimentin and transforming growth factor alpha are all involved in digestion of the basement membrane and extracellular matrix. Degraded extracellular matrix is replaced by fibronectin and other proteins that are recognized by

integrins that are expressed on cancer cells. Unlike epithelial cells, malignant cells show mesenchymal characteristics in which intermediate filament production consists of keratins and vimentin, which promote the fluid structure required for motility, rather than simply keratin subtypes, which are characteristic of fixed epithelial cells.

In an effort to characterize the metastatic genotype of bladder cancer Nicholson et al (Nicholson, Frierson et al. 2004) profiled gene expression changes in an in vivo bladder cancer lung-metastasis model in nude mice and compared results with gene expression profiles of 23 primary human bladder tumours, and found that the expression of four genes were consistently and progressively upregulated. Three of these genes, uPA, matrix metalloproteinase 14 (MMP14) and tissue inhibitor of metalloproteinase (TIMP2) have been shown to be hypoxia regulated (Graham, Forsdike et al. 1999; Ottino, Finley et al. 2004). In vitro invasiveness of MDA-231 breast cancer cells was increased by exposure to hypoxia and inhibition of uPA receptor inhibited this increase in invasion (Graham, Forsdike et al. 1999). Measurement of uPA protein and its receptor in plasma in 51 cancer cases prior to cystectomy and 44 men without cancer showed uPA to be independently associated with metastasis, disease progression and death from bladder cancer (Shariat, Monoski et al. 2003). Immunohistochemistry and in situ hybridization have shown higher expression of uPA to correspond with higher stage and grade in bladder cancer (Bhuvaramurthy, Schroeder et al. 2004).

MMP14 is better known as membrane-type 1 matrix metalloproteinase (MT-1 MMP) and is a major activator of pro-matrixmetalloproteinase-2 (pro-MMP2) on the cell surface and TIMP2 is known to inhibit this activation (Jo, Yeon et al. 2000; Oblander, Zhou et al. 2005). MT-1 MMP has been shown to be a target of HIF-2 in renal cell carcinoma cells defective for VHL (Petrella, Lohi et al. 2005). MT-1 MMP deficient mice display a deficiency in growth and maintenance of the skeleton and soft tissues post-natally due to a loss of collagenolysis activity (Holmbeck, Bianco et al. 2004). Deleting or suppressing expression of MT1-MMP, in fibroblasts or tumor cells results in a loss of collagenolytic and invasive activity in vitro or in vivo and occurs independently of plasminogen, the gelatinase A/TIMP-2 axis, gelatinase B, collagenase-3, collagenase-2, or stromelysin-1 (Sabeh, Ota et al. 2004).

Other in vitro and nude mouse experiments also suggest a role of hypoxia in invasion. Over-expression of HIF-1 by transfection in vitro colon carcinoma assays stimulates invasion in matrigel (Krishnamachary, Berg-Dixon et al. 2003). Prior hypoxic exposure of melanoma cells before xenografting increases lymph node metastasis (Rofstad and Danielsen 1999; Rofstad, Rasmussen et al. 2002). Finally more hypoxic xenografts of a fibrosarcoma are significantly more likely to form early pulmonary metastases (De Jaeger, Kavanagh et al. 2001).

## 1.7 Angiogenesis.



Angiogenesis is the growth of new blood vessels from the existing vasculature. One of the most potent angiogenic factors, often measured in picogram amounts, is vascular endothelial growth factor (VEGF). VEGF is induced by hypoxia in most cell types. Angiogenesis is a normal physiological process, vital in embryogenesis, wound healing and the female menstrual cycle. In addition to cancer, angiogenesis plays a role in the pathogenesis of diabetes, atherosclerosis, endometriosis and arthritis. Nearly a century ago it was observed that angiogenesis occurs around tumours (Goldman, 1907). Thirty years ago Judah Folkman proposed that 'the population of tumour cells and the population of capillary endothelial cells within a neoplasm may constitute a highly integrated ecosystem' (Folkman 1971). Folkman went on to suggest that if tumours are absolutely dependent on angiogenesis, then angiogenesis is a very attractive therapeutic target. A considerable body of evidence now supports his hypothesis (Folkman 1990), some of the most important insights are listed below:

1. The growth of tumours in an environment that does not permit vessel proliferation is limited to 1-2mm<sup>3</sup>. Rapid expansion occurs on transplantation to a permissive environment.
2. Studies of hepatic metastases in rats show they are avascular prior to reaching 1mm<sup>3</sup> in size. Thereafter they are vascularised.
3. Until vascularisation occurs at the base of human melanomas, metastases are rare.



4. Transfection of pro-angiogenic factors into tumour cells results in enhanced tumourgenicity.
5. Inhibition of angiogenic stimulators results in reduced tumour growth in experimental situations.

Microvessel counting in histopathological sections has also demonstrated angiogenesis to be an independent prognostic factor on multivariate analysis for breast cancer (Fox, Leek et al. 1995) and lung cancer (Macchiarini, Fontanini et al. 1992), prostate cancer (Jones and Fujiyama 1999) and muscle-invasive bladder cancer (Dickinson, Fox et al. 1994; Bochner, Cote et al. 1995; Jaeger, Weidner et al. 1995; Inoue, Slaton et al. 2000).

Some positive results have been obtained for antiangiogenic approaches in clinical trials: In a randomised trial of an anti-VEGF antibody (bevacizumab) in metastatic renal cell cancer, the drug significantly prolonged time to progression of disease (Yang, Haworth et al. 2003). In a similar randomised trial in metastatic colorectal cancer there was a significantly longer median survival in the patients receiving the drug with standard chemotherapy compared to chemotherapy alone (Kabbinar, Hurwitz et al. 2003).



## 1.8 Hypoxia, angiogenesis and bladder cancer

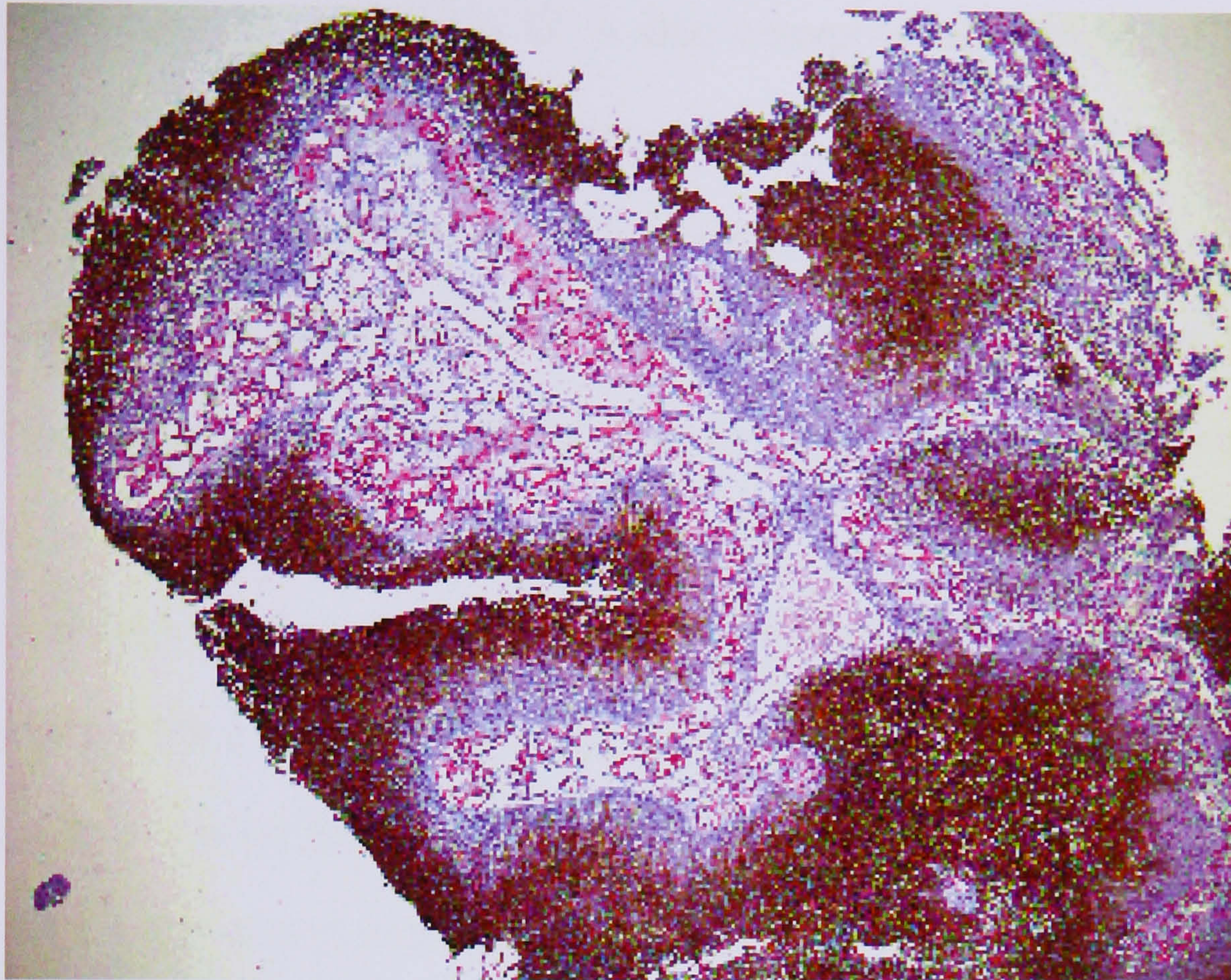


Fig 1.7 Superficial bladder cancer stained with CA IX (brown) and CD34 (red) (to show blood vessels). Note how the area of hypoxia starts at some distance from the vessels and goes right to the luminal surface.

There are no direct measurements of tissue oxygen levels in human bladder cancer in vivo. Eppendorf electrode measures have not been performed probably due to the difficulty of accessing the tumour through a cystoscope. MRI measures of in vivo hypoxia are promising (Cooper, Carrington et al. 2000) but not yet performed in bladder cancer. However hypoxia markers demonstrate that bladder cancers are hypoxic at the luminal surface and around areas of necrosis. Clinical studies correlate expression of CA IX with staining of bladder tumours by pimonidazole (Wykoff, Beasley et al. 2000). The latter is a



recognised marker of tumour hypoxia given to patients preoperatively and metabolised in hypoxic areas to an adduct that can be detected histochemically. In superficial bladder cancer CA IX is also coexpressed with VEGF (Wykoff, Beasley et al. 2000). The urine in the bladder is hypoxic and this luminal localisation of hypoxia induced genes in cancer has encouraged us to look for more such proteins that might be secreted into the urine. Since they are adjacent to the urine on the luminal surface they may be useful markers of recurrence as well as important mechanistically.

Bladder cancers have been analysed in a similar way that Gray analysed histological sections of squamous lung carcinomata. Double staining for CA IX as a surrogate marker for hypoxia and CD 34 as a marker of blood vessels in 12 bladder cancer cases showed that hypoxic areas were a mean distance of 80 $\mu$ m away from 54 separate blood vessels stained (standard deviation = 44 $\mu$ m) (Turner, Crew et al. 2002). This distance of 80 $\mu$ m fits well with the upper limit of the distance between normal capillaries. In the lungs the alveolocapillary barrier is only 1.5 $\mu$ m for speedy gas exchange, whereas the intercapillary distance in ventricular muscle is 25 $\mu$ m, in brain 40 $\mu$ m and in resting skeletal muscle 80 $\mu$ m.

HIF-1 $\alpha$  and HIF-2 $\alpha$  proteins were highly induced by hypoxia in four different bladder cancer cell lines (Jones, Fujiyama et al. 2001). All four cell lines (RT4, RT112, 253J and EJ28) upregulate HIF-1 $\alpha$  protein in hypoxia on western blot.

. Three of these cell lines also induce significant levels of HIF-2 $\alpha$  with

hypoxia, and also during exponential growth in normoxia (EJ28). However immunohistochemistry and in situ hybridization (Onita, Ji et al. 2002) of 67 cases showed that HIF-2 $\alpha$  was not found in bladder cancer cells or normal tissues. Staining of serial sections with CD68 confirmed that stromal cells staining for HIF-2 $\alpha$  were tumour associated macrophages and probably fibroblasts. No association with angiogenesis was found but they were significantly associated with perinecrotic areas.

VEGF measured in the urine or in the tumour, and platelet-derived endothelial cell growth factor (also known as thymidine phosphorylase) have been shown to be significant factors in mediating tumour angiogenesis in bladder cancer (O'Brien, Cranston et al. 1995). VEGF levels have been related to recurrence rates in superficial bladder cancer stage T1 or less (Crew, O'Brien et al. 1999). Thymidine phosphorylase activity is an independent prognostic factor in bladder cancer (Arima, Imazono et al. 2000), promotes VEGF expression (Brown, Jones et al. 2000), and promotes invasion (Jones, Fujiyama et al. 2002). Thymidine phosphorylase activity causes cellular oxidative stress which augments the levels of HIF1 produced by hypoxia (Brown, Streeter et al. 2005).

Although angiogenesis correlates with grade of bladder cancer, assessment of angiogenesis did not help predict recurrence in 80 cases of superficial bladder cancer (Sagol, Yorukoglu et al. 2001).





### 1.8.2 Hypoxia, angiogenesis and invasive bladder cancer

Angiogenesis is a prognostic factor independent of stage and grade in invasive bladder cancer and this correlation has been found in multiple studies (Dickinson, Fox et al. 1994; Bochner, Cote et al. 1995; Jaeger, Weidner et al. 1995; Inoue, Slaton et al. 2000). The reason for this correlation is not clear but one hypothesis is that bladder tumours with high microvessel counts also have high levels of hypoxia and necrosis. There is good evidence from a study of 109 breast tumours (Leek, Landers et al. 1999) and 56 gliomas (Chan, Leung et al. 1998) that microvessel density correlates with necrosis. Tumours with high levels of angiogenesis are not necessarily well oxygenated. Blood vessels of tumours are not thought to be as functionally competent as their normal counterparts. They are known to be more permeable than normal blood vessels. Furthermore microvessel counting by the commonly used Chalkley method involves assessing angiogenesis at vessel hotspots, which may exist between areas of hypoxia and necrosis. Perinecrotic areas of tumours are known to be hypoxic and secrete VEGF boosting the VEGF secretion already occurring inside tumours due to growth factor stimulated synthesis of HIF-1 $\alpha$ . Presence of hypoxia and necrosis may be the primary reason why some invasive bladder tumours have high levels of angiogenesis.

The colocalisation of CA IX and VEGF near the luminal surface and in perinecrotic areas in bladder cancer suggests the mechanism for the upregulation of VEGF in bladder cancer is hypoxia. However the increased expression of eukaryotic translation initiation factor eIF-4E was also

correlated with VEGF protein: mRNA ratios in superficial and invasive bladder cancers (Crew, Fuggle et al. 2000). eIF-4E affects the rate of translation of a subset of mRNAs within the cell including HIF-1 $\alpha$  (see figure earlier). eIF-4E is downstream of growth factor signalling pathways. This suggests that VEGF may be upregulated in bladder cancer by growth factor stimulation of HIF-1 $\alpha$  protein synthesis and not merely by hypoxia. Many of the genetic changes, which are known to increase HIF synthesis, occur in bladder cancer. 50% of invasive bladder cancers have p53 mutation, 30% have lost PTEN, and up to 45% have H-ras mutation. 49% also stain strongly for EGF receptor (EGFR). (Neal, Sharples et al. 1990; Brandau and Bohle 2001). Autocrine growth stimulation involving HIF-1 may also occur in bladder cancer since HIF-1 transcriptionally activates TGF  $\alpha$  which binds to EGFR.

### 1.8.3 Hypoxia and the selection of malignant cells with an aggressive phenotype in invasive bladder cancer

Lack of oxygen at the centre of tumours induces cell death and Vogelstein likened (Kinzler and Vogelstein 1996) the inside of solid tumours to a scene from Dante's Inferno with cells being born amidst the decaying remains of their ancestors. In such a hypoxic/necrotic environment cells that can resist death by hypoxia will be selected out. Hypoxia has been shown to induce p53 protein in non-mutant cells which may lead to apoptosis (Graeber, Peterson et al. 1994). In hypoxia therefore a p53 competent cell will be at a selective



disadvantage. Cycles of hypoxia and normoxia have been shown to select for p53 mutant cells in vitro (Graeber, Osmanian et al. 1996). p53 mutations are commonly found in invasive bladder cancer, are independently prognostic, (Esrig, Spruck et al. 1993) (Chatterjee, Datar et al. 2004) and show a significant association with angiogenesis (Bochner, Esrig et al. 1997). In superficial bladder cancer p53 mutations are rare, several studies have failed to find prognostic value for p53 independent of tumour stage and grade, and there is no correlation with angiogenesis (Reiher, Ozer et al. 2002). This suggests that p53 mutation and angiogenesis in invasive bladder cancer may be linked. A directly causal relationship seems unlikely when 27% of tumors that showed no evidence of p53 alterations exhibited high microvessel counts, and 26% of tumors with evidence of p53 alterations had low microvessel counts (Bochner, Esrig et al. 1997). A study in superficial bladder cancer also found a weak association between p53 status and microvessel counts (Reiher, Ozer et al. 2002). Furthermore p53 status modulation in normal urothelial and bladder cancer cells does not affect VEGF or thrombospondin-1 (TSP-1) levels (Reiher, Ivanovich et al. 2001). Upregulation of VEGF and loss of TSP-1 are thought to be the prime regulators of angiogenesis in bladder cancer. The development of hypoxia and necrosis in invasive bladder cancer however could cause the selection of p53 mutants and angiogenesis.

As previously mentioned one study of differentiated cells (murine embryonic fibroblasts and splenic B lymphocytes) has shown that loss of HIF-1 $\alpha$  by



knockout abolished hypoxia-induced growth arrest and did this in a p53 independent fashion (Goda, Ryan et al. 2003). Hypoxia caused an increase in the cyclin-dependent kinase inhibitor p21, and it was also found that hypophosphorylation of retinoblastoma protein in hypoxia is HIF-1 $\alpha$  dependent. P53, p21 and retinoblastoma protein have all been shown to have prognostic value in invasive bladder cancer, an effect that is additive, (Chatterjee, Datar et al. 2004) such that if all three mutations were present 5 year survival was only 8%, if no such mutations are present 5 year survival was 70%. Because all three of these proteins normally interact with HIF-1 $\alpha$ , it is highly likely that these mutations represent evolution within the tumour of mutations that can resist hypoxic growth arrest and/or death. It follows that the hypoxic and necrotic environment and downstream pathways of HIF-1 $\alpha$  in the bladder may be highly relevant to the evolution of an aggressive phenotype in bladder cancer.

### 1.9 Gene expression profiling of hypoxia pathways

DNA chips or microarrays are a sensitive and comprehensive way of investigating global gene expression at the RNA level but are not without pitfalls depending on their method of production and quality assessment (Knight 2001). The first application of a two-colour fluorescence hybridization scheme for expression profiling was carried out on a study of 45 genes of the flowering plant, *Arabidopsis thaliana* (Schena, Shalon et al. 1995). Several groups



have used Genomic approaches to screen for genes upregulated by hypoxia and/or VHL deficiency. Early arrays screened less than 1000 genes in cell lines derived from foetal hepatic cells, hepatocellular carcinoma, and squamous cell carcinoma of the cervix and pharynx (Koong, Denko et al. 2000; Fink, Ebbesen et al. 2001; Scandurro, Weldon et al. 2001). Later arrays have screened progressively larger numbers of genes in cell lines from glioblastoma, pancreatic cancer and renal cell carcinoma (Lal, Peters et al. 2001) (Yoon, Buchler et al. 2001) (Wykoff, Pugh et al. 2000). Untransformed cells; keratinocytes, cervical cells and stromal fibroblasts (Denko, Fontana et al. 2003) and renal proximal tubule cells (Jiang, Zhang et al. 2003), and their transformed counterparts have also been studied.

The results of all these studies conclude:

1. There is HIF-1 target genes, such as vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT1) and phosphoglycerate kinase 1 (PGK1) that are induced by hypoxia in most cell types.

2. There is significant heterogeneity in the transcriptional response to hypoxia between different cell types. Not only between different cancer types but also among cell lines that have been derived from cancers of the same histopathological type (e.g. 786-0 cells and RCC4 cell in renal cancer (Wykoff, Pugh et al. 2000), and among transformed and non-transformed cells of the same tissue type (Denko, Fontana et al. 2003) (Jiang, Zhang et al. 2003). In fact renal carcinoma cells seem to have evolved an alternative hypoxia signalling pathway compared with normal cells.



3. Hypoxia-induced pathways demonstrate different degrees of VHL-dependent and VHL-independent induction. For example for the renal cell line RCC4, the majority but not all genes induced by VHL loss investigated on northern blot were also regulated by oxygen (8 of 12 (Wykoff, Pugh et al. 2000)). However in the renal cell line 786-0, using a genome approach, the vast majority (122 of 160) of genes induced by hypoxia in 786-0 VHL+ cells were not upregulated in the same cells by mutating VHL (Jiang, Zhang et al. 2003). However this may be because 786-0 cells do not express HIF-1 $\alpha$ .

For HIF-1 target genes therefore expression is induced by hypoxia in a cell-type-specific manner. HIF-1 alone cannot account for this cell-type-specific gene expression. Rather, it is the functional interaction of HIF-1 with other transcription factors that determines the subgroup of HIF-1 target genes that is inactivated in any particular hypoxic cell. Similar findings have been reported for p53-dependent gene expression (Yu, Zhang et al. 1999).

#### 1.10 Physiological and pathological hypoxia in the non-cancerous bladder

Research into the tissue-specific hypoxia response of urothelium may also be of relevance to noncancerous bladder physiology and pathology. Normal physiologic bladder wall is probably not hypoxic as shown by negative CA IX staining in humans, blood flow measurements using fluorescent microspheres in rats and Doppler laser probes in pigs (Turner, Crew et al. 2002)



(Ghafar, Shabsigh et al. 2002) (Greenland and Brading 2001). However the normal bladder does experience periods of hypoxia on voiding. Studies of experimentally obstructed rat, dog and rabbit and pig bladders using implanted laser Doppler fibres show that on normal voiding blood flow to the bladder is temporarily reduced to an ischaemic level. In obstructed bladders this ischaemic period is significantly prolonged and this insult may be the cause of biochemical and neuronal alterations seen in such bladders. (Zhao, Wein et al. 1995; Greenland and Brading 2001; Ghafar, Shabsigh et al. 2002). One well controlled study which measured blood flow in rats and rabbits utilising a fluorescent microsphere technique and measured hypoxia by immunostaining for an injected Hypoxyprobe-1 (a chemical probe for hypoxia) showed that in the 1-2 weeks after obstruction the blood flow to the bladder wall and the bladder weight increases along with the appearance of hypoxia. This hypoxia is shown to affect first the mucosa, progressing through submucosa, lamina propria and into smooth muscle by 2 weeks of obstruction and continued for the four weeks duration of the experiment (Ghafar, Shabsigh et al. 2002). This supports the idea that hypoxia can drive angiogenesis and that such pathological angiogenesis may not be enough to correct the original hypoxia. In rabbits in such experiments a third phase of bladder decompensation is seen, where bladder weight reduces and scar tissue replaces the damaged muscle.



1.11 Bladder cancer trials of potential relevance to this thesis at the time of thesis submission (May 2008)

<b>UK Trials</b>		
Name of trial	Reference or Code of trial	Note
Phase II ARCON accelerated radiotherapy carbogen and nicotinamide.	(Hoskin, Saunders et al. 1997; Hoskin, Rojas et al. 2005).	Completed trial, morbidity and treatment response no worse with ARCON compared with historical controls of conventional radiotherapy. Phase III trials underway in the UK to formally assess cancer treatment efficacy. UK trial (Mount Vernon Hospital)
Trial of radiotherapy vs intravesical BCG or observation for T1G3 bladder cancer +/- CIS.	(Harland, Kynaston et al. 2007)	Completed trial conclusion – radiotherapy is not superior to more conservative treatment for T1G3 ‘high risk’ superficial bladder cancer.



Standard or reduced volume radiotherapy, with or without chemotherapy for localised invasive bladder cancer	BC2001	Starts 03/08/2001 Ends 30/04/2008 Birmingham UK
A trial comparing surgery with treatment that may help people to keep their bladder after invasive bladder cancer	SPARE	Starts 01/07/2007 Ends 30/06/2014 A pilot study and will recruit 110 people.
Antioxidant dietary supplements Selenium and vitamin E	SELENIB	
Suramin intravesical for superficial bladder cancer	Phase I (Ord, Streeter et al. 2005)	*See note at end



<b>USA trials</b>		
Name of trial	Reference or Code of trial	Note
Trial of Single-Dose Intravesical EOquin® as a Surgical Adjuvant Instilled in the Early Postoperative Period in Patients Undergoing TUR-BT	SPI-611 NCT00461591 Phase III	EOquin® is a bioreductive prodrug activated by reductase enzymes that are overexpressed by cancer cells to form the active alkylating agent.
Monoclonal antibody inhibitors of VEGF (USA)		
Cisplatin, Gemcitabine and Bevacizumab in Combination for Metastatic Transitional Cell Cancer	Phase II  HOG GU04-75 NCT00234494	



<p>Cisplatin, Bevacizumab, and Gemcitabine Followed by Surgery, Bevacizumab, and Paclitaxel in Treating Patients With Locally Advanced Nonmetastatic Bladder Cancer That Can Be Removed By Surgery</p>	<p>Phase II</p> <p>MUSC-AVF-3312  MUSC-HR-15537,  GENENTECH-AVF-3312,  NCT00268450, MUSC-CTO-100892</p>	
<p>Trial of Gemcitabine, Carboplatin, and Bevacizumab in Chemotherapy Naive Patients With Advanced/Metastatic Urothelial Carcinoma</p>	<p>Phase II</p> <p>06-006  NCT00588666</p>	
<p>Sorafenib and Bevacizumab in Treating Patients With Refractory, Metastatic, or Unresectable Solid Tumors</p>	<p>NCI-05-C-0022  6750, NCI-6750,  NCT00098592  Phase I</p>	



Small molecule inhibitors of tyrosine kinase		
Sunitinib in Treating Patients With Progressive Metastatic Transitional Cell Cancer of the Urothelium	Phase II MSKCC-06081 memorial Sloane Kettering NCT00397488	
Sunitinib in Treating Patients With Locally Advanced Bladder Cancer	Phase II CASE-24806 CASE 24806, PFIZER- GA6180CV	
VEGF Trap in Treating Patients With Recurrent, Locally Advanced, or Metastatic Cancer of the Urothelium	Phase II CCC-PHII-76	
Small molecule inhibitor of raf kinase and VEGF receptor (Sorafenib)		
Trial of Gemcitabine, Carboplatin, and Sorafenib in Chemotherapy-Naive Patients With Advanced/Metastatic Bladder Carcinoma	Phase II  0609001823 NCT00461851	



The use of VEGF inhibitors and tyrosine kinase inhibitors have had promising results and Professor R K Jain at Harvard medical school has postulated that VEGF inhibitors may work synergistically with chemotherapy not by rendering the tumour anoxic and so killing tumour cells as first suggested by Judah Folkman but by repairing tumour blood vessels made leaky by excess VEGF (which used to be called vascular permeability factor), reducing interstitial pressure and increasing the penetration of chemotherapeutic drugs and oxygen vital for radiotherapy. (Jain, Duda et al. 2006; Jain 2008)

Since publication of this trial in 2005, there have been two papers one based on cell line work and one based on a mouse model of bladder cancer which strongly suggest that drugs to inhibit VEGF and or FGF will work synergistically with standard intravesical treatment mitomycin. The effect of adding such drugs to mitomycin halved the IC50 in vitro and increased absolute tumour shrinkage in the mouse model by 50% more. This is a very promising line of research. (Krause, Forster et al. 2005; Xin, Lyness et al. 2005)



## 1.12 Rational and objectives

The hypothesis behind this thesis was proposed by an article entitled 'Life (and death) in a malignant tumour.' by Dr B Vogelstein MD of Johns Hopkins who has been credited with the discovery of the multistep pathway of carcinogenesis in colorectal cancer (Kinzler and Vogelstein 1996).

In this article it was suggested that as a cancer grows it becomes hypoxic and the lack of oxygen at the centre of tumours induces cell death, and that it is the adverse internal environment of the tumour that results in the selection out of individual cells with a locally aggressive and metastatic phenotype.

Hence the objectives of the thesis were:

1. Investigate the transcriptional response of an aggressive (grade three) bladder cancer cell line to severe hypoxia to find out how it is adapted to the hypoxic environment. And to validate the findings of this microarray in a panel of cell lines and untransformed cells for comparison. (Chapter Three)
2. Investigate genes of potential in vivo relevance by cross-referencing the hypoxia-regulated transcriptome of the aggressive cell line (from 1) with an in vivo microarray of primary bladder cancer (39 cases data courtesy of E Streeter). (Chapter Four)
3. Relate transcriptional changes of genes to histopathological evidence of necrosis and hypoxia and to levels of any secreted proteins in the urine of bladder cancer patients. (Chapter Four and Chapter Five)
4. Investigate whether or not cancer cell lines are better at surviving severe hypoxia than a normal untransformed cell type (Chapter Five)
5. Investigate whether or not a variety of markers of a hypoxic or



necrotic tumour environment is or is not associated with an independently worse prognosis by multivariate analysis of a series of high grade and invasive bladder cancers. (Chapter Six) Also for superficial bladder cancer (Appendix VI).

6. Investigate the transcriptional response of normal urothelium to hypoxia to see if it does differ in anyway from that in cancer cells. And also to see how other normal cell types (e.g. peripheral blood monocytes and T cells) differ in their response to hypoxia.



# **Chapter Two**

## **Materials and methods**



## 2.1 Cell Culture

### 2.1.1 Bladder cancer and HUVE cells

Bladder cancer cells were maintained on polystyrene dishes in Dulbecco's Modified Eagles Medium (DMEM) and HEPES (EJ28,253J, 2T10) or RPMI 1% (RT112) supplemented with 1 mM glutamine (GibcoBRL, UK), 0.002mg/ml puromycin and 10% fetal bovine serum (FBS) (Globepharm, UK). Cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Cells were plated at a minimum density of 5000cells/cm<sup>2</sup> to achieve exponential growth. Cells were passaged by washing with phosphate buffered saline (PBS: 150 mM NaH<sub>2</sub>PO<sub>4</sub> and 150 mM NaCl), detached with trypsin (4 mg/ml trypsin in PBS).

Human umbilical vein endothelial cells (HUVE cells) were grown on plates coated with 0.2% gelatine maintained in MCDB 131 (Invitrogen) containing 20% fetal calf serum (Sigma-Aldrich), 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 5 IU/ml heparin, and 50 µg/ml endothelial cell growth supplement (Sigma, Dorset, UK). HUVE cells were purchased from Clonetics BioWhittaker (Wokingham, Berkshire, UK) and were used up to the 8th passage.

Hypoxia experiments were performed with the cells at 80-90% confluence. Cells were exposed to hypoxia by incubation in a humidified atmosphere of 0.1% O<sub>2</sub>, 5% CO<sub>2</sub> and the remainder N<sub>2</sub> in a NAPCO 7301 incubator (Precision Scientific, USA).

### 2.1.2 Normal Urothelial Culture

A strip of ice cooled fresh normal human ureter from a patient undergoing nephrectomy for renal cell cancer was placed in stripper medium (0.1% EDTA, 10mM HEPES, Trasylol 500,000 IU in 500ml Hanks Balanced Salt Solution minus calcium and magnesium) for maximum 6 hours at 4C. Over 6 hours and the cells tended to die or become infected. The urothelial lining was then



scraped off, digested with collagenase for 30 mins and plated on collagen coated plates (Becton Dickinson, NJ) in Keratinocyte serum free medium with additives (25mg Bovine Pituitary Extract, 2.5ug Epidermal Growth Factor in 500ml medium (Gibco, Invitrogen) and Cholera toxin 30ng/ml to prevent fibroblast growth). Penicillin 5000U/ml and Streptomycin 5mg/ml at 1:100 and Gentamicin 5ug/ml were also necessary in the medium. Cells were passaged five times with EDTA 0.1% and trypsin versene before being exposed to hypoxia and RNA extracted as above. Morphology of the cultured urothelia fitted well with previous descriptions (Mackillop, Bizarri et al. 1985). In order to confirm the urothelial origin of the cultured cells cytopins were performed and cells stained for URO5 antibody, Cytokeratin 7; and Cytokeratin 20 confirming urothelium as the cell of origin (Chapter 4) (Cordon-Cardo, Bander et al. 1984; Dubeau and Jones 1987; Schaafsma, Ramaekers et al. 1989).

### 2.1.3 Guards against cell line contamination, bladder cancer cell lines used and their provenance.

Cross-contamination of cell lines continues to be a widespread problem despite the fact that techniques have been available for researchers to check the identity of their cell lines. Cross-contamination can occur in one of two ways: Clerical error (mislabelling frozen stocks or specimens) or poor culture technique when for some reason two cell lines get into the same culture. Cross contamination can be detected by karyotyping, isozyme analysis, HLA (human leucocyte antigen) typing, DNA fingerprinting (restriction fragment length polymorphism analysis), or more recently and conveniently DNA profiling of STR (short tandem repeats). (Masters 2000; Masters 2002)

The widespread problem of cross contamination of human cell lines was demonstrated in one large study of 252 cell lines studied by cytogenetics (N markers present and retained) and DNA fingerprinting. Cross-contamination occurred in 18%. 45 cell lines were contaminated with other cell lines, mostly human (in 42 cases). Bladder cancer cell line T24 was among the worst with 4 different identifiable cross contaminants none of which were even



bladder cell lines (MacLeod, Dirks et al. 1999).

EJ was the cell line used in this paper for the main hypoxic microarray analysis. This cell line was supplied by the CRUK (Cancer Research UK) facility where authenticity is verified by DNA profiling. However by the criteria of HLA-A-B-C typing and isozyme analysis EJ cells were shown in 1983 to be T24 cells (O'Toole, Povey et al. 1983). Both cells share HLA type A1,A3/B18/Cw5. Analysis of 15 different isozyme subtypes by starch gel electrophoresis in T24 were identical to that in EJ a combination that would be expected in less than 1% of the population. Bladder cancer cell lines RT4 and RT112 (also used in this thesis) were clearly distinct from T24 by HLA typing (O'Toole, Tiptaft et al. 1982).

T24 cells were established from a Swedish female patient C M aged 82 with long diagnosed bladder cancer. The tumour was grade III histologically when processed and cultured in February 1970 in Stockholm. Full characterisation was performed 18 months later with a generation time of 19 hours based on 3H-thymidine labelling. Electron microscopy, cytology and karyotyping were also performed along with implantation into hamsters to confirm their malignant character (Bubenik, Baresova et al. 1973). In this same paper two other cell lines T15 and T22 were cultured for a short time but were not established in long-term culture.

253J was established from a multiple TCC grade IV specimen in 1974 (Elliott, Cleveland et al. 1974). After the demonstration that many cell lines were contaminated with HeLa cells in 1974 by Walter Nelson-Rees, karyotyping in 1977 proved that 253J had not been cross contaminated with HeLa cells (Elliott, Bronson et al. 1978).

RT4 was established and described in 1970 (Rigby and Franks 1970).



#### 2.1.4 Extraction and purification of Peripheral Blood Monocytes (PMBC) and T-cells (performed by Sarah Harten, Hammersmith Hospital, London)

Blood (500mL) was taken from a willing volunteer (Dr Peter Hill) into tubes with heparin. 1ul heparin was used per ml of blood. Blood was then mixed 1:1 with serum free medium (RPMI-sigma or AIM-V lymphocyte medium from GIBCO Invitrogen) and carefully layered 1:1 over a lymphoprep (Axis Shield , Oslo) gradient. Tubes were then centrifuged at 2000rpm for 30 minutes with no brake. After centrifugation the PBMC interface layer was harvested, resuspended in medium and washed 2/3 times at 1800rpm. After washing the PBMCs were resuspended in a suitable volume of medium and placed in T75 plastic flasks (Helena) and incubated at 37 degrees temperature for 45 minutes. After this incubation the medium containing non-adherent cells was removed and the flask washed to remove any further non-adherent cells. The non-adherent cells were then spun down at 1800rpm and resuspended in a small volume (500ul). Selection antibodies were then added to the non adherent cells; anti CD8, CD19, CD33, Cd14, CD16 and CD56 (Diaclone and Caltag). The cells were incubated with the antibodies for 20 minutes at 4 degrees. After this incubation the cells were washed again (as above) and anti mouse-Ig Dynabeads were added (Dynal), amount depending on the number of cells at this stage (assuming four beads per CD4+ cell). Cells were incubated with beads for 45 minutes with rotation, again at 4 degrees. Finally beads were removed from the solution using a bead magnet. The cells were washed once more and resuspended in a suitable volume. CD4 cells could then be counted and purity assessed by FACS. Purity was 90% or above. Activation of cells was done at this stage, by addition of CD3/CD28 beads, amount depending on the number of CD4+ cells. Cells were exposed to hypoxia by placing in a Concept 1000 Ruskin hypoxia workstation set to 1% O<sub>2</sub> immediately after activation, for 16 hrs.

## 2.2 RNA



### 2.2.1 Extraction and Storage

Both normoxic and hypoxic cell plates were washed in ice-cold PBS and placed on ice immediately on being removed from the incubator. RNA was extracted using 7ml Trireagent (Sigma, U.K.) per 75 cm<sup>2</sup> dish. Concentrations of RNA and purity were assessed on a mass spectrometer. RNA was stored initially in 75% alcohol at –80°C (Sanger Array experiments) but then latterly for convenience in DEPC water at –80°C. Tumours samples were collected with informed consent and approval from the Local Regional Ethics Committee. Samples were rapidly defrosted and homogenised in ice-cold Tri-Reagent (Sigma, UK). Phenol-chloroform based extraction of RNA and protein was performed according to the method of Chomczynski (Chomczynski 1993).

### 2.2.2 Purity and Integrity

RNA purity was assessed for the Sanger Arrays by ultraviolet spectrometry and integrity by running 1µg of total RNA on a 1% agarose / Tris-borate-EDTA (TBE) gel prepared with 0.002% ethidium bromide. All samples were checked and any samples not showing two clear bands were excluded. Latterly for the Affymetrix arrays purity and integrity was assessed more thoroughly using the RNA 6000 Nano Assay in an Agilent 2100 bioanalyser (Agilent Technologies). Again any samples not showing two clear peaks of ribosomal RNA with 28S taller than 18S were not used.

## 2.3 Sanger Microarrays

### 2.3.1 Chips and Analysis

Hver 1.2.1 cDNA microarrays were obtained from the Microarray Consortium, Sanger Centre, Hinxton, Cambridge. These contain 9,932 spotted samples of cDNA originating from the I.M.A.G.E. collections of the Human Genome mapping Project and Research Genetics, Hinxton, UK, and 468 chromosome 22 gene-specific PCR products from the Sanger Centre, representing approximately 6000 human genes in total. The PCR products used are between 0.2 and 1kb in length. A Biorobotics MGII split pin arrayer cDNA spots of



between 150 $\mu$ m and 250 $\mu$ m in size, with the cDNA binding covalently to the glass slide via a 5-prime aminolink incorporated into the first strand cDNA during PCR. Complementary second strand cDNA removed by subsequently boiling the slides and washing in ammonium solution, to leave single strand cDNA ready for hybridisation. The use of bacterial control cDNA distributed regularly throughout the slide allows sensitivity to be monitored over the whole surface. The limit of sensitivity estimated to be one cDNA molecule in 500,000. A GSI Lumionics Scan Array 400 array-reader with Scanarray and Quantarray<sup>®</sup> software was used to analyse the slides. The analysed data was imported into GeneSpring<sup>®</sup> software (Silicon Genetics, USA). Data was normalised internally by region on each chip (regional normalisation) to compensate for systematic variation over the surface of the array. The mathematical Lowess correction was applied to allow for non-linear fluorescence characteristics of the dyes.

### 2.3.2 Generation of Fluorescently Labelled Single Stranded cDNA and Competitive Hybridization of Labelled Single-stranded cDNA

Between 25-50 $\mu$ g total RNA in 75% ethanol was mixed with 1 $\mu$ l of the bacterial RNA cocktail and precipitated by the addition of 1/40<sup>th</sup> volume of 3M sodium acetate and storage at -70<sup>o</sup> C for 30 minutes. The RNA pellet was washed in 70% ethanol and dried. The fluorescent dyes Cy3 and Cy5 come readily attached to the pyrimidine cytosine and this is mixed with the remaining 3 nucleotides along with 30  $\mu$ g of the total RNA sample and reverse transcriptase (Superscript II 200U/ $\mu$ l Gibco BRL). Selection of mRNA for reverse transcription is achieved by initially priming with anchored oligo-dT<sub>17</sub> primers (17 thymine with a terminal adenine, guanine or cytosine) that hybridises with the poly-A tail found on mRNA. Reverse transcription is performed at 42<sup>o</sup>C using Superscript II reverse transcriptase (Gibco), with 60% of the added cytosine nucleotides labelled with Cy3 or Cy5 dyes (Amersham), thus fluorescence was incorporated into the reverse transcribed cDNA. At the end of this period the RNA hydrolysed by incubation at 70<sup>o</sup>C for 20 mins. Filtration to remove nucleotides and short oligomers performed using AutoSeq G-50 columns. The resulting single<sub>71</sub>



stranded cDNA was mixed with a second labelled cDNA from 30µg RNA sample 2 along with PolyA (2µg/ml) and cot1 DNA (1µg/ml). This mix was precipitated in sodium acetate at -70C for 20 mins and the pellet formed resuspended in hybridisation buffer (5x SSC, 6x Denhardt's solution, 60mM Tris HCL pH7.6, 0.12% sarkosyl, 48% formamide). This mix applied to the microarray slide. This slide placed in a hybridisation chamber humidified with hybridisation buffer. Incubation occurred at 47°C for 12-24 hours. The slides then placed in a slide rack and washed in darkness in 3 different solutions containing SSC and 0.1% SDS on a tilting table for 1 hour. Slides dried by centrifugation.



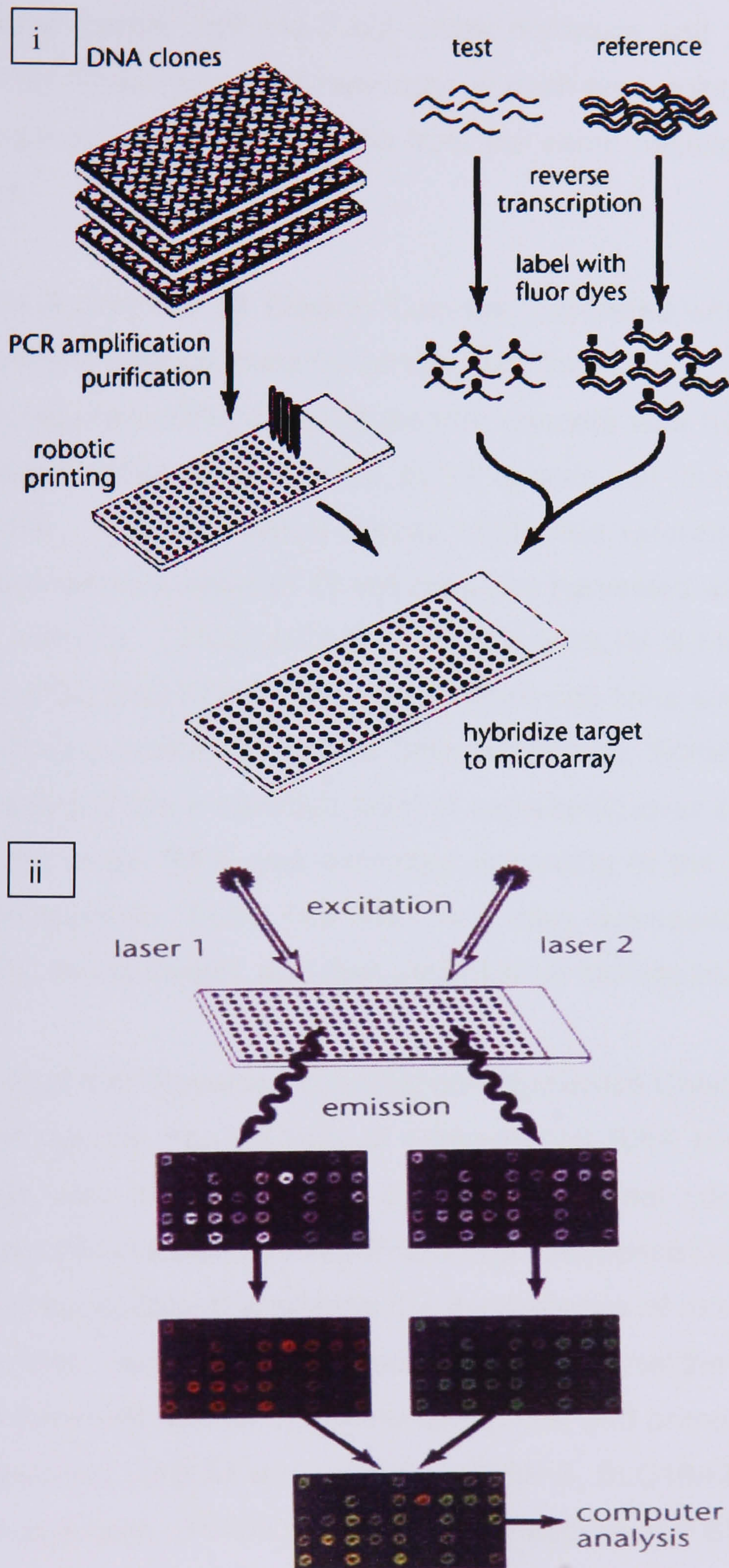


Figure 2.1 Sanger cDNA microarrays.

(i) Microarray slides are constructed by a split-pin microarrayer, which applies spots of cloned cDNA 200 microns in diameter, 50 microns apart. A total of 9932 elements for human genes, with 728 control points are used. cDNA is generated from sample and control RNA by reverse transcription, incorporating Cy3 or Cy5 fluorescently labelled cytidine. cDNA from sample and reference RNA is competitively hybridised overnight onto the arrays.

(ii) The hybridised array is passed through a laser scanner, with the fluorescence spectrum due to the two dyes allowing distinction between paired sample and reference cDNA.



### 2.3.3 Analysis of Genes Upregulated by Hypoxia (0.1%) in Cell Line EJ28

Using Sanger cDNA chips we analysed gene expression in an aggressive bladder cancer cell line EJ28 under normoxia and 16 and 24 hours hypoxia (0.1%). There were four replicates at each time point grown in separate plates compared with normoxic cells from the same passage harvested at the same time.

### 2.3.4 Analysis of 38 Bladder Cancers Compared with Cancer Cell Line Panel (work and data courtesy Ed Streeter MD thesis)

The labelled cDNA from 38 bladder cancers was hybridised to the chip with labelled cDNA from a panel of 11 cancer cell lines from different types of cancer. This provided a strictly controlled reference for each competitive hybridisation, a panel of 11 cell lines was harvested whilst in exponential growth in culture (SK23, NCI-H1385, NCI-H69, IM-9, MCF-7, OVCAR-3, MOLT-4, HEPG2, SW620, HT1376, W38). These cell lines were selected according to previously published work at Stanford (Perou, Sorlie et al. 2000), in order to reliably provide a constant level of expression over the vast majority of genes on the array. RNA was extracted according to the method of Chomczynski (Chomczynski 1993). The RNA was then resuspended in equal amounts in DEPC-treated water, and then aliquoted for storage at  $-80^{\circ}\text{C}$ .

### 2.4 Real time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

After reverse transcription of experimental RNA (SuperScript II, Invitrogen) cDNA was diluted down to 0.5 ug/ul and final concentrations checked on spectrophotometer. The ABI Prism 7700 Sequence Detection System was used which is capable of analysing the fluorescence of more than one dye in a tube at a time. Sequencher software used to analyse the results and set baseline and threshold values. A commercial probe and primer set supplied by Applied Biosystems (U.S.A) was used for IGFBP-3, SLC16A3, RNase4 MARCKS and Peroxiredoxin (PRDX5). The sequences of IGFBP3 and SCL16A3 were checked by BLAST search on Ensembl and NCBI databases (Table 2.1).



Both probes hit the gene of interest, the SLC16A3 probe was seen to hit the 3' untranslated region of the gene.

IGFBP3	5'-GCGCCGCCAGCTCCAGGAAATGCTA-3'
SLC16A3	5'-CCCCTTCGGGAGGGCAAACCTCCTGGA-3'
RNAse4	5'-TCTGAGGCCGAGGGCACCTCTAAGAT-3'
MARCKS	5'-AGCGAACGGACAGGAGAATGGCCAC-3'
PRDX5	5'-CCCCAATCAAGGTGGGAGATGCCAT-3'

Table 2.1 Sequences used for Real Time Probes

The probes of the genes of interest had FAM reporter dyes. Beta-2-microglobulin was chosen as a control gene as this is not known to be upregulated by hypoxia in the literature and was not upregulated by hypoxia in the cell line on the array. Subsequently RTPCR demonstrated no change under hypoxia. This gene was also sold as a control probe with primers limited which in theory prevents the relative abundance of this control gene affecting the assay of other genes of interest when used in the same tube. The relative technique of real time analysis was used looking at changes in cycle threshold between 3 normoxic replicates and 3 hypoxic replicates. Two to the power of the difference in average cycle threshold normoxia to hypoxia calculated the fold change. Each individual result was required to be within 0.5 cycles of each of the 3 replicates otherwise it was discarded. In addition if the cycle threshold of the 3 replicates of the control gene in normoxia differed significantly from hypoxia the result was also discarded. The most likely reason of this would be different starting amounts of cDNA in the normoxic and hypoxic master mix. This method is in contrast to other investigators that have used a varying control to normalise the probe to the amount of cDNA in each tube (Girault, Lerebours et al. 2002). PCR reactions were performed with the gene of interest and control probe in the same and separate tubes to be sure the probes were not interacting.



## 2.5 Tissue Arrays

### 2.5.1 Tumour Material and Array Construction

Tissue arrays are accepted forms of rapidly assessing antibodies on a panel of tumours. The cores are significantly representative of the whole block so long as at least 2 cores are used and regional differences in staining are taken into account when selecting cores (Camp, Charette et al. 2000; Nocito, Bubendorf et al. 2001).

98 consecutive cases of radical cystectomy for primary invasive transitional cell carcinoma of the bladder were retrieved with ethical approval and made into an array (coded TA 20 and TA 29). To gain an accurate assessment of necrosis in the three cystectomy stage pT0 tumours, the previous transurethral resection specimens were reviewed and stained. A tissue array was also made from paraffin sections of the 39 superficial and invasive cases analysed on the gene microarray (coded TA 19).

Whole sections of bladder cancer stained with haematoxylin and eosin (H&E) were reviewed under light microscopy. All representative tumour regions were encircled on stained slides by a consultant histopathologist (I.R.) specialising in urological cancer and one researcher (J.O). Carbonic anhydrase IX staining is known to be regional in bladder cancer and to occur more frequently around areas of necrosis (Wykoff, Beasley et al. 2000). We therefore selected two 1mm cores from representative areas of tumour and if any necrotic areas existed another 2 perinecrotic cores.

Cylinders with a diameter of 1.0mm were punched from the donor block and brought into a recipient paraffin block using a custom-made precision instrument (Beecher Instruments, Silver Springs, MD, USA). Up to 120 cores were placed in a single block in a standardized pattern with a core of tonsil tissue to mark the top left corner. Pairs of cores from the same tumour were placed in different array blocks. Sections of the resulting TMA blocks (5mm) were transferred to glass slides.



### 2.5.2 Staining and Scoring of Tissue Microarray (CAIX, HIF1 $\alpha$ , HIF2 $\alpha$ , NIP3)

For pre-staining slide preparation, all slides were heated at 60°C for 15 mins then dewaxed and rehydrated through citrate and graded alcohol (100% ethanol then 50% ethanol) and finally placed in 1xPBS for 5 mins. Endogenous peroxidase was blocked with hydrogen peroxidase from the EnVision kit (DAKO) for 5 mins. After any pre-treatment and primary antibody, secondary ChemMate (DAKO) polymer was added neat for 30 mins. Visualisation of staining was by DAB substrate. Slides were counterstained with haematoxylin before mounting in Aquamount (BDH, UK). CA IX: 10% normal human serum was applied in PBS for 15 mins to block. The CA IX antibody M75 (Pastorekova, Parkkila et al. 1997) (1:50) was applied for 30 mins at room temperature. HIF 1 $\alpha$ : Primary antibody ESEE122 (1:30) applied for 60 mins. Pressure cook pre-treatment at 120°C 15lbs/sq in, in Tris EDTA pH 9.0 for 3 mins. Triton X 100 0.2% in PBS for 10 mins. HIF 2 $\alpha$ : As HIF1a except antibody used was EP190. NIP3: 0.2% Triton X 100 0.2% in PBS 10 mins. 10% Normal Human Serum (NHS) in PBS. NHS tipped off slide. Rabbit polyclonal antibody to NIP3 described previously was (Ray, Chen et al. 2000) applied at 1:400 in 10% NHS 30min.

Scoring of whole H&E sections was done for necrosis at the time the cores were marked out (0=none, 1=comedo or <5mm microscopic necrosis, 2=>5mm or gross necrosis). CA IX scoring was based on the intensity and area of membrane staining and a score of 0-3 was given, if the majority of the core showed intense membrane staining a score of 3 was given. Scoring of tissue arrays was done separately by two people, scores were compared and an agreement reached. The scores from two or more cores were both averaged and the maximum score was taken. Results were then analysed using both scores.

### 2.5.3 Statistical Analysis

Statistical analysis of associations between markers and tumour characteristics employed Chi squared test and Fisher's exact test. Survival curves used



the method of Kaplan and Meier. When comparing two survival curves the logrank test was used. Two tailed t-tests were used; p-values of less than 0.05 were considered significant. If the logrank test showed a significant difference between groups then univariate and multivariate analysis was performed using nonlinear regression. This uses a statistical model to generate hazard ratios. Multivariate analysis was performed by a statistician (CH).

## 2.6 Urine Collection

Urine was collected with informed consent fresh from patients with histologically confirmed bladder cancer an average of 1 week before operation. Protease inhibitor cocktail (80µg 4-[2-aminoethyl]-benzenesulphonylfluoride (AEBSF), 200µg EDTA, 0.2µg leupeptin, 0.2µg pepstatin) was added and the sample spun at 3000g for 10 minutes to remove debris. The supernatant was aliquoted and stored at  $-70^{\circ}\text{C}$  until required. IGFBP-3 ELISA was performed using a commercial kit (Diagnostic Systems Laboratories). There were 71 superficial cancers, 18 invasive, 4 carcinoma-in situ, 30 patients who had previously had bladder cancer resected and were now clear, and 30 controls. Half of the controls were 43-80 years who had full investigations for haematuria with no bladder cancer found, the other half were medical student volunteers age 18-30 with no blood in the urine on dipstix.

## 2.7 Affymetrix Arrays

### 2.7.1 HG-U133 Target

The Human Genome U133 Set (HG-U133 A and HG-U133 B) is comprised of two microarrays containing over 1,000,000 unique oligonucleotide features (18 mm in size, oligonucleotide length 25mers) covering more than 39,000 transcript variants, which in turn represent greater than 33,000 of the best characterised genes. For each sequence represented in the array there are 11 probe pairs.



### 2.7.2 Oligonucleotide Probe Manufacture

Affymetrix oligonucleotide probes are manufactured in situ on the chip through a combination of photolithography and combinatorial chemistry using technologies adapted from the semiconductor industry. Manufacturing begins with a 5-inch square quartz wafer. Because quartz is naturally hydroxylated, it provides an excellent substrate for the attachment of chemicals by covalent bonding. Each array holds over 500,000 probe locations, or features, within 1.28 square centimetres. Each of these features harbours millions of identical DNA molecules. Probe synthesis occurs in parallel, resulting in the addition of an A, C, T, or G nucleotide to multiple growing chains simultaneously.

### 2.7.3 Generation of biotin-labelled cRNA

7-10 $\mu$ g total RNA at a minimum concentration of 1mg/ml was used per sample. Reverse transcription was performed using Superscript II and a T7-dT24 primer to yield ss-cDNA. (T7-dT24 sequence = 5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)<sub>24</sub>-3'). Second strand synthesis was completed using Superscript ds-cDNA synthesis kit (Gibco), followed by a DNA phenol-chloroform-isoamyl alcohol purification step to exclude residual proteins. The dscDNA was then used to transcribe biotin-labelled cRNA via a five hour incubation at 37°C with T7 RNA polymerase and biotin-labelled ribonucleotides (BioArray high yield RNA transcript labelling kit (Enzo)). RNA was purified using selective elution from a silica-gel based membrane (RNeasy mini kit (Qiagen)). This typically yielded 40-60 $\mu$ g cRNA. RNA fragmentation was performed at 94°C for 35 minutes in 1M Tris acetate pH8.1, 500mM KOAc, 150mM MgOAc. Fragmented cRNA was mixed with eukaryotic hybridisation controls (Gene Chip eukaryotic hybridisation control kit (ENZO)) (Figure 2.2).

### 2.7.4 Array Hybridisation

This was kindly performed by Tracy Chaplin, at St Bartholomew's Hospital,



London. Samples were incubated on Affymetrix HU133-A and B arrays for 16 hours at 45°C. Washing, and scanning were also performed by the microarray facility in London.



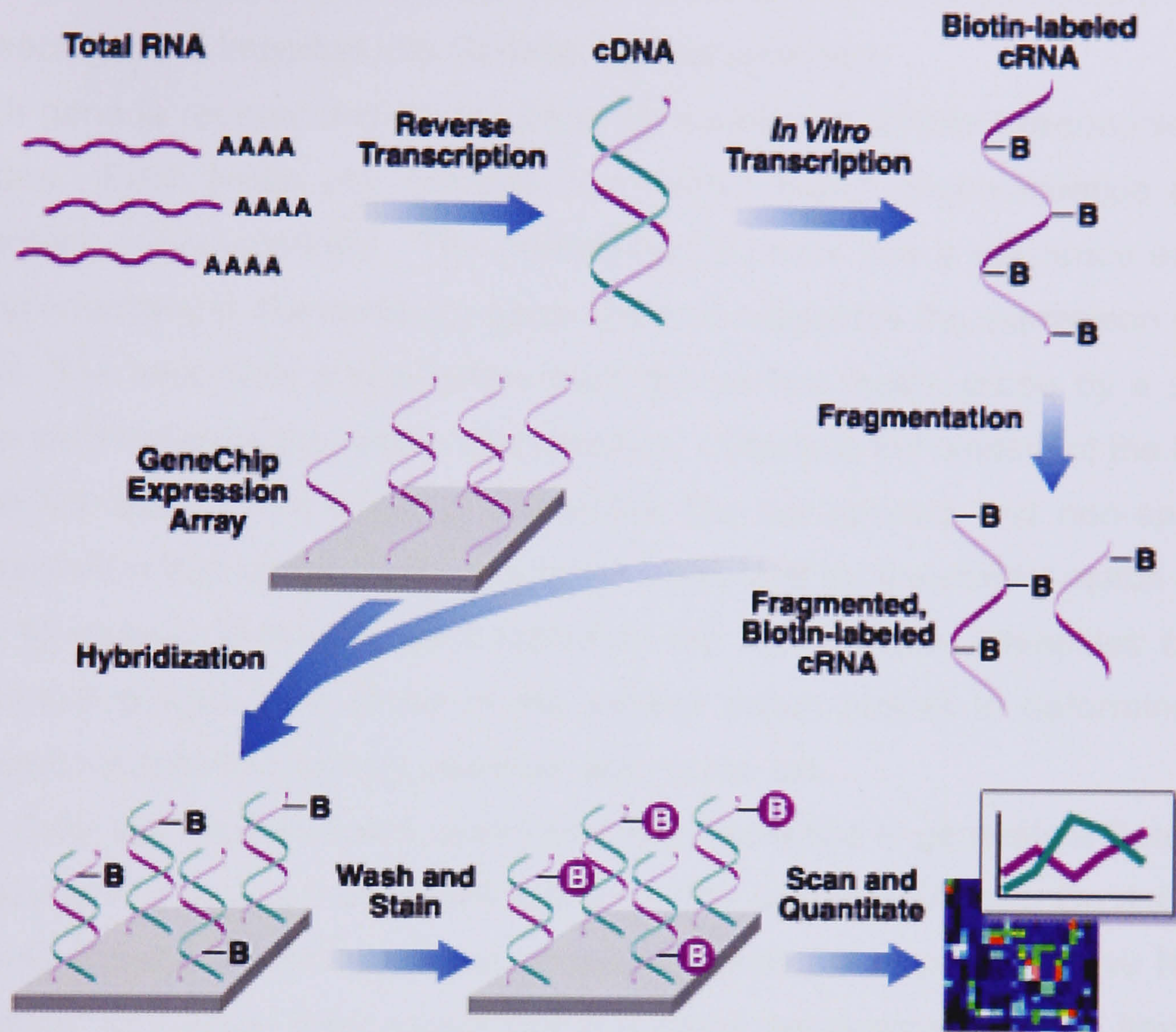


Figure 2.2 Affymetrix arrays labelled cRNA targets derived from the mRNA of an experimental sample are hybridized to nucleic acid probes attached to the solid support. By monitoring the amount of label associated with each DNA location, it is possible to infer the abundance of each mRNA species represented.

### 2.7.5 Affymetrix Analysis

The Array analysis generates a Detection p-value, which is evaluated against user-definable cut-offs to determine the Detection call. This call indicates whether a transcript is reliably detected (Present) or not detected



(Absent). Additionally, a signal value is calculated which assigns a relative measure of abundance to the transcript. Absolute intensities and calls (P or A) for each gene is imported into Genespring and analysed.

Each gene is represented on the array by a series of different oligonucleotide probes. Each probe pair consists of a perfect match oligonucleotide and a mismatch oligonucleotide. The perfect match probe has a sequence exactly complementary to the particular gene and thus measures the expression of the gene. The mismatch probe differs from the perfect match probe by a single base substitution at the centre base position, disturbing the binding of the target gene transcript. This helps to determine the background and non-specific hybridization that contributes to the signal measured for the perfect match oligo. The Microarray Suite software subtracts the hybridization intensities of the mismatch probes from those of the perfect match probes to determine the absolute or specific intensity value for each probe set.

The Detection algorithm also uses probe pair intensities to generate a Detection p-value and assign a Present (P), Marginal (M), or Absent(A) call. On average, as with Sanger Arrays sequences deemed significant or present have higher absolute intensities. Each probe pair in a probe set is considered as having a potential vote in determining whether the measured transcript is detected (Present) or not detected (Absent). The vote is described by a value called the Discrimination score [R]. The score is calculated for each probe pair and is compared to a predefined threshold Tau. Probe pairs with scores higher than Tau vote for the presence of the transcript. Probe pairs with scores lower than Tau vote for the absence of the transcript. The voting result is summarized as a p-value. The higher the discrimination scores are above Tau, the smaller the p-value and the more likely the transcript will be Present (defined as  $p < 0.05$ ). The lower the discrimination score below Tau, the larger the p-value and the more likely the transcript will be Absent (defined as  $p > 0.065$ ). The p-value associated with this test reflects the confidence of the Detection call.



## **Chapter Three**

**Genearray analysis of an in vitro hypoxia transcriptome  
in a bladder cancer cell line and validation in a panel of  
cell lines and untransformed cells**



### 3.1 Introduction

In this chapter the results of experiments to define an in vitro hypoxia transcriptome are detailed and analysed. The anaplastic bladder cancer cell line EJ28 was chosen as a previous smaller array experiment looking in this cell line had identified genes that were upregulated by hypoxia and this had been confirmed by northern blot (Wykoff, Pugh et al. 2000). Genes that are known to be upregulated by hypoxia in other cell lines could also be expected to be upregulated. In a hypoxia time course experiment the anaplastic bladder cancer cell line EJ28 was exposed to 0.1% hypoxia for 16, and 24 hours, the mRNA extracted and competitively hybridised with mRNA taken from the same cells grown under normoxia to Sanger microarray chips. There were two biological replicates and two dye reversed replicates at each time point as well as a self-self control.

### 3.2 Results

#### 3.2.1 Genearray results

Bladder cancer cell line EJ28 which is derived from an invasive tumour was examined in vitro hypoxia at 0.1% oxygen. In order to minimize false positive results and generate a reliable shortlist, only those genes with raw absolute values of >1000, upregulated >2-fold in at least 6 of 8 replicates (four at 16 hours and four at 24 hours) were selected. This would ensure a reliable result but perhaps at the cost of a lower sensitivity. From scatter plots of the self-self control it is clear that at lower intensities the fold-regulation has more random error. Therefore a relatively high cut-off point for intensity of 1000 units was chosen.



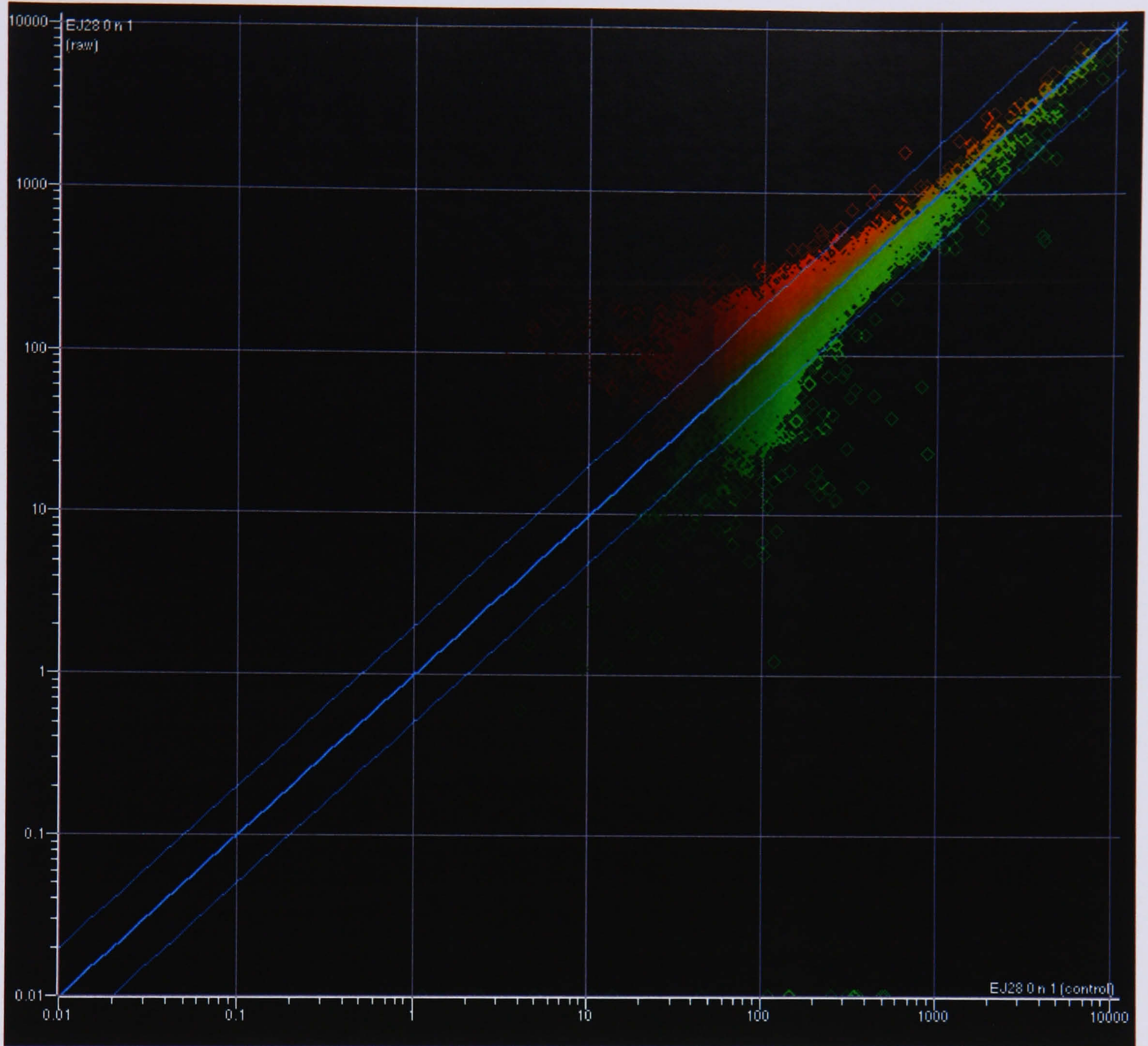


Figure 3.1 Self/self control test of the Sanger array log scale. cDNA from identical EJ28 bladder cancer cell line samples one labeled with Cy5 (green) the other with Cy3 (red) were competitively hybridized. At lower intensities (<1000) there is visible variation around the true value of 1 represented by the blue diagonal line .



Of 6000 genes analyzed only a small subset of 32 (0.53%) were repeatably observed to be upregulated on the gene array (Table 3.1). Of these 32 genes were two of the three previously known to be upregulated by hypoxia in this cell line on Northern blot (Cyclin G2 and DEC1) as well as genes known to be upregulated by hypoxia in other cell lines (e.g. VEGF). Adrenomedullin (ADM) was upregulated 17-fold and Insulin-like growth factor binding protein 3 (IGFBP-3) was upregulated 12-fold. They were the most upregulated mRNAs with VEGF less at 2.5 fold upregulation. Nearly all genes either increased or retained the upregulation found at 16 hours at 24 hours. These genes divided up into functional groups typical of hypoxia induced genes (Harris 2002) (Table 3.1). Several genes not previously known to be upregulated by hypoxia included Ribonuclease 4 RNase4 3.1-fold, monocarboxylate transporter family member 4 (SLC16A3 also called MCT4), Preferentially expressed antigen of melanoma PRAME 4.9-fold, and Importin Beta 3 4.1-fold. Not listed because it was not validated later was the protein kinase C substrate MARCKS which appeared upregulated 2.2 fold at 16 hours. The ESTs do not relate to any known genes (last checked August 2005).

Table 3.1 (next 2 pages) Mean fold changes of all 32 genes upregulated more than 2-fold by hypoxia (0.1%) at 16 and 24 hours in cell line EJ28. Microarray result of 8 biological replicates; genes had to be upregulated more than 2-fold with in cell line EJ28. Microarray result of 8 biological replicates; genes had to be upregulated more than 2-fold with high raw signal intensity (>1000 units) in more than 6 of 8 replicates to avoid false positives and be included in this list (ECMM=Extracellular Matrix/Collagen Metabolism).



No.	Fold-change at hypoxia		GENE	FUNCTIONAL GROUP
	16 Hours	24 Hours		
<b>Known to be hypoxia-upregulated in other cell lines</b>				
1	3.4	17	Adrenomedullin (ADM)	Vascular Tone
			Insulin-like growth factor binding protein 3 (IGFBP-3)	Apoptosis
2	2.6	12.1		
3	2.9	8.2	n-myc downstream regulated protein 1 (NDRG1)	Cell Cycle
4	5.3	6.6	BCL2 binding protein NIP3 (NIP3)	Apoptosis
			Inducible 6-phosphofructo-2-kinase (PFKFB3)	Glucose Metabolism
5	2.7	4.7		
			Lysyl Hydroxylase (PLOD2)	Tissue remodeling
6	2.4	4.7		
			MAX interacting protein 1 (MAX 1)	Transcription factor
7	2.2	4.4		
			Glucose Transporter 1 (GLUT 1)	Glucose metabolism
8	3.1	4.2		
			1,4-Alpha Glucan Branching Enzyme (HGBE)	Glucose Metabolism
9	2.3	3.9		
10	2.2	3.8	Cyclin G2 (CCNG2)	Cell Cycle
			Proline-4-hydroxylase (P4HA1)	Oxygen sensing/ECMM
11	2.5	3.7		
			Adenylate Kinase 4	Nucleotide metabolism
12	3.2	3.2		
			Aldolase C (ALDO C)	Glucose Metabolism
13	2.9	3.1		
14	1.9	3	Prolyl-4-hydroxylase alpha subunit	Oxygen sensing
15	1.8	2.6	Haem oxygenase 1 (HMOX1)	Vascular Tone
16	2.5	2.5	EGL9 Homologue 1	Oxygen sensing
17	2.1	2.5	Testis-specific lactate dehydrogenase (LDH C)	Acid Base
18	1.9	2.5	VEGF	Angiogenesis
			Adenylate Kinase 3 (AK3)	Nucleotide metabolism
19	2.4	2.5		
			Dual Specificity Phosphatase 1 (DUSP1/CL100/MKP1)	Cell signalling
20	1.7	2.4		
21	1.7	2.1	DEC 1	Differentiation
			Glucose-6-Phosphate isomerase (GPI)	Glucose Metabolism
22	2.6	2.1		
			Fructose-Bisphosphate Aldolase A	Glucose Metabolism
23	2.1	2.1		

Continued on next page



No.	Fold-change at hypoxia		GENE	FUNCTIONAL GROUP
	16 Hours	24 Hours		
<b>Not previously known to be hypoxia regulable</b>				
24	2.8	8.2	Solute carrier family 16 member 3 (SLC16A3)	Acid Base
			Preferentially expressed antigen of melanoma (PRAME)	Antigen
25	4.5	4.9	Importin Beta 3	Nuclear pore protein
26	2.8	4.1	RNAse 4	Antimicrobial ? Angiogenic like Angiogenin family
27	1.9	3.1		
28	2.2	2.9	Protooncogene ABL1	Cell cycle
<b>ESTs</b>				
29	3	3.9	50378_A	EST
30	3.2	3.3	50022_A	EST
31	3.5	3.3	31564_B	EST
32	2.1	2.4	21875_A	EST

Table 3.1 Mean fold changes of all 32 genes upregulated more than 2-fold by hypoxia (0.1%) at 16 and 24 hours in cell line EJ28. Microarray result of 8 biological replicates; genes had to be upregulated more than 2-fold with in cell line EJ28. Microarray result of 8 biological replicates; genes had to be upregulated more than 2-fold with high raw signal intensity (>1000 units) in more than 6 of 8 replicates to avoid false positives and be included in this list (ECMM=Extracellular Matrix/Collagen Metabolism).



### 3.2.2 Validation of microarray results

In order to validate the results of array analysis the technique of Reverse Transcription Polymerase Chain Reaction (RT-PCR) was initially used (Figure 3.2a and b). Products of PCR amplification of cDNA prepared from RNA extracted from cell lines was compared on an agarose gel after a fixed number of PCR cycles. However many of the gene fold changes were in the range 2-8 fold and RT-PCR was found to be too insensitive to reliably detect these changes.



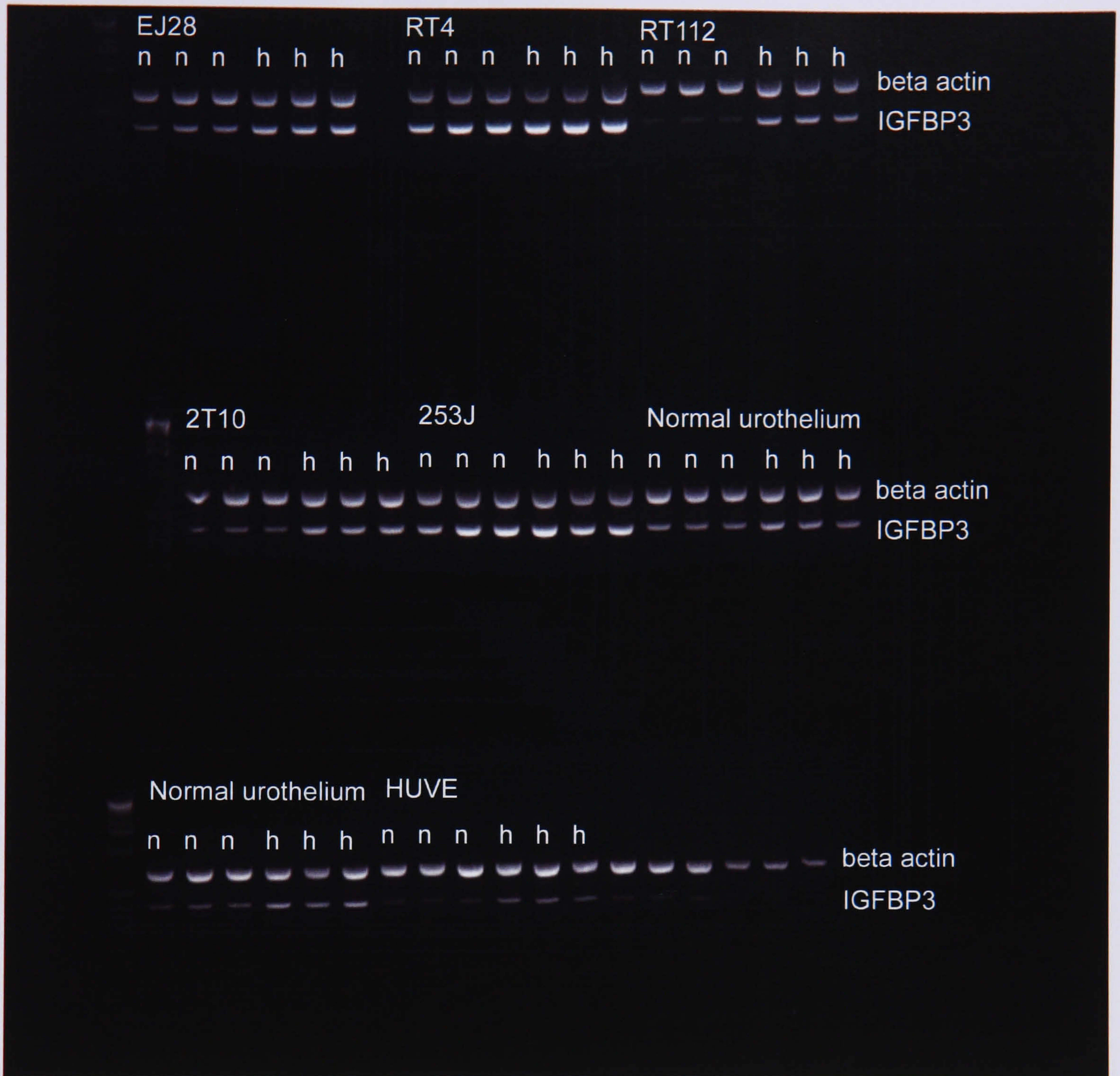


Figure 3.2a An example of a RT-PCR result. n=normoxia replicate in sets of 3, h=hypoxia replicate in sets of 3. IGFBP3 with beta actin control (reaction stopped at 32 cycles) confirms upregulation of IGFBP3 in bladder cancer cell lines EJ28 RT4 RT112 and 2T10 253J(equivocal), normal urothelium and HUVE cells. 2% agarose gell with ethidium bromide.



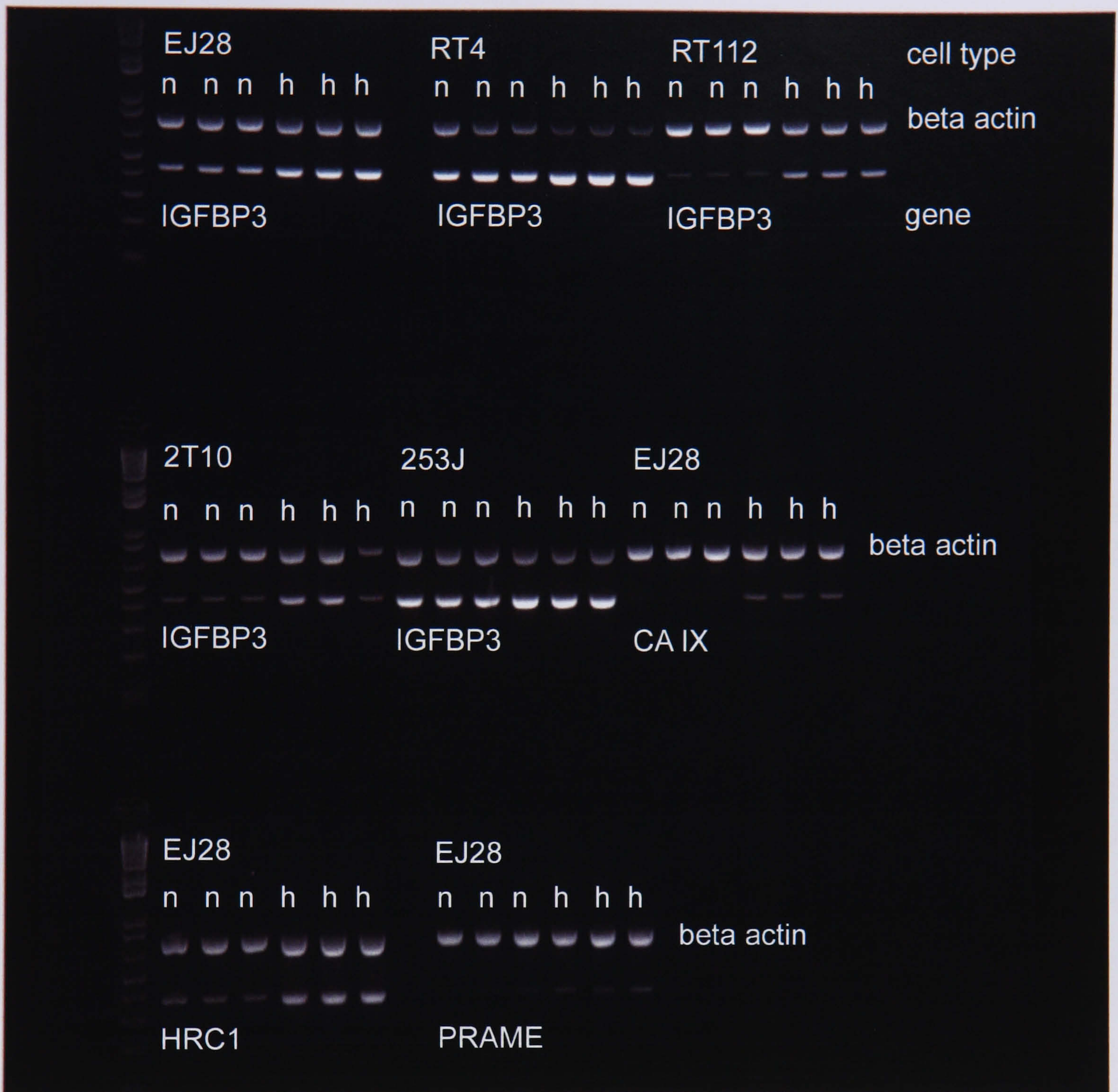


Figure 3.2b An example of a RT-PCR result. n=normoxia replicate in sets of 3, h=hypoxia replicate in sets of 3. IGFBP3 with beta actin control (reaction stopped at 32 cycles) also confirms upregulation of IGFBP3 in bladder cancer cell lines EJ28 RT4 RT112 and 2T10. Remaining three genes at 38 cycles: CA IX, HRC1 (HRAS associated gene (Weitzel, Kasperczyk et al. 1992)) and PRAME confirmed as upregulated by hypoxia in EJ28. 2% agarose gell with ethidium bromide.



Furthermore actin was used as a control in these experiments and there has been a report that actin is induced by hypoxia (Zhong and Simons 1999). Commercially available genes to act as controls for Real-time RTPCR were literature searched to ensure there were no reports of hypoxia regulation, data from the hypoxia array also suggested that the genes Beta-2-Microglobulin (Figure 3.3) and Peroxiredoxin would be suitable; neither was upregulated by hypoxia on the in vitro array.

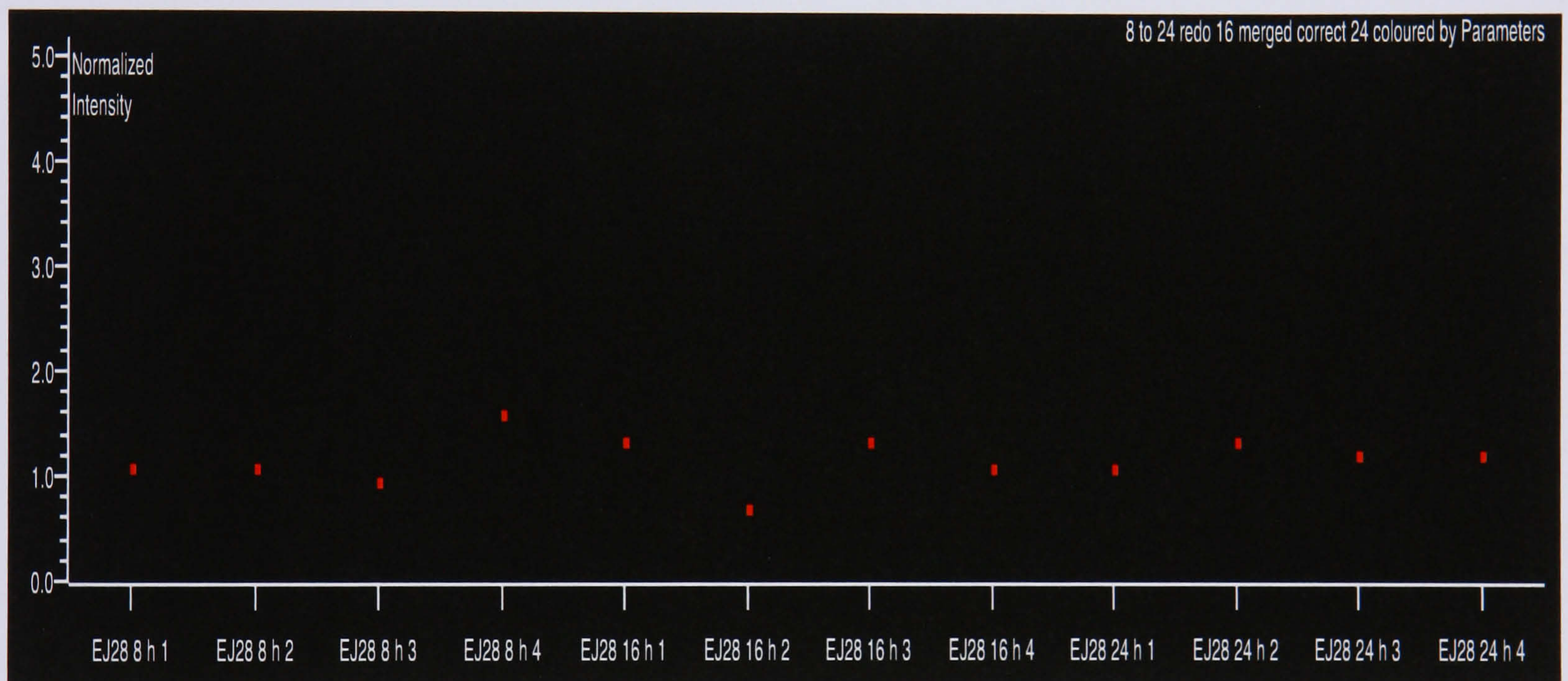


Figure 3.3 Hypoxia array result for fold change in Beta-2 microglobulin under hypoxia. Four replicates at 8,16 and 24 hours all showed no significant change.

Apart from the two control genes four genes in total were selected for validation and further investigation in other cell lines; IGFBP-3, RNase4, SLC16A3, MARCKS. IGFBP3 was selected because it was the most upregulated gene on the in vitro and in vivo arrays (see Chapter 4). RNase4, SLC16A3 and MARCKS were selected because they have not been previously reported to be upregulated by hypoxia in cell lines and were significantly upregulated in

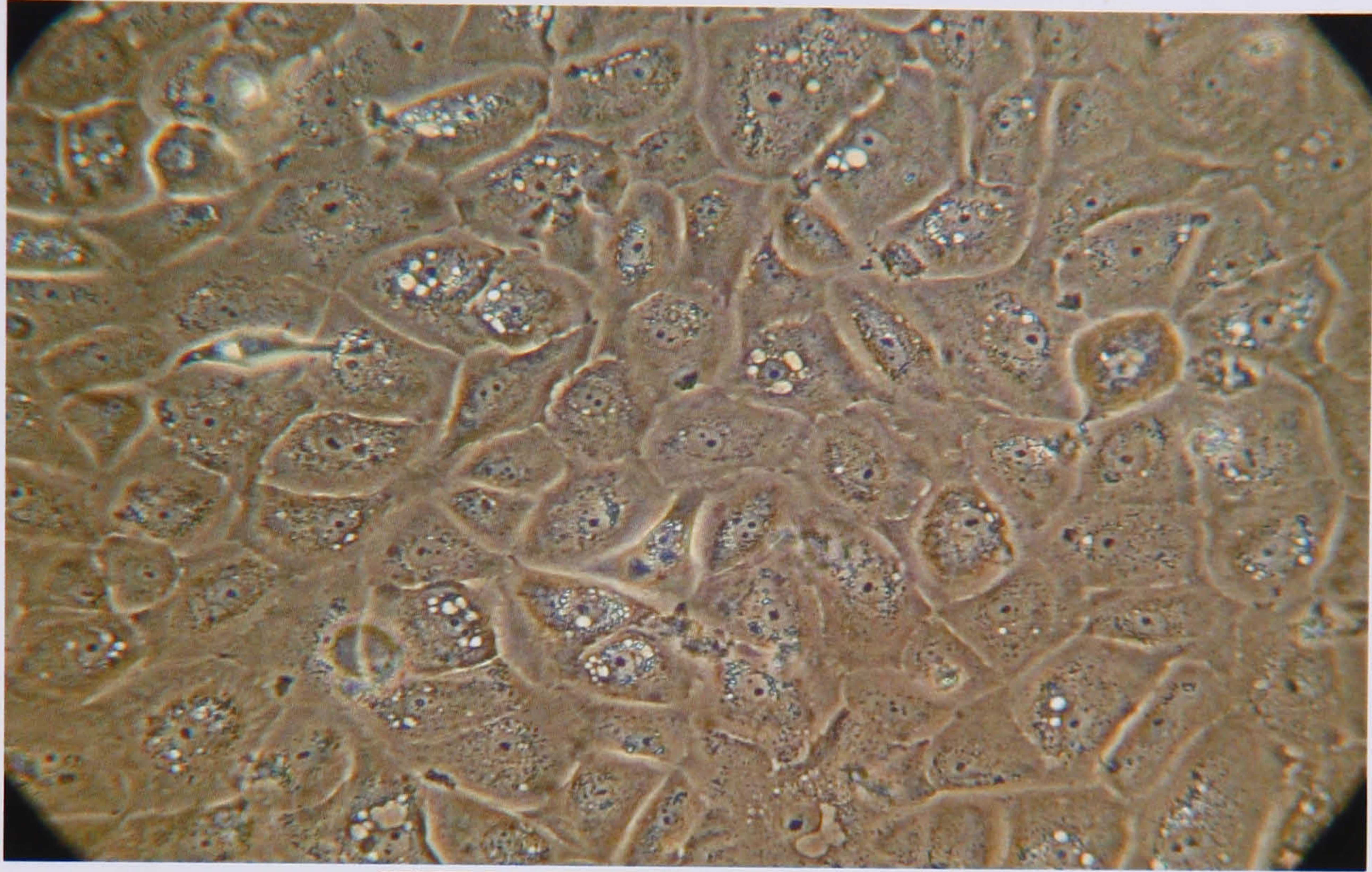


some bladder cancers on the in vivo array. RNAse 4 comes from the same family as the archetypal angiogenic factor Angiogenin which is known to be upregulated in the blood of bladder cancer patients, and SLC16A3 is a lactate transporter. MARCKS was upregulated by hypoxia in EJ28 only 2.1 fold but appeared strongly upregulated on the in vivo array giving reason for further investigation.

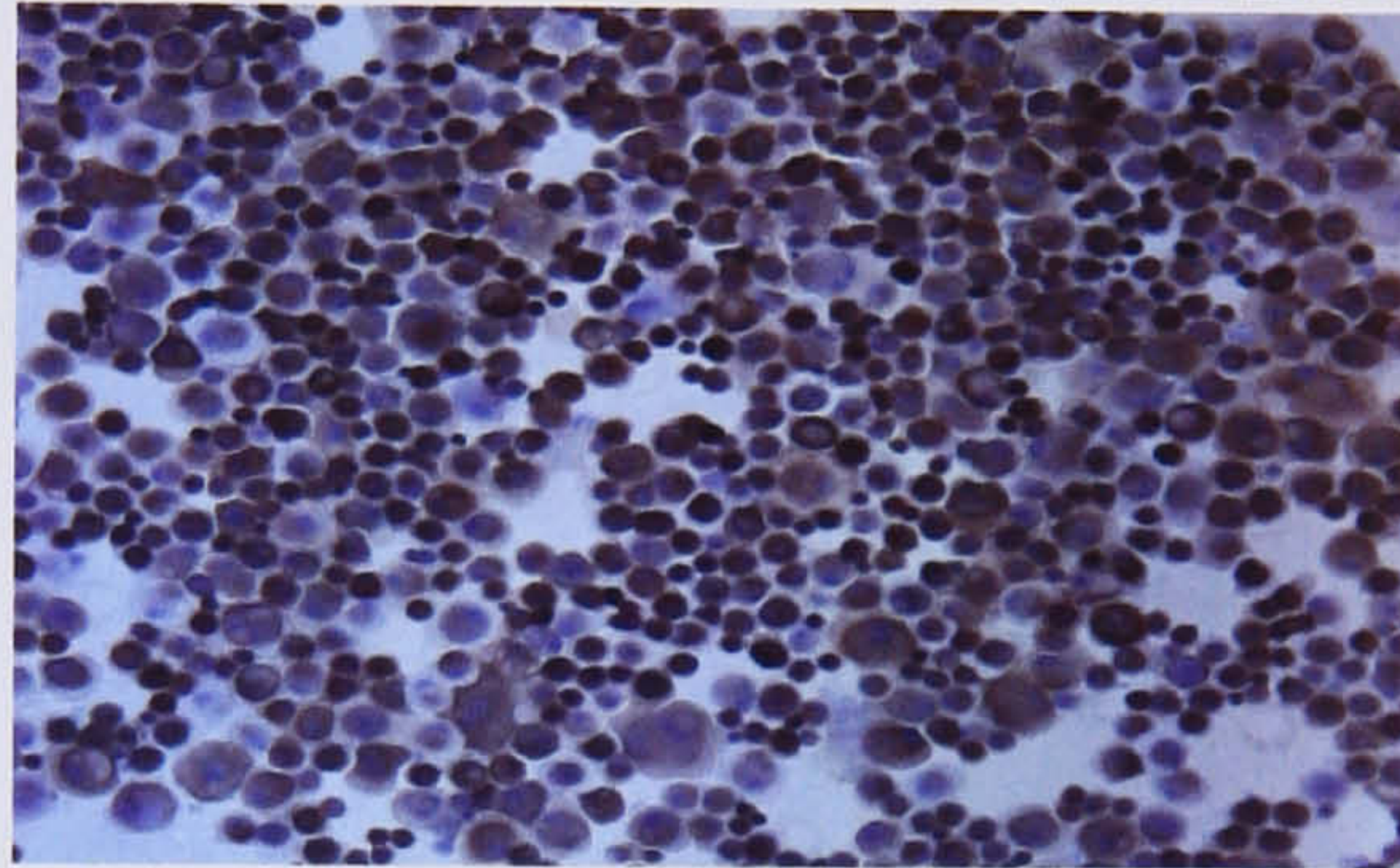
Once real-time RTPCR had confirmed the upregulation by hypoxia in EJ28 other bladder cell lines, cultured normal urothelium, breast cell lines, human umbilical vein cells and fresh monocytes were also investigated. The culturing of human normal urothelial cells was performed by the current investigator and required several attempts. Initial cultures were killed by fungal or bacterial infections. Normal urothelia was grown to 6 passages. The origin of the cells as normal urothelia was confirmed by immunohistochemical staining of cytopun lifted cells with cytokeratin 7, 20 and antibody Uro 5 (Cordon-Cardo, Bander et al. 1984; Dubeau and Jones 1987; Schaafsma, Ramaekers et al. 1989) (Figure 3.4). Fresh monocytes were prepared courtesy of Dr P Maxwell.



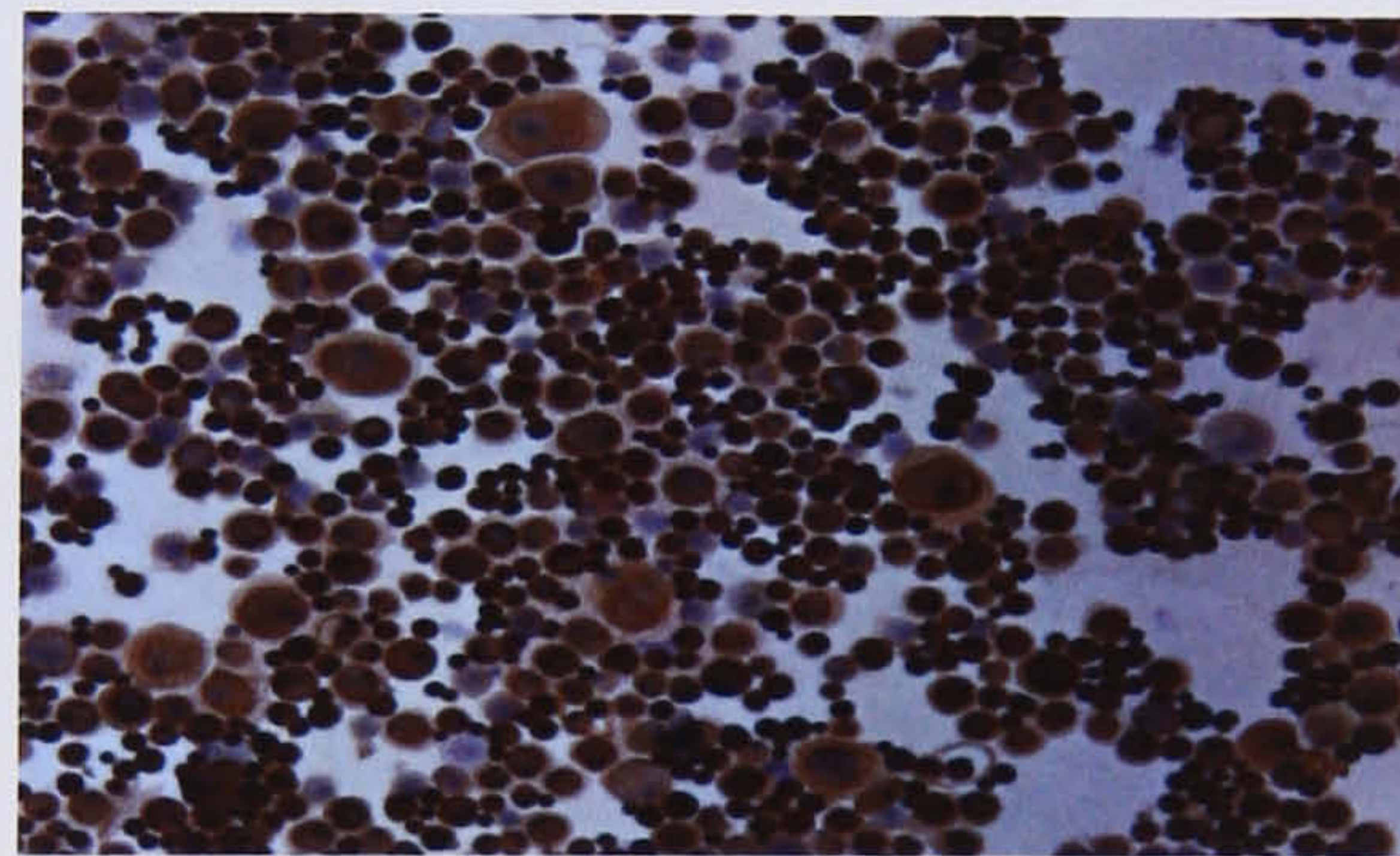
A



B



C



D

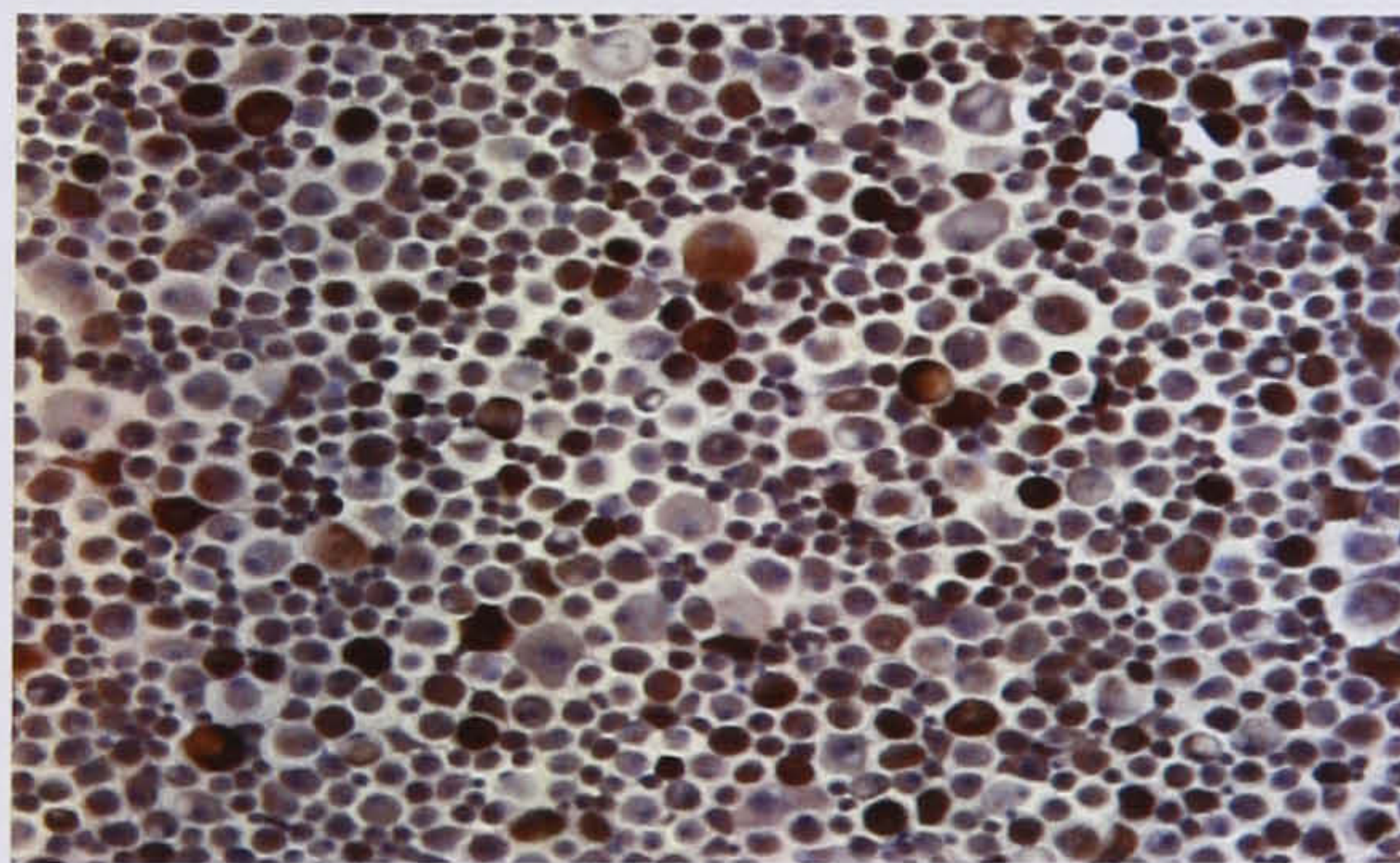


Figure 3.4 A Cultured normal urothelia grown from fresh normal ureter from a nephrectomy specimen. B,C,D Cytospin samples of the cultured cells was positive for Uro 5 (B) Cytokeratin 7 (C) and Cytokeratin 20 (D) confirming urothelium as the cell of origin (see text for references).



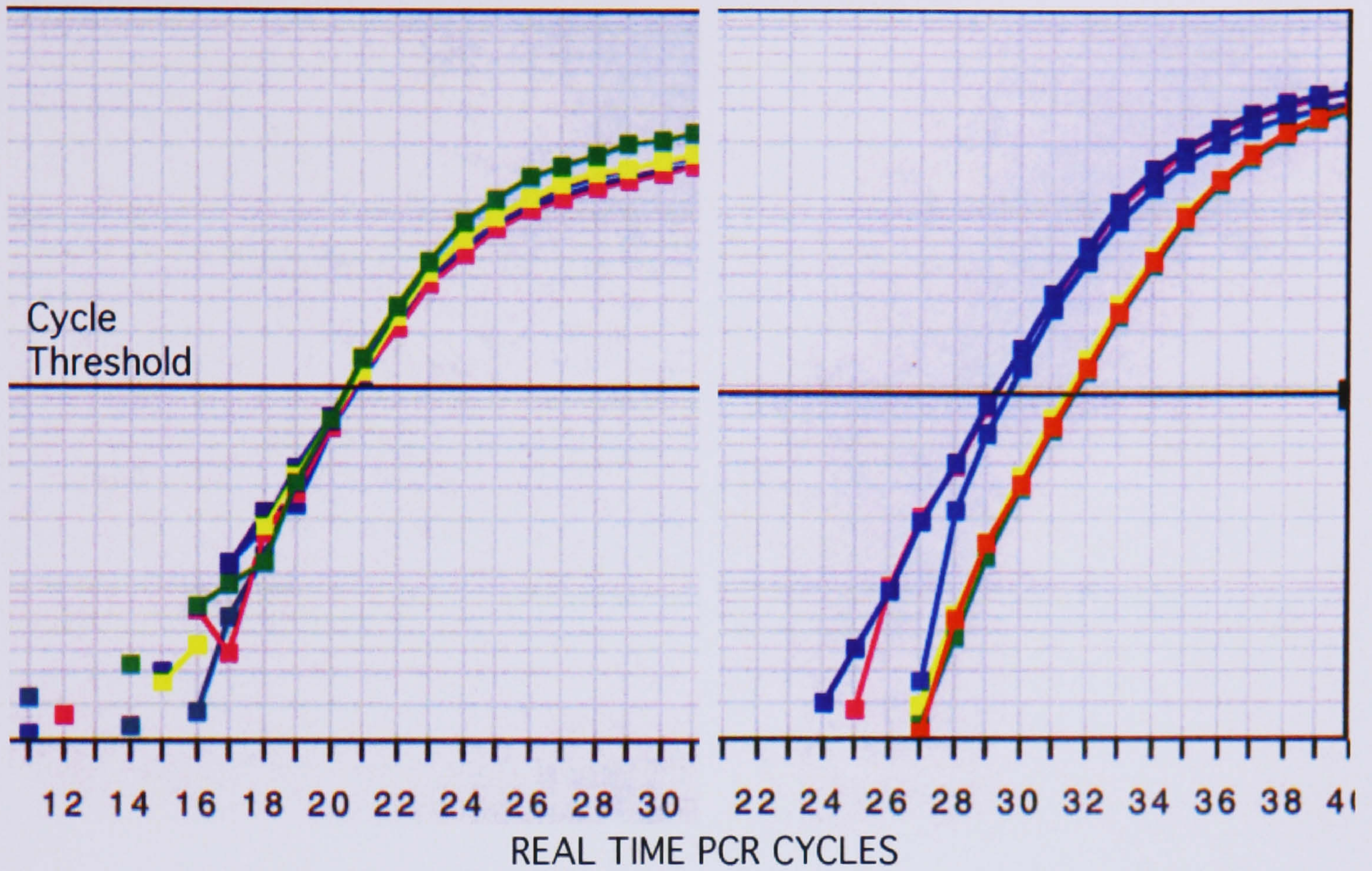


Figure 3.5 An example of a real time RT-PCR result. Graphs were produced but results were analysed based on raw numerical data. Normal urothelium grown in culture. Right graph; total of six IGFBP-3 probe replicates normoxia vs. hypoxia show a significant ( $p=0.0003$ ) average difference of 2.1 cycles at the threshold (equating to 4.6 fold upregulation). Left graph; six beta-2-microglobulin replicates performed with the identical experimental samples, reagents and processing in separate experimental tubes (three normoxia and three hypoxia) show no significant change.



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**BLADDER CANCER CELL LINE EJ28 - FOLD CHANGES IN RNA LEVELS OF GENES**

GENE NAME	HOURS OF HYPOXIA	REAL TIME RESULTS		SANGER ARRAY RESULTS
		PROBE	B-2-M (CONTROL)	
RNASE4	16	3.6	1	1.9
	24	6.5	1	3
SLC16A3	16	5.35	1	2.8
	24	6.4	1	8.1
IGFBP3	16			2.8
	24	5.5	1.2	8.9
MARCKS	16	1.2	1	2.2
	24	1.2	1.4	1.6
PEROXI (2nd CONTROL)	16	1.2	1	1

Table 3.2 Real time RTPCR results for genes in bladder cancer cell line EJ28 compared with results on the Sanger array. MARCKS was not confirmed as being upregulated under hypoxia. Beta-2-microglobulin (B-2-M) and Peroxiredoxin (PEROXI) both were established as useful controls as neither were upregulated by hypoxia.



CELL TYPE	HOURS OF											
	CELL LINE	EJ28	EJ28	2T10	253J	RT112	MDA 468	T47D	NORMAL UROTHELIUM	ENDOTHELIAL CELLS	HUVEC	FRESH MONOCYTES
	HYPOXIA	IGFBP-3	CONTROL	RNASE4	CONTROL	SLC16A3	CONTROL					
BLADDER CELL LINE	EJ28	16	3.6	1	5.35	1	1					
	EJ28	24	5.5	1.2	6.5	1	1	6.4	1			
	2T10	16	14.5	1.3	3.4	1	1	5.5	1			
	253J	16	3.4	1.1	3.9	1.1	1.1	2.1	1.1			
	RT112	16	38	1.4	3.8	1.3	1.3	3.8	1.4			
BREAST CELL LINES	MDA 468	16						3.8	-1.1			
	T47D	16						10.8	1.1			
NORMAL UROTHELIUM		16	4.6	1.1	3.7	1	1	-1.3	1			
ENDOTHELIAL CELLS	HUVEC	16	7	-1.4				64	1.6			
FRESH MONOCYTES		16						-1.3	-1.1			

Table 3.3 Fold changes of IGFBP-3 RNAse4 and SLC16A3 by hypoxia (0.1%) in bladder cancer cell lines, normal cultured human urothelia and HUVE cells analysed by real time RT-PCR. 3 replicates of each experiment were performed and if replicates were more than 0.5 cycles different the result was not accepted. The control gene beta-2-microglobulin in each experiment did not differ significantly from normoxia to hypoxia but any trend to change of the control gene is demonstrated by minimal fold-changes (range -1.4 to 1.6). Fold-change calculated on the basis of 2 to the power of the change in cycle number at a set threshold. Differences in gene of interest in normoxia and hypoxia all showed a significant difference in average cycle threshold (p<0.01) unless indicated. Negative values indicate down regulation.



IGFBP3, RNase4 and SLC16A3 were confirmed to be upregulated by hypoxia in all bladder cancer cell lines examined and other cell lines (Table 3.3). Of the untransformed cells SLC16A3 RNA was markedly upregulated by hypoxia in HUVE cells but not in cultured normal urothelium nor in fresh monocytes. RNase 4 like IGFBP-3 was upregulated by hypoxia in the cell lines and normal urothelial cells. A typical result for RTPCR is shown (Figure 3.4). The control gene Beta-2-microglobulin showed no significant change by comparison. Nor did a second control gene Peroxiredoxin selected from the hypoxia array by its lack of fold-change (Table 3.2). MARCKS was upregulated by hypoxia 2.1-fold on the in vitro array and also more than 2-fold in more than 20 tumours on the in vivo array. However MARCKS upregulation in the cell line panel was not confirmed (Table 3.2)

### 3.3 Discussion

Genes up regulated in EJ28 could be classified into groups typical of hypoxia inducible genes (Table 3.4). EJ28 readily upregulated genes involved in glycolysis and nucleotide metabolism as expected as hypoxia increases demands on glycolysis and its energy transfer molecules AMP and ADP. Cell cycle and putative apoptotic genes (CCNG2 IGFBP3 NIP3 ABL1) were also upregulated but it is not clear whether some of these genes (e.g. IGFBP3) promote apoptosis or proliferation in vivo (vide infra). EJ28 also markedly upregulated genes involved in acid base regulation, angiogenesis and vascular tone.

#### 3.3.1 NDRG1

N-myc downstream-regulated protein 1 (NDRG1) was upregulated 8-fold. NDRG1 is known to be upregulated by hypoxia (Lachat, Shaw et al. 2002) and in many human cancers (Cangul 2004). C-myc is an oncogene whose normal physiological function is the normal proliferative maintenance of and wound healing in skin, as has been recently demonstrated in skin



epithelium-specific c-myc knock-out mice (Zanet, Pibre et al. 2005). C-myc upregulation has also been demonstrated to be physiologically upregulated in healing skin wounds in rats (Fu, Gu et al. 2003). C-myc and n-myc are members of the same family and similar enough for n-myc to replace c-myc in mice with no adverse effects on development or adulthood (Malynn, de Alboran et al. 2000). C-myc amplifications are known to occur in approximately 40% of bladder cancers and are strongly associated with markers of biological aggressiveness such as stage and grade and proliferation index (Sauter, Carroll et al. 1995). It is tempting to speculate that NDRG1 has a role in the biological aggressiveness of bladder cancer too.



<b><u>Functional group</u></b>	<b><u>Gene</u></b>	<b><u>Fold change</u></b>
Vascular Tone	Adrenomedullin (ADM)	17
	Haem oxygenase 1 (HMOX1)	2.6
Apoptosis	Insulin-like growth factor binding protein 3 (IGFBP-3)	12.1
	Bcl-2 binding protein NIP3	6.6
Cell Cycle	N-myc downstream regulated protein 1 (NDRG1)	8.2
	Cyclin G2 (CCNG2)	3.8
	Protooncogene ABL1	2.9
Acid Base	Solute carrier family 16 member 3 (SLC16A3)	8.2
	Testis-specific lactate dehydrogenase (LDH-C)	2.5
Glucose Metabolism	Inducible 6-phosphofructo-2-kinase (PFKFB3)	4.7
	Glucose Transporter 1 (GLUT 1)	4.2
	1,4-Alpha Glucan Branching Enzyme	3.9
	Aldolase C	3.1
	Glucose-6-Phosphate isomerase	2.1
	Fructose-Bisphosphate Aldolase A	2.1
Oxygen sensing/ECMM	Lysyl Hydroxylase (PLOD2)	4.7
	Proline-4-hydroxylase (P4HA1)	3.7
	Prolyl-4-hydroxylase alpha subunit	3
	EGL9 Homologue 1	2.5
Nucleotide metabolism	Adenylate Kinase 4	3.2
	Adenylate Kinase 3	2.5
Antigen	Preferentially expressed antigen of melanoma (PRAME)	4.9
Transcription factor	MAX interacting protein 1	4.4
Nuclear pore protein	Importin Beta 3	4.1
Antimicrobial / Angiogenesis?	RNAse 4	3.1
Angiogenesis	VEGF	2.5
Cell signalling	Dual Specificity Phosphatase 1 (DUSP1/CL100/MKP1)	2.4
Differentiation	Dec-01	2.1

Table 3.4 Functional groupings of the 32 hypoxia inducible genes in bladder cancer cell line EJ 28.



### 3.3.2 IGFBP-3

IGFBP-3 mRNA is known to be hypoxia-inducible in a number of cell lines and these results show that this is also true of bladder cell lines with upregulation 5 to 38 fold. Nor is this upregulation specific to cancer, with upregulation in human cultured urothelium (4.6 fold) and HUVE cells (7-fold).

Insulin-like growth factor (IGF) binding protein-3 (IGFBP-3) is known to block IGF action and inhibit cell growth (Baxter 2001; Firth and Baxter 2002). IGFBP-3 is thought to act by sequestering free IGFs or, possibly, acts via a novel IGF-independent mechanism. Supporting its role as a primary growth inhibitor, IGFBP-3 production has been shown to be increased by cell growth-inhibitory agents, such as transforming growth factor-beta (TGF-beta), and the tumor suppressor gene p53 (Rajah, Valentinis et al. 1997). IGFBP-3 has also been shown to have an apoptotic function independent of IGFs and IGF receptor and p53 in the p53 negative prostate cancer cell line PC-3. In the same cell line TGFbeta1, a known apoptosis-inducing factor was shown to induce apoptosis by the induction of IGFBP3 (Rajah, Valentinis et al. 1997).

However other in vitro studies suggest IGFBP3 is involved with stimulation of proliferation and DNA synthesis. IGF-1 on its own has no nuclear localisation signal but IGFBP-3 does. Endogenously expressed IGFBP3 in transfected bovine mammary cell culture makes them more sensitive to IGF-1 stimulation of DNA synthesis than vector only transfected controls (Grill and Cohick 2000). In experimental wounds caused by scratching a confluent layer of opossum kidney cells, the IGFBP-3/IGF-1 complex locates to the nucleus in proliferating cells near the wound edge but not in resting cells at a distance from the wound (Li, Fawcett et al. 1997). IGFBP-3 has been shown to have a stimulatory effect on the growth of sheep smooth



muscle bladder cells (Weinzimer, Gibson et al. 2001); an intriguing finding given the link between bladder outflow obstruction, hypoxia and bladder muscle hypertrophy (see introduction). Topical administration of IGFBP-3 and IGF-1 together reverse the defect caused by corticosteroids in healing skin wounds of rats better than IGF-1 alone (Hamon, Hunt et al. 1993). Thus IGFBP-3 may potentiate or sensitise cells to the proliferative effects of IGF-1.

Nuclear translocation and phosphorylation of IGFBP-3 is described (Schedlich, Nilsen et al. 2003) (Schedlich, Young et al. 1998), with phosphorylation increasing nuclear import of IGFBP-3, and decreasing its affinity for IGF with release of IGF. The import mechanism involves interaction with Importin Beta (Schedlich, Le Page et al. 2000). Interestingly this gene was also in our list of 32 genes upregulated (4.1 fold) by hypoxia on the array and is not previously reported to be upregulated by hypoxia. Thus IGFBP-3/Importin Beta may be an important pathway upregulated by hypoxia in bladder urothelium.

Animal studies suggest that the initial physiological response of urothelium after 24 hours of hypoxic stress is proliferation of the basal cell layer with a concomitant decrease in TGF beta levels. TGF beta is known to be associated with differentiation of prostate cancer cell lines and its down regulation in proliferating bladder urothelium suggests a similar role in bladder. IGFBP-3 antiproliferative signaling appears to require an active transforming growth factor beta (TGF-beta) signaling pathway, and IGFBP-3 stimulates phosphorylation of the TGF-beta signaling intermediates Smad2 and Smad3. (Baxter 2001)

IGFBP-3 may play a significant role in endothelium. In a mouse xenograft model IGFBP-3 RNA was overexpressed in tumour endothelial cells (Schmid, Bisoffi et al. 2003). Addition of IGFBP-3 in vitro reverses VEGF-



induced proliferation of HUVE cells in an IGF-independent fashion (Franklin, Ferry et al. 2003). Thus IGFBP-3 may act as a direct apoptotic response to hypoxia, balancing the proliferation of endothelium caused by VEGF in hypoxic areas. If so, this implies that there are major selective mechanisms in vivo to overcome this pathway and suggests that antagonising the IGF1 receptor could have high selectivity in cancer, with endogenous IGFBP-3 synergising with such inhibition.

As IGFBP3 is a secreted protein its levels were also measured in the urine of bladder cancer patients (Chapter 5).

### 3.3.3 RNase4

The upregulation of RNase4 by hypoxia was confirmed. Upregulation by hypoxia does not appear to be cancer-specific as we also found RNA levels induced in normal urothelium. The human enzyme is secreted into plasma (Terzyan, Peracaula et al. 1999). But very little about its function is known. The gene (Rosenberg and Dyer 1995) shares various identical 5'-untranslated regions on its mRNA with angiogenin, suggesting that expression of these proteins is under similar control, with obvious implications for their biological activities (Strydom 1998). Angiogenin is known to stimulate angiogenesis (Folkman and Klagsbrun 1987) but has recently been found to play an antimicrobial role as part of the innate immune system as well (Hooper, Stappenbeck et al. 2003). Angiogenin protein is upregulated by hypoxia in cervical and melanoma cell lines (Hartmann, Kunz et al. 1999; Pilch, Schlenger et al. 2001) and is raised in the blood and tumours of patients with bladder cancer (Miyake, Hara et al.



1999). Since the surface of bladder cancers is hypoxic the levels of angiogenin in urine were also looked at (see Chapter 5).

#### 3.3.4 SLC16A3

The lactate transporter SLC16A3 is also confirmed to be a hypoxia-regulated gene. This occurred in all six cell lines examined. Of the untransformed cells SLC16A3 was markedly upregulated in human umbilical vein cells 68-fold but not in cultured normal urothelium nor fresh monocytes. This suggests a possible physiological role for SLC16A3 in endothelial cells under hypoxia. It is a member of the proton-linked transmembrane monocarboxylate transporter (MCT) family with high affinity for lactate. It is also known as MCT4 (previously known as MCT3)(Halestrap and Price 1999). Transfection of a monkey kidney epithelial cell line (COS) and *Xenopus* oocytes with MCT4 stimulates lactate transport. (Kirk, Wilson et al. 2000; Manning Fox, Meredith et al. 2000). MCT 4 is in most tissues but is particularly high in the white skeletal muscle, white blood cells and cell lines suggesting it may be of particular importance in tissues that rely on high levels of glycolysis for ATP production and hence lactate production (Wilson, Jackson et al. 1998; Halestrap and Price 1999) (Juel and Halestrap 1999).

#### 3.3.5 HRC1

In an earlier analysis of the array data HRC1 was one gene that was upregulated on the in vitro array but in less than 7 of 8 replicates. It was however confirmed as upregulated by hypoxia on standard reverse



transcription-PCR (see Figure 3.1). Molecular genetic alterations of chromosome 11p15.5 are a common finding in human cancer and this region contains HRC1, the protooncogene HRAS1 and the ribonuclease-angiogenin inhibitor RNH (Weitzel and Patel 1994). Furthermore HRAS1 and HRC1 are known to be upregulated in the EJ28 bladder carcinoma cell line (Weitzel, Kasperczyk et al. 1992). This is the first report that HRC1 is hypoxia inducible.



## **Chapter Four**

**The comparison of in vitro data with in vivo array data and in vivo staining**



## 4.1 Introduction

This thesis benefited much from in vivo array data courtesy of Mr E Streeter. The in vivo array data came from samples of 39 transitional cell carcinomas of the bladder competitively hybridised to a Sanger microarray chip with reference RNA pooled from 11 cell lines from a panel of various cancers (see method for details). As the same type of Sanger chips were used for the in vivo array there was data available for all genes identified as hypoxia-inducible on the in vitro array. Both superficial (Ta, T1) and invasive (T2,T3) tumours were used on this array as listed in Table 4.1. Of 6000 genes on the in vivo array, 70 (1.2%) were upregulated more than 2-fold in over 30 tumours. The results for hypoxia-regulated genes identified in EJ28 are analysed in this chapter.

## 4.2 Results

### 4.2.1 Hypoxia inducible genes upregulated on the in vivo array

Upregulation on the in vivo array data was defined as upregulation more than 2-fold, in more than 5 of the 39 tumours. As with the in vitro array, data with raw signal intensity <1000 was excluded to reduce erroneous fold-changes caused by the increased random error seen at lower intensities. Of the 32 genes upregulated by hypoxia in vitro, 8 genes were upregulated more than 2-fold in more than 5 of the 39 tumours (red in Figure 4.1). VEGF, which is known to be expressed in bladder cancer was upregulated 2.5-fold by hypoxia and 2 to 18-fold in 31/39 tumours making it the second most frequently upregulated hypoxia-inducible gene on the in vivo array. The most highly and frequently upregulated gene in both arrays however was the mRNA IGFBP-3. This was upregulated up to 12-fold by hypoxia in vitro and from 2 to 100-fold in 33/39 tumours compared to the cell line



panel. The other 6 genes with more than 2-fold upregulation in more than 5 tumours arrayed were CCNG2 in 30 tumours, NDRG1 in 18 tumours, PFKFB3 in 17 tumours, RNAse4 in 10 tumours, Adrenomedullin (ADM) in 8 tumours and Glucose transporter 1 (GLUT-1) in 6 tumours. The cumulative fold changes for all 8 genes are plotted for each tumour (Figure 4.2). It can be seen that fold changes for IGFBP-3 were greater than for other genes.

Of the genes not previously reported to be upregulated by hypoxia were RNAse4 and SLC16A3. RNAse4 was significantly upregulated on the in vivo array in 10 tumours. Although signal was low for SLC16A3 in tumours it was upregulated more than 2-fold in 7 tumours. The mean fold-change across all 39 tumours was 1.4 (lower and upper quartiles 1.1 and 1.7). A paired t-test of the raw data showed significant upregulation (mean control signal 520, mean raw signal 690, ratio 1.3,  $p=0.0003$ ).



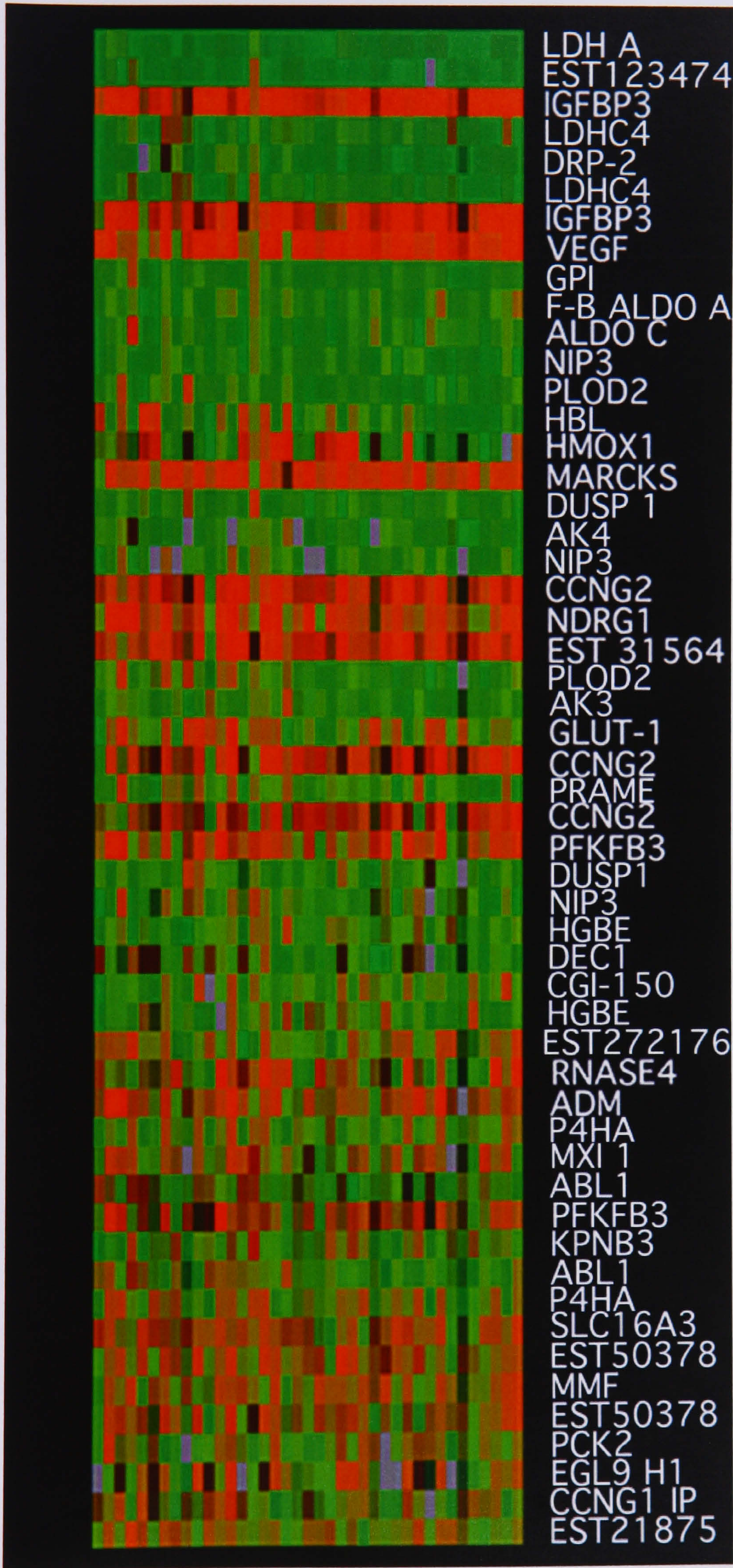


Figure 4.1 Graphic representation of array results of in vivo hypoxia gene expression in bladder cancers, for all genes induced by hypoxia in vitro (as listed in Table 3.1). In order of Genespring 'Interest' function which places the most statistically trustworthy and greatest fold-changes towards the top of the list. Some of the 32 genes are represented by more than one row as they were represented by more than one spot on the array eg CyclinG2 (CCNG2). One columns = One tumour sample. Samples/columns (starting on the left) 1 to 26 = superficial, samples/columns (on the right) 27-39 = muscle invasive. One row=one gene as labelled. Tumour cDNA was competitively hybridised against a panel of 11 cancer cell lines. Red=2-fold or more upregulation, green = down regulation, brown equals no change. Brightness=statistical trust. Gene abbreviations are listed in Table 3.1.



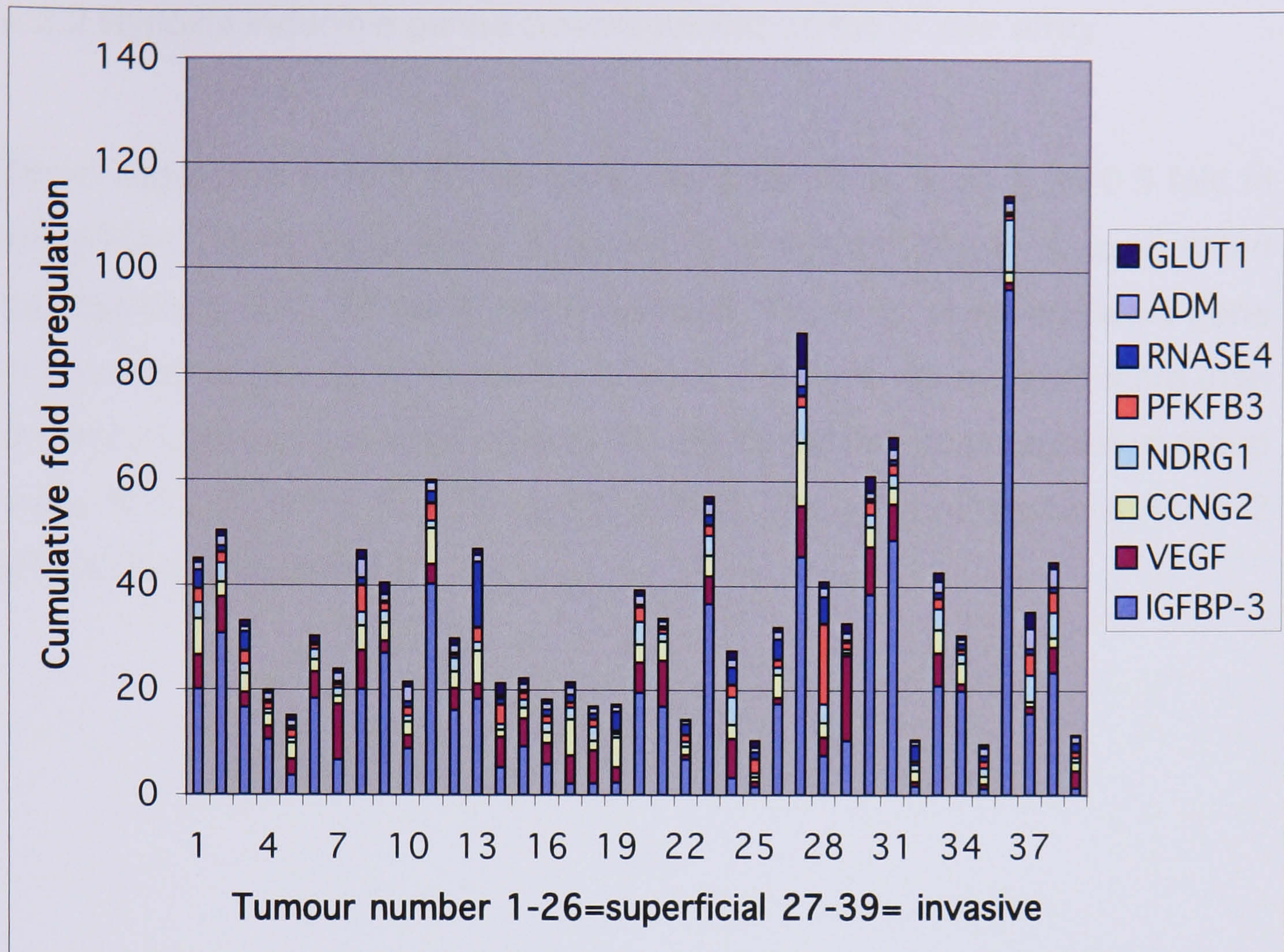


Figure 4.2 Fold changes of hypoxia-inducible genes based on in vivo array data. IGFBP-3 makes the largest fold contribution and is upregulated more than 2-fold in the majority of samples.



#### 4.2.2 Hypoxia inducible genes downregulated on the in vivo array

Down regulation on the in vivo data was defined as more than 0.5 fold in more than 5 tumours (green in Figure 4.1) in vivo. Of 32 genes upregulated by hypoxia in vitro, 24 were downregulated. The most downregulated gene in vivo was lactate dehydrogenase. Genes that were upregulated more than 3-fold by hypoxia, yet were among the top ten of this downregulated group were Bcl-2 Binding Protein NIP3 (NIP3), lysyl hydroxylase isoform 2 (PLOD2) and Aldolase C.



#### 4.2.3 Scoring of tumours for necrosis and hypoxia

To correlate the in vivo transcriptome of these tumours with tumour hypoxia, we scored paraffin sections of the tumours using CA IX stain as a marker of hypoxia. We also scored tumours for the presence of intratumoural necrosis seen with haematoxylin and eosin stain. 31 tumours were available for both scores (Table 4.1). CAIX positive staining was present in 45% of all tumours and appeared to be equally prevalent in superficiales as in invasives. Necrosis became more prevalent with increasing grade and stage. Surprisingly only two of seven necrotic invasive tumours (stage T2 or T3) stained positive for CA IX, suggesting that a significant proportion of invasive bladder cancers do not upregulate CA IX in the perinecrotic regions. Overall 71% of bladder tumours had either CA IX staining or necrosis.



<b>Stage/grade</b>	<b>Total Number</b>	<b>CA9 Positive</b>	<b>%</b>
Ta	8	5	63
T1/G2	7	4	57
T1/G3	6	2	33
T2/G2	4	2	50
T2/G3	4	0	0
T3	4	2	50
<b>All</b>	<b>33</b>	<b>15</b>	<b>45</b>

<b>Stage/grade</b>	<b>Total Number</b>	<b>Necrosis Positive</b>	<b>%</b>
Ta	7	0	0
T1/G2	6	0	0
T1/G3	7	1	14
T2/G2	4	1	25
T2/G3	4	3	75
T3	4	3	75
<b>All</b>	<b>32</b>	<b>8</b>	<b>25</b>

<b>Stage/grade</b>	<b>Total Number</b>	<b>Necrosis and CA9 Positive</b>	<b>%</b>
Ta	7	0	0
T1/G2	6	0	0
T1/G3	6	0	0
T2/G2	4	1	25
T2/G3	4	0	0
T3	4	1	25
<b>All</b>	<b>31</b>	<b>2</b>	<b>6</b>

<b>Stage/grade</b>	<b>Total Number</b>	<b>Necrosis and/or CA9 Positive</b>	<b>%</b>
Ta	7	5	71
T1/G2	7	5	71
T1/G3	6	3	50
T2/G2	4	2	50
T2/G3	4	3	75
T3	4	4	100
<b>All</b>	<b>31</b>	<b>22</b>	<b>71</b>

Table 4.1. Presence of CA IX positive immunohistochemistry and necrosis by stage and grade in bladder tumours taken from the 39 tumours used on the in vivo genearray.



<i>HIF1 score</i>	0	1	2	Total
Ta-T1	6	9	8	23
T2-T3	4	6	1	11
Total	10	15	9	34

Table 4.2 Presence of HIF1 $\alpha$  positive immunohistochemistry by stage in bladder tumours taken from the 39 tumours used on the in vivo genearray. Score 0 means no HIF staining, score 1 and 2 intermediate and strong HIF cytoplasmic staining respectively. There was no significant difference between the proportion of superficial and invasive bladder cancers staining for HIF1 (p=0.26 Chi square test) in terms of the numbers.



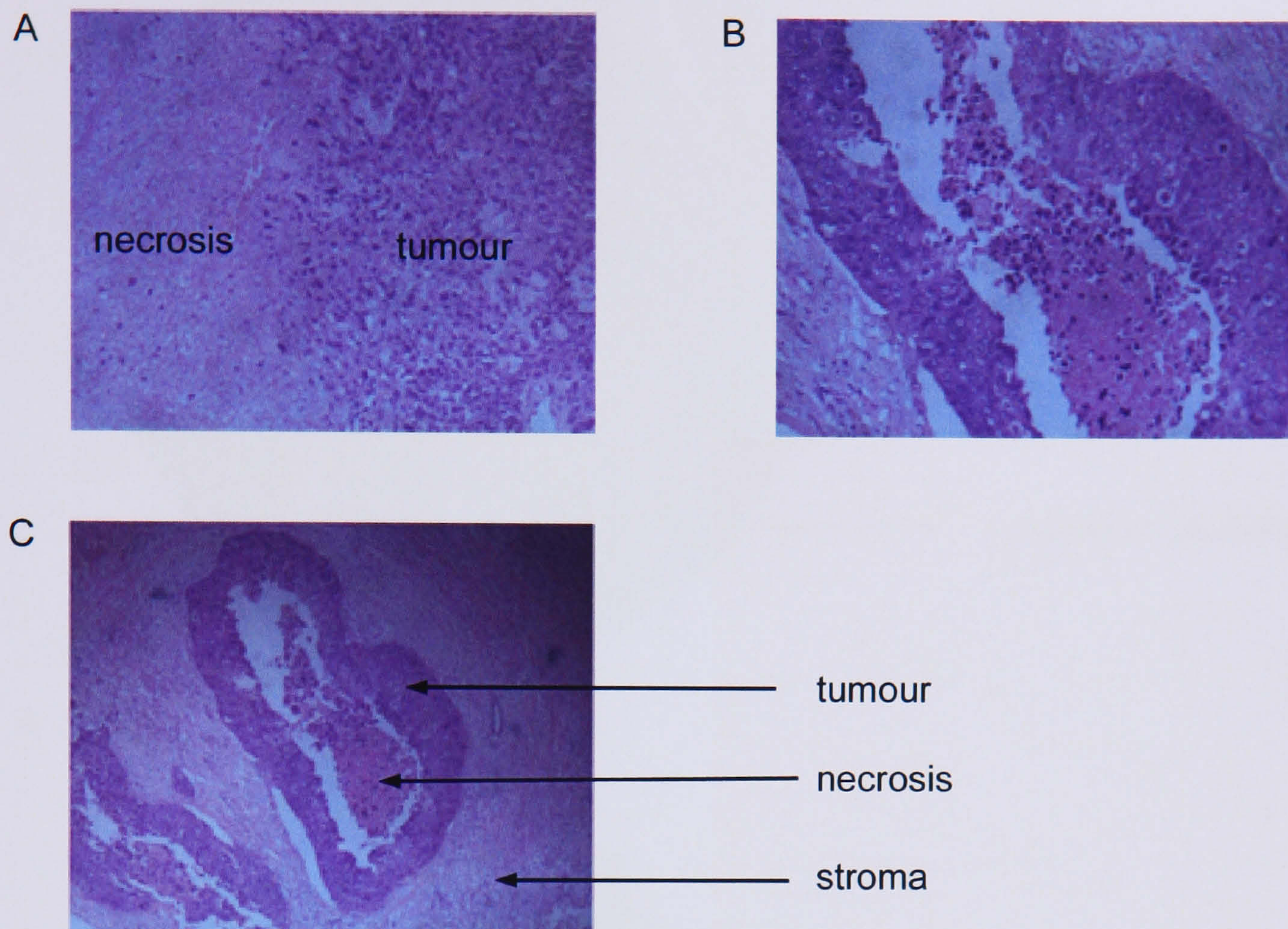
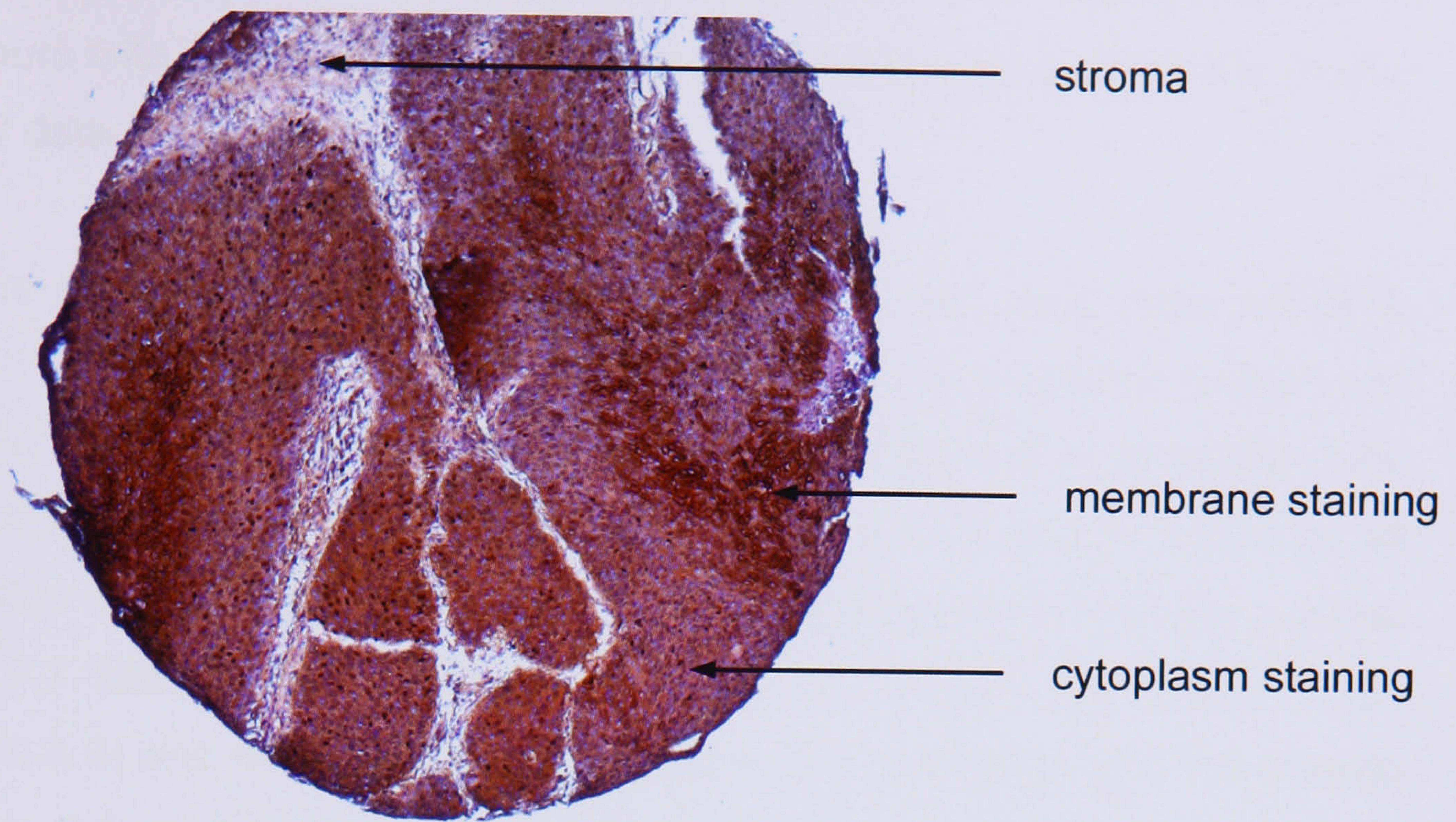
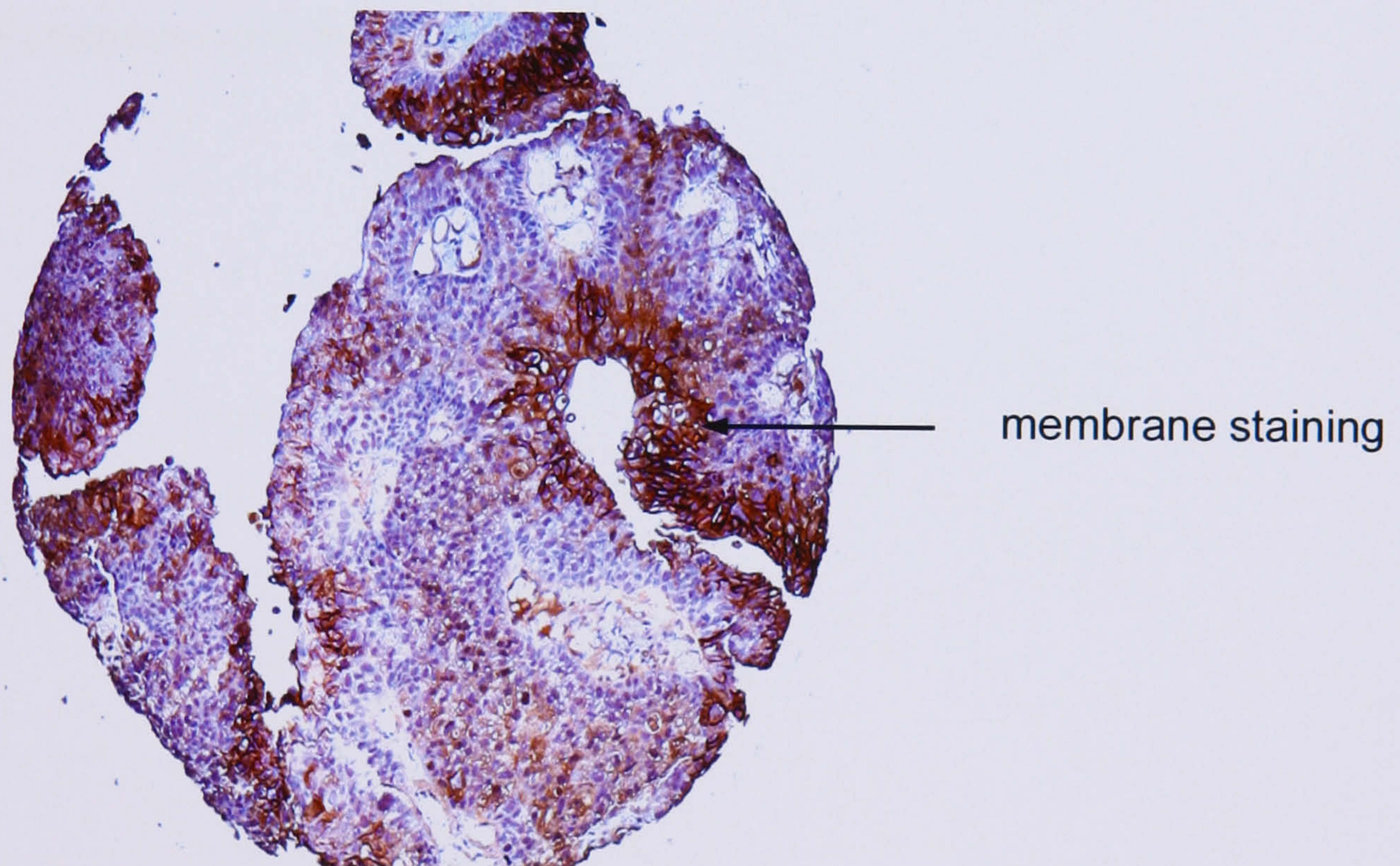


Figure 4.3 Examples of necrosis as seen on haematoxylin and eosin staining. A shows gross necrosis score 2/2. B and C show the same area of comedo necrosis (magnified in B) necrosis score 1 defined as an area <5mm. Necrosis positive tumours were defined as scoring 1 or 2.





A



B

Figure 4.4 Examples of tissue array staining for CA IX both showing areas of intense lattice-like membrane staining. A also demonstrates scattered nuclear staining and intense widespread cytoplasmic staining. Stroma did not stain for CA IX.



#### 4.2.4 Correlation of immunohistochemical CA IX and necrosis scores of tumours with fold-upregulation of hypoxia inducible genes from the in vivo array data

Of the eight most upregulated genes on the in vivo array data; IGFBP3, VEGF, CCNG2, NDRG1, PFKFB3, RNase4, Adrenomedullin (ADM) and Glucose transporter 1 (GLUT-1), only one gene showed a correlation with CA IX scores. None of the gene fold-changes individually or combined all together correlated with necrosis or HIF1 immunohistochemistry scores. GLUT-1 fold-changes correlated with CA IX scores ( $p=0.008$   $r^2=0.21$ , Figure 4.5) and were significantly higher in CA IX positive tumours (means 0.9 vs 1.8  $p=0.03$  2-tailed t-test variances not assumed to be equal). GLUT1 fold changes significantly correlated with ADM fold-changes (Figure 4.5). CA IX itself was not on the genearray to test a correlation with immunohistochemistry or other genes.



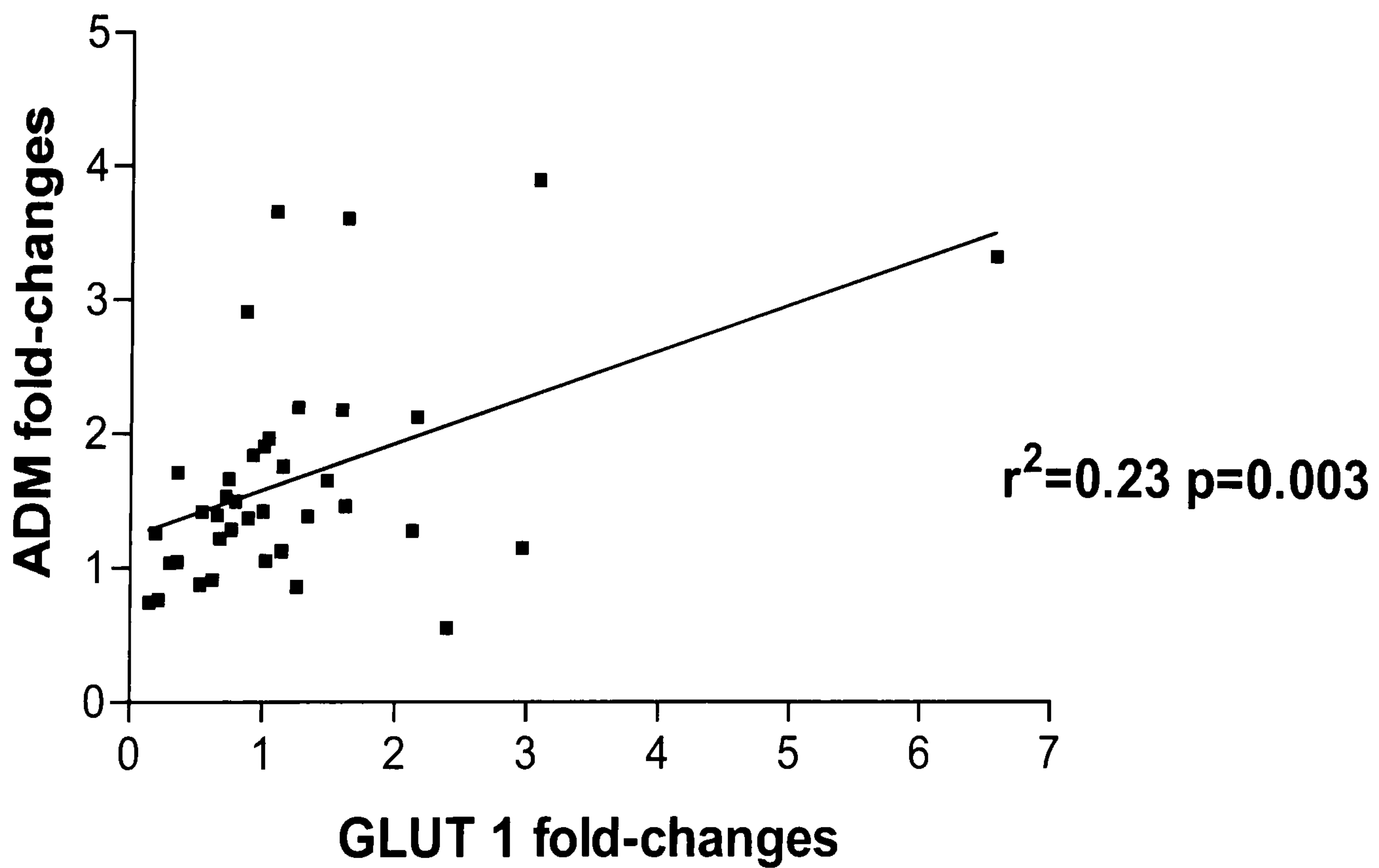
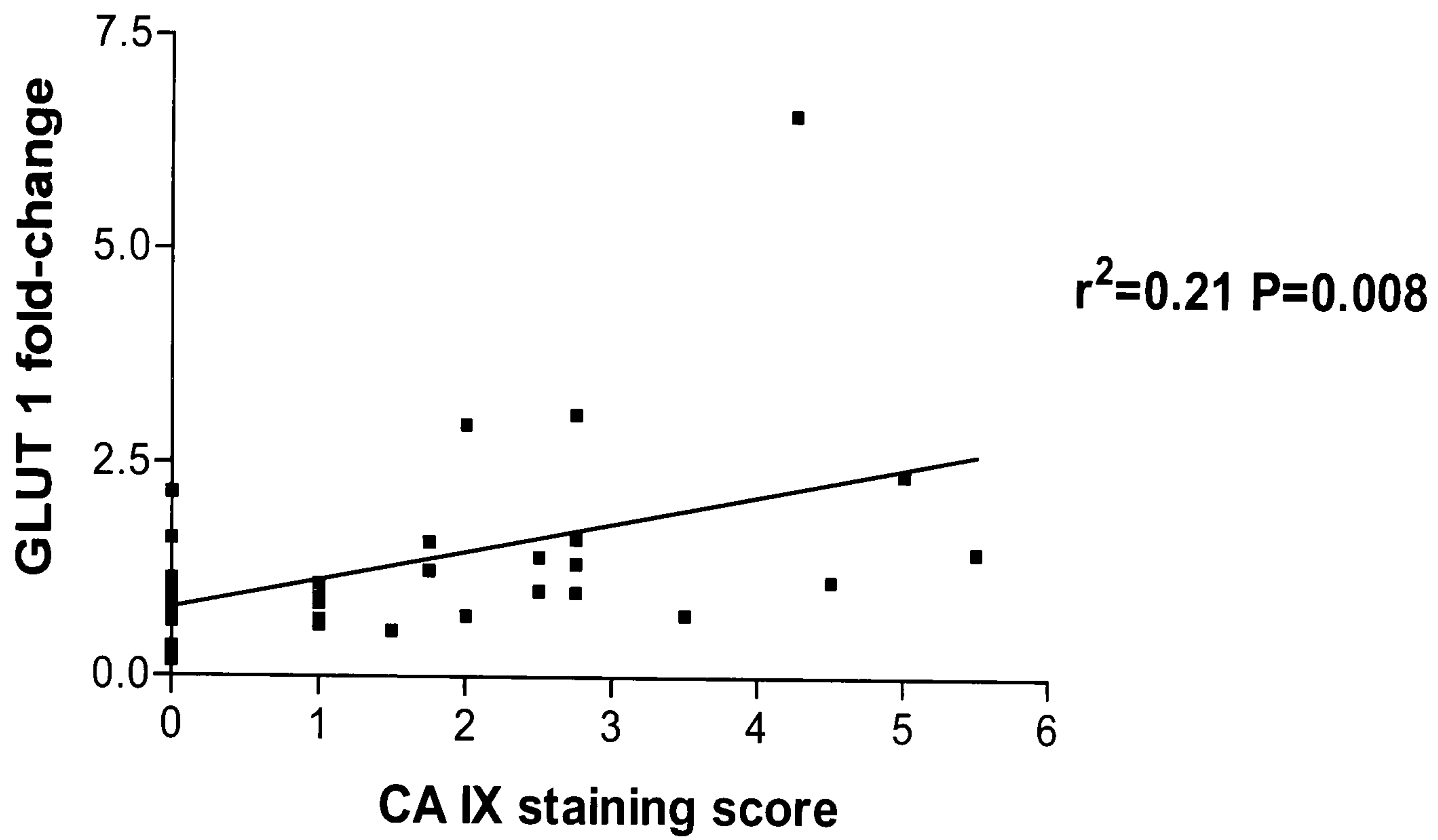


Figure 4.5 GLUT-1 fold-changes in vivo correlated with scores for CA IX staining of tumours by immunohistochemistry as a surrogate marker of hypoxia. ADM fold-changes correlated with GLUT-1 fold-changes. CA IX itself was not on the array.



#### 4.2.5 Analysis of genes upregulated in CA IX positive tumours

Having defined which tumours were CA IX positive, it was possible to split the tumours into two groups and by performing a two-tailed t-test (variances not assumed to be equal) on each gene, and look for genes differentially regulated between the two groups. The result for genes with p values  $<0.25$  is shown in Figure 3.4, Table 3.2. Very few of these genes were upregulated in the CA IX group. By performing a t-test 6000 times, by chance 0.05% or 300 genes will come up as false positive. So although individually there were many significant genes ( $p < 0.05$ ), when the Benjamin and Hochberg correction for multiple t-tests was performed the p values were all  $>0.05$ .



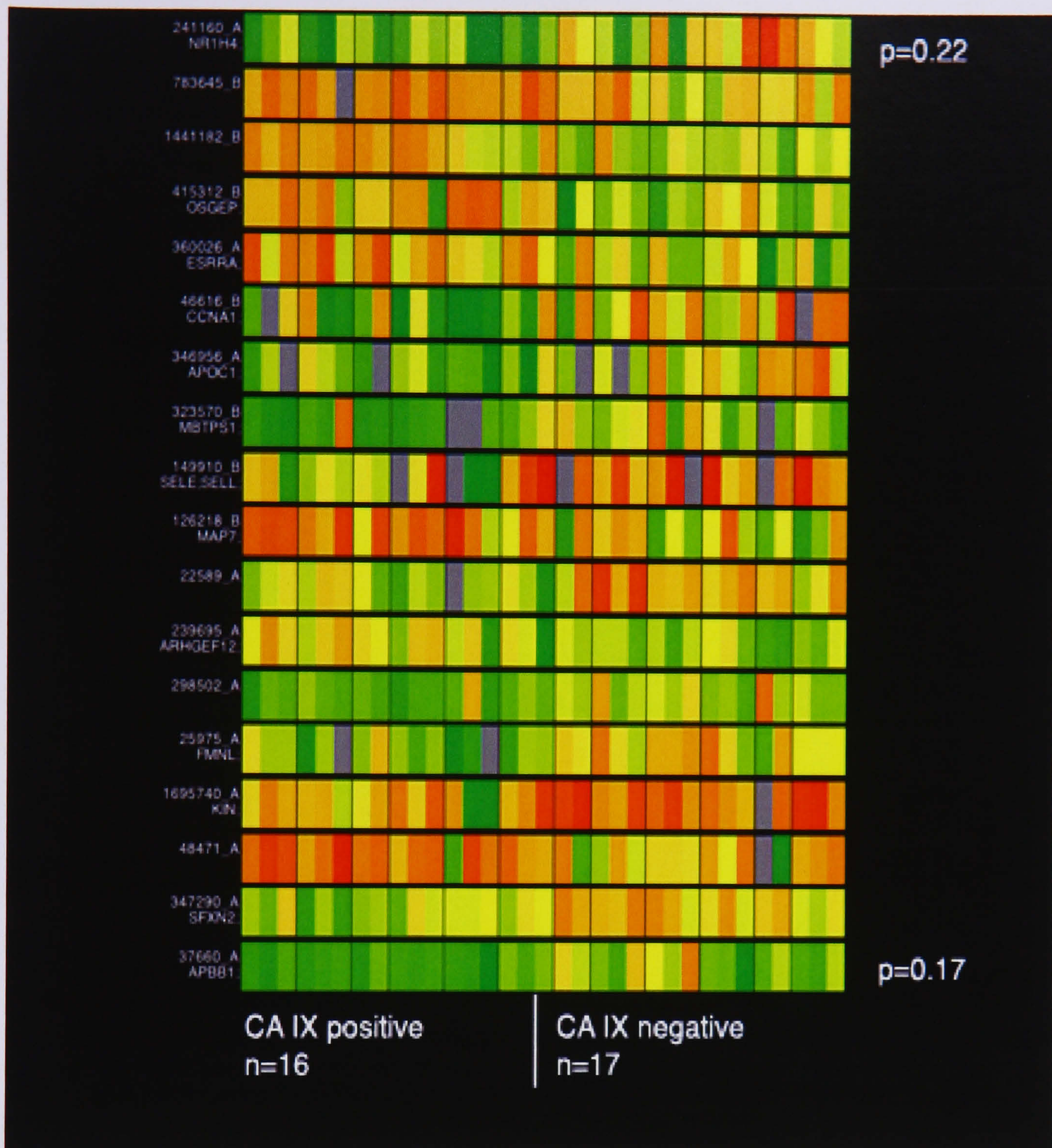


Figure 4.6 Genes differentially regulated in CA IX positive bladder tumours compared with CA IX negative tumours. p values shown (p<0.25 illustrated)

Table 4.2. (next page) Tabulated results of Figure 4.6. Genes upregulated in CA IX positive bladder tumours compared with CA IX negative tumours (total n=33). Superficial and invasive tumours included. p value cut off 0.25. Non-parametric test. Genes upregulated in CA IX positive tumours are in red.



<b>Sanger annotation number</b>	<b>Common name</b>	<b>p value</b>	<b>CA IX negative group average fold-change (range)</b>	<b>CA IX positive group average fold change (range)</b>	<b>GENE</b>
347290_A	SFXN2;	0.17	1.251 (0.78 to 1.817)	0.797 (0.353 to 1.132)	SIDEROFLEXIN 2.
37660_A	APBB1;	0.17	0.767 (0.0585 to 1.84)	0.411 (0 to 0.682)	AMYLOID BETA A4 PRECURSOR PROTEIN-BINDING FAMILY B MEMBER 1
1695740_A	KIN;	0.18	2.588 (1.297 to 4.395)	1.262 (0 to 2.374)	HSKIN17 PROTEIN
48471_A		0.18	1.191 (0 to 1.93)	2.191 (0.457 to 4.545)	POLYPOSIS LOCUS PROTEIN 1 (TB2 PROTEIN).
25975_A	FMNL;	0.21	1.201 (0.624 to 2.069)	0.631 (0 to 1.278)	FORMIN-LIKE PROTEIN (PROTEIN C17ORF1).
298502_A		0.21	0.877 (0.482 to 2.086)	0.562 (0.258 to 1.325)	ENSESTG00002582999
1441182_B		0.23	0.889 (0.466 to 1.559)	1.385 (0.691 to 1.909)	
415312_B	OSGEP;	0.23	0.822 (0 to 1.509)	1.483 (0.348 to 2.459)	O-SIALOGLYCOPROTEIN ENDOPEPTIDASE
360026_A	ESRRA;	0.23	0.857 (0 to 1.655)	1.647 (0.849 to 3.148)	STEROID HORMONE RECEPTOR ERR1 (ESTROGEN-RELATED RECEPTOR, ALPHA) CYCLIN A1.
46616_B	CCNA1;	0.23	1.498 (0.373 to 3.559)	0.557 (0 to 1.605)	
346956_A	APOC1;	0.23	1.195 (0.645 to 2.516)	0.592 (0 to 1.088)	APOLIPOPROTEIN C-I PRECURSOR (APO-CI).
323570_B	MBTPS1;	0.23	0.912 (0.421 to 2.17)	0.541 (0.187 to 2.106)	MEMBRANE-BOUND TRANSCRIPTION FACTOR SITE-1
149910_B	SELE;SEL L;	0.23	5.509 (1.177 to 32.56)	1.253 (0 to 4.46)	E-SELECTIN PRECURSOR (ELAM-1) (LECAM2) (CD62E).
126218_B	MAP7;	0.23	1.052 (0.315 to 2.446)	2.15 (0.841 to 4.283)	MICROTUBULE-ASSOCIATED PROTEIN 7
22589_A		0.23	1.521 (0.22 to 3.599)	0.851 (0.457 to 1.231)	LYSOSOMAL-ASSOCIATED MULTITRANSMEMBRANE PROTEIN(HA1520).
239695_A	ARHGEF1 2;	0.23	0.696 (0 to 1.075)	1.043 (0.317 to 1.587)	RHO GUANINE NUCLEOTIDE EXCHANGE FACTOR (GEF) 12; LEUKEMIA-ASSOCIATED
241160_A	NR1H4;	0.23	1.53 (0.161 to 7.337)	0.519 (0 to 0.95)	BILE ACID RECEPTOR (RXR-INTERACTING PROTEIN 14).
783645_B		0.23	1.266 (0.632 to 2.174)	1.853 (1.331 to 2.865)	ENSG000000119707;



#### 4.2.5 Analysis of genes upregulated in necrotic invasive bladder tumours

Similarly, genes upregulated in necrotic tumours were looked for. Since necrosis was a feature only in invasive cancer (bar 1 superficial cancer), to avoid confounding differences between different stages of disease, this analysis was performed only for invasive cancers. Results in Figure 3.5, Table 3.3 again show genes with significance values  $p < 0.25$ . Again, as with CA IX, after correcting for multiple t-tests, none of the genes show a significant difference ( $p < 0.05$ ).



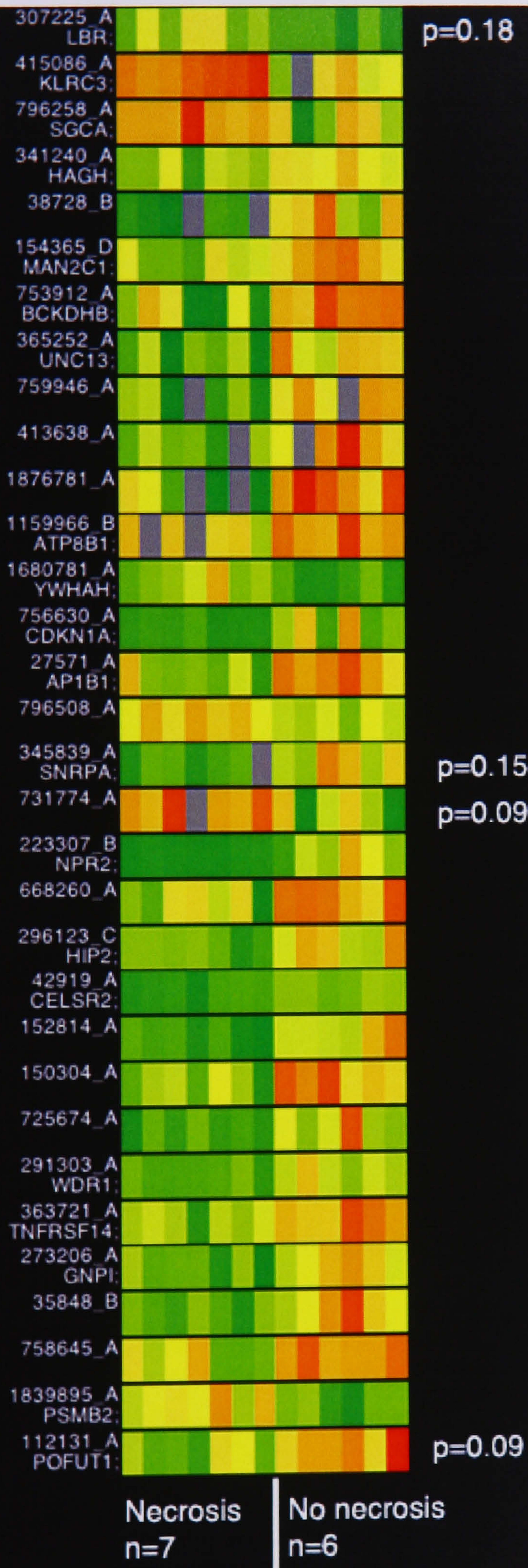


Figure 4.7 Genes differentially regulated in necrotic invasive tumours compared with invasive tumours with no necrosis. Nonparametric test with multiple testing correction. p values shown (p<0.25 illustrated)



<b>Sanger annotation number</b>	<b>Common name</b>	<b>p value</b>	<b>Fold change</b>	<b>No necrosis group average fold change (range)</b>	<b>Necrosis group average fold change (range)</b>	<b>GENE</b>
1839895_A	PSMB2;	0.09	2.00	0.516 (0.234 to 0.718)	1.042 (0.752 to 1.572)	PROTEASOME SUBUNIT BETA TYPE 2
758645_A		0.09		1.645 (1.393 to 2.313)	0.805 (0.556 to 1.328)	RING FINGER PROTEIN 3
363721_A	TNFRSF14;	0.09		1.522 (1.136 to 2.692)	0.735 (0.332 to 0.96)	TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY MEMBER 14).
112131_A	POFUT1;	0.09		1.743 (1.095 to 6.198)	0.704 (0.44 to 1.074)	GDP-FUCOSE PROTEIN O-FUCOSYLTRANSFERASE 1 (O-FUCT-1)
150304_A		0.09		1.577 (1.07 to 2.888)	0.622 (0.242 to 0.97)	ENSESTG00001361480;
296123_C	HIP2;	0.09		1.091 (0.78 to 1.654)	0.564 (0.297 to 0.711)	UBIQUITIN-CONJUGATING ENZYME E2-25 (HIP-2)
35848_B		0.09		1.277 (0.808 to 3.033)	0.54 (0.295 to 0.699)	ALPHA GLUCOSIDASE II ALPHA SUBUNIT
291303_A	WDR1;	0.09		0.876 (0.692 to 1.189)	0.53 (0.393 to 0.675)	WD-REPEAT PROTEIN 1 (ACTIN INTERACTING PROTEIN 1) (AIP1)
668260_A		0.09		1.728 (1.109 to 2.562)	0.452 (0.01 to 1.095)	
725674_A		0.09		0.985 (0.697 to 2.536)	0.425 (0.231 to 0.629)	MUF1 PROTEIN
42919_A	CELSR2;	0.09		0.685 (0.591 to 0.769)	0.337 (0.127 to 0.504)	CADHERIN EGF LAG SEVEN-PASS G-TYPE RECEPTOR 2
273206_A	GNPI;	0.09		1.103 (0.833 to 1.572)	0.316 (0.01 to 0.782)	GLUCOSAMINE-6-PHOSPHATE ISOMERASE
152814_A		0.09		1.079 (0.825 to 1.862)	0.234 (0.01 to 0.581)	
223307_B	NPR2;	0.09		0.792 (0.408 to 1.36)	0.165 (0.107 to 0.248)	ATRIAL NATRIURETIC PEPTIDE RECEPTOR B PRECURSOR(ANPRB)
731774_A		0.15	4.30	0.412 (0.045 to 1.222)	1.777 (1.251 to 3.481)	ENSG00000156787;



345839_A	SNRPA;	0.15	1.01 (0.687 to 1.733)	0.384 (0.215 to 0.558)	U1 SMALL NUCLEAR RIBONUCLEOPROTEIN A
415086_A	KLRC3;	0.18	0.997 (0.687 to 1.387)	2.448 (1.521 to 4.571)	NKG2-E TYPE II MEMBRANE PROTEIN (NK CELL RECEPTOR E)
796258_A	SGCA;	0.18	0.752 (0.2 to 1.339)	1.885 (1.263 to 10.58)	ALPHA-SARCOGLYCAN PRECURSOR (ADHALIN) (50DAG)
796508_A		0.18	0.828 (0.622 to 0.996)	1.172 (0.975 to 1.434)	ENSG00000052749;
1159966_B	ATP8B1;	0.18	1.757 (1.323 to 3.613)	1.072 (0.754 to 1.251)	POTENTIAL PHOSPHOLIPID-TRANSPORTING ATPASE IC
307225_A	LBR;	0.18	0.443 (0.233 to 0.631)	0.797 (0.596 to 1.04)	LAMIN B RECEPTOR (NUCLEAR ENVELOPE PROTEIN) (LMN2R).
154365_D	MAN2C1;	0.18	1.458 (1.059 to 2.168)	0.785 (0.502 to 1.071)	ALPHA-MANNOSIDASE 2C1
1680781_A	YWHAH;	0.18	0.37 (0.221 to 0.619)	0.769 (0.534 to 1.324)	14-3-3 PROTEIN ETA (PROTEIN AS1).
341240_A	HAGH;	0.18	1.05 (0.958 to 1.277)	0.733 (0.428 to 0.973)	HYDROXYACYLGLUTATHIONE HYDROLASE
27571_A	AP1B1;	0.18	1.67 (1.048 to 2.786)	0.673 (0.346 to 1.253)	ADAPTER-RELATED PROTEIN COMPLEX 1 (BETA-ADAPTIN 1)
413638_A		0.18	1.932 (0.969 to 10.73)	0.614 (0.34 to 0.9)	ENSESTG00002918787;
756630_A	CDKN1A;	0.18	0.779 (0.454 to 1.605)	0.329 (0.209 to 0.478)	CYCLIN-DEPENDENT KINASE INHIBITOR 1 (P21) (MDA-6).
38728_B		0.18	1.111 (0.641 to 2.163)	0.302 (0.11 to 0.507)	TRAF2 AND NCK INTERACTING KINASE
753912_A	BCKDHB;	0.18	1.815 (1.194 to 3.145)	0.278 (0.01 to 1.259)	2-OXOISVALERATE DEHYDROGENASE BETA SUBUNIT
759946_A		0.18	1.303 (1.02 to 1.599)	0.274 (0.01 to 0.866)	
365252_A	UNC13;	0.18	1.188 (0.841 to 1.912)	0.205 (0.01 to 0.893)	UNC13 (C. ELEGANS)-LIKE; HOMOLOG OF RAT MUNC13).
1876781_A		0.18	2.274 (1.105 to 6.7)	0.141 (0.01 to 1.095)	ENSESTG00001227010;



Table 4.3 Tabulated results of Figure 4.7. Genes differentially regulated in necrotic invasive tumours compared with invasive tumours with no necrosis. Nonparametric multiple t-tests with multiple testing correction, corrected p values shown ( $p < 0.25$  illustrated). Genes upregulated in necrosis in red.



### 4.3 Discussion

In this chapter 32 hypoxia-upregulated genes in vitro have been further investigated for their role in bladder cancer biology by analyses of in vivo array data and in vivo staining for necrosis and hypoxia marker CA IX.

#### 4.3.1 Hypoxia inducible genes upregulated on the in vivo array

On the tumour array, 70 of 6000 (1.2%) genes were upregulated more than 2-fold in over 30 tumours. 3 of these 70 genes (4.3%) were hypoxia-inducible on the in vitro array (IGFBP-3, VEGF, CCNG2). Since only 32 of 6000 (0.53%) genes on the in vitro array were upregulated by hypoxia, hypoxia-induced genes are over-represented in the group of genes most upregulated on the tumour array ( $p < 0.01$  Chi<sup>2</sup> test). If the 32 hypoxia-induced genes were distributed at random we would have expected only 0.53% of 70 i.e. less than one gene to appear in this group of 70. Genes upregulated on the in vivo array were also the most hypoxia inducible genes on the list (Figure 4.4)

IGFBP3 is discussed elsewhere (see chapter 3 and 5) and VEGF is well described in bladder cancer, However the in vivo array data here highlighted the potential importance of Cyclin G2 in bladder cancer. Cyclin G2 is known to be regulated by the VHL/hypoxia pathway (Wykoff, Beasley et al. 2000). It is an unconventional cyclin highly expressed in the immune system (spleen and thymus) as well as kidney, cerebellum and prostate (Horne, Goolsby et al. 1996). It is thought to be a negative regulator of the cell cycle with peak expression in S phase. Transfection of Chinese Hamster Ovary (CHO) cells and HEK293 fibroblasts with it stimulates apoptosis (Bennin, Don et al. 2002). In murine B cells expression is upregulated by DNA damaging agents (Bates, Rowan et al. 1996) and negative growth factors independent of p53 (Horne, Donaldson et al. 1997). Cyclin G2 RNA levels have been shown to



be significantly expressed at a lower level in stage T2 bladder tumours than in Ta by microarray analysis validated by immunohistochemistry on a tissue array (Dyrskjot, Thykjaer et al. 2003). Cyclin G2 was also upregulated in Ta tumours compared to normal tissue. This contrasts with the result on the tumour array here, where there was no significant difference in Cyclin G2 mRNA fold change from Ta to T2 tumours.

The top three genes upregulated in vivo were upregulated more than 2-fold in more than 30 of 39 tumours each. As 71% of the tumours on staining had some evidence of hypoxia (necrosis or CA IX staining) it is possible that the upregulation of these genes is driven by intratumoural hypoxia. However the upregulation seen across all tumours (Figure 4.1) suggests that other factors may play a role.

Oncogenic activation of these genes may occur in bladder cancer via HIF or HIF independent mechanisms. Upregulation of HIF-1 $\alpha$  is known to occur by tumour suppressor genes (mutant p53 or PTEN) and oncogenes (H-ras). The HER2 oncogene, which overexpresses a tyrosine-kinase domain on the EGF receptor, is one potential candidate as the protein is over expressed in 57% of invasive bladder cancers (Latif, Watters et al. 2003; Latif, Watters et al. 2004) although gene amplification occurs only in 5%. In vivo array data here demonstrated HER2 upregulation more than 2-fold in 22 of 39 of the bladder tumour samples. Interestingly in an in vitro genearray study of HER2-overexpressing breast carcinoma cells the hypoxia inducible genes IGFBP-3 NDRG1 and P4HA were all upregulated (Mackay, Jones et al. 2003). These three genes are clearly hypoxia upregulated in our bladder cancer cell line and IGFBP3 and NDRG1 are clearly upregulated on the in vivo array data. In vitro data on breast cell lines has shown that overexpressing HER2 increases expression of HIF-1 $\alpha$  and VEGF (Laughner, Taghavi et al. 2001). Immunohistochemical staining for HER2 in breast cancer is associated with HIF1 staining and high levels of angiogenesis, however HER2 breast



cancers only conferred a significantly poorer prognosis if they also stained for HIF-1 $\alpha$  (Giatromanolaki, Koukourakis et al. 2004). If activation of HER2 in humans results in overexpression of HIF-1 $\alpha$  independently of conditions of hypoxia, as occur in experimental studies, this interaction may represent a main pathway conferring clinical aggressiveness to HER2 positive bladder tumors.

The across the board upregulation on these genes on the in vivo array may be highlighting gene expression differences between monolayer culture and the three-dimensional tumour environment where there are more complex influences on gene expression present. Three-dimensional geometry in in vitro cell growth alone has been shown to have dramatic influences on gene expression profile (Dangles, Lazar et al. 2002) responses of cells to differing stimuli and biological function (Mansbridge, Ausserer et al. 1994; Dangles, Femenia et al. 1997; Huang and Ingber 1999; Lang, Sharrard et al. 2001). Primary tumours have added complexity from angiogenesis, stromal interactions and inflammation.

#### 4.3.2 Hypoxia inducible genes downregulated on the in vivo array

Of 32 genes upregulated by hypoxia in vitro, 24 were downregulated more than 0.5-fold in more than 5 tumour samples (green) on the in vivo array (Figure 4.1, Figure 4.4). That this occurred even in tumours where necrosis and CA IX staining were present was surprising. LDH-C, NIP3, Aldolase C, and PLOD2 were the most downregulated genes. LDH-C is the testis-specific member of the family of LDH that is expressed in normal testicular tissue but not in other normal tissues. However in cancer and cell lines it has significant expression levels in virtually all human tumours tested (Koslowski, Tureci et al. 2002). Although bladder cancer was not tested true down regulation of LDH-C in bladder cancer is unlikely. LDH A is known to be hypoxically regulated, with a gene promoter that contains an essential binding site for



HIF (Semenza, Jiang et al. 1996). This raises the question of why these genes are downregulated compared to the normoxic cell line panel. The cell lines may have basal upregulation of hypoxia-inducible genes in normoxia due to oncogenic changes. The in vivo downregulated genes were in fact the hypoxically lesser upregulated genes (Table 4.4), so if they were only mildly basally upregulated in the cell lines this would obscure their in vivo upregulation.

Aberrant spots on the array could also produce an artifactual result, however three of these most downregulated genes were represented by 2 or 3 array spots each, so that is unlikely.

Table 4.4 (next page) Replication of Table 3.1 showing the genes upregulated on the in vivo array in red. Genes downregulated on the in vivo array are in black. Clearly the genes that are more sensitive to hypoxia (greater fold upregulation near the top of the list) were more likely to be upregulated on the in vivo array.



No.	Fold-change at hypoxia		GENE	FUNCTIONAL GROUP
	16 Hours	24 Hours		
<b>Known to be hypoxia-upregulated in other cell lines</b>				
1	3.4	17	Adrenomedullin (ADM)	Vascular Tone
2	2.6	12.1	Insulin-like growth factor binding protein 3 (IGFBP-3)	Apoptosis
3	2.9	8.2	n-myc downstream regulated protein 1 (NDRG1)	Cell Cycle
4	5.3	6.6	BCL2 binding protein NIP3 (NIP3)	Apoptosis
5	2.7	4.7	Inducible 6-phosphofructo-2-kinase (PFKFB3)	Glucose Metabolism
6	2.4	4.7	Lysyl Hydroxylase (PLOD2)	Tissue remodeling
7	2.2	4.4	MAX interacting protein 1 (MAX 1)	Transcription factor
8	3.1	4.2	Glucose Transporter 1 (GLUT 1)	Glucose metabolism
9	2.3	3.9	1,4-Alpha Glucan Branching Enzyme (HGBE)	Glucose Metabolism
10	2.2	3.8	Cyclin G2 (CCNG2)	Cell Cycle
11	2.5	3.7	Proline-4-hydroxylase (P4HA1)	Oxygen sensing/ECMM
12	3.2	3.2	Adenylate Kinase 4	Nucleotide metabolism
13	2.9	3.1	Aldolase C (ALDO C)	Glucose Metabolism
14	1.9	3	Prolyl-4-hydroxylase alpha subunit	Oxygen sensing
15	1.8	2.6	Haem oxygenase 1 (HMOX1)	Vascular Tone
16	2.5	2.5	EGL9 Homologue 1	Oxygen sensing
17	2.1	2.5	Testis-specific lactate dehydrogenase (LDH C)	Acid Base
18	1.9	2.5	VEGF	Angiogenesis
19	2.4	2.5	Adenylate Kinase 3 (AK3)	Nucleotide metabolism
20	1.7	2.4	Dual Specificity Phosphatase 1 (DUSP1/CL100/MKP1)	Cell signalling
21	1.7	2.1	DEC 1	Differentiation
22	2.6	2.1	Glucose-6-Phosphate isomerase (GPI)	Glucose Metabolism
23	2.1	2.1	Fructose-Bisphosphate Aldolase A	Glucose Metabolism
<b>Not previously known to be hypoxia regulable</b>				
24	2.8	8.2	Solute carrier family 16 member 3 (SLC16A3)	Acid Base
25	4.5	4.9	Preferentially expressed antigen of melanoma (PRAME)	Antigen
26	2.8	4.1	Importin Beta 3	Nuclear pore protein
27	1.9	3.1	RNAse 4	Antimicrobial/?Angiogenic
28	2.2	2.9	Protooncogene ABL1	Cell cycle



ESTs				
29	3	3.9	50378_A	EST
30	3.2	3.3	50022_A	EST
31	3.5	3.3	31564_B	EST
32	2.1	2.4	21875_A	EST

Table 4.4 (above) Replication of Table 3.1 showing the genes upregulated on the in vivo array in red. Genes downregulated on the in vivo array are in black. Clearly the genes that are more sensitive to hypoxia (greater fold upregulation near the top of the list) were more likely to be upregulated on the in vivo array.

#### 4.3.3 Correlation of immunohistochemical CA IX and necrosis scores of tumours with fold-upregulation of hypoxia inducible genes from the in vivo array data

GLUT1 and showed a significant correlation with CA IX score as a surrogate marker of hypoxia. GLUT1 and ADM also showed significant correlation with each other. GLUT 1 protein immunohistochemistry has previously been shown to correlate with CA IX staining in cervical cancer and, like CA IX, has been shown to be a marker of tumour hypoxia (Airley, Loncaster et al. 2001; Airley, Loncaster et al. 2003). The correlation here shows that the same is likely also to be true for RNA transcripts in bladder cancer. ADM, a secreted protein, may also prove to be useful as a marker of hypoxic tumours in the urine. The other genes did not correlate with tumour hypoxia and necrosis scores and were upregulated in many more tumours. This was surprising as we previously showed that VEGF protein staining correlated with CA IX



staining in bladder cancer (Turner, Crew et al. 2002). We might have expected all 8 genes significantly upregulated by hypoxia in vitro and in vivo to correlate with tumour scores of hypoxia and necrosis in vivo. However as discussed in 4.3.1 oncogenic changes specific to bladder cancer, but not found in the majority of the cell line panel, may contribute; as well as the complex gene expression difference between monolayer cell culture and the three-dimensional tumour environment: In particular the lack of blood vessels, stromal interactions and inflammation in monolayer cell culture.

These results show that there is significant concordance of genes induced by hypoxia in vitro and in vivo in spite of differences in tissue complexity and that there is concordance in vivo for a subset of hypoxia-induced genes (ADM, GLUT1 and CA IX protein), which may provide a hypoxia signature for analysis of gene array data. Hypoxia-regulated genes are disproportionately upregulated in primary bladder cancer. The induction of putative pro-apoptotic, cell cycle arrest pathways commonly occurs (IGFBP-3 and CCNG2) as well as angiogenic pathways (VEGF). Many hypoxia-induced genes (NIP3, PLOD2, Aldolase C) appear downregulated in vivo but this may be due to basal upregulation in cancer cell lines.

#### 4.3.4 Addendum to Chapter Four added June 2008:

Since working on this thesis there has been a recent publication on identifying hypoxia regulated genes on in vivo cancer samples. In agreement with Chapter Four in this thesis, they found that grouping tumours according to immunohistochemical CA IX and HIF 1 staining was not fruitful in identifying known hypoxia related genes. However by clustering genes around a group of 10 well characterised hypoxia regulated genes in vitro, 99 correlated genes were identified 27% of which were known to be hypoxia regulated from the literature (validating the method). Furthermore on multivariate analysis this cluster of 99 genes was found to have independent prognostic value in 59 head and neck cancers (RR 1.96 CI 1.16 to 3.33) with



16 months follow-up and in 245 breast cancers (RR 1.38 CI 1.11 to 1.70) with 7 years follow-up. Pearson correlation was used within the framework of Significance Analysis of Microarrays (SAM). Genes were considered significantly correlated to the core of 10 genes if they appeared in more than 50% of the clusters. (Winter, Buffa et al. 2007).

Subsequent to this paper Moller-Levet has robustly described a new form of statistical analysis called Enhanced correlation, which outperforms Pearson correlation for microarray data. Essentially this method exploits the information from biological samples more efficiently by first calculating inter-sample similarities within a dataset and then integrating this additional information into the original gene expression data. (Moller-Levet, West et al. 2007)



## **Chapter Five**

**Measurement of IGFBP-3 and Angiogenin protein in the urine of bladder cancer patients, measurement of IGFBP-3 in cell culture supernatants, and in vitro viability of bladder cancer cell line RT112 and HUVE cells under hypoxia**



## 5.1 Introduction

The results of the preceding two chapters confirmed that mRNA for IGFBP3 and RNase4 was induced by hypoxia in all bladder cancer cell lines tested and in normal urothelium. This chapter concentrates on the measurement of protein. Since IGFBP3 is a secreted protein, levels for it were assessed in the urine of bladder cancer patients. RNase4 protein may also be secreted however no commercially available ELISA for it exists. However it shares similar 5'untranslated region on its mRNA with the secreted protein Angiogenin with implications for the similar transcriptional control. Angiogenin like RNase4, is an RNase that has been described as hypoxia inducible in other cell lines. It seemed likely therefore that Angiogenin protein would also be upregulated in the urine of bladder cancer patients. Furthermore a commercial ELISA did exist for Angiogenin. Levels of IGFBP3 protein were also measured in cell culture supernatant of RT112 cells as this cell line had showed the greatest fold upregulation of its mRNA (38-fold). HUVEC cells were also examined as an example of an untransformed cell line in which upregulation of IGFBP3 mRNA had also been confirmed (7-fold). In case of any confounding effect of cell death under hypoxia the cell viability under hypoxia of these two cell lines was also assessed by the MTS assay.



## 5.2 Results

### 5.2.1 Validation of the IGFBP3 ELISA on urine

In order to ensure that urine was not affecting the recovery of IGFBP3 protein by ELISA, neat IGFBP3 protein was added to deionised water, cell media and urine (Figure 5.1). The fetal calf serum in the cell media may have contained IGFBP3, which would account for the small rise above baseline. The urine contained approximately 20 ng/ml IGFBP3 and recovery paralleled that seen in deionised water.

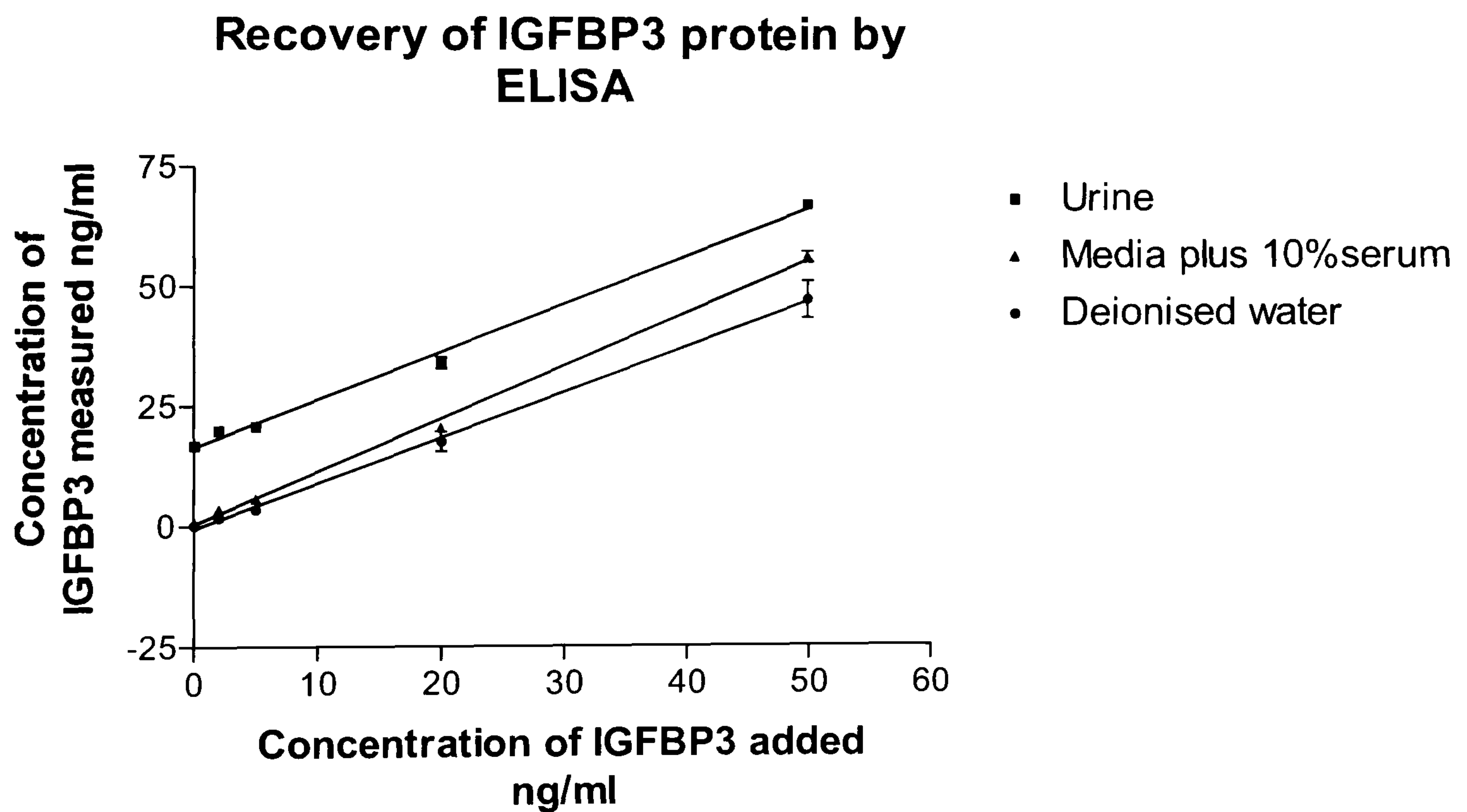


Figure 5.1 Recovery of IGFBP3 proteins by ELISA.



### 5.2.2 Levels of IGFBP3 in the urine of bladder cancer patients

IGFBP-3 at the transcript level was upregulated higher and more frequently than VEGF in both in vitro and in vivo arrays. We therefore measured levels of IGFBP-3 protein in the urine (Figure 5.2). Urine from 157 bladder cancer patients and controls was collected prospectively and IGFBP-3 levels measured by ELISA. There was a significant increase in urinary IGFBP-3 concentration from control/clear groups to superficial cancers to invasive cancers,  $p < 0.01$  for superficial vs. control/clear,  $p < 0.01$  for invasive vs. control/clear [IGFBP-3 ng/ml mean (standard deviation); controls 15.2ng/ml (7.55); clears 14.6ng/ml (8.1); superficial 27.7ng/ml (29); invasive 55.1ng/ml (40)]. After correction for urinary creatinine significance ( $p < 0.01$ ) was retained for invasive tumours, but not for the superficial group. There were 15 stage T1G3 tumours in the superficial group. There was no significant difference between levels in the urine of T1G3 tumours and other superficial tumours or with the invasive group. If a single exceptionally high ( $>4$  standard deviations) level in the superficial group is excluded the mean corrected levels of urine IGFBP-3 increase with stage and grade; superficial stage T1G2 or lower, 351 ng/mmol Cr; superficial stage T1G3, 479 ng/mmol Cr; invasive stage T2-T4, 733 ng/mmol Cr.

One of the anonymously taken medical student controls had a level of IGFBP-3 five standard deviations higher than the rest of this group. Despite this the significant difference between this group and the bladder cancer group held. All medical student control urines dipped negative for blood so an undiagnosed cancer is unlikely in such a young person. The likely explanation is an exercise induced rise in IGFBP-3 (Manetta, Brun et al. 2002).



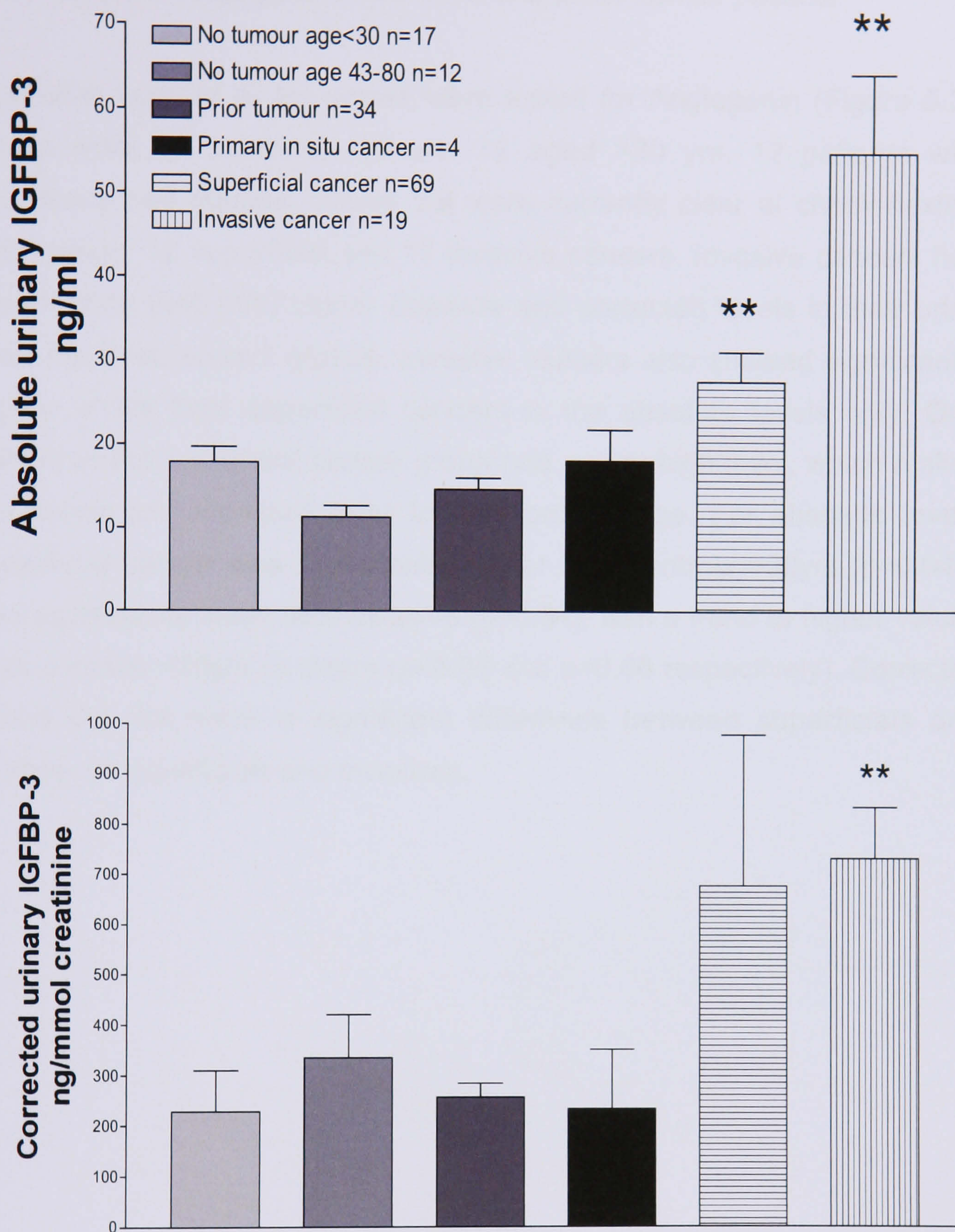


Figure 5.2 Levels of IGFBP-3 in urine: TOP absolute level, BOTTOM corrected level for urine creatinine. Invasive significantly higher than all control groups in both cases. Superficial significantly higher than control groups for absolute levels only. (\*\*=p<0.01). Error bar = standard error.



### 5.2.3 Levels of Angiogenin in the urine of bladder cancer patients

A smaller number of the urines were tested for Angiogenin (Figure 5.3). There were 17 controls <30 yrs, 12 aged >30 yrs, 12 patients who previously had bladder cancer but were currently clear at check flexible cystoscopy, 12 superficial and 17 invasive cancers. Invasive cancers had significantly ( $p < 0.001$ ) higher absolute and corrected levels in their urine than the three control groups. Invasive cancers also showed significantly higher levels than superficial cancers in the absolute levels only. One patient in the superficial cancer group had a very high level, which makes the mean and standard error for this group large. For absolute levels superficial cancer was significantly higher than controls >30yrs ( $p = 0.045$ ) and significantly lower than invasive ( $p = 0.04$ ), with a trend to higher values than controls <30yrs or clears ( $p = 0.05$  and  $p = 0.06$  respectively). Corrected levels did not show a significant difference between superficiales and controls or superficiales and invasives.



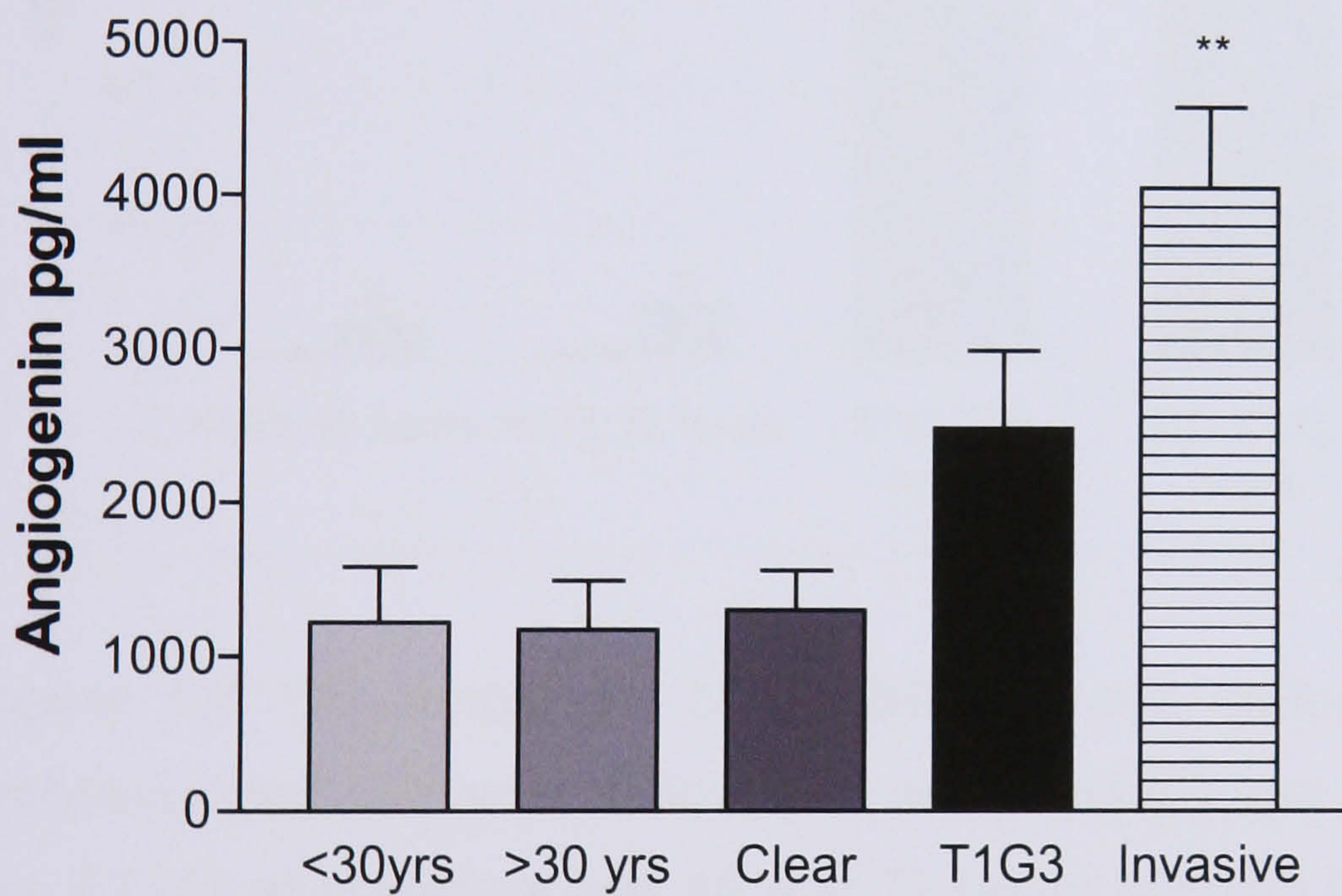
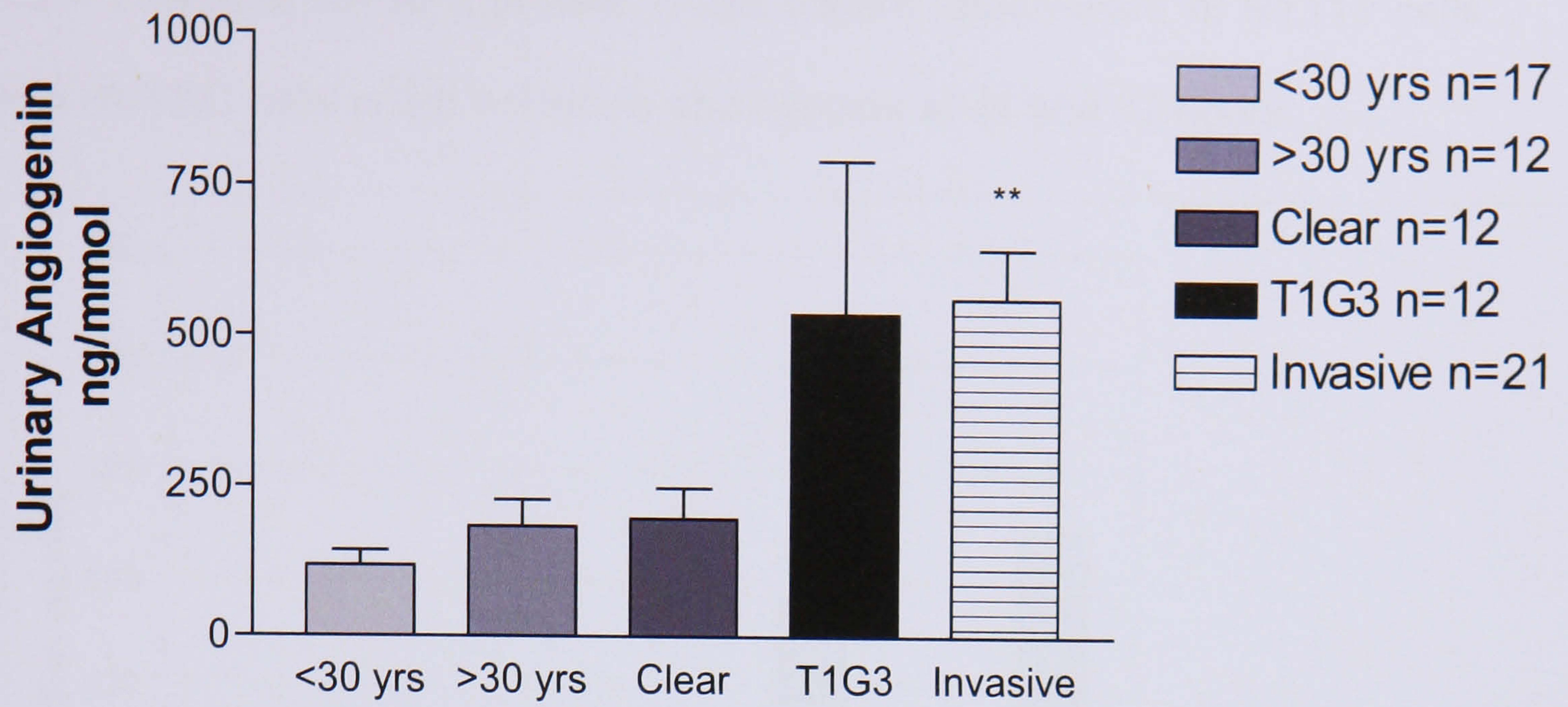


Figure 5.3 Levels of Angiogenin in urine: BOTTOM absolute level, TOP corrected level for urine creatinine. Invasive significantly higher than all control groups in both cases (\*\*= $p < 0.01$ ). T1G3 significantly higher than control group (>30 yrs old) for absolute levels only ( $p = 0.04$ ). Error bar = standard error.



#### 5.2.4 Levels of IGFBP3 protein in cell culture supernatant of RT112 cells and HUVEC cells under normoxia and hypoxia at 42 and 72 hours

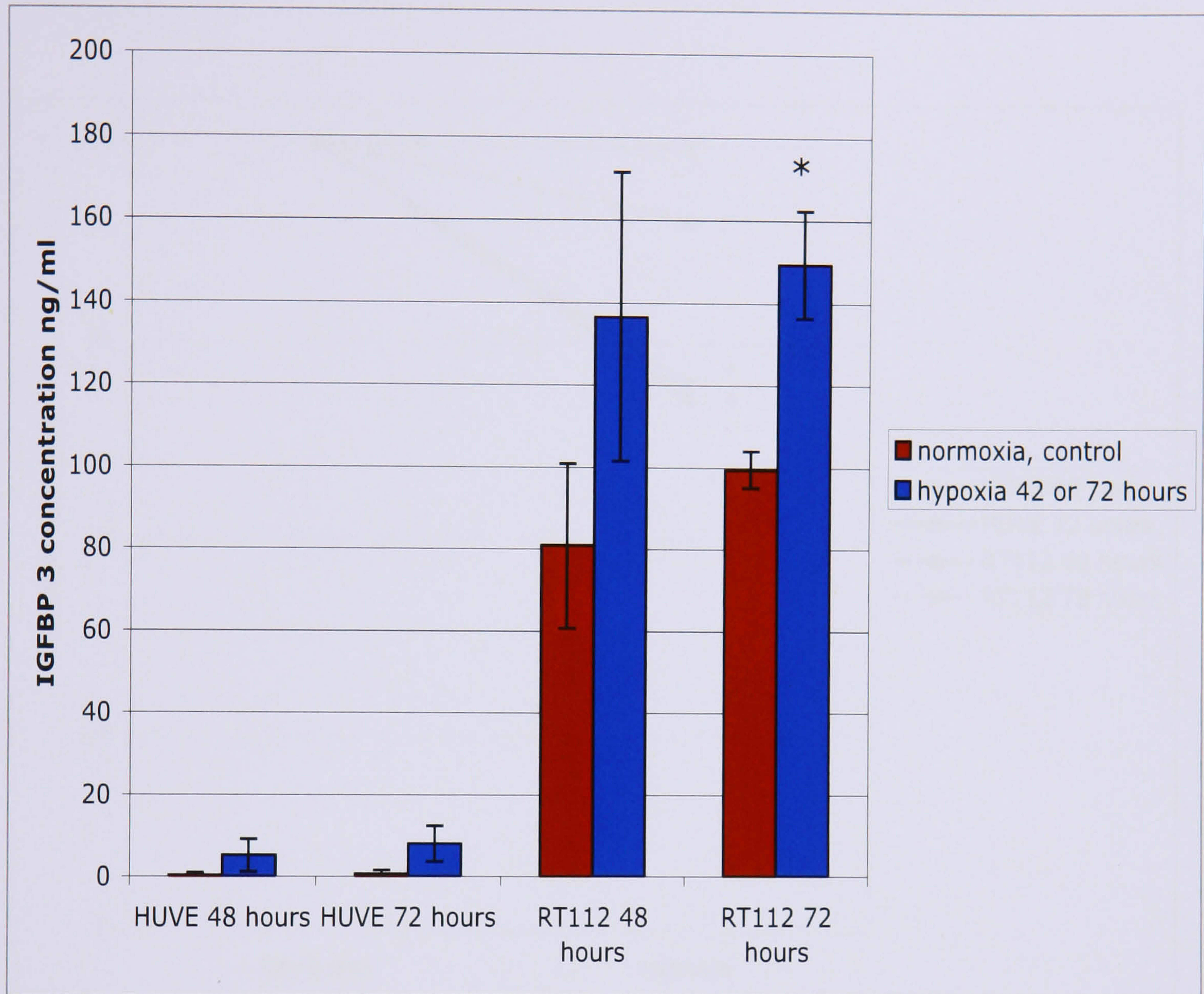


Figure 5.4 Concentration of IGFBP3 in cell culture supernatant of untransformed human umbilical vein cells (HUVE) and bladder cancer cell line RT112 at normoxia and 48 and 72 hours hypoxia (0.1% oxygen). The normoxic and hypoxic levels of IGFBP3 in HUVE supernatant was significantly smaller than that found in RT112. Both cell types were seeded at 5000 cells per well making a difference in confluence status unlikely as an explanation. In both cell lines the protein showed a trend to upregulation by hypoxia and for RT112 at 72 hours this difference was statistically significant (\*= $P < 0.05$ , error bar = 1 standard deviation, 3 replicates for each bar).



### 5.2.5 Cell viability under hypoxia of RT112 and HUVEC cells at 42 and 72 hours

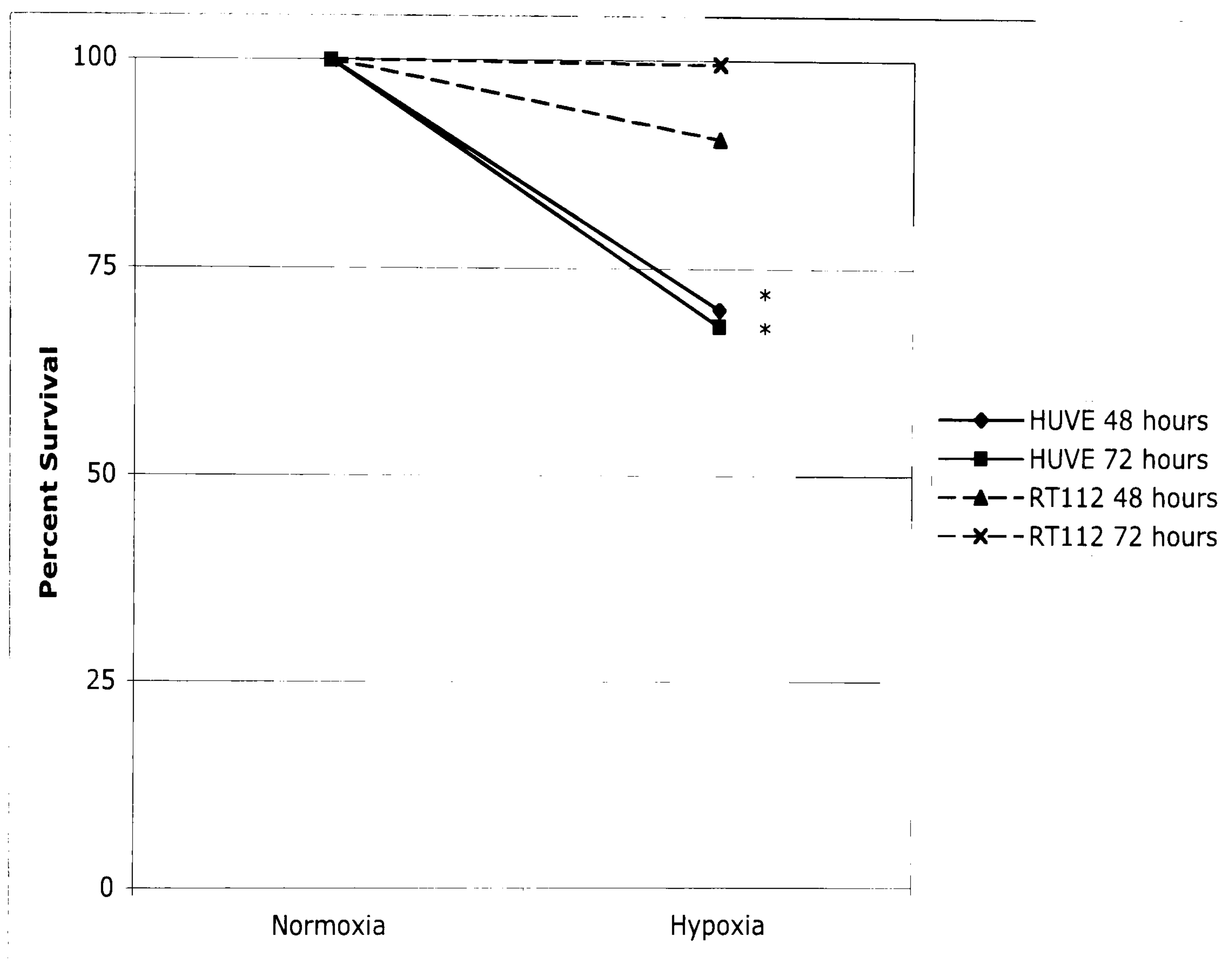


Figure 5.5 Percentage survival of HUVE cells and RT112 cells under hypoxia (0.1% oxygen) compared to normoxic controls seeded at the same number per well and grown for the same time under normoxia. Viability was measured using the MTS assay by light absorbance at 490nm. RT112 cells grew as well under hypoxia as normoxia whereas HUVE cells grew significantly less well. (3 replicates per condition \* =  $p < 0.05$  significance).



### 5.3 Discussion

The finding of raised IGFBP-3 in the urine of bladder cancer patients has not been previously noted. In prostate cancer, levels have been shown to be significantly lower in the plasma of patients than in controls (Zhao, Grossman et al. 2003). In vitro experiments on cell growth and IGFBP-3 make it likely that the levels of IGFBP-3 seen here in urine are of clinical significance. Levels as low as 10ng/ml and more commonly 30-50 ng/ml have been shown to have inhibitory effects on growth of neoplastic cells in vitro. (Martin and Baxter 1999; Ewton, Kansra et al. 2002). Significantly elevated levels in the urine have also been described in interstitial cystitis, with levels in bacterial cystitis lower than this. However the mean absolute level in interstitial cystitis is lower than seen in these cancer patients with greater overlap with controls (mean level in interstitial cystitis 13.2ng/ml) (Keay, Zhang et al. 1997). It must also be noted that the ELISA measures both proteolysed fragments and intact IGFBP-3 (Diamandi, Mistry et al. 2000). The rise we measured may reflect increased proteolysis of IGFBP-3 that would make more IGF available to the tumour. However the data suggests at least that direct upregulation of mRNA levels of IGFBP-3 occur with or without increased proteolysis.

If IGFBP3 were acting predominantly as an apoptotic factor in bladder cancer, significantly raised levels would be expected in higher stages and grades where apoptosis is more common. In transitional cell cancer of the bladder levels of apoptosis, as assessed by light microscopy, correlate with increased stage, grade and markers of proliferation (Ki-67). However apoptosis has no useful independent prognostic value over stage and grade on multivariate analysis (Lipponen and Aaltomaa 1994).

As a screening tool for bladder cancer urinary IGFBP-3 lacks the sensitivity of a direct cystoscopy and a significant number of cancer patients had values similar to age-matched controls. This may be because small tumours



of low grade secrete less protein, which is the likely problem with many bladder tumour markers (Boman, Hedelin et al. 2002).

Raised levels of angiogenin were also found in the urine of bladder cancer patients. Specifically, like IGFBP3, very significantly raised levels were found in invasive tumours. This would fit with the increased levels of angiogenesis that are found in invasive tumours where high levels of angiogenesis have been related independently to prognosis. The connection between angiogenin and RNAse4 has already been discussed in Chapter 3.

Significant levels of IGFBP3 were measured in the supernatant of bladder cancer cell line RT112 even in normoxia raising the possibility that levels of IGFBP3 found in the urine of bladder cancer patients may not be related to levels of hypoxia in the tumour. However even this high level in vitro was increased 150% by hypoxia. The untransformed HUVE cells secreted significantly less IGFBP3 in normoxia and hypoxia than RT112

Cell viability under hypoxia was interestingly not affected in the RT112 cancer cell line even after 72 hours and despite the upregulation of IGFBP3, which is a likely apoptotic factor. This suggests either that RT112 proliferates so fast that it outpaces any increased apoptotic rate stimulated by the increased IGFBP3 or that this cell line has molecular pathways to avoid any apoptotic effects of IGFBP3.

HUVE cells significantly lost viability in hypoxia. Ideally normal urothelia would have been used in this experiment but supplies were limited. In some senses this loss of viability in these untransformed cells under hypoxia is surprising as previous study (Graeber, Osmanian et al. 1996) in untransformed cells (mouse embryonic fibroblasts) suggested that they maintain viability in hypoxia at 48 hours. However that same study found that mouse embryonic fibroblasts transformed by adenovirus early region 1A



(E1A) and activated Ha-ras dramatically enhanced their susceptibility to apoptosis under hypoxia. Although these HUVE cells were not transformed, they were stimulated to growth by exogenously applied epidermal cell growth stimulant (ECGS) for the duration of the experiment, and this stimulation could have rendered them more susceptible to hypoxia induced apoptosis. Physiological data from healing wounds strongly suggests that apoptosis occurs more readily in cells that are activated to proliferate rather than in quiescent cells (Kane and Greenhalgh 2000; Takahashi, Aoshiba et al. 2002; Akasaka, Ono et al. 2004). Clinically, wounds that are ischaemic or infected are well known not to heal and this maybe due to increased rates of apoptosis caused by oxidative stress and/or hypoxia. Recent in vitro investigation of wound healing in normal human urothelial cells in the United Kingdom (Varley, Hill et al. 2005) has found that whereas normal human urothelial cells are mitotically quiescent and G0 arrested in situ, they rapidly enter the cell cycle upon seeding in primary culture and show reversible growth arrest at confluence. Growth was propagated in the absence of exogenous epidermal growth factor, but autocrine signaling through HER-1 via the MAPK and PI3-kinase pathways was essential for normal proliferation and migration during urothelial wound repair.

The role of IGFBP3 could be further tested by the use of IGFBP3 blocking antibodies (Kamanga-Sollo, Pampusch et al. 2005) or the technique of RNAi to IGFBP3, and the addition of IGF I and II.



## **Chapter Six**

**An investigation into the prognostic significance of necrosis and hypoxia in high grade and invasive bladder cancer**



## 6.1 Introduction

The purpose of this chapter was to investigate hypoxia and necrosis in high grade and invasive bladder cancer and to relate this to prognosis. The study method used was immunohistochemistry of 98 primary cystectomy specimens stained and scored for necrosis, and a range of hypoxia-associated markers ; Carbonic anhydrase IX (CA IX), Hypoxia-inducible factor 1 $\alpha$  ( HIF1 $\alpha$  ), HIF2 and Bcl2/adenovirus E1B 19kD-interacting protein 3 (NIP3). The advantage of using cystectomy specimens is that they are more accurately staged than endoscopically resected tumours, which are inaccurately staged in 40% of cases (Dutta, Smith et al. 2001).

Cystectomy studies large enough to conduct multivariate analysis of prognostic factors in invasive bladder cancer universally agree that stage and nodal status are independent prognostic factors (Bassi, Ferrante et al. 1999; Bella, Stitt et al. 2003; Frank, Cheville et al. 2003; Stein, Cai et al. 2003). Tumour size in T2 cancer (Cheng, Neumann et al. 1999); blood vessel invasion (Leissner, Koeppen et al. 2003); nonTCC non SCC subtype (Rogers, Palapattu et al. 2006); and microvessel density as a measure of angiogenesis have also been shown to be independent prognostic indicators. Angiogenesis is highly associated with the presence of lymph node metastasis (Jaeger, Weidner et al. 1995) (Bochner, Cote et al. 1995).



Patients who develop metastasis do so at a mean of 11 months from diagnosis (Babaian, Johnson et al. 1980). Therefore it was important that patients in this study had an average follow-up of at least 11 months.

At the outset of this thesis there were no other studies on necrosis and HIF-1 $\alpha$  in transitional cell carcinoma of the bladder. Since completion one group has published two papers on HIF-1 $\alpha$  in bladder cancer and there have been three large studies on necrosis three other types of cancer (transitional cell cancer of the upper renal tract, renal cell carcinoma and sarcoma). These studies are described in the discussion at the end of this chapter.

HIF-1 $\alpha$  has been described in the introduction to this thesis. Any upregulation of HIF-1 $\alpha$  in bladder cancer could occur by oxygen-dependent (i.e. in perinecrotic or hypoxic areas) or by oxygen-independent means. Growth factors, cytokines and other signaling molecules stimulate HIF-1 $\alpha$  synthesis in a cell-type-specific manner. HIF-1 $\alpha$  protein is likely to be particularly sensitive to changes in the rate of synthesis because of its extremely short half-life under non-hypoxic conditions. Many genetic changes, which are known to increase HIF1 $\alpha$  synthesis, occur in bladder cancer. 50% of invasive bladder cancers have p53 mutation, 30% have lost PTEN, and up to 45% have H-ras mutation. 49% also stain strongly for EGF receptor (EGFR) (Neal, Sharples et al. 1990; Brandau and Bohle 2001). Autocrine growth stimulation involving HIF1 $\alpha$  may also occur in bladder



cancer since HIF1 $\alpha$  transcriptionally activates transforming growth factor  $\alpha$  (TGF  $\alpha$ ) which binds to EGFR.

HIF2 $\alpha$  is a protein that can also dimerise with HIF-1 $\beta$ . Experiments in control and hypoxic rats showed that HIF2 $\alpha$ , like HIF1 $\alpha$  was induced by hypoxia in all organs investigated. In some organs induction of HIF2 $\alpha$  was exclusively nonparenchymal (kidney) but predominantly parenchymal in others (liver and intestine) (Wiesener, Jurgensen et al. 2003). In many tumors including bladder, HIF2 $\alpha$  staining is predominantly in tumor-associated macrophages (Talks, Turley et al. 2000; Onita, Ji et al. 2002).

NIP3 is a cell death protein (Ray, Chen et al. 2000) regulated by HIF 1 $\alpha$  (Sowter, Ratcliffe et al. 2001) that has been shown to cause necrosis rather than apoptosis.



## 6.2 Results

98 cases were analysed histopathologically. Mean age at cystectomy was 69.2 years. Two cases were squamous cell histology, the rest transitional cell. Only one patient had neoadjuvant chemotherapy. There were 18 stage T0/1, 20 T2, 46 T3, and 14 T4. Initial statistical analysis was by the chi test to look for associations between markers and pathological stage, grade and nodal status.

Clinical follow-up data was available in 96. There were 5 operative deaths within 30 days of surgery, these cases were excluded from survival analysis, as it is not known whether they would have died from cancer or not. 91 cases were therefore available for survival analysis. Median follow-up was 22 months (interquartile range 8-35). There were 18 stage T0/1, 19 T2, 42 T3, and 12 T4. The prevalence of necrosis in bladder cancer was high and increased with stage (T0-1 17%, T2 30%, T3 66%, T4 67%).



### 6.2.1 Results: Chi test analysis of staining associations between necrosis, HIF1 $\alpha$ , HIF2 $\alpha$ , CA IX, NIP3

Necrosis occurred in small (defined as less than 5mm) islands of comedo necrosis and in larger areas of gross necrosis (defined as larger than 5mm). HIF1 $\alpha$  staining was cytoplasmic and in a very few intense cases both cytoplasmic and nuclear. CA IX staining was largely cytoplasmic but also membranous. HIF2 $\alpha$  staining was predominantly in tumor associated stromal cells previously identified largely as macrophages. Bcl2/adenovirus E1B 19kD-interacting protein 3 (NIP3) staining was cytoplasmic. (Figures 6.1,6.2,6.3,6.4)

Necrosis showed a significant association with higher stage disease and positive nodes. CA IX showed a significant association with higher stage disease and positive nodes. There was no association between HIF 1 $\alpha$  and stage or grade or nodal status. HIF2 $\alpha$  also showed no association with stage or grade or nodal status. NIP3 showed a significant association with higher stage and (paradoxically) with lower grade. CAIX and HIF1 $\alpha$  were significantly associated with necrosis. HIF 2 $\alpha$  and NIP3 were not. None of the immunohistochemical markers were significantly associated with HIF1 $\alpha$ . (Tables 6.1,6.2,6.3,6.4,6.5,6.6,6.7)



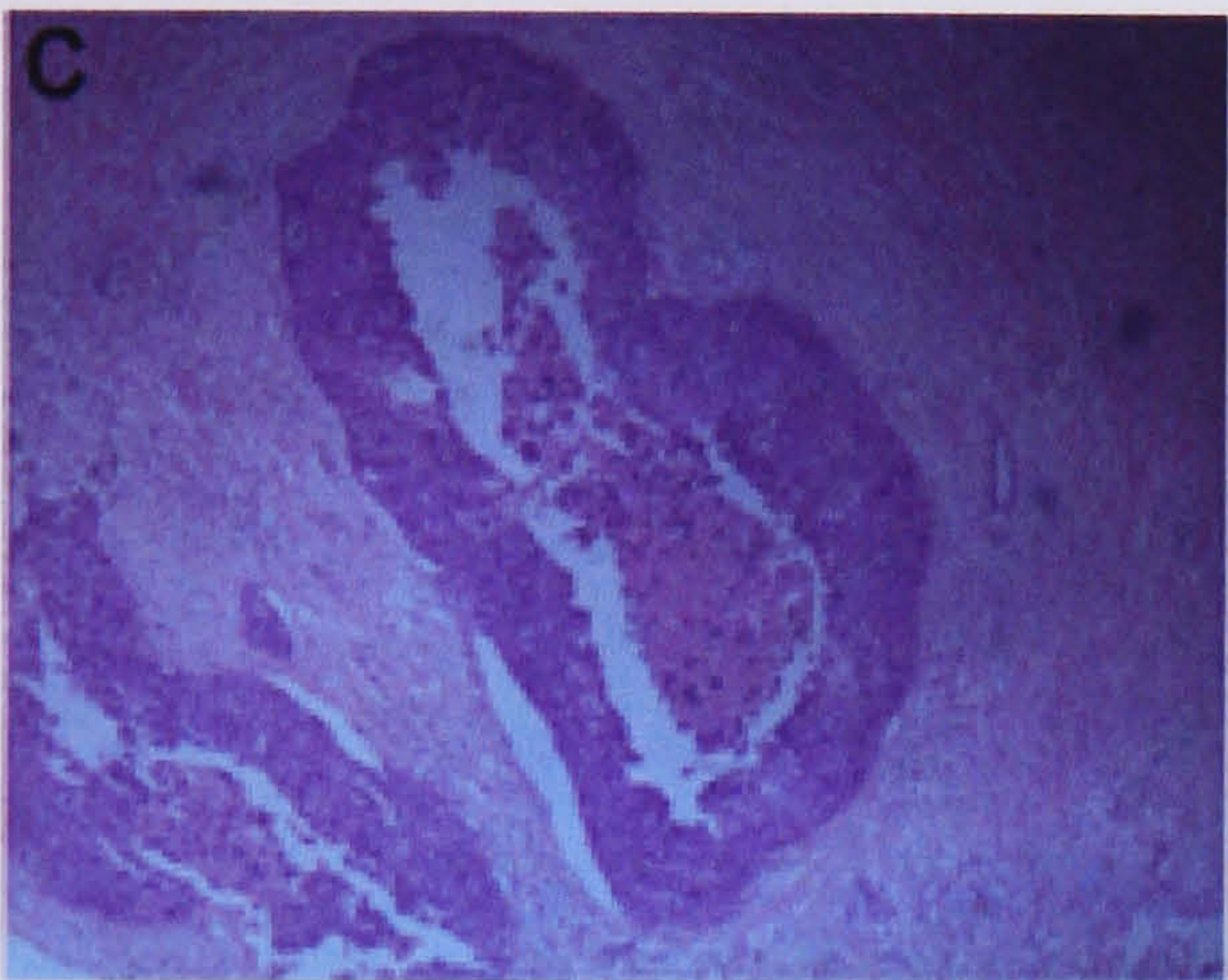
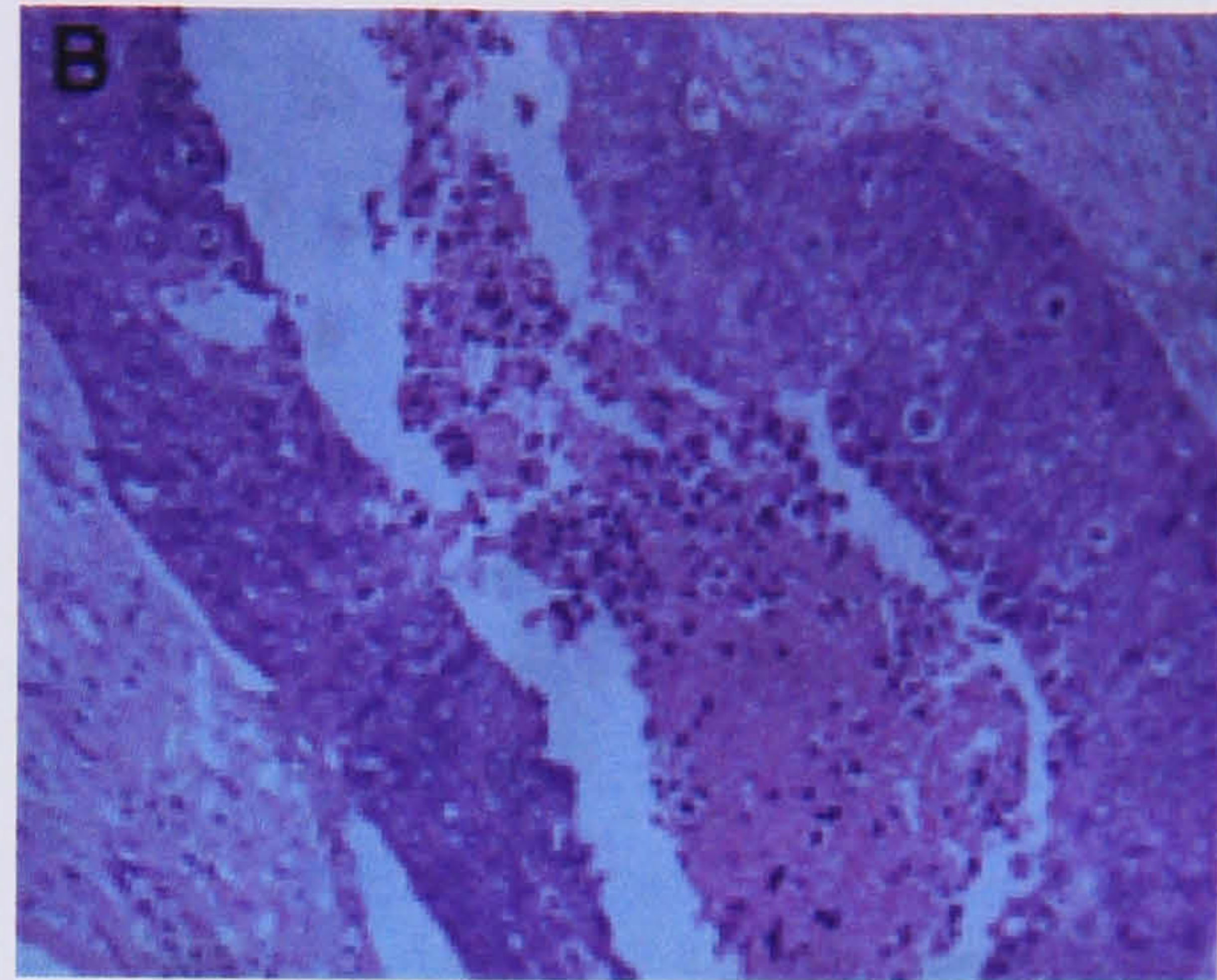
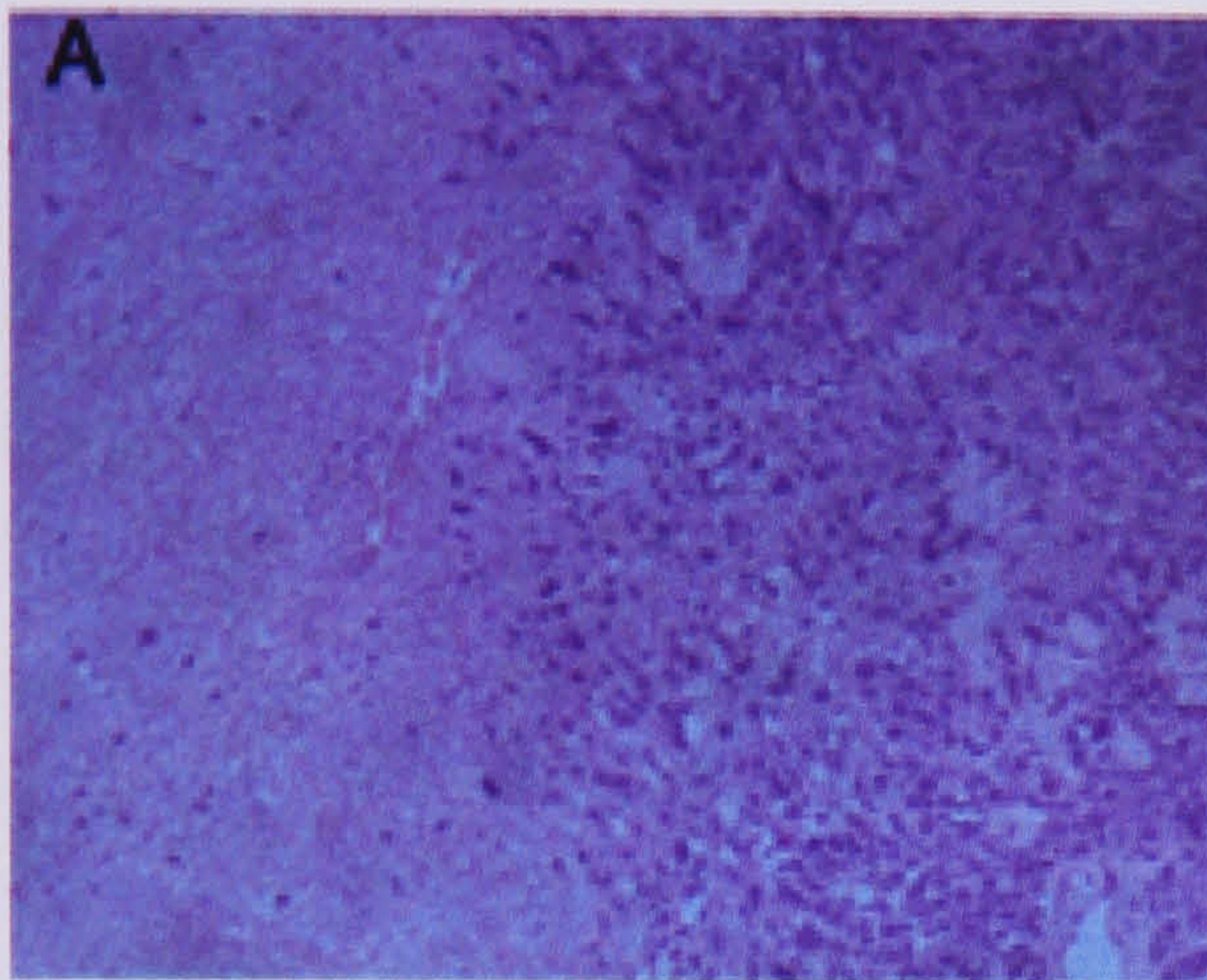


Figure 6.1 Haematoxylin and eosin staining of necrotic areas seen in transitional cell carcinoma of the bladder whole sections.

A gross necrosis score 2, x20

B comedo necrosis score 1, x20

C comedo necrosis score 1, x10



	<b>Necrosis negative</b>	<b>Necrosis positive (Comedo or gross)</b>	<b>p-value</b>
<b>Total No.</b>	47	51	<b>chitest</b>
<b>Stage:</b>			<b>0.0001***</b>
<b>T0-1</b>	15	3	
<b>T2</b>	14	6	
<b>T3</b>	14	32	
<b>T4</b>	4	10	
<b>Grade:</b>			<b>0.58</b>
<b>II</b>	9	8	
<b>III</b>	38	43	
<b>Nodal status:</b>			<b>0.016*</b>
<b>Neg.</b>	46	41	
<b>Pos.</b>	1	10	
<b>Cancer specific survival</b>			<b>logrank</b>
<b>0 vs 1/2</b>			<b>0.01*</b>
<b>0 vs 1 vs 2</b>			<b>0.0001***</b>

Table 6.1 Association between necrosis and stage, grade, nodal status, clinical follow-up (univariate analysis). Necrosis showed a significant association with higher stage disease and positive nodes.



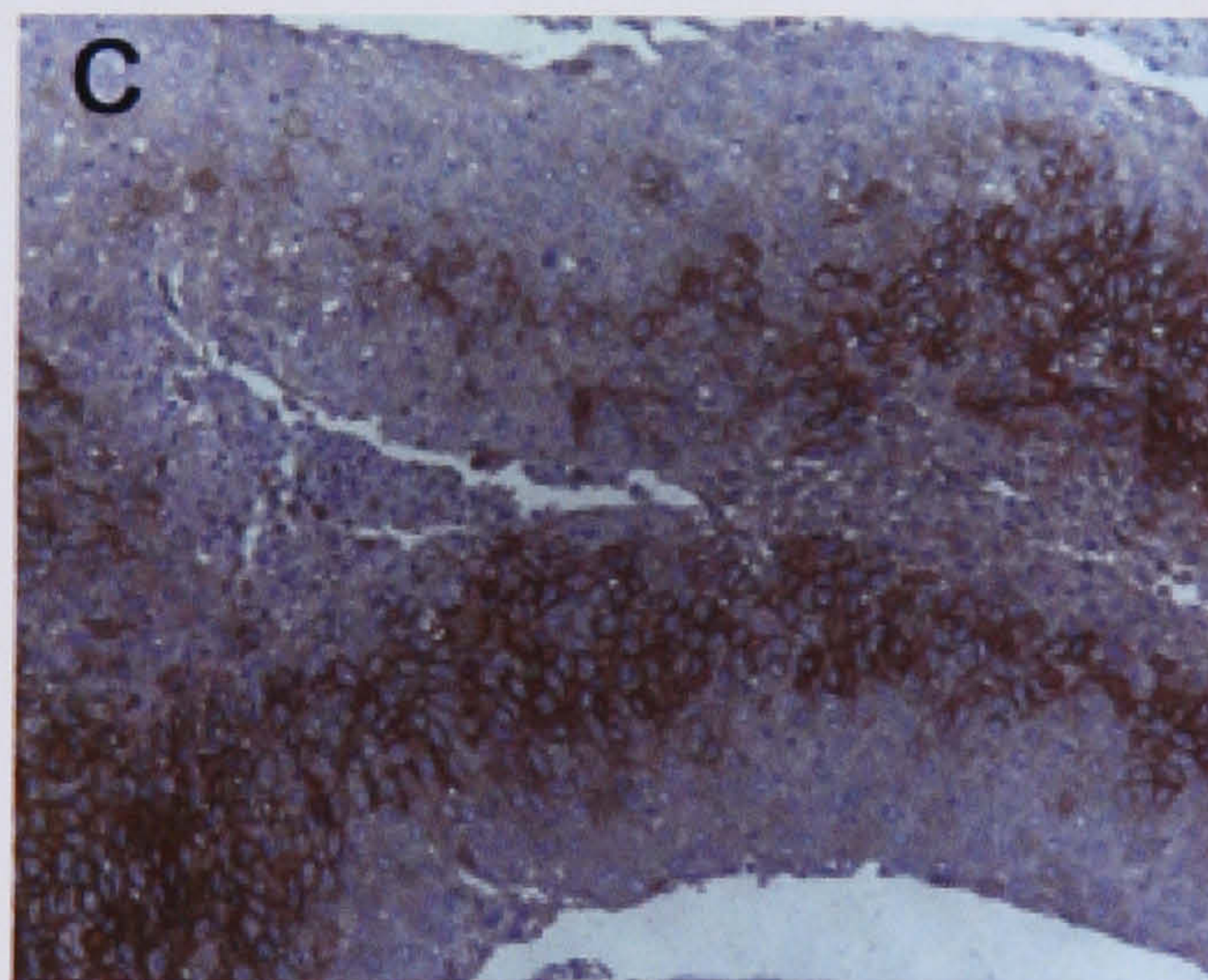
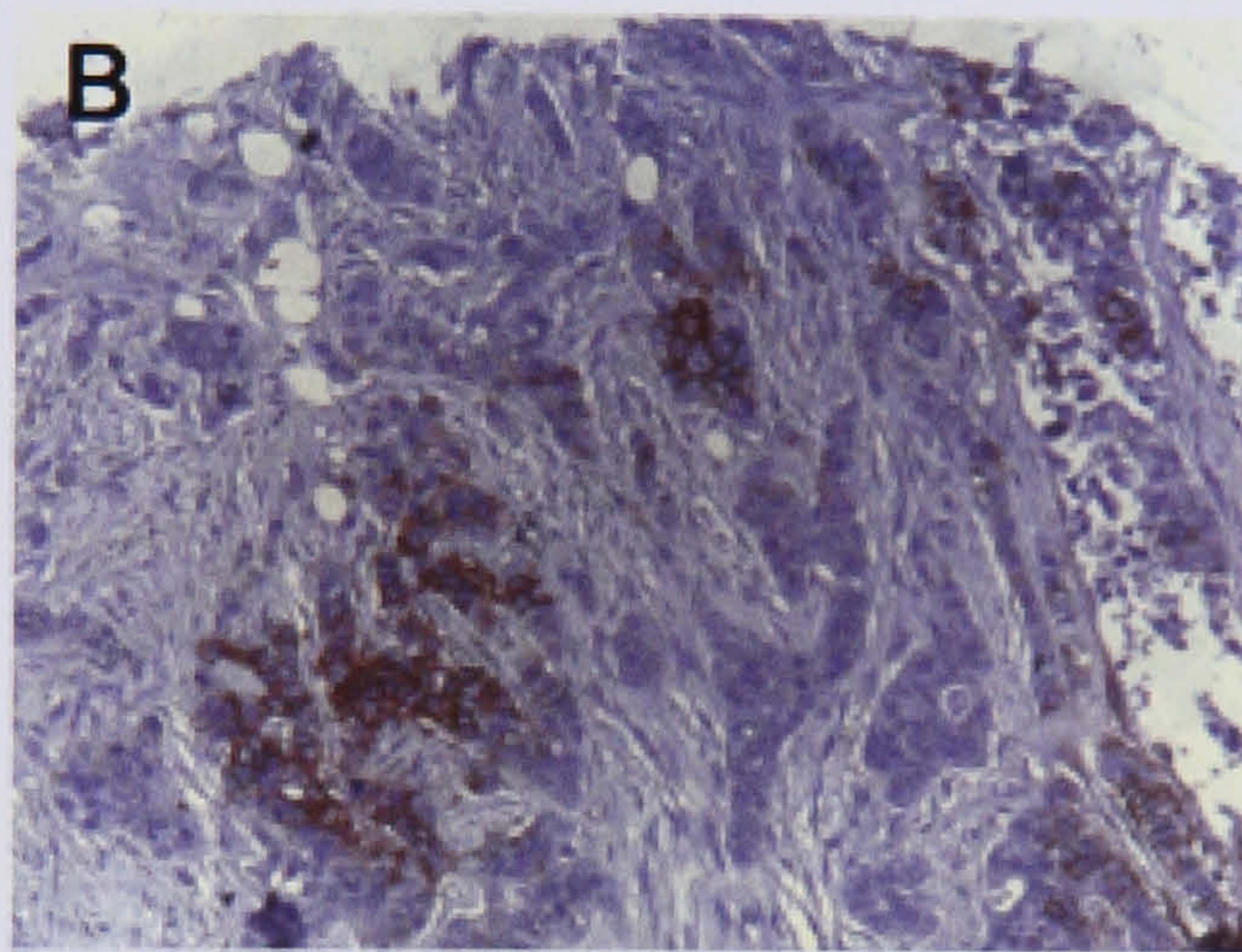
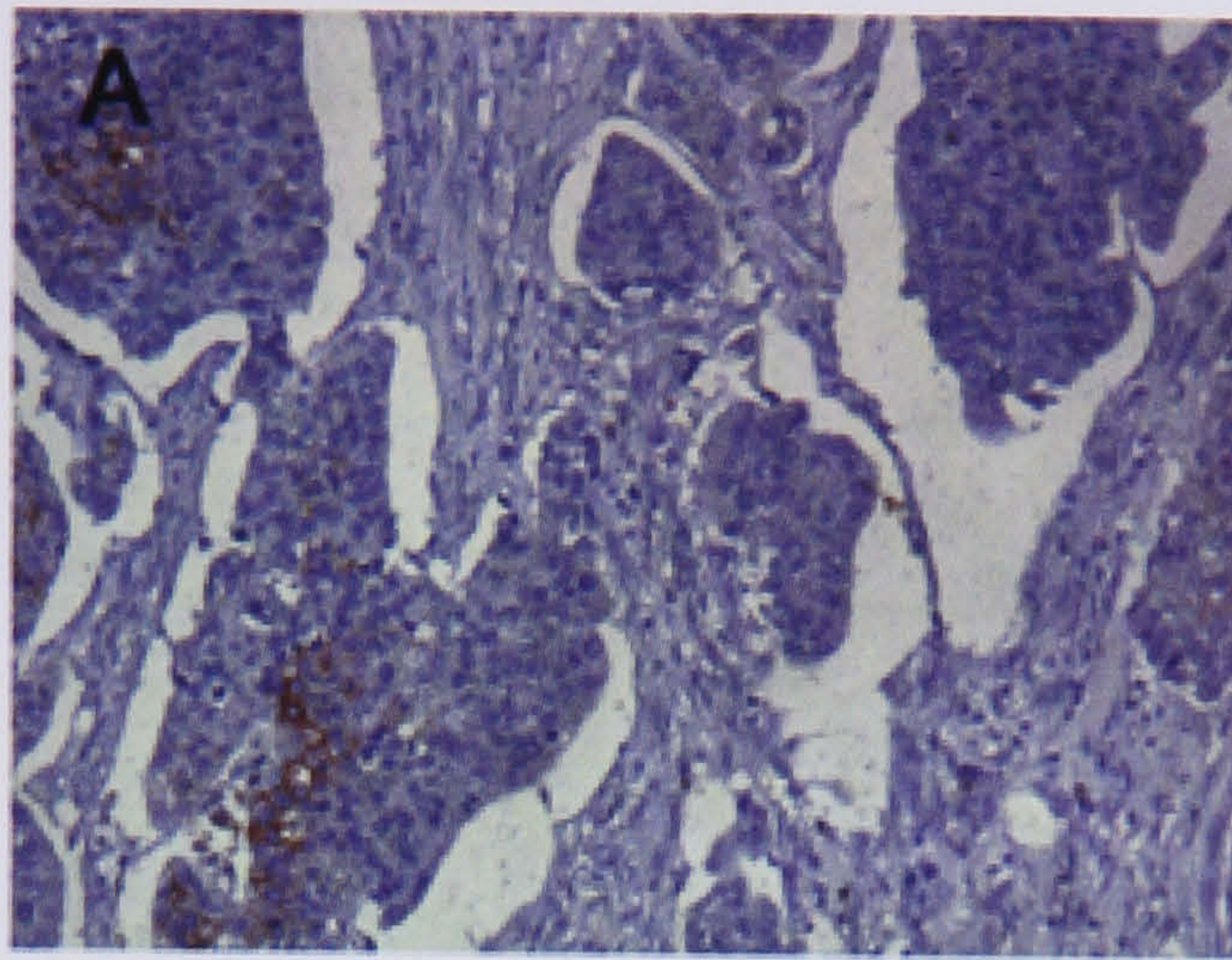


Figure 6.2 CA IX staining of bladder tissue array, x20  
A Score 1  
B Score 2  
C Score 3



	CA IX negative (CA IX score = 0)	CA IX positive (CA IX score = 123)	p-value
<b>Total:</b>	55	43	
<b>Stage:</b>			<b>0.01*</b>
<b>T0-1</b>	14	4	
<b>T2</b>	14	6	
<b>T3</b>	18	28	
<b>T4</b>	9	5	
<b>Grade:</b>			
<b>II</b>	6	11	0.06
<b>III</b>	49	32	
<b>Nodal status:</b>			
<b>Neg.</b>	52	35	<b>0.046*</b>
<b>Pos.</b>	3	8	
<b>Cancer specific survival</b>			0.55

Table 6.2 Association between CA IX and stage, grade and nodal status, clinical follow-up (univariate analysis). CA IX showed a significant association with higher stage disease and positive nodes.



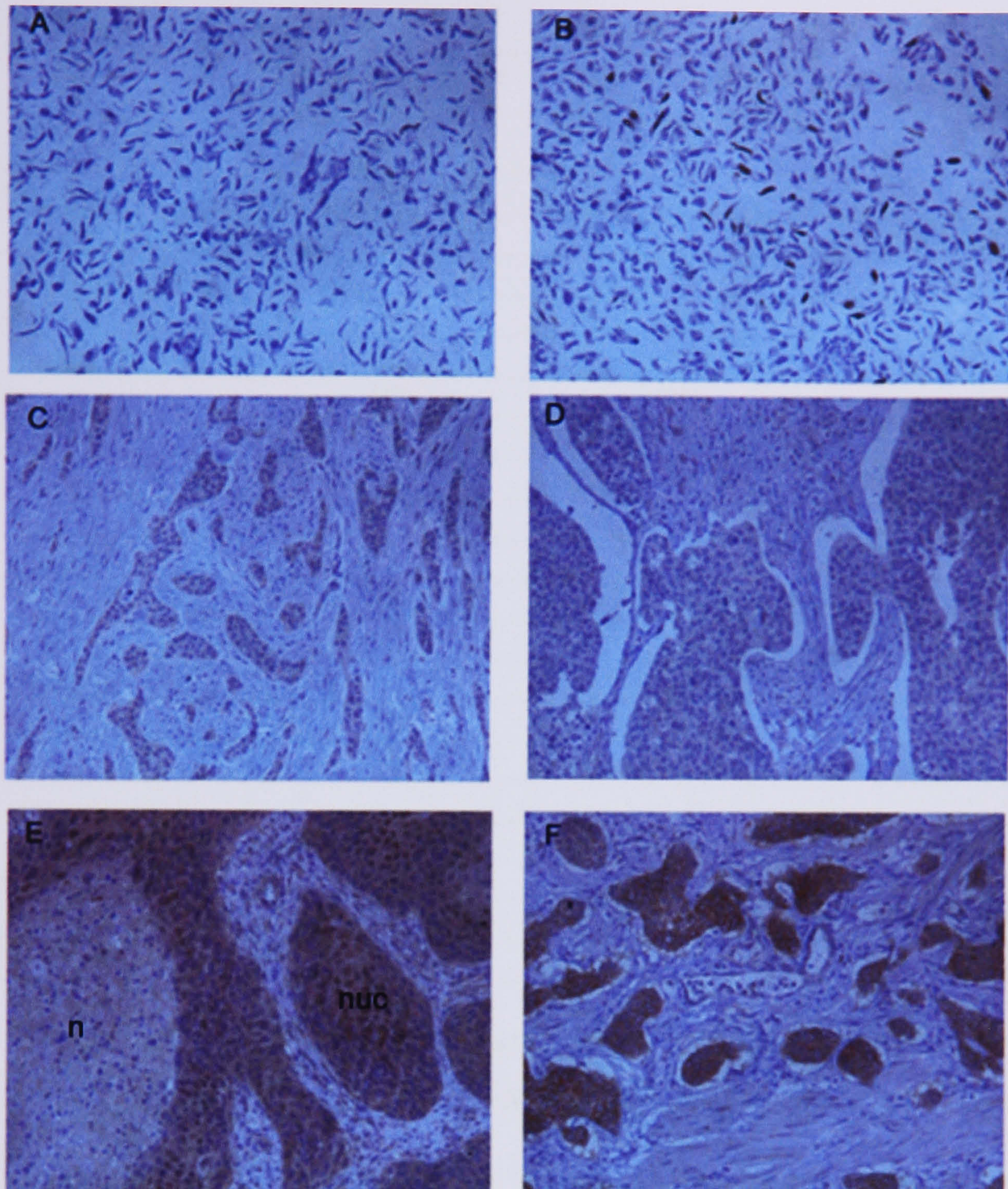


Figure 6.3 HIF-1 $\alpha$  staining x20

A, B Control cell line transfected with HIF-1 $\alpha$

A no primary antibody applied (negative control)

B primary antibody applied (positive control)

C, D, E, F Bladder tissue array:

C Score 1

D Score 2

E Score 3 with area of necrosis (n) and nuclear staining (nuc)

F Score 3, no necrosis associated, invasion of muscularis propria



	HIF 1 negative (HIF 1 score = 0-1)	HIF 1 positive (HIF 1 score = 2- 3)	p-value
<b>Total No.</b>	47	51	
			<b>chi test</b>
<b>Stage:</b>			0.8
<b>T0-1</b>	9	9	
<b>T2</b>	10	10	
<b>T3</b>	23	23	
<b>T4</b>	5	9	
<b>Grade:</b>			0.09
<b>II</b>	5	12	
<b>III</b>	42	39	
<b>Nodal status:</b>			
<b>Neg.</b>	43	44	0.4
<b>Pos.</b>	4	7	
<b>Cancer specific survival</b>			<b>logrank test</b>
<b>HIF1 score 0-1 vs 2-3</b>			0.13
<b>HIF1 score 012 vs 3</b>			<b>0.049*</b>

Table 6.3 Association between HIF 1 $\alpha$  and stage, grade and nodal status, clinical follow-up (univariate analysis). HIF 1 $\alpha$  showed no association with stage nor grade nor nodal status.



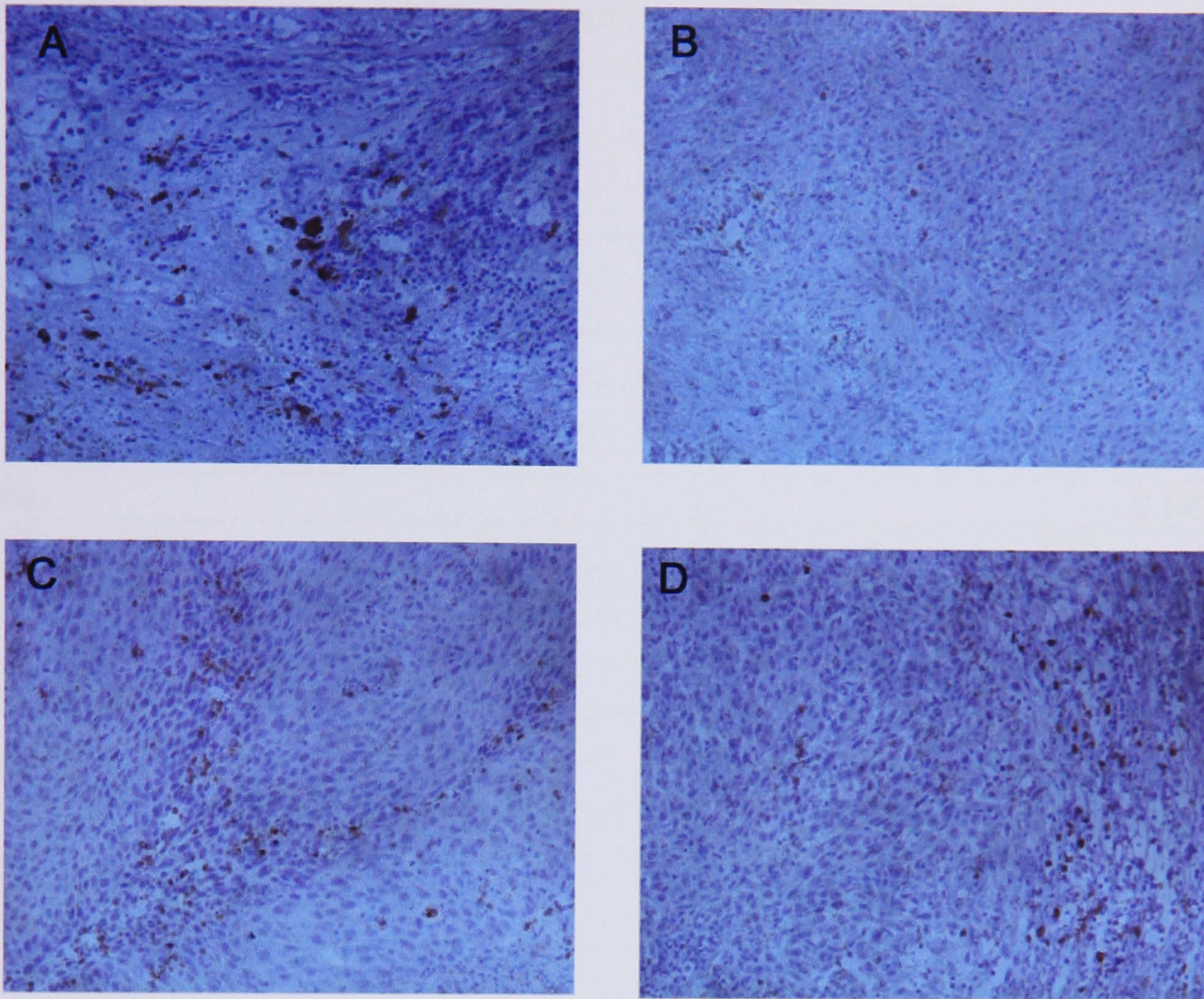


Figure 6.4 HIF 2 $\alpha$  staining, x20.

A Positive control, clear cell renal cell carcinoma with an area of necrosis.

B, C, D Bladder tumour cores:

B Score 1

C Score 2

D Score 3



	HIF2 $\alpha$ negative (HIF2 $\alpha$ max = 0-1)	HIF2 $\alpha$ positive (HIF2 $\alpha$ max = 2-3)	p-value
<b>Total No.</b>	69	29	
<b>Stage:</b>			
<b>T0-1</b>	12	6	0.57
<b>T2</b>	13	7	
<b>T3</b>	32	14	
<b>T4</b>	12	2	
<b>Grade:</b>			0.08
<b>II</b>	9	8	
<b>III</b>	60	21	
<b>Nodal status:</b>			
<b>Neg.</b>	60	27	0.37
<b>Pos.</b>	9	2	
<b>Cancer specific survival</b>			0.057

Table 6.4 Association between HIF2 $\alpha$  and stage, grade and nodal status and follow-up (Univariate analysis). HIF2 $\alpha$  showed no association with stage nor grade nor nodal status.



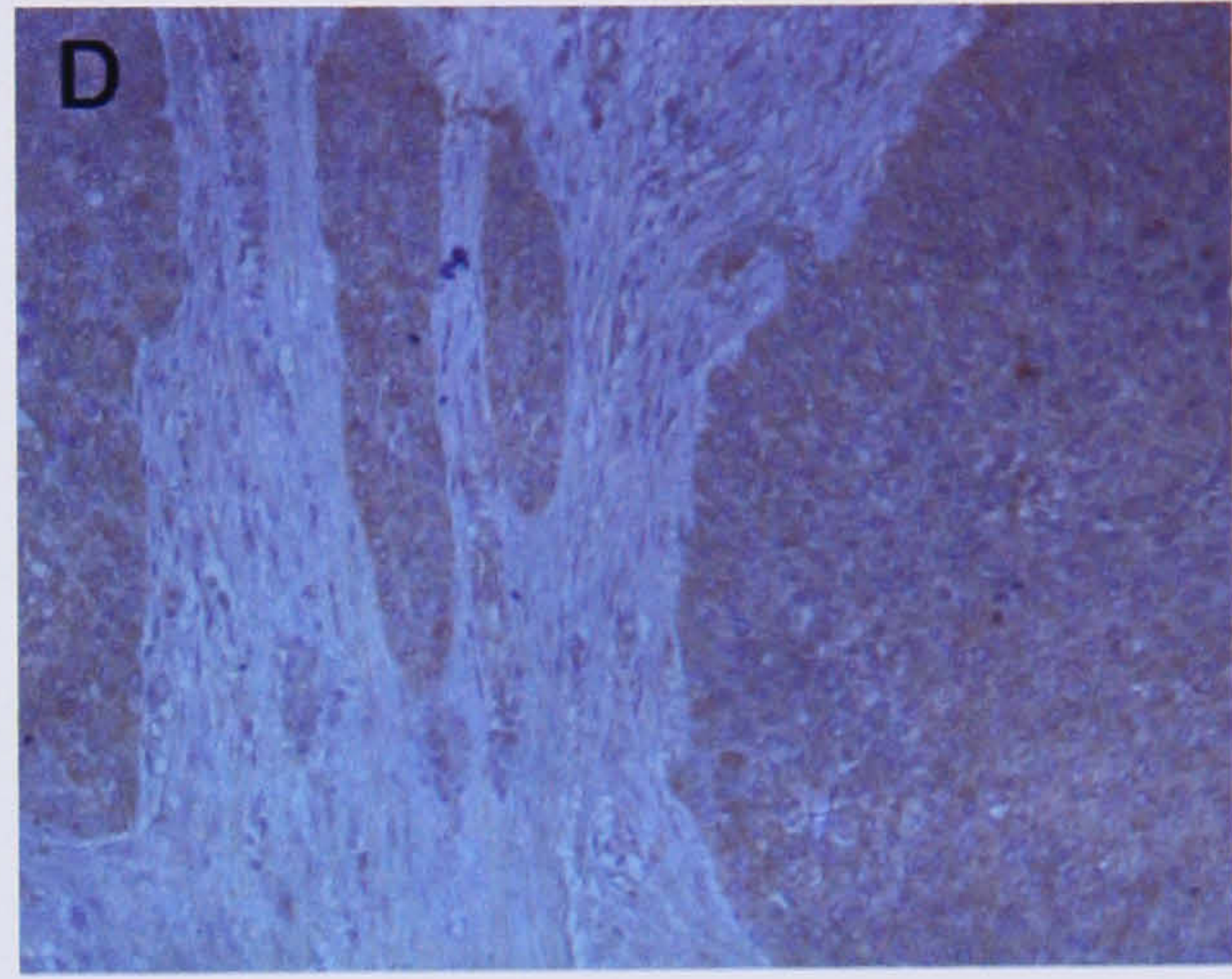
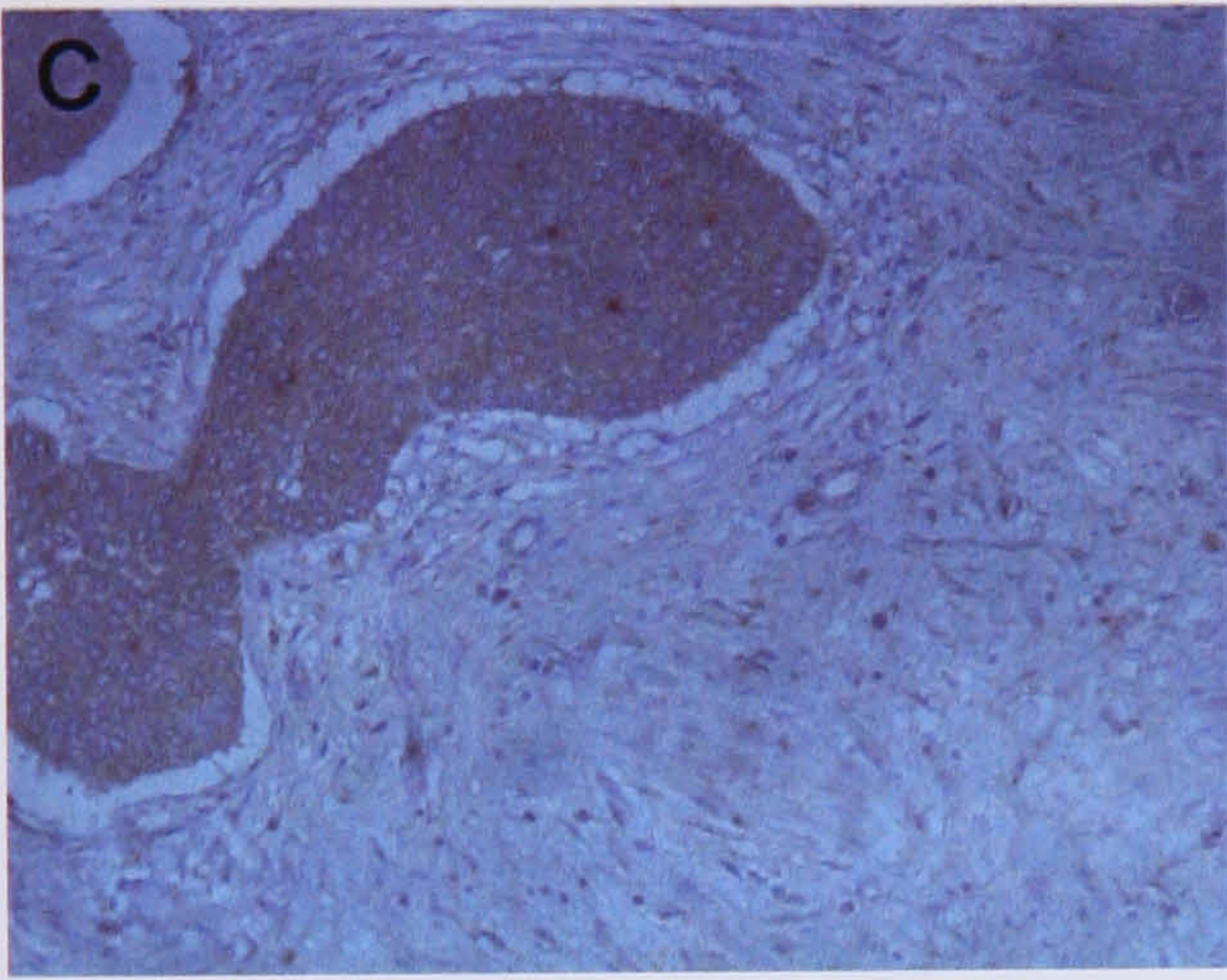
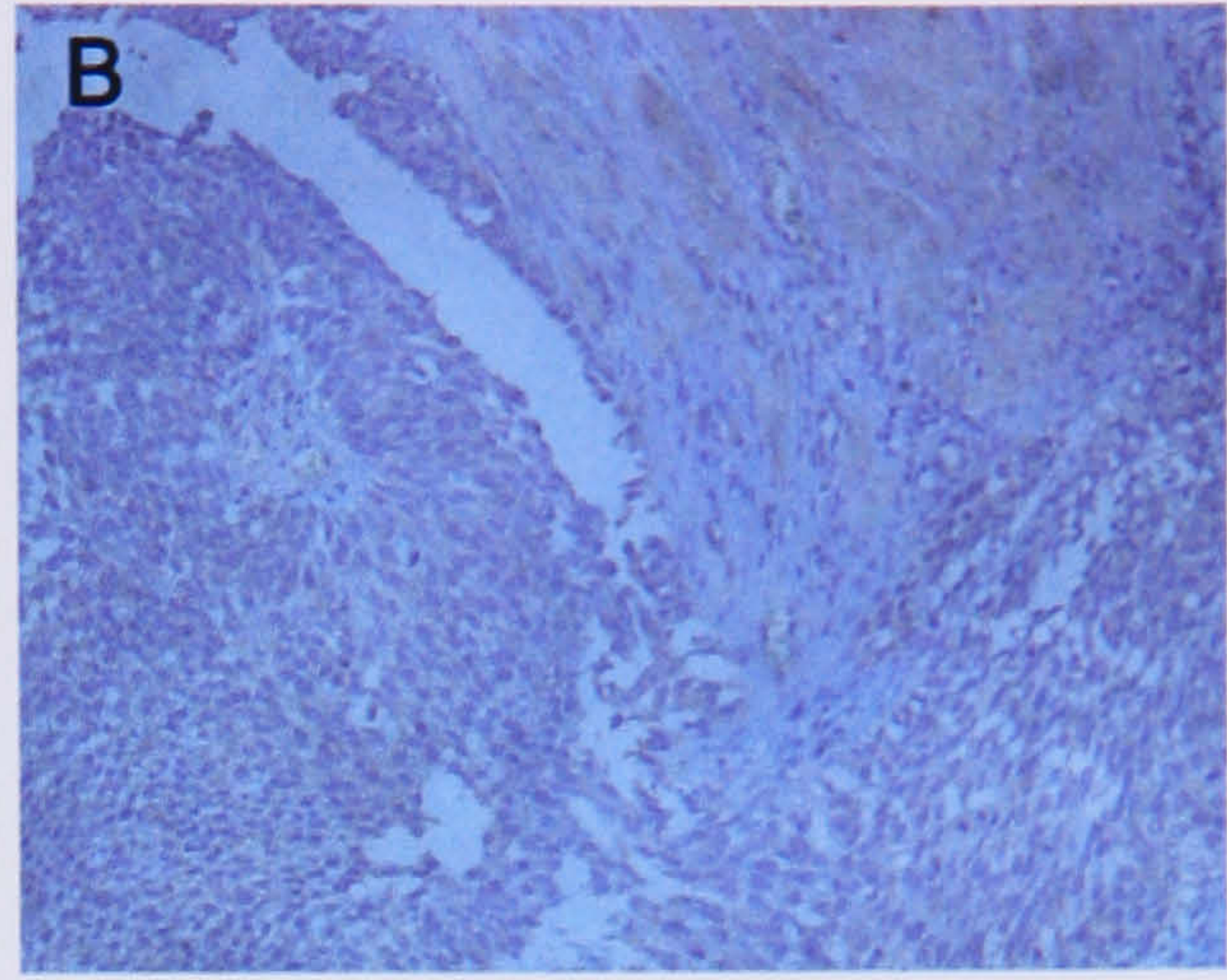
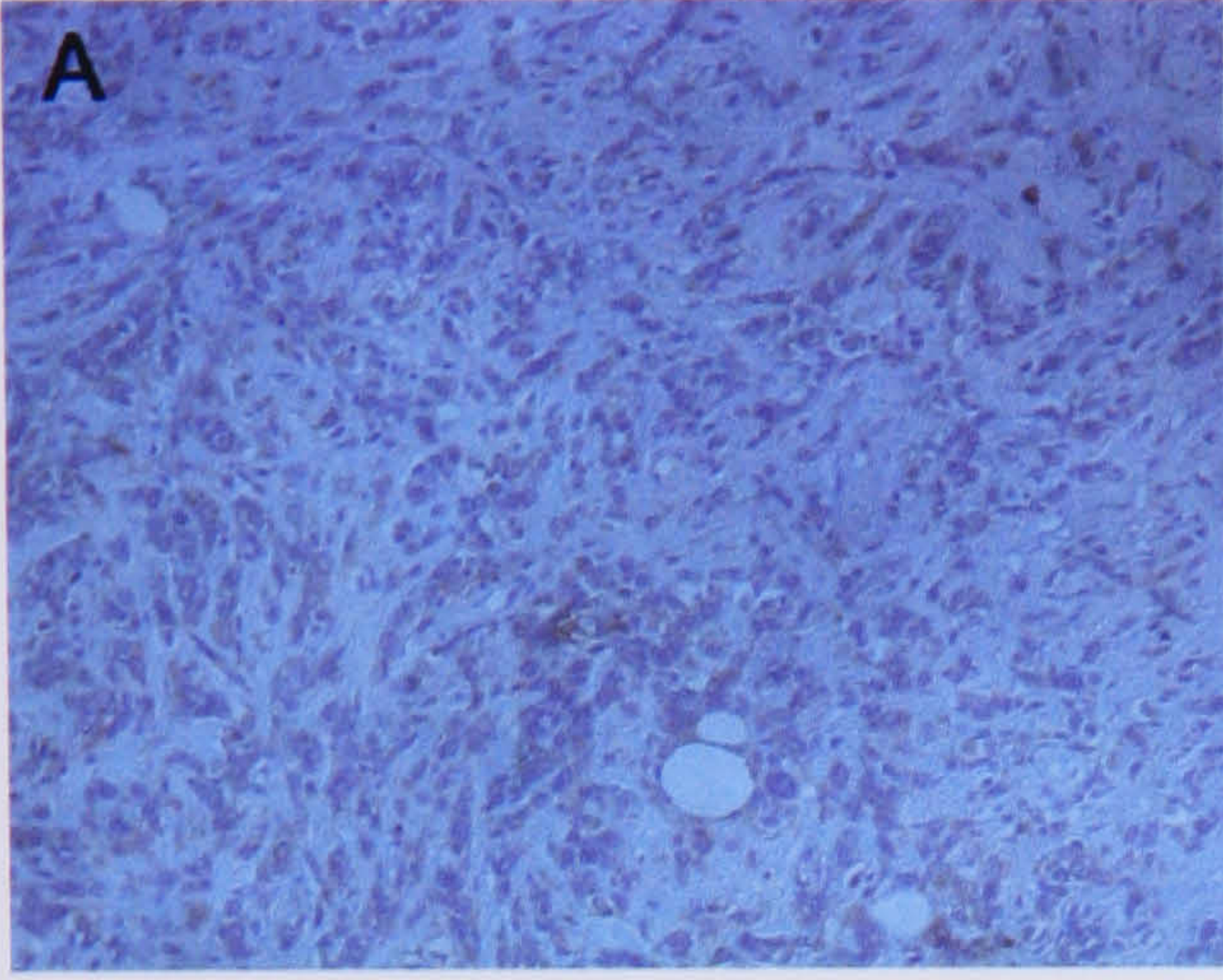


Figure 6.5 Staining for NIP3, x20.

A Score 1

B Score 1

C Score 3

D Score 3



### 6.2.5 NIP3

	<b>NIP negative (NIP max = 0-1)</b>	<b>NIP positive (NIP max = 2-3)</b>	<b>p-value</b>
<b>Total No.</b>	42	56	
<b>Stage:</b>			
<b>T0-1</b>	13	5	<b>0.03*</b>
<b>T2</b>	7	13	
<b>T3</b>	15	31	
<b>T4</b>	7	7	
<b>Grade:</b>			
<b>II</b>	3	14	<b>0.02*</b>
<b>III</b>	39	42	
<b>Nodal status:</b>			
<b>Neg.</b>	37	50	0.9
<b>Pos.</b>	5	6	
<b>Cancer specific survival</b>			0.96

Table 6.5 Association between NIP3 and stage, grade, nodal status and follow-up. <sup>a</sup> Fischer's Exact test. NIP3 showed a significant association with higher stage and (paradoxically) with lower grade.



	<b>Necrosis negative</b>	<b>Necrosis positive (Comedo or gross)</b>	<b>Chi Test p-value</b>
<b>Total No.</b>	47	51	
<b>CA IX</b>			<b>0.0003***</b>
0	37	22	
1-2-3	10	29	
<b>HIF 1<math>\alpha</math></b>			<b>0.0047**</b>
0-1	30	18	
2-3	17	33	
<b>HIF 1<math>\alpha</math></b>			0.28
0-1-2	43	43	
3	4	8	
<b>HIF 2<math>\alpha</math></b>			0.4
0-1	35	34	
2-3	12	17	
<b>NIP 3</b>			0.17
0-1	24	33	
2-3	23	18	

Table 6.6 Association between immunohistochemical markers and necrosis. CAIX and HIF1 $\alpha$  (when split by the median score) were significantly associated with necrosis. HIF 2 $\alpha$  and NIP3 were not.



6.2.7 Association of markers to HIF 1 $\alpha$

	HIF 1 $\alpha$ score		p-value
	0-1	2-3	
<b>Total No.</b>	47	51	
<b>CA IX</b>			
<b>0</b>	29	26	0.29
<b>1-2-3</b>	18	25	
<b>HIF 2<math>\alpha</math></b>			
<b>0-1</b>	33	36	0.96
<b>2-3</b>	14	15	
<b>NIP 3</b>			
<b>0-1</b>	23	17	0.11
<b>2-3</b>	24	34	

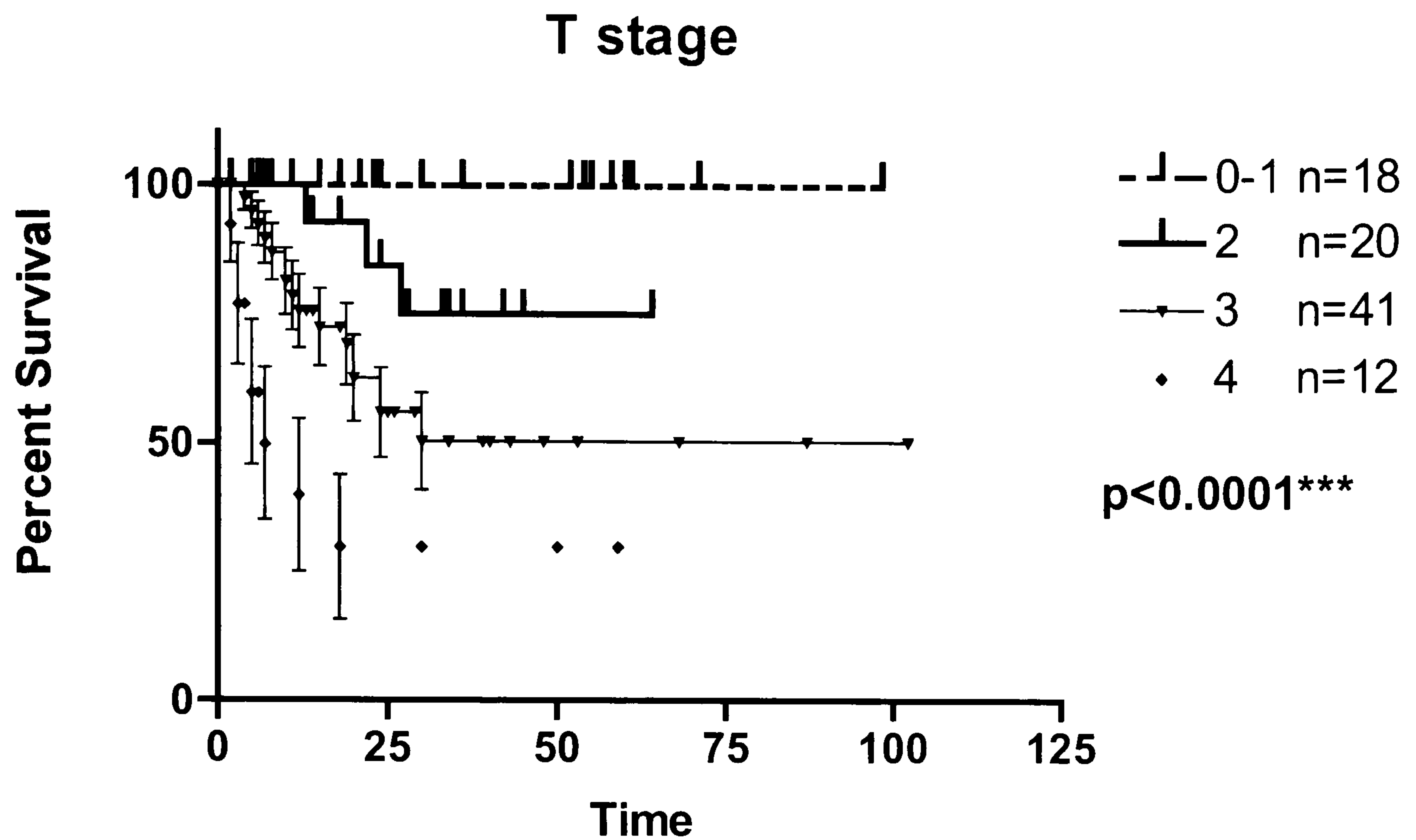
Table 6.7 Association of immunohistochemical markers to HIF1 $\alpha$  immunohistochemistry. None of the immunohistochemical markers were significantly associated with HIF1 $\alpha$



### 6.2.2 Results: Survival analysis: Necrosis, HIF1 $\alpha$ , HIF2 $\alpha$ , CA IX, NIP3

Stage ( $p < 0.0001$ ), necrosis ( $p < 0.0001$ ) and HIF 1 $\alpha$  (intensity score 3) ( $p = 0.048$ ) were the only significant prognostic factors on univariate analysis. Stage ( $p < 0.001$  HR 3.29 95% CI 1.80 – 6.04) and necrosis ( $p = 0.04$ , HR 1.92 95% CI 1.05 – 3.51) were independent prognostic factors on multivariate analysis; HIF 1 $\alpha$  just lost significance ( $p = 0.07$  HR 1.36 CI 0.98 – 1.88). Node status and grade were not prognostic factors possibly due to the low rate of node reporting (45%) and the small proportion of grade 2 tumours (17%). (Graphs 6.1,6.2,6.3.1, 6.3.2,6.4,6.5,6.6 Table 6.8,6.9)



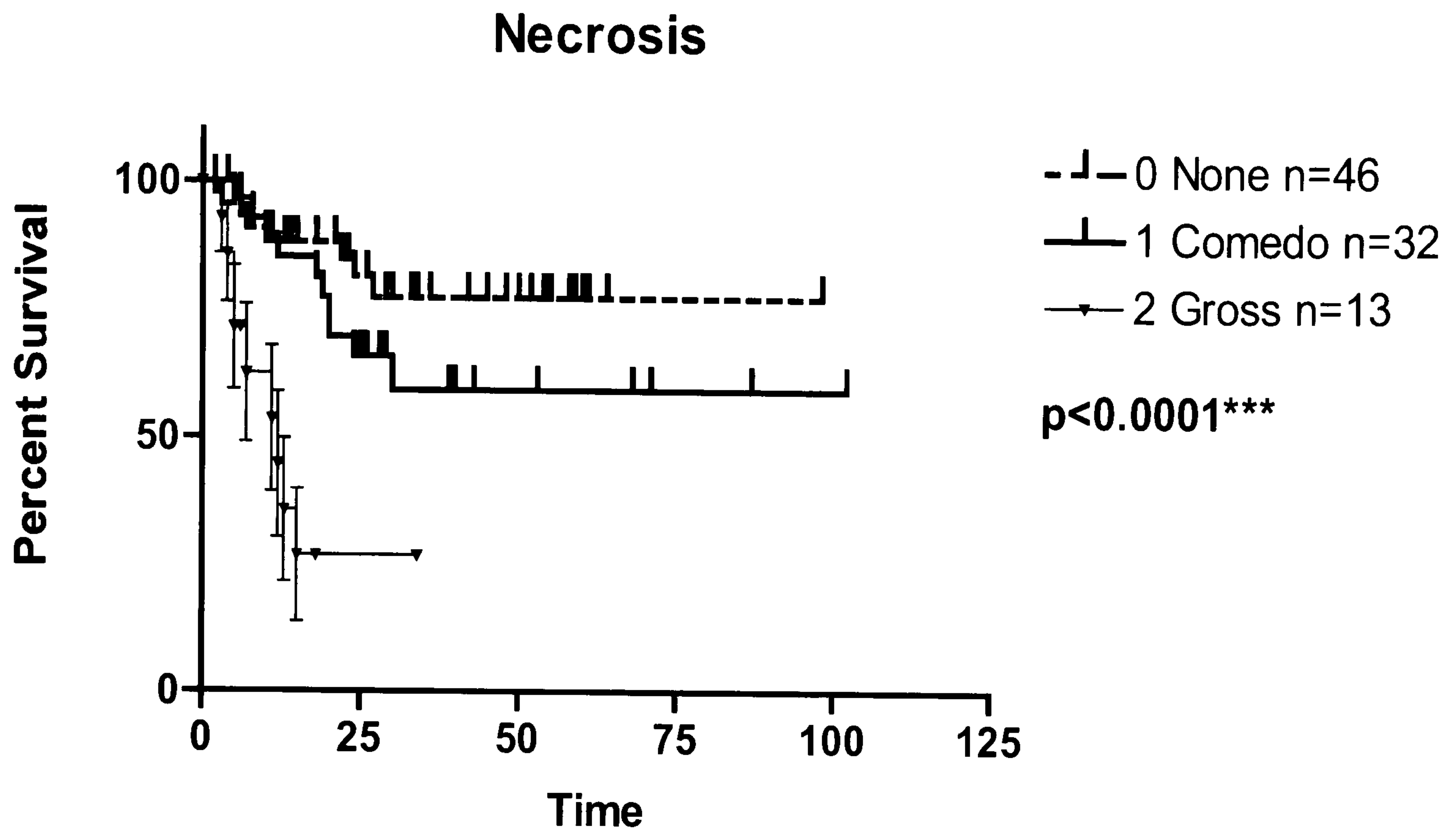


Graph 6.1 Kaplan Meyer plot of cancer specific survival by stage

	Hazard Ratio	SE	P-value	95% CI
Stage	3.29	1.02	<0.001	1.80 – 6.04
Necrosis (0 vs.1 vs. 2)	1.92	0.59	0.04	1.05 – 3.51
HIF1 (0,1,2 vs. 3)	1.36	0.23	0.07	0.98 – 1.88

Table 6.8 Multivariate cancer specific survival analysis (SE Standard Error, CI Confidence Interval)



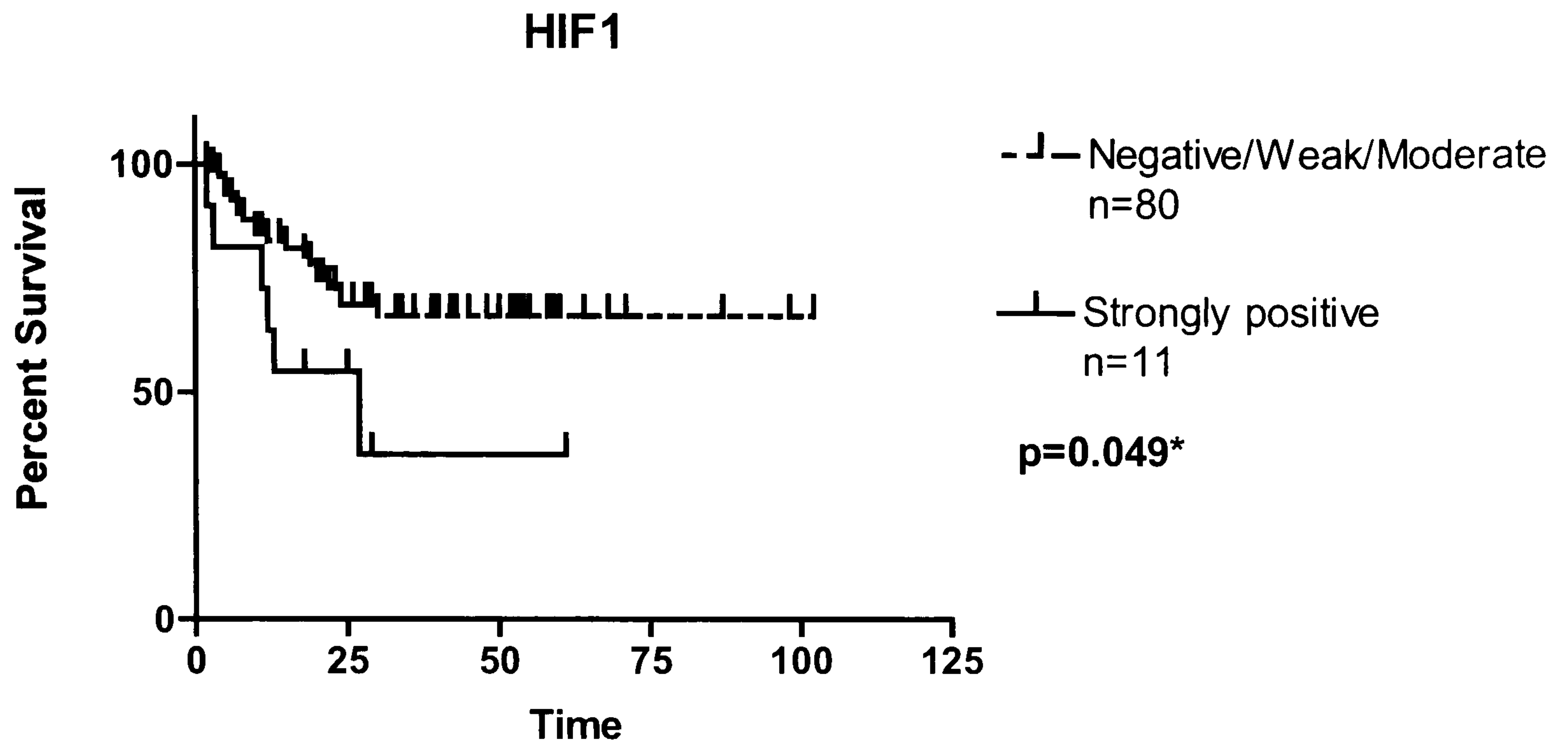


Graph 6.2 Kaplan Meyer plot of cancer specific survival by amount of necrosis Univariate analysis. See table 6.9 below

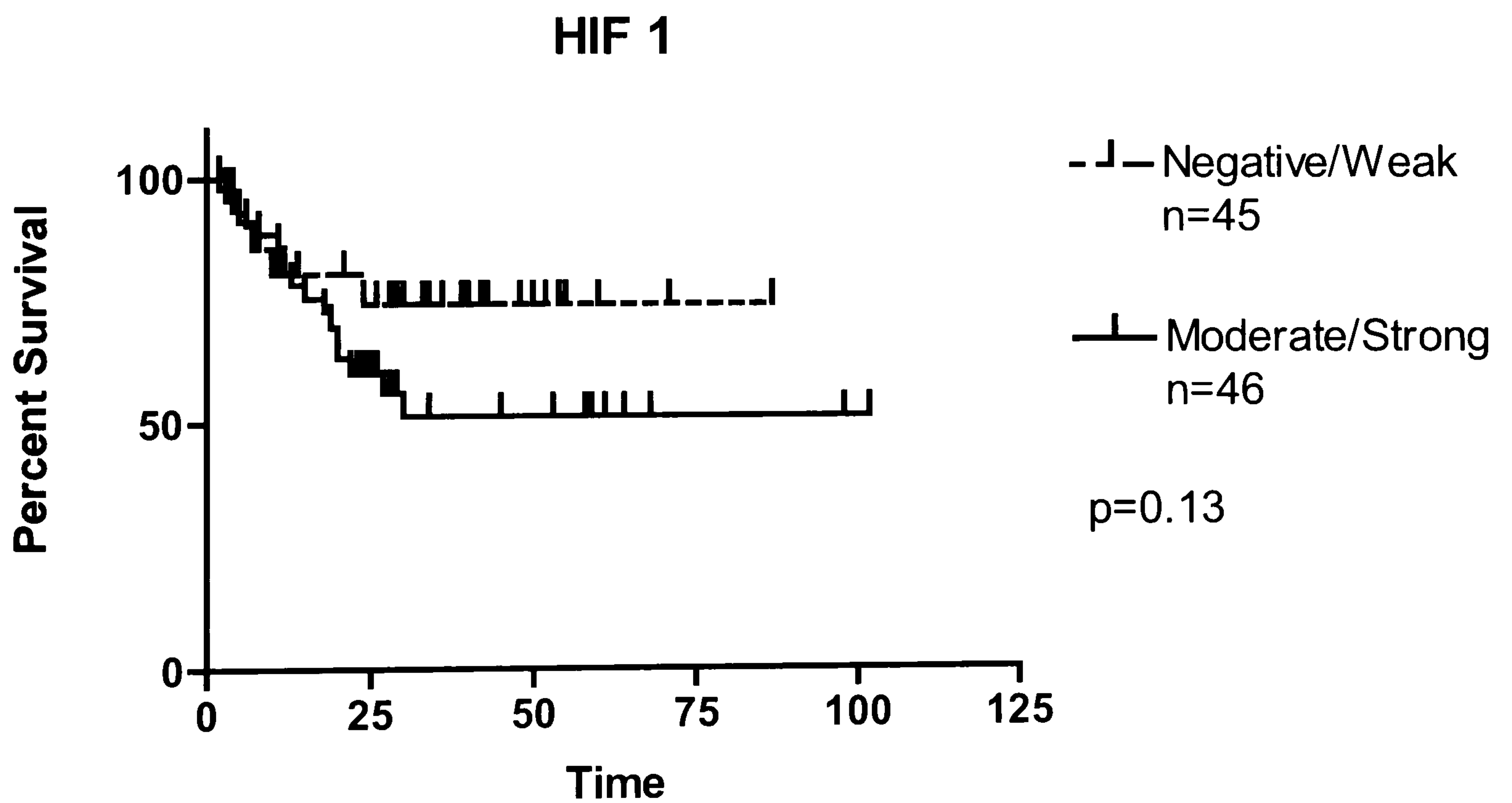
Stage	N	No necrosis	Comedo necrosis	Gross necrosis
T0	3	2	1	0
T1	15	13	1 (7%)	1 (7%)
T2	19	13	4 (21%)	2 (10%)
T3	42	14	21 (50%)	7 (16%)
T4	12	4	5 (42%)	3 (25%)
<b>Totals</b>	<b>91</b>	<b>46</b>	<b>32</b>	<b>13</b>

Table 6.9 Breakdown of the stages and amount of necrosis in the 91 cases with clinical follow up used to construct survival Graph 6.2





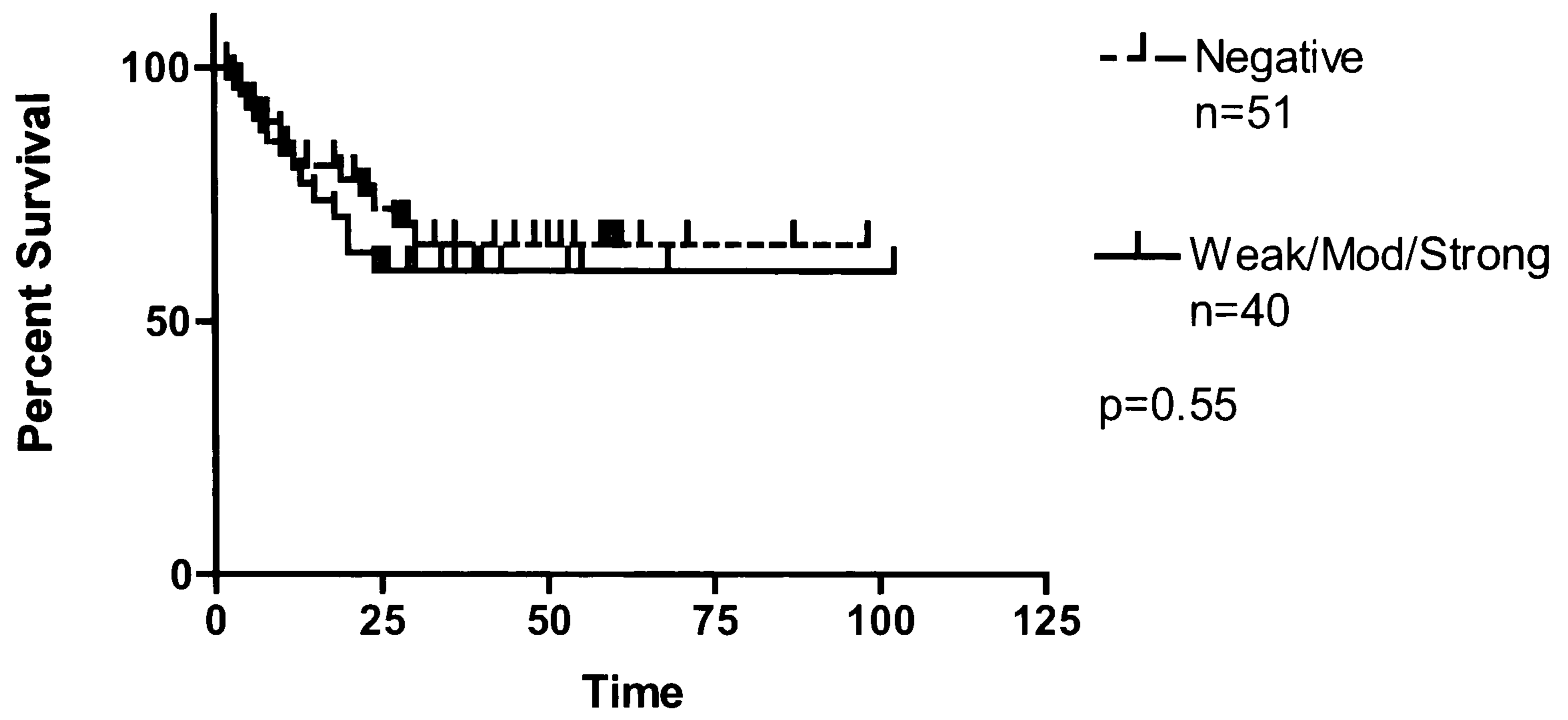
Graph 6.3.1 Kaplan Meyer plot of cancer specific survival, HIF1 $\alpha$



Graph 6.3.2 Kaplan Meyer plot of cancer specific survival, HIF1 $\alpha$



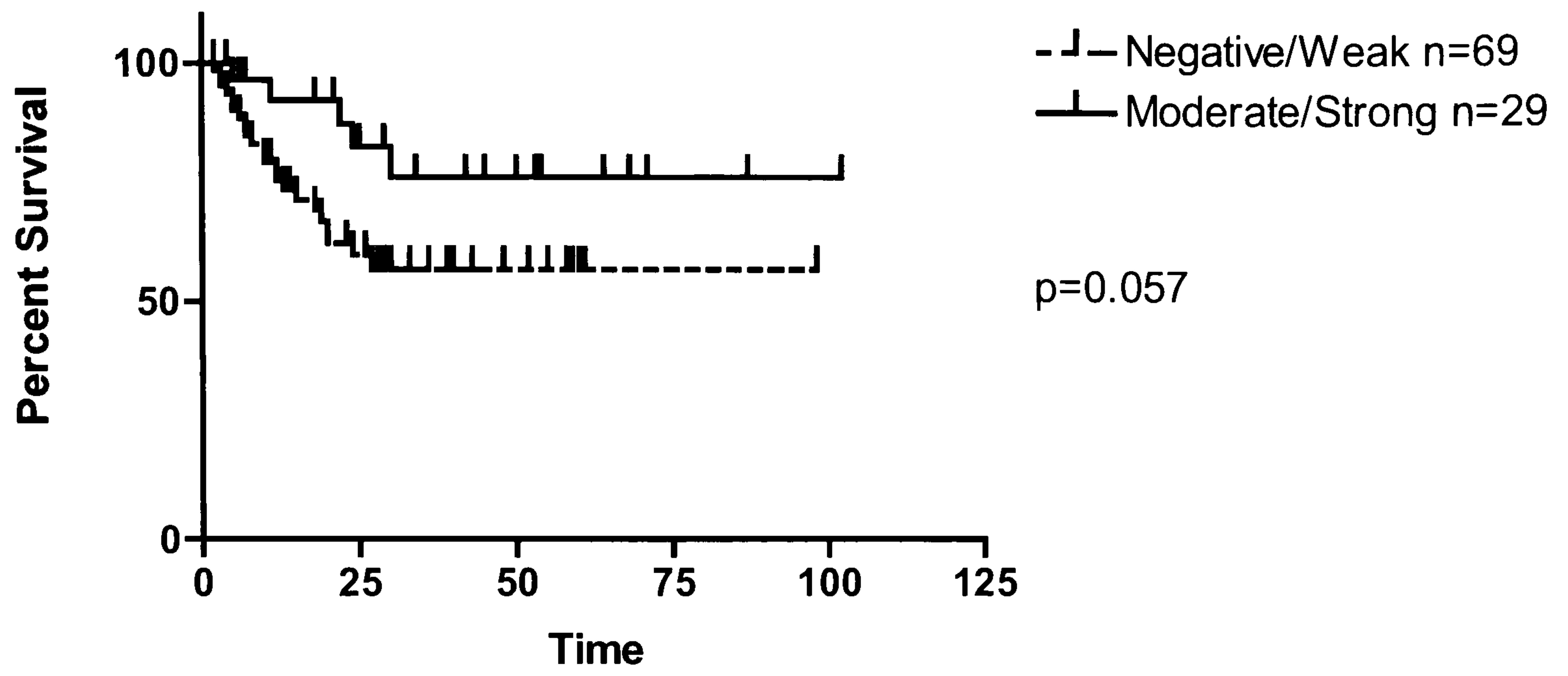
# CA IX



Graph 6.4 Kaplan Meyer plot of cancer specific survival for CA IX

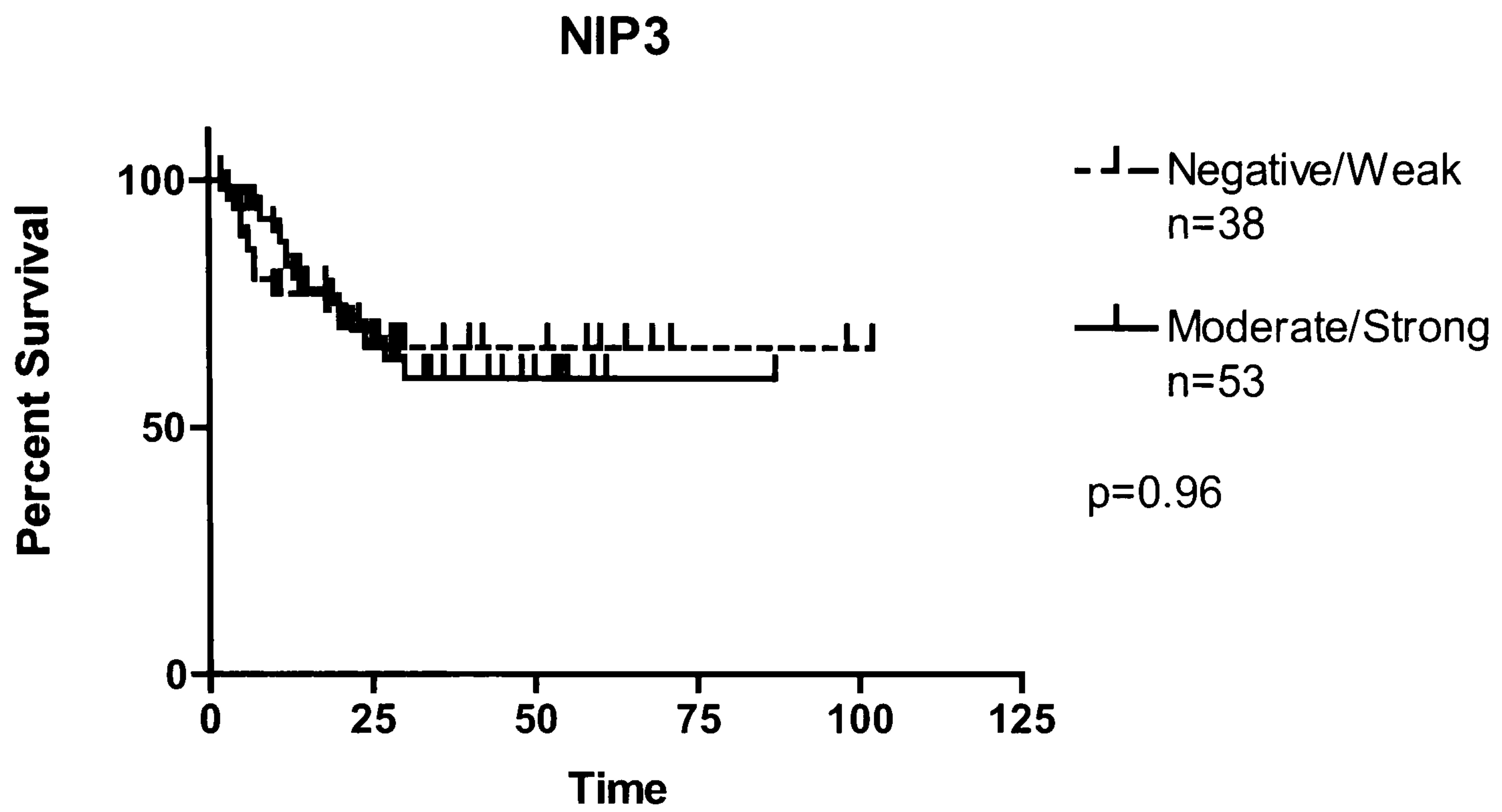


## HIF 2



Graph 6.5 Kaplan Meyer plot of cancer specific survival for HIF2 $\alpha$





Graph 6.6 Kaplan Meyer plot of cancer specific survival for NIP3



### 6.3 Discussion

In this study only transitional cell bladder tumors from primary cystectomies rather than transurethral resected specimens were used to ensure accurate staging (Dutta, Smith et al. 2001). None had previously had radiotherapy. A median follow-up of 22 months ensured that most patients who were going to develop metastasis had done so by the time of analysis.

The results show that although hypoxia related factors CA IX and HIF1 $\alpha$  were significantly associated with necrosis, the presence of necrosis itself was the independent prognostic factor. Areas of necrosis are thought to represent areas of chronic severe hypoxia, so this study supports the assertion that the development of chronic severe hypoxia in bladder cancer is independently associated with a worse prognosis. Hypoxia associated markers CA IX and HIF1 $\alpha$  were not associated with a worse prognosis but were significantly associated with necrosis.

This study demonstrates the high prevalence of necrosis in bladder cancer, and suggests that necrosis is an independent prognostic factor. Since this study was completed three larger in studies in other cancers have shown necrosis to have independent prognostic value (Table 6.10). The results in transitional cell cancer of the upper renal tract are of particular relevance and show good concordance with the present study's results. In that study, similar



to the present study, they also found a relationship between the amount of necrosis and progressively worse prognosis. Instead of comedo and gross necrosis they defined 'focal' and 'extensive' necrosis.

Cancer type <sup>x</sup>	n	Median Follow-up / years	Definition of necrosis	% with necrosis	Multivariate analysis		
					P value	95% CI	RR
Upper tract transitional cell carcinoma <sup>1</sup>	268	5	Semiquantitative microscopic on low magnification of available blocks. Focal = <10% tumour area, Extensive = >10% tumour area.	42%	0.02	1.09-3.05	1.8
Clear cell renal cell carcinoma <sup>2</sup>	485	4.2	Macroscopically on gross examination. Only if >10% tumour area, Confirmed as necrosis microscopically.	27%	0.004	1.36-4.89	2.58
Sarcoma <sup>3</sup>	338	7	Microscopic present or absent. No 10% cut off.	57%	0.01	1.2-3.0	1.9

Table 6.10 Studies showing necrosis to be an independent prognostic risk factor in solid tumours to date (July 2007). Studies <sup>1</sup>(Langner, Hutterer et al. 2006), <sup>2</sup>(Lee, Byun et al. 2006), <sup>3</sup>(Engellau, Anderson et al. 2004)



In bladder cancer a single study to date has found HIF1 $\alpha$  to be an independent prognostic indicator (p=0.02) and associated with microvessel density (p<0.001) (Theodoropoulos, Lazaris et al. 2004). However this study comprised only 93 predominantly endoscopically resected tumours that may have been inaccurately staged, and did not study necrosis. Furthermore one year after this study the same group published a larger study (n=140) in superficial cancer showing that HIF1 $\alpha$  positivity on its own was not an independent prognostic factor, but was only when present with p53 positivity (Theodoropoulos, Lazaris et al. 2005). This study supports the hypothesis that cells with mutations that enable survival in areas of chronic hypoxia are more biologically aggressive.

This was a retrospective study with the potential for selection bias. Six different surgeons performed at three different sites, with potential for individual variation in case selection and lymphadenectomy. The number of nodes removed and whether positive or negative is known to be an independent prognostic factor in multiple other large studies, but this was not found in this study. However nodal status was reported in only 41 of 91 patients and statistical analysis was performed assuming the remaining 50 were node negative. It is therefore more accurate to say that this study cannot comment on node status as a prognostic factor. Only after the period from which this cohort was taken (1999-2003) was the importance of pelvic



lymphadenectomy in bladder cancer shown convincingly in large studies (Konety, Joslyn et al. 2003) and has been more routinely performed since 2003.

Tumour adaptation and/or clonal selection might explain why necrosis is an independent prognostic risk factor (giving more information than stage alone). Adaptation to a hypoxic environment occurs with for example the secretion of angiogenic factors and upregulation of HIF1 $\alpha$  in perinecrotic areas. Such changes are associated with a more aggressive phenotype. However hypoxia severe enough to cause necrosis may also result in selection of cells resistant to hypoxic cell death for example p53 mutants and this effect has been demonstrated convincingly in vitro (Graeber, Osmanian et al. 1996). This effect may explain why p53 mutants are common in invasive cancer where necrosis is common but rare in superficial bladder cancer where necrosis rare. The exception being CIS where presumably the initiating event is a de novo p53 mutation.

HIF1 $\alpha$  staining was significantly associated with areas of necrosis but a significant minority (3/12 or 25%) of strongly HIF1 $\alpha$  positive tumors were completely negative for necrosis and CA IX staining. In these tumors HIF1 $\alpha$  may be predominantly under oxygen-independent regulation by growth factors and oncogenes. Indeed it has been shown since this thesis was started that bladder cancer cell line EJ when transfected with HIF1 and



grown as xenografts in nude mice, form tumours of a similar size but with less necrosis and greater microvessel density compared to vector-only transfected controls (Kondo, Hamada et al. 2005). Both hepatoma and renal cell carcinoma cell xenografts grow larger and with more blood vessels when transfected with HIF1 (Kondo, Hamada et al. 2005) (Maxwell, Dachs et al. 1997).

HIF1 $\alpha$  has been proposed as a target for therapy (Giaccia, Siim et al. 2003). The effects of antagonising HIF1 $\alpha$  in vivo are unknown. HIF1 $\alpha$  upregulation by hypoxia in untransformed cells in vitro causes cell cycle arrest (Goda, Ryan et al. 2003) (Carmeliet, Dor et al. 1998) inhibiting proliferation and apoptosis. Antagonising HIF1 $\alpha$  in cancer may cause cancer cells in hypoxic areas that have been made quiescent by HIF 1 $\alpha$  to re-enter the cell cycle or to apoptose. Cells re-entering the cell cycle would be more prone to chemotherapy or radiotherapy.

Tumors with HIF2 $\alpha$  staining showed a trend to a better prognosis ( $p=0.05$ ) and did not show any association with necrosis. This is in contrast to another study (Onita, Ji et al. 2002) of 67 cases which found an association of HIF2 $\alpha$  with a worse prognosis and necrosis. One possible explanation is that macrophages express HIF2 $\alpha$  in areas of inflammation as well as in perinecrotic areas. Inflammation in bladder cancer has been shown to be an independent marker of good prognosis in a study of 107 tumors (Offersen,



Knap et al. 2002). Possibly in this study by chance the HIF2 $\alpha$  staining was more in inflamed tumors than necrotic ones. Another possibility is that these studies were inaccurate as tumors were staged by endoscopic resection only. Areas of comedo/microscopic gross/macrosopic necrosis as identified in this study were not distinguished in these other two papers.

NIP3 is a cell death protein (Ray, Chen et al. 2000) regulated by HIF 1 $\alpha$  (Sowter, Ratcliffe et al. 2001) that has been shown to cause necrosis rather than apoptosis. There was surprisingly no relationship found with necrosis suggesting the presence of necrosis in bladder cancer is not primarily driven by HIF upregulation of NIP3 activity. Although there was a significant positive association with stage there was a significant inverse association with grade that is difficult to interpret.

Angiogenesis is an independent prognostic factor on multivariate analysis for breast cancer (Fox, Leek et al. 1995) and lung cancer (Macchiarini, Fontanini et al. 1992) and prostate cancer (Jones and Fujiyama 1999). That angiogenic invasive tumors have a poorer prognosis does not imply that these tumors are well oxygenated. Microvessel counts are assessed at vessel hotspots. Between the hotspots there may still be significant areas of hypoxia and necrosis. High levels of angiogenesis correlated with high levels of necrosis in breast tumours and gliomas (Chan, Leung et al. 1998; Leek, Landers et al. 1999). The same may be true in bladder cancer.



This study found a high prevalence of gross necrosis in advanced bladder cancer (7% of T1, 10% T2, 16% of T3 and 25% of T4 tumours). Necrosis has been shown here to give prognostic information to tumours treated surgically. The presence of necrosis is also relevant to radiotherapy. For over fifty years (Gray, Conger et al. 1953) it has been known that the effectiveness of radiotherapy is directly proportional to tissue oxygen concentrations. Radiotherapy in bladder cancer is known to be more successful in lower stages (T2), where necrosis is less prevalent, and where a more complete endoscopic resection is performed before treatment, perhaps due to debridement of necrotic areas prior to treatment.

The present study gives good evidence that gross necrosis and probably also comedo or microscopic necrosis is an independent prognostic factor in high-grade transitional cell carcinoma of the bladder. A more recent study of cystectomy specimens with more complete lymphadenectomies would be useful to see if presence of necrosis is still an independent prognostic factor when lymph node status is included in the statistical model. However this finding has potential use in prognosticating superficial high-grade bladder cancer after endoscopic resection (when no information on node status and limited information on stage is available) and hence selecting and counseling patients whether or not to have a radical cystectomy. Currently the presence or absence of necrosis is not part of any prognostic staging system, as it is



merely believed to be an epiphenomenon of higher stage tumours. These results also suggest that patients with necrosis in their tumours should be selected for trial of more intensive adjuvant therapies, both standard drugs and those targeting hypoxic cells.

### 6.3.1 The clinical relevance of biomarkers and the structured analysis of biomarker studies

TNM is the staging system used in bladder cancer to predict prognosis. On the basis of such staging patients will either be treated endoscopically (with or without intravesical chemotherapy) or with radical cystectomy which carries major risks of mortality (3%) and morbidity (30%). The TNM staging system however is not accurate enough to predict which tumours will progress to metastasis and which will not. Hence for high risk superficial bladder cancer some patients could be safely spared a cystectomy, equally for invasive disease some patients could be selected for novel or alternative treatments if it was known that they would fail conventional treatment. For a long time mutations in p53 has been known to be one of the commonest mutations in cancer and mutated p53 occurs in 50% of invasive bladder cancers and carcinoma in situ. P53's physiological role at the G1/S checkpoint is to arrest the cell cycle or trigger apoptosis in conditions of cellular stress such as hypoxia or DNA damage. Hence mutation of p53 would allow a cell to divide unchecked even in the adverse environment of a tumour. The great interest in p53 as a biomarker is indicated by the fact that at least 117 different studies of p53 in bladder cancer have been carried out. However a metanalysis of these studies concluded that there is insufficient evidence to use p53 as a prognostic marker in bladder cancer. Most if not all studies were retrospective. 70% of the studies contained less than 100 patients, which is a minimum requirement for multivariate analysis. There was great heterogeneity in the laboratory and statistical methods used. The



method used to look for p53 mutation was the most convenient (immunohistochemistry) but not the most accurate, false negatives and positives occur. Secondly neoplastic change is a heterogenous and complex process involving many different pathways with many different molecules and it is perhaps naive to expect a single molecule in a single pathway to add predictive value to all bladder cancers. However the authors acknowledged that there was sufficient evidence that p53 could have a role to play in bladder cancer management. However they recommended that a formal process of development of biomarkers be followed before introducing them into clinical practice, and that laboratory methods and statistical analysis should be centralized (Malats, Bustos et al. 2005).

The National Cancer Institute has recommended a strategy for delineating the full development of biological determinants. (Cordon-Cardo 2004). The scheme follows a similar one as for drug trials: Phase I (pilot studies and assay development), Phase II (Retrospective clinical analysis) Phase III (Prospective confirmatory analysis) and Phase IV (Multi-institutional validation trials). Studies in bladder cancer are now proceeding down this path slowly A recent retrospective study of 191 bladder cancer patients qualifies as Phase II of the NCI strategy (Shariat, Karakiewicz et al. 2008). The study employed five related biomarkers (p53, pRB, p21, p27, and cyclin E1) and demonstrated a significant 11% increase in predictive accuracy over predictive nomograms based on the TNM system alone. For cancer specific survival the presence of 4 or 5 altered biomarkers was a stronger predictor of death from bladder cancer than stage as indicated by their relative risks in multivariate analysis (RR12.5 for 4 or more biomarkers vs RR4.8 for stage T1 vs stage T3).







## **Chapter Seven**

**Genearray experiments on normal human urothelial cells, peripheral blood monocytes and T-cells under hypoxia, and conclusion**



## 7.1 Introduction

In this chapter the in vitro hypoxia profile of genes upregulated in bladder cancer cell line EJ28 is compared with genearray profiles of hypoxia upregulated genes at 16 hours in normal human urothelial cells, peripheral blood monocytes and T-cells. These experiments were performed as single array experiments each. These experiments benefited from the increased array size and accuracy of Affymetrix arrays (HGU 133 A and B) which contain a total of 44,000 probes.

There are no published papers on hypoxia upregulated genes in normal human urothelium, and having studied the hypoxia profile of cancer cell lines in culture, a natural comparison of interest would be normal human urothelium to see if there were any major differences.

As demonstrated in Chapter Six of this thesis and observed by others HIF  $2\alpha$  has been located to tumour-associated macrophages in bladder cancer (Onita, Ji et al. 2002) and in tumour associated macrophages of many solid tumour types (Talks, Turley et al. 2000). As such the gene profile of monocytes to hypoxia is relevant to this thesis. A significant number of bladder cancers have areas of inflammation and coordinating the macrophage response are helper T-cells (CD4) typically in a Type IV cell mediated hypersensitivity reaction. Such a reaction may occur within bladder tumours naturally but also occurs when bladder tumours are treated with intravesical BCG (bacillus calmette-guerin), a commonly used treatment for high-risk superficial bladder cancer. Macrophages have also been proposed as a novel way of delivering gene therapy to areas of pathological hypoxia (Griffiths, Binley et al. 2000). HIF  $2\alpha$  has been shown in experiments exposing normal rats to hypoxia to be inducible by hypoxia in many normal tissues both parenchymal and non-parenchymal, and its role seems to be complementary to that of HIF  $1\alpha$  (Wiesener, Jurgensen et al. 2003).



Normal human urothelial cells were extracted and grown in culture by the author, human peripheral blood monocytes and CD4 T-cells were cultured, purified and T-cells were activated by addition of CD3/CD28 beads by Patrick Maxwell's laboratory (see Chapter 2).



## 7.2 Results

The results displayed in Table 7.1 compare genes upregulated by hypoxia at 16 and 24 hours in bladder cancer cell line EJ28 on the Sanger array to genes upregulated by hypoxia at 16 hours in 3 non-transformed cell types analysed on Affymetrix arrays. (see also Appendix II-VI of this thesis for the actual lists of genes).

Table 7.1 (On the next two pages) Genes upregulated by hypoxia at 16 hours in cultured normal urothelium, peripheral blood monocytes and T-cells compared to the original 32 genes identified as hypoxia regulable in bladder cancer cell line EJ28. Genes highlighted in yellow were upregulated in all cell types examined.



No.	Affy. probe	GENE	Sanger Array		Affymetrix HGU133 A and B Arrays			
			EJ 28 n=32	EJ 28 n=32	Normal Urothelium n=198	Peripheral Blood Monocytes n=492	T cell unactivated n=12	T cell activated n=333
Total upregulated by hypoxia >2 fold			16	24	16	16	16	16
Hours in hypoxia								
<b>Known to be hypoxia-upregulated in other cell lines (1-23)</b>								
1	202998_s_at	Adrenomedullin (ADM)	3.4	17	2.1	10		
2	210095_s_at	Insulin-like growth factor binding protein 3 (IGFBP-3)	2.6	12.1	2.9	15		
3		n-myc downstream regulated protein 1 (NDRG1)	2.9	8.2	1.4		3	
4	201849_at	NIP3	5.3	6.6	2.4	7.4	5.5	15
5	202464_s_at	Inducible 6-phosphofructo-2-kinase (PFKFB3)	2.7	4.7	4	2.6		
6	202998_s_at	Lysyl Hydroxylase (PLOD2)	2.4	4.7	2			
7	202364_at	MAX interacting protein 1	2.2	4.4	2	4		2.3
8	201250_s_at	Glucose Transporter 1 (GLUT 1)	3.1	4.2	2.4	10.5	4.6	4
9		1,4-Alpha Glucan Branching Enzyme	2.3	3.9	1.3	6.8	4.9	2.2
10	202770_s_at	Cyclin G2	2.2	3.8	1.8	2.2		
11	207543_s_at	Proline-4-hydroxylase (P4HA1)	2.5	3.7	1.3	12	7	4.6
12		Adenylate Kinase 4	3.2	3.2				
13	202022_at	Aldolase C	2.9	3.1	1.6			
14		Prolyl-4-hydroxylase alpha subunit	1.9	3				
15	203665_at	Haem oxygenase 1	1.8	2.6				
16		EGL9 Homologue 1	2.5	2.5	3.5			
17	207022_s_at	Testis-specific lactate dehydrogenase	2.1	2.5		?5.5		
18		VEGF	1.9	2.5	2.9			6
19	204348_s_at	Adenylate Kinase 3	2.4	2.5	1.2			
20	201041_s_at	Dual Specificity Phosphatase 1 (DUSP1/CL100/MKP1)	1.7	2.4	1			4
21	220781_at	Dec-01	1.7	2.1				



No. Affy. probe	GENE	Sanger Array		Affymetrix HGU133 A and B Arrays			
		EJ 28	EJ 28	Normal Urothelium	Peripheral Blood Monocytes	T cell unactivated	T cell activated
Total upregulated by hypoxia >2 fold		n=32	n=32	n=198	n=492	n=12	n=333
Hours in hypoxia		16	24	16	16	16	16

**Known to be hypoxia-upregulated in other cell lines (no.1-23)**

22	208308_s_at	Glucose-6-Phosphate isomerase	2.6	2.1	1.1	11	7	2.5
23	200966_x_at	Fructose-Bisphosphate Aldolase A	2.1	2.1	1	5		

**Not previously known to be hypoxia regulable (no.24-28)**

24	202856_s_at	Solute carrier family 16 member 3 (SLC16A3)	2.8	8.2	0.8	2		5.4
25	204086_at	Preferentially expressed antigen of melanoma (PRAME)	4.5	4.9	no data			
26	211955_at	Importin Beta 3	2.8	4.1	1			
27	205158_at	RNAse 4	1.9	3.1	no data			
28	202123_s_at	Protooncogene ABL1	2.2	2.9	1.1			

**ESTs (no.29-32)**

29	50378_A		3	3.9				
30	50022_A		3.2	3.3				
31	31564_B		3.5	3.3				
32	21875_A		2.1	2.4				

**Others genes of interest upregulated only in normal human urothelium in culture (no probes present for these on the Sanger array) (no.33-37)**

33	230746_s_at	stanniocalcin 1			6.8			
34	222847_s_at	egl nine homolog 3 (C. elegans)			3.55			
35	227271_at	Fibroblast Growth Factor 11			3.5			
36	205199_at	Carbonic anhydrase IX			3.4			
37	225912_at	P53 protein			2			

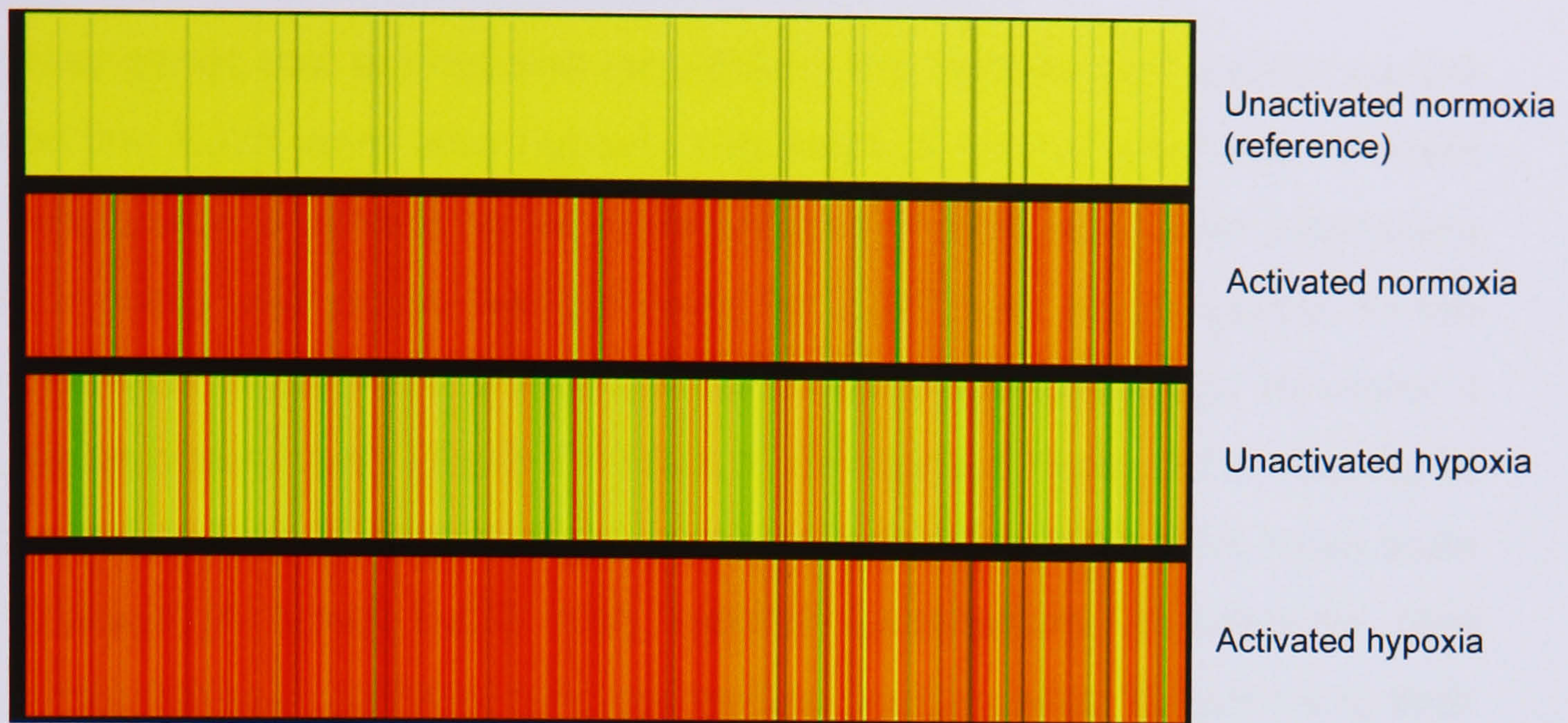
188



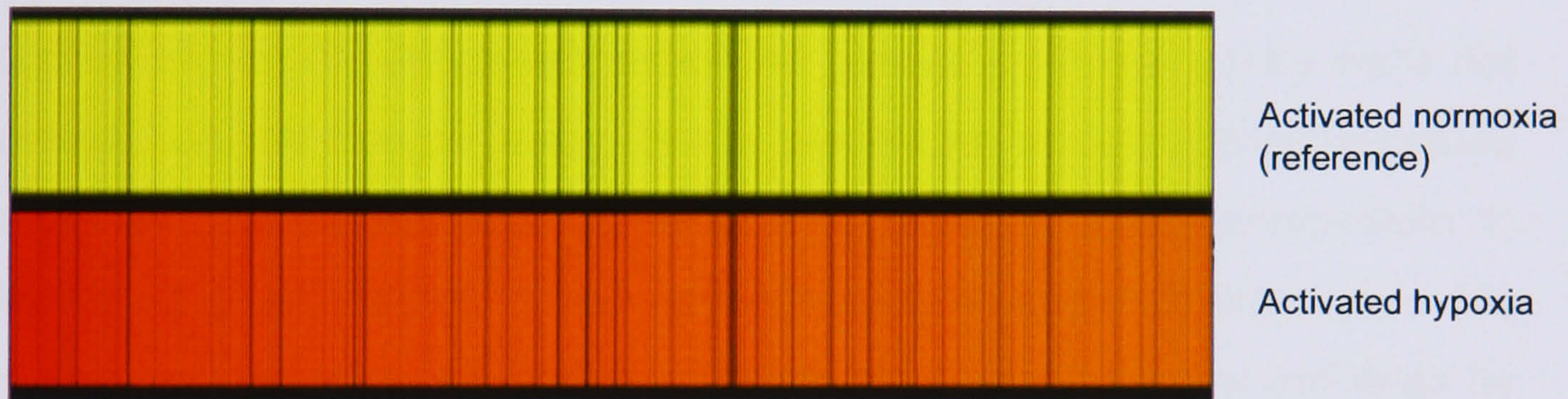
Affy probe number	Gene
1 226464_at	Homo sapiens, clone MGC:33365 IMAGE:5267770, mRNA, complete cds
2 226348_at	EST, Weakly similar to JC5238 galactosylceramide-like protein, GCP - human [H.sapiens]
3 226347_at	EST, Weakly similar to JC5238 galactosylceramide-like protein, GCP - human [H.sapiens]
4 224605_at	EST, Weakly similar to T02670 probable thromboxane A2 receptor isoform beta - human [H.sapiens]
5 221497_x_at	gb:BC005369.1 /DEF=Homo sapiens, chromosome 1 open reading frame 12, clone MGC:12484, mRNA, complete cds. /FEA=FLJ21620
6 219862_s_at	gb:NM_012336.1 /DEF=Homo sapiens nuclear prelamin A recognition factor (NARF), mRNA. /FEA=mRNA /GEN=NARF /PROD=FLJ21620
7 219232_s_at	gb:NM_022073.1 /DEF=Homo sapiens hypothetical protein FLJ21620 (FLJ21620), mRNA. /FEA=mRNA /GEN=FLJ21620 /PROD=FLJ21620
8 218149_s_at	gb:NM_017606.1 /DEF=Homo sapiens hypothetical protein DKFZp434K1210 (DKFZp434K1210), mRNA. /FEA=mRNA /GEN=DKFZp434K1210
9 217047_s_at	Consensus includes gb:AK027138.1 /DEF=Homo sapiens cDNA: FLJ23485 fis, clone KAI05211. /FEA=mRNA /DB_XREF=gi:1022073
10 209122_at	gb:BC005127.1 /DEF=Homo sapiens, adipose differentiation-related protein, clone MGC:10598, mRNA, complete cds. /FEA=mRNA /GEN=ADIRP
11 202973_x_at	gb:NM_014883.1 /DEF=Homo sapiens KIAA0914 gene product (KIAA0914), mRNA. /FEA=mRNA /GEN=KIAA0914 /PROD=KIAA0914
12 202464_s_at	gb:NM_004566.1 /DEF=Homo sapiens 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), mRNA. /FEA=mRNA /GEN=PFKFB3
13 201313_at	gb:NM_001975.1 /DEF=Homo sapiens enolase 2, (gamma, neuronal) (ENO2), mRNA. /FEA=mRNA /GEN=ENO2 /PROD=enolase 2
14 201250_s_at	gb:NM_006516.1 /DEF=Homo sapiens solute carrier family 2 (facilitated glucose transporter), member 1 (SLC2A1), mRNA. /FEA=mRNA /GEN=SLC2A1
15 201849_at	gb:NM_004052.2 /DEF=Homo sapiens BCL2adenovirus E1B 19kD-interacting protein 3 (BNIP3), nuclear gene encoding mitochondrial protein 3 /FL=gb:AF002697.1 gb:U15174.1 gb:NM_004052.2

Table 7.2 The small number of genes (15) upregulated by hypoxia in common between normal urothelium (198 genes total) and peripheral blood monocytes (492 genes total).





A



B

Figure 7.1 Graphic representation of 333 genes upregulated by hypoxia in activated T cells. A single vertical line represents each gene; intensity of red colour indicates fold upregulation (most intense red = 8 fold upregulation, yellow = no fold change). In both (A) and (B) genes are ranked by fold upregulation of activated T-cells in hypoxia (B).

(A) With unactivated normoxic levels as reference. Pure activation of T-cells in normoxia upregulates many more genes than the effect of pure hypoxia on unactivated T-cells. Without activation only relatively few genes are upregulated by hypoxia (12 genes in red on the left). Activating T-cells in normoxia upregulates many genes (333).

(B) With activated normoxic levels as reference, this figure illustrates that once activated, all of the 333 activated genes also acquired the property of being hypoxia inducible >2 fold.



### 7.3 Discussion

Many genes that are hypoxia regulable in the transformed bladder cancer cell line EJ28 were also hypoxia regulable in normal urothelia. A direct comparison is difficult as two different array types were used (Affymetrix for normal urothelium, Sanger array for EJ28) and not all genes on the Affymetrix array were present on the smaller Sanger array. However it can be clearly seen (Table 7.1) that at 16 hours of hypoxia the majority of the more strongly inducible genes in the upper half of the table have been induced in both EJ28 and normal urothelium suggesting that untransformed urothelial cells upregulate similar genes in hypoxia to their transformed counterparts.

Several genes of interest were noted to be upregulated in normal human urothelium on the Affymetrix array but probes for these genes were not present on the Sanger array. Some are known to be hypoxia regulable such as p53 protein and carbonic anhydrase IX and stanniocalcin 1; fibroblast growth factor 11 was not previously reported. Stanniocalcin 1 is a calcium homeostasis protein upregulated in many tumour cell lines by hypoxia and hence is probably also upregulated in bladder cancer cell lines (Yeung, Lai et al. 2005). It also is known to play a likely physiological role, dependent on cytokine interleukin 6 (IL-6), in protection against ischaemia in untransformed cells in the brain and heart (Westberg, Serlachius et al. 2007) (Westberg, Serlachius et al. 2007). Of particular interest is the unreported hypoxic upregulation of fibroblast growth factor 11. Recently activating mutations in the FGF receptor 3 (FGFR3) has been found to be present in the majority of stage Ta low-grade superficial bladder cancers. The same mutations in FGFR3 have been described in developmental disorders of human cartilaginous growth plates (e.g. achondroplasia, hypochondroplasia) (van Rhijn, Lurkin et al. 2001; van Rhijn, van Tilborg et al. 2002). Interestingly the cartilaginous growth plate is one avascular area where HIF has been



shown to be essential for development, in a cartilage-specific HIF1 knock-out mouse (Schipani, Ryan et al. 2001).

The gene most upregulated by hypoxia (22-fold) in peripheral blood leucocytes was interleukin-1 (IL-1) which is the archetypal proinflammatory cytokine. Tumour necrosis factor (TNF) receptor and converting enzyme (2.5 and 2 fold, see appendix III), another proinflammatory cytokine was also upregulated. These two genes are known to work synergistically in the inflammatory response whether induced by an infection, trauma, ischaemia, immune-activated T cells, or toxins (Warrell 2004). Unlike IL-1, TNF induces cell death, particularly in tumour cells. TNF has been injected as part of anticancer protocols, either systemically or regionally, in order to bring about death of tumour cells. Similar to the effects of IL-1 in humans, TNF induces fever, headaches, myalgias, nausea, vomiting, and profound hypotension. Similar to IL-1 administration, elevated levels of IL-6, other cytokines, soluble cytokine receptors, and acute phase proteins are observed. In fact, the systemic responses to TNF and IL-1 are mostly indistinguishable. However, activation of the coagulation pathways is observed with TNF but not IL-1. In human subjects, TNF induces a short-term increase in circulating plasminogen activator activity with rises in the antigenic levels of urokinase-type plasminogen activator, a gene which transcript was also upregulated 2 fold on this array. Hence in monocytes we see a cluster of functionally related genes all being hypoxia regulable, turning on an inflammatory response to ischaemia.

Comparing the untransformed cell types of normal human urothelium and peripheral blood monocytes which were both studied on the larger Affymetrix array, less than 7% (15 genes) of hypoxia upregulated genes were in common between the two cell types. This suggests that there can be a significant difference in the hypoxic response between different cell types.



Unactivated CD4 T-cells upregulate only a few genes under hypoxia, but when activated by the influence of CD3/CD28 activation, all the genes that were activated by CD3/CD28 also then became hypoxia regulable. This suggests that even within a single cell type other molecular pathways and the immune environment of the cell may have a dramatic effect on the scale and specificity of the transcriptional response to hypoxia.

Some genes (highlighted in yellow in Table 7.1) were upregulated by hypoxia in all cell types. These mostly related to glycolysis but NIP3 upregulation was also common to all cell types examined.

SLC16A3 the lactate transporter has already been dealt with in Chapter 3 but at least here we see that the Affymetrix array result in this chapter (no upregulation in normal urothelium) agrees with the result of the real-time RT-PCR used in Chapter 3.

This chapter truly reveals a much greater heterogeneity in hypoxia response pathways than previously recognised. Each cell type may have different functions under hypoxia to conserve or activate which will determine the profile, and the extent of hypoxia interaction with the microenvironment may be highly tissue specific.

### 7.3 Conclusion

Perhaps the most significant finding of this thesis is that necrosis is an independent prognostic risk factor in bladder cancer (Chapter 6). This is the first published report of this, but another large multivariate study in transitional cell carcinoma of the upper renal tract has come to the same conclusion and indeed produced very similar survival curves and hazard ratios. As have large multivariate studies of necrosis in renal cell carcinoma and sarcoma (see table 6.10 pp161). This supports the



hypothesis of this thesis that the development of chronic hypoxia within bladder cancer is itself not a mere epiphenomenon of tumour growth but is associated with biologically more aggressive disease, invasion and metastasis.

New pathways of relevance to hypoxia in bladder cancer have been defined by comparing two microarray experiments; a set of 8 replicates of a cancer cell line exposed to hypoxia and a set of 39 tumours against a reference panel of a variety of cancer cell lines in normoxia. The eight genes that were significantly upregulated on both arrays (Chapter 4, figure 4.4). Validating this method of investigating bladder cancer was the discovery that VEGF was one of the 8 genes picked out from the 6000 transcripts. VEGF is already known to be the key hypoxia regulable angiogenic factor in bladder cancer (Crew, O'Brien et al. 1999; Reiher, Ivanovich et al. 2001). The results of a few of the most upregulated transcripts on both arrays (e.g. IGFBP3, SLC16A3, RNase 4 in Chapter 3) were validated in cell lines and untransformed cells by realtime RT-PCR and by the finding of raised levels of IGFBP3 and Angiogenin in the urine of bladder cancer patients (Chapter 5). It would have been interesting to see if levels of IGFBP3 in the urine correlated with amount of necrosis in patients' tumours but without reviewing another 157 sets of slides this was not possible. However from Chapter 6 we discovered that necrosis becomes more common with invasive disease, suggesting a possible reason why levels of IGFBP3 were significantly higher in invasive bladder cancer patients than in superficial disease. The function of IGFBP3 in hypoxia whether stimulating or inhibiting the IGF growth factor pathway is controversial and would require further experiments with inhibitory RNA (RNAi), blocking antibodies to IGFBP3 and addition of IGF I and II to the cell lines. The lactate transporter SLC16A3 was a gene of particular interest as it was not upregulated by hypoxia in normal urothelial cells or peripheral blood monocytes but was upregulated in all cancer cell lines examined and most of all (64 fold) in cultured endothelial cells. This pattern of upregulation was unusual, IGFBP3 mRNA for example was upregulated by hypoxia in normal urothelium and



endothelial cells and more so in bladder cancer cell lines. Consistent with this IGFBP3 protein was found in much greater baseline and hypoxic concentrations in cell-line supernatant compared with endothelial cell supernatant (Chapter 5). Levels of the protein were measured at 48 and 72 hours compared with the 16 and 24 hour measurements made on the arrays to see if protein levels were present after the rise in mRNA transcript. IGFBP3 may play a normal physiological role in the sequestration of IGF to the nucleus (via importin beta 3 also found to be upregulated in hypoxia on the array) promoting proliferation. This baseline level of IGFBP3 may be upregulated in bladder cancer cells and enhanced by hypoxia both in normal urothelium, endothelium and bladder cancer cells.

IGFBP3 has recently been shown to have antiangiogenic and apoptotic properties in a human prostate cancer xenograft model. Tumour size and number of blood vessels was reduced. Antiangiogenic properties were confirmed in three other in vitro assays and were found to be IGF independent (Liu, Lee et al. 2007)

Leek has recently shed light on the role of HIF1 in tumours by growing a HIF1 competent and incompetent cell line as tumour spheroids. (Leek, Stratford et al. 2005). Both spheroids developed central necrosis showing that HIF1 does not protect against that. Oxygenation and nutrition and acidity are normal at the outer surface of the tumour spheroid where it comes into contact with the medium. Like a tumour however there is an increasing gradient of hypoxia and nutrition poor environment towards the centre of the spheroid. The HIF1 competent cells grew into larger tumour spheroids from day 3 largely because in the intermediate zone between rim and necrotic centre HIF1 protected the cells against apoptosis by inducing cell cycle arrest. The HIF incompetent cell line by comparison had higher apoptotic indices in this area. Hence the role of HIF 1 appears as stated in the introduction to be important to survival of cells (cancerous or normal cells) in conditions of cellular stress. Hence it is believed that inhibiting HIF1 in cancer could make cells more susceptible to apoptosis



from chemotherapy by preventing cell cycle arrest under conditions of cellular stress.

It has subsequently been shown after work on this thesis was completed that very low oxygen tensions of 0.01% i.e. anoxia are required to cause apoptosis and this anoxic cell death occurs independent of any HIF1 pathway such as NIP3. Oxygen tensions of 0.5% do not kill human tumour cells. (Papandreou, Krishna et al. 2005).

In Chapter 4 individual fold changes and combined fold changes of transcripts on the in vivo microarray of these eight hypoxia inducible genes were found not to show a close correlation with microscopic or gross tumour necrosis or hypoxia-associated marker CA IX. Only GLUT-1 fold changes on the in vivo array showed a correlation with CA IX scores. Unfortunately CA IX itself was not on the Sanger array. This suggests that attempting to define a hypoxia profile for individual tumours based on a microarray analysis is not as simple as it seems at first. There are multiple possible reasons for this: Firstly the heterogeneity within a tumour is large with well vascularised areas and necrotic areas coexisting in many tumours. There are multiple cell types found in tumours, whether stroma or normal epithelium or neoplastic cells or inflammatory cells. Bladder cancers are thought to be oligoclonal so not all neoplastic cells will share a similar array profile. Cancers from different individuals and from different stages may have significantly different profiles. Some hypoxia inducible genes are controlled mostly at the protein level; HIF1 $\alpha$  for example oxygen-dependent regulation is at the level of the protein rather than at transcript level. Finally this study relied on the Sanger array that is not as semi quantitative as the Affymetrix array, and requires a comparator reference RNA. The selection of the comparator reference RNA has to be carefully considered.

In Chapter 5 an attempt was made to discover a microarray hypoxia profile for necrotic/hypoxic tumours by firstly grouping tumours by necrosis or CAIX staining and then performing multiple t-tests across all



the thousands of transcripts on the array. However even with 32 tumours to analyze there were not enough replicates to allow for a statistically significant result. Simple t-tests for each transcript produced significant results but this method requires sophisticated statistical analysis to correct for the use of multiple t-tests. If such a study were performed again more replicates would be required of bladder cancers of a similar stage, perhaps with several samples from the same individual, with the amount of necrosis on histology carefully documented, and perhaps the selection of tissue used for analysis performed by laser microdissection.

The finding in Chapter 6 that necrotic tumours have an independently poorer prognosis is an intriguing one. Chapter 5 provided some evidence that an untransformed cell line dies more readily in hypoxia than a cancer cell line, suggesting that perhaps one reason tumours with necrosis are more aggressive could be that in these tumours the chronically hypoxic environment has selected out cancer cells which can survive the adverse environment and that such cancer cells have a more aggressive phenotype. The selection out of p53 mutations by hypoxia in vitro (Graeber, Osmanian et al. 1996) and the recent finding that the HIF1 $\alpha$ <sup>+</sup>/p53<sup>+</sup> immunohistochemical phenotype is independently associated with an adverse prognosis in bladder cancer (Theodoropoulos, Lazaris et al. 2005) fits with this hypothesis. It would be interesting to see if p53<sup>+</sup> is associated with necrosis in bladder cancer. There are no studies on this to date. Levels of apoptosis and P53 on its own however have not proved consistent or independent predictors of prognosis in bladder cancer.

Chapter 7 used purely Affymetrix microarrays to look at the heterogeneity in the hypoxic response of specific cell types (normal human urothelium in culture, normal human monocytes, normal human purified CD4<sup>+</sup> Tcells activated and unactivated). The Affymetrix arrays used were larger and more precise than the Sanger arrays however a limited comparison was made with the Sanger array data from Chapter 3. Most genes that were



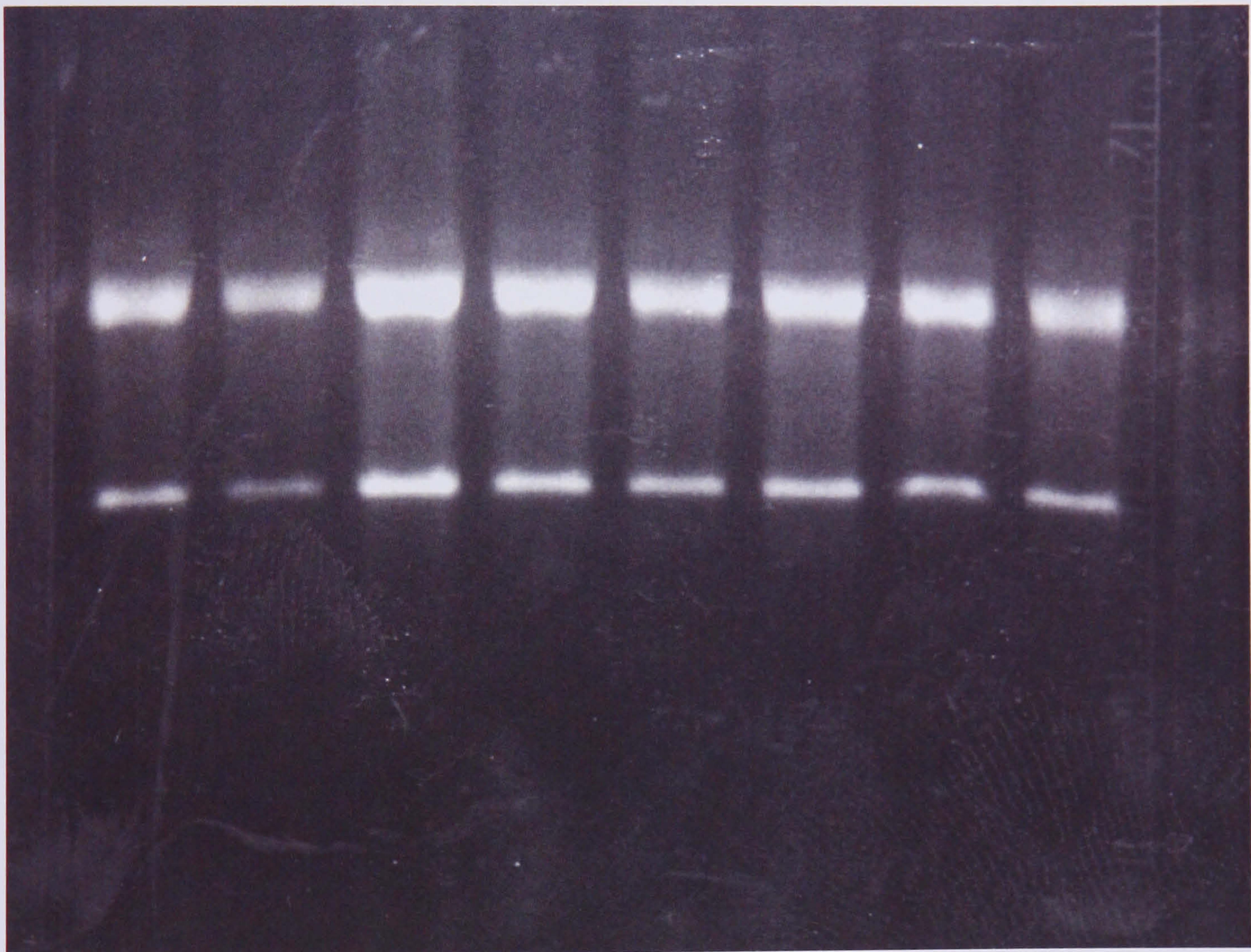
hypoxia regulable in the transformed bladder cancer cell line EJ28 on the Sanger array were also hypoxia regulable in normal urothelia. The Affymetrix array also identified p53 protein, carbonic anhydrase IX, stanniocalcin 1 (previously known to be hypoxia regulable in cancer) but also fibroblast growth factor 11 previously not known to be hypoxia regulable but of particular interest in bladder cancer superficial disease given the common mutation of FGFR3 recently discovered in superficial disease. Analysis and comparison of the other cell lines revealed a much greater heterogeneity in hypoxia response pathways than previously recognised. Each cell type may have different functions under hypoxia to conserve or activate which will determine the profile, and the extent of hypoxia interaction with the microenvironment may be highly tissue specific.



## Appendix I

### Purity of total RNA (tRNA) used in array experiments

Figure AI.1 Purity of tRNA extracted from EJ28 biological replicates grown in normoxia (3 samples on left) and hypoxia (five samples on right). Agarose gel 1% with ethidium bromide, 1ug of RNA loaded in each lane, showing two clear bands of 18s and 28s tRNA.





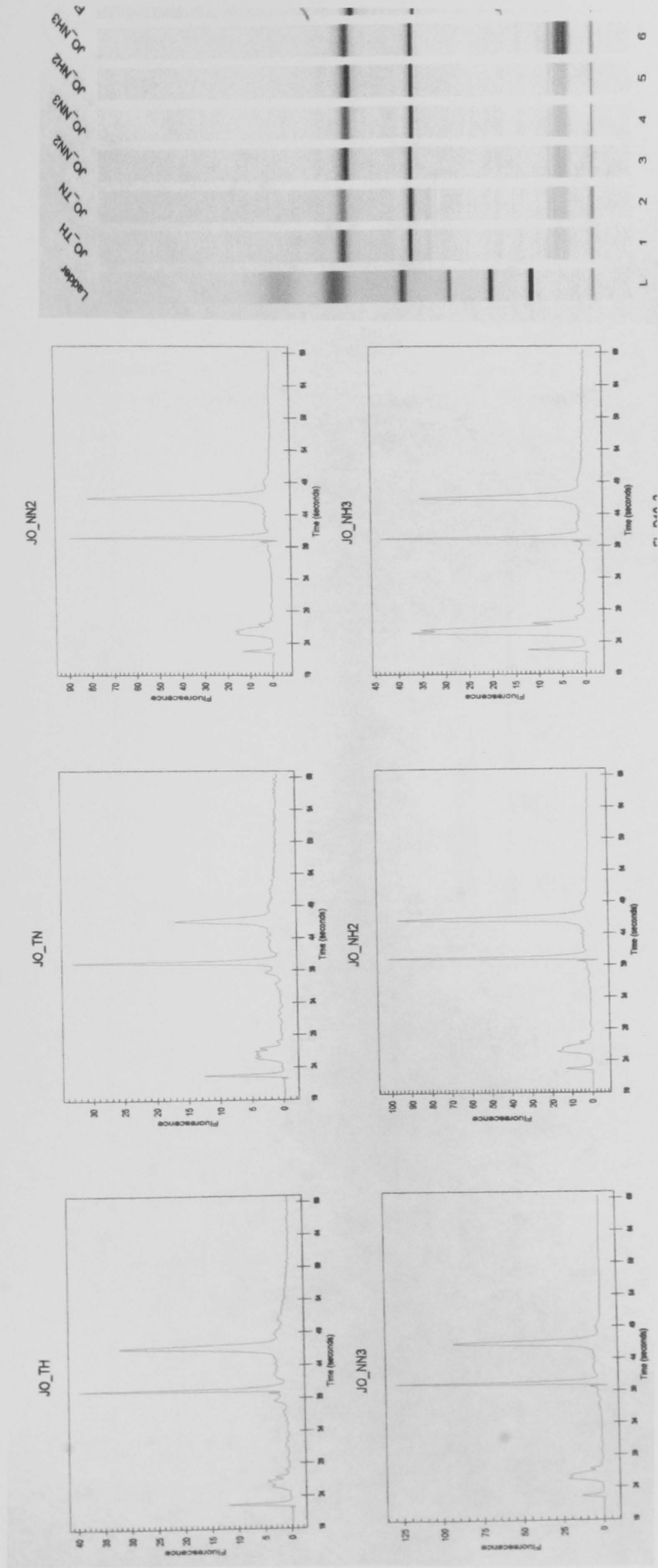


Figure A1.2 For Affymetrix chips RNA purity was analysed using an Agilent 2100 bioanalyser. Agilent analysis of total RNA extracted from normal urothelial cells grown in culture. The gel on the right is a virtual one. Samples N2 and H2 were used for the microarray chip as they had the best purity.



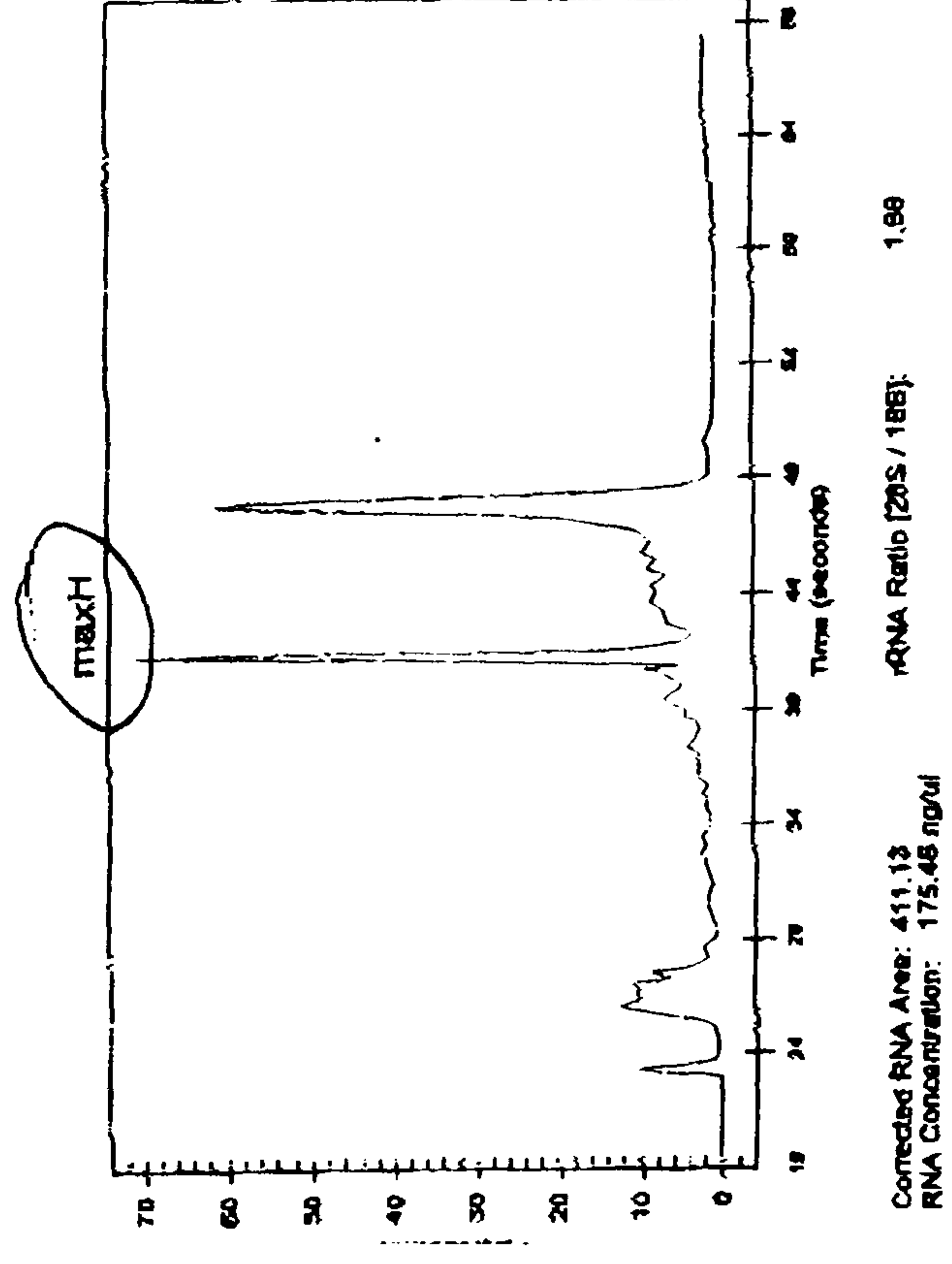
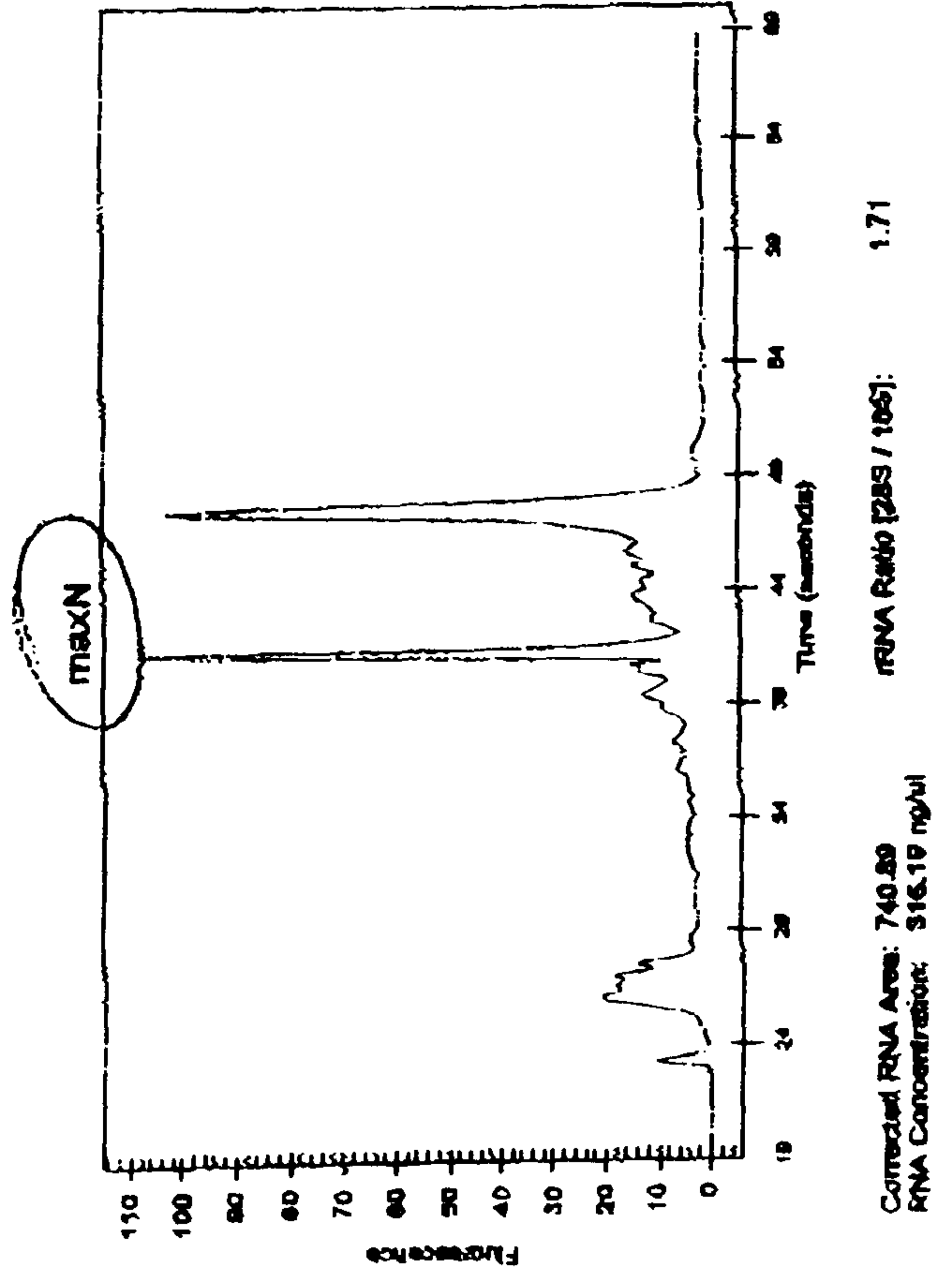


Figure A1.3 Agilent analysis of tRNA extracted by P. Maxwell from fresh peripheral blood mononuclear cells (PMBC). The peaks for 18s and 28s ribosomal RNA are sufficiently clear



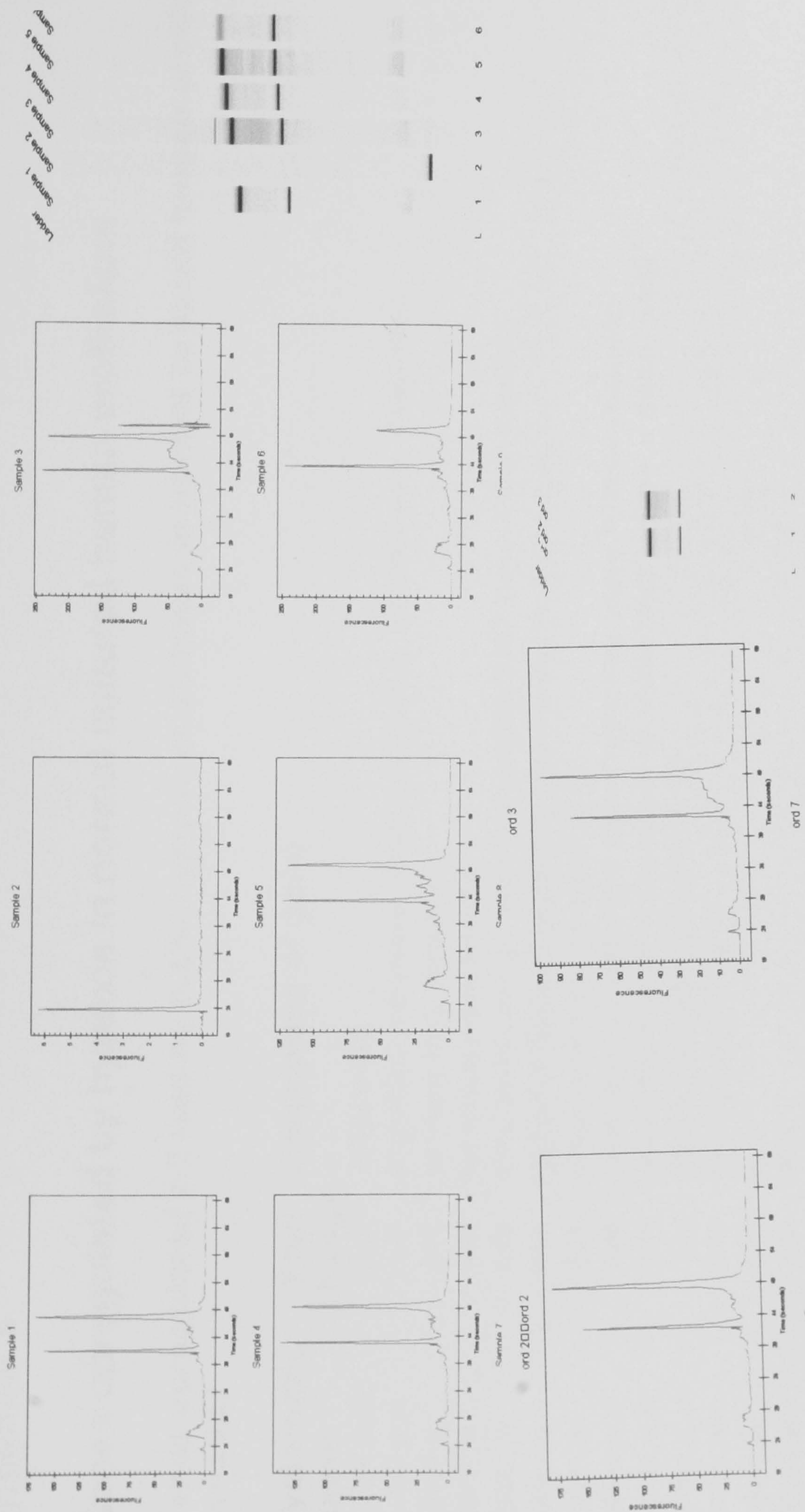


Figure A1.2 Agilent analysis of total RNA extracted from fresh T cells extracted from human peripheral blood. The gel on the right is a virtual one. Samples 2 and 3 were repeated below and were found to be of good quality



## Appendix II

### Genes upregulated by hypoxia in normal cultured human urothelium

#### Genes upregulated >2 fold by 0.1% hypoxia at 16 hours in normal cultured human urothelium (n=198)

<b>Systematic Name</b>	<b>Fold change</b>	<b>Description of gene</b>
230746_s_at	6.83	stanniocalcin 1
210947_s_at	5.47	gb:J04810.1 /DEF=Human MSH3 gene, complete cds. /FEA=mRNA /PROD=MSH3
232035_at	4.76	H4 histone family, member H
227337_at	4.51	hypothetical protein FLJ11200
230267_at	4.08	ESTs, Weakly similar to hypothetical protein FLJ20489 [Homo sapiens] [H.sapiens]
228499_at	4.01	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4
223276_at	3.71	putative small membrane protein NID67
219232_s_at	3.66	gb:NM_022073.1 /DEF=Homo sapiens hypothetical protein FLJ21620 (FLJ21620)
208180_s_at	3.59	gb:NM_003543.2 /DEF=Homo sapiens H4 histone family, member H (H4FH), mRNA.
222847_s_at	3.55	egl nine homolog 3 (C. elegans)
227271_at	3.49	fibroblast growth factor 11
228255_at	3.39	ESTs
235756_at	3.32	ESTs
204284_at	3.26	protein phosphatase 1, regulatory (inhibitor) subunit 3C
221805_at	3.25	Consensus includes gb:AL537457 neurofilament, light polypeptide (68kD)
209732_at	3.24	gb:BC005254.1 /DEF=Homo sapiens, Similar to C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 2 (activation-induced)



217731\_s\_at 3.23 gb:NM\_021999.1 /DEF=Homo sapiens integral membrane protein 2B (ITM2B)  
201313\_at 3.20 gb:NM\_001975.1 /DEF=Homo sapiens enolase 2, (gamma, neuronal) (ENO2), mRNA.  
209546\_s\_at 3.16 gb:AF323540.1 /DEF=Homo sapiens apolipoprotein L-I mRNA, splice variant B,  
214717\_at 3.14 Consensus includes gb:AL137534.1 /DEF=Homo sapiens mRNA  
201513\_at 3.13 Consensus includes gb:AI659180 /FEA=EST  
205199\_at 3.11 gb:NM\_001216.1 /DEF=Homo sapiens carbonic anhydrase IX (CA9), mRNA  
218149\_s\_at 3.10 gb:NM\_017606.1 /DEF=Homo sapiens hypothetical protein DKFZp434K1210  
223383\_at 3.10 nerve injury gene 283  
209006\_s\_at 3.09 gb:AF247168.1 /DEF=Homo sapiens NP014 (NPD014) mRNA, complete cds.  
229174\_at 3.06 Homo sapiens, Similar to RIKEN cDNA 4930453N24 gene  
225346\_at 3.01 transcription termination factor-like protein  
201416\_at 3.01 Consensus includes gb:BG528420 /FEA=EST /DB\_XREF=gi:  
220748\_s\_at 2.98 gb:NM\_016202.1 /DEF=Homo sapiens LDL induced EC protein (LOC51157), mRNA.  
221646\_s\_at 2.96 gb:AF267859.1 /DEF=Homo sapiens GL013 mRNA, complete cds.  
231086\_at 2.96 beta-site APP-cleaving enzyme  
213624\_at 2.94 acid sphingomyelinase-like phosphodiesterase  
226348\_at 2.92 EST, Weakly similar to JC5238 galactosylceramide-like protein, GCP - human [H.sapiens]  
208370\_s\_at 2.86 gb:NM\_004414.2 /DEF=Homo sapiens Down syndrome critical region gene 1 (DSCR1),  
203595\_s\_at 2.84 retinoic acid- and interferon-inducible protein (58kD)  
228023\_x\_at 2.81 amylase, alpha 2B; pancreatic  
224605\_at 2.80 EST, Weakly similar to T02670 probable thromboxane A2 receptor isoform beta  
205060\_at 2.80 gb:NM\_003631.1 /DEF=Homo sapiens poly (ADP-ribose) glycohydrolase (PARG)  
209596\_at 2.77 gb:AF245505.1 /DEF=Homo sapiens adlican mRNA, complete cds.  
219327\_s\_at 2.76 gb:NM\_022036.1 /DEF=Homo sapiens G protein-coupled receptor, family C, group 5  
212143\_s\_at 2.75 Consensus includes gb:BF340228 insulin-like growth factor binding protein 3  
213668\_s\_at 2.72 SRY (sex determining region Y)-box 4  
202358\_s\_at 2.72 KIAA0254 gene product



202379\_s\_at 2.71 natural killer-tumor recognition sequence  
 221497\_x\_at 2.71 gb:BC005369.1 /DEF=Homo sapiens, chromosome 1 open reading frame 12  
 239845\_at 2.70 ESTs  
 205174\_s\_at 2.66 gb:NM\_012413.2 /DEF=Homo sapiens glutaminyl-peptide cyclotransferase (glutaminyl cyclase) (QPCT)  
 210095\_s\_at 2.66 gb:M31159.1 /DEF=Human growth hormone-dependent insulin-like growth factor-binding protein mRNA insulin-like growth factor binding protein 3 /FL=gb:BC000013.1 gb:M31159.1  
 209652\_s\_at 2.65 gb:BC001422.1 /DEF=Homo sapiens, Similar to placental growth factor, vascular endothelial growth factor-related protein, 2.1  
 202124\_s\_at 2.65 amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 3  
 202464\_s\_at 2.61 gb:NM\_004566.1 /DEF=Homo sapiens 6-phosphofructo-2,6-biphosphatase 3 (PFKFB3 3)  
 202023\_at 2.60 gb:NM\_004428.1 /DEF=Homo sapiens ephrin-A1 (EFNA1), mRNA. /FEA=mRNA /GEN=EFNA1 /PROD=ephrin A1 precursor /DB\_XREF=gi:4758245 /UG=Hs.1624 ephrin-A1 /FL=gb:M57730.1 gb:NM\_004428.1  
 240221\_at 2.59 ESTs, Moderately similar to hypothetical protein FLJ20489 [Homo sapiens] [H.sapiens]  
 220232\_at 2.57 gb:NM\_024906.1 /DEF=Homo sapiens hypothetical protein FLJ21032 (FLJ21032), mRNA. /FEA=mRNA /GEN=FLJ21032 /PROD=hypothetical protein FLJ21032 /DB\_XREF=gi:13376362 /UG=Hs.247474 hypothetical protein FLJ21032 /FL=gb:NM\_024906.1  
 226753\_at 2.55 ESTs, Weakly similar to A43932 mucin 2 precursor, intestinal - human (fragments) [H.sapiens]  
 220643\_s\_at 2.55 gb:NM\_018147.1 /DEF=Homo sapiens hypothetical protein FLJ10582 (FLJ10582), mRNA  
 241721\_at 2.54 ESTs, Weakly similar to L1 repeat, Tf subfamily, member 18 [Mus musculus] [M.musculus]  
 228843\_at 2.53 ESTs, Moderately similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]  
 211675\_s\_at 2.53 gb:AF054589.1 /DEF=Homo sapiens HIC protein isoform p40 and HIC protein isoform DKFZP566F2124 protein  
 225838\_at 2.50  
 209015\_s\_at 2.49 gb:BC002446.1 /DEF=Homo sapiens, MRJ gene for a member of the DNAJ protein family  
 209122\_at 2.49 gb:BC005127.1 /DEF=Homo sapiens, adipose differentiation-related protein, polybromo 1  
 223400\_s\_at 2.48 Homo sapiens, clone MGC:33365 IMAGE:5267770, mRNA, complete cds  
 226464\_at 2.48 Consensus includes gb:AW138159 /FEA=EST /DB\_XREF=gi:6142559.3B)  
 211998\_at 2.48 Consensus includes gb:AK027138.1 /DEF=Homo sapiens cDNA: FLJ23485 fis,  
 217047\_s\_at 2.48 natural killer-tumor recognition sequence  
 215338\_s\_at 2.46



228914_at	2.45	ESTs
201418_s_at	2.44	gb:NM_003107.1 /DEF=Homo sapiens SRY (sex determining region Y)-box 4 (SOX4)
232451_at	2.43	EST
201325_s_at	2.42	gb:NM_001423.1 /DEF=Homo sapiens epithelial membrane protein 1 (EMP1), mRNA.
200762_at	2.42	gb:NM_001386.1 /DEF=Homo sapiens dihydropyrimidinase-like 2
202973_x_at	2.40	gb:NM_014883.1 /DEF=Homo sapiens KIAA0914 gene product
226113_at	2.39	KIAA1710 protein
229841_at	2.39	eukaryotic translation initiation factor 2C, 2
212775_at	2.39	KIAA0657 protein
209373_at	2.38	gb:BC003179.1 /DEF=Homo sapiens, clone MGC:4419, mRNA, complete cds.
213664_at	2.38	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag)
226986_at	2.38	DKFZP434J154 protein
223183_at	2.37	1-acylglycerol-3-phosphate O-acyltransferase 3
202980_s_at	2.36	seven in absentia homolog 1 (Drosophila)
224593_at	2.35	hypothetical protein DKFZp761B128
209598_at	2.34	gb:AB020690.1 /DEF=Homo sapiens mRNA for KIAA0883 protein, complete cds.
226782_at	2.33	ESTs
203155_at	2.33	gb:NM_012432.1 /DEF=Homo sapiens SET domain, bifurcated 1 (SETDB1),
226542_at	2.33	ESTs, Weakly similar to neuronal thread protein [Homo sapiens] [H.sapiens]
204354_at	2.33	gb:NM_015450.1 /DEF=Homo sapiens DKFZP586D211 protein (DKFZP586D211),
223553_s_at	2.32	hypothetical protein FLJ22570
218138_at	2.32	gb:NM_018848.1 /DEF=Homo sapiens McKusick-Kaufman syndrome (MKKS),
202014_at	2.31	gb:NM_014330.2 /DEF=Homo sapiens growth arrest and DNA-damage-inducible 34 (GADD34)
228145_s_at	2.31	zinc finger protein 398
219303_at	2.30	gb:NM_024546.1 /DEF=Homo sapiens hypothetical protein
203438_at	2.30	stanniocalcin 2
229400_at	2.30	homeo box D10



228638\_at 2.29 hypothetical protein MGC34648  
 214974\_x\_at 2.29 Consensus includes gb:AK026546.1 /DEF=Homo sapiens cDNA: FLJ222893  
 230194\_at 2.29 ESTs, Weakly similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]  
 223912\_s\_at 2.29 ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)  
 210776\_x\_at 2.28 gb:M31222.1 /DEF=Human e12 protein (E2A) mRNA, complete cds. GEN=TCF3  
 (E2A immunoglobulin enhancer binding factors E12E47) /FL=gb:M31222.1  
 209905\_at 2.27 homeo box A9  
 233814\_at 2.27 EST  
 225667\_s\_at 2.26 NSE1  
 221756\_at 2.25 Consensus includes gb:AL540260 /FEA=EST /DB\_XREF=gi:12870241  
 227410\_at 2.25 hypothetical protein FLJ90022  
 226745\_at 2.25 ESTs, Weakly similar to A43932 mucin 2 precursor, intestinal - human (fragments)  
 237252\_at 2.24 thrombomodulin  
 228919\_at 2.24 ESTs  
 204668\_at 2.24 Consensus includes gb:AL031670 /DEF=Human from clone RP4-681N20  
 225021\_at 2.24 zinc finger protein  
 225835\_at 2.23 EST, Moderately similar to ZN91\_HUMAN Zinc finger protein 91 (Zinc finger protein HTF10)  
 238902\_at 2.23 ESTs  
 229450\_at 2.22 ESTs, Moderately similar to hypothetical protein FLJ20489 [Homo sapiens] [H.sapiens]  
 204523\_at 2.22 gb:NM\_003440.1 /DEF=Homo sapiens zinc finger protein 140 (clone PHZ-39) (ZNF140)  
 201250\_s\_at 2.22 gb:NM\_006516.1 /DEF=Homo sapiens solute carrier family 2 (facilitated glucose  
 transporter), member 1 (SLC2A1), mRNA. /FEA=mRNA /GEN=SLC2A1  
 similar to RIKEN cDNA 1200014N16 gene  
 228280\_at 2.22  
 228452\_at 2.21 ESTs  
 235940\_at 2.20 RC3-HN0002-230300-011-a11 HN0002 Homo sapiens cDNA, mRNA sequence.  
 202265\_at 2.20 gb:NM\_005180.1 /DEF=Homo sapiens murine leukemia viral (bmi-1)  
 211711\_s\_at 2.20 gb:BC005821.1 /DEF=Homo sapiens, phosphatase and tensin homolog  
 (mutated in multiple advanced cancers 1)  
 235202\_x\_at 2.19 hypothetical protein FLJ31051



235542\_at hypothetical protein MGC22014

202066\_at Consensus includes gb:AA195259 /FEA=EST /DB\_XREF=gi:1784959

233005\_at ESTs

201849\_at gb:NM\_004052.2 /DEF=Homo sapiens BCL2adenovirus E1B 19kD-interacting protein 3 (BNIP3), nuclear gene encoding mitochondrial protein, mRNA.

213243\_at KIAA0532 protein

204064\_at gb:NM\_005131.1 /DEF=Homo sapiens nuclear matrix protein p84 (P84),

230571\_at transmembrane 4 superfamily member 11 (plasmolipin)

226512\_at zinc finger protein 198

208933\_s\_at Fc fragment of IgG, low affinity IIb, receptor for (CD32)

200990\_at gb:NM\_005762.1 /DEF=Homo sapiens KRAB-associated protein 1 (TIF1B)

220617\_s\_at gb:NM\_018181.1 /DEF=Homo sapiens hypothetical protein FLJ10697 (FLJ10697)

227250\_at ESTs

229997\_at vang-like 1 (van gogh, Drosophila)

226347\_at EST, Weakly similar to JC5238 galactosylceramide-like protein, GCP - human

225363\_at EST

222565\_s\_at protein kinase C, nu

209538\_at gb:U69645.1 /DEF=Human zinc finger protein mRNA, complete cds.

223707\_at EST

202729\_s\_at gb:NM\_000627.1 /DEF=Homo sapiens latent transforming growth factor beta binding protein 1 (LTBP1),

226562\_at hypothetical protein FLJ35867

218507\_at gb:NM\_013332.1 /DEF=Homo sapiens hypoxia-inducible protein 2 (HIG2), mRNA

227717\_at ESTs, Weakly similar to 2004399A chromosomal protein [Homo sapiens] [H.sapiens]

203642\_s\_at gb:NM\_014900.1 /DEF=Homo sapiens KIAA0977 protein (KIAA0977), mRNA.

203986\_at gb:NM\_003943.1 /DEF=Homo sapiens genethonin 1 (GENX-3414), mRNA. /FEA=mRNA

225683\_x\_at HSPC141 protein

209535\_s\_at gb:AF127481.1 /DEF=Homo sapiens non-oncogenic Rho GTPase-specific GTP exchange factor (proto-LBC) mRNA lymphoid blast crisis oncogene



227428_at	2.12	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F6
229140_at	2.12	hypothetical protein FLJ35453
212493_s_at	2.12	Huntingtin interacting protein B
235924_at	2.12	ESTs
228391_at	2.11	ESTs, Weakly similar to A43932 mucin 2 precursor, intestinal - human (fragments) [H.sapiens]
235222_x_at	2.11	ESTs, Moderately similar to hypothetical protein FLJ20294 [Homo sapiens] [H.sapiens]
207030_s_at	2.11	gb:NM_001321.1 /DEF=Homo sapiens cysteine and glycine-rich protein 2 (CSRP2),
244758_at	2.11	zv41h03.s1 Soares ovary tumor NbHOT Homo sapiens cDNA clone IMAGE:756245 3'
222789_at	2.11	hypothetical protein FLJ11220
218179_s_at	2.10	gb:NM_021942.1 /DEF=Homo sapiens hypothetical protein FLJ12716 (FLJ12716), mRNA
214104_at	2.10	G-protein coupled receptor
225299_at	2.10	myosin VB
212919_at	2.10	KIAA1096 protein
218142_s_at	2.10	gb:NM_016302.1 /DEF=Homo sapiens protein x 0001 (LOC51185), mRNA. /FEA=mRNA
208103_s_at	2.10	gb:NM_030920.1 /DEF=Homo sapiens hypothetical protein MGC5350 (MGC5350), mRNA
225897_at	2.10	myristoylated alanine-rich protein kinase C substrate
227518_at	2.10	hypothetical protein FLJ14251
221009_s_at	2.10	gb:NM_016109.1 /DEF=Homo sapiens PPAR(gamma) angiotensin related protein (PGAR
238507_at	2.09	ESTs, Weakly similar to neuronal thread protein [Homo sapiens] [H.sapiens]
219410_at	2.08	gb:NM_018004.1 /DEF=Homo sapiens hypothetical protein FLJ10134 (FLJ10134), mRNA.
224963_at	2.08	EST, Moderately similar to T02670 probable thromboxane A2 receptor isoform beta - human
212407_at	2.08	Consensus includes gb:AL049669.1 /DEF=Human gene from PAC 612B18, chromosome 1.
228857_at	2.08	Homo sapiens mRNA; cDNA DKFZp547E128 (from clone DKFZp547E128)
242273_at	2.08	ESTs
241798_at	2.07	ESTs, Weakly similar to hypothetical protein FLJ20294 [Homo sapiens] [H.sapiens]
218775_s_at	2.07	gb:NM_024949.1 /DEF=Homo sapiens hypothetical protein FLJ22029 (FLJ22029),
229553_at	2.06	Homo sapiens cDNA FLJ32029 fis, clone NTONG1000264



202127_at	2.06	PRP4 pre-mRNA processing factor 4 homolog B (yeast)
227924_at	2.06	ESTs, Weakly similar to A43932 mucin 2 precursor, intestinal - human (fragments) [H.sapiens]
219225_at	2.05	gb:NM_024554.1 /DEF=Homo sapiens hypothetical protein FLJ11413 (FLJ11413),
235352_at	2.04	ESTs
219862_s_at	2.04	gb:NM_012336.1 /DEF=Homo sapiens nuclear prelamin A recognition factor (NARF),
228091_at	2.04	ESTs
202998_s_at	2.04	gb:NM_002318.1 /DEF=Homo sapiens lysyl oxidase-like 2 (LOXL2), mRNA.
222719_s_at	2.04	platelet derived growth factor C
208153_s_at	2.03	gb:NM_001447.1 /DEF=Homo sapiens FAT tumor suppressor (Drosophila) homolog 2
223593_at	2.03	L-kynurenine/alpha-aminoadipate aminotransferase
218367_x_at	2.03	gb:NM_012475.1 /DEF=Homo sapiens ubiquitin specific protease 21 (USP21), mRNA
228221_at	2.03	hypothetical protein MGC45474
209230_s_at	2.03	gb:AF135266.1 /DEF=Homo sapiens p8 protein homolog (COM1)
215108_x_at	2.02	Consensus includes gb:U80736.1 /DEF=Homo sapiens CAGF9 mRNA,
225509_at	2.02	ESTs, Weakly similar to TRHY_HUMAN Trichohyalin [H.sapiens]
227354_at	2.02	Homo sapiens cDNA FLJ37858 fis, clone BRSSN2015238
223027_at	2.01	sorting nexin 9
205079_s_at	2.01	gb:NM_003829.1 /DEF=Homo sapiens multiple PDZ domain protein
228630_at	2.01	ESTs
242539_at	2.01	hypothetical protein MGC42174
208118_x_at	2.01	gb:NM_031211.1 /DEF=Homo sapiens LAT1-3TM protein (LAT1-3TM)
227990_at	2.01	ESTs, Moderately similar to hypothetical protein FLJ20234 [Homo sapiens] [H.sapiens]
224825_at	2.01	Consensus includes gb:AL050348 similar to heterogenous nuclear ribonucleoprotein A1
229420_at	2.00	ribosomal protein L23a
225912_at	2.00	tumor protein p53 inducible nuclear protein 1



## **Appendix III**

### **Genes upregulated by hypoxia in peripheral blood monocytes**



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## Genes upregulated by 1% hypoxia at 16 hours in peripheral blood monocytes (n=494)

Affy Probe	Fold change	Gene
212657_s_at	22.17	interleukin 1 receptor antagonist
211571_s_at	20.25	gb:D32039.1 /DEF=Human pgH3 mRNA for proteoglycan PG-M(V3), complete cds. /FEA=mRNA /GEN=pgH3 /PROD=proteoglycan PG-M(V3) /DB_XREF=gi:1008912 /UG=Hs.81800 chondroitin sulfate proteoglycan 2 (versican) /FL=gb:D32039.1
218507_at	20.21	gb:NM_013332.1 /DEF=Homo sapiens hypoxia-inducible protein 2 (HIG2), mRNA. /FEA=mRNA /GEN=HIG2 /PROD=hypoxia-inducible protein 2 /DB_XREF=gi:7019408 /UG=Hs.61762 hypoxia-inducible protein 2 /FL=gb:BC001863.1 gb:AF144755.1 gb:NM_013332.1
209875_s_at	19.82	gb:M83248.1 /DEF=Human nephropontin mRNA, complete cds. /FEA=mRNA /GEN=nephropontin /PROD=nephropontin /DB_XREF=gi:189150 /UG=Hs.313 secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1) /FL=gb:M83248.1
200632_s_at	15.11	gb:NM_006096.1 /DEF=Homo sapiens N-myc downstream regulated (NDRG1), mRNA. /FEA=mRNA /GEN=NDRG1 /PROD=N-myc downstream regulated /DB_XREF=gi:5174656 /UG=Hs.75789 N-myc downstream regulated /FL=gb:BC003175.1 gb:D87953.1 gb:AF004162.1 gb:NM_006096.1
215646_s_at	15.08	Homo sapiens versican Vint isoform, mRNA, partial cds
202887_s_at	14.49	gb:NM_019058.1 /DEF=Homo sapiens hypothetical protein (FLJ20500), mRNA. /FEA=mRNA /GEN=FLJ20500 /PROD=hypothetical protein /DB_XREF=gi:9506686 /UG=Hs.111244 hypothetical protein /FL=gb:AL136668.1 gb:NM_019058.1
207543_s_at	11.78	gb:NM_000917.1 /DEF=Homo sapiens procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I (P4HA1), mRNA. /FEA=mRNA /GEN=P4HA1 /PROD=procollagen-proline, 2-oxoglutarate4-dioxygenase (proline 4-hydroxylase), alpha polypeptideI /DB_XREF=gi:4505564 /UG=Hs.76768 procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I /FL=gb:M24486.1 gb:NM_000917.1
202499_s_at	11.77	gb:NM_006931.1 /DEF=Homo sapiens solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3), mRNA. /FEA=mRNA /GEN=SLC2A3 /PROD=solute carrier family 2 (facilitated glucosetransporter), member 3 /DB_XREF=gi:5902089 /UG=Hs.7594 solute carrier family 2 (facilitated glucose transporter), member 3 /FL=gb:M20681.1 gb:NM_006931.1
232693_s_at	11.60	Homo sapiens cDNA FLJ11788 fis, clone HEMBA1006081.
221731_x_at	11.13	Consensus includes gb:BF218922 /FEA=EST /DB_XREF=gi:11112418 /DB_XREF=est:601885091F1 /CLONE=IMAGE:4103447 /UG=Hs.81800 chondroitin sulfate proteoglycan 2 (versican)
216236_s_at	10.97	Consensus includes gb:AL110298.1 /DEF=Homo sapiens mRNA; cDNA DKFZp564K1672 (from clone DKFZp564K1672); partial cds. /FEA=mRNA /GEN=DKFZp564K1672 /PROD=hypothetical protein /DB_XREF=gi:5817258 /UG=Hs.7594 solute carrier family 2 (facilitated glucose transporter), member 3
204620_s_at	10.97	gb:NM_004385.1 /DEF=Homo sapiens chondroitin sulfate proteoglycan 2 (versican) (CSPG2), mRNA. /FEA=mRNA /GEN=CSPG2



210889\_s\_at 10.78 /PROD=chondroitin sulfate proteoglycan 2 (versican) /DB\_XREF=gi:4758081 /UG=Hs.81800 chondroitin sulfate proteoglycan 2 (versican) /FL=gb:NM\_004385.1

208308\_s\_at 10.78 gb:M31933.1 /DEF=Human IgG low affinity Fc fragment receptor (FcRIIb3) mRNA, complete cds. /FEA=mRNA /DB\_XREF=gi:182598 /UG=Hs.278443 Fc fragment of IgG, low affinity IIB, receptor for (CD32) /FL=gb:M31933.1

222088\_s\_at 10.51 gb:NM\_000175.1 /DEF=Homo sapiens glucose phosphate isomerase (GPI), mRNA. /FEA=mRNA /GEN=GPI /PROD=glucose phosphate isomerase /DB\_XREF=gi:4504086 /UG=Hs.180532 glucose phosphate isomerase /FL=gb:BC004982.1 gb:K03515.1

202912\_at 10.01 gb:NM\_000175.1 /DEF=Homo sapiens glucose phosphate isomerase (GPI), mRNA. /FEA=mRNA /GEN=GPI /PROD=glucose phosphate isomerase /DB\_XREF=gi:4504086 /UG=Hs.180532 glucose phosphate isomerase /FL=gb:BC004982.1 gb:K03515.1

201313\_at 9.38 solute carrier family 2 (facilitated glucose transporter), member 3

211506\_s\_at 9.23 gb:NM\_001124.1 /DEF=Homo sapiens adrenomedullin (ADM), mRNA. /FEA=mRNA /GEN=ADM /PROD=adrenomedullin /DB\_XREF=gi:4501944 /UG=Hs.394 adrenomedullin /FL=gb:NM\_001124.1 gb:D14874.1

228483\_s\_at 9.18 gb:NM\_001975.1 /DEF=Homo sapiens enolase 2, (gamma, neuronal) (ENO2), mRNA. /FEA=mRNA /GEN=ENO2 /PROD=enolase 2, (gamma, neuronal) /DB\_XREF=gi:5803010 /UG=Hs.146580 enolase 2, (gamma, neuronal) /FL=gb:BC002745.1

219232\_s\_at 7.90 gb:NM\_001975.1 gb:M22349.1

223193\_x\_at 7.45 gb:AF043337.1 /DEF=Homo sapiens interleukin 8 C-terminal variant (IL8) mRNA, complete cds. /FEA=mRNA /GEN=IL8 /PROD=interleukin 8 C-terminal variant /DB\_XREF=gi:12641914 /UG=Hs.624 interleukin 8 /FL=gb:AF043337.1

201848\_s\_at 7.42 TAF9-like RNA polymerase II, TATA box binding protein (TBP)-associated factor, 31kDa

218149\_s\_at 7.36 gb:NM\_022073.1 /DEF=Homo sapiens hypothetical protein FLJ21620 (FLJ21620), mRNA. /FEA=mRNA /GEN=FLJ21620 /PROD=hypothetical protein FLJ21620 /DB\_XREF=gi:11545786 /UG=Hs.18878 hypothetical protein FLJ21620 /FL=gb:NM\_022073.1

226452\_at 7.34 hypothetical protein, estradiol-induced

217871\_s\_at 7.05 gb:U15174.1 /DEF=Homo sapiens BCL2adenovirus E1B 19kD-interacting protein 3 (BNIP3) mRNA, complete cds. /FEA=mRNA /GEN=BNIP3 /PROD=BCL2adenovirus E1B 19kD-interacting protein 3 /DB\_XREF=gi:558845 /UG=Hs.79428 BCL2adenovirus E1B 19kD-interacting protein 3 /FL=gb:AF002697.1 gb:U15174.1 gb:NM\_004052.2

225262\_at 6.96 gb:NM\_017606.1 /DEF=Homo sapiens hypothetical protein DKFZp434K1210 (DKFZp434K1210), mRNA. /FEA=mRNA /GEN=DKFZp434K1210 /PROD=hypothetical protein DKFZp434K1210 /DB\_XREF=gi:8922146 /UG=Hs.32352 hypothetical protein DKFZp434K1210 /FL=gb:NM\_017606.1

210854\_x\_at 6.89 pyruvate dehydrogenase kinase, isoenzyme 1

203282\_at 6.88 gb:NM\_002415.1 /DEF=Homo sapiens macrophage migration inhibitory factor (glycosylation-inhibiting factor) (MIF), mRNA. /FEA=mRNA /GEN=MIF /PROD=macrophage migration inhibitory factor(glycosylation-inhibiting factor) /DB\_XREF=gi:4505184 /UG=Hs.73798 macrophage migration inhibitory factor (glycosylation-inhibiting factor) /FL=gb:BC000447.1 gb:M25639.1

225262\_at 6.96 hypothetical protein FLJ23306

210854\_x\_at 6.89 gb:U17986.1 /DEF=Human GABAnoradrenaline transporter mRNA, complete cds. /FEA=mRNA /PROD=GABAnoradrenaline transporter /DB\_XREF=gi:602433 /UG=Hs.187958 solute carrier family 6 (neurotransmitter transporter, creatine), member 8 /FL=gb:U17986.1

203282\_at 6.88 gb:NM\_000158.1 /DEF=Homo sapiens glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme, Andersen disease, glycogen storage disease type IV) (GBE1), mRNA. /FEA=mRNA /GEN=GBE1 /PROD=glucan (1,4-alpha-), branching enzyme



221479\_s\_at 6.88 1(glycogen branching enzyme) /DB\_XREF=gi:4557618 /UG=Hs.1691 glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme, Andersen disease, glycogen storage disease type IV) /FL=gb:L07956.1 gb:NM\_000158.1 gb:AF060922.1 /DEF=Homo sapiens clone 016a05 My020 protein mRNA, complete cds. /FEA=mRNA /PROD=My020 protein /DB\_XREF=gi:12001981 /UG=Hs.132955 BCL2adenovirus E1B 19kD-interacting protein 3-like /FL=gb:AF060922.1 gb:AB004788.1 gb:AF067396.1 gb:NM\_004331.1 gb:AL132665.1

231274\_s\_at 6.73 likely ortholog of mouse mitochondrial solute carrier protein

222646\_s\_at 6.70 ERO1-like (*S. cerevisiae*)

214183\_s\_at 6.51 Consensus includes gb:X91817.1 /DEF=H.sapiens mRNA for transketolase-like protein (2418 bp). /FEA=mRNA /PROD=transketolase /DB\_XREF=gi:1232174 /UG=Hs.102866 transketolase-like 1

217356\_s\_at 6.50 Consensus includes gb:S81916.1 /DEF=phosphoglycerate kinase {alternatively spliced} human, phosphoglycerate kinase deficient patient with episodes of muscl, mRNA Partial Mutant, 307 nt. /FEA=mRNA /PROD=phosphoglycerate kinase /DB\_XREF=gi:1470308 /UG=Hs.169313 Phosphoglycerate kinase {alternatively spliced} human, phosphoglycerate kinase deficient patient with episodes of muscl, mRNA Partial Mutant, 307 nt

218280\_x\_at 6.48 gb:NM\_003516.1 /DEF=Homo sapiens H2A histone family, member O (H2AFO), mRNA. /FEA=mRNA /GEN=H2AFO /PROD=H2A histone family, member O /DB\_XREF=gi:4504250 /UG=Hs.795 H2A histone family, member O /FL=gb:BC001629.1 gb:L19779.1 gb:NM\_003516.1

224345\_x\_at 6.45 gb:AF107495.1 /DEF=Homo sapiens FWP001 and putative FWP002 mRNA, complete cds. /FEA=mRNA /PROD=putative FWP002; FWP001 /DB\_XREF=gi:11640556 /FL=gb:AF107495.1

226347\_at 6.40 EST, Weakly similar to JC5238 galactosylceramide-like protein, GCP - human [H.sapiens]

212689\_s\_at 6.37 Human putative zinc finger protein mRNA

220942\_x\_at 6.29 gb:NM\_014367.1 /DEF=Homo sapiens hypothetical protein, estradiol-induced (E2IG5), mRNA. /FEA=mRNA /GEN=E2IG5 /PROD=hypothetical protein, estradiol-induced /DB\_XREF=gi:7657049 /UG=Hs.5243 hypothetical protein, estradiol-induced /FL=gb:AF191020.1 gb:NM\_014367.1

207850\_at 6.22 gb:NM\_002090.1 /DEF=Homo sapiens GRO3 oncogene (GRO3), mRNA. /FEA=mRNA /GEN=GRO3 /PROD=GRO3 oncogene /DB\_XREF=gi:4504156 /UG=Hs.89690 GRO3 oncogene /FL=gb:M36821.1 gb:NM\_002090.1

214290\_s\_at 6.14 H2A histone family, member O

236571\_at 6.03 ESTs

239798\_at 6.02 ESTs

221478\_at 5.92 Consensus includes gb:AL132665.1 /DEF=Homo sapiens mRNA; cDNA DKFZp566E034 (from clone DKFZp566E034); complete cds. /FEA=mRNA /DB\_XREF=gi:6137021 /UG=Hs.132955 BCL2adenovirus E1B 19kD-interacting protein 3-like /FL=gb:AF060922.1 gb:AB004788.1 gb:AF067396.1 gb:NM\_004331.1 gb:AL132665.1

200737\_at 5.88 gb:NM\_000291.1 /DEF=Homo sapiens phosphoglycerate kinase 1 (PGK1), mRNA. /FEA=mRNA /GEN=PGK1 /PROD=phosphoglycerate kinase 1 /DB\_XREF=gi:4505762 /UG=Hs.78771 phosphoglycerate kinase 1 /FL=gb:NM\_000291.1

203939\_at 5.68 gb:NM\_002526.1 /DEF=Homo sapiens 5 nucleotidase (CD73) (NT5), mRNA. /FEA=mRNA /GEN=NT5 /PROD=5 nucleotidase /DB\_XREF=gi:4505466 /UG=Hs.153952 5 nucleotidase (CD73) /FL=gb:NM\_002526.1



223709\_s\_at 5.67 wingless-type MMTV integration site family, member 10A

221123\_x\_at 5.60 gb:NM\_018660.1 /DEF=Homo sapiens papillomavirus regulatory factor PRF-1 (LOC55893), mRNA. /FEA=mRNA /GEN=LOC55893 /PROD=papillomavirus regulatory factor PRF-1 /DB\_XREF=gi:8923886 /UG=Hs.27410 papillomavirus regulatory factor PRF-1 /FL=gb:AF263928.1 gb:NM\_018660.1

200650\_s\_at 5.52 gb:NM\_005566.1 /DEF=Homo sapiens lactate dehydrogenase A (LDHA), mRNA. /FEA=mRNA /GEN=LDHA /PROD=LDHA /DB\_XREF=gi:5031856 /UG=Hs.2795 lactate dehydrogenase A /FL=gb:BC001829.1 gb:NM\_005566.1

227068\_at 5.45 phosphoglycerate kinase 1

224605\_at 5.42 EST, Weakly similar to T02670 probable thromboxane A2 receptor isoform beta - human [H.sapiens]

217984\_at 5.19 gb:NM\_003730.2 /DEF=Homo sapiens ribonuclease 6 precursor (RNASE6PL), mRNA. /FEA=mRNA /GEN=RNASE6PL /PROD=ribonuclease 6 precursor /DB\_XREF=gi:5231227 /UG=Hs.8297 ribonuclease 6 precursor /FL=gb:BC001660.1 gb:BC001819.1 gb:U85625.2 gb:NM\_003730.2

213843\_x\_at 5.19 solute carrier family 6 (neurotransmitter transporter, creatine), member 8

217047\_s\_at 5.16 Consensus includes gb:AK027138.1 /DEF=Homo sapiens cDNA: FLJ23485 fis, clone KAIA05211. /FEA=mRNA /DB\_XREF=gi:10440190 /UG=Hs.177664 KIAA0914 gene product

200738\_s\_at 5.04 gb:NM\_000291.1 /DEF=Homo sapiens phosphoglycerate kinase 1 (PGK1), mRNA. /FEA=mRNA /GEN=PGK1 /PROD=phosphoglycerate kinase 1 /DB\_XREF=gi:4505762 /UG=Hs.78771 phosphoglycerate kinase 1 /FL=gb:NM\_000291.1

200966\_x\_at 5.01 gb:NM\_000034.1 /DEF=Homo sapiens aldolase A, fructose-bisphosphate (ALDOA), mRNA. /FEA=mRNA /GEN=ALDOA /PROD=aldolase A /DB\_XREF=gi:4557304 /UG=Hs.273415 aldolase A, fructose-bisphosphate /FL=gb:BC004333.1 gb:M11560.1 gb:NM\_000034.1

205443\_at 4.98 gb:NM\_003082.1 /DEF=Homo sapiens small nuclear RNA activating complex, polypeptide 1, 43kD (SNAPC1), mRNA. /FEA=mRNA /GEN=SNAPC1 /PROD=small nuclear RNA activating complex,polypeptide 1, 43kD /DB\_XREF=gi:4507100 /UG=Hs.179312 small nuclear RNA activating complex, polypeptide 1, 43kD /FL=gb:U44754.1 gb:NM\_003082.1

217738\_at 4.91 pre-B-cell colony-enhancing factor

230747\_s\_at 4.91 Homo sapiens cDNA FLJ90394 fis, clone NT2RP2005632

213011\_s\_at 4.84 triosephosphate isomerase 1

226348\_at 4.81 EST, Weakly similar to JC5238 galactosylceramide-like protein, GCP - human [H.sapiens]

224602\_at 4.78 EST, Weakly similar to T02670 probable thromboxane A2 receptor isoform beta - human [H.sapiens]

243020\_at 4.77 ESTs

202973\_x\_at 4.40 gb:NM\_014883.1 /DEF=Homo sapiens KIAA0914 gene product (KIAA0914), mRNA. /FEA=mRNA /GEN=KIAA0914 /PROD=KIAA0914 gene product /DB\_XREF=gi:7662375 /UG=Hs.177664 KIAA0914 gene product /FL=gb:AB020721.1 gb:NM\_014883.1

201626\_at 4.33 insulin induced gene 1

235035\_at 4.27 hypothetical protein FLJ14251



214687_x_at	4.26	Consensus includes gb:AK026577.1 /DEF=Homo sapiens cDNA: FLJ22924 fis, clone KAT06977, highly similar to HSALDAR Human fibroblast mRNA for aldolase A. /FEA=mRNA /DB_XREF=gi:10439461 /UG=Hs.273415 aldolase A, fructose-bisphosphate
209774_x_at	4.25	gb:M57731.1 /DEF=Human gro-beta mRNA, complete cds. /FEA=mRNA /GEN=gro-beta /PROD=cytokine gro-beta /DB_XREF=gi:183626 /UG=Hs.75765 GRO2 oncogene /FL=gb:M57731.1 gb:M36820.1 gb:NM_002089.1
239619_at	4.21	ESTs
228549_at	4.18	ESTs, Weakly similar to T02670 probable thromboxane A2 receptor isoform beta - human [H.sapiens]
224604_at	4.15	EST, Weakly similar to T02670 probable thromboxane A2 receptor isoform beta - human [H.sapiens]
202934_at	4.07	hexokinase 2
214092_x_at	4.07	KIAA0365 gene product
AFFX- HUMGAPDH/M33197 _M_at	4.06	glyceraldehyde-3-phosphate dehydrogenase
202364_at	4.04	gb:NM_005962.1 /DEF=Homo sapiens MAX-interacting protein 1 (MXI1), mRNA. /FEA=mRNA /GEN=MXI1 /PROD=MAX-interacting protein 1 /DB_XREF=gi:5174596 /UG=Hs.118630 MAX-interacting protein 1 /FL=gb:L07648.1 gb:NM_005962.1 gb:D63940.1
217294_s_at	4.03	Consensus includes gb:U88968.1 /DEF=Human alpha enolase like 1 (ENO1L1) mRNA, partial cds. /FEA=mRNA /GEN=ENO1L1 /PROD=alpha enolase like 1 /DB_XREF=gi:3282242 /UG=Hs.254105 enolase 1, (alpha)
204496_at	4.01	gb:NM_014574.1 /DEF=Homo sapiens nuclear autoantigen (GS2NA), mRNA. /FEA=mRNA /GEN=GS2NA /PROD=nuclear autoantigen /DB_XREF=gi:11128016 /UG=Hs.183105 nuclear autoantigen /FL=gb:NM_014574.1 gb:U17989.1
221748_s_at	3.98	tensin
223172_s_at	3.91	hypothetical protein HSPC242
220742_s_at	3.89	gb:NM_018297.1 /DEF=Homo sapiens hypothetical protein FLJ11005 (FLJ11005), mRNA. /FEA=mRNA /GEN=FLJ11005 /PROD=hypothetical protein FLJ11005 /DB_XREF=gi:8922817 /UG=Hs.63657 hypothetical protein FLJ11005 /FL=gb:NM_018297.1
213453_x_at	3.88	glyceraldehyde-3-phosphate dehydrogenase
238423_at	3.85	ESTs, Weakly similar to synaptotagmin-like 4 (granuphilin-a) [Homo sapiens] [H.sapiens]
201261_x_at	3.84	gb:BC002416.1 /DEF=Homo sapiens, biglycan, clone MGC:2298, mRNA, complete cds. /FEA=mRNA /PROD=biglycan /DB_XREF=gi:12803216 /UG=Hs.821 biglycan /FL=gb:BC002416.1 gb:BC004244.1 gb:J04599.1 gb:NM_001711.1
201231_s_at	3.79	gb:NM_001428.1 /DEF=Homo sapiens enolase 1, (alpha) (ENO1), mRNA. /FEA=mRNA /GEN=ENO1 /PROD=enolase 1, (alpha) /DB_XREF=gi:4503570 /UG=Hs.254105 enolase 1, (alpha) /FL=gb:BC001810.1 gb:BC004458.1 gb:M14328.1 gb:NM_001428.1
212958_x_at	3.78	peptidylglycine alpha-amidating monoxygenase
203234_at	3.77	gb:NM_003364.1 /DEF=Homo sapiens uridine phosphorylase (UP), mRNA. /FEA=mRNA /GEN=UP /PROD=uridine phosphorylase /DB_XREF=gi:4507838 /UG=Hs.77573 uridine phosphorylase /FL=gb:BC001405.1 gb:NM_003364.1
212399_s_at	3.77	Consensus includes gb:D50911.2 /DEF=Homo sapiens mRNA for KIAA0121 protein, partial cds. /FEA=mRNA /GEN=KIAA0121 /PROD=KIAA0121 protein /DB_XREF=gi:6633996 /UG=Hs.155584 KIAA0121 gene product



205567\_at 3.77 gb:NM\_003654.1 /DEF=Homo sapiens carbohydrate (chondroitin 6keratan) sulfotransferase 1 (CHST1), mRNA. /FEA=mRNA /GEN=CHST1 /PROD=carbohydrate (chondroitin 6keratan)sulfotransferase 1 /DB\_XREF=gi:4502840 /UG=Hs.104576 carbohydrate (keratan sulfate Gal-6) sulfotransferase 1 /FL=gb:U65637.1 gb:AB003791.1 gb:AF090137.1 gb:NM\_003654.1

212688\_at 3.71 Consensus includes gb:BC003393.1 /DEF=Homo sapiens, clone IMAGE:3447173, mRNA, partial cds. /FEA=mRNA /PROD=Unknown (protein for IMAGE:3447173) /DB\_XREF=gi:13097275 /UG=Hs.239818 phosphoinositide-3-kinase, catalytic, beta polypeptide /FL=gb:NM\_006219.1

209122\_at 3.71 gb:BC005127.1 /DEF=Homo sapiens, adipose differentiation-related protein, clone MGC:10598, mRNA, complete cds. /FEA=mRNA /PROD=adipose differentiation-related protein /DB\_XREF=gi:13477306 /UG=Hs.3416 adipose differentiation-related protein /FL=gb:BC005127.1 gb:NM\_001122.1

223774\_at 3.70 PNAS-123

AFFX- 3.67 glyceraldehyde-3-phosphate dehydrogenase

HUMGAPDH/M33197\_5\_at 3.67

227868\_at 3.67 wp61g11.x1 NCI\_CGAP\_Brn25 Homo sapiens cDNA clone IMAGE:2466308 3', mRNA sequence.

210269\_s\_at 3.67 gb:M99578.1 /DEF=Human lymphocyte surface protein exons 1-5, complete cds. /FEA=mRNA /DB\_XREF=gi:187241 /UG=Hs.321160 Human lymphocyte surface protein exons 1-5, complete cds /FL=gb:M99578.1

225342\_at 3.64 EST

212581\_x\_at 3.64 glyceraldehyde-3-phosphate dehydrogenase

223046\_at 3.64 egl nine homolog 1 (C. elegans)

217507\_at 3.63 solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1

202859\_x\_at 3.61 gb:NM\_000584.1 /DEF=Homo sapiens interleukin 8 (IL8), mRNA. /FEA=mRNA /GEN=IL8 /PROD=interleukin 8 /DB\_XREF=gi:10834977 /UG=Hs.624 interleukin 8 /FL=gb:NM\_000584.1 gb:M17017.1 gb:M26383.1

238320\_at 3.56 ESTs

239697\_x\_at 3.55 ESTs, Highly similar to S22655 translation elongation factor eEF-1 gamma chain - human [H.sapiens]

212977\_at 3.55 G protein-coupled receptor

200822\_x\_at 3.53 gb:NM\_000365.1 /DEF=Homo sapiens triosephosphate isomerase 1 (TPI1), mRNA. /FEA=mRNA /GEN=TPI1 /PROD=triosephosphate isomerase 1 /DB\_XREF=gi:4507644 /UG=Hs.83848 triosephosphate isomerase 1 /FL=gb:BC004230.1 gb:NM\_000365.1

208798\_x\_at 3.53 gb:AF204231.1 /DEF=Homo sapiens 88-kDa Golgi protein (GM88) mRNA, complete cds. /FEA=mRNA /GEN=GM88 /PROD=88-kDa Golgi protein /DB\_XREF=gi:6808610 /UG=Hs.182982 golgin-67 /FL=gb:NM\_015003.1 gb:AF204231.1

219941\_at 3.53 gb:NM\_018279.1 /DEF=Homo sapiens hypothetical protein FLJ10936 (FLJ10936), mRNA. /FEA=mRNA /GEN=FLJ10936 /PROD=hypothetical protein FLJ10936 /DB\_XREF=gi:8922782 /UG=Hs.7337 hypothetical protein FLJ10936 /FL=gb:NM\_018279.1

225750\_at 3.47 ESTs, Weakly similar to PRO0478 protein [Homo sapiens] [H.sapiens]



229390\_at 3.45 ESTs, Highly similar to JC5276 HXC-26 protein - human [H.sapiens]

AFFX- HUMGAPDH/M33197 3.44 glyceraldehyde-3-phosphate dehydrogenase

AFFX- HUMGAPDH/M33197 3.44 glyceraldehyde-3-phosphate dehydrogenase

217983\_s\_at 3.44 gb:NM\_003730.2 /DEF=Homo sapiens ribonuclease 6 precursor (RNASE6PL), mRNA. /FEA=mRNA /GEN=RNASE6PL /PROD=ribonuclease 6 precursor /DB\_XREF=gi:5231227 /UG=Hs.8297 ribonuclease 6 precursor /FL=gb:BC001660.1

214004\_s\_at 3.43 ubiquitin activating enzyme E1-like protein

225557\_at 3.41 AXIN1 up-regulated 1

225756\_at 3.39 casein kinase 1, epsilon

212561\_at 3.38 RAB6 interacting protein 1

200886\_s\_at 3.37 gb:NM\_002629.1 /DEF=Homo sapiens phosphoglycerate mutase 1 (brain) (PGAM1), mRNA. /FEA=mRNA /GEN=PGAM1 /PROD=phosphoglycerate mutase 1 (brain) /DB\_XREF=gi:4505752 /UG=Hs.181013 phosphoglycerate mutase 1 (brain) /FL=gb:BC000455.1 gb:NM\_002629.1 gb:J04173.1

217398\_x\_at 3.34 Consensus includes gb:AK026525.1 /DEF=Homo sapiens cDNA: FLJ22872 fis, clone KAT02551, highly similar to HUMGAPDH Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. /FEA=mRNA /DB\_XREF=gi:10439402 /UG=Hs.169476 glyceraldehyde-3-phosphate dehydrogenase

218136\_s\_at 3.34 gb:NM\_018579.1 /DEF=Homo sapiens mitochondrial solute carrier (LOC51312), mRNA. /FEA=mRNA /GEN=LOC51312 /PROD=hypothetical protein PRO1278 /DB\_XREF=gi:8924027 /UG=Hs.300496 mitochondrial solute carrier /FL=gb:AF155660.1 gb:AF116630.1 gb:NM\_018579.1

221497\_x\_at 3.33 gb:BC005369.1 /DEF=Homo sapiens, chromosome 1 open reading frame 12, clone MGC:12484, mRNA, complete cds. /FEA=mRNA /PROD=chromosome 1 open reading frame 12 /DB\_XREF=gi:13529208 /UG=Hs.6523 chromosome 1 open reading frame 12 /FL=gb:AF229245.1 gb:AF277176.1 gb:NM\_022051.1 gb:BC005369.1

201968\_s\_at 3.33 gb:NM\_002633.1 /DEF=Homo sapiens phosphoglucomutase 1 (PGM1), mRNA. /FEA=mRNA /GEN=PGM1 /PROD=phosphoglucomutase 1 /DB\_XREF=gi:4505764 /UG=Hs.1869 phosphoglucomutase 1 /FL=gb:BC001756.1 gb:M83088.1 gb:NM\_002633.1

220066\_at 3.31 gb:NM\_022162.1 /DEF=Homo sapiens NOD2 protein (NOD2), mRNA. /FEA=mRNA /GEN=NOD2 /PROD=NOD2 protein /DB\_XREF=gi:11545911 /UG=Hs.135201 NOD2 protein /FL=gb:AF178930.1 gb:NM\_022162.1

205685\_at 3.30 CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)

217739\_s\_at 3.28 gb:NM\_005746.1 /DEF=Homo sapiens pre-B-cell colony-enhancing factor (PBECF), mRNA. /FEA=mRNA /GEN=PBECF /PROD=pre-B-cell colony-enhancing factor /DB\_XREF=gi:5031976 /UG=Hs.239138 pre-B-cell colony-enhancing factor /FL=gb:U02020.1 gb:NM\_005746.1



214482\_at 3.27 Consensus includes gb:NM\_006977.1 /DEF=Homo sapiens zinc finger protein 46 (KUP) (ZNF46), mRNA. /FEA=CDS /GEN=ZNF46 /PROD=zinc finger protein 46 (KUP) /DB\_XREF=gi:5902163 /UG=Hs.164347 zinc finger protein 46 (KUP) /FL=gb:NM\_006977.1

212902\_at 3.24 GTP-binding protein Sara

214057\_at 3.24 myeloid cell leukemia sequence 1 (BCL2-related)

232722\_at 3.24 ribonuclease 6 precursor

221747\_at 3.23 tensin

201625\_s\_at 3.21 insulin induced gene 1

205349\_at 3.21 gb:NM\_002068.1 /DEF=Homo sapiens guanine nucleotide binding protein (G protein), alpha 15 (Gq class) (GNA15), mRNA. /FEA=mRNA /GEN=GNA15 /PROD=guanine nucleotide binding protein (G protein),alpha 15 (Gq class) /DB\_XREF=gi:4504038 /UG=Hs.73797 guanine nucleotide binding protein (G protein), alpha 15 (Gq class) /FL=gb:M63904.1 gb:NM\_002068.1

AFFX-r2-  
Hs28SrRNA-3\_at 3.20 Human 28S ribosomal RNA gene.

202506\_at 3.19 gb:NM\_006751.1 /DEF=Homo sapiens sperm specific antigen 2 (SSFA2), mRNA. /FEA=mRNA /GEN=SSFA2 /PROD=sperm specific antigen 2 /DB\_XREF=gi:5803178 /UG=Hs.82767 sperm specific antigen 2 /FL=gb:M61199.1 gb:NM\_006751.1

201690\_s\_at 3.19 tumor protein D52

201013\_s\_at 3.18 phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase

217620\_s\_at 3.17 phosphoinositide-3-kinase, catalytic, beta polypeptide

227295\_at 3.13 hypothetical protein FLJ31051

201548\_s\_at 3.13 putative DNA/chromatin binding motif

204243\_at 3.13 gb:NM\_012421.1 /DEF=Homo sapiens rearranged L-myc fusion sequence (RLF), mRNA. /FEA=mRNA /GEN=RLF /PROD=rearranged L-myc fusion sequence /DB\_XREF=gi:6912631 /UG=Hs.13321 rearranged L-myc fusion sequence /FL=gb:U22377.1 gb:NM\_012421.1

233500\_x\_at 3.13 lectin-like NK cell receptor

221617\_at 3.11 Consensus includes gb:AF077053.1 /DEF=Homo sapiens neuronal cell death-related protein mRNA, complete cds. /FEA=mRNA /PROD=neuronal cell death-related protein /DB\_XREF=gi:4689153 /UG=Hs.171723 neuronal cell death-related protein /FL=gb:AF077053.1 gb:NM\_015975.1 gb:AF220509.1

212366\_at 3.11 hypothetical protein FLJ13564

207712\_at 3.10 gb:NM\_001187.1 /DEF=Homo sapiens B melanoma antigen (BAGE), mRNA. /FEA=mRNA /GEN=BAGE /PROD=B melanoma antigen /DB\_XREF=gi:4557346 /UG=Hs.2355 B melanoma antigen /FL=gb:NM\_001187.1 gb:U19180.1

205372\_at 3.09 gb:NM\_002655.1 /DEF=Homo sapiens pleiomorphic adenoma gene 1 (PLAG1), mRNA. /FEA=mRNA /GEN=PLAG1 /PROD=pleiomorphic adenoma gene 1 /DB\_XREF=gi:4505854 /UG=Hs.14968 pleiomorphic adenoma gene 1 /FL=gb:U65002.1 gb:NM\_002655.1



215101\_s\_at 3.07 chemokine (C-X-C motif) ligand 5

221203\_s\_at 3.06 gb:NM\_018023.2 /DEF=Homo sapiens hypothetical protein FLJ10201 (FLJ10201), mRNA. /FEA=mRNA /GEN=FLJ10201 /PROD=hypothetical protein FLJ10201 /DB\_XREF=gi:13492976 /UG=Hs.318127 hypothetical protein FLJ10201 /FL=gb:NM\_018023.2

212368\_at 3.06 hypothetical protein FLJ13564

206525\_at 3.05 gb:NM\_002042.1 /DEF=Homo sapiens gamma-aminobutyric acid (GABA) receptor, rho 1 (GABRR1), mRNA. /FEA=mRNA /GEN=GABRR1 /PROD=gamma-aminobutyric acid (GABA) receptor, rho 1precursor /DB\_XREF=gi:4503868 /UG=Hs.1438 gamma-aminobutyric acid (GABA) receptor, rho 1 /FL=gb:M62400.1 gb:NM\_002042.1

AFFX- HUMGAPDH/M33197 3.04 glyceraldehyde-3-phosphate dehydrogenase

\_3\_at

225239\_at 3.03 sprouty homolog 4 (Drosophila)

216511\_s\_at 2.98 Consensus includes gb:AJ270770 /DEF=Homo sapiens partial TCF-4 gene for T-cell transcription factor-4, exon 1 and joined CDS features /FEA=mRNA\_1 /DB\_XREF=gi:9188625 /UG=Hs.283857 Homo sapiens partial TCF-4 gene for T-cell transcription factor-4, exon 1 and joined CDS features

36711\_at 2.98 Cluster Incl. AL021977:bk447C4.1 (novel MAFF (v-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein F) LIKE protein) /cds=(0,494) /gb=AL021977 /gi=4914526 /ug=Hs.51305 /len=2128

AFFX-r2- Human 28S ribosomal RNA gene.

Hs28SrRNA-3\_at 2.97

223484\_at 2.95 normal mucosa of esophagus specific 1

233952\_s\_at 2.93 zinc finger protein 295

227765\_at 2.92 ESTs, Weakly similar to hypothetical protein FLJ10884 [Homo sapiens] [H.sapiens]

205001\_s\_at 2.92 gb:AF000985.1 /DEF=Homo sapiens dead box, Y isoform (DBY) mRNA, alternative transcript 1, complete cds. /FEA=mRNA /GEN=DBY /PROD=dead box, Y isoform /DB\_XREF=gi:2580555 /UG=Hs.99120 DEADH (Asp-Glu-Ala-AspHis) box polypeptide, Y chromosome /FL=gb:NM\_004660.2 gb:AF000984.1 gb:AF000985.1

229354\_at 2.92 arylhydrocarbon receptor repressor

228291\_s\_at 2.88 chromosome 20 open reading frame 19

236354\_at 2.88 ESTs

AFFX- HUMGAPDH/M33197 2.86 glyceraldehyde-3-phosphate dehydrogenase

\_3\_at

219862\_s\_at 2.85 gb:NM\_012336.1 /DEF=Homo sapiens nuclear prelamin A recognition factor (NARF), mRNA. /FEA=mRNA /GEN=NARF /PROD=nuclear prelamin A recognition factor /DB\_XREF=gi:6912523 /UG=Hs.256526 nuclear prelamin A recognition factor /FL=gb:AF128406.1 gb:NM\_012336.1



210004\_at 2.84 gb:AF035776.1 /DEF=Homo sapiens oxidized low-density lipoprotein receptor mRNA, complete cds. /FEA=mRNA /PROD=oxidized low-density lipoprotein receptor /DB\_XREF=gi:3941299 /UG=Hs.77729 oxidised low density lipoprotein (lectin-like) receptor 1 /FL=gb:AB010710.1 gb:AF035776.1 gb:NM\_002543.1

204998\_s\_at 2.83 gb:NM\_012068.2 /DEF=Homo sapiens activating transcription factor 5 (ATF5), mRNA. /FEA=mRNA /GEN=ATF5 /PROD=activating transcription factor 5 /DB\_XREF=gi:12597624 /UG=Hs.9754 activating transcription factor 5 /FL=gb:AF305687.1 gb:AB021663.2 gb:NM\_012068.2 gb:BC005174.1

214775\_at 2.83 Nedd4 binding protein 3

242117\_at 2.82 ESTs

209017\_s\_at 2.82 gb:U02389.1 /DEF=Human hLON ATP-dependent protease mRNA, nuclear gene encoding mitochondrial protein, complete cds. /FEA=mRNA /PROD=hLON ATP-dependent protease /DB\_XREF=gi:639426 /UG=Hs.278614 protease, serine, 15 /FL=gb:BC000235.1 gb:NM\_004793.1 gb:U02389.1

210465\_s\_at 2.82 gb:U71300.1 /DEF=Human snRNA activating protein complex 50kD subunit (SNAP50) mRNA, complete cds. /FEA=mRNA /GEN=SNAP50 /PROD=snRNA activating protein complex 50kD subunit /DB\_XREF=gi:1619945 /UG=Hs.164915 small nuclear RNA activating complex, polypeptide 3, 50kD /FL=gb:U71300.1

211202\_s\_at 2.80 gb:AF087481.1 /DEF=Homo sapiens retinoblastoma binding protein 2 homolog 1 (RBBP2H1) mRNA, complete cds. /FEA=mRNA /GEN=RBBP2H1 /PROD=retinoblastoma binding protein 2 homolog 1 /DB\_XREF=gi:4322487 /UG=Hs.143323 putative DNACHROMATIN binding motif /FL=gb:AF087481.1

204900\_x\_at 2.80 gb:NM\_003864.1 /DEF=Homo sapiens sin3-associated polypeptide, 30kD (SAP30), mRNA. /FEA=mRNA /GEN=SAP30 /PROD=sin3 associated polypeptide p30 /DB\_XREF=gi:4506782 /UG=Hs.20985 sin3-associated polypeptide, 30kD /FL=gb:AF055993.1 gb:NM\_003864.1

219434\_at 2.80 gb:NM\_018643.1 /DEF=Homo sapiens triggering receptor expressed on myeloid cells 1 (TREM1), mRNA. /FEA=mRNA /GEN=TREM1 /PROD=triggering receptor expressed on myeloid cells1 /DB\_XREF=gi:8924261 /UG=Hs.283022 triggering receptor expressed on myeloid cells 1 /FL=gb:AF196329.1 gb:NM\_018643.1 gb:AF287008.1

228680\_at 2.79 ESTs, Weakly similar to I78885 serine/threonine-specific protein kinase (EC 2.7.1.-) STK2 - human [H.sapiens]

223261\_at 2.79 polymerase (DNA directed) kappa

212360\_at 2.79 adenosine monophosphate deaminase 2 (isoform L)

227772\_at 2.78 ESTs, Weakly similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]

205045\_at 2.78 gb:NM\_007202.1 /DEF=Homo sapiens A kinase (PRKA) anchor protein 10 (AKAP10), mRNA. /FEA=mRNA /GEN=AKAP10 /PROD=A kinase (PRKA) anchor protein 10 /DB\_XREF=gi:6005706 /UG=Hs.75456 A kinase (PRKA) anchor protein 10 /FL=gb:AF037439.1 gb:NM\_007202.1

205495\_s\_at 2.78 gb:NM\_006433.2 /DEF=Homo sapiens granulysin (GNLY), transcript variant NKG5, mRNA. /FEA=mRNA /GEN=GNLY /PROD=granulysin, isoform NKG5 /DB\_XREF=gi:7108343 /UG=Hs.105806 granulysin /FL=gb:NM\_006433.2

212495\_at 2.77 KIAA0876 protein

223263\_s\_at 2.76 DKFZP564O1863 protein



238692\_at 2.76 hypothetical protein FLJ33957

201952\_at 2.76 Consensus includes gb:AA156721 /FEA=EST /DB\_XREF=gi:1728335 /DB\_XREF=est:z118b04.s1 /CLONE=IMAGE:502255 /UG=Hs.10247 activated leucocyte cell adhesion molecule /FL=gb:NM\_001627.1 gb:L38608.1

229391\_s\_at 2.75 ESTs, Highly similar to JC5276 HXC-26 protein - human [H.sapiens]

201942\_s\_at 2.73 gb:D85390.1 /DEF=Homo sapiens mRNA for gp180-carboxypeptidase D-like enzyme, complete cds. /FEA=mRNA /PROD=gp180-carboxypeptidase D-like enzyme /DB\_XREF=gi:3641620 /UG=Hs.5057 carboxypeptidase D /FL=gb:U65090.1 gb:D85390.1 gb:NM\_001304.2

232055\_at 2.72 ESTs

220661\_s\_at 2.72 gb:NM\_017865.1 /DEF=Homo sapiens hypothetical protein FLJ20531 (FLJ20531), mRNA. /FEA=mRNA /GEN=FLJ20531 /PROD=hypothetical protein FLJ20531 /DB\_XREF=gi:8923497 /UG=Hs.23617 hypothetical protein FLJ20531 /FL=gb:NM\_017865.1

227062\_at 2.72 plectin 1, intermediate filament binding protein 500kDa

AFFX-r2- 2.72 Human 18S rRNA gene, complete.

Hs18SrRNA-5\_at 2.72

229700\_at 2.72 Homo sapiens, similar to hypothetical protein FLJ13659, clone MGC:26923 IMAGE:4838161, mRNA, complete cds

234989\_at 2.71 ESTs, Weakly similar to 1207289A reverse transcriptase related protein [H.sapiens] [H.sapiens]

201455\_s\_at 2.71 Consensus includes gb:AJ132583.1 /DEF=Homo sapiens mRNA for puromycin sensitive aminopeptidase, partial. /FEA=mRNA /PROD=puromycin sensitive aminopeptidase /DB\_XREF=gi:4210725 /UG=Hs.293007 aminopeptidase puromycin sensitive /FL=gb:NM\_006310.1

208891\_at 2.71 gb:BC003143.1 /DEF=Homo sapiens, dual specificity phosphatase 6, clone MGC:3789, mRNA, complete cds. /FEA=mRNA /PROD=dual specificity phosphatase 6 /DB\_XREF=gi:13111942 /UG=Hs.180383 dual specificity phosphatase 6 /FL=gb:BC003562.1 gb:BC003143.1 gb:BC005047.1 gb:AB013382.1 gb:NM\_001946.1

209403\_at 2.71 gb:AL136860.1 /DEF=Homo sapiens mRNA; cDNA DKFZp434P2235 (from clone DKFZp434P2235); complete cds. /FEA=mRNA /GEN=DKFZp434P2235 /PROD=hypothetical protein /DB\_XREF=gi:12053224 /UG=Hs.105891 Homo sapiens mRNA; cDNA DKFZp434P2235 (from clone DKFZp434P2235); complete cds /FL=gb:AL136860.1

219957\_at 2.71 gb:NM\_017987.1 /DEF=Homo sapiens hypothetical protein FLJ10063 (FLJ10063), mRNA. /FEA=mRNA /GEN=FLJ10063 /PROD=hypothetical protein FLJ10063 /DB\_XREF=gi:8922215 /UG=Hs.154091 hypothetical protein FLJ10063 /FL=gb:NM\_017987.1

201170\_s\_at 2.71 gb:NM\_003670.1 /DEF=Homo sapiens basic helix-loop-helix domain containing, class B, 2 (BHLHB2), mRNA. /FEA=mRNA /GEN=BHLHB2 /PROD=differentiated embryo chondrocyte expressed gene1 /DB\_XREF=gi:4503298 /UG=Hs.171825 basic helix-loop-helix domain containing, class B, 2 /FL=gb:AB004066.1 gb:NM\_003670.1

231377\_at 2.70 ESTs

221103\_s\_at 2.69 gb:NM\_018338.1 /DEF=Homo sapiens hypothetical protein FLJ11142 (FLJ11142), mRNA. /FEA=mRNA /GEN=FLJ11142 /PROD=hypothetical protein FLJ11142 /DB\_XREF=gi:8922896 /UG=Hs.272244 hypothetical protein FLJ11142 /FL=gb:NM\_018338.1

224571\_at 2.68 Homo sapiens, clone IMAGE:3882977, mRNA, partial cds

222662\_at 2.68 Ac-like transposable element



226392\_at 2.67 ESTs

243747\_at 2.67 ESTs

209822\_s\_at 2.66 gb:L22431.1 /DEF=Human very low density lipoprotein receptor, complete cds. /FEA=mRNA /PROD=very low density lipoprotein receptor /DB\_XREF=gi:437386 /UG=Hs.73729 very low density lipoprotein receptor /FL=gb:D16493.1 gb:L22431.1 gb:NM\_003383.1

211245\_x\_at 2.66 gb:AF002256.1 /DEF=Homo sapiens killer cell inhibitory receptor homolog cl-9 mRNA, complete cds. /FEA=mRNA /PROD=cl-9 /DB\_XREF=gi:2197058 /UG=Hs.166085 killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4 /FL=gb:AF002256.1

204924\_at 2.65 gb:NM\_003264.1 /DEF=Homo sapiens toll-like receptor 2 (TLR2), mRNA. /FEA=mRNA /GEN=TLR2 /PROD=toll-like receptor2 /DB\_XREF=gi:4507528 /UG=Hs.63668 toll-like receptor 2 /FL=gb:U88878.1 gb:AF051152.1 gb:NM\_003264.1

227391\_x\_at 2.65 ESTs

202972\_s\_at 2.64 KIAA0914 gene product

215775\_at 2.64 QV4-CT0491-140900-398-e06 CT0491 Homo sapiens cDNA, mRNA sequence.

205180\_s\_at 2.63 gb:NM\_001109.1 /DEF=Homo sapiens a disintegrin and metalloproteinase domain 8 (ADAM8), mRNA. /FEA=mRNA /GEN=ADAM8 /PROD=a disintegrin and metalloproteinase domain 8precursor /DB\_XREF=gi:4557252 /UG=Hs.86947 a disintegrin and metalloproteinase domain 8 /FL=gb:D26579.1 gb:NM\_001109.1

228770\_at 2.62 hypothetical protein BC014241

217473\_x\_at 2.62 Consensus includes gb:AF229163 /DEF=Homo sapiens natural resistance-associated macrophage protein 1 (SLC11A1) gene, complete cds, alternatively spliced; and nuclear LIM interactor-interacting factor (NLI-IF) gene, complete cds /FEA=mRNA\_3 /DB\_XREF=gi:10257408 /UG=Hs.182611 solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1

202140\_s\_at 2.61 gb:NM\_003992.1 /DEF=Homo sapiens CDC-like kinase 3 (CLK3), transcript variant phck3, mRNA. /FEA=mRNA /GEN=CLK3 /PROD=CDC-like kinase 3 isoform hck3 /DB\_XREF=gi:4502884 /UG=Hs.73987 CDC-like kinase 3 /FL=gb:BC002555.1 gb:NM\_003992.1 gb:L29217.1

225486\_at 2.60 KIAA1557 protein

204999\_s\_at 2.60 gb:BC005174.1 /DEF=Homo sapiens, activating transcription factor 5, clone MGC:842, mRNA, complete cds. /FEA=mRNA /PROD=activating transcription factor 5 /DB\_XREF=gi:13477389 /UG=Hs.9754 activating transcription factor 5 /FL=gb:AF305687.1 gb:AB021663.2 gb:NM\_012068.2 gb:BC005174.1

205673\_s\_at 2.59 gb:NM\_024087.1 /DEF=Homo sapiens DKFZP564L0862 protein (DKFZP564L0862), mRNA. /FEA=mRNA /GEN=DKFZP564L0862 /PROD=hypothetical protein FLJ20636 /DB\_XREF=gi:13443013 /UG=Hs.19404 DKFZP564L0862 protein /FL=gb:NM\_024087.1

201977\_s\_at 2.59 KIAA0141 gene product

215825\_at 2.59 Consensus includes gb:AF070579.1 /DEF=Homo sapiens clone 24487 mRNA sequence. /FEA=mRNA /DB\_XREF=gi:3387951 /UG=Hs.283819 Homo sapiens clone 24487 mRNA sequence

225898\_at 2.58 hypothetical protein FLJ12953 similar to Mus musculus D3Mm3e

201642\_at 2.58 gb:NM\_005534.1 /DEF=Homo sapiens interferon gamma receptor 2 (interferon gamma transducer 1) (IFNGR2), mRNA.



202464\_s\_at 2.58 /FEA=mRNA /GEN=IFNGR2 /PROD=interferon gamma receptor 2 (interferon gamma receptor 2 (interferon gamma receptor 1) /DB\_XREF=gi:5031782 /UG=Hs.177559 interferon gamma receptor 2 (interferon gamma transducer 1) /FL=gb:BC003624.1 gb:U05875.1 gb:U05877.1 gb:NM\_005534.1  
 gb:NM\_004566.1 /DEF=Homo sapiens 6-phosphofructo-2-kinasefructose-2,6-biphosphatase 3 (PFKFB3), mRNA. /FEA=mRNA /GEN=PFKFB3 /PROD=6-phosphofructo-2-kinasefructose-2,6-biphosphatase 3 /DB\_XREF=gi:4758899 /UG=Hs.195471 6-phosphofructo-2-kinasefructose-2,6-biphosphatase 3 /FL=gb:D49817.1 gb:AF109735.1 gb:NM\_004566.1  
 ESTs  
 235593\_at 2.58 hypothetical protein FLJ10252  
 242224\_at 2.57 Consensus includes gb:BF594446 /FEA=EST /DB\_XREF=gi:11686770 /DB\_XREF=est:7i06c12.x1 /CLONE=IMAGE:3324694 /UG=Hs.86386 myeloid cell leukemia sequence 1 (BCL2-related) /FL=gb:NM\_021960.1 gb:AF118124.1  
 200796\_s\_at 2.56 gb:NM\_005542.1 /DEF=Homo sapiens insulin induced gene 1 (INSIG1), mRNA. /FEA=mRNA /GEN=INSIG1 /PROD=insulin induced gene 1 /DB\_XREF=gi:5031800 /UG=Hs.56205 insulin induced gene 1 /FL=gb:NM\_005542.1  
 201627\_s\_at 2.55 Consensus includes gb:AI493245 /FEA=EST /DB\_XREF=gi:4394248 /DB\_XREF=est:ti30d08.x1 /CLONE=IMAGE:2131983 /UG=Hs.169610 CD44 antigen (homing function and Indian blood group system)  
 212014\_x\_at 2.55 type 1 tumor necrosis factor receptor shedding aminopeptidase regulator  
 214034\_at 2.55 gb:NM\_023941.1 /DEF=Homo sapiens hypothetical protein MGC3032 (MGC3032), mRNA. /FEA=mRNA /GEN=MGC3032 /PROD=hypothetical protein MGC3032 /DB\_XREF=gi:13027613 /UG=Hs.300383 hypothetical protein MGC3032 /FL=gb:BC000572.1 gb:NM\_023941.1  
 203553\_s\_at 2.54 gb:NM\_006575.1 /DEF=Homo sapiens mitogen-activated protein kinase kinase 5 (MAP4K5), mRNA. /FEA=mRNA /GEN=MAP4K5 /PROD=mitogen-activated protein kinase kinase /DB\_XREF=gi:5729890 /UG=Hs.246970 mitogen-activated protein kinase kinase 5 /FL=gb:U77129.1 gb:NM\_006575.1  
 226062\_x\_at 2.54 hypothetical protein FLJ11280  
 226100\_at 2.54 myeloid/lymphoid or mixed-lineage leukemia 5 (trithorax homolog, Drosophila)  
 228812\_at 2.54 ESTs, Weakly similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]  
 31845\_at 2.53 E74-like factor 4 (ets domain transcription factor)  
 219093\_at 2.53 gb:NM\_017933.1 /DEF=Homo sapiens hypothetical protein FLJ20701 (FLJ20701), mRNA. /FEA=mRNA /GEN=FLJ20701 /PROD=hypothetical protein FLJ20701 /DB\_XREF=gi:8923631 /UG=Hs.169764 hypothetical protein FLJ20701 /FL=gb:NM\_017933.1  
 AFFX-r2- 2.53 Human 18S rRNA gene, complete.  
 Hs18SrRNA-5\_at 2.53 ESTs  
 241851\_x\_at 2.53 Homo sapiens, clone IMAGE:3865769, mRNA, partial cds  
 226201\_at 2.53 Consensus includes gb:N24643 /FEA=EST /DB\_XREF=gi:1138793 /DB\_XREF=est:yx89f11.s1 /CLONE=IMAGE:268941 /UG=Hs.187991 DKFZP564A122 protein /FL=gb:AF106684.1 gb:NM\_015626.1  
 201294\_s\_at 2.52 Consensus includes gb:X02761.1 /DEF=Human mRNA for fibronectin (FN precursor). /FEA=mRNA /PROD=fibronectin precursor  
 212464\_s\_at 2.52



/DB\_XREF=gi:31396 /UG=Hs.287820 fibronectin 1  
 225066\_at 2.52 uncharacterized hematopoietic stem/progenitor cells protein MDS026  
 225951\_s\_at 2.52 ESTs, Weakly similar to CGHU3B collagen alpha 3(IV) chain precursor, long splice form - human [H.sapiens]  
 201250\_s\_at 2.52 gb:NM\_006516.1 /DEF=Homo sapiens solute carrier family 2 (facilitated glucose transporter), member 1 (SLC2A1), mRNA. /FEA=mRNA /GEN=SLC2A1 /PROD=solute carrier family 2 (facilitated glucosetransporter), member 1 /DB\_XREF=gi:5730050 /UG=Hs.169902 solute carrier family 2 (facilitated glucose transporter), member 1 /FL=gb:K03195.1 gb:NM\_006516.1  
 220436\_at 2.51 gb:NM\_024879.1 /DEF=Homo sapiens hypothetical protein FLJ14195 (FLJ14195), mRNA. /FEA=mRNA /GEN=FLJ14195 /PROD=hypothetical protein FLJ14195 /DB\_XREF=gi:13376319 /UG=Hs.212839 hypothetical protein FLJ14195; KIAA1714 protein /FL=gb:NM\_024879.1  
 202670\_at 2.51 mitogen-activated protein kinase kinase 1  
 215607\_x\_at 2.51 AU144530 HEMBA1 Homo sapiens cDNA clone HEMBA1002189 3', mRNA sequence.  
 211337\_s\_at 2.50 gb:BC000966.2 /DEF=Homo sapiens, Similar to gamma tubulin ring complex protein (76p gene), clone MGC:4930, mRNA, complete cds. /FEA=mRNA /PROD=Similar to gamma tubulin ring complex protein(76p gene) /DB\_XREF=gi:12803021 /UG=Hs.20621 gamma tubulin ring complex protein (76p gene) /FL=gb:BC000966.2  
 37145\_at 2.50 Homo sapiens NKG5 gene, complete cds.  
 204604\_at 2.49 gb:NM\_012395.1 /DEF=Homo sapiens PFTAIRE protein kinase 1 (PFTK1), mRNA. /FEA=mRNA /GEN=PFTK1 /PROD=PFTAIRE protein kinase 1 /DB\_XREF=gi:6912583 /UG=Hs.57856 PFTAIRE protein kinase 1 /FL=gb:AB020641.1 gb:NM\_012395.1  
 225569\_at 2.48 eukaryotic translation initiation factor 2C, 2  
 202328\_s\_at 2.48 gb:NM\_000296.1 /DEF=Homo sapiens polycystic kidney disease 1 (autosomal dominant) (PKD1), mRNA. /FEA=mRNA /GEN=PKD1 /PROD=polycystic kidney disease 1 (autosomaldominant) /DB\_XREF=gi:4505832 /UG=Hs.75813 polycystic kidney disease 1 (autosomal dominant) /FL=gb:NM\_000296.1 gb:U24497.1 gb:L33243.1  
 225827\_at 2.48 eukaryotic translation initiation factor 2C, 2  
 222669\_s\_at 2.47 CGI-97 protein  
 213300\_at 2.47 KIAA0404 protein  
 221139\_s\_at 2.47 gb:NM\_015989.1 /DEF=Homo sapiens cysteine sulfinic acid decarboxylase-relatedprotein 2 (CSAD), mRNA. /FEA=mRNA /GEN=CSAD /PROD=cysteine sulfinic aciddecarboxylase-relatedprotein 2 /DB\_XREF=gi:7705333 /UG=Hs.279815 cysteine sulfinic acid decarboxylase-relatedprotein 2 /FL=gb:AF116546.1 gb:NM\_015989.1  
 212492\_s\_at 2.47 KIAA0876 protein  
 204490\_s\_at 2.47 gb:M24915.1 /DEF=Human CDw44 antigen, complete cds. /FEA=mRNA /DB\_XREF=gi:180196 /UG=Hs.169610 CD44 antigen (homing function and Indian blood group system) /FL=gb:NM\_000610.1 gb:U40373.1 gb:M59040.1 gb:M24915.1  
 225517\_at 2.46 PRO1914 protein  
 223199\_at 2.46 MAP kinase-interacting serine/threonine kinase 2



224918_x_at	2.45	microsomal glutathione S-transferase 1
227934_at	2.45	ESTs
215524_x_at	2.45	immunoglobulin heavy constant mu
209446_s_at	2.44	gb:BC001743.1 /DEF=Homo sapiens, Similar to hypothetical protein FLJ10803, clone MGC:933, mRNA, complete cds. /FEA=mRNA /PROD=Similar to hypothetical protein FLJ10803 /DB_XREF=gi:12804636 /UG=Hs.8173 hypothetical protein FLJ10803 /FL=gb:BC001743.1
223584_s_at	2.44	DKFZP566C134 protein
224137_at	2.44	calcium channel, voltage-dependent, gamma subunit 7
214620_x_at	2.44	peptidylglycine alpha-amidating monooxygenase
230795_at	2.44	H4 histone, family 2
225939_at	2.44	Homo sapiens, similar to RIKEN cDNA 1300018P11 gene, clone MGC:39820 IMAGE:5299499, mRNA, complete cds
207780_at	2.43	gb:NM_001340.1 /DEF=Homo sapiens cylicin, basic protein of sperm head cytoskeleton 2 (CYLC2), mRNA. /FEA=mRNA /GEN=CYLC2 /PROD=cylicin 2 /DB_XREF=gi:4557508 /UG=Hs.3232 cylicin, basic protein of sperm head cytoskeleton 2 /FL=gb:NM_001340.1
229543_at	2.43	ESTs, Highly similar to JC5276 HXC-26 protein - human [H.sapiens]
209706_at	2.42	gb:AF247704.1 /DEF=Homo sapiens homeobox protein NKX3.1 mRNA, complete cds. /FEA=mRNA /PROD=homeobox protein NKX3.1 /DB_XREF=gi:9963969 /UG=Hs.55999 NK homeobox (Drosophila), family 3, A /FL=gb:AF249670.1 gb:AF249672.1 gb:U80669.1 gb:U91540.1 gb:NM_006167.1 gb:AF247704.1
208426_x_at	2.42	gb:NM_002255.1 /DEF=Homo sapiens killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4 (KIR2DL4), mRNA. /FEA=mRNA /GEN=KIR2DL4 /PROD=killer cell immunoglobulin-like receptor, twodomains, long cytoplasmic tail, 4 /DB_XREF=gi:4504870 /UG=Hs.166085 killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4 /FL=gb:AF002981.1 gb:NM_002255.1
207483_s_at	2.42	gb:NM_018448.1 /DEF=Homo sapiens TIP120 protein (TIP120), mRNA. /FEA=mRNA /GEN=TIP120 /PROD=TIP120 protein /DB_XREF=gi:8924259 /UG=Hs.283668 TIP120 protein /FL=gb:AF157326.1 gb:NM_018448.1
219966_x_at	2.42	gb:NM_017869.1 /DEF=Homo sapiens BANP homolog, SMAR1 homolog (FLJ20538), mRNA. /FEA=mRNA /GEN=FLJ20538 /PROD=BANP homolog, SMAR1 homolog /DB_XREF=gi:8923506 /UG=Hs.194637 BANP homolog, SMAR1 homolog /FL=gb:NM_017869.1
225945_at	2.41	vav-1 interacting Kruppel-like protein
213700_s_at	2.41	ESTs, Weakly similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]
208039_at	2.41	gb:NM_003048.1 /DEF=Homo sapiens solute carrier family 9 (sodiumhydrogen exchanger), isoform 2 (SLC9A2), mRNA. /FEA=mRNA /GEN=SLC9A2 /PROD=solute carrier family 9 (sodiumhydrogenexchanger), isoform 2 /DB_XREF=gi:4507058 /UG=Hs.103132 solute carrier family 9 (sodiumhydrogen exchanger), isoform 2 /FL=gb:NM_003048.1
241894_at	2.40	ESTs, Weakly similar to T26708 hypothetical protein Y38H6C.19 - Caenorhabditis elegans [C.elegans]



225502\_at 2.40 Consensus includes gb:AL161725 /DEF=Human DNA sequence from clone RP11-165F24 on chromosome 9. Contains the 3 end of the gene for a novel protein (similar to Drosophila CG6630 and CG11376, KIAA1058, rat TRG), an RPL12 (60S ribosomal protein L12) pseudogene, ESTs, STSs, GSSs and a C... /FEA=mRNA\_1 /DB\_XREF=gi:10045359 /UG=Hs.171118 hypothetical protein FLJ00026

210895\_s\_at 2.40 gb:L25259.1 /DEF=Human CTLA4 counter-receptor (B7-2) mRNA, complete cds. /FEA=mRNA /GEN=B7-2 /PROD=CTLA4 counter-receptor /DB\_XREF=gi:416368 /UG=Hs.27954 CD86 antigen (CD28 antigen ligand 2, B7-2 antigen) /FL=gb:L25259.1 ESTs

236202\_at 2.40

202284\_s\_at 2.40 gb:NM\_000389.1 /DEF=Homo sapiens cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A), mRNA. /FEA=mRNA /GEN=CDKN1A /PROD=cyclin-dependent kinase inhibitor 1A (p21,Cip1) /DB\_XREF=gi:11386202 /UG=Hs.179665 cyclin-dependent kinase inhibitor 1A (p21, Cip1) /FL=gb:NM\_000389.1 gb:BC000275.1 gb:U03106.1 gb:L26165.1 gb:L25610.1 gb:U09579.1 ESTs

235174\_s\_at 2.40

226675\_s\_at 2.39 Human heat shock protein 86 mRNA, 5'end

218073\_s\_at 2.39 gb:NM\_018087.1 /DEF=Homo sapiens hypothetical protein FLJ10407 (FLJ10407), mRNA. /FEA=mRNA /GEN=FLJ10407 /PROD=hypothetical protein FLJ10407 /DB\_XREF=gi:8922408 /UG=Hs.30738 hypothetical protein FLJ10407 /FL=gb:BC003082.1 gb:NM\_018087.1 ESTs

239843\_at 2.38

210424\_s\_at 2.38 gb:AF163441.1 /DEF=Homo sapiens golgin 67 mRNA, complete cds. /FEA=mRNA /PROD=golgin 67 /DB\_XREF=gi:6969979 /UG=Hs.182982 golgin-67 /FL=gb:AF163441.1 gb:AF164622.1

210797\_s\_at 2.38 gb:AF063612.1 /DEF=Homo sapiens 2-5oligoadenylate synthetase-related protein p30 (OASL) mRNA, alternatively spliced, complete cds. /FEA=mRNA /GEN=OASL /PROD=2-5oligoadenylate synthetase-related proteinp30 /DB\_XREF=gi:4731858 /UG=Hs.118633 2-5oligoadenylate synthetase-like /FL=gb:AF063612.1

212311\_at 2.38 Consensus includes gb:AA522514 /FEA=EST /DB\_XREF=est:ni38c01.s1 /CLONE=IMAGE:979104 /UG=Hs.49500 KIAA0746 protein

212190\_at 2.38 serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2

240655\_at 2.38 ESTs

221995\_s\_at 2.38 mitochondrial ribosomal protein 63

220244\_at 2.37 gb:NM\_013343.1 /DEF=Homo sapiens NAG-7 protein (NAG-7), mRNA. /FEA=mRNA /GEN=NAG-7 /PROD=NAG-7 protein /DB\_XREF=gi:7106370 /UG=Hs.278951 NAG-7 protein /FL=gb:AF086709.2 gb:NM\_013343.1

225142\_at 2.37 KIAA1718 protein

244811\_at 2.36 eukaryotic translation elongation factor 1 epsilon 1

227539\_at 2.36 guanine nucleotide binding protein (G protein), alpha 13

210426\_x\_at 2.36 gb:U04897.1 /DEF=Human orphan hormone nuclear receptor RORalpha1 mRNA, complete cds. /FEA=mRNA /PROD=RORalpha1 /DB\_XREF=gi:451563 /UG=Hs.2156 RAR-related orphan receptor A /FL=gb:U04897.1



209215\_at 2.35 gb:L11669.1 /DEF=Human tetracycline transporter-like protein mRNA, complete cds. /FEA=mRNA /PROD=tetracycline transporter-like protein /DB\_XREF=gi:307501 /UG=Hs.157145 tetracycline transporter-like protein /FL=gb:L11669.1 gb:NM\_001120.1 ESTs

230263\_s\_at 2.35 procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase) 2

202619\_s\_at 2.35 gb:NM\_021016.1 /DEF=Homo sapiens pregnancy specific beta-1-glycoprotein 3 (PSG3), mRNA. /FEA=mRNA /GEN=PSG3 /PROD=pregnancy specific beta-1-glycoprotein 3 /DB\_XREF=gi:11036637 /UG=Hs.282847 pregnancy specific beta-1-glycoprotein 3 /FL=gb:NM\_021016.1 gb:M34420.1 gb:M23575.1 gb:M93061.1

AFFX-r2- 2.34 Human 18S rRNA gene, complete.

Hs18SrRNA-M\_x\_at 2.34 hypothetical protein FLJ31951

226077\_at 2.34 gb:NM\_013252.1 /DEF=Homo sapiens C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 5 (CLECSF5), mRNA. /FEA=mRNA /GEN=CLECSF5 /PROD=C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 5 /DB\_XREF=gi:10281668 /UG=Hs.126355 C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 5 /FL=gb:NM\_013252.1 gb:AF139768.1

219890\_at 2.34 splicing factor, arginine/serine-rich 1 (splicing factor 2, alternate splicing factor)

226419\_s\_at 2.34 Consensus includes gb:NM\_005504.1 /DEF=Homo sapiens branched chain aminotransferase 1, cytosolic (BCAT1), mRNA. /FEA=CDS /GEN=BCAT1 /PROD=branched chain aminotransferase 1, cytosolic /DB\_XREF=gi:5031606 /UG=Hs.157205 branched chain aminotransferase 1, cytosolic /FL=gb:U21551.1 gb:NM\_005504.1

227432\_s\_at 2.32 insulin receptor

235410\_at 2.32 ESTs

218205\_s\_at 2.32 gb:NM\_017572.1 /DEF=Homo sapiens G protein-coupled receptor kinase 7 (GPRK7), mRNA. /FEA=mRNA /GEN=GPRK7 /PROD=G protein-coupled receptor kinase 7 /DB\_XREF=gi:9994196 /UG=Hs.261828 G protein-coupled receptor kinase 7 /FL=gb:AF237776.1 gb:AF125532.1 gb:NM\_017572.1

201348\_at 2.31 gb:NM\_002084.2 /DEF=Homo sapiens glutathione peroxidase 3 (plasma) (GPX3), mRNA. /FEA=mRNA /GEN=GPX3 /PROD=plasma glutathione peroxidase 3 precursor /DB\_XREF=gi:6006000 /UG=Hs.172153 glutathione peroxidase 3 (plasma) /FL=gb:D00632.1 gb:NM\_002084.2 gb:AF217787.1

224797\_at 2.31 KIAA1376 protein

227073\_at 2.31 ESTs, Highly similar to R3HU16 ribosomal protein S16, cytosolic - human [H.sapiens]

227685\_at 2.31 ESTs

240344\_x\_at 2.31 Homo sapiens, clone MGC:24679 IMAGE:4270959, mRNA, complete cds

209933\_s\_at 2.31 gb:AF020314.1 /DEF=Homo sapiens CMRF-35-H9 mRNA, complete cds. /FEA=mRNA /PROD=CMRF-35-H9 /DB\_XREF=gi:4103065 /UG=Hs.9688 leukocyte membrane antigen /FL=gb:AF020314.1

242693\_at 2.30 ESTs



225786\_at 2.30 Homo sapiens, clone MGC:21581 IMAGE:4476038, mRNA, complete cds

225368\_at 2.30 ESTs, Weakly similar to CA11\_HUMAN Collagen alpha 1(I) chain precursor [H.sapiens]

212722\_s\_at 2.30 Consensus includes gb:AK021780.1 /DEF=Homo sapiens cDNA FLJ11718 fis, clone HEMBA1005252, highly similar to Homo sapiens mRNA for KIAA0585 protein. /FEA=mRNA /DB\_XREF=gi:10433034 /UG=Hs.72660 phosphatidylserine receptor

228641\_at 2.30 Homo sapiens, clone IMAGE:4182081, mRNA, partial cds

205684\_s\_at 2.29 gb:NM\_017925.1 /DEF=Homo sapiens hypothetical protein FLJ20686 (FLJ20686), mRNA. /FEA=mRNA /GEN=FLJ20686 /PROD=hypothetical protein FLJ20686 /DB\_XREF=gi:8923616 /UG=Hs.271480 hypothetical protein FLJ20686 /FL=gb:NM\_017925.1

226503\_at 2.29 Homo sapiens cDNA FLJ23769 fis, clone HEP19294

235789\_at 2.29 KIAA0876 protein

231736\_x\_at 2.28 microsomal glutathione S-transferase 1

239804\_at 2.28 ESTs

212743\_at 2.28 Consensus includes gb:AV694093 /FEA=EST /DB\_XREF=gi:10295956 /DB\_XREF=est:AV694093 /CLONE=GKCDSD10 /UG=Hs.48297 DKFZP586C1620 protein

201549\_x\_at 2.27 gb:NM\_006618.1 /DEF=Homo sapiens putative DNACHromatin binding motif (PLU-1), mRNA. /FEA=mRNA /GEN=PLU-1 /PROD=putative DNACHromatin binding motif /DB\_XREF=gi:5729977 /UG=Hs.143323 putative DNACHromatin binding motif /FL=gb:NM\_006618.1

211559\_s\_at 2.27 gb:L49506.1 /DEF=Homo sapiens cyclin G2 mRNA, complete cds. /FEA=mRNA /PROD=cyclin G2 /DB\_XREF=gi:1236234 /UG=Hs.79069 cyclin G2 /FL=gb:L49506.1

218380\_at 2.26 gb:NM\_021730.1 /DEF=Homo sapiens hypothetical protein PP1044 (PP1044), mRNA. /FEA=mRNA /GEN=PP1044 /PROD=hypothetical protein PP1044 /DB\_XREF=gi:11119423 /UG=Hs.7212 hypothetical protein PP1044 /FL=gb:NM\_021730.1

217578\_at 2.26 ESTs, Moderately similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]

218999\_at 2.26 gb:NM\_018295.1 /DEF=Homo sapiens hypothetical protein FLJ11000 (FLJ11000), mRNA. /FEA=mRNA /GEN=FLJ11000 /PROD=hypothetical protein FLJ11000 /DB\_XREF=gi:8922813 /UG=Hs.302981 hypothetical protein FLJ11000 /FL=gb:NM\_018295.1

217559\_at 2.26 ribosomal protein L10-like

203317\_at 2.25 gb:NM\_012455.1 /DEF=Homo sapiens SEC7 homolog (TIC), mRNA. /FEA=mRNA /GEN=TIC /PROD=SEC7 homolog /DB\_XREF=gi:6912705 /UG=Hs.110121 SEC7 homolog /FL=gb:U63127.1 gb:NM\_012455.1

206708\_at 2.25 gb:NM\_002158.1 /DEF=Homo sapiens human T-cell leukemia virus enhancer factor (HTLF), mRNA. /FEA=mRNA /GEN=HTLF /PROD=T-cell leukemia virus enhancer factor /DB\_XREF=gi:4504526 /UG=Hs.103126 human T-cell leukemia virus enhancer factor /FL=gb:U57029.1 gb:NM\_002158.1

218472\_s\_at 2.25 gb:NM\_015946.1 /DEF=Homo sapiens pelota (Drosophila) homolog (PELO), mRNA. /FEA=mRNA /GEN=PELO /PROD=CGI-17 protein /DB\_XREF=gi:7705599 /UG=Hs.5798 pelota (Drosophila) homolog /FL=gb:AF139828.1 gb:AF132951.1 gb:NM\_015946.1

201404\_x\_at 2.25 gb:BC000268.1 /DEF=Homo sapiens, proteasome (prosome, macropain) subunit, beta type, 2, clone MGC:1664, mRNA, complete cds. /FEA=mRNA /PROD=proteasome (prosome, macropain) subunit, betatype, 2 /DB\_XREF=gi:12653014 /UG=Hs.1390 proteasome (prosome, macropain) subunit, beta type, 2 /FL=gb:BC000268.1 gb:NM\_002794.1 gb:D26599.1



223014\_at 2.25 ubiquitin-conjugating enzyme UBC3B

236128\_at 2.25 ESTs

225950\_at 2.25 ESTs, Weakly similar to unnamed protein product [Homo sapiens] [H.sapiens]

240538\_at 2.24 y182a11.s1 Soares placenta Nb2HP Homo sapiens cDNA clone IMAGE:145724 3', mRNA sequence.

210144\_at 2.24 Consensus includes gb:AK000851.1 /DEF=Homo sapiens cDNA FLJ20844 fis, clone ADKA01904. /FEA=mRNA /DB\_XREF=gi:7021173 /UG=Hs.20017 chromosome 22 open reading frame 4 /FL=gb:BC002743.1

229025\_s\_at 2.24 hypothetical protein FLJ25059

204521\_at 2.24 gb:NM\_013300.1 /DEF=Homo sapiens protein predicted by clone 23733 (HSU79274), mRNA. /FEA=mRNA /GEN=HSU79274 /PROD=protein predicted by clone 23733 /DB\_XREF=gi:9558740 /UG=Hs.150555 protein predicted by clone 23733 /FL=gb:U79274.1 gb:NM\_013300.1

211289\_x\_at 2.24 gb:AF067524.1 /DEF=Homo sapiens PITSLRE protein kinase beta SV12 isoform (CDC2L2) mRNA, complete cds. /FEA=mRNA /GEN=CDC2L2 /PROD=PITSLRE protein kinase beta SV12 isoform /DB\_XREF=gi:3850327 /UG=Hs.183418 cell division cycle 2-like 1 (PITSLRE proteins) /FL=gb:AF067524.1

202979\_s\_at 2.23 gb:NM\_021212.1 /DEF=Homo sapiens HCF-binding transcription factor Zhangfei (ZF), mRNA. /FEA=mRNA /GEN=ZF /PROD=HCF-binding transcription factor Zhangfei /DB\_XREF=gi:10864024 /UG=Hs.29417 HCF-binding transcription factor Zhangfei /FL=gb:NM\_021212.1 gb:AF039942.1

232297\_at 2.23 EST, Moderately similar to ZN91\_HUMAN Zinc finger protein 91 (Zinc finger protein HTF10) (HPF7) [H.sapiens]

202683\_s\_at 2.23 gb:NM\_003799.1 /DEF=Homo sapiens RNA (guanine-7-) methyltransferase (RNMT), mRNA. /FEA=mRNA /GEN=RNMT /PROD=RNA (guanine-7-) methyltransferase /DB\_XREF=gi:4506566 /UG=Hs.8086 RNA (guanine-7-) methyltransferase /FL=gb:AB007858.1

225097\_at 2.22 gb:AF067791.1 gb:AB022604.1 gb:NM\_003799.1 gb:AB020966.1

230928\_at 2.22 ESTs

225222\_at 2.22 likely ortholog of mouse hippocampus abundant gene transcript 1

244728\_at 2.22 ESTs

216383\_at 2.22 Homo sapiens chromosome X clone Qc-7G6, QLL-F1720, QLL-C1335, Qc-8B7, Qc-11H12, Qc-7F6, QLL-E153, Qc-10B7 map q28, complete sequence.

212762\_s\_at 2.21 transcription factor 7-like 2 (T-cell specific, HMG-box)

233252\_s\_at 2.21 EST

230500\_at 2.20 phosphodiesterase 7A

240165\_at 2.20 ESTs, Weakly similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]

207086\_x\_at 2.20 gb:NM\_001474.1 /DEF=Homo sapiens G antigen 4 (GAGE4), mRNA. /FEA=mRNA /GEN=GAGE4 /PROD=G antigen 4 /DB\_XREF=gi:4503882 /UG=Hs.183199 G antigen 4 /FL=gb:NM\_001474.1 gb:U19145.1



241644_at	2.19	ESTs
224806_at	2.19	ESTs, Weakly similar to MUC2_HUMAN Mucin 2 precursor (Intestinal mucin 2) [H.sapiens]
221978_at	2.19	major histocompatibility complex, class I, F
215985_at	2.19	Consensus includes gb:X92110.1 /DEF=H.sapiens mRNA for hcgVIII protein. /FEA=mRNA /DB_XREF=gi:1216163 /UG=Hs.153618 HCGVIII-1 protein
225539_at	2.19	Consensus includes gb:AP001745 /DEF=Homo sapiens genomic DNA, chromosome 21q, section 89105 /FEA=mRNA_2 /DB_XREF=gi:7768737 /UG=Hs.157079 zinc finger protein 295 /FL=gb:Nm_020727.1
222574_s_at	2.19	hypothetical protein FLJ22060
223915_at	2.19	BCL-6 interacting corepressor
235000_at	2.19	ESTs
225834_at	2.18	ESTs, Weakly similar to A43932 mucin 2 precursor, intestinal - human (fragments) [H.sapiens]
201310_s_at	2.18	gb:NM_004772.1 /DEF=Homo sapiens P311 protein (P311), mRNA. /FEA=mRNA /GEN=P311 /PROD=P311 protein /DB_XREF=gi:4758865 /UG=Hs.142827 P311 protein /FL=gb:U36189.1 gb:Nm_004772.1 gb:U30521.1
200916_at	2.18	gb:NM_003564.1 /DEF=Homo sapiens transgelin 2 (TAGLN2), mRNA. /FEA=mRNA /GEN=TAGLN2 /PROD=transgelin 2 /DB_XREF=gi:4507356 /UG=Hs.75725 transgelin 2 /FL=gb:D21261.1 gb:Nm_003564.1
243515_at	2.18	ESTs
201369_s_at	2.17	gb:NM_006887.1 /DEF=Homo sapiens butyrate response factor 2 (EGF-response factor 2) (BRF2), mRNA. /FEA=mRNA /GEN=BRF2 /PROD=butyrate response factor 2 (EGF-response factor2) /DB_XREF=gi:5901899 /UG=Hs.78909 butyrate response factor 2 (EGF-response factor 2) /FL=gb:BC005010.1 gb:Nm_006887.1
205294_at	2.17	gb:NM_017450.1 /DEF=Homo sapiens BAI1-associated protein 2 (BAIAP2), transcript variant 1, mRNA. /FEA=mRNA /GEN=BAIAP2 /PROD=BAI1-associated protein 2, isoform 1 /DB_XREF=gi:9257196 /UG=Hs.7936 BAI1-associated protein 2 /FL=gb:AB015019.1 gb:AB017120.1 gb:Nm_017450.1
235374_at	2.17	ESTs, Moderately similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]
207196_s_at	2.17	gb:NM_006058.1 /DEF=Homo sapiens Nef-associated factor 1 (NAF1), mRNA. /FEA=mRNA /GEN=NAF1 /PROD=Nef-associated factor 1 /DB_XREF=gi:5174608 /UG=Hs.109281 Nef-associated factor 1 /FL=gb:NM_006058.1
225825_at	2.16	l(3)mbt-like (Drosophila)
225213_at	2.16	T-cell activation protein phosphatase 2C
222559_s_at	2.16	hypothetical protein FLJ10656
224558_s_at	2.16	PRO1073 protein
244654_at	2.16	Homo sapiens mRNA for FLJ00121 protein
216037_x_at	2.16	transcription factor 7-like 2 (T-cell specific, HMG-box)



202696\_at 2.15 gb:NM\_005109.1 /DEF=Homo sapiens oxidative-stress responsive 1 (OSR1), mRNA. /FEA=mRNA /GEN=OSR1 /PROD=oxidative-stress responsive 1 /DB\_XREF=gi:4826877 /UG=Hs.95220 oxidative-stress responsive 1 /FL=gb:AB017642.1 gb:NM\_005109.1 gb:AB029024.1

225663\_at 2.15 endozepine-related protein precursor

243790\_at 2.15 Homo sapiens cDNA FLJ31827 fis, clone NT2RP6000100, moderately similar to ZINC FINGER PROTEIN 41

201689\_s\_at 2.15 tumor protein D52

203887\_s\_at 2.15 gb:NM\_000361.1 /DEF=Homo sapiens thrombomodulin (THBD), mRNA. /FEA=mRNA /GEN=THBD /PROD=thrombomodulin /DB\_XREF=gi:4507482 /UG=Hs.2030 thrombomodulin /FL=gb:M16552.1 gb:NM\_000361.1

212552\_at 2.14 hippocalcin-like 1

218373\_at 2.14 gb:NM\_022476.1 /DEF=Homo sapiens hypothetical protein FLJ13258 similar to fused toes (FLJ13258), mRNA. /FEA=mRNA /GEN=FLJ13258 /PROD=hypothetical protein FLJ13258 similar to fusedtoes /DB\_XREF=gi:11968026 /UG=Hs.288929 hypothetical protein FLJ13258 similar to fused toes /FL=gb:NM\_022476.1

215460\_x\_at 2.14 Consensus includes gb:AL080149.1 /DEF=Homo sapiens mRNA; cDNA DKFZp434B094 (from clone DKFZp434B094); partial cds. /FEA=mRNA /GEN=DKFZp434B094 /PROD=hypothetical protein /DB\_XREF=gi:5262602 /UG=Hs.127950 bromodomain-containing 1

214364\_at 2.14 Homo sapiens HSPC096 mRNA, partial cds

229316\_at 2.13 hypothetical protein MGC2749

210803\_at 2.13 gb:AF201385.1 /DEF=Homo sapiens mitochondrial thioredoxin reductase (TRXR2A) mRNA, complete cds, alternatively spliced; nuclear gene for mitochondrial product. /FEA=mRNA /GEN=TRXR2A /PROD=mitochondrial thioredoxin reductase /DB\_XREF=gi:12043737 /UG=Hs.12971 thioredoxin reductase beta /FL=gb:AF201385.1

239131\_at 2.13 ESTs

222459\_at 2.13 hypothetical protein FLJ12666

208797\_s\_at 2.13 golgin-67

208438\_s\_at 2.13 gb:NM\_005248.1 /DEF=Homo sapiens Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog (FGR), mRNA. /FEA=mRNA /GEN=FGR /PROD=Gardner-Rasheed feline sarcoma viral (v-fgr)oncogene homolog /DB\_XREF=gi:4885234 /UG=Hs.1422 Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog /FL=gb:M19722.1 gb:NM\_005248.1

226464\_at 2.12 Homo sapiens, clone MGC:33365 IMAGE:5267770, mRNA, complete cds

238379\_x\_at 2.12 ESTs

228077\_at 2.12 EST, Moderately similar to T02670 probable thromboxane A2 receptor isoform beta - human [H.sapiens]

202030\_at 2.11 gb:NM\_005881.1 /DEF=Homo sapiens branched chain alpha-ketoacid dehydrogenase kinase (BCKDK), mRNA. /FEA=mRNA /GEN=BCKDK /PROD=branched chain alpha-ketoacid dehydrogenasekinase /DB\_XREF=gi:5031608 /UG=Hs.20644 branched chain alpha-ketoacid dehydrogenase kinase /FL=gb:AF026548.1 gb:NM\_005881.1

223940\_x\_at 2.11 predicted protein of HQ1859; Homo sapiens PRO1859 mRNA, complete cds.



235116_at	2.11	TNF receptor-associated factor 1
221820_s_at	2.11	Consensus includes gb:AK024102.1 /DEF=Homo sapiens cDNA FLJ14040 fis, clone HEMBA1005513, weakly similar to MALES-ABSENT ON THE FIRST PROTEIN (EC 2.3.1.-). /FEA=mRNA /DB_XREF=gi:10436399 /UG=Hs.42343 member of MYST family histone acetyl transferases, homolog of Drosophila MOF
226742_at	2.11	ESTs, Weakly similar to hypothetical protein FLJ20489 [Homo sapiens] [H.sapiens]
225231_at	2.11	Homo sapiens cDNA FLJ34981 fis, clone OCBBF2000982
235716_at	2.11	ESTs, Moderately similar to hypothetical protein FLJ20489 [Homo sapiens] [H.sapiens]
224698_at	2.10	KIAA1228 protein
230528_s_at	2.10	ESTs, Moderately similar to hypothetical protein FLJ20619 [Homo sapiens] [H.sapiens]
200832_s_at	2.10	gb:AB032261.1 /DEF=Homo sapiens Scd mRNA for stearoyl-CoA desaturase, complete cds. /FEA=mRNA /GEN=Scd /PROD=stearoyl-CoA desaturase /DB_XREF=gi:7415720 /UG=Hs.119597 stearoyl-CoA desaturase (delta-9-desaturase) /FL=gb:AF097514.1 gb:NM_005063.1 gb:AB032261.1
226015_at	2.10	ESTs
239381_at	2.10	kallikrein 7 (chymotryptic, stratum corneum)
225992_at	2.10	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)
244251_at	2.10	ESTs
209863_s_at	2.10	gb:AF091627.1 /DEF=Homo sapiens CUSP mRNA, complete cds. /FEA=mRNA /PROD=CUSP /DB_XREF=gi:3644039 /UG=Hs.137569 tumor protein 63 kDa with strong homology to p53 /FL=gb:AF091627.1 gb:AF075431.1 gb:AF075433.1
225116_at	2.10	ESTs
203341_at	2.09	gb:NM_005760.1 /DEF=Homo sapiens CCAAT-box-binding transcription factor (CBF2), mRNA. /FEA=mRNA /GEN=CBF2 /PROD=CCAAT-box-binding transcription factor /DB_XREF=gi:5031624 /UG=Hs.184760 CCAAT-box-binding transcription factor /FL=gb:M37197.1 gb:NM_005760.1
241871_at	2.09	ESTs
238040_at	2.09	ESTs, Weakly similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]
231902_at	2.09	Homo sapiens cDNA FLJ33811 fis, clone CTONG2002095
212549_at	2.09	Consensus includes gb:BE645861 /FEA=EST /DB_XREF=gi:9970172 /DB_XREF=est:7e77d05.x1 /CLONE=IMAGE:3288489 /UG=Hs.24064 Homo sapiens mRNA; cDNA DKFZp586N1323 (from clone DKFZp586N1323)
205626_s_at	2.09	gb:NM_004929.2 /DEF=Homo sapiens calbindin 1, (28kD) (CALB1), mRNA. /FEA=mRNA /GEN=CALB1 /PROD=calbindin 1 /DB_XREF=gi:5579451 /UG=Hs.65425 calbindin 1, (28kD) /FL=gb:NM_004929.2
210879_s_at	2.09	gb:AF334812.1 /DEF=Homo sapiens Rab11 interacting protein Rip11a mRNA, complete cds. /FEA=mRNA /PROD=Rab11 interacting protein Rip11a /DB_XREF=gi:13377896 /UG=Hs.24557 KIAA0857 protein /FL=gb:AF334812.1
228343_at	2.08	POU domain, class 2, transcription factor 2



223679\_at 2.08 predicted protein of HQ2286; Homo sapiens clone FLB8503 PRO2286 mRNA, complete cds.

229145\_at 2.08 Homo sapiens, RIKEN cDNA 2310005G07 gene, clone MGC:10049 IMAGE:3890955, mRNA, complete cds

242270\_at 2.08 ESTs, Weakly similar to neuronal thread protein [Homo sapiens] [H.sapiens]

227220\_at 2.08 hypothetical protein LOC152518

230703\_at 2.08 ESTs

219133\_at 2.08 gb:NM\_017897.1 /DEF=Homo sapiens hypothetical protein FLJ20604 (FLJ20604), mRNA. /FEA=mRNA /GEN=FLJ20604 /PROD=hypothetical protein FLJ20604 /DB\_XREF=gi:89233558 /UG=Hs.55781 hypothetical protein FLJ20604 /FL=gb:NM\_017897.1

205548\_s\_at 2.08 gb:NM\_006806.1 /DEF=Homo sapiens BTG family, member 3 (BTG3), mRNA. /FEA=mRNA /GEN=BTG3 /PROD=BTG family, member 3 /DB\_XREF=gi:5802989 /UG=Hs.77311 BTG family, member 3 /FL=gb:D64110.1 gb:NM\_006806.1

222762\_x\_at 2.07 LIM domains containing 1

201951\_at 2.07 Consensus includes gb:BF242905 /FEA=EST /DB\_XREF=gi:11156833 /DB\_XREF=est:601877949F1 /CLONE=IMAGE:4106028 /UG=Hs.10247 activated leucocyte cell adhesion molecule /FL=gb:NM\_001627.1 gb:L38608.1

238327\_at 2.07 endothelial cell growth factor 1 (platelet-derived)

236079\_at 2.07 hypothetical protein DKFZp667E0512

213974\_at 2.07 Consensus includes gb:AB033059.1 /DEF=Homo sapiens mRNA for KIAA1233 protein, partial cds. /FEA=mRNA /GEN=KIAA1233 /PROD=KIAA1233 protein /DB\_XREF=gi:6330728 /UG=Hs.18705 KIAA1233 protein

230621\_at 2.07 a disintegrin and metalloproteinase domain 17 (tumor necrosis factor, alpha, converting enzyme)

205013\_s\_at 2.07 gb:NM\_000675.2 /DEF=Homo sapiens adenosine A2a receptor (ADORA2A), mRNA. /FEA=mRNA /GEN=ADORA2A /PROD=adenosine A2a receptor /DB\_XREF=gi:5921991 /UG=Hs.1613 adenosine A2a receptor /FL=gb:M97370.1 gb:NM\_000675.2

242377\_x\_at 2.07 DKFZP434F091 protein

239963\_at 2.07 ESTs

204131\_s\_at 2.07 forkhead box O3A

202769\_at 2.07 cyclin G2

211924\_s\_at 2.07 gb:AY029180.1 /DEF=Homo sapiens soluble urokinase plasminogen activator receptor precursor (SUPAR) mRNA, complete cds. /FEA=CDS /GEN=SUPAR /PROD=soluble urokinase plasminogen activator receptorprecursor /DB\_XREF=gi:13641308 /FL=gb:AY029180.1

226457\_at 2.07 ESTs

204774\_at 2.06 gb:NM\_014210.1 /DEF=Homo sapiens ecotropic viral integration site 2A (EVI2A), mRNA. /FEA=mRNA /GEN=EVI2A /PROD=ecotropic viral integration site 2A /DB\_XREF=gi:7657074 /UG=Hs.70499 ecotropic viral integration site 2A /FL=gb:NM\_014210.1

227510\_x\_at 2.06 ESTs, Highly similar to A Chain A, Solution Structure Of The Second Rna-Binding Domain Of Hu2af65 [H.sapiens]



205590\_at 2.06 gb:NM\_005739.2 /DEF=Homo sapiens RAS guanyl releasing protein 1 (calcium and DAG-regulated) (RASGRP1), mRNA. /FEA=mRNA /GEN=RASGRP1 /PROD=RASGRP1 /DB\_XREF=gi:6382080 /UG=Hs.182591 RAS guanyl releasing protein 1 (calcium and DAG-regulated) /FL=gb:AF081195.1 gb:AF106071.1 gb:NM\_005739.2

239355\_at 2.06 hypothetical protein FLJ13057 similar to germ cell-less

243485\_at 2.06 ESTs

242798\_at 2.06 ESTs

224791\_at 2.05 development and differentiation enhancing factor 1

231247\_s\_at 2.05 Homo sapiens cDNA FLJ39739 fis, clone SMINT2016440

228528\_at 2.05 ESTs

213475\_s\_at 2.05 Consensus includes gb:AC002310 /DEF=Human Chromosome 16 BAC clone CIT987SK-A-635H12 /FEA=mRNA\_2 /DB\_XREF=gi:2576342 /UG=Hs.174103 integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen 1; alpha polypeptide)

202855\_s\_at 2.05 solute carrier family 16 (monocarboxylic acid transporters), member 3

201798\_s\_at 2.05 gb:NM\_013451.1 /DEF=Homo sapiens fer-1 (C.elegans)-like 3 (myoferlin) (FER1L3), mRNA. /FEA=mRNA /GEN=FER1L3 /PROD=fer-1 (C.elegans)-like 3 (myoferlin) /DB\_XREF=gi:7305052 /UG=Hs.234680 fer-1 (C.elegans)-like 3 (myoferlin) /FL=gb:AF182316.1 gb:NM\_013451.1

226117\_at 2.05 hypothetical protein MGC20791

235980\_at 2.05 potassium large conductance calcium-activated channel, subfamily M beta member 3

231735\_s\_at 2.05 Consensus includes gb:NM\_014086.1 /DEF=Homo sapiens PRO1073 protein (PRO1073), mRNA. /FEA=CDS /GEN=PRO1073 /PROD=PRO1073 protein /DB\_XREF=gi:7662589 /UG=Hs.6975 PRO1073 protein /FL=gb:NM\_014086.1

204194\_at 2.05 gb:NM\_001186.1 /DEF=Homo sapiens BTB and CNC homology 1, basic leucine zipper transcription factor 1 (BACH1), mRNA. /FEA=mRNA /GEN=BACH1 /PROD=BTB and CNC homology 1, basic leucine zippertranscription factor 1 /DB\_XREF=gi:4502352 /UG=Hs.154276 BTB and CNC homology 1, basic leucine zipper transcription factor 1 /FL=gb:AB002803.1 gb:NM\_001186.1

AFFX-M27830\_5\_at 2.04 Human 28S ribosomal RNA gene, complete cds.

224276\_at 2.04 Homo sapiens brain my041 protein mRNA, complete cds

227450\_at 2.04 hypothetical protein FLJ32115

229410\_at 2.04 hypothetical protein FLJ14251

235566\_at 2.04 ESTs, Moderately similar to hypothetical protein FLJ20489 [Homo sapiens] [H.sapiens]

222846\_at 2.04 RAB-8b protein

228959\_at 2.04 ESTs

231918\_s\_at 2.04 hexosaminidase B (beta polypeptide)



203910\_at 2.04 gb:NM\_004815.1 /DEF=Homo sapiens PTPL1-associated RhoGAP 1 (PARG1), mRNA. /FEA=mRNA /GEN=PARG1 /PROD=PTPL1-associated RhoGAP 1 /DB\_XREF=gi:4758881 /UG=Hs.70983 PTPL1-associated RhoGAP 1 /FL=gb:U90920.1 gb:NM\_004815.1

212102\_s\_at 2.03 Consensus includes gb:AK002111.1 /DEF=Homo sapiens cDNA FLJ11249 fis, clone PLACE1008790, highly similar to Homo sapiens importin alpha 7 subunit mRNA. /FEA=mRNA /DB\_XREF=gi:7023792 /UG=Hs.301553 karyopherin alpha 6 (importin alpha 7) /FL=gb:AF060543.1 gb:NM\_012316.1

206245\_s\_at 2.03 gb:NM\_006469.1 /DEF=Homo sapiens NS1-binding protein (NS1-BP), mRNA. /FEA=mRNA /GEN=NS1-BP /PROD=NS1-binding protein /DB\_XREF=gi:5453803 /UG=Hs.197298 NS1-binding protein /FL=gb:NM\_006469.1

212613\_at 2.03 butyrophilin, subfamily 3, member A2

236704\_at 2.03 ESTs, Weakly similar to S29857 nonhistone chromosomal protein HMG-1 - human [H.sapiens]

228991\_at 2.03 ESTs

220853\_at 2.03 gb:NM\_014118.1 /DEF=Homo sapiens PRO0159 protein (PRO0159), mRNA. /FEA=mRNA /GEN=PRO0159 /PROD=PRO0159 protein /DB\_XREF=gi:7662529 /UG=Hs.278931 PRO0159 protein /FL=gb:AF090899.1 gb:NM\_014118.1

224577\_at 2.03 KIAA1181 protein

214508\_x\_at 2.03 Consensus includes gb:U44836.1 /DEF=Human inducible cAMP early repressor (CREM) mRNA, complete cds. /FEA=CDS /GEN=CREM /PROD=inducible cAMP early repressor /DB\_XREF=gi:1177861 /UG=Hs.155924 cAMP responsive element modulator /FL=gb:U44836.1

227228\_s\_at 2.03 KIAA1509 protein

212634\_at 2.03 KIAA0776 protein

236610\_at 2.02 ESTs

226958\_s\_at 2.02 chemokine (C-X-C motif) ligand 16

223797\_at 2.02 hypothetical protein PRO2852

200920\_s\_at 2.02 B-cell translocation gene 1, anti-proliferative

36129\_at 2.02 KIAA0397 gene product

202877\_s\_at 2.02 complement component 1, q subcomponent, receptor 1

202130\_at 2.02 sudD suppressor of bimD6 homolog (A. nidulans)

233075\_at 2.02 Homo sapiens HERC2P7 pseudogene, partial mRNA sequence.

240383\_at 2.02 ESTs, Weakly similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]

204715\_at 2.02 gb:NM\_015368.1 /DEF=Homo sapiens pannexin 1 (PANX1), mRNA. /FEA=mRNA /GEN=PANX1 /PROD=pannexin 1 /DB\_XREF=gi:7662507 /UG=Hs.30985 pannexin 1 /FL=gb:AF093239.1 gb:NM\_015368.1

243912\_x\_at 2.01 ESTs

243262\_at 2.01 ESTs



201251_at	2.01	gb:NM_002654.1 /DEF=Homo sapiens pyruvate kinase, muscle (PKM2), mRNA. /FEA=mRNA /GEN=PKM2 /PROD=pyruvate kinase, muscle /DB_XREF=gi:4505838 /UG=Hs.198281 pyruvate kinase, muscle /FL=gb:BC000481.1 gb:M23725.1 gb:M26252.1 gb:NM_002654.1
205981_s_at	2.01	gb:NM_001564.1 /DEF=Homo sapiens inhibitor of growth family, member 1-like (ING1L), mRNA. /FEA=mRNA /GEN=ING1L /PROD=inhibitor of growth 1-like /DB_XREF=gi:4504694 /UG=Hs.107153 inhibitor of growth family, member 1-like /FL=gb:AB012853.1 gb:NM_001564.1 gb:AF053537.1
237096_at	2.01	ESTs
213376_at	2.01	KIAA0997 protein
204665_at	2.01	Consensus includes gb:NM_025073.1 /DEF=Homo sapiens hypothetical protein FLJ21168 (FLJ21168), mRNA. /FEA=mRNA /GEN=FLJ21168 /PROD=hypothetical protein FLJ21168 /DB_XREF=gi:13376618 /UG=Hs.288106 hypothetical protein FLJ21168 /FL=gb:NM_025073.1
242124_at	2.01	ESTs
230707_at	2.00	ESTs, Highly similar to SORL_HUMAN Sortilin-related receptor precursor (Sorting protein-related receptor containing LDLR class A repeats) (SorLA) (SorLA-1) (Low-density lipoprotein receptor relative with 11 ligand-binding repeats) (LDLR relative with 11 ligand-binding repeats) (LR11) [H.sapiens]
201624_at	2.00	gb:NM_001349.1 /DEF=Homo sapiens aspartyl-tRNA synthetase (DARS), mRNA. /FEA=mRNA /GEN=DARS /PROD=aspartyl-tRNA synthetase /DB_XREF=gi:4557512 /UG=Hs.80758 aspartyl-tRNA synthetase /FL=gb:BC000629.1 gb:J05032.1 gb:NM_001349.1
238909_at	2.00	ESTs
239486_at	2.00	ESTs, Weakly similar to MAPB_HUMAN Microtubule-associated protein 1B (MAP 1B) [Contains: MAP1 light chain LC1] [H.sapiens]

## Appendix IV



## Genes upregulated by hypoxia in unactivated T cells

### Genes upregulated by hypoxia 1% >2 fold in unactivated T cells (n=12)

Affy probe no.	Fold upregulation	Gene name
208308_s_at	7.29	gb:NM_000175.1 /DEF=Homo sapiens glucose phosphate isomerase (GPI), mRNA. /FEA=mRNA /GEN=GPI /PROD=glucose phosphate isomerase /DB_XREF=gi:4504086 /UG=Hs.180532 glucose phosphate isomerase /FL=gb:BC004982.1 gb:K03515.1 gb:NM_000175.1
207543_s_at	7.28	gb:NM_000917.1 /DEF=Homo sapiens procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I (P4HA1), mRNA. /FEA=mRNA /GEN=P4HA1 /PROD=procollagen-proline, 2-oxoglutarate4-dioxygenase (proline 4-hydroxylase), alpha polypeptideI /DB_XREF=gi:4505564 /UG=Hs.76768 procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I /FL=gb:M24486.1 gb:NM_000917.1
213011_s_at	6.32	triosephosphate isomerase 1
221478_at	5.96	Consensus includes gb:AL132665.1 /DEF=Homo sapiens mRNA; cDNA DKFZp566E034 (from clone DKFZp566E034); complete cds. /FEA=mRNA /DB_XREF=gi:6137021 /UG=Hs.132955 BCL2adenovirus E1B 19kD-interacting protein 3-like /FL=gb:AF060922.1 gb:AB004788.1 gb:AF067396.1 gb:NM_004331.1 gb:AL132665.1
202499_s_at	5.36	gb:NM_006931.1 /DEF=Homo sapiens solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3), mRNA. /FEA=mRNA /GEN=SLC2A3 /PROD=solute carrier family 2 (facilitated glucosetransporter), member 3 /DB_XREF=gi:5902089 /UG=Hs.7594 solute carrier family 2 (facilitated glucose transporter), member 3 /FL=gb:M20681.1 gb:NM_006931.1
203282_at	4.91	gb:NM_000158.1 /DEF=Homo sapiens glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme, Andersen disease, glycogen storage disease type IV) (GBE1), mRNA. /FEA=mRNA /GEN=GBE1 /PROD=glucan (1,4-alpha-), branching enzyme 1(glycogen branching enzyme) /DB_XREF=gi:4557618 /UG=Hs.1691 glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme, Andersen disease, glycogen storage disease type IV) /FL=gb:L07956.1 gb:NM_000158.1
221479_s_at	4.84	gb:AF060922.1 /DEF=Homo sapiens clone 016a05 My020 protein mRNA, complete cds. /FEA=mRNA /PROD=My020 protein /DB_XREF=gi:12001981 /UG=Hs.132955 BCL2adenovirus E1B 19kD-interacting protein 3-like /FL=gb:AF060922.1 gb:AB004788.1 gb:AF067396.1 gb:NM_004331.1 gb:AL132665.1
202497_x_at	4.59	Consensus includes gb:AI631159 /FEA=EST /DB_XREF=gi:4682489 /DB_XREF=est:ts93d05.x1 /CLONE=IMAGE:2238825 /UG=Hs.7594 solute carrier family 2 (facilitated glucose transporter), member 3 /FL=gb:M20681.1 gb:NM_006931.1
207785_s_at	2.75	gb:NM_015874.1 /DEF=Homo sapiens H-2K binding factor-2 (LOC51580), mRNA. /FEA=mRNA /GEN=LOC51580 /PROD=H-2K binding factor-2 /DB_XREF=gi:7706215 /UG=Hs.327138 H-2K binding factor-2 /FL=gb:D14041.1 gb:NM_015874.1



222088_s_at	2.65	solute carrier family 2 (facilitated glucose transporter), member 3
222139_at	2.61	KIAA1466 protein
212172_at	2.06	Consensus includes gb:AW277253 /FEA=EST /DB_XREF=gi:6664283 /DB_XREF=est:xq80f09.x1 /CLONE=IMAGE:2756969 /UG=Hs.171811 adenylate kinase 2

## Appendix V



## Genes upregulated by hypoxia in activated T cells

Genes upregulated >2 fold by hypoxia 1% in activated T cells (n=333 total top 200 displayed)

Affy probe no.	Fold upregulation	Gene name
213164_at	8.25	ESTs, Weakly similar to A43932 mucin 2 precursor, intestinal - human (fragments) [H.sapiens]
203946_s_at	8.12	gb:U75667.1 /DEF=Human arginase II mRNA, complete cds. /FEA=mRNA /PROD=arginase II /DB_XREF=gi:1763757 /UG=Hs.172851 arginase, type II /FL=gb:NM_001172.2 gb:BC001350.1 gb:D86724.1 gb:U75667.1 gb:U82256.1
201105_at	8.00	gb:NM_002305.2 /DEF=Homo sapiens lectin, galactoside-binding, soluble, 1 (galectin 1) (LGALS1), mRNA. /FEA=mRNA /GEN=LGALS1 /PROD=beta-galactosidase binding lectin precursor /DB_XREF=gi:6006015 /UG=Hs.227751 lectin, galactoside-binding, soluble, 1 (galectin 1) /FL=gb:BC001693.1 gb:J04456.1 gb:NM_002305.2
212944_at	7.84	Consensus includes gb:AK024896.1 /DEF=Homo sapiens cDNA: FLJ21243 fis, clone COL01164. /FEA=mRNA /DB_XREF=gi:10437310 /UG=Hs.268016 Homo sapiens cDNA: FLJ21243 fis, clone COL01164
217028_at	7.44	Homo sapiens CXCR4 gene encoding receptor CXCR4.
212689_s_at	7.25	Human putative zinc finger protein mRNA
201849_at	6.70	gb:NM_004052.2 /DEF=Homo sapiens BCL2adenovirus E1B 19kD-interacting protein 3 (BNIP3), nuclear gene encoding mitochondrial protein, mRNA. /FEA=mRNA /GEN=BNIP3 /PROD=BCL2adenovirus E1B 19kD-interacting protein 3 /DB_XREF=gi:7669480 /UG=Hs.79428 BCL2adenovirus E1B 19kD-interacting protein 3 /FL=gb:AF002697.1 gb:U15174.1 gb:NM_004052.2
204472_at	6.64	gb:NM_005261.1 /DEF=Homo sapiens GTP-binding protein overexpressed in skeletal muscle (GEM), mRNA. /FEA=mRNA /GEN=GEM /PROD=GTP-binding protein overexpressed in skeletal muscle /DB_XREF=gi:4885262 /UG=Hs.79022 GTP-binding protein overexpressed in skeletal muscle /FL=gb:NM_005261.1 gb:U13052.1 gb:U10550.1
201012_at	6.37	gb:NM_000700.1 /DEF=Homo sapiens annexin A1 (ANXA1), mRNA. /FEA=mRNA /GEN=ANXA1 /PROD=annexin I /DB_XREF=gi:4502100 /UG=Hs.78225 annexin A1 /FL=gb:BC001275.1 gb:NM_000700.1
210512_s_at	5.88	gb:AF022375.1 /DEF=Homo sapiens vascular endothelial growth factor mRNA, complete cds. /FEA=mRNA /PROD=vascular endothelial growth factor /DB_XREF=gi:3719220 /UG=Hs.73793 vascular endothelial growth factor /FL=gb:M32977.1 gb:AF022375.1 gb:NM_003376.1 gb:AB021221.1 gb:AF091352.1
203158_s_at	5.80	gb:AF097493.1 /DEF=Homo sapiens glutaminase kidney isoform mRNA, complete cds. /FEA=mRNA /PROD=glutaminase kidney isoform /DB_XREF=gi:6002672 /UG=Hs.239189 glutaminase /FL=gb:AF327434.1 gb:AB020645.1 gb:AF097493.1 gb:AF223943.1 gb:NM_014905.1
202856_s_at	5.40	gb:NM_004207.1 /DEF=Homo sapiens solute carrier family 16 (monocarboxylic acid transporters), member 3 (SLC16A3), mRNA. /FEA=mRNA /GEN=SLC16A3 /PROD=solute carrier family 16 (monocarboxylic acid transporters), member 3 /DB_XREF=gi:4759111 /UG=Hs.85838 solute carrier family 16 (monocarboxylic acid transporters), member 3



/FL=gb:U81800.1 gb:NM\_004207.1  
 209034\_at 5.25 gb:AF279899.1 /DEF=Homo sapiens PNAS-145 mRNA, complete cds. /FEA=mRNA /PROD=PNAS-145  
 /DB\_XREF=gi:12751123 /UG=Hs.75969 proline-rich protein with nuclear targeting signal /FL=gb:AF279899.1 gb:U03105.1  
 gb:NM\_006813.1  
 213191\_at 4.89 Consensus includes gb:AF070530.1 /DEF=Homo sapiens clone 24751 unknown mRNA. /FEA=mRNA /PROD=unknown  
 /DB\_XREF=gi:3387885 /UG=Hs.29344 hypothetical protein, clone 24751  
 210592\_s\_at 4.70 gb:M55580.1 /DEF=Human spermidinase mRNA, complete cds. /FEA=mRNA  
 /GEN=spermidinase N1-acetyltransferase /PROD=spermidinase N1-acetyltransferase /DB\_XREF=gi:338335  
 /UG=Hs.28491 spermidinase N1-acetyltransferase /FL=gb:M55580.1  
 207543\_s\_at 4.64 gb:NM\_000917.1 /DEF=Homo sapiens procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha  
 polypeptide I (P4HA1), mRNA. /FEA=mRNA /GEN=P4HA1 /PROD=procollagen-proline, 2-oxoglutarate4-dioxygenase (proline  
 4-hydroxylase), alpha polypeptideI /DB\_XREF=gi:4505564 /UG=Hs.76768 procollagen-proline, 2-oxoglutarate 4-  
 dioxygenase (proline 4-hydroxylase), alpha polypeptide I /FL=gb:M24486.1 gb:NM\_000917.1  
 203045\_at 4.60 gb:NM\_004148.1 /DEF=Homo sapiens ninjurin 1 (NINJ1), mRNA. /FEA=mRNA /GEN=NINJ1 /PROD=ninjurin 1  
 /DB\_XREF=gi:4758809 /UG=Hs.11342 ninjurin 1 /FL=gb:BC004440.1 gb:U72661.1 gb:U91512.1 gb:NM\_004148.1  
 204243\_at 4.58 gb:NM\_012421.1 /DEF=Homo sapiens rearranged L-myc fusion sequence (RLF), mRNA. /FEA=mRNA /GEN=RLF  
 /PROD=rearranged L-myc fusion sequence /DB\_XREF=gi:6912631 /UG=Hs.13321 rearranged L-myc fusion sequence  
 /FL=gb:U22377.1 gb:NM\_012421.1  
 218183\_at 4.56 gb:NM\_013399.1 /DEF=Homo sapiens chromosome 16 open reading frame 5 (C16orf5), mRNA. /FEA=mRNA  
 /GEN=C16orf5 /PROD=chromosome 16 open reading frame 5 /DB\_XREF=gi:7019336 /UG=Hs.7765 chromosome 16 open  
 reading frame 5 /FL=gb:AL136698.1 gb:BC002882.1 gb:AF131218.1 gb:NM\_013399.1  
 201848\_s\_at 4.41 gb:U15174.1 /DEF=Homo sapiens BCL2adenovirus E1B 19kD-interacting protein 3 (BNIP3) mRNA, complete cds.  
 /FEA=mRNA /GEN=BNIP3 /PROD=BCL2adenovirus E1B 19kD-interacting protein 3 /DB\_XREF=gi:558845 /UG=Hs.79428  
 BCL2adenovirus E1B 19kD-interacting protein 3 /FL=gb:AF002697.1 gb:U15174.1 gb:NM\_004052.2  
 218570\_at 4.37 gb:NM\_018095.1 /DEF=Homo sapiens hypothetical protein FLJ10450 (FLJ10450), mRNA. /FEA=mRNA /GEN=FLJ10450  
 /PROD=hypothetical protein FLJ10450 /DB\_XREF=gi:8922425 /UG=Hs.267604 hypothetical protein FLJ10450  
 /FL=gb:BC002736.1 gb:NM\_018095.1  
 203140\_at 4.16 gb:NM\_001706.1 /DEF=Homo sapiens B-cell CLLlymphoma 6 (zinc finger protein 51) (BCL6), mRNA. /FEA=mRNA  
 /GEN=BCL6 /PROD=B-cell CLLlymphoma 6 (zinc finger protein 51) /DB\_XREF=gi:4502382 /UG=Hs.155024 B-cell  
 CLLlymphoma 6 (zinc finger protein 51) /FL=gb:U00115.1 gb:NM\_001706.1  
 202499\_s\_at 4.14 gb:NM\_006931.1 /DEF=Homo sapiens solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3),  
 mRNA. /FEA=mRNA /GEN=SLC2A3 /PROD=solute carrier family 2 (facilitated glucosetransporter), member 3  
 /DB\_XREF=gi:5902089 /UG=Hs.7594 solute carrier family 2 (facilitated glucose transporter), member 3  
 /FL=gb:M20681.1 gb:NM\_006931.1  
 203159\_at 4.14 gb:NM\_014905.1 /DEF=Homo sapiens glutaminase (GLS), mRNA. /FEA=mRNA /GEN=GLS /PROD=glutaminase C  
 /DB\_XREF=gi:7662327 /UG=Hs.239189 glutaminase /FL=gb:AF327434.1 gb:AB020645.1 gb:AF097493.1 gb:AF223943.1  
 gb:NM\_014905.1



218507\_at 4.14 gb:NM\_013332.1 /DEF=Homo sapiens hypoxia-inducible protein 2 (HIG2), mRNA. /FEA=mRNA /GEN=HIG2 /PROD=hypoxia-inducible protein 2 /DB\_XREF=gi:7019408 /UG=Hs.61762 hypoxia-inducible protein 2 /FL=gb:BC001863.1 gb:AF144755.1 gb:NM\_013332.1

202497\_x\_at 4.04 Consensus includes gb:AI631159 /FEA=EST /DB\_XREF=gi:4682489 /DB\_XREF=est:ts93d05.x1 /CLONE=IMAGE:2238825 /UG=Hs.7594 solute carrier family 2 (facilitated glucose transporter), member 3 /FL=gb:M20681.1 gb:NM\_006931.1

221497\_x\_at 4.00 gb:BC005369.1 /DEF=Homo sapiens, chromosome 1 open reading frame 12, clone MGC:12484, mRNA, complete cds. /FEA=mRNA /PROD=chromosome 1 open reading frame 12 /DB\_XREF=gi:13529208 /UG=Hs.6523 chromosome 1 open reading frame 12 /FL=gb:AF277176.1 gb:AF277176.1 gb:NM\_022051.1 gb:BC005369.1

216236\_s\_at 4.00 Consensus includes gb:AL110298.1 /DEF=Homo sapiens mRNA; cDNA DKFZp564K1672 (from clone DKFZp564K1672); partial cds. /FEA=mRNA /GEN=DKFZp564K1672 /PROD=hypothetical protein /DB\_XREF=gi:5817258 /UG=Hs.7594 solute carrier family 2 (facilitated glucose transporter), member 3

201041\_s\_at 4.00 gb:NM\_004417.2 /DEF=Homo sapiens dual specificity phosphatase 1 (DUSP1), mRNA. /FEA=mRNA /GEN=DUSP1 /PROD=dual specificity phosphatase 1 /DB\_XREF=gi:7108342 /UG=Hs.171695 dual specificity phosphatase 1 /FL=gb:NM\_004417.2

201242\_s\_at 3.99 gb:BC000006.1 /DEF=Homo sapiens, ATPase, Na+K+ transporting, beta 1 polypeptide, clone MGC:1798, mRNA, complete cds. /FEA=mRNA /PROD=ATPase, Na+K+ transporting, beta 1 polypeptide /DB\_XREF=gi:12652534 /UG=Hs.78629 ATPase, Na+K+ transporting, beta 1 polypeptide /FL=gb:BC000006.1 gb:NM\_001677.1

202149\_at 3.98 match: proteins: Tr:Q61140 Tr:Q63766 Tr:Q35177 Tr:Q14511 Tr:Q9YHC0; Human DNA sequence from clone RP4-761I2 on chromosome 6 Contains 3' part of the gene for enhancer of filamentation (HEF1), ESTs, STSs and CpG islands, complete sequence.

36711\_at 3.73 Cluster Incl. AL021977:bK447C4.1 (novel MAFF (v-maf musculoaponeurotic fibrosarcoma (avian) oncogene family, protein F) LIKE protein) /cds=(0,494) /gb=AL021977 /gi=4914526 /ug=Hs.51305 /len=2128

221523\_s\_at 3.67 Consensus includes gb:AL138717 /DEF=Human DNA sequence from clone RP11-11D8 on chromosome 6 Contains the 5 end of the gene for a yeast ubiquitin conjugating enzyme UBC6 homolog, the gene for a possible GTP binding protein, a NACA (nascent-polypeptide-associated complex alpha polypeptide... /FEA=mRNA\_2 /DB\_XREF=gi:8894207 /UG=Hs.238679 Rag D protein /FL=gb:NM\_021244.1 gb:AF272036.1 gb:BC003088.1

202127\_at 3.65 PRP4 pre-mRNA processing factor 4 homolog B (yeast)

208103\_s\_at 3.60 gb:NM\_030920.1 /DEF=Homo sapiens hypothetical protein MGC5350 (MGC5350), mRNA. /FEA=mRNA /GEN=MGC5350 /PROD=hypothetical protein MGC5350 /DB\_XREF=gi:13569878 /FL=gb:NM\_030920.1

212399\_s\_at 3.57 Consensus includes gb:D50911.2 /DEF=Homo sapiens mRNA for KIAA0121 protein, partial cds. /FEA=mRNA /GEN=KIAA0121 /PROD=KIAA0121 protein /DB\_XREF=gi:6633996 /UG=Hs.155584 KIAA0121 gene product

220924\_s\_at 3.55 gb:NM\_018976.1 /DEF=Homo sapiens amino acid transporter 2 (KIAA1382), mRNA. /FEA=mRNA /GEN=KIAA1382 /PROD=amino acid transporter 2 /DB\_XREF=gi:9506836 /UG=Hs.298275 amino acid transporter 2 /FL=gb:NM\_018976.1

217783\_s\_at 3.53 gb:NM\_016061.1 /DEF=Homo sapiens CGI-127 protein (LOC51646), mRNA. /FEA=mRNA /GEN=LOC51646 /PROD=CGI-127 protein /DB\_XREF=gi:7706340 /UG=Hs.184542 CGI-127 protein /FL=gb:BC000836.1 gb:AF151885.1 gb:NM\_016061.1

219862\_s\_at 3.53 gb:NM\_012336.1 /DEF=Homo sapiens nuclear prelamin A recognition factor (NARF), mRNA. /FEA=mRNA /GEN=NARF /PROD=nuclear prelamin A recognition factor /DB\_XREF=gi:6912523 /UG=Hs.256526 nuclear prelamin A recognition



212366\_at 3.51 factor /FL=gb:AF128406.1 gb:NM\_012336.1  
hypothetical protein FLJ13564

214696\_at 3.49 Consensus includes gb:AF070569.1 /DEF=Homo sapiens clone 24659 mRNA sequence. /FEA=mRNA  
/DB\_XREF=gi:3387938 /UG=Hs.29206 Homo sapiens clone 24659 mRNA sequence

202855\_s\_at 3.47 solute carrier family 16 (monocarboxylic acid transporters), member 3

212907\_at 3.47 ESTs

201307\_at 3.47 hypothetical protein FLJ10849

215001\_s\_at 3.42 Consensus includes gb:AL161952.1 /DEF=Homo sapiens mRNA; cDNA DKFZp434M0813 (from clone DKFZp434M0813);  
partial cds. /FEA=mRNA /GEN=DKFZp434M0813 /PROD=hypothetical protein /DB\_XREF=gi:7328002 /UG=Hs.170171  
glutamate-ammonia ligase (glutamine synthase)

210426\_x\_at 3.41 gb:U04897.1 /DEF=Human orphan hormone nuclear receptor RORalpha1 mRNA, complete cds. /FEA=mRNA  
/PROD=RORalpha1 /DB\_XREF=gi:451563 /UG=Hs.2156 RAR-related orphan receptor A /FL=gb:U04897.1

218041\_x\_at 3.41 gb:NM\_018573.1 /DEF=Homo sapiens hypothetical protein PRO1068 (PRO1068), mRNA. /FEA=mRNA /GEN=PRO1068  
/PROD=hypothetical protein PRO1068 /DB\_XREF=gi:8924006 /UG=Hs.321158 hypothetical protein PRO1068  
/FL=gb:AF116620.1 gb:NM\_018573.1

203157\_s\_at 3.38 gb:AB020645.1 /DEF=Homo sapiens mRNA for KIAA0838 protein, complete cds. /FEA=mRNA /GEN=KIAA0838  
/PROD=KIAA0838 protein /DB\_XREF=gi:4240164 /UG=Hs.239189 glutaminase /FL=gb:AF327434.1 gb:AB020645.1  
gb:AF097493.1 gb:AF223943.1 gb:NM\_014905.1

212561\_at 3.34 RAB6 interacting protein 1

204900\_x\_at 3.32 gb:NM\_003864.1 /DEF=Homo sapiens sin3-associated polypeptide, 30kD (SAP30), mRNA. /FEA=mRNA /GEN=SAP30  
/PROD=sin3 associated polypeptide p30 /DB\_XREF=gi:4506782 /UG=Hs.20985 sin3-associated polypeptide, 30kD  
/FL=gb:AF055993.1 gb:NM\_003864.1

202670\_at 3.30 mitogen-activated protein kinase kinase 1

212492\_s\_at 3.29 KIAA0876 protein

210479\_s\_at 3.27 gb:L14611.1 /DEF=Human transcription factor RZR-alpha mRNA, complete cds. /FEA=mRNA /PROD=transcription factor  
/DB\_XREF=gi:348240 /UG=Hs.2156 RAR-related orphan receptor A /FL=gb:L14611.1 gb:NM\_002943.1 gb:U04899.1

206848\_at 3.25 gb:NM\_006896.1 /DEF=Homo sapiens homeo box A7 (HOXA7), mRNA. /FEA=mRNA /GEN=HOXA7 /PROD=homeo box A7  
/DB\_XREF=gi:5901971 /UG=Hs.70954 homeo box A7 /FL=gb:AF026397.1 gb:NM\_006896.1

211742\_s\_at 3.20 gb:BC005926.1 /DEF=Homo sapiens, ecotropic viral integration site 2B, clone MGC:14529, mRNA, complete cds.  
/FEA=mRNA /PROD=ecotropic viral integration site 2B /DB\_XREF=gi:13543535 /FL=gb:BC005926.1

220232\_at 3.20 gb:NM\_024906.1 /DEF=Homo sapiens hypothetical protein FLJ21032 (FLJ21032), mRNA. /FEA=mRNA /GEN=FLJ21032  
/PROD=hypothetical protein FLJ21032 /DB\_XREF=gi:13376362 /UG=Hs.247474 hypothetical protein FLJ21032  
/FL=gb:NM\_024906.1



221676\_s\_at 3.19 gb:BC002342.1 /DEF=Homo sapiens, coronin, actin-binding protein, 1C, clone MGC:8518, mRNA, complete cds. /FEA=mRNA /PROD=coronin, actin-binding protein, 1C /DB\_XREF=gi:12803080 /UG=Hs.17377 coronin, actin-binding protein, 1C /FL=gb:BC002342.1

201368\_at 3.18 zinc finger protein 36, C3H type-like 2

221011\_s\_at 3.16 gb:NM\_030915.1 /DEF=Homo sapiens hypothetical protein DKFZp566J091 (DKFZP566J091), mRNA. /FEA=mRNA /GEN=DKFZP566J091 /PROD=hypothetical protein DKFZp566J091 /DB\_XREF=gi:13569871 /FL=gb:NM\_030915.1 hypothetical protein FLJ13564

212368\_at 3.15

205981\_s\_at 3.15 gb:NM\_001564.1 /DEF=Homo sapiens inhibitor of growth family, member 1-like (ING1L), mRNA. /FEA=mRNA /GEN=ING1L /PROD=inhibitor of growth 1-like /DB\_XREF=gi:4504694 /UG=Hs.107153 inhibitor of growth family, member 1-like /FL=gb:AB012853.1 gb:NM\_001564.1 gb:AF053537.1

204563\_at 3.13 gb:NM\_000655.2 /DEF=Homo sapiens selectin L (lymphocyte adhesion molecule 1) (SELL), mRNA. /FEA=mRNA /GEN=SELL /PROD=selectin L /DB\_XREF=gi:5713320 /UG=Hs.82848 selectin L (lymphocyte adhesion molecule 1) /FL=gb:M25280.1 gb:NM\_000655.2

218878\_s\_at 3.13 gb:NM\_012238.3 /DEF=Homo sapiens sirtuin (silent mating type information regulation 2, *S. cerevisiae*, homolog) 1 (SIRT1), mRNA. /FEA=mRNA /GEN=SIRT1 /PROD=sirtuin 1 /DB\_XREF=gi:13775598 /UG=Hs.31176 sirtuin (silent mating type information regulation 2, *S. cerevisiae*, homolog) 1 /FL=gb:NM\_012238.3 gb:AF083106.2

217945\_at 3.10 gb:NM\_025238.1 /DEF=Homo sapiens BTB (POZ) domain containing 1 (BTBD1), mRNA. /FEA=mRNA /GEN=BTBD1 /PROD=BTB (POZ) domain containing 1 /DB\_XREF=gi:13376847 /UG=Hs.21332 BTB (POZ) domain containing 1 /FL=gb:AL136853.1 gb:AF257241.1 gb:NM\_025238.1 gb:AF355402.1

208798\_x\_at 3.09 gb:AF204231.1 /DEF=Homo sapiens 88-kDa Golgi protein (GM88) mRNA, complete cds. /FEA=mRNA /GEN=GM88 /PROD=88-kDa Golgi protein /DB\_XREF=gi:6808610 /UG=Hs.182982 golgin-67 /FL=gb:NM\_015003.1 gb:AF204231.1

201938\_at 3.09 gb:NM\_004642.1 /DEF=Homo sapiens deleted in oral cancer (mouse, homolog) 1 (DOC1), mRNA. /FEA=mRNA /GEN=DOC1 /PROD=deleted in oral cancer (mouse, homolog) 1 /DB\_XREF=gi:4758187 /UG=Hs.3436 deleted in oral cancer (mouse, homolog) 1 /FL=gb:AB006077.1 gb:AF006484.1 gb:NM\_004642.1

203791\_at 3.08 gb:NM\_005509.2 /DEF=Homo sapiens Dmx-like 1 (DMXL1), mRNA. /FEA=mRNA /GEN=DMXL1 /PROD=Dmx-like 1 /DB\_XREF=gi:9961348 /UG=Hs.181042 Dmx-like 1 /FL=gb:NM\_005509.2

203455\_s\_at 3.08 gb:NM\_002970.1 /DEF=Homo sapiens spermidinespermine N1-acetyltransferase (SAT), mRNA. /FEA=mRNA /GEN=SAT /PROD=spermidinespermine N1-acetyltransferase /DB\_XREF=gi:4506788 /UG=Hs.28491 spermidinespermine N1-acetyltransferase /FL=gb:BC002503.1 gb:M77693.1 gb:NM\_002970.1

208936\_x\_at 3.07 gb:AF074000.1 /DEF=Homo sapiens Po66 carbohydrate binding protein mRNA, complete cds. /FEA=mRNA /PROD=Po66 carbohydrate binding protein /DB\_XREF=gi:5577965 /UG=Hs.4082 lectin, galactoside-binding, soluble, 8 (galectin 8) /FL=gb:AF342815.1 gb:L78132.1 gb:AF074000.1 gb:NM\_006499.1

209606\_at 3.05 gb:L06633.1 /DEF=Human transcription factor mRNA, complete cds. /FEA=mRNA /PROD=transcription factor /DB\_XREF=gi:431327 /UG=Hs.270 pleckstrin homology, Sec7 and coiledcoil domains, binding protein /FL=gb:AF068836.1 gb:L06633.1 gb:NM\_004288.2

202523\_s\_at 3.00 KIAA0275 gene product



200632\_s\_at 2.99 gb:NM\_006096.1 /DEF=Homo sapiens N-myc downstream regulated (NDRG1), mRNA. /FEA=mRNA /GEN=NDRG1 /PROD=N-myc downstream regulated /DB\_XREF=gi:5174656 /UG=Hs.75789 N-myc downstream regulated /FL=gb:BC003175.1 gb:D87953.1 gb:AF004162.1 gb:NM\_006096.1

200782\_at 2.97 gb:NM\_001154.2 /DEF=Homo sapiens annexin A5 (ANXA5), mRNA. /FEA=mRNA /GEN=ANXA5 /PROD=annexin V /DB\_XREF=gi:4809273 /UG=Hs.300711 annexin A5 /FL=gb:BC001429.1 gb:BC004993.1 gb:M18366.1 gb:J03745.1 gb:M21731.1 gb:M19384.1 gb:D00172.1 gb:NM\_001154.2

202693\_s\_at 2.97 Consensus includes gb:AW194730 /FEA=EST /DB\_XREF=gi:6473630 /DB\_XREF=est:xn43d11.x1 /CLONE=IMAGE:2696469 /UG=Hs.9075 serinethreonine kinase 17a (apoptosis-inducing) /FL=gb:AB011420.1 gb:NM\_004760.1

216841\_s\_at 2.96 Consensus includes gb:X15132.1 /DEF=Human mRNA for manganese containing superoxide dismutase (EC 1.15.1.1). /FEA=mRNA /DB\_XREF=gi:34794 /UG=Hs.318885 superoxide dismutase 2, mitochondrial

212171\_x\_at 2.95 vascular endothelial growth factor

219279\_at 2.95 gb:NM\_017718.1 /DEF=Homo sapiens hypothetical protein FLJ20220 (FLJ20220), mRNA. /FEA=mRNA /GEN=FLJ20220 /PROD=hypothetical protein FLJ20220 /DB\_XREF=gi:8923209 /UG=Hs.21126 hypothetical protein FLJ20220 /FL=gb:NM\_017718.1

218319\_at 2.95 gb:NM\_020651.2 /DEF=Homo sapiens pellino (Drosophila) homolog 1 (PELI1), mRNA. /FEA=mRNA /GEN=PELI1 /PROD=pellino protein /DB\_XREF=gi:11037062 /UG=Hs.7886 pellino (Drosophila) homolog 1 /FL=gb:AF302505.1 gb:AF300987.1 gb:NM\_020651.2

202704\_at 2.94 transducer of ERBB2, 1

200983\_x\_at 2.92 Consensus includes gb:BF983379 /FEA=EST /DB\_XREF=gi:12386191 /DB\_XREF=est:602305270F1 /CLONE=IMAGE:4396576 /UG=Hs.119663 CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, EJ30, EL32 and G344) /FL=gb:NM\_000611.1 gb:M34671.1

201057\_s\_at 2.91 gb:NM\_004487.1 /DEF=Homo sapiens golgi autoantigen, golgin subfamily b, macrogolgin (with transmembrane signal), 1 (GOLGB1), mRNA. /FEA=mRNA /GEN=GOLGB1 /PROD=golgi autoantigen, golgin subfamily b, macrogolgin (with transmembrane signal), 1 /DB\_XREF=gi:4758453 /UG=Hs.7844 golgi autoantigen, golgin subfamily b, macrogolgin (with transmembrane signal), 1 /FL=gb:NM\_004487.1

214430\_at 2.90 Consensus includes gb:NM\_000169.1 /DEF=Homo sapiens galactosidase, alpha (GLA), mRNA. /FEA=CDS /GEN=GLA /PROD=galactosidase, alpha /DB\_XREF=gi:4504008 /UG=Hs.69089 galactosidase, alpha /FL=gb:BC002689.1 gb:NM\_000169.1

210425\_x\_at 2.88 gb:AF164622.1 /DEF=Homo sapiens golgin-67 (GOLGA5) mRNA, complete cds. /FEA=mRNA /GEN=GOLGA5 /PROD=golgin-67 /DB\_XREF=gi:7211437 /UG=Hs.182982 golgin-67 /FL=gb:AF163441.1 gb:AF164622.1

212902\_at 2.87 GTP-binding protein Sara

212812\_at 2.86 ESTs

218764\_at 2.86 gb:NM\_024064.1 /DEF=Homo sapiens hypothetical protein MGC5363 (MGC5363), mRNA. /FEA=mRNA /GEN=MGC5363 /PROD=hypothetical protein MGC5363 /DB\_XREF=gi:13129041 /UG=Hs.1880 hypothetical protein MGC5363 /FL=gb:BC001000.2 gb:NM\_024064.1



220418\_at 2.83 gb:NM\_018961.1 /DEF=Homo sapiens ubiquitin associated and SH3 domain containing, A (UBASH3A), mRNA. /FEA=mRNA /GEN=UBASH3A /PROD=ubiquitin associated and SH3 domain containing,A /DB\_XREF=gi:9507220 /UG=Hs.183924 ubiquitin associated and SH3 domain containing, A /FL=gb:NM\_018961.1

218364\_at 2.82 gb:NM\_017724.1 /DEF=Homo sapiens leucine rich repeat (in FLII) interacting protein 2 (LRRFIP2), mRNA. /FEA=mRNA /GEN=LRRFIP2 /PROD=leucine rich repeat (in FLII) interactingprotein 2 /DB\_XREF=gi:8923223 /UG=Hs.57672 leucine rich repeat (in FLII) interacting protein 2 /FL=gb:NM\_017724.1

202638\_s\_at 2.82 gb:NM\_000201.1 /DEF=Homo sapiens intercellular adhesion molecule 1 (CD54), human rhinovirus receptor (ICAM1), mRNA. /FEA=mRNA /GEN=ICAM1 /PROD=intercellular adhesion molecule 1 precursor /DB\_XREF=gi:4557877 /UG=Hs.168383 intercellular adhesion molecule 1 (CD54), human rhinovirus receptor /FL=gb:M24283.1 gb:J03132.1 gb:NM\_000201.1

37145\_at 2.82 Homo sapiens NKG5 gene, complete cds.

209007\_s\_at 2.81 gb:AF267856.1 /DEF=Homo sapiens HT033 mRNA, complete cds. /FEA=mRNA /PROD=HT033 /DB\_XREF=gi:12006038 /UG=Hs.8084 hypothetical protein dJ465N24.2.1 /FL=gb:AF247168.1 gb:AF267856.1

218149\_s\_at 2.81 gb:NM\_017606.1 /DEF=Homo sapiens hypothetical protein DKFZp434K1210 (DKFZp434K1210), mRNA. /FEA=mRNA /GEN=DKFZp434K1210 /PROD=hypothetical protein DKFZp434K1210 /DB\_XREF=gi:89222146 /UG=Hs.32352 hypothetical protein DKFZp434K1210 /FL=gb:NM\_017606.1

209446\_s\_at 2.80 gb:BC001743.1 /DEF=Homo sapiens, Similar to hypothetical protein FLJ10803, clone MGC:933, mRNA, complete cds. /FEA=mRNA /PROD=Similar to hypothetical protein FLJ10803 /DB\_XREF=gi:12804636 /UG=Hs.8173 hypothetical protein FLJ10803 /FL=gb:BC001743.1

209085\_x\_at 2.79 gb:L14922.1 /DEF=Homo sapiens DNA-binding protein (PO-GA) mRNA, complete cds. /FEA=mRNA /PROD=DNA-binding protein /DB\_XREF=gi:307337 /UG=Hs.166563 replication factor C (activator 1) 1 (145kD) /FL=gb:AF040250.1 gb:L14922.1

202604\_x\_at 2.78 gb:NM\_001110.1 /DEF=Homo sapiens a disintegrin and metalloproteinase domain 10 (ADAM10), mRNA. /FEA=mRNA /GEN=ADAM10 /PROD=a disintegrin and metalloprotease domain 10 /DB\_XREF=gi:4557250 /UG=Hs.172028 a disintegrin and metalloproteinase domain 10 /FL=gb:AF009615.1 gb:NM\_001110.1

210029\_at 2.76 gb:M34455.1 /DEF=Human interferon-gamma-inducible indoleamine 2,3-dioxygenase (IDO) mRNA, complete cds. /FEA=mRNA /DB\_XREF=gi:185790 /UG=Hs.840 indoleamine-pyrrole 2,3 dioxygenase /FL=gb:M34455.1 gb:NM\_002164.1

203367\_at 2.76 gb:NM\_007026.1 /DEF=Homo sapiens MKP-1 like protein tyrosine phosphatase (MKP-L), mRNA. /FEA=mRNA /GEN=MKP-L /PROD=MKP-1 like protein tyrosine phosphatase /DB\_XREF=gi:5902001 /UG=Hs.91448 MKP-1 like protein tyrosine phosphatase /FL=gb:BC001894.1 gb:BC004448.1 gb:AF038844.1 gb:NM\_007026.1 gb:AF120032.1

212817\_at 2.76 Consensus includes gb:AK023253.1 /DEF=Homo sapiens cDNA FLJ13191 fis, clone NT2RP3004262, highly similar to Homo sapiens heat shock protein hsp40-3 mRNA. /FEA=mRNA /DB\_XREF=gi:10435102 /UG=Hs.237506 DnaJ (Hsp40) homolog, subfamily B, member 5

219557\_s\_at 2.75 gb:NM\_020645.1 /DEF=Homo sapiens chromosome 11 open reading frame 14 (C11ORF14), mRNA. /FEA=mRNA /GEN=C11ORF14 /PROD=chromosome 11 open reading frame 14 /DB\_XREF=gi:11034818 /UG=Hs.32017 chromosome 11 open reading frame 14 /FL=gb:NM\_020645.1

212589\_at 2.73 related RAS viral (r-ras) oncogene homolog 2



202859\_x\_at 2.72 gb:NM\_000584.1 /DEF=Homo sapiens interleukin 8 (IL8), mRNA. /FEA=mRNA /GEN=IL8 /PROD=interleukin 8 /DB\_XREF=gi:10834977 /UG=Hs.624 interleukin 8 /FL=gb:NM\_000584.1 gb:M17017.1 gb:M26383.1

217775\_s\_at 2.72 gb:NM\_016026.1 /DEF=Homo sapiens CGI-82 protein (LOC51109), mRNA. /FEA=mRNA /GEN=LOC51109 /PROD=CGI-82 protein /DB\_XREF=gi:7705790 /UG=Hs.179817 CGI-82 protein /FL=gb:BC000112.1 gb:AF151840.1 gb:NM\_016026.1 gb:AF167438.1

222088\_s\_at 2.72 solute carrier family 2 (facilitated glucose transporter), member 3

202336\_s\_at 2.72 gb:NM\_000919.1 /DEF=Homo sapiens peptidylglycine alpha-amidating monoxygenase (PAM), mRNA. /FEA=mRNA /GEN=PAM /PROD=peptidylglycine alpha-amidating monoxygenase /DB\_XREF=gi:4505602 /UG=Hs.83920 peptidylglycine alpha-amidating monoxygenase /FL=gb:M37721.1 gb:NM\_000919.1

200697\_at 2.71 gb:NM\_000188.1 /DEF=Homo sapiens hexokinase 1 (HK1), nuclear gene encoding mitochondrial protein, mRNA. /FEA=mRNA /GEN=HK1 /PROD=hexokinase 1 /DB\_XREF=gi:4504390 /UG=Hs.118625 hexokinase 1 /FL=gb:M75126.1 gb:NM\_000188.1

217911\_s\_at 2.70 gb:NM\_004281.1 /DEF=Homo sapiens BCL2-associated athanogene 3 (BAG3), mRNA. /FEA=mRNA /GEN=BAG3 /PROD=BCL2-associated athanogene 3 /DB\_XREF=gi:6631072 /UG=Hs.15259 BCL2-associated athanogene 3 /FL=gb:AF095193.2 gb:NM\_004281.1 gb:AF127139.1 gb:AF071218.2

212803\_at 2.69 signal transducer and activator of transcription 6, interleukin-4 induced

204566\_at 2.69 gb:NM\_003620.1 /DEF=Homo sapiens protein phosphatase 1D magnesium-dependent, delta isoform (PPM1D), mRNA. /FEA=mRNA /GEN=PPM1D /PROD=protein phosphatase 1D magnesium-dependent,delta isoform /DB\_XREF=gi:4505996 /UG=Hs.100980 protein phosphatase 1D magnesium-dependent, delta isoform /FL=gb:U78305.1 gb:NM\_003620.1

205349\_at 2.68 gb:NM\_002068.1 /DEF=Homo sapiens guanine nucleotide binding protein (G protein), alpha 15 (Gq class) (GNA15), mRNA. /FEA=mRNA /GEN=GNA15 /PROD=guanine nucleotide binding protein (G protein),alpha 15 (Gq class) /DB\_XREF=gi:4504038 /UG=Hs.73797 guanine nucleotide binding protein (G protein), alpha 15 (Gq class) /FL=gb:M63904.1 gb:NM\_002068.1

201739\_at 2.67 gb:NM\_005627.1 /DEF=Homo sapiens serumglucocorticoid regulated kinase (SGK), mRNA. /FEA=mRNA /GEN=SGK /PROD=serumglucocorticoid regulated kinase /DB\_XREF=gi:5032090 /UG=Hs.296323 serumglucocorticoid regulated kinase /FL=gb:BC001263.1 gb:NM\_005627.1 gb:AF153609.1

206545\_at 2.67 gb:NM\_006139.1 /DEF=Homo sapiens CD28 antigen (Tp44) (CD28), mRNA. /FEA=mRNA /GEN=CD28 /PROD=CD28 antigen (Tp44) /DB\_XREF=gi:5453610 /UG=Hs.1987 CD28 antigen (Tp44) /FL=gb:J02988.1 gb:NM\_006139.1 gb:AF222342.1

203966\_s\_at 2.66 gb:NM\_021003.1 /DEF=Homo sapiens protein phosphatase 1A (formerly 2C), magnesium-dependent, alpha isoform (PPM1A), mRNA. /FEA=mRNA /GEN=PPM1A /PROD=protein phosphatase 1A (formerly 2C),magnesium-dependent, alpha isoform /DB\_XREF=gi:10337594 /UG=Hs.57764 protein phosphatase 1A (formerly 2C), magnesium-dependent, alpha isoform /FL=gb:NM\_021003.1

208933\_s\_at 2.65 Fc fragment of IgG, low affinity IIb, receptor for (CD32)

210031\_at 2.65 gb:J04132.1 /DEF=Human T cell receptor zeta-chain mRNA, complete cds. /FEA=mRNA /PROD=T-cell receptor zeta chain /DB\_XREF=gi:623041 /UG=Hs.97087 CD3Z antigen, zeta polypeptide (TIT3 complex) /FL=gb:NM\_000734.1 gb:J04132.1



212221\_x\_at 2.64 Consensus includes gb:AV703259 /FEA=EST /DB\_XREF=gi:10720588 /DB\_XREF=est:AV703259 /CLONE=ADBCRE12 /UG=Hs.303154 popeye protein 3

219492\_at 2.64 gb:NM\_012110.1 /DEF=Homo sapiens cysteine-rich hydrophobic domain 2 (CHIC2), mRNA. /FEA=mRNA /GEN=CHIC2 /PROD=cysteine-rich hydrophobic domain 2 /DB\_XREF=gi:6912275 /UG=Hs.119488 cysteine-rich hydrophobic domain 2 /FL=gb:AF159423.1 gb:NM\_012110.1

35626\_at 2.63 N-sulfoglucosamine sulfohydrolase (sulfamidase)

213750\_at 2.63 ESTs, Weakly similar to hypothetical protein FLJ20489 [Homo sapiens] [H.sapiens]

215223\_s\_at 2.63 superoxide dismutase 2, mitochondrial

74694\_s\_at 2.62 hypothetical protein FLJ23282

209122\_at 2.61 gb:BC005127.1 /DEF=Homo sapiens, adipose differentiation-related protein, clone MGC:10598, mRNA, complete cds. /FEA=mRNA /PROD=adipose differentiation-related protein /DB\_XREF=gi:13477306 /UG=Hs.3416 adipose differentiation-related protein /FL=gb:BC005127.1 gb:NM\_001122.1

211414\_at 2.61 gb:AF097495.1 /DEF=Homo sapiens glutaminase isoform M precursor, mRNA, complete cds. /FEA=mRNA /PROD=glutaminase isoform M precursor /DB\_XREF=gi:6002675 /UG=Hs.284195 Homo sapiens glutaminase isoform M precursor, mRNA, complete cds /FL=gb:AF097495.1

206095\_s\_at 2.61 gb:NM\_006625.2 /DEF=Homo sapiens TLS-associated serine-arginine protein 1 (TASR1), mRNA. /FEA=mRNA /GEN=TASR1 /PROD=TLS-associated serine-arginine protein 1 /DB\_XREF=gi:12056474 /UG=Hs.288038 TLS-associated serine-arginine protein 1 /FL=gb:NM\_006625.2

203132\_at 2.60 gb:NM\_000321.1 /DEF=Homo sapiens retinoblastoma 1 (including osteosarcoma) (RB1), mRNA. /FEA=mRNA /GEN=RB1 /PROD=retinoblastoma 1 (including osteosarcoma) /DB\_XREF=gi:4506434 /UG=Hs.75770 retinoblastoma 1 (including osteosarcoma) /FL=gb:M33647.1 gb:M15400.1 gb:M28419.1 gb:NM\_000321.1

209566\_at 2.60 Consensus includes gb:AL080184.1 /DEF=Homo sapiens mRNA; cDNA DKFZp434O071 (from clone DKFZp434O071). /FEA=mRNA /DB\_XREF=gi:5262661 /UG=Hs.7089 insulin induced protein 2 /FL=gb:AF125392.1

202771\_at 2.60 gb:NM\_014745.1 /DEF=Homo sapiens KIAA0233 gene product (KIAA0233), mRNA. /FEA=mRNA /GEN=KIAA0233 /PROD=KIAA0233 gene product /DB\_XREF=gi:7662013 /UG=Hs.79077 KIAA0233 gene product /FL=gb:D87071.1 gb:NM\_014745.1

202727\_s\_at 2.60 gb:NM\_000416.1 /DEF=Homo sapiens interferon gamma receptor 1 (IFNGR1), mRNA. /FEA=mRNA /GEN=IFNGR1 /PROD=interferon gamma receptor 1 /DB\_XREF=gi:4557879 /UG=Hs.180866 interferon gamma receptor 1 /FL=gb:BC005333.1 gb:J03143.1 gb:NM\_000416.1

205462\_s\_at 2.59 gb:NM\_002149.1 /DEF=Homo sapiens hippocalcin-like 1 (HPCAL1), mRNA. /FEA=mRNA /GEN=HPCAL1 /PROD=hippocalcin-like 1 /DB\_XREF=gi:4504474 /UG=Hs.3618 hippocalcin-like 1 /FL=gb:NM\_002149.1 gb:D16227.1

208836\_at 2.57 gb:U51478.1 /DEF=Human sodiumpotassium-transporting ATPase beta-3 subunit mRNA, complete cds. /FEA=mRNA /PROD=sodiumpotassium-transporting ATPase beta-3subunit /DB\_XREF=gi:1522634 /UG=Hs.76941 ATPase, Na+K+ transporting, beta 3 polypeptide /FL=gb:U51478.1 gb:NM\_001679.1

209112\_at 2.57 gb:BC001971.1 /DEF=Homo sapiens, Similar to cyclin-dependent kinase inhibitor 1B (p27, Kip1), clone MGC:5304, mRNA, complete cds. /FEA=mRNA /PROD=Similar to cyclin-dependent kinase inhibitor 1B(p27, Kip1)



203581\_at 2.57 /DB\_XREF=gi:12805034 /UG=Hs.238990 cyclin-dependent kinase inhibitor 1B (p27, Kip1) /FL=gb:BC001971.1  
gb:NM\_004064.1 gb:U10906.1 gb:AF247551.1 gb:AY004255.1  
gb:BC002438.1 /DEF=Homo sapiens, RAB4, member RAS oncogene family, clone MGC:1486, mRNA, complete cds.  
/FEA=mRNA /PROD=RAB4, member RAS oncogene family /DB\_XREF=gi:12803248 /UG=Hs.119007 RAB4, member RAS  
oncogene family /FL=gb:BC002438.1 gb:BC004309.1 gb:NM\_004578.1 gb:M28211.1  
Homo sapiens, Similar to N-terminal Asn amidase, clone MGC:29626 IMAGE:4872717, mRNA, complete cds

213061\_s\_at 2.57 gb:NM\_005493.1 /DEF=Homo sapiens RAN binding protein 9 (RANBP9), mRNA. /FEA=mRNA /GEN=RANBP9 /PROD=RAN  
binding protein 9 /DB\_XREF=gi:4885570 /UG=Hs.279886 RAN binding protein 9 /FL=gb:AF306510.1 gb:AB008515.1  
gb:NM\_005493.1

202583\_s\_at 2.57 Homo sapiens mannosidase, alpha, class 1A, member 1 (MAN1A1), mRNA

221760\_at 2.57 gb:NM\_006407.2 /DEF=Homo sapiens vitamin A responsive; cytoskeleton related (JWA), mRNA. /FEA=mRNA /GEN=JWA  
/PROD=vitamin A responsive; cytoskeleton related /DB\_XREF=gi:7669496 /UG=Hs.92384 vitamin A responsive;  
cytoskeleton related /FL=gb:BC005143.1 gb:AF070523.1 gb:AF125530.1 gb:AF161476.1 gb:NM\_006407.2  
ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1

214132\_at 2.56 gb:AF044221.1 /DEF=Homo sapiens HCG-1 protein (HCG-1) mRNA, complete cds. /FEA=mRNA /GEN=HCG-1  
/PROD=HCG-1 protein /DB\_XREF=gi:4105251 /UG=Hs.173091 ubiquitin-like 3 /FL=gb:AF044221.1 gb:AL080177.1  
gb:NM\_007106.1

218723\_s\_at 2.55 gb:NM\_014059.1 /DEF=Homo sapiens RGC32 protein (RGC32), mRNA. /FEA=mRNA /GEN=RGC32 /PROD=RGC32 protein  
/DB\_XREF=gi:7662650 /UG=Hs.76640 RGC32 protein /FL=gb:AF036549.1 gb:NM\_014059.1

201296\_s\_at 2.55 gb:NM\_015626.1 /DEF=Homo sapiens DKFZP564A122 protein (DKFZP564A122), mRNA. /FEA=mRNA /GEN=DKFZP564A122  
/PROD=DKFZP564A122 protein /DB\_XREF=gi:7661595 /UG=Hs.187991 DKFZP564A122 protein /FL=gb:AF106684.1  
gb:NM\_015626.1

206513\_at 2.55 gb:NM\_004833.1 /DEF=Homo sapiens absent in melanoma 2 (AIM2), mRNA. /FEA=mRNA /GEN=AIM2 /PROD=absent in  
melanoma 2 /DB\_XREF=gi:4757733 /UG=Hs.105115 absent in melanoma 2 /FL=gb:AF024714.1 gb:NM\_004833.1  
Consensus includes gb:AL049390.1 /DEF=Homo sapiens mRNA; cDNA DKFZp586O1318 (from clone DKFZp586O1318).  
/FEA=mRNA /DB\_XREF=gi:4500184 /UG=Hs.22689 Homo sapiens mRNA; cDNA DKFZp586O1318 (from clone  
DKFZp586O1318)

215528\_at 2.54 Consensus includes gb:AL117515.1 /DEF=Homo sapiens mRNA; cDNA DKFZp434L0735 (from clone DKFZp434L0735);  
partial cds. /FEA=mRNA /GEN=DKFZp434L0735 /PROD=hypothetical protein /DB\_XREF=gi:5912029 /UG=Hs.54886  
phospholipase C, epsilon 2  
ESTs

213309\_at 2.54 Consensus includes gb:AK026548.1 /DEF=Homo sapiens cDNA: FLJ22895 fis, clone KAT04940. /FEA=mRNA  
/DB\_XREF=gi:10439429 /UG=Hs.76847 KIAA0088 protein /FL=gb:AF144074.1  
cathepsin B

221840\_at 2.54 dJ406P24.1 (Thioredoxin-like pseudogene); Human DNA sequence from clone RP3-406P24 on chromosome 6 Contains a

214626\_s\_at 2.53

213274\_s\_at 2.53

216532\_x\_at 2.53



thioredoxin-like pseudogene, 2 CpG islands, ESTs, STSs and GSSs, complete sequence.

203578\_s\_at 2.53 Consensus includes gb:BG230586 /FEA=EST /DB\_XREF=gi:12725619 /DB\_XREF=est:naf40g01.x1 /CLONE=IMAGE:4143552 /UG=Hs.10315 solute carrier family 7 (cationic amino acid transporter, y+ system), member 6 /FL=gb:D87432.1 gb:NM\_003983.1

221938\_x\_at 2.53 thyroid hormone receptor-associated protein, 95-kD subunit

204286\_s\_at 2.53 gb:NM\_021127.1 /DEF=Homo sapiens phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1), mRNA. /FEA=mRNA /GEN=PMAIP1 /PROD=phorbol-12-myristate-13-acetate-induced protein1 /DB\_XREF=gi:10863922 /UG=Hs.96 phorbol-12-myristate-13-acetate-induced protein 1 /FL=gb:NM\_021127.1

206907\_at 2.53 gb:NM\_003811.1 /DEF=Homo sapiens tumor necrosis factor (ligand) superfamily, member 9 (TNFSF9), mRNA. /FEA=mRNA /GEN=TNFSF9 /PROD=tumor necrosis factor (ligand) superfamily,member 9 /DB\_XREF=gi:4507608 /UG=Hs.1524 tumor necrosis factor (ligand) superfamily, member 9 /FL=gb:NM\_003811.1 gb:U03398.1

216421\_at 2.53 Consensus includes gb:AL121886 /DEF=Human DNA sequence from clone RP5-1028D15 on chromosome 20. Contains the 3 end of the gene for CGI-53 protein (ortholog of rodent NGD5), a (possibly pseudo) gene for a protein similar to RPL27A (ribosomal protein L27a), the MYBL2 gene for v-myb a... /FEA=mRNA\_1 /DB\_XREF=gi:8247022 /UG=Hs.287772 Human DNA sequence from clone RP5-1028D15 on chromosome 20. Contains the 3 end of the gene for CGI-53 protein (ortholog of rodent NGD5), a (possibly pseudo) gene for a protein similar to RPL27A (ribosomal protein L27a), the MYBL2 gene for v-myb avian mye muscleblind-like protein MBL39

203640\_at 2.52

200915\_x\_at 2.52 gb:NM\_004986.1 /DEF=Homo sapiens kinectin 1 (kinesin receptor) (KTN1), mRNA. /FEA=mRNA /GEN=KTN1 /PROD=kinectin 1 (kinesin receptor) /DB\_XREF=gi:4826813 /UG=Hs.211577 kinectin 1 (kinesin receptor) /FL=gb:D13629.1 gb:L25616.1 gb:NM\_004986.1

213650\_at 2.52 golgin-67

221763\_at 2.52 hypothetical protein MGC14425

215015\_at 2.52 ESTs, Weakly similar to arginine-glutamic acid dipeptide (RE) repeats; atrophin 1-like; arginine glutamic acid dipeptide RE repeats [Homo sapiens] [H.sapiens]

208308\_s\_at 2.51 gb:NM\_000175.1 /DEF=Homo sapiens glucose phosphate isomerase (GPI), mRNA. /FEA=mRNA /GEN=GPI /PROD=glucose phosphate isomerase /DB\_XREF=gi:4504086 /UG=Hs.180532 glucose phosphate isomerase /FL=gb:BC004982.1 gb:K03515.1 gb:NM\_000175.1

206099\_at 2.51 gb:NM\_006255.1 /DEF=Homo sapiens protein kinase C, eta (PRKCH), mRNA. /FEA=mRNA /GEN=PRKCH /PROD=protein kinase C, eta /DB\_XREF=gi:5453971 /UG=Hs.315366 protein kinase C, eta /FL=gb:M55284.1 gb:NM\_006255.1

205194\_at 2.51 gb:NM\_004577.1 /DEF=Homo sapiens phosphoserine phosphatase (PSPH), mRNA. /FEA=mRNA /GEN=PSPH /PROD=phosphoserine phosphatase /DB\_XREF=gi:4758971 /UG=Hs.56407 phosphoserine phosphatase /FL=gb:NM\_004577.1

62212\_at 2.50 hypothetical protein MGC955



204170\_s\_at 2.50 gb:NM\_001827.1 /DEF=Homo sapiens CDC28 protein kinase 2 (CKS2), mRNA. /FEA=mRNA /GEN=CKS2 /PROD=CDC28 protein kinase 2 /DB\_XREF=gi:4502858 /UG=Hs.83758 CDC28 protein kinase 2 /FL=gb:NM\_001827.1 optineurin

202073\_at 2.50

204129\_at 2.49 gb:NM\_004326.1 /DEF=Homo sapiens B-cell CLLlymphoma 9 (BCL9), mRNA. /FEA=mRNA /GEN=BCL9 /PROD=B-cell CLLlymphoma 9 /DB\_XREF=gi:4757845 /UG=Hs.122607 B-cell CLLlymphoma 9 /FL=gb:NM\_004326.1 cell division cycle 27

217880\_at 2.49

200985\_s\_at 2.48 gb:NM\_000611.1 /DEF=Homo sapiens CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, EJ30, EL32 and G344) (CD59), mRNA. /FEA=mRNA /GEN=CD59 /PROD=CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, EJ30, EL32 and G344) /DB\_XREF=gi:10835164 /UG=Hs.119663 CD59 antigen p18-20 (antigen identified by monoclonal antibodies 16.3A5, EJ16, EJ30, EL32 and G344) /FL=gb:NM\_000611.1 gb:M34671.1

212553\_at 2.48 Consensus includes gb:AK026120.1 /DEF=Homo sapiens cDNA: FLJ22467 fis, clone HRC10345. /FEA=mRNA /DB\_XREF=gi:10438867 /UG=Hs.29956 KIAA0460 protein

212172\_at 2.48 Consensus includes gb:AW277253 /FEA=EST /DB\_XREF=gi:6664283 /DB\_XREF=est:xq80f09.x1 /CLONE=IMAGE:2756969 /UG=Hs.171811 adenylate kinase 2

218289\_s\_at 2.48 gb:NM\_024818.1 /DEF=Homo sapiens hypothetical protein FLJ23251 (FLJ23251), mRNA. /FEA=mRNA /GEN=FLJ23251 /PROD=hypothetical protein FLJ23251 /DB\_XREF=gi:13376211 /UG=Hs.170737 hypothetical protein FLJ23251 /FL=gb:AL136757.1 gb:NM\_024818.1

209342\_s\_at 2.48 gb:AF080158.1 /DEF=Homo sapiens Ikb kinase-b (IKK-beta) mRNA, complete cds. /FEA=mRNA /GEN=IKK-beta /PROD=Ikb kinase-b /DB\_XREF=gi:4185274 /UG=Hs.226573 inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta /FL=gb:AF031416.1 gb:AF080158.1

218871\_x\_at 2.47 gb:NM\_018590.1 /DEF=Homo sapiens hypothetical protein PRO0082 (PRO0082), mRNA. /FEA=mRNA /GEN=PRO0082 /PROD=hypothetical protein PRO0082 /DB\_XREF=gi:8923965 /UG=Hs.180758 hypothetical protein PRO0082 /FL=gb:AF116646.1 gb:NM\_018590.1

202111\_at 2.47 gb:NM\_003040.1 /DEF=Homo sapiens solute carrier family 4, anion exchanger, member 2 (erythrocyte membrane protein band 3-like 1) (SLC4A2), mRNA. /FEA=mRNA /GEN=SLC4A2 /PROD=solute carrier family 4, anion exchanger, member2 (erythrocyte membrane protein band 3-like 1) /DB\_XREF=gi:4507022 /UG=Hs.79410 solute carrier family 4, anion exchanger, member 2 (erythrocyte membrane protein band 3-like 1) /FL=gb:U62531.1 gb:NM\_003040.1

219615\_s\_at 2.47 gb:NM\_003740.1 /DEF=Homo sapiens potassium channel, subfamily K, member 5 (TASK-2) (KCNK5), mRNA. /FEA=mRNA /GEN=KCNK5 /PROD=potassium channel, subfamily K, member 5 (TASK-2) /DB\_XREF=gi:4504850 /UG=Hs.127007 potassium channel, subfamily K, member 5 (TASK-2) /FL=gb:AF084830.1 gb:NM\_003740.1

210346\_s\_at 2.46 gb:AF212224.1 /DEF=Homo sapiens CLK4 mRNA, complete cds. /FEA=mRNA /PROD=CLK4 /DB\_XREF=gi:9437514 /UG=Hs.295231 Homo sapiens CLK4 mRNA, complete cds /FL=gb:AF212224.1

201272\_at 2.46 gb:NM\_001628.1 /DEF=Homo sapiens aldo-keto reductase family 1, member B1 (aldose reductase) (AKR1B1), mRNA. /FEA=mRNA /GEN=AKR1B1 /PROD=aldo-keto reductase family 1, member B1 (aldose reductase) /DB\_XREF=gi:4502048 /UG=Hs.75313 aldo-keto reductase family 1, member B1 (aldose reductase) /FL=gb:BC000260.1 gb:BC005387.1 gb:J04795.1 gb:J05017.1 gb:J05474.1 gb:M34720.1 gb:NM\_001628.1



202814\_s\_at 2.46 HMBA-inducible

218696\_at 2.46 gb:NM\_004836.1 /DEF=Homo sapiens eukaryotic translation initiation factor 2-alpha kinase 3 (EIF2AK3), mRNA. /FEA=mRNA /GEN=EIF2AK3 /PROD=eukaryotic translation initiation factor 2-alpha kinase 3 /DB\_XREF=gi:4758891 /UG=Hs.102506 eukaryotic translation initiation factor 2-alpha kinase 3 /FL=gb:AF110146.1 gb:NM\_004836.1 gb:AF1933339.1

202637\_s\_at 2.45 intercellular adhesion molecule 1 (CD54), human rhinovirus receptor

217678\_at 2.45 ESTs, Moderately similar to hypothetical protein FLJ20489 [Homo sapiens] [H.sapiens]

205266\_at 2.44 gb:NM\_002309.2 /DEF=Homo sapiens leukemia inhibitory factor (cholinergic differentiation factor) (LIF), mRNA. /FEA=mRNA /GEN=LIF /PROD=leukemia inhibitory factor (cholinergic differentiation factor) /DB\_XREF=gi:6006018 /UG=Hs.2250 leukemia inhibitory factor (cholinergic differentiation factor) /FL=gb:NM\_002309.2

212934\_at 2.44 ESTs, Weakly similar to p47 [Homo sapiens] [H.sapiens]

209340\_at 2.44 gb:S73498.1 /DEF=Homo sapiens AgX-1 antigen mRNA, complete cds. /FEA=mRNA /PROD=AgX-1 antigen /DB\_XREF=gi:688010 /UG=Hs.21293 UDP-N-acetylglucosamine pyrophosphorylase 1 /FL=gb:AB011004.1 gb:NM\_003115.1 gb:S73498.1

202079\_s\_at 2.44 KIAA1042 protein

32541\_at 2.42 protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform (calcineurin A gamma)

200832\_s\_at 2.41 gb:AB032261.1 /DEF=Homo sapiens Scd mRNA for stearoyl-CoA desaturase, complete cds. /FEA=mRNA /GEN=Scd /PROD=stearoyl-CoA desaturase /DB\_XREF=gi:7415720 /UG=Hs.119597 stearoyl-CoA desaturase (delta-9-desaturase) /FL=gb:AF097514.1 gb:NM\_005063.1 gb:AB032261.1

208456\_s\_at 2.41 gb:NM\_012250.1 /DEF=Homo sapiens oncogene TC21 (TC21), mRNA. /FEA=CDS /GEN=TC21 /PROD=oncogene TC21 /DB\_XREF=gi:6912697 /UG=Hs.206097 oncogene TC21 /FL=gb:M31468.1 gb:NM\_012250.1

213376\_at 2.41 KIAA0997 protein

212579\_at 2.40 KIAA0650 protein

221260\_s\_at 2.40 gb:NM\_030809.1 /DEF=Homo sapiens chromosome 12 open reading frame 2 (C12orf2), mRNA. /FEA=mRNA /GEN=C12orf2 /PROD=chromosome 12 open reading frame 2 /DB\_XREF=gi:13540601 /FL=gb:NM\_030809.1

221778\_at 2.39 KIAA1718 protein

202695\_s\_at 2.39 gb:NM\_004760.1 /DEF=Homo sapiens serinethreonine kinase 17a (apoptosis-inducing) (STK17A), mRNA. /FEA=mRNA /GEN=STK17A /PROD=serinethreonine kinase 17a(apoptosis-inducing) /DB\_XREF=gi:4758191 /UG=Hs.9075 serinethreonine kinase 17a (apoptosis-inducing) /FL=gb:AB011420.1 gb:NM\_004760.1

209657\_s\_at 2.39 gb:M65217.1 /DEF=Human heat shock factor 2 (HSF2) mRNA, complete cds. /FEA=mRNA /GEN=heat shock factor 2 /PROD=HSF2 /DB\_XREF=gi:184404 /UG=Hs.158195 heat shock transcription factor 2 /FL=gb:M65217.1 gb:NM\_004506.2

219549\_s\_at 2.39 gb:NM\_006054.1 /DEF=Homo sapiens reticulon 3 (RTN3), mRNA. /FEA=mRNA /GEN=RTN3 /PROD=reticulon 3 /DB\_XREF=gi:5174654 /UG=Hs.252831 reticulon 3 /FL=gb:BC000634.1 gb:AF059524.1 gb:AF119297.1 gb:NM\_006054.1



217743\_s\_at 2.39 gb:NM\_018247.1 /DEF=Homo sapiens hypothetical protein FLJ10856 (FLJ10856), mRNA. /FEA=mRNA /GEN=FLJ10856 /PROD=hypothetical protein FLJ10856 /DB\_XREF=gi:89222719 /UG=Hs.108530 hypothetical protein FLJ10856 /FL=gb:NM\_018247.1

220702\_at 2.39 gb:NM\_018616.1 /DEF=Homo sapiens hypothetical protein PRO2037 (PRO2037), mRNA. /FEA=mRNA /GEN=PRO2037 /PROD=hypothetical protein PRO2037 /DB\_XREF=gi:8924113 /UG=Hs.283067 hypothetical protein PRO2037 /FL=gb:AF116684.1 gb:NM\_018616.1

212458\_at 2.38 ESTs, Weakly similar to SPY3\_HUMAN Sprouty homolog 3 (Spry-3) [H.sapiens]

203200\_s\_at 2.38 gb:NM\_024010.1 /DEF=Homo sapiens 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR), transcript variant 2, mRNA. /FEA=mRNA /GEN=MTRR /PROD=methionine synthase reductase, isoform 2 /DB\_XREF=gi:13325067 /UG=Hs.153792 5-methyltetrahydrofolate-homocysteine methyltransferase reductase /FL=gb:NM\_024010.1 gb:AF121214.1

200934\_at 2.38 gb:NM\_003472.1 /DEF=Homo sapiens DEK oncogene (DNA binding) (DEK), mRNA. /FEA=mRNA /GEN=DEK /PROD=DEK oncogene (DNA binding) /DB\_XREF=gi:4503248 /UG=Hs.110713 DEK oncogene (DNA binding) /FL=gb:NM\_003472.1

219237\_s\_at 2.37 gb:NM\_024920.1 /DEF=Homo sapiens hypothetical protein FLJ14281 (FLJ14281), mRNA. /FEA=mRNA /GEN=FLJ14281 /PROD=hypothetical protein FLJ14281 /DB\_XREF=gi:13376391 /UG=Hs.266957 hypothetical protein FLJ14281 /FL=gb:NM\_024920.1

200602\_at 2.36 gb:NM\_000484.1 /DEF=Homo sapiens amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease) (APP), mRNA. /FEA=mRNA /GEN=APP /PROD=amyloid beta (A4) precursor protein (proteas nexin-II, Alzheimer disease) /DB\_XREF=gi:4502166 /UG=Hs.177486 amyloid beta (A4) precursor protein (protease nexin-II, Alzheimer disease) /FL=gb:NM\_000484.1

216901\_s\_at 2.36 Consensus includes gb:S80876.1 /DEF=IKAROS=hIk1 {alternatively spliced} human, Jurkat T cell line, mRNA Partial, 1788 nt. /FEA=CDS /GEN=IKAROS /PROD=hIk1 /DB\_XREF=gi:1911482 /UG=Hs.54452 zinc finger protein, subfamily 1A, 1 (Ikaros)

201200\_at 2.35 gb:NM\_003851.1 /DEF=Homo sapiens cellular repressor of E1A-stimulated genes (CREG), mRNA. /FEA=mRNA /GEN=CREG /PROD=cellular repressor of E1A-stimulated genes /DB\_XREF=gi:4503036 /UG=Hs.5710 cellular repressor of E1A-stimulated genes /FL=gb:AF084523.1 gb:NM\_003851.1

202364\_at 2.35 gb:NM\_005962.1 /DEF=Homo sapiens MAX-interacting protein 1 (MXI1), mRNA. /FEA=mRNA /GEN=MXI1 /PROD=MAX-interacting protein 1 /DB\_XREF=gi:5174596 /UG=Hs.118630 MAX-interacting protein 1 /FL=gb:L07648.1 gb:NM\_005962.1 gb:D63940.1

203579\_s\_at 2.34 Consensus includes gb:AI660619 /FEA=EST /DB\_XREF=gi:4764202 /DB\_XREF=est:wf21h04.x1 /CLONE=IMAGE:2351287 /UG=Hs.10315 solute carrier family 7 (cationic amino acid transporter, y+ system), member 6 /FL=gb:D87432.1 gb:NM\_003983.1

201752\_s\_at 2.34 adducin 3 (gamma)







## Appendix VI

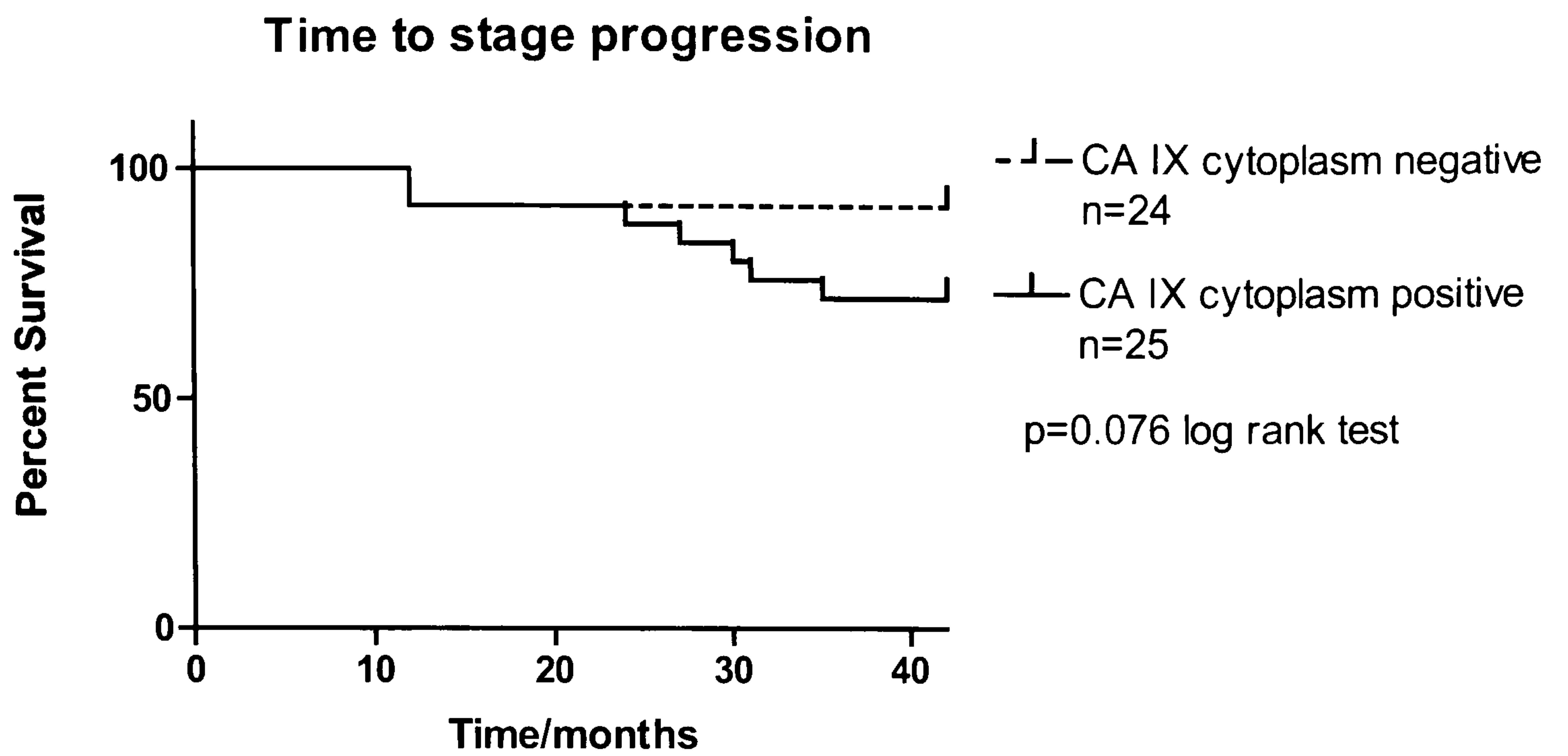
### A study on carbonic anhydrase IX staining in superficial bladder cancer

49 stage pTa to pT1 bladder cancers resected at TURBT were stained and scored for the hypoxia marker carbonic anhydrase IX. Intensity was scored from 0 to 3 (intense) and the percentage of tumour cells staining was also estimated to the nearest 10%. Scoring was performed separately for membrane staining and cytoplasm staining. Groups were split according to the median score. Although the study was under-powered there was a trend for tumours with more intense cytoplasm staining to have a worse prognosis, whereas membrane staining (although it is more striking) showed no significant adverse prognosis.

	CA IX cytoplasm	
	Negative	Positive
Ta G1	4	6
Ta G2	2	3
T1 G1	2	3
T1 G2	14	10
T1 G3	3	2
<b>Total</b>	<b>25</b>	<b>24</b>

Table AVI.1 Carbonic anhydrase IX cytoplasm staining in 49 Ta-T1 bladder tumours. There was no association with stage or grade

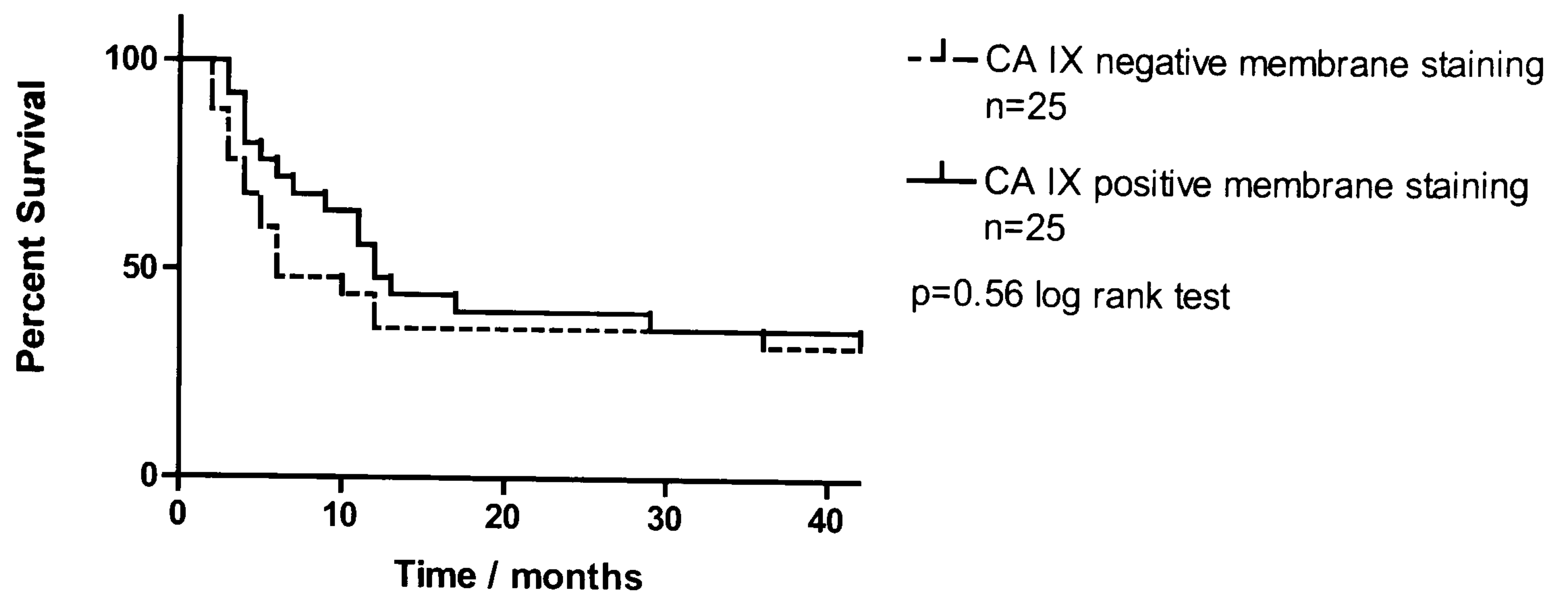




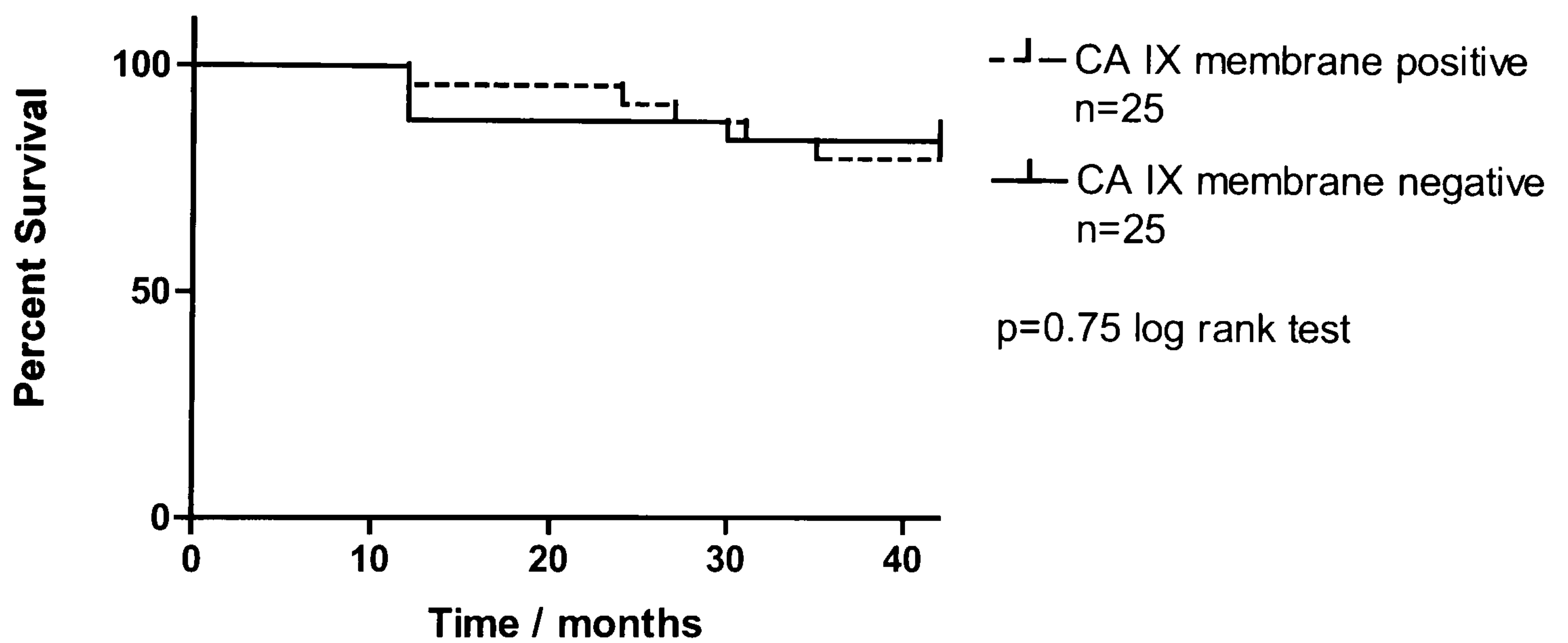
Graph AVI. 1 Progression-free survival (defined as an increase in T stage) over 40 months of follow-up. There was a trend to worse prognosis in the CA IX staining group



**Time to first recurrence,  
superficial bladder cancer**



**Time to stage progression in  
superficial bladder cancer**



Graph AVI.2 Membrane staining for CA IX showed no relationship or trend with time to first recurrence or prognosis



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