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Chapter 5

Diagnostic of CNS neoplasia – AFP and IGF-I targets

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Introduction

It was demonstrated that alpha-fetoprotein, AFP, an oncoprotein is present in different neoplastic or cancer tissues [1]. In 1963, Abelev and his coworkers discovered the reappearence of this fetal globulin in the sera of mice bearing primary hepatomas [1]. AFP is also present in normal developing tissues [2]. It is now well established that in early post-implantation embryos of mammals, the ability to synthetize AFP is restricted to the visceral endoderm cells around the embryonic region of the egg cylinder

[3]. Later in development, AFP is predominantly produced by the yolk sac and the fetal liver [4]. In addition, a number of fetal structures that do not synthetize AFP have, a high affinity for the protein though. Thus, Benno and Williams [5] and Trojan and Uriel [6]. have drawn attention to the presence of AFP in the developing rat brain, and they have demonstrated the presence of the protein in several embryonic and fetal tissues in the developing rat, mouse, and monkey as well as in the chicken embryo [2]. The presence of AFP seems to be related **to** the stage of cell and tissue differentiation. AFP is absent from either undifferentiated or fully differentiated cells [2].

We have compared the localization of AFP was compared with that of another oncoprotein — serum albumin, SA. The distribution of SA and AFP and their mRNAs was investigated in primitive neuroectoblastic structures of rat and mouse embryos, and of the teratocarcinomas presenting comparative neoplastic structures. SA-mRNA gave a strong signal in differentiating structures as well as in undifferentiated cell clusters. AFP-mRNA was observed only in differentiating structures [7]. In teratocarcinoma-bearing mice injected intraperitoneally with J-125 radiolabeled SA and AFP, significant accumulations of both SA and AFP were demonstrated in the tumors, SA being about 3-fold higher than that of AFP after normalization to quantity of uptake in liver. In the case of comparatively studied neuroblastoma presenting only neuroblastic components (different from teratocarcinoma containing both neuroectoblastic and neuroblastic elements), the accumulation of radiolabelled SA and AFP showed relationship 1:1. External *in vivo* photoscanning confirmed this relationship of accumulated radiolabelled proteins in both studied tumors; the last observations were useful for differential diagnosis of tumors [7]. In the paragraph ‘’AFP target’’ we have described in details this technique — injection of radiolabeled AFP, as the tool for tumor diagnosis, using a model of mouse neuroblastoma .

In 1992, Trojan and his co-workers have demonstrated that another oncode-velopmental antigen, an insulin like-growth factor, IGF-1 [8-12], is present in glioma cells but absent in neuroblastoma cells [13]. Using teratocarcinoma model, Trojan and his co-workers have shown that neoplastic hepatocytes express IGF-1 and IGF-II, and neuroblastic cells express IGF-II [14]. These observations permitted to study separately, using IGF-I and IGF-II as the oncoprotein markers, different tumors, especially glial and neural tumors [8-11,15-19]. IGF-I and -II are actually recognized as the most important growth factor related to the differentiation and maturation of developing normal and neoplastic tissues, especially nervous system tissues (as it was suggested earlier [11,20].

In another paragraph – ''IGF-I target'', we have described up to date use of IGF-I as biomarker of brain tumors and other nervous system pathologies.

AFP

Generality

A number of cell surface antigens of neuroblastomas are expressed also by cells in mature brain [21]. On the other hand, foetal onconeural antigens have been described which are expressed by both neuroblastoma and foetal neural cells [22], including AFP and IGF-II [13]. Immunocytochemical work has shown the intracellular presence of alpha-foetoprotein (AFP) and also of serum albumin (SA) in most neural a transitory period of their maturation pathways. Several *in vitro* and *in vi*studies support the conclusion that the presence of AFP, and perhaps of SA, results from protein uptake as opposed to eventual *in situ* synthesis [25-27]. The ability to incorporate AFP, common to many tissues during ontogenesis may reappear in neoplastic cells [28]. We have tested two models: the C-1300 neuroblastoma cell line, and derivatived solid tumor of neuroblastoma, and comparatively PCC4 embryonal carcinoma cell line, and derivatived solid tumor of teratocarcinoma [14,29] for its potentiality to internalize AFP and SA, both *in vitro* and *in vivo* for diagnosis puropose.

Material and methods

Protein preparations

Mouse AFP was isolated from a PBS-homogenate of 17 day old mouse foetuses as previously described [30]. Rat serum albumin was from Nordic (the Netherlands) and ovalbumin from Sigma (USA). Mouse AFP, rat SA and OA were conjugated to fluorescein isothiocyanate (FITC) following the technique described previously [28]. A fluorescein-lysine conjugate (FITC-lys) was prepared by coupling 1 ml of 0.2 M L-lysine with 0.4 mg of FITC and used as a control. Nuclei were counterstained with p-phenylenediamine [31]. Proteins (20 pg) were labelled with 1 mCi of either ^{125}J or ^{131}J by the chloramine T method [32]. Specific activities ranged from 2 to 15 pCi pg^{-1} of protein.

Cell culture

The C-1300and PCC4 uncloned cell lines were routinely maintained in Eagle's medium (MEM enriched with non essential amino acids; Seromed, West Germany) containg 10% foetal calf serum (FCS) inactivated at 56°C for 30 min, penicillin and streptomycin (100 U/100 pg $m1^{-1}$). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The average population doubling time was 24 h. Cell viability was determined by trypan blue exclusion. Cultures were trypsinized before attaining confluency and replated in plastic tissue culture dishes (35 mm; Falcon) at a density of 7 x 10^4 cells per dish in 1.5 ml of growth medium and cultured for 48 h. After incubation for 48 h, the medium was removed and the plates incubated for 1 h in serum-free medium to deplete cells of endogenous bovine AFP. Then, 1 ml per plate of fresh medium containing 100 pg of fluorescein conjugates of mouse AFP (FITC-AFP), rat serum albumin (FITC-SA) or ovalbumin (FITC-OA) was added. The cells were incubated in this medium for 4 h at 37°C. They were washed 3 times with sterile PBS before being fixed in acid ethanol (ethanol 70% in PBS, acetic acid 1%) at room temperature, mounted in 30% glycerol phosphate buffer 0.05 M pH 7.6 and examined with a microscope equiped with fluorescein optics and epi-illumination. Alternatively, after acid-alcohol fixation, cultures were processed for immunocytochemical labelling. Control dishes containing no FITC-proteins or FITC-lysine were treated in parallel.

Immunocytochemistry

Anti-mouse AFP was produced in rabbits as previously described [30]. Rabbit antisera to rat SA and to ovalbumin (OA) were obtained from Nordic (the Netherlands). Vectastain ABC kit was purchased from Vector Lab., USA. No cross reactivity was found by immunodiffusion methods between anti-mouse AFP or anti-rat SA antibodies and calf serum proteins. Experimental and control dishes were treated with either rabbit anti-AFP, anti-SA or anti-OA (1/200 v/v) for 45 min at room temperature and then processed by the ABC immunoperoxidase technique [6,33,34].

Tumours

Male A/J mice, and male 129 SV mice, weighting 20 to 25g were inoculated s.c. in the scapular region with 0.5 ml of a suspension containing 10^6 viable tumour cells. All animals were examined daily for the appearance

of palpable tumours. Mice injected as well with neuroblastoma cells as with PCC4 embryonal carcinoma cells developed neuroblastoma and teratocarcinom tumors, respectively, within 15-20 days after injection. When the tumours measured 9 mm in diameter, 3 pg each of ^{125}J-SA or ^{125}J-AFP or ^{125}J 0A were injected i.p. Three to four days after injection, mice were anaesthetized with ether and perfused at 37°C through the left ventricule with 50-60 ml of 10 mM K-phosphate, 150 mM NaC1 and 1 mM EDTA buffer, pH 7.4. Perfusion was carried out with a peristaltic pump after section of the jugular vein before perfusion was started. Tumour and aliquots of other normal solid tissues (spleen, lung, brain, heart and liver) were rapidly dissected, washed in PBS, weighed and measured for radioactivity in a y-counter. Fragments of all organs were fixed for 3 days in cold ethanol/acetic acid (98/2; v/v) or Bouin's fixative, embedded in paraffin and sectioned at 3-4 pm for a haematoxylin-eosin observation or autoradiography. Blood, liver and tumour samples were homogenized with PBS (1/2; w/v) and precipitated with trichloracetic acid (TCA, 10% final concentration). Concentration values in nCi g^{-1} of tissue were estimated, and tumour to liver ratios were calculated by dividing nCi g^{-1} values in the tumour by those in the liver. For a comparison of ^{125}J-AFP, ^{125}J-SA and ^{125}J-0A distribution in mice specificity indices were obtained by dividing individual nCi g^{-1} values for AFP or SA by those obtained for OA.

In order to test the possibility of tumour localization of radiolabelled AFP by external photoscanning, mice were injected i.p withp ^{131}J-AFP (20-40 pCi, i.e. 0.5-1.0µg AFP) or with ^{131}J-0A (40 pCi; 4 pg-OA). Images were obtained 3-6 days after injection with a standard y-camera linked to a computer with data display. During photoscanning, mice were anaesthetized with sodium pentobarbital and immobilized in the prone position. Counts were calculated at different regions of interest including total body and tumor.

Results – AFP

Morphology

The majority of neuroblastoma cells in culture had round or ovoid bodies of 15-30 pm in diameter, with a single nucleus of 12-20 pm. Variation in

number, length, diameter and arborization of cells was noted. Large flattened cells with diameters up to 100pm were also observed; these cells often appeared to be multinucleated. Tumours consisted of masses of round cells separated by small quantities of intercellular substance. The rounded nuclei were centrally located, displayed a thin border of heterochromatin and often contained several prominent nucleoli. The undifferentiated tumour cell typically displayed a high nuclear: cytoplasmic ratio. Multi-nucleated cells were rare. The PCC4 cells and derivatived tumors were described earlier [29,35]. FITC-conjugates of AFP, SA or OA were added as described above. After a 4 h incubation at 37°C, specific fluorescence for AFP and SA could be observed in a large number of cells: the fluorescence appeared to be intracytoplasmic and often extended into the pseudoneuronal processes. No positive labelling could be observed for the FITC conjugated OA. Control cultures containing the FITC-lysine also appeared negative. AFP positive cells revealed with antibodies to AFP are shown in Figure l. Here too, the incorporation appeared to be intracytoplasmic and extended to cell processes. Although, as indicated above, some heterogeneity was noticed in cell morphology, AFP staining was indistinguishably positive in the whole population. Cell nuclei were systematically AFP negative (Figure 1). The same localization was observed in cells incubated with SA and revealed with anti-SA antibodies. No significant staining was revealed in cultures treated with OA. When neither AFP, SA or OA was added, control cultures appeared totally negative.

Autoradiographs

Table I shows the tissue distribution of ^{125}J-AFP after injection into tumour bearing animals. Radioactivity concentration (mean value+ s.e.) in the tumour was the highest among all solid tissues examined. Tumour-to-liver radioactivity ratios were clearly positive (mean value 3.8 + 0.6) and ratios of tumour AFP content versus brain, spleen, heart and lung confirmed the significant accumulation of the protein in the tumour. The radioactivity recovered in TCA precipitates from tissue homogenates averaged 72% for liver samples and respectively 87 and 93% for tumour and blood.

Examination of autoradiographs from tumours and other normal solid tissue sections confirmed the selective accumulation of radioiodinated AFP in the tumour. The localization was mainly cytoplasmic (Figure 1). While quantitative variations could be observed among all tumour sections observed, the quantitative tumour-to-liver staining ratio always appeared positive. Some areas, corresponding to small local necroses, were not considered.

Scintigraphic imaging of mice bearing teratocarcinoma and neuroblastomas

Four mice bearing tumors, either neuroblastoma or teratocarcinoma, were injected with ^{125}J-AFP and one with ^{131}J-0A. About fifty thousand total counts were collected over 10 to 30 min. In mice injected with ^{131}J-AFP a selective accumulation of radioactivity could be detected by external photo-scanning in areas corresponding to tumour location. By contrast, no tumour imaging was obtained in the mouse injected with ^{131}J-OA. The images of mice injected with ^{131}J-AFP are shown in Figures 2 and 3. The localization of the tumour is clearly seen.

Discussion – AFP

The results presented here show that C-1300 neuroblastoma cells [36-40], possess *in vitro,* similar to PCC4 cells, the ability to incorporate exogenous AFP, as was previously described for other normal and neoplastic cell systems [25,28]. After grafting into syngeneic hosts, the developed tumours retained the property of AFP uptake, as did the mouse mammary carcinomas previously studied [41]. The described here study shows that rat SA, like AFP, is internalized by neuroblastoma and teratocarcinoma tumour cells (embryonal carcinoma) *in vitro.* The previous observations have shown that the intracellular presence of SA in the central nervous system of developing animals follows the same pattern of cell and tissue localization as does that of AFP [6,42,43]. Like that we have used AFP and SA as radiotracers for teratocarcinoma and neuroblastoma localization. Morphologically, mouse neuroblastoma constitutes the homologue of neuroepithelial proliferation observed in differentiating mouse teratocarcinoma [14,44]. At this stage of

differentiation, the intensity of staining for both AFP and SA in mouse teratocarcinoma is similar [7,35]. No significant uptake could be demonstrated for OA, a low mol. wt protein (43,000) as compared to AFP (73,000).

The great variability observed in the individual AFP tumour-to-liver ratios (Table I) could be due, at least in part, to the degree of differentiation associated with the presence of heterogeneous cell populations in single tumours [40]. Previous work with primary cultures of dissociated foetal brain cells and organotypic cultures of sensory dorsal root ganglia demonstrated that AFP uptake is not displayed by undifferentiated cell precursors, but seems restricted to elements with phenotypic characteristics of maturing neurons [25,45]. Immunocytochemical work has shown that the intracellular presence of AFP and SA during development is also associated with a certain degree of cell and tissue differentiation [2,7,29]. Neither undifferentiated nor fully differentiated cells incorporate AFP.

As compared to monoclonal or polyclonal antibodies to tumour antigens, AFP may be used to advantage in radiotracing experiments, since this isologous protein is not expected to induce hypersensitivity reactions. On the other hand, and contrary to SA, the extremely low serum levels of AFP in adult individuals should minimize effects due to competition with endogenous protein. This makes AFP a good candidate for tumour biomarker by imaging techniques. The diagnosis and therapies of CNS tumors including neuroblastoma are always a subject of discussion [29,46-48].

IGF-I: target in progress

Alphafetoprotein, serum albumin [6], as well as Growth Hormone, growth factors especially IGF (Insulin-like Growth Factor type I and type II)[20,21] reappear in neoplastic developing tissues including brain [13,34,49-54]. Comparative studies of AFP, IGF-I, IGF-II presence in neoplastic cells [7,13,55] have demonstrated that IGF-I constitutes an essential target for genetic testing and therapy purpose. IGF-I, similarly to AFP, is involved in tissue development and differentiation, especially in the development of the nervous system [56-58] as a mediator of Growth Hormone and glucose metabolism;

acting locally with autocrine/paracrine, with a predominant role compared to other growth factors [9,19,58-62]. According to Baserga [12], IGF-I is one of the most important growth factors related to normal and neoplastic differentiation, and its overproduction is considered to be a participating factor in cancer development [61,63-65].

IGF-I reconstitutes the first step of the following signal transduction pathway: IRS/PI3K-PKC/PDK1/AKT-Bcl2/GSK3/GS [66,67]. The elements of said IGF-I related transduction pathway were also considered as targets for diagnostic and therapy purposes [58,66,68-77]. The relationship between IGF-I and IGF binding proteins are being introduced in clinical diagnostics as one of the indicators of precancerous development [78]. Also, as far as the relationship between brain tumor disease and depression is concerned, elevated IGF-I serum levels have been found to be significantly associated with depression [79].

Considering *IGF-1 gene*, an over-expression of this gene in mature tissues is a sign of neoplastic processes, especially brain tumors [20] (Figures 4,5,6). Molecular testing could be also useful in congenital malformations involving the central nervous system (CNS). Primary malformations go hand-in-hand with genetic intrinsic diseases, and the increase of intracytoplasmic IGF-I is associated with CNS malformations. IGF-I function is parallel to the commonly used marker of alpha-fetoprotein (AFP), and IGF-I becomes useful in molecular diagnostic of neonatal CNS malformations and tumors [18,48,58,80-82]. These observations enabled the testing of IGF-I as the oncoprotein and genetic marker. Diagnosis and treatment should logically be related, at first using *IGF-I gene* testing for diagnosis [83-85], and then targeting *IGF-I gene* through special therapy, such as cancer gene therapy, especially therapy of gliomas [20,86-90].

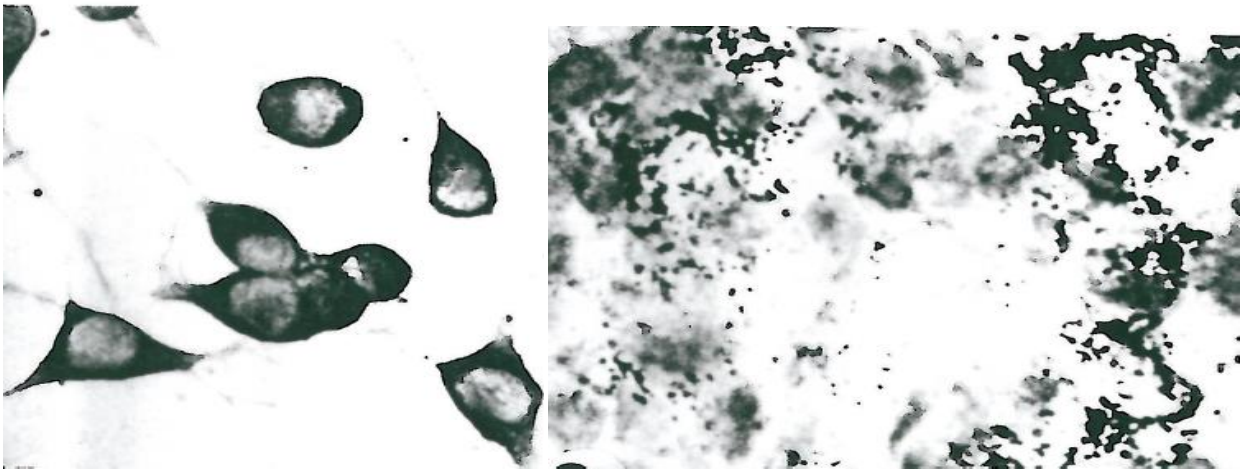

Figure 1 Neuroblastoma C-1300 cells. (left) *In vitro* experiment**.** Cells incubated at 37°C with mouse FITC-AFP (100 pg). Immunocytoperoxidase staining using anti AFP antibodies. Nuclei slightly counterstained with haematoxylin. (right) *In vivo* experiment. Autoradiographs counterstained with haematoxylin: sections of a neuroblastoma tumour developed in a mouse injected s.c. with C-1300 cells. The animal was injected with ^{125}J-AFP (20 pCi) and killed 4 days after. Sections (3-6 um thickness) of the tumour mounted on glass slides and covered with Ilford K5 photographic emulsion were examined after 3 weeks standing at +4°C. (a) Silver grains concentrated in the cytoplasm of elements arranged in neuroepithelial, vesicle-like structure constituted by hyperchromatic cells surrounding a cavity (x 400).

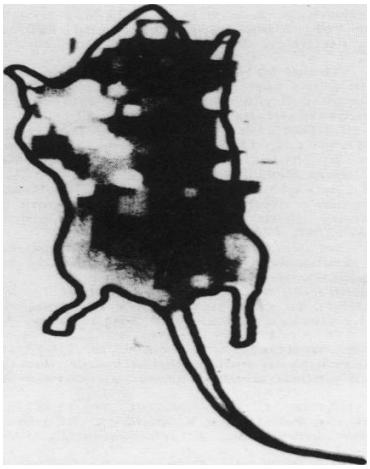

Figure 2. External photoscanning of a mouse bearing a single (large) neuroblastoma tumour in the upper left part of the dorsal region. The mouse was injected with ^{131}J-AFP (30 /Xi) i.p. 4 days before tumour imaging. The contour of the mouse has been positioned over the scan. The image was performed with an Informatck Simis 3 computer and was not corrected by data subtraction. The picture presented is a black and white copy from a negative colour film.

Table 1. Distribution of ^{125}J-AFP 3 to 4 days after injection into neuroblastoma tumour bearing animals*

	nCi AFP g^{-1} tissue							
Mouse no	*Blood*	*Tumor*	*Liver*	*Brain*	*Spleen*	*Lung*	*Heart*	*Tumor/liver ratio*
1	157	32.2	6.6	0.99	20	21	15	4.8
2	67	9.7	4.5	0.35	9.2	6.3	3.8	2.16
3	44	15.2	8.5				1.4	1.77
4	22.9	10.7	6	0.34			1.1	1.78
5	176	80	14.9		22	22	11.7	5.4
6	160	39.6	17.4	0.5	12.7	2.2	2.9	2.3
7	162	57.4	6.5		17	1.72	5.4	8.7
8	151	43.2	8.1	2.1	22	1.7	7.2	5.3
9	156	39.4	14	0.33	15.7	4.5	6.8	2.8
10	106	51.8	10.5	0.25	19	16.6	7.3	5
11	52	21.7	12		11	2.9	6.3	1.8
Mean values	114	36.4	9.7	0.69	16.5	8.7	6.2	3.8
±s.e.	±17	±3.3	±1.2	±0.25	±1.5	±2.8	±1.2	±0.6
(N)	(11)	(11)	(11)	(7)	(9)	(9)	(11)	(11)

*****Tumour-to-liver ratios were calculated by dividing nCi values in the tumour by those in the liver.

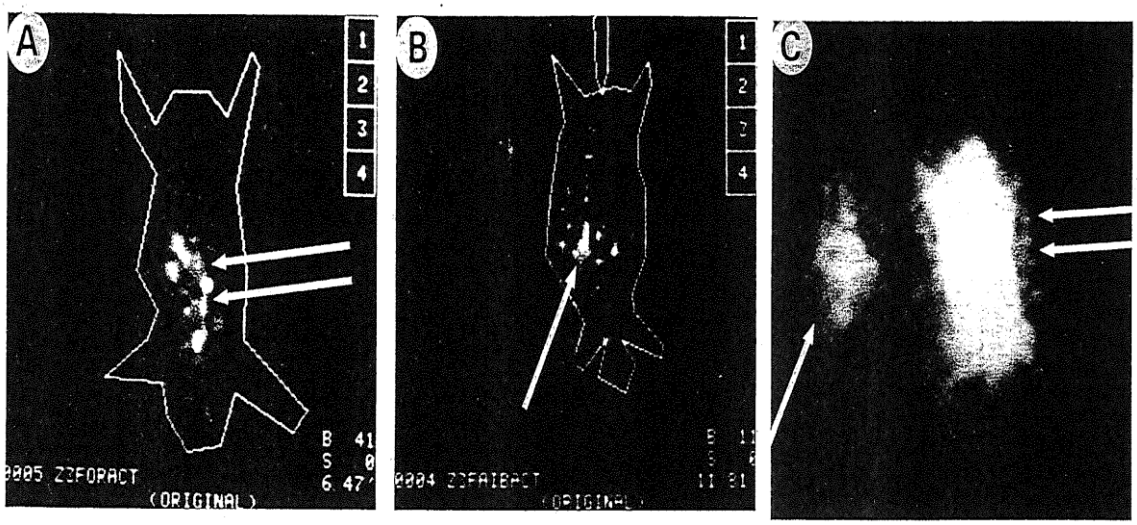

Figure 3. Visualization of differential SA and AFP uptake by scintigraphy. Mice bearing a single teratocarcinoma tumor (in the upper right part of the dorsal region) were injected with ^{131}J-SA (A) or ^{131}J-AFP (B), 3 days before tumor imaging. Images were obtained with a standard y-camera linked to a computer with data display. The contour of the mouse has been positioned over the scan. The position of tumors is indicated by arrows (double arrow SA; single arrow AFP). C: Computerized comparison of images given by tumors after injection of AFP (single arrow) and SA (double arrow) corrected for camera exposure time.

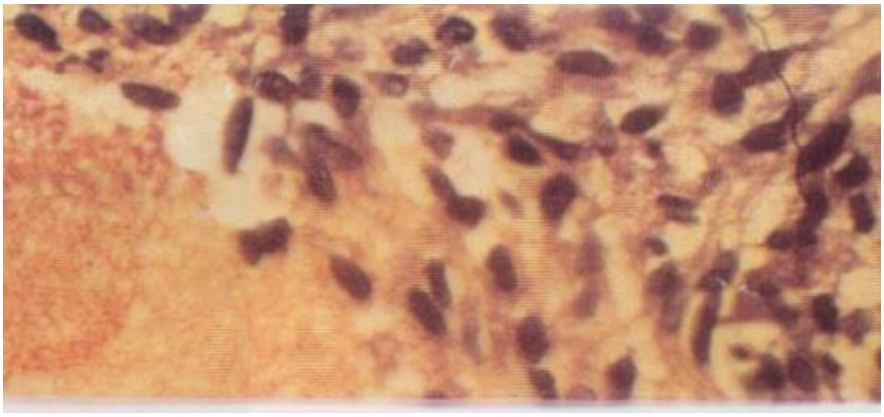

Figure 4. Histopathologic slide of solid tumor of glioma. On the left down: normal brain tissue. On the right: neoplastic proliferation of glioma tissue.

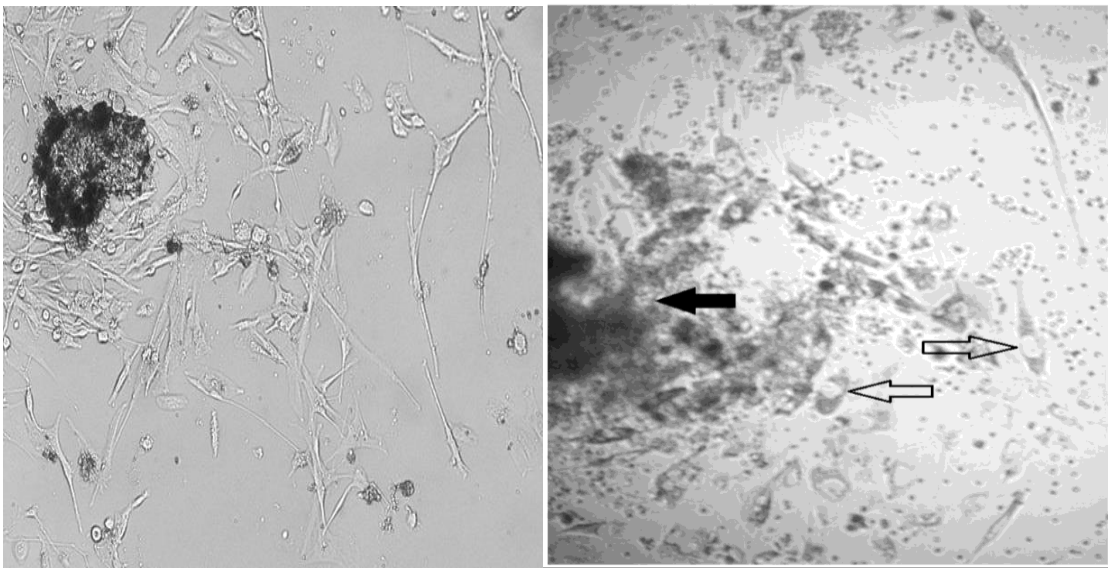

Figure 5. (left) Sixth day of culture established from human glioblastoma biopsy. (right) *In vitro* staining of IGF-1 biomarker in glioma cell culture. Note the cells (empty arrows) proliferating from compact tissue of biopsy (black arrow). The tissue and cells are stained for IGF-1 using anti IGF-1 antibodies applied in immunoperoxidase technique: note dark cytoplasm. (x200)

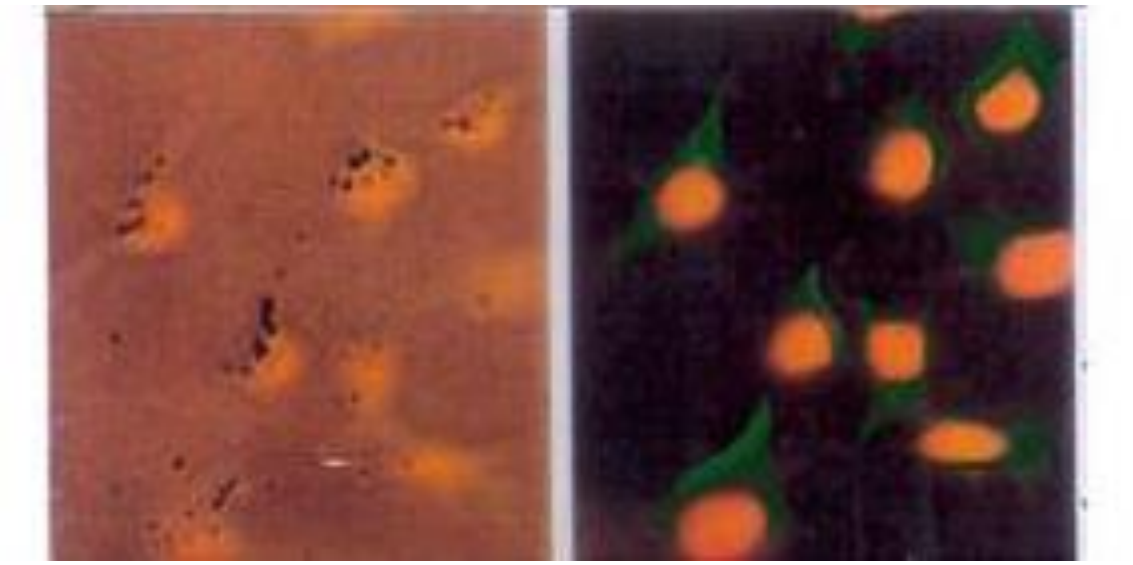

Figure 6. C6 glioma cell culture. On the left: in situ hybridisation of IGF-I mRNA using molecular probe of IGF-I antisense RNA. On the right: immunofuorescence staining of the same area using anti IGF-I – FITC antibodies. (400)

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