213 Characterization of novel recombinant human single chain cancer-specific antibodies

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Background: Our overall goal is to identify antibodies for possible use in diagnostics and therapy utilizing phage-display technology and cell specific antibody selection.
Material and Methods: A breast cancer cell line, PM-1, and the colorectal cancer (CRC) cell lines HCT-116 and HT-29 were used to screen for breast and CRC specific antibodies, respectively. Three unique recombinant antibodies were screened for binding to a panel of cancer cell lines and normal cells using immuno-magnetic bead selection (IMS) or flow cytometry. Cancer cell specific binding was investigated using immunohistochemistry (IHC) on frozen normal tissue and tumour tissue of different histology.
Results: IMS and flow cytometry analyses revealed differences in binding patterns comparing the three antibodies. CRC-1, one of the anti-CRC antibodies, bound to cancer cell lines of various origins. Endothelial cells were negative for CRC-1 binding. However, binding was observed when testing vascular smooth muscle cells and mononuclear cells (MNC) isolated from normal bone marrow. The other CRC antibody only bound to CRC cell lines and only a fraction of the cells displayed positive binding. The breast cancer cell binding antibody BC-1 bound to most of the cancer cell lines tested, and did not bind to endothelial cells or normal bone marrow MNC.
In IHC positive CRC-1 staining was observed in clinical cancer samples and in normal tissue. Surprisingly, cancer epithelia were not stained, blood vessels stained positive, and myo-epithelial cell staining was observed in breast tissue samples (cancer and normal). Positive BC-1 staining was on the other hand highly cancer specific. The BC-1 antigen is identified as ALCAM and the antibody inhibited cancer cell invasion through Matrigel and reduced tumour growth in nude mice.
Conclusion: We have characterized novel recombinant human single chain antibodies recognizing differentially expressed antigens in cancer cell lines. Additionally, one antigen is expressed also on vascular smooth muscle cells and in normal bone marrow cells, possibly perivascular progenitor cells. The anti-ALCAM antibody is highly cancer specific and inhibits cancer cell invasion and tumour growth.

214 Molecular characterization of patient tumour-derived breast cancer xenografts shows a strong genomic and gene expression stability

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Background: Identifying new therapeutic agents for breast cancer (BC) treatment requires preclinical models that recapitulate the molecular characteristics of their respective clinical tumours. In this work, we analyzed the genomic and expression profiles of human BC xenografts and their corresponding patient's tumours.
Material and Methods: 18 breast cancer xenografts (2 HER2 positive, 6 estrogen receptor positive (ER+), and 10 triple-negative) were obtained by grafting tumour fragments from patients into Swiss nude mice. The molecular characterization of patient's and xenograft's tumours was performed by DNA copy number analysis using a home-made BAC array (aCGH) as well as gene expression profiling using Affymetrix HGU133 Plus 2.0 Microarrays.
Results: We compared the genomic profiles of BC xenografts with those of respective patient's donor tumours by calculating the correlation coefficient based on the status of the probes on each pair of chromosomes. We found that 14/18 paired tumours (78%) had a correlation coefficient higher than 0.50. aCGH based unsupervised hierarchical clustering analysis showed that 16/18 pairs segregated together and revealed the presence of the different molecular classes (ER+, triple negative and HER2+).We next analyzed the gene expression profile of the paired primary tumours and xenografts as described in molecular subtype's classification and found that they showed no or few variations. Interestingly, we identified 558 Probesets corresponding to 371 unique genes differentially expressed in more than 84% of paired tumours. 536 were under-expressed and 22 over-expressed in the xenografts. Immune response, response to wounding, extracellular matrix component, cell adhesion, and angiogenesis constituted the main categories thus identified. An analysis in a public dataset of 1143 breast cancer samples evaluated the prognostic value of the 558 Probe Sets and showed a significant enrichment in genes with prognostic ability. The underexpression of human stromal compartment related genes in the xenografts may highlight its involvement in the prognosis of breast cancer patients. Finally, further analyses on xenografts at different tumour passages did not reveal important changes in the genomic rearrangements, neither in the gene expression profiles. These data suggest a striking genetic stability of the models across the time.
Conclusions: This panel of human BC xenografts accurately maintains the genomic and the expression profiles of their corresponding patient's tumours and are stable across sequential in vivo passages. Consequently, these xenografts represent an ideal model for preclinical investigation of new therapeutic agents. In addition, the lack of human stromal compartment in the xenograft model gave indirectly access to the stromal gene expression profiles of the primary tumour and confirmed its major involvement in the prognosis of breast cancer patients.

215 Study of the regulation of DUSP6 expression as a chemorresistance mechanism in exocrine pancreatic cancer

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Several reasons can explain the poor survival associated to pancreatic cancer: A late diagnosis, a high methastatical potential, and the failure of conventional antineoplasic treatments. We are particularly interested in the search of new therapeutical approaches for the treatment of patients affected with pancreatic cancer. We had previously generated pancreatic cancer cellular models resistant to gemcitabin, antineoplasic drug that has been considered the standard treatment for this type of tumour. IMIM-PC1, IMIM-PC2, and RWP1 pancreatic cancer cells were treated with increasing concentrations of gemcitabin. The RNA from the cells resistant to gemcitabin 500 nM was isolated and, the differential gene expression associated to gemcitabin resistance, was determined by DNA microarrays analysis, comparing gene expression between the parental gemcitabin sensitive cell lines and the resistant cell lines. Several genes were affected in their expression in response to gemcitabin resistance. One of the genes commonly overexpressed in the resistant cell lines is the dual phosphatase DUSP6, enzyme that inactivates the MAP kinases through dephosphorylation in serine and threonine residues. The inhibitory effect of DUSP6 on the MAP kinases-signalling pathways, suggest an important role of this phosphatase in the regulation of a high variety of physiological processes. We analysed in other pancreatic cancer cells (HS-766T, HPAF-II, SKPC-1), the level of expression of DUSP6 and the degree of resistance to gemcitabin, finding a good correlation between both parameters.
We obtained a pancreatic cancer cell line (IMIM-PC2/src) stably transfected with a constitutively active mutant of the tyrosine kinase src; this cell line shows an increased resistance to gemcitabin and high levels of DUSP6 mRNA with respect to the parental untransfected IMIM-PC2 cell line, indicating again a relationship between DUSP6 expression and gemcitabin resistance.
In order to determine the role of DUSP6 in gemcitabin resistance in pancreatic cancer, HS-766T and HPAF-II cells have been transfected with a plasmid that expresses a small interference RNA for DUSP6. We will determine if the decrease in DUSP6 expression correlates with an increase in the sensitivity to gemcitabin in the transfected cell lines. If this is the case, we can consider that the regulation of DUSP6 expression can be considered as a target for the improvement of sensitivity to gemcitabin in pancreatic cancer patients.

216 Selective cytotoxicity of parvovirus H-1 infection for human neuroblastoma and medulloblastoma cells

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Background: In paediatric neuro-oncology, patients with high-risk neuroblastoma and metastasized medulloblastoma both represent subgroups of poor prognosis. Thus, new modalities such as oncolytic virotherapy are urgently required for the treatment of these patients. The rodent parvovirus H-1 (H-1PV) is a non-recombinant wildtype virus apathogenic in animals and in humans. For its efficient cytopathogenicity and full life cycle H-1PV depends on the transformed phenotype of cells. From the aspect of clinical safety, this natural oncoselectivity renders H-1PV a very interesting virus for oncolytic virotherapy in children.
Recently it has been shown that H-1PV selectively kills glioma cells *in vitro* and is oncolytic for gliomas in animal glioma models (Geletneky et. al., 2010). In order to determine its oncolytic potential in paediatric tumours we analyzed cytotoxicity of H-1PV in neuroblastoma and medulloblastoma cells *in vitro*.
Materials and Methods: Neuroblastoma cell lines with different MYCN status (n = 11), medulloblastoma cell lines (n = 7), and normal primary brain cells were infected with H-1PV. We determined efficiency of infection, viral replication, and effects of H1-PV on the cell cycle and cell viability *in vitro*.
Results: Non-neoplastic infant cells (glia cells, astrocytes and neuronal cells in short term culture) were unaffected in viability and morphology after H1-PV infection.

In contrast, all neuroblastoma cell lines analyzed proved to be infectable with H-1PV, with complete viral replication yielding virus titer increase of up to 10.000-fold within 48 to 96 h after infection. Lytic infection was observed at TCIDs50 between 0.001 and 10 pfu/cell. Cell killing was independent of the status of MYCN amplification in the respective cell lines.
As with neuroblastoma cells, all medulloblastoma cell lines were efficiently infectable with H-1PV, displaying a dose-dependent cytotoxicity with TCIDs50 between 0.001 and 5 pfu/cell. Similar to the results with neuroblastoma cells, efficient viral replication could be shown in medulloblastoma cells by an increase of virus titers in a range of two log-steps within 72 to 96 h after infection.
In both, neuroblastoma and medulloblastoma cells, H-1PV induced a G2-arrest and subsequent apoptosis.
Conclusions: The data infer that application of oncolytic H1-PV may be a promising treatment option for embryonic tumours of neuroectodermal origin. This, however, has to be substantiated by a pre-clinical evaluation of the therapeutic efficacy for these tumours *in vivo*.

217 Testing of a poly(ADP-ribose) polymerase (PARP) inhibitor on human BRCA2 heterozygous cell lines

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The PARP inhibitor, AZD2281, olaparib®, is a promising targeted anti-cancer agent for patients with specific DNA-repair defects such as found in BRCA1 and BRCA2 mutation carriers. Early clinical trials suggest that this targeted drug is effective on tumour cells and well tolerated by normal tissues in mutation carriers.
Materials and Methods: Response to olaparib, was tested on three heterozygous mammary epithelial cell lines derived from carriers of a 999del5 BRCA2 founder mutation (A176, A240, A256) and one non-BRCA cell line (D492) transformed in the same way, as well as the commercially available BRCA2 deficient pancreatic cell line Capan-1 and the mammary cell line MCF7. Heterozygous cell lines were examined for BRCA2 allel loss using TaqMan qRT-PCR and copy number changes on CGH arrays (385K aCGH; NimbleGen Systems). CellTiter 96® Aqueous One Solution Cell Proliferation assay (MTS assay) was used to estimate survival and determine the maximum tolerated dose of olaparib and IC_{50} values for all cell lines. Cell death was also assessed with annexin-V and probidium iodide staining. Immunostaining for Rad51 and γH2AX was carried out to evaluate DNA double stand breaks and DNA repair.
Results from olaparib testing using the MTS assay show that the heterozygous cell-lines A176, A240 and A256 have similar IC_{50} values as both the non-BRCA cell lines, D492 and MCF-7. Whereas, Capan-1 shows increased sensitivity to the inhibitor. Annexin-V and PI staining show that the Capan-1 cell line goes through apoptosis at low dosages. Only at exposure to high dosages did the heterozygous cell lines show PI staining. Immunostaining with γH2A.X and RAD51 antibodies indicates that the Capan-1 cell-line has loss of γH2AX/RAD51 colocalization after treatment with olaparib, whereas the heterozygous BRCA2 cell-lines show colocalization after treatment.
In conclusion: Human mammary cell lines heterozygous for a BRCA2 mutation that have retained the second BRCA2 allel are not more sensitive to PARP inhibitor olaparib treatment than non-BRCA2 mammary cell line controls.

218 L-Asparaginase-loaded red blood cells: a promising therapy in solid tumours

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Tumour cells deficient in asparagine synthetase (ASNS) are unable to synthesize enough L-asparagine (L-Asn) to meet metabolic demands and, therefore, depend upon circulating L-Asn in the plasma for survival. L-asparaginase (L-aspa), which depletes L-Asn via deamination, has been used in the treatment of acute lymphoblastic leukemia (ALL) for over 40 years, since the majority of such tumours are deficient in ASNS. Patients able to tolerate more than 25 weeks of L-aspa experience significantly increased survival, but side effects often preclude that duration of use. However, in a phase II clinical trial, entrapment of L-aspa inside red blood cells (GRASPA®) has been found to strongly reduce side effects and also to improve L-aspa pharmacokinetics. Recent evidence suggests that subsets of solid tumours (head and neck, ovarian and pancreatic) are deficient in ASNS, providing rationale for testing L-aspa against such tumours.
To demonstrate the utility of L-aspa in solid tumours, we investigated:
- Expression of ASNS on human cell lines and tumour samples by immunohistochemistry and western blot
- IC50 and GI50 of L-aspa and cell viability in L-Asn deprived medium
- *In vivo* studies in subcutaneous and intraperitoneal metastasis models.

Four pancreatic and 4 ovarian cell lines were studied, as well as MOLT-4, a lymphoblastic leukemia cell line used as a negative ASNS expressing control.
In several patient tumour samples, the healthy tissues highly expressed ASNS in opposite to cancerous tissues which weakly expressed ASNS. With cell lines, ASNS expression was variable regardless of the detection method used, but only MOLT-4 was totally negative.
L-aspa was efficient *in vitro* in all cell lines studied (IC50 between 0.1 and 0.6 IU/mL). In addition, we confirmed that cytotoxicity was essentially due to L-Asn deprivation from the medium.
In vivo, a pancreatic model in mice displayed sensitivity to GRASPA in combination or not with gemcitabine. However, this sensitivity was dependent on the treatment schedule.
The results indicate that L-Asn depletion can be an effective strategy for treating solid tumours. GRASPA®, a better tolerated L-aspa formulation is under evaluation in a phase I clinical trial for pancreatic cancers and a biomarker assay for ASNS expression is being developed for patient stratification.

219 Cytotoxicity and cell death signaling in stem cell like AML cells

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Background: Acute myeloid leukemia (AML) is a result of uncontrolled proliferation of white blood cells in the myeloid line of cell differentiation in the bone marrow. High dose chemotherapy is the current treatment option but a large proportion of the patients relapse within 1−2 years and the 5-year survival threshold is low, particularly in elderly patients. Thus, new effective therapeutic drugs are highly desirable. We evaluate the cytotoxic potential of two novel treatments, gemtuzumab ozogamicin (GO), a monoclonal antibody linked to a toxin causing DNA double strand breaks and J1, an alkalyting prodrug of melphalan, in AML cell lines of different maturation stages. The aim of this study was to understand if and how these novel agents may work in different AML maturation stages with comparison to the conventional therapies daunorubicin or cytarabine.
Material and Methods: Two AML cell lines with different maturation stages were used. The partly differentiated HL60 cells were compared to the immature Kasumi-1 cells, both expressing high levels of CD33 on their surface and both positive for CD13. The cytoxic responses of the agents were compared using MTT cell viability assay. Activation of the proapoptotic proteins caspase 3 and Bax were determined using activation specific antibodies and was evaluated using FACS analysis.
Results: Treating the AML cells with GO, J1 daunorubicin or cytarabine revealed significant differences in their cytotoxic potential in HL60 and Kasumi-1 cells. Thus, daunorubicin (100 nM) and cytarabine (1 μM) were found to cause 50% growth inhibition at 48 h post drug addition in HL60 cells whereas in Kasumi-1 cells no more than 20% growth inhibition was observed. This was consistent with a lack of Bax and caspase-3 activation in Kasumi-1 cells. Comparison of GO sensitivity revealed a 50% growth inhibition in HL60 cells after 48 h of treatment with 100 ng/ml GO while no inhibition was observed in Kasumi-1 cells. This was not caused by different expression of the target CD33 on the cells, but may be explained by less efficient activation of caspase-3. A comparison of J1-induced cytotoxicity in HL60 and Kasumi 1 cells revealed a 50% growth inhibition after 48 h treatment with 0.5 μM J1 in HL60 cells whereas the Kasumi-1 cells needed 5 μM to achieve similar growth reduction. Analysis of apoptotic signaling is ongoing.
Conclusions: We demonstrate that the maturation state of the AML cells influences their responsiveness to both novel and conventional chemotherapeutics. The immature Kasumi-1 cells were more resistant to all drugs tested and only J1 was able to induce a proper cytotoxic response. Our data suggest that the capacity to trigger apoptotic signaling may explain why the drugs have a diverse cytotoxic potential in different maturation stages of AML. Further analyses of Kasumi-1 cells may reveal novel therapeutic targets that might have profound therapeutic implications for AML.

220 Identification of resistance mechanisms to EGFR inhibitors in non-small cell lung cancer cells

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Non-small cell lung cancer (NSCLC) patients are routinely treated with small molecule EGFR tyrosine kinase inhibitors, such as erlotinib and gefitinib. These inhibitors compete reversibly with ATP to bind the intracellular catalytic domain