Isolation and genetic characterisation of CD133+ve cell population from a paediatric astrocytoma short-term cell culture

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List of Abbreviations

β-ME - β-mercaptoethanol

µl - Microlitres

BSA – Bovine Serum Albumin

CD133 – prominin-1

CD133+ve - CD133 positive

CD133-ve – CD133 negative

CNAs - Copy Number Aberrations

CNS – Central Nervous System

DAPI - 4,6-diamidino-2-phenylindole

DMSO - Dimethyl Sulfoxide

DNA - Deoxyribonucleic Acid

FCS – Foetal Calf Serum

GBM – Glioblastoma Multiforme

HBSS – Hank's Balanced Salts Buffer

mins - Minutes

ml - Millilitre

nm - Nanometres

P - Passage number

PBS – Phosphate buffer solution

RNA - Ribonucleic Acid

rpm – Revolutions per minute

secs – Seconds

WHO – World Health Organisation

Abstract

Each year in the UK 4,400 people are diagnosed with primary brain tumours. The most common of these are astrocytomas with the most malignant having poor prognosis despite aggressive treatments. Genetic studies have shown chromosomal changes in adult high-grade astrocytomas which differ to those seen within the paediatric population. In recent years the tumour stem cell hypothesis has been put forward which suggests that tumours have a sub-population of cells similar to adult stem cells. These tumour stem cells can be isolated from other cells in a tumour from their expression of CD133 protein. The aim of this study was to investigate DNA copy number aberrations of CD133 positive and CD133 negative cells found in a paediatric glioblastoma multiforme. Cells expressing CD133 were isolated using a monoclonal antibody for CD133 labelled with APC and the expression of CD133 was confirmed with fluorescence microscopy. DNA was extracted from both CD133 positive and CD133 negative cells and copy number aberrations analysed using a high-density oligonucleotide 244k array. DNA copy number aberrations were found to differ between the CD133 positive and CD133 negative cells. Further genetic research of these cell sub-populations may alter the way in which high-grade paediatric brain tumours are treated in the future.

Introduction

Astrocytomas

Primary brain tumours are malignancies of the central nervous system (CNS). In the UK 4,400 people are diagnosed each year (Office for National Statistics, 2007) of these, 350 are within the paediatric population (Cancer Backup, 2008). Astrocytoma are the most common brain tumour and in children account for two fifths of all childhood brain tumours (Figure 1) (Cancer Research UK, 2005).

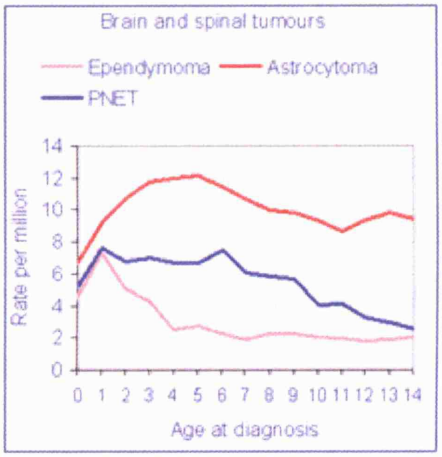


Figure 1. This graph shows the breakdown of brain tumour types in the paediatric population. Astrocytomas as a group, including all tumour grades, account for the majority of childhood brain tumours (adapted from Cancer Research UK, 2005).

Astrocytomas are graded using a classification scheme provided by the World Health Organisation (WHO) into four grades from I-IV. According to Louis *et al,* each grade has different features and, more importantly, a different prognosis. WHO grade I astrocytomas are benign, slow-growing and have little potential for proliferation. In contrast, WHO grade III and IV tumours are malignant with signs of anaplasia and mitotic activity. Additional characteristics of WHO grade IV astrocytomas (GBM) include microvascular proliferation, necrosis and angiogenesis. The prognosis of GBM is very poor with less than half of patients surviving more than 12 months (Louis *et al*, 2007). In children, approximately 80% of astrocytomas will be classified as low-grade tumours (WHO grade I – II) and, 20% will be

diagnosed as high-grade tumours (WHO grade III – IV) (Marchese and Chang, 1990).

Treatment

Treatment options for astrocytoma include surgery, chemotherapy and radiotherapy. Low-grade astrocytoma (WHO grade I-II) are best treated by surgical resection (Packer, 1999). If surgery is not a viable option then chemotherapy treatment is used, with successful results (Prados, 1997; Khaw, 2007). Radiotherapy is often postponed in young children due to radiation-induced side effects such as developmental abnormalities, increased risk of vasculopathies and secondary brain tumours (Kortmann *et al*, 2003). Overall survival in the paediatric population is excellent, with 83% of cases having an overall survival rate of 10 years (Fisher *et al,* 2008). In contrast, treatment for high-grade astrocytomas is often palliative due to the aggressive nature of the tumour. GBM, in particular, shows radioresistance (Masuda *et al*, 1983), which is partly thought to arise from stem cell-like cells found in GBM (Bao *et al*, 2006).

Tumour genetics

Many genetic changes have been found in adult GBM. Early studies showed that in more than 60% of GBM there has been loss of chromosome 9 as well as loss of 10 alongside the gain of chromosome 7 (Bigner *et al*, 1988). These findings have been confirmed by many studies (Schr□ck *et al*, 1994; Venkatraj *et al*, 1998; Vranová *et al*, 2007). Genetic changes have led to the identification of genes involved in the development of brain tumours. Many of these genes are involved in important pathways, such as the Rb and p53 pathways. Genes involved in these pathways, for

example *Rb1, CDK4, CDK6* (in the Rb pathway) and *p53* (in the p53 pathway) are commonly found in areas of chromosomal aberrations in GBM (Furnari *et al*, 2007). Although many DNA abnormalities have been established in adult GBM, little is known of abnormalities in paediatric GBM and aberrations found differ to those seen in the adult population (Bigner *et al*, 1997).

Tumour stem cell hypothesis

The tumour stem cell hypothesis suggests that tumours are formed of a population of cells which are similar to adult stem cells (Tan *et al*, 2006). Normal adult stem cells are cells that are able to self-renew and differentiate to produce mature specialised cells (Reya *et al*, 2001). In recent years a sub-population of stem cell-like cells has been found within a number of different cancers, including prostate, colon, leukaemia and brain tumours (Chiou *et al*, 2008). These tumour stem cells are characterised by their ability to induce tumourigenesis and self-renewal (Clement *et al*, 2007). They have similar traits to normal adult stem cells in that they differentiate and proliferate (Dell'Albani, 2008). They have also been shown to promote tumour growth *in vitro* and *in vivo* (Singh *et al*, 2004a). In 2000, Uchida *et al*, isolated stem cells from foetal brain tissue using antibodies specific to cell surface markers, including CD133 (Uchida *et al,* 2000). Many studies involving brain tumours have focused on genetic differences between tumour and normal tissue (Bigner *et al*, 1997; Wiltshire *et al,* 2000; Warr *et al*, 2001). However, since the tumour stem cell hypothesis was suggested there has been a flurry of research into the genetic differences between tumour stem cells and other tumour cell populations (Joo *et al*, 2008; Shepherd *et al,* 2008; Ieta *et al,* 2008).

Stem cell markers

Over the years, many stem cell markers have been identified. These include nestin (Lendahl *et al*, 1990), CD133 (Uchida *et al*, 2000), Lex/ssea-1 (Capela & Temple, 2002), musashi (Sakakibara *et al*, 2002) and Sox2 (D'Amour & Gage, 2003). Of these, CD133 has been found to be expressed by brain tumour stem cell populations (Singh *et al*, 2003). It is the marker of choice in brain tumours because CD133+ve cells from brain tumour biopsies from can give rise to brain tumours in mice models (Singh *et al*, 2004b)

CD133, also known as prominin-1, is a pentaspan membrane protein expressed on stem cells (Uchida *et al,* 2000) and tumour stem cells (Mizrak *et al*, 2008). The gene expressing CD133, *Prominin-1*, is located on chromosome 4p15.32 but the function of the protein is unknown (Jászai *et al*, 2007). Tumour stem cells expressing CD133 exhibit radioresistance (Bao *et al*, 2006) and chemoresistance (Liu *et al*, 2006). Therefore they are of great interest in the search for new and better treatments for brain tumours.

Aims & Objectives

Hypothesis

Sub-populations of CD133+ve and CD133-ve cells in a paediatric astrocytoma will show distinct DNA copy number aberrations.

Aims & Objectives

1. Identify a sub-population of CD133+ve cells in a paediatric astrocytoma

To do this we will isolate CD133+ve and CD133-ve cell populations from the short-term cell culture of IN699. This will be completed with Dynabeads and DynaMag magnets (Invitrogen), using CD133 antibody (CD133/1 (AC133)). Immunofluorescence staining of cells will be carried out to confirm the presence of CD133.

2. Identify DNA copy number aberrations in the sub-populations of CD133+ve and CD133-ve cells in a paediatric astrocytoma

DNA will be extracted from the original short-term cell culture and sub-populations of CD133+ve and CD133-ve cells using a Flexigene DNA Kit (Qiagen). DNA CNAs of the original short-term cell culture and the sub-populations of CD133+ve and CD133-ve cells will be completed using a high-density oligonucleotide 244k array (Agilent).

Methods & Materials

Cells

The short term cell culture used in this study (IN699) was originally derived in the laboratory from a GBM (WHO grade IV) biopsy sample from a 15 year old male. The sample was collected from Great Ormond Street with consent from the patient. Further details in regards to the patient's condition are unknown as he was lost to follow-up. The short-term cell culture investigated here was passage (P) 21.

The immortalised retinoblastoma Weri cell line (Weri-Rb-1) cells and Normal Human Astrocytes (NHA) were commercially purchased from LGC Promochem and Cambrex, respectively. These cells were cultured and used as positive and negative controls in fluorescence microscopy.

Cell Culture

Thawing Frozen Cell Stocks

A frozen vial containing the desired cells was removed from liquid nitrogen and placed into lukewarm water. Once thawed the cell suspension was added to a sterile universal tube containing 10 millilitres (ml) of Ham's nutrient media and 10% foetal calf serum (FCS). This was then centrifuged at 1,000 revolutions per minute (rpm) for 5 minutes (min) in order to pellet cells. Once centrifuged, the supernatant was aspirated with a sterile glass pipette and the cells re-suspended in 10ml of Ham's/FCS media. This solution was then transferred to a $25cm^3$ culture flask containing 10ml Ham's/FCS media and incubated at 37°C until 80% confluent.

Feeding Cells

Cells were fed to remove waste products and replace nutrients. The accumulation of waste products results in a media colour change from orange to yellow. Cells were fed on a weekly basis or sooner if the Ham's/FCS media turned yellow. The media was aspirated with a sterile glass pipette and replaced with the same amount of fresh Ham's/FCS media; 10ml, 13ml, or 30ml for small ($25cm^3$), medium ($80cm^3$) or large ($175cm^3$) flasks respectively.

Passaging Cells

Cells were passaged for sub-culturing or freezing down once they had reached approximately 80% confluence. When sub-culturing, cells were harvested (refer to cell harvesting) and re-suspended in 3-9ml of Ham's/FCS media. This was added to either: three small, one medium or one large culture flask, depending on the size of the original cell culture flask.

Harvesting Cells

To harvest the cells the Ham's/FCS media was aspirated from the culture flask using a sterile glass pipette and the cells were washed with 10ml of Hank's Balanced Salts Buffer (HBSS). This procedure was then repeated. Incubation at 37°C for 5mins with 3ml of trypsin detached the cells from the flask, followed by the addition of 7ml of Ham's/FCS media to inhibit the action of the trypsin. The resulting cell suspension was transferred into a sterile universal which was centrifuged at 1,000rpm for 5min. The supernatant was aspirated with a sterile glass pipette and the cell pellet ready for further application.

Freezing down

Cells were harvested and re-suspended in 10ml of Ham's/FCS media. The cell number was counted (refer to cell counting) and the cell suspension centrifuged at 1,000rpm for 5 min. The supernatant was aspirated and the cell pellet re-suspended in FCS and 10% Dimethyl Sulfoxide (DMSO) at one million per ml. This was then divided into 1ml labelled cryovials which were stored at -70°C for approximately 3 days prior to being placed in a liquid nitrogen store.

Cell Counting

Cell counting was completed prior to freezing down of cells using a Coulter Counter following the manufacturer's instructions (Beckman Coulter). To 19.6ml of isoton (0.15M sodium chloride), 0.4ml of a 10ml cell suspension was added and the total number of cells counted by the cell counter.

Mycoplasma Detection

Mycoplasma are small prokaryotes which can infect cell cultures without causing cell death. Cultures infected with mycoplasma exhibit reduced cell proliferation and changes in gene expression compared to uncontaminated cultures (Miller *et al*, 2003). Therefore it is important to ensure all cultures for study are mycoplasma free. Detection of mycoplasma was performed using a MycoAlert kit following the manufacturer's instructions (Cambrex). Prior to testing, the MycoAlert Reagent and Substrate were prepared. This involved adding 600 microlitres (µl) of MycoAlert Assay Buffer to both lyophilised MycoAlert Reagent and Substrate. These were left at room temperature for a minimum of 15min. Testing was performed using a 96 well microplate and Mediators PhL luminometer (ImmTech Inc.). Briefly, 2ml of

supernatant was removed from the culture flask, centrifuged at 1,500rpm for 5min to remove any debris and 100µl of the cleared supernatant added to a 96 well microplate and a measure of luminescence taken as a control. To this was added 100µl of the MycoAlert Reagent and a luminescence reading taken after 5min (reading A). Finally, 100µl of MycoAlert Substrate was added and a reading taken after 10min (reading B). A ratio of reading B to reading A below 1 indicated a negative test i.e. no contamination by mycoplasma, whereas a ratio greater than 1 showed a positive test.

Immunofluorescence Staining

Samples for immunofluorescence staining were the original sample of IN699 and sub-populations of CD133+ve and CD133-ve cells. In addition, positive and negative controls in the form of Weri and NHA cells were used to ensure that staining occurred correctly. Cells were cytospun at 40,000 cells per slide. A circle was etched around the cells and the samples fixed by adding 200µl of 2% paraformaldehyde at room temperature for 15mins. The paraformaldehyde was aspirated, 200µl of 0.1M glycine added and the sample incubated at 4°C for 30mins. The cells were then blocked with 200µl of dilution buffer at 4°C for 60mins. The dilution buffer was aspirated and 200µl of primary antibody in dilution buffer was added to each slide. The slides were then incubated in a humid slide chamber in the dark at 4°C overnight. The slides were washed in phosphate buffer solution (PBS) three times for 5min. 4,6-diamidino-2-phenylindole (DAPI) nuclei counter stain was added to each slide and the cells mounted by adding a cover-slip. Slides were left at room temperature for 15mins and the cover-slip edges sealed. Slides were stored in the dark at 4°C until ready for viewing. Slides were viewed with an Olympus BX41

Epifluorescence microscope equipped with a Hamamatsu ORCA-ER digital camera and images captured with Smart Capture software (Digital Scientific, Cambridge UK).

Separating Cells

CD133+ve and CD133-ve cells were isolated using CELLection Pan Mouse IgG Kit following the manufacturer's instructions (Invitrogen). The Dynabeads were re-suspended and 50µl placed into a tube. The Dynabeads were washed using 1ml of Buffer 1 and placed into a magnet for 1min. The supernatant was then removed and the Dynabeads re-suspended in 50µl of Buffer 1. The Dynabeads were coated by mixing with 20µl of primary antibody (mouse IgG) and incubating at 4°C for 30mins with gentle tilting and rotation. The dynabeads were then placed into a magnet for 1min and the supernatant discarded. The Dynabeads were washed twice using 2ml of Buffer 1 and re-suspended in 50µl of Buffer 1.

A large culture flask was harvested (refer to cell harvesting) of the short-term cell culture IN699, p21, and approximately 2 million cells re-suspended in 1ml of Buffer 2. To this solution, 50µl of Dynabeads was added, followed by an incubation at 4°C for 20mins with gentle tilting and rotation. The tube was then placed into a magnet for 3mins and the supernatant containing the CD133-ve cells collected. The bead-bound cells were washed 3 times using 1ml of Buffer 1. The sample was re-suspended in 400µl of Buffer 3 pre-heated to 37°C and 8µl of Releasing Buffer (DNase I). (For immune-staining, 20µl of primary antibody was mixed with 180µl of Buffer 1 and incubated at 4°C for 10mins, prior to adding the releasing buffer). This was incubated at room temperature for 15mins with gentle tilting and rotation before vigorous pippetting to release cells from the dynabeads. The suspension was placed

into a magnet for 2mins and the CD133+ve cells collected and placed into a clean tube. The dynabeads were re-suspended in 400µl of Buffer 3, pipette vigorously, placed into a magnet for 2mins, cells collected and added to the previously collected cells. To ensure maximum dynabeads were removed from the cell sample, the cell suspension was placed into a magnet for 1min, the supernatant placed into a clean tube and the step repeated. The final supernatant was collected and placed into a tube which had been pre-coated with Buffer 3.

DNA extraction

DNA extraction and purification from cells in culture was performed using a FlexiGene DNA Kit and carried out following the manufacturer's instructions (Qiagen). Prior to completing the extraction, 0.3ml of lyophilised QIAGEN Protease was re-suspended in 50ml of hydration buffer (Buffer FG3). Cells were harvested from a large culture flask (refer to cell harvesting) and re-suspended in 10ml of Ham's/FCS media. A cell count was performed and two million cells were re-suspended in 300µl of Buffer FG1. To 300µl of denaturation buffer (Buffer FG2), 3µl reconstituted QIAGEN Protease was added and this solution mixed with the FG1 buffer and cell mix by inversion and vortexing for 5 seconds (secs). The suspension was incubated in a water bath at 65°C for 10min before adding 600µl of 100% isopropanol. This solution was then mixed by inversion until the DNA precipitate became visible and centrifuged for 5min at 13,000rpm to collect the DNA. The supernatant was removed by tube inverting, the remaining DNA pellet washed using 600µl of 70% ethanol and centrifuged for 3min at 13,000rpm. The ethanol was removed and the DNA pellet left to air-dry for 10mins. Once dry, 30µl of deionised water was added, the tube vortexed for 5secs at a low speed and incubated at 65°C

for 30min in order to dissolve the DNA. Once dissolved the quality and quantity of the DNA was assessed using a nanodrop (refer to DNA quality & quantity assessment).

DNA Quality & Quantity Assessment

DNA quality and quantity was assessed using a Nanodrop ND-1000 spectrophotometer. Briefly, the sampling arm was opened and 1µl of sample placed onto the measuring platform. The sampling arm was carefully closed and a measurement taken. Quality measurement results were displayed as 230/280 and 260/280 nanometre (nm) ratios. All measurements were taken in duplicates and a mean calculated.

Microarray

Labelling DNA

Following DNA extraction, DNA was labelled for use with a high-density oligonucleotide 244k array. A sample of 2µg of DNA was re-suspended in 16µl nuclease free water. To this was added 2µl of fluorescent dye, ULS-Cy5, and 2µl of 10 x labelling solution. A control sample was also prepared using normal male DNA labelled with fluorescent dye ULS-Cy3. These were transferred to a PCR machine and incubated at 85°C for 30mins. The samples were then incubated on ice for 3mins and spun at 2,000rpm for 1min to collect the sample at the bottom of the tube.

Laballed DNA was washed using KREApure columns. The columns were re-suspended by vortexing, the lids were loosened, the bottom closure snapped off and the columns placed into 2ml collection tubes prior to spinning for 2mins at

13,000rpm. The flow-through was discarded, 300µl of nuclease free water added and the column spun for 1.5mins at 13,000rpm. The column was placed into a clean 1.5ml collection tube. The ULS-labelled DNA was added onto the column and spun for 2mins at 13,000rpm. The flow-through was collected and 1.2µl used to determine the degree of labelling using the Nanodrop ND-1000 Spectrophotometer (refer to DNA quality and quantity assessment).

Preparing labelled DNA for Hybridisation

Labelled DNA was hybridised to normal male DNA, whilst the labelled control DNA was hybridised to normal female DNA. The labelled DNA mixture was reduced to 28µl using a Speed-vac DNA110 (Savant). To the labelled DNA was added 50µl of Cot-1 DNA, 52µl of Agilent 10 x Blocking Agent and 260µl of Agilent 2 x Hybridisation Buffer. This was mixed by pipetting and spun briefly to collect contents of the tube. The sample was then incubated at 95°C for 3mins, followed by incubation at 37°C for 30mins. Briefly, 130µl of Agilent-CGH Block was added and the sample spun for 1min at 13,000rpm.

Microarray Hybridisation

A clean gasket slide was placed into the Agilent SureHyb chamber base with gasket label facing upwards. Onto the slide was placed 490µl of hybridisation sample mixture and microarray was placed "active-side" down onto the SureHyb gasket slide. The SureHyb chamber cover was placed onto the slides and the clamp tightened onto the chamber. The assembled chamber was vertically rotated in order to wet the slides and to remove stationary air bubbles. The slide chambers were

placed into a rotator rack (rotating at 20rpm) in a hybridisation oven at 65°C for 40 hours.

Microarray Washing

Chamber covers were removed and sandwiched slides taken from chamber base and submerged in Oligo aCGH wash buffer 1 at room temperature. Slides were prised apart gently whilst submerged and the microarray slide placed into a slide rack, in a dish filled with wash buffer 1. A magnetic stirrer was added to the dish and this was placed onto a magnetic stir plate. This was stirred at room temperature for 5mins. The slide rack was then transferred to a dish filled with Oligo aCGH wash buffer 2 and stirred at 37°C for 1min. The slide rack was transferred to a dish containing acetonitrile and stirred at room temperature for 1min. The slide rack was then transferred to a dish containing the stabilisation and drying solution and stirred at room temperature for 30secs. The slide rack was slowly removed from the dish and slides stored in a slide box ready for scanning.

Microarray Analysis

Slides were scanned using an InnoScan 700 microarray scanner (Stretton Scientific) and Mapix software. Analysis of results was performed using Imagene and Nexus software.

Results

Mycoplasma testing

All cell cultures used in this study tested negative for mycoplasma infection.

DNA Extraction & Assessment

DNA was extracted from samples of IN699 CD133+ve and CD133-ve cells and from the original IN699 short-term cell culture. These were assessed using a Nanodrop. Results from this can be seen in Table 1.

Sample	ng/µl	260/280	260/230
IN699 (a)	345.15	1.95	1.78
IN699 (b)	334.58	1.90	1.69
mean	**339.87**	**1.93**	**1.74**
CD133+ve (a)	320.20	1.92	1.84
CD133+ve (b)	317.4	1.91	1.67
mean	**318.80**	**1.92**	**1.76**
CD133-ve (a)	373.3	2.04	1.85
CD133-ve (b)	379.8	2.00	1.85
mean	**376.55**	**2.02**	**1.85**

Table 1. Table showing quantity and quality of DNA from IN699, CD133+ve and CD133-ve cells.

CD133 Mixed Cell Population

DNA from the original IN699 short-term cell culture was analysed for CNAs using a high-density oligonucleotide 244k array. DNA CNAs can clearly be seen in the short term cell culture of IN699 compared to the normal control (Figure 2a-b). CNAs of IN699 compared to the normal control include gains at 1p, 7q, 9q, 17p and losses at 3p, 6p, 9p, 13q, 15q and 18q.

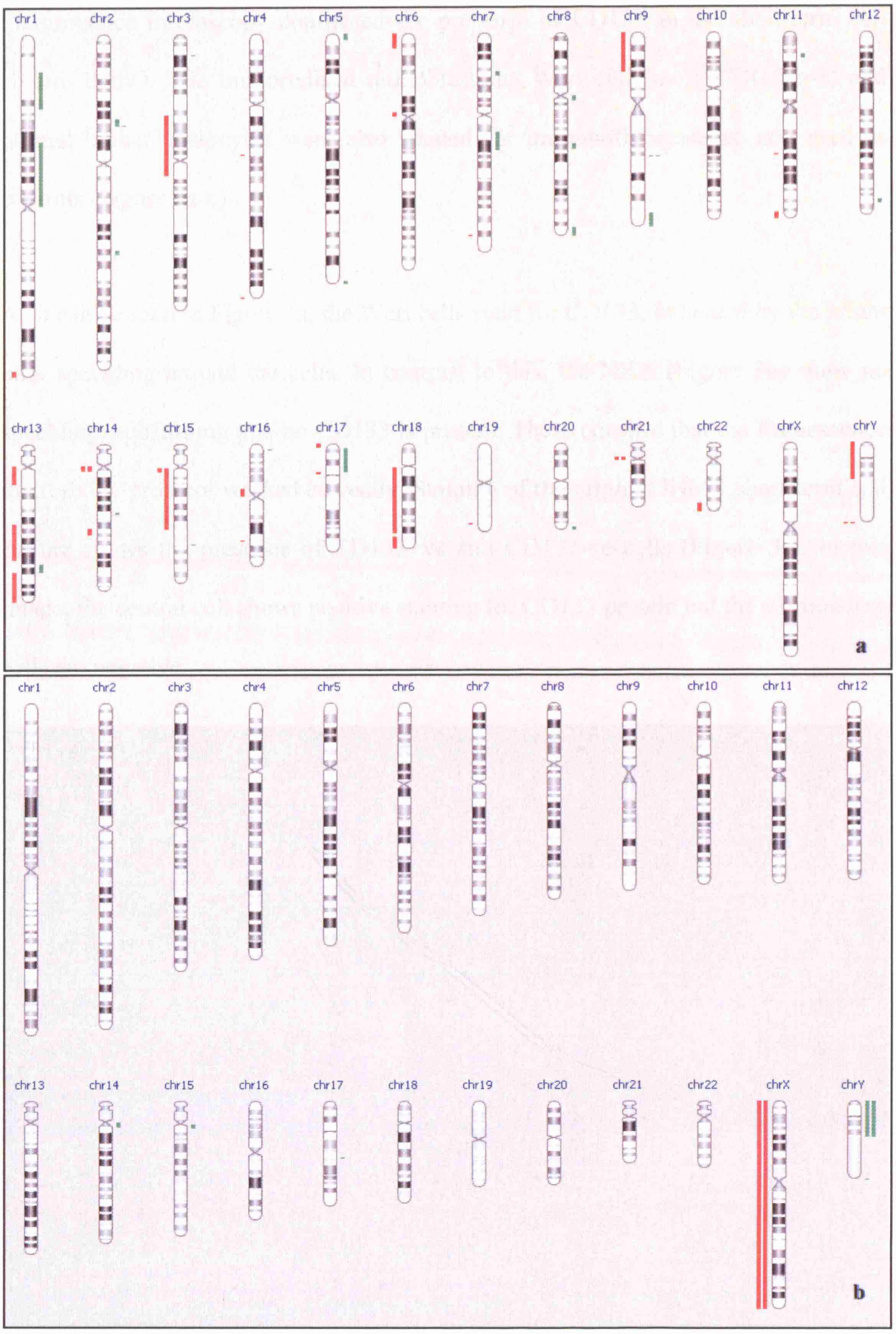


Figure 2a DNA CNAs seen in the short-term cell culture of IN699 **2b**. DNA CNAs seen in the normal control group. In all cases green lines to the right of chromosomes indicates gain and red lines to the left indicate loss

Fluorescence microscopy confirmed the presence of CD133 in the short-term cell culture IN699. The immortalised retinoblastoma Weri cell line (WERI-Rb-1) and normal human astrocytes were also stained for immunofluorescence and used as controls (Figure 3a-c).

As it can be seen in Figure 3a, the Weri cells stain for CD133, indicated by the bright pink speckling around the cells. In contrast to this, the NHA (Figure 3b) show no speckling, confirming that no CD133 is present. These confirm that the fluorescence microscopy protocol worked correctly. Staining of the original IN699 short-term cell culture shows the presence of CD133+ve and CD133-ve cells (Figure 3c). In this image, the central cell shows positive staining for CD133 protein but the surrounding cells are negative.

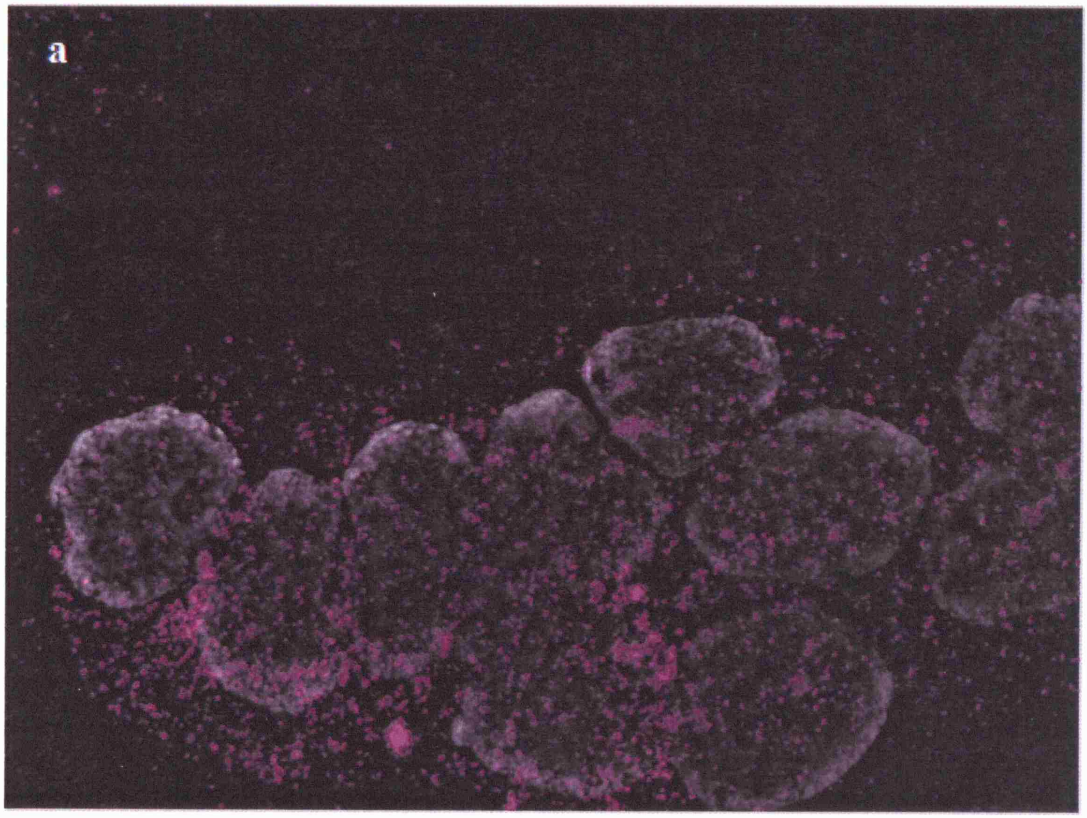
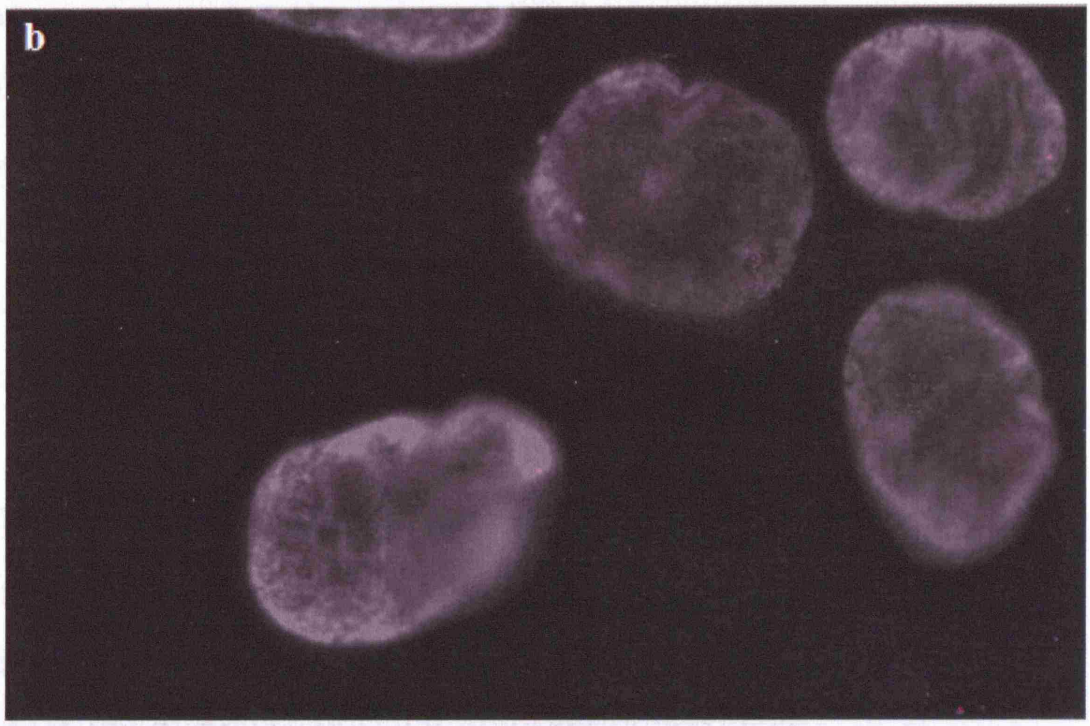


Figure 3a Positive control: immunofluorescent staining of the immortalized retinoblastoma Weri cell line (WERI-Rb-1). Bright pink speckling indicates the presence of CD133 protein.



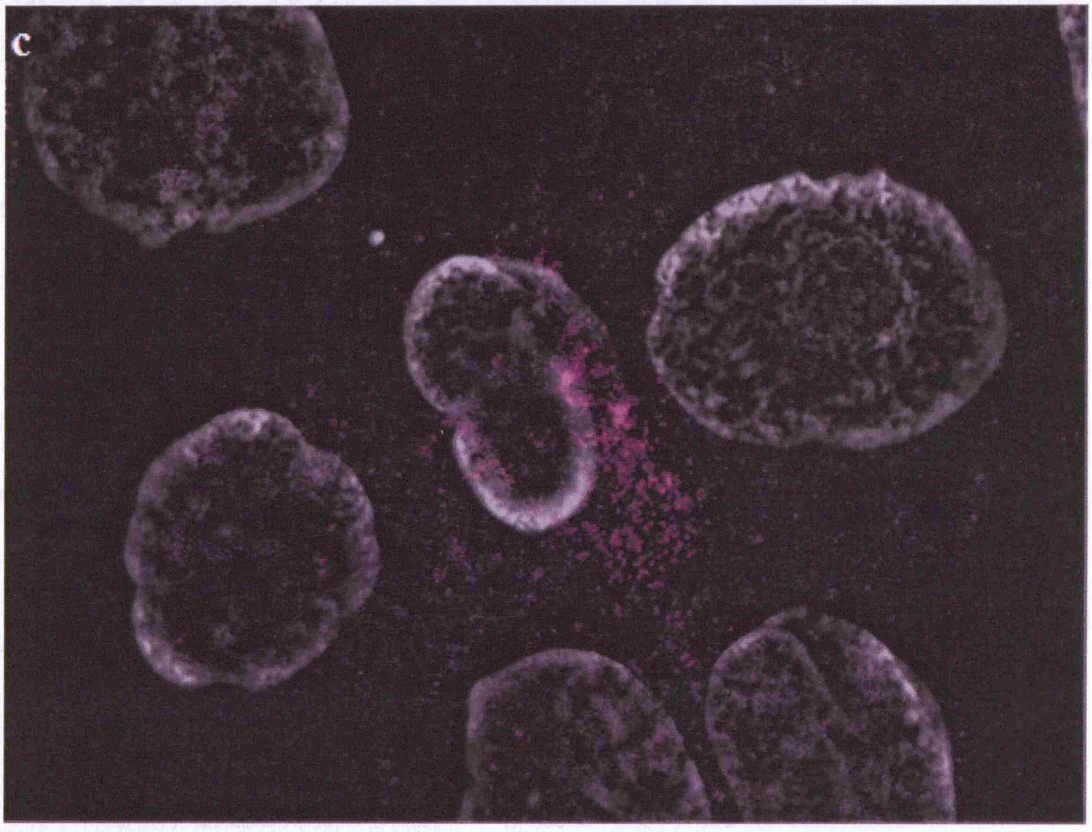


Figure 3b Negative control: immunofluorescent staining of normal human astrocytes with no speckling. **3c** Immunofluorescence staining of the original IN699 short-term cell culture showing a combination of CD133+ve and CD133-ve cells

Sub-populations of CD133+ve & CD133-ve Cells

Sub-populations of CD133+ve and CD133-ve cells were successfully isolated from the short-term cell culture of IN699 with the use of dynabeads. The positive staining of the CD133+ve cells, indicated by pink speckling in Figure 4a, confirms the expression of CD133 in this cell population. The negative staining of the CD133-ve cells confirms that CD133 is not expressed by these cells (Figure 4b).

DNA from sub-populations of CD133+ve and CD133-ve cells was analysed using a high-density oligonucleotide 244k array. DNA CNAs can be seen in both populations of cells. However, CD133+ve cells (Figure 5a) show large areas of gain at 1p, 5p, 7p, 7q, 9q, 13q, 17p and Xq. In addition there are large areas of loss at 3p, 4p, 4q, 5q, 6p, 9p, 11p, 13q, 15q, 18p and 18q. In comparison, the CD133-ve cells (Figure 5b) show small multiple regions of gain at 1p, 2p, 2q, 3q, 4q, 5p, 6q, 7p, 7q, 8p, 8q, 10p, 10q, 12q, 13p, 16p, 20q, 21q and gain of one copy of chromosome X. Small regions of loses can be seen at 2p, 9p, 9q, 16p, 18p and 21p.

Chromosomal gains and losses are seen in CD133+ve and CD133-ve cells. However, there are distinct DNA CNAs in these two sub-populations.

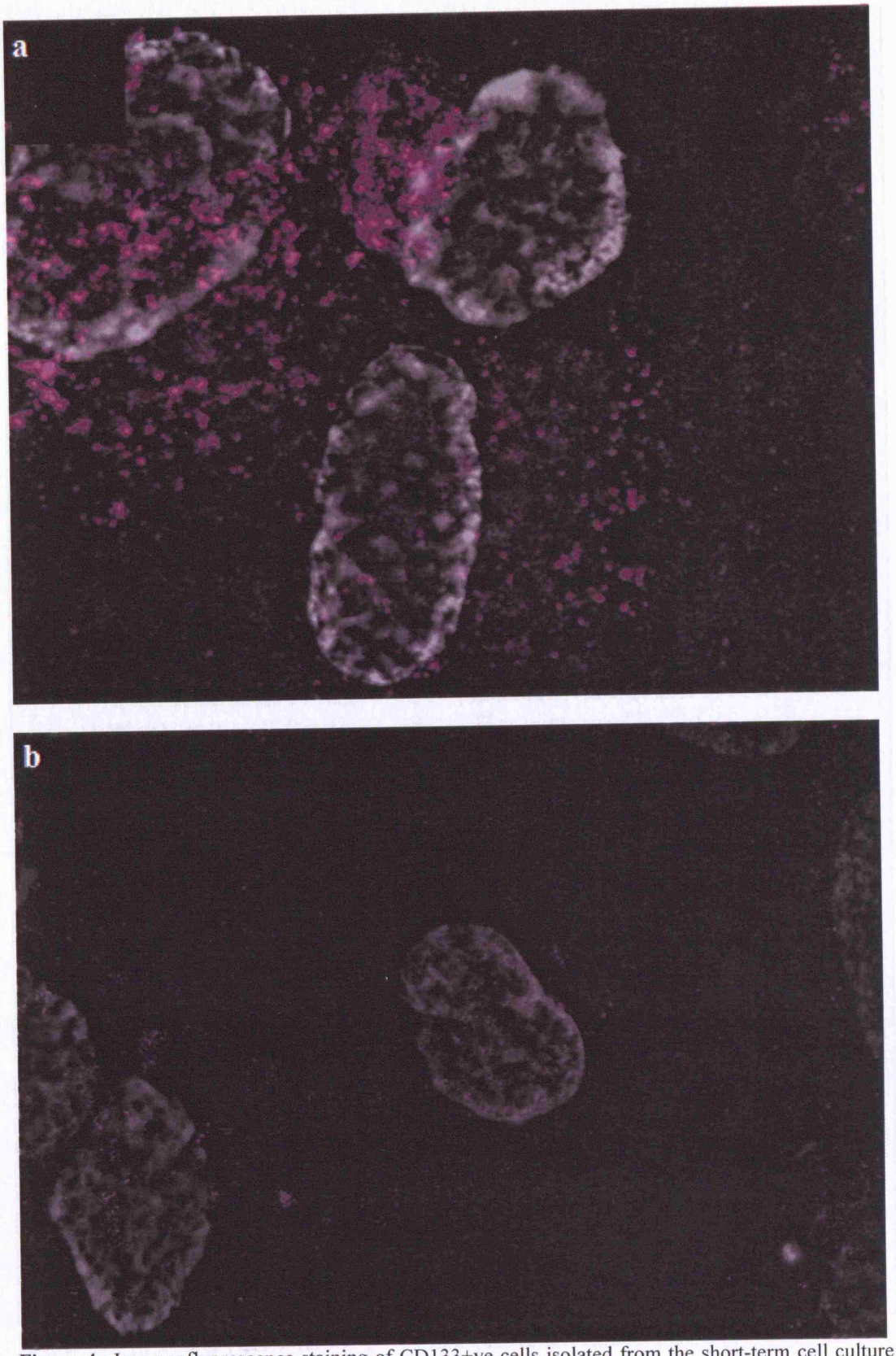


Figure 4a Immunofluorescence staining of CD133+ve cells isolated from the short-term cell culture IN699. **4b** Immunofluorescence staining of CD133-ve cells isolated from the short-term cell culture IN699.

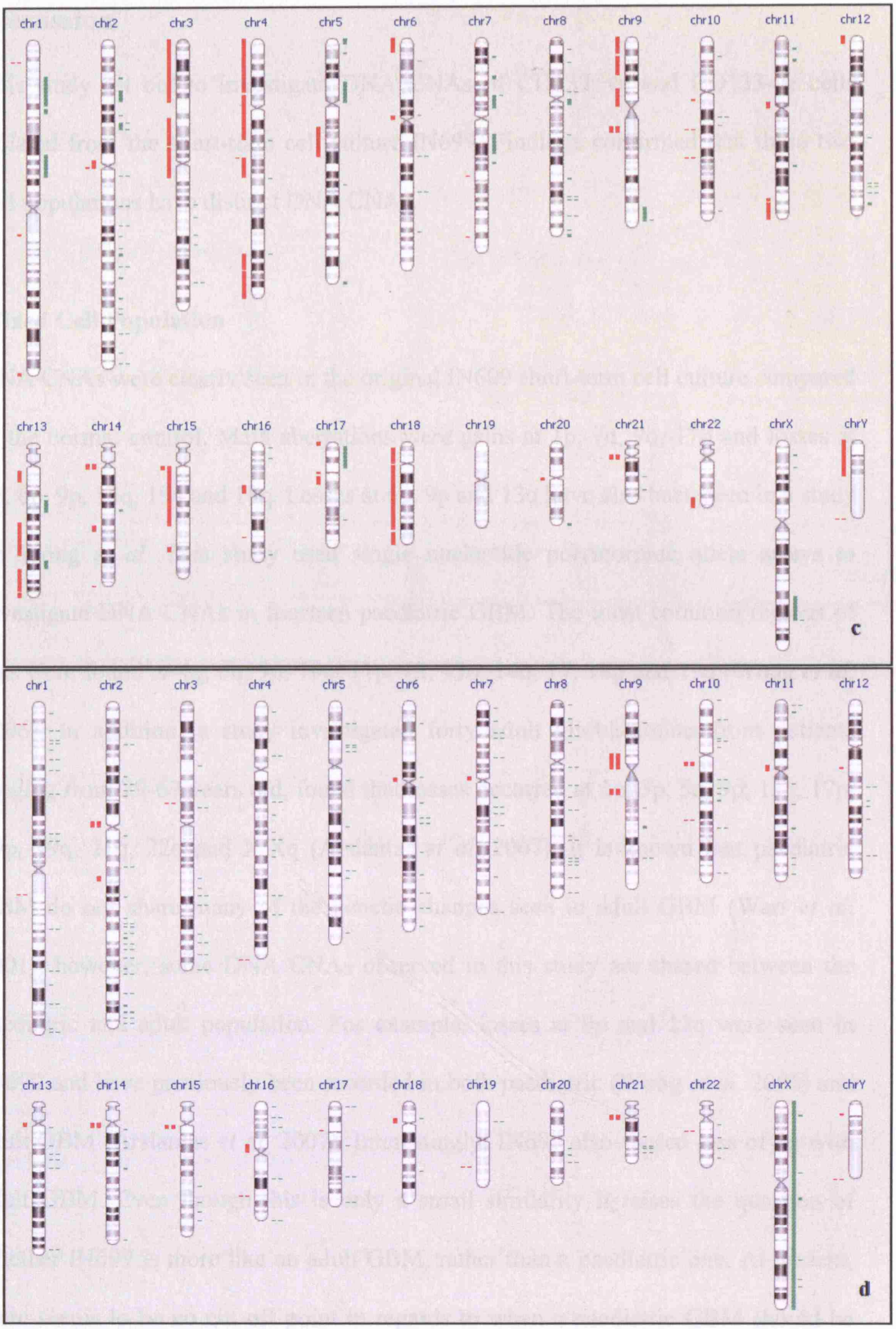


Figure 5a DNA CNAs seen in CD133+ve cells. **5b** DNA CNAs seen in CD133-ve cells. Differences in DNA CNAs can be seen between these two cell populations.

Discussion

This study set out to investigate DNA CNAs of CD133+ve and CD133-ve cells isolated from the short-term cell culture IN699. Findings confirmed that these two cell populations have distinct DNA CNAs.

Mixed Cell Population

DNA CNAs were clearly seen in the original IN699 short-term cell culture compared to the normal control. Main aberrations were gains at 1p, 7q, 9q, 17p and losses at 3p, 6p, 9p, 13q, 15q and 18q. Losses at 6p, 9p and 13q have also been seen in a study by Wong *et al.* This study used single nucleotide polymorphic allele arrays to investigate DNA CNAs in fourteen paediatric GBM. The most common regions of loss were found at 4q, 6q, 9p, 10q, 11p, 12, 13q, 14q, 17, 18p and 19q (Wong *et al*, 2006). In addition, a study investigated forty adult glioblastomas from patients ranging from 29-69 years old, found that losses occurred at 1p, 3p, 5q, 9p, 13q, 17p, 19p, 19q, 21q, 22q and X/Xq (Arslantas *et al*, 2007). It is known that paediatric GBM do not share many of the genetic changes seen in adult GBM (Warr *et al*, 2001), however, some DNA CNAs observed in this study are shared between the paediatric and adult population. For example, losses at 9p and 13q were seen in IN699 and have previously been recorded in both paediatric (Wong *et al,* 2006) and adult GBM (Arslantas *et al,* 2007). Interestingly, IN699 also shared loss of 3p with adult GBM. Even though this is only a small similarity it raises the question of whether IN699 is more like an adult GBM, rather than a paediatric one. At present, there seems to be no cut off point in regards to when a paediatric GBM should be classed as an adult GBM. Studies of paediatric GBM vary widely in the age ranges of participants, with a study by Liang *et al* including patients as old as 19 years of

age (Liang *et al*, 2008). Perhaps GBM should be classed as adult or paediatric depending on its genetic make-up. Research suggests that there may be less consistency in chromosomal changes in paediatric GBM compared to adult GBM (Bigner *et al*, 1997), so in theory it may be possible to classify an adult GBM by common genetic aberrations.

CD133+ve & CD133-ve cells

Different DNA CNAs were observed in the CD133+ve and CD133-ve cells of the short-term cell culture IN699. The most distinctive differences of CD133+ve cells compared to CD133-ve were, gain at 9q, 13q, 17p and losses at 3p, 4, 5q, 6p, 11p, 13q, 15q and 18q. Interestingly, a number of tumour associated genes are located in some of these regions. One of these is *CDKN2A*, located on chromosome 17. This gene is located in areas of CNAs within the CD133+ve cells but not the CD133-ve. *CDKN2A* is a tumour suppressor gene which is commonly deleted in adult GBM (Kraus *et al,* 2002) but less often deleted in paediatric GBM (Raffel *et al*, 1999).

Most of the CNAs of the CD133+ve cells in this study have been reported in GBM as a whole. This suggests that CD133+ve cells are mainly responsible for the chromosomal changes common to GBM. This has large implications for future treatment options. There are many genes of possible interest in the areas with CNAs in CD133+ve cells. Gene expression profiling could be carried out in order to find genes which are over or under-expressed. In addition, important pathways in the development and progression of brain tumours can be explored. Novel treatments can include disrupting these pathways, or developing drugs which specifically target the tumour stem cells expressing CD133. Potential treatments may include tailor-made regimes for each individual depending on the genetics of their particular brain

tumour. It must be noted that CD133-ve cells have also been found to have properties common to normal adult stem cells (Beier *et al*, 2007) but they do not show similar DNA CNAs seen in CD133+ve cells. The constant development of the technologies used in this study will be of great aid in investigating this further.

This study was limited, in that it only investigated a single brain tumour. Repeat studies should include many more samples to gain a better representation of distinct CNAs of CD133+ve and CD133-ve cells which are common to paediatric GBM. In addition, the short-term cell culture used in this study was at P21. There is some evidence to suggest that genetic changes can occur in some cell cultures with high passage number (O'Driscoll *et al*, 2006). Ideally, passage levels should be kept as low as possible in future studies in order to avoid the risk of "artificial" genetic changes.

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