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H-MR Spectroscopic Imaging-guided Surgery of Brain Tumors and correlation with Neuropathology.

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Purpose: Evaluate H-MR spectroscopic imaging in detecting tumor heterogeneity and infiltration to be used for guiding biopsy or surgical resection. Determine whether elevated choline correlates with cellular density, proliferation and/or grade.

Methods and Materials:

Twenty-eight patients (M:F=16:12; mean age: 44 yrs.) with a new untreated intracranial mass were evaluated pre-operatively with H-MRSI using the multivoxel PRESS volume selection technique (TR/TE=1500/136; 2D phase-encoding). H-MRSI coordinates of surgical specimens were recorded using an intraoperative neuronavigation device. Biopsy slides were stained with HE, GFAP, vimentin and MIB1. In few patients genetic markers were investigated. The following histopathological diagnosis were made: 20 high grade gliomas, 6 low grade gliomas, 1 PNET, 1 metastatic carcinoma. Areas of choline, creatine and NAA signals were integrated and normalized to contralateral normal spectra. The Cho/NAA, Cho/Cr and Cr/NAA ratios for voxels within and around the T2-w. signal abnormality were calculated. Lesions were classified in homogeneous or heterogeneous, well-defined or infiltrating/multicenter by H-MRSI and MRI.

Results: Cho was elevated in all types of tumors with nCho values in the range between 1.5 and 5.4. In 5/28 cases we unexpectedly found moderate Cr elevation (Cho/Cr near or less than one): all 5 cases were astrocytomas (grade II and III) with intensive GFAP positive cells. Low grade gliomas had homogeneous abnormal spectra with well-defined borders; anaplastic astrocytomas showed an infiltrating appearance. Glioblastoma multiforme subdivided in two groups: with “well-defined borders”; and with infiltrating appearance. Elevated nCho best correlated with cellular density.

Conclusion: 1. H-MRSI is a sensitive tool to depict tumor heterogeneity and borders and it is more specific than conventional MRI.

2. H-MRSI-guidance may improve pre-operative planning in heterogeneous, infiltrating lesions.

3. A new H-MRS profile was identified: the elevation of Cr with Cho was seen only in astrocytomas and it may suggest a specific tumor type.

4. Choline is elevated in all tumors and best correlates with cellular density.

O-2

Expression of neural specific kinesins in neuronal and glial tumours

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Kinesins are a superfamily of molecular motors that use the energy of ATP hydrolysis to move different types of cargoes (membranes, protein complexes or filaments) along microtubules (MTs). The founding member of the kinesin I family is conventional kinesin, composed of two identical kinesin heavy chains containing the motor domain and two identical light chains. Kinesin II is a more recently described motor consisting of two distinct subunits complexed with a third nonmotor kinesin associated protein. nKHC (neuronal kinesin) and KIF3C are kinesin motors of the kinesin I and the kinesin II family, respectively. We have recently shown that both proteins are selectively expressed in the adult and the developing nervous system and that their expression levels are up-regulated during neuroblastoma differentiation, suggesting a role for these motors during maturation of neuronal cells. Comparison of the expression of these neural kinesins in neuronal and glial cells in culture indicates a different pattern of expression for the two motors: while both nKHC and KIF3C are present in SH-SY5Y human neuroblastoma cells, only KIF3C, and not nKHC, can be detected in primary cultures of rat hippocampal astrocytes. The specificity of expression of nKHC in neurons became more interesting when samples obtained from tumours of the central nervous system were analysed for the presence of nKHC and KIF3C. Our results indicate that the expression of nKHC is strikingly induced in tumours of astrocytic lineage, although it cannot be detected in glial cells of normal human cortex. In addition, the quantitative expression of this kinesin appears to be higher in more malignant tumours, although a considerable heterogeneity of expression is found among samples of tumours of the same grade. Similar results were obtained for the presence of KIF3C. For comparison, the expression of the ubiquitously present uKHC was analysed in parallel and was found to have little variability among tumours of different malignancy. To better understand the possible correlation between the levels of expression of neural specific kinesins and the malignancy of glial tumours, we used astrocytoma and glioblastoma cell lines as an in vitro model for cancer cells characterized by distinct differentiation and motility properties. We found that all three kinesins are expressed in glioblastoma cells and that nKHC, but not uKHC, can be up-regulated in conditions that are known to modify their adhesive properties, such as serum deprivation.

O-3

Genetic evidence for a precursor lesion to astrocytoma WHO grade II in a patient with a germline TP53 mutation and astrocytoma progression

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Little is known about the early events of human tumorigenesis. We used clonal analysis to determine the order of occurrence of three p53 mutations during cell transformation in a p53 germline patient with recurrent astrocytoma (II-IV). The primary tumor contained two genetically distinct cell populations: the first showed R267W in the R283H germline allele and, the second, which clonally derived from the first, acquired E258D in the remaining WT allele. This provides the first genetic evidence for a precursor lesion to WHO grade II astrocytoma, not clinically detected to date. The germline p53R283H had partial loss of function since it could transactivate the *CDKN1a*(p21,WAF1,cip1) but not the *BAX* genes, whereas p53R267W+R283H and p53E258D were incapable of transactivating either promoter. Modeling of p53 interaction with DNA suggests that R283H mutation might weaken the interaction of K120 with the *BAX* p53 responsive element (p53RE). This indicates that tumor initiation and progression can result from monoclonal evolution of cell populations losing consecutively pro-apoptotic and growth arrest functions of p53 due to sequential accumulation of mutations in single *TP53* alleles.

O-4

The role of technology in maximizing the efficacy of traditional therapy: image guided neurosurgery and conformal stereotactic radiotherapy.

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The evolution of traditional treatment of cerebral malignancies, i.e. surgery and radiation therapy, has scored a number of important goals in the last few years. The common target has been to improve the impact on tumor control by enhancing the precision with which tissue removal or tissue irradiation is accomplished. Even if gliomas are a diffuse entity, still they tend to recur locally; for this reason it is important to remove safely as much tumor as possible. In order to obtain this, the surgeon needs to be guided, by means of well known "neuronavigation" devices that register MR or CT studies in the surgical frame of reference. These devices, though, are known to lose their efficacy as soon as tissue manipulation and removal takes place. For this reason we have developed an intraoperative volumetric ultrasound device that upgrades the anatomy of the operative region during surgery, telling the surgeon where tumor is still found. The design of this proprietary device, based on the movement of a single quartz, is focused on obtaining high tissue localization, as compared to the "phased array" based devices that are commonly employed, although suitable to diagnostic, rather than surgical applications. Residual tumor or recurrences are commonly treated with radiotherapy. Here too stereotactic concepts have been borrowed, to improve the dose distribution to the target volume. Highly conformal plans can be designed to cover the contrast enhancing volume plus a margin of tissue surrounding it. New devices have been designed, to shape the X-ray beam from a Linear Accelerator. A special arc modulation procedure developed by our group, allows the design of treatment plans that can effectively cover lesions with a very complicated geometry or in proximity of radiosensitive eloquent areas.

O-5

Nestin is a neuroepithelial target gene of TTF-1, a homeodomain-containing transcription factor required for forebrain organogenesis.

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Thyroid Transcription Factor-1 (TTF-1), also known as NKX2.1 and T/EBP, is a member of the NKX family of homeodomain-containing genes related to NK Drosophila genes. TTF-1 plays a fundamental role in the tissue-specific expression of several thyroid- and lung-specific genes. TTF-1 is expressed at the onset of thyroid and lung organogenesis, and in restricted areas of the developing forebrain, namely within the diencephalon (i.e., in the hypothalamus and neurohypophysis) and the telencephalon (i.e., in the medial ganglionic eminence). TTF-1 ^{-/-} mutant mice are born dead and lack completely the thyroid gland, lung parenchima, the entire pituitary, and extensive defects were found in the ventral region of the forebrain. In addition, it has been reported that in TTF-1 knockout mice a ventral-to-dorsal transformation of the pallidum primordium into a striatal-like anlage takes place. Nestin is an intermediate filament protein strongly expressed in multipotential neuroepithelial stem cells and rapidly down-regulated during post-natal life.

We show that upon transfection with an expression vector encoding for TTF-1, fibroblasts acquire a phenotype reminiscent of neuroepithelial cells in culture and up-regulate the endogenous nestin gene. TTF-1 transactivates in HeLa and NIH3T3 cells a reporter gene driven by a CNS-specific enhancer element from the second intron of the rat nestin gene, where it recognizes a DNA-binding site (5'-TGAGGTCA-3', NestBS) whose sequence resembles a nuclear hormone/cAMP responsive element, quite different from canonical TTF-1 binding sites.

Nuclear extracts from the head of mouse embryos form a retarded complex with NestBS of the same mobility of the extracts obtained from TTF1-expressing clones, which is either abolished or supershifted in the presence of two different antibodies recognizing the TTF-1 protein.

Thus, the neuroepithelial marker nestin is a neural-specific target gene of TTF-1, suggesting that it might be the effector through which TTF-1 plays its role in the organogenesis of the forebrain.

O-6

Co-localization of murine neural progenitor cells in focal and disseminated neoplastic disease of the brain.

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Cerebral metastasis represent the most common type of brain tumors. Multiple metastasis have not been subject of modern therapeutic concepts because they represent a systemic, non-focal disease that is difficult to target. Neural stem / progenitor cells (NSC) have been used as vectors for glioma therapy to deliver or activate therapeutic genes. We were interested in the migration capacity of NSC for targeting of disseminated as well as focal tumors.

Three different murine cerebral tumor models were tested : 1) direct intra-cerebral deposition of tumor cells and concomitant intra-arterial delivery of NSC, 2) implantation of tumor cell spheroids and NSC spheroids in a modified cranial window, and 3) co-injection of metastatic tumor cells and NSC into the carotid artery. Intra-arterial delivery of NSC via the internal carotid artery showed preferential deposition of NSC both in metastatic and focal tumors. In a modified cranial window fluorescently labelled tumor cell spheroids and NSC spheroids were juxtaposed subpially. Intravital monitoring revealed directional invasion of tumor spheroids and developing tumors by NSC.

Neural stem cells target cerebral tumors irrespective of their location and method of delivery.

O-7

Ad.d24-RGD, a conditionally replicative infectivity-enhanced adenovirus, shows improved oncolytic activity compared to wild-type and E1B-mutated adenoviruses which can be further enhanced when combined with radiotherapy

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The use of conditionally replicating adenoviruses for cancer therapy is currently receiving widespread attention. Ad.d24 that carries a 24-bp deletion in E1 was shown to produce a strong anti-glioma effect. In this study we demonstrate that Ad.d24 is more effective than wild-type and E1B-mutated adenoviruses in a panel of glioma cells. Average viability of established glioma cell lines and primary glioma cell cultures was reduced to 44% and 47% of controls, respectively, 6 days after MOI 10 Ad.d24 infection.

Expression of the primary adenovirus receptor CAR has been shown to be limited in glioma. The tropism of adenovirus was expanded towards integrin-expressing cells by insertion of RGD into the fiber knob of Ad.d24 (Ad.d24-RGD). On a broad panel of glioma cells, Ad.d24-RGD was found to have greater oncolytic activity than the non-RGD expressing CRAds. Average viability of glioma cell lines and primary glioma cells was reduced to 19% and 29% of controls, respectively, 6 days after MOI 10 Ad.d24-RGD infection. The effects of Ad.d24-RGD were also assessed in organotypic glioma spheroids made from a panel of glioma samples. Ad.d24-RGD decreased the viability of these spheroids more efficiently than wild-type adenovirus, confirming cell culture data. In a subcutaneous human glioma xenograft model, 10^7 pfu Ad.d24-RGD was injected intratumorally on 5 consecutive days in pre-established tumors. By day 17 all PBS-treated mice were sacrificed due to continued tumor growth. In the Ad.d24.RGD-treated group tumor regression was noted in 10 out of 10 animals. Regressed tumors were followed over 2 months without evidence of regrowth in 9 out of 10 cases.

Clinical trials of replication-competent adenovirus have shown more promising results when combined with conventional therapeutics than as a single agent treatment. We therefore assessed the effects of Ad.d24-RGD in combination with radiotherapy on primary glioma cell cultures and glioma cell lines. Low-dose irradiation prior to Ad.d24.RGD infection decreased viability of glioma cells more effectively than Ad.d24-RGD alone. We are currently assessing the effects of combination therapy in human glioma xenografts.

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PRETARGETED ADJUVANT RADIOIMMUNOTHERAPY WITH YTTRIUM-90-BIOTIN IN GLIOMA PATIENTS

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In a previous study we applied a three-step avidin-biotin pretargeting approach to target ^{90}Y -biotin to the tumor of in patients with recurrent high grade glioma. The encouraging results obtained in this phase I-II study prompted us to apply the same approach in an adjuvant setting.

We enrolled 35 high grade glioma patients, 17 with anaplastic astrocytoma (AA) and 18 with glioblastoma (GBL), in a controlled open non-randomized study. All patients received surgery and radiotherapy and were disease-free by neuroradiological examinations. Nineteen patients (treated) received adjuvant treatment with radioimmunotherapy (RIT) using a three-step protocol in which biotin radiolabelled with 2.2 GBq/m^2 of ^{90}Y was injected as third step.

In the treated GBL patients, median disease-free interval (DFI) was 28.5 months (range 9-41); median survival was 36 months and three out eight patients are still without evidence of disease. All 10 control GBL patients died after a median survival from diagnosis of eight months.

In the treated AA patients DFI and survival cannot be calculated as eight of 11 have not progressed; however six of the eight have been followed for more than 30 months. Three-step RIT promises to have an important role as adjuvant treatment in high grade gliomas, particularly glioblastoma where it interferes with progression, prolonging time to relapse and overall survival.

Keywords: radioimmunotherapy; glioma; avidin-biotin.

Are human-brain-derived "neurospheres" composed by neural stem cells or by cells endowed with stemness property?

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In this study we tested human-brain-derived "neurospheres" for the presence of some extra-neural markers. We performed RT-PCR studies on "neurospheres" probed with primers for well known endothelial /hematopoietic stem cells/progenitor markers: Flt4, ecNOS, CD31, KDR and Flt1. Representative data from 5 DIV spheres show that these markers are clearly present in the nestin-positive neurospheres (thus confirming the maintenance of their central nervous system origin). Hematopoietic/endothelial markers expression in "neurospheres" was also tested by immunocytochemistry and cytofluorimetric analysis. A strong signal respectively for CD31, CD34, Tek and Flk1 was detected. Endothelial markers CD31 and CD34 were completely lost or concentrated in just a few cells during differentiation; on the contrary, Tek and KDR/Flk1 signals could be still distinguished clearly, even in terminally differentiated cells. After 9 DIV, all the cells have a clear neuron and glia morphology.

Our data support the idea that *non-neural* receptors are expressed on NSCs surfaces. Since angioblasts, hematopoietic stem cells and NSCs share certain antigenic determinants, these progenitor cells may derive from a common progenitor; otherwise, as we think likely, "neurospheres" may be composed by an heterogeneous progenitors population characterized by typical stem cell features (stemness property) and different antigen expression. This last hypothesis is supported by preliminary confocal microscopy data, showing that NCSs differ in size, mitochondrial activity, intracellular calcium content and distribution, reaction to stimulating substances.

"Neurosphere" appears to be an unsuitable definition to identify such an heterogeneous cell population: it doesn't seem to be absolutely "neural", at least until progenitor cells, which "neurospheres" are composed by, completely differentiate into mature phenotypes.