Nr 4. Lipid transfer in hypoxic tumor microenvironment promotes cancer survival and tumor growth after re-oxygenation

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We have identified recently a new mechanism of tumor adaptation to sunitinib and sorafenib, two anti-angiogenics of the tyrosine kinase receptor inhibitor (RTKI) family, actually used in clinic. This adaptation relies on metabolic reprogramming towards a glycolysis and lactate production during treatment and *de novo* lipogenesis after treatment cessation. However, the mechanism of this adaptive response of tumor to RTKI is not fully deciphered, yet. Tumor metabolic reprogramming was further investigated in harsh tumor microenvironment (TME) lacking oxygen and nutrients when blood vessels are shut down with treatment. First, we analyzed the timing of metabolic shift after treatment withdrawal in MDA-MB231 and LLC xenografts by immunohistochemical analyses (IHC), western blot and RT-PCR in tumors collected at different time points. FASN expression was increased at day 14 and day 2 after angiogenic therapy cessation in MDA-MB231 and LLC xenografts, respectively. FASN expression correlated with increase in mature adipocytes stained for perilipin, 14 and 21 days post treatment. IHC staining with pref-1 antibody, a marker of pre-adipocytes showed a significant increase in pre-adipocytes during treatment, which decreases and inversely correlates with mature adipocytes after treatment cessation. This prompted us to investigate the origin of adipocytes by conducting a GFP+ bone marrow (BM) transplantation from GFP-C57/bl6 mouse strain into irradiated C57bl6 mice, 5 weeks later, mice were transplanted with LLC tumors and challenged with sunitinib treatment and withdrawal. Confocal microscopy analysis and double IHC staining with GFP antibody and perilipin-1, Pref-1, adipophilin or FASN revealed that adipocytes in tumors are not derived from BM-MSC. These data suggest that cancer cells are the main sources of lipids, which are either stored in lipid droplets (LDs) in the cytoplasm of cancer cells. Alternatively, lipids could be produced or uptaken by resident adipocytes. By exploring deeply the role of lipid metabolism in cancer adaptation to a metabolic stress induced by hypoxic TME (sunitinib condition), we found that hypoxic tumors increase their absorption of lipids from the TME as demonstrated by increased number of LDs stained by adipophilin (Perilipin-II) and overexpression of lipid transporter, FABP4 in tumors. In contrast, after tumor re-oxygenation adipophilin expression is reduced but the level of perilipin-I, the marker of mature adipocytes is up-regulated. Interestingly, FABP-4 expression level was kept increased from hypoxic to re-oxygenation state. In vivo inhibition of FABP4 by pharmacological inhibitor resulted in delayed tumor growth and prolonged effect angiogenesis inhibitor after treatment cessation. All together FABP4 plays a key role in lipid transfer, and acts as a shuttle between stroma and cancer cells and inhibition of FABP4 prolonged the benefit of angiogenesis inhibitors after treatment cessation.

Nr 5. **Androgen receptor profiling in circulating tumour cells from patients with castration-resistant prostate cancer reveals novel splice variant expression patterns.**

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Background: The androgen receptor (AR) splice variant 7 (AR-V7) is associated with resistance to abiraterone acetate and enzalutamide in patients with castration-resistant prostate cancer (CRPC). However, a large fraction of AR-V7 negative patients remain unresponsive to these endocrine therapies.

Objective: To investigate the feasibility to generate an AR splice variant (ARV) profile from enriched circulating tumour cells (CTCs), encompassing seven AR isoforms. And to assess the predictive value of the generated profile in the context of second line endocrine therapy.

Design, settings and participants: We collected peripheral blood samples from patients (n=26) with CRPC before (n=18) and at progressive disease (n=12) on abiraterone or enzalutamide. Four cases had sampling at both baseline and progression. Circulating tumour cells (CTCs) were enumerated and enriched using the FDA-cleared CellSearch platform. Enriched CTC fractions were subjected to RNA isolation, cDNA synthesis and targeted RNA sequencing to infer ARV expression profiles. Results were validated by qRT-PCR.

Outcome measurements and statistical analysis: ARV expression patterns were correlated to clinic-pathological parameters.

Results and limitations: On average, targeted RNA sequencing resulted in 3672 reads (IQR: 1272 – 5970). AR splice variants were detected in 17/30 (56.6%) analysed samples, resulting in 15/26 (57.7%) patients being ARV-positive, of whom 13/15 (86,7%) had less than 6 months benefit from their therapy (Fisher exact: p = 0.0115). The majority of ARV-positive patients (10/15 (66.7%)) expressed multiple ARVs, where AR-V3 was the most abundantly expressed, demonstrating 3.5-fold higher median expression levels compared to AR-V7 (Wilcox signed rank, p = 0.0029). In addition, two poorly responding patients were AR-V7 negative whilst expressing AR-V3. Baseline ARV and follow-up data were available in 17 patients. At 10-12 weeks, 30% and 50% PSA response rates were not statistically different between patients with (n=8) and without (n=9) ARV (Fisher exact test: p = 0.15 and p = 0.29, respectively). Presence of AR splice variants was associated with progression-free survival, as measured by time to no longer clinically benefitting (hazard ratio: 4.53; 95% CI, 1.424–14.41; p = 0.0105).

Conclusions: ARV profiling is feasible from liquid biopsies and may provide additional information to stratify patients, compared to assays targeting AR-V7 expression alone.

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Nr 7. Tumor metabolic and functional imaging modifications after fractionated Neoadjuvant radiotherapy.

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Purpose: Most of cancer patients resort to radiotherapy (RT) during their treatment. Neoadjuvant RT, applied before surgery, aims at improving tumor local control and patient overall survival. In clinical practice, there is a need to develop new tools predicting the tumor response to treatment, the best timing to perform surgery and some possible adjuvant treatments. We previously observed an impact of the timing of surgery on metastatic spreading in a preclinical model. In this study, we analyzed tumor microenvironment modifications by functional imaging and metabolomics following neoadjuvant RT.

Materials and methods: Tumor-bearing mice (SCID or BalbC mice implanted with MDA-MB-231 or 4T1 tumor cells) were locally irradiated (2x5Gy) with a specific irradiator dedicated for small animals. Tumors were imaged by fMRI every days and collected 4 or 11 days after the end of RT. We performed DW-MRI with FSEMS (Fast Spin Echo MultiSlice) sequences, with 9 different B-values (from 40 to 1000) and B0, in the 3 main directions. We performed IVIM (IntraVoxel Incoherent Motion) analysis to obtain information on intravascular diffusion, related to perfusion (*F*: perfusion factor) and subsequently tumor vessel perfusion. Tumor extracts were homogenized in deuterated phosphate buffer and supplemented with maleic acid and TMSP before Nuclear Magnetic Resonance (NMR) analyses. Data were analyzed with powerful statistical tool (supervised and multivariate analyses).

Results: *In vivo* with fMRI, we observed a significant peak of *F* (60% increase of the basal value, n=6, $p<0,05$) at day 6 after irradiation for MDA-MB 231 tumors. We observed similar results at day 3 with 4T1 tumors (55% increase of the basal value, n=10, $p<0,05$). All other DW-MRI parameters (D and ADC) were not modified by the treatment. We also performed surgery based on F peak obtained by functional imaging in the MDA-MB 231. Surprisingly, we observed a decrease of the metastatic burden when surgery was performed on the *F* peak compared to surgery performed at day 4 or day 11. To study the tumor microenvironment, we analyzed *ex vivo* tumor extracts with NMR and observed some differences in the metabolome of irradiated and non-irradiated tumors. This difference was more marked at day 11 for MDA-MB31 tumors and at day 4 for 4T1 tumors. Intriguingly, some metabolites (i.e. glutamate, taurine, glycine, betaine) were decreased following RT in both models. An increase in lipid signal in irradiated tumors was also noticed compared to non-irradiated tumors. On the other hand, a correlation between metabolic profile and metastatic profile for individuals in the different groups of the "MDA-MB231 cells/ SCID mice" model was remarked.

Conclusion: For the first time, we demonstrate the feasibility of repetitive fMRI imaging in preclinical models after neoadjuvant RT. With these models, we show a significant modification in perfusion-related parameter (F) at a specific time point depending of the tumor cells. These modifications are correlated to a decrease of metastasis spreading related to the surgery procedure. NMR analyses and discriminant analyses showed an impact of neoadjuvant RT on tumor microenvironment which could be correlated to the metastatic profile. These results open new perspectives in the personalized medicine and MRI guided surgery timing after neoadjuvant RT.

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NR 11. RADIOSENSITIZING POTENTIAL OF PHENFORMIN IN A PRECLINICAL COLORECTAL CANCER MODEL.

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Background and Purpose: Radiotherapy is the treatment of choice for colorectal cancer. Tumor hypoxia is a negative prognostic factor, due in part to its role in causing resistance to radiotherapy. Hypoxia arises in tumor regions distal to blood vessels as oxygen is consumed by more proximal tumor cells. Reducing the rate of oxygen consumption is therefore a potential strategy to reduce tumor hypoxia. We hypothesize that Phenformin, a biguanide, which reduces oxygen consumption through inhibition of mitochondrial complex I, would improve radiation. Moreover, may Phenformin exert antitumor effects via activation of AMPK. In this study we investigated the beneficial effect of combination of Phenformin and radiotherapy on colon cancer.

Material and Methods: Two human (DLD1 and HCT116) and one mice (CT26) colorectal cancer cell lines were subjected to metabolic hypoxia and analyzed for radiosensitivity by clonogenic assay. Their oxygen consumption was measured by Seahorse and expression of AMPK by Western blotting. Furthermore, were CT26 cells injected in Balb/c mice and treated with Phenformin through oral gavage in combination or alone with radiation therapy.

Results: Phenformin radiosensitizes all the three cell lines subjected to metabolic hypoxia. *In vivo* we observed tumor growth delay in the CT26 tumors after treatment with Phenformin with radiation, there was also some tumor growth delay after treatment with Phenformin alone.

Conclusion: Phenformin has a clear radiosensitizing effect both *in vitro* as *in vivo* on colorectal cancer cell lines.

Nr 16. Mouse Ear Sponge Assay: a new tool to investigate lymphovascular pre-metastatic niche

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Background: Most of solid tumors first spread via lymphatic network to form distant metastasis. Lymphangiogenesis, the formation of new lymphatic vessels, occurs in primary tumors but also in draining lymph nodes leading to pre-metastatic niche formation. Reliable *in vivo* models are becoming instrumental for investigating alterations occurring in lymph nodes before tumor cell arrival. Most of these models reproduce experimental metastasis with a direct drainage by lymphatic vessels without all steps appearing during cancer progression.

Methods and results: In this study, we demonstrate that B16F10 melanoma cell encapsulation in a biomaterial (gelatin sponge covered in collagen), and implantation in the mouse ear, prevent their rapid lymphatic spread observed when cells are directly injected in the ear. Vascular remodeling in the lymph node was detected two weeks after sponge implantation, while their colonization by tumor cells occurred two weeks later. In this model, a huge lymphangiogenic response was induced in primary tumors but also in pre-metastatic and metastatic lymph nodes. In control lymph nodes, lymphatic vessels were confined to the cortex. In contrast, an enlargement and expansion of lymphatic vessels towards paracortical and medullar areas occurred in pre-metastatic lymph nodes. We designed an original computerized quantification method to examine the lymphatic vessel structure and the spatial distribution.

Conclusion: This new reliable and accurate model is suitable for *in vivo* studies of lymphangiogenesis and holds promise for unraveling the mechanisms underlying pre-metastatic niche formation in lymph nodes. The ear sponge assay will contribute to give new mechanistic insights in lymphatic metastases and provide new tools for drug testing.

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Nr 19. CRISPR/Cas9 mediated knockout of *rb1* and *rbl1* leads to rapid and penetrant retinoblastoma development in *Xenopus tropicalis*

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Retinoblastoma is a pediatric eye tumor in which bi-allelic inactivation of the *Retinoblastoma 1* (*RB1*) gene is the initiating genetic lesion. Although recently curative rates of retinoblastoma have increased, there are at this time no molecular targeted therapies available. This is, in part, due to the lack of highly penetrant and rapid retinoblastoma animal models that facilitate rapid identification of targets that allow therapeutic intervention. Different mouse models are available, all based on genetic deactivation of both *Rb1* and *Retinoblastoma-like 1* (*Rbl1)*, and each showing different kinetics of retinoblastoma development. Here, we show by CRISPR/Cas9 techniques that similar to the mouse, neither *rb1* nor *rbl1* single mosaic mutant *Xenopus tropicalis* develop tumors, whereas *rb1/rbl1* double mosaic mutant tadpoles rapidly develop retinoblastoma. Moreover, occasionally presence of pinealoblastoma (trilateral retinoblastoma) was detected. We thus present the first CRISPR/Cas9 mediated cancer model in *Xenopus tropicalis* and the first genuine genetic non-mammalian retinoblastoma model. The rapid kinetics of our model paves the way for use as a pre-clinical model. Additionally, this retinoblastoma model provides unique possibilities for fast elucidation of novel drug targets by triple multiplex CRISPR/Cas9 gRNA injections (*rb1+rbl1+modifier gene)* in order to address the clinically unmet need of targeted retinoblastoma therapy.

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Nr 20. Characterization of the tumor microenvironment and investigation of immune checkpoint expression in malignant pleural mesothelioma

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BACKGROUND: Malignant pleural mesothelioma (MPM) is an aggressive cancer with a poor prognosis and an increasing incidence, for which novel therapeutic strategies are urgently required. Since the immune system has been described to play a role in protection against MPM, characterization of its tumor immune microenvironment (TME) and immune checkpoints might help to identify new immunotherapeutic targets and their predictive and/or prognostic value.

METHODS: Immunohistochemistry (IHC) was performed on tissue samples of untreated (n=40) and chemotherapy-pretreated (n=14) MPM patients. Different subsets if immune cells were identified based on staining for CD4, CD8, FoxP3, CD68, CD45RO and granzyme B. The expression of the immune checkpoints TIM-3, LAG-3, PD-1 and its ligand PD-L1 was also investigated. The relationship between the immunological parameters and survival, as well as response to chemotherapy was analyzed using the R statistical software.

RESULTS: All patients had CD8+ tumor infiltrating lymphocytes (TILs), CD68+ histiocytes and macrophages and CD45RO+ T cells in their stroma, with CD8+ TILs being the predominant cell type of the immune infiltrate. Stromal CD4+ TILs were found in 75% of the untreated and 71% of the pretreated samples. A subset of those cells was also FoxP3+ and these CD4+FoxP3+ cells were positively correlated with stromal CD4 expression ($p<0.001$). Less than half of the samples showed positivity for granzyme B. Both, untreated and pretreated patients had PD-1+ TILs, while only 10% of the untreated patients also had PD-1+ tumor cells. PD-L1 positivity on lymphocytes and/or tumor cells was observed for more than half of the patients, with significant differences according to the histological subtype ($p<0.001$). Patients with a sarcomatoid histology showed the most PD-1 expression. TIM-3 was expressed in tumor cells, stromal lymphocytes and plasma cells, less often in pretreated samples compared to untreated samples. All samples were negative for LAG-3. After multivariate analysis stromal CD45RO expression was found to be an independent negative predictive factor for response to chemotherapy ($p=0.017$) and expression of CD4 and TIM-3 in lymphoid aggregates were good prognostic factors ($p=0.008$; $p=0.001$).

CONCLUSION: Our data reveal the diversity of immune cells present in MPM and point to TIM-3 as a new target in mesothelioma. Administering chemotherapy before or together with PD-1/PD-L1/TIM-3 blocking agents may not be the best combination sequence and further research on the predictive value of CD45RO in the stroma might guide patient selection for chemotherapy.

Nr 24. P53 and MITF/Bcl-2 identified as key pathways in the resistance of NRAS mutant melanoma to MEK inhibition

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Background
Activating NRAS mutations are found in 15-30% of melanomas and are associated with a poor prognosis. Nevertheless, there is lack of effective targeted therapies for NRAS mutant melanoma. Recently, the MEK inhibitor pimasertib showed a clinical benefit in patients with NRAS-mutated melanoma. However, used as single agents, MEK inhibitors had limited efficacy.

Objectives
We aimed to identify and investigate signaling pathways linked to the resistance to MEK inhibitors in a panel of $^{Q61K/L/R}$NRAS mutated melanoma cells, with a special attention to MITF, the master transcription factor regulating cell growth and differentiation in melanocytes and the common alteration of p53 signaling.

Results
First, we showed that pimasertib inhibited cell proliferation with IC50 ranging from 0.01 to 0.05 µM in four $^{Q61K/L/R}$NRAS melanoma cell lines but without any significant effect on apoptosis. On the other hand, we developed a line with acquired resistance to pimasertib and found that such resistance was associated with a substantial activation of MITF-Bcl-2 pathway. Likewise, the stimulation of MITF expression by cAMP conferred resistance to pimasertib and stimulated Bcl-2 expression in a panel of NRAS mutant cell lines.
Accordingly, we used ABT-199 to specifically inhibit Bcl-2 or PRIMA-1^{Met} to reactivate p53 and obtained a very significant synergistic effect on apoptosis in both conditions, thus breaking the resistance to the drug. p53 reactivation, also monitored by p21 expression, was accompanied by PTEN increase and a consequent inhibition of AKT phosphorylation.

Conclusion
We identified cAMP-MITF-Bcl-2 pathway activation as a main mechanism of resistance to MEK inhibition in NRAS mutant melanoma. This particular anti-apoptotic mechanism warrants further preclinical investigation to evaluate the benefit of combining MEK inhibition to p53 reactivation or to Bcl-2 inhibition as a promising therapeutic strategy.

Nr 26. Tumour Biobanking in Belgium: a central role for the BVT, the nationwide Belgian Virtual Tumourbank

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Biobanks play a critical role in cancer research by providing high quality biological samples for research. However, the availability of tumour samples in single research institutions is often limited, especially for rare cancers. To facilitate the search for samples scattered among different institutions, data from material of human tumours are collected in one central database, the so-called Belgian Virtual Tumourbank (BVT). The samples themselves, however, remain physically located at eleven Belgian university hospital biobanks.

The BVT-project was launched in March 2008 by the Belgian Minister of Health, Ms. Laurette Onkelinx (initiative 27 of the Belgian Cancer Plan) and assigned to the Belgian Cancer Registry.

The BVT web application for tumour sample annotation and traceability went live in January 2012. The BVT collects data on tumour samples via a custom-made application for registration i.e. the BVTr. After quality control, the registered data are made available for researchers via an online catalogue (i.e. the BVTc) containing medical, technical, and patient details (but excluding identifying information to ensure privacy of individuals). The BVTc allows scientists to perform queries using specific search criteria and to trace the samples of interest to the local biobanks of the BVT network (December 2016: more than 61,000 registrations available). Data quality control of the BVT includes control measures at every stage of the data process guaranteeing a high quality of the data on the samples requested by scientists working in translational research in oncology.

Any researcher active in the broad field of oncology (both from non-profit and profit organisations), can easily verify the number of samples of interest registered in the BVTc by completing the sample availability request form on the BVT website. Belgian researchers of non-profit research organisations can also obtain full access to the BVTc, allowing them to browse through the BVTc with detailed sample level information, by completing the catalogue application form available on the BVT website.

More detailed information can be found at www.virtualtumourbank.be

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Nr 27. The prognostic and predictive effect of TILs is not determined by B cells or PD-L1-expression in inflammatory breast cancer.

Background

There is increasing evidence that the tumor stroma with tumor infiltrating lymphocytes (TILs) plays a crucial role in the aggressive inflammatory breast cancer (IBC) phenotype. However, it remains an enigma how infiltrating immune cells are able to determine the IBC phenotype. In both IBC and nIBC, tumors are infiltrated by B cells, but their role in regulating anti-tumor immunity is not well understood and evidence suggests that tumor educated B cells acquire PD-L1 expression. In this study we look at the differences and outcome effects of TILS, $CD79\alpha^+$ B cells and PD-L1-expression in the immune infiltrate of 169 IBC and 235 non-inflammatory breast cancer (nIBC) patients.

Methods

TIL and B cell scoring was done according to international guidelines (Salgado et al., 2015) (fig. 1). PDL1 scoring was based on the percentage of positive tumor cells (TC) and positive immune cells (IC) occupying the tumor area. All obtained data were analyzed using SPSS 24 (IBM). Analyses were 2-sided and p-values of < 0.05 were considered statistically significant. Cohen's kappa test showed a good interobserver agreement (TIL: $\kappa = 0.694$, PD-L1: $\kappa = 0.718$).

Results

Most of our IBC patients presented with a grade 3 (67.7%) ductal (90.5%) carcinoma. The mean TIL score was comparable between IBC (18.02%, 15.78 – 20.46) and nIBC (19.77%, 17.19 – 22.36). However, in the hormone receptor positive (HR^+) group TILs were significantly higher in IBC patients (15.60%, 12.62 – 18.59 vs. 11.45%, 9.75 – 13.16 ; p= 0,011). Peri- and intratumoral CD79α scores were not significantly different between IBC and nIBC.

In IBC, 45.0% (76/169) of the patients were PD-L1 positive while only 17.9% (42/235) of the patients were positive in the nIBC group ($p < 0,001$). This difference remained significant in all subtypes, except for the HR^-Her2^+ subtype. Only 1.8% of the IBC patients and 1.3% of the nIBC group showed TC positivity. PD-L1 expression in IBC correlated with higher intratumoral CD79α (p= 0.029) and TIL (p= 0.001) scores in multivariate analysis; but not with HR status, molecular subtype, grade or nodal status. In contrast, PD-L1 expression in nIBC correlated with HR^- subtypes and tertiary lymphoid structures (TLS).

Univariate analysis showed that achieving pCR was significantly associated with more TIL infiltration (p= 0.002), PD-L1 IC expression (p= 0.011) and intratumoral $CD79\alpha^+$ cells (p= 0.049) in IBC. However, in multivariate analysis only TIL infiltration was an independent predictor of pCR. Survival analysis showed a significant beneficial effect of TILs, especially in the HR^+ subtypes; but not for PD-L1 or $CD79\alpha^+$ in IBC.

Conclusion

A high number of TILs correlates with a better pCR and a longer OS in IBC, especially in the HR^+ subtypes. Characterization of the infiltrate shows that $CD79\alpha^+$ cells and PD-L1-expression do not contribute to this effect. B cells seem not to determine the IBC phenotype since the numbers of $CD79\alpha^+$ cells in IBC and nIBC are comparable, however PD-L1-expression is significantly increased in IBC compared to nIBC. This expression is associated with intratumoral $CD79\alpha^+$ cells in IBC and TLS presence in nIBC.

Figures

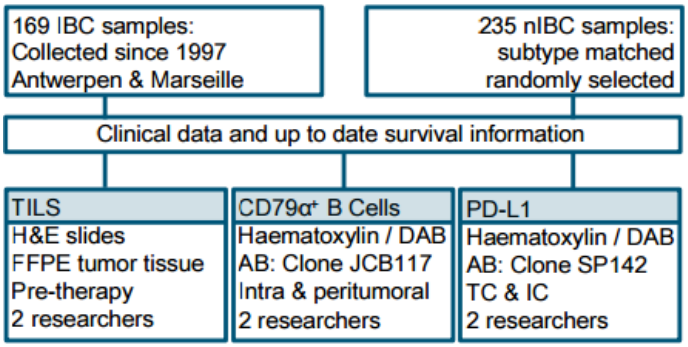

Figure 1: Study protocol.

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Nr 29.

RADIOSYNTHESIS AND VALIDATION OF A PET TRACER OF LACTATE TO MONITOR OXIDATIVE LACTATE METABOLISM AND ITS INHIBITION IN CANCER

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Cancer cells develop a variety of metabolic strategies to cope with the harsh conditions of their microenvironment characterized by hypoxia, limited nutrient bioavailability and exposure to anticancer treatments. Among these strategies, metabolic symbiosis optimizes the bioavailability of glucose in the hypoxic cancer cell compartment where cancer cells primarily rely on a glycolytic metabolism with glucose as a necessary fuel. This symbiosis is based on the exchange of lactate between hypoxic/glycolytic cancer cells that convert glucose to lactate and oxidative cancer cells that preferentially use lactate instead of glucose as an oxidative fuel. Such metabolic cooperation is found in a variety of human cancers of different histological types, and can be established as a mode of resistance to anti-angiogenic therapies. Overall, it depends on the expression and activity of monocarboxylate transporters (MCTs) at the cell membrane: MCT4 is the main facilitator of lactate export by glycolytic cancer cells, and MCT1 primarily conveys lactate uptake by oxidative cancer cells. Consequently, MCT inhibitors have been developed for cancer therapy, among which AZD3965 is a MCT1 inhibitor currently tested in phase I clinical trials. In this context, predicting and monitoring a response to the inhibition of lactate uptake is still an unmet clinical need. To this end, we developed and validated *in vitro* and *in vivo* [^{18}F]-3-fluorolactate (^{18}FLac) as a tracer of lactate for positron emission tomography (PET). ^{18}FLac offers the possibility to monitor MCT1-dependent lactate uptake and inhibition in tumors *in vivo*, which we validated in mice bearing SiHa human cervix tumors and SQD9 human head and neck tumors that were treated with MCT1 inhibitors AR-C155858 and AZD3965. The new tracer was produced in clinical settings.

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Submitted for an oral presentation

Nr 30. Modeling molecular subgroups of medulloblastoma, in *Xenopus tropicalis* by CRISPR/Cas9.

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Medulloblastoma (MB) is a pediatric malignancy originating in the cerebellum that accounts for 20% of central nervous system tumors. MB is subdivided in 4 groups – Wnt, Shh, Group 3 and Group 4 – based on their molecular signature. CRISPR/Cas9 is applied in our lab in the diploid vertebrate *Xenopus tropicalis*, and demonstrated great efficiency in modeling human cancer, like Familial Adenomatous Polyposis (FAP) and retinoblastoma (RB). Our direct goal is to establish models for all MB subgroups in *X. tropicalis*. We demonstrated that co-injection of embryos with sgRNAs targeting *APC* and *TP53* induced tumors in the brain of developing tadpoles after 6 weeks, with an incidence ratio of over 10%. These tumors exhibited the histological hallmarks of human MB. Our direct aim is to optimize this Wnt tumor model by increasing incidence ratio and reduce latency period. For Wnt – MB model, *APC* was injected in a *TP53* null background and injected animals will be analyzed for tumor development. Furthermore we aim to identify effector genes in Wnt – MB formation. Besides modeling Wnt – MB, we attempt to create models for Shh – as well as Group 4 – MB by disrupting essential genes, like *PTCH1* and *KDM6A* respectively. Embryos that were injected with sgRNAs targeting *PTCH1* and *TP53*, demonstrated tumor formation after 2,5 months. This Shh – MB model will be further optimized by injecting *PTCH1* in a *TP53* null background. Co-injections of embryos with *KDM6A* and *ZMYM3* did not result in tumor formation. However, ectopic tissue in the 4th ventricle was observed in histological sections of one tadpole, 4 weeks old. This sample is currently analyzed by Laser Capture Microdissection (LCM). Finally a Group 3 – MB will be generated by activating proto-oncogene *GFI1B* expression. We will use multiplexed sgRNA injections targeting *DDX31* and *GFI1B* in order to create a targeted large chromosomal deletion. This chromosomal rearrangement should result in enhancer hijacking mediated activation of the proto-oncogene *GFI1B*, a molecular event occurring in Group 3 – MB. Our *Xenopus* tumor models should expand the experimental portfolio for identification of genes essential for medulloblastoma formation and provide a platform for pre-clinical testing of novel therapeutic compounds.

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Nr 31. Search for substrates and partners of the pro-metastatic MT4-MMP

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Introduction :
Triple negative breast cancers (TNBC) represent about 15 to 20% of newly diagnosed breast cancers. This cancer affects young women in the reproductive age and follows an aggressive clinical course with a high rate of early-occurring metastasis and poor treatment options.
The high expression level of EGF receptor (EGFR) in most of TNBC provides an option for a potential targeted therapy. EGFR interacts with several cell surface molecules including integrins. Interestingly, we recently discovered that MT4-MMP, a membrane-type matrix metalloprotease expressed at the surface of breast cancer cells colocalizes and interacts with EGFR in triple negative breast carcinomas. MT4-MMP exerts pro-angiogenic and pro-metastatic effects through its proteolytic activity, and promotes tumor growth and proliferation through its interaction with EGFR in a non-proteolytic manner.

- Aims :
The main goal here is to investigate in-depth the molecular mechanisms underlying the dual function of MT4-MMP, namely 1) its capacity to exert pro-angiogenic effects through its proteolytic activity, and 2) its impact on the EGFR pathway activation, which does not rely on its proteolytic function. In this context, we aim at identifying the different substrates of MT4-MMP, which are unknown yet, and its putative partners involved in the complex formed by EGFR and MT4-MMP.

- Methods and results :
To deeply decipher the mechanisms underlying its dual functions we have applied the state of the art N-TAILS (N-Terminal Amine Isotopic Labeling of Substrates) method for the identification of MT4-MMP substrates. By using this method on conditioned medium by MDA-MB231 cells expressing MT4-MMP or its inactive form MT4-E249A, we identified 26 proteins as potential substrates. Among these substrates, syndecan-1 (SDC1) and syndecan-4 (SDC4) are potential interesting candidates since they are known to be involved in cancer progression. SDC1 plays a role in cell spreading and invasion, while SDC4 also has a major role in regulating the matrix structure and cell adhesion/migration.
These data are validated by the identification of a tri-molecular complex of EGFR/syndecan/MT4-MMP by a co-immunoprecipitation (co-IP) experiments and confocal analysis of an immunofluorence (IF).
In the continuation of this work, we will explore the functional significance of syndecans in MT4-MMP-dependant proliferation, EGFR activation and metastasis. For that, studies are underway to study the role of syndecans in EGFR proliferation in vitro by using siRNA inhibition experiment in a 3D matrigel matrix.
We also plan to study the interaction mechanisms of MT4-MMP with EGFR and syndecans and the impact of this complex on the signaling pathway of EGFR. For this, various constructions are generated to identify the key areas of MT4-MMP that regulate its dimerization and interaction with EGFR.

- Conclusions :
These data provide evidence for an unexpected crosstalk between MT4-MMP, syndecans and the EGF receptor that might have implication on EGFR inhibition strategies for breast cancers and other cancers including at least colorectal, head and neck, and lung cancers.

Nr 32. Tumor hypoxia causes DNA hypermethylation by reducing TET activity

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Abstract:

Hypermethylation of tumor suppressor gene (TSG) promoters confers growth advantages to cancer cells, but how these changes arise is poorly understood. Here, we report that tumor hypoxia reduces the activity of oxygen-dependent TET enzymes, which catalyze DNA de-methylation through 5-methylcytosine oxidation. This occurs independently of hypoxia-associated alterations in *TET* expression, proliferation, metabolism, HIF activity or reactive oxygen, but directly depends on oxygen shortage. Hypoxia-induced loss of TET activity increases hypermethylation at gene promoters *in vitro*. Also in patients, TSG promoters are markedly more methylated in hypoxic tumors, independently of proliferation, stromal cell infiltration and tumor characteristics. Our data suggest cellular selection of hypermethylation events, with almost half of them being ascribable to hypoxia across tumor types. Accordingly, increased hypoxia after vessel pruning in murine breast tumors increases hypermethylation, while restored tumor oxygenation by vessel normalization abrogates this effect. Tumor hypoxia thus acts as a novel regulator underlying DNA methylation.

Nr 33. Robustness of scoring stromal features in ductal carcinoma in situ of the breast: an inter-observer variability study.

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Aim. The goal of this study is to determine a cut-off for assessment of stromal architecture in ductal carcinoma in situ (DCIS), based on inter-observer concordance. Robustness of scoring myxoid stroma is compared with reliability of other histopathological features.

Materials and methods. Hematoxylin/eosin stained tissue sections of 213 DCIS lesions were retrieved from the archives of the Department of Pathology of Leuven University Hospitals, Leuven, Belgium. The following histopathological features were independently assessed by two pathologists: nuclear grade, DCIS architecture, intraductal calcifications, extensive comedonecrosis, stromal architecture and stromal inflammation. Nuclear grade was scored as low, intermediate or high grade. DCIS architecture was classified as non-solid or solid, with a cut-off at 50% of ducts presenting with solid growth. Intraductal calcifications were scored as either absent or present. Extensive comedonecrosis was defined as the presence of confluent eosinophilic necrotic debris in >50% of ductal lumina. Myxoid stromal architecture was defined as loosely arranged collagen fibers interspersed with an amorphous, slightly basophilic substance. Stromal architecture was divided into 4 categories (0%, 1-33%, 33-66% or >66% of ducts surrounded by myxoid stroma). Stromal inflammation

was semi-quantitatively categorized into absent, mild, moderate or extensive periductal inflammation, which integrated information on both the extent and the density of the stromal inflammatory infiltrate. All features were dichotomized, using different cut-offs. Kappa values were determined to assess inter-rater variability.

Results. When dichotomized as grade 1/2 versus grade 3, scoring nuclear atypia showed moderate agreement (κ0,524). The kappa value for myxoid stromal architecture was highest by dichotomization with a 33% cut-off (κ0,604), compared to cut-offs of 1% (κ,494) and 66% (κ0,578), respectively. The highest kappa value (κ0,731) for stromal inflammation was obtained by similar dichotomization as 'absent to mild' versus 'moderate to extensive' inflammation. Scores for necrosis showed substantial agreement (κ0,618) at a 50% cut-off. Scores for solid versus non-solid DCIS architecture (κ0,495) and presence of calcifications (κ0,624) showed moderate and substantial agreement, respectively.

Conclusion. Adequate prognostic markers require robustness of assessment, i.e. high inter-rater concordance and thus high reproducibility. The dichotomous assessment of stromal features in DCIS resulted in similar or even higher kappa values compared to the dichotomous scoring of other histopathological features. This study demonstrates the robustness of dichotomous assessment of both stromal architecture and stromal inflammation in DCIS.

* I agree to have the abstract released on the BACR website before the conference in January 2017.

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Nr 34. *In Vivo* Imaging of Xenografts Targeted by RGD-Magnetoliposomes

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Using targeted nanoparticles to deliver cytotoxic drugs to tumors is a promising strategy. Successful targeting depends on the probe's cellular binding specificities. The Arg-Gly-Aps (RGD) peptide has a small molecular weight and low propensity to cause an immune response. It can be used as a ligand that binds integrins in the surface of endothelial cells, *e.g.* hepatic stellate cells, but also cancer cells, *e.g.* glioblastoma. Here, we have used magnetic resonance (MR) and *in vivo* and *ex vivo* fluorescence imaging (FLI) to show targeted binding of the RGD-coupled magnetoliposomes (MLs) to ovarian (SKOV-3) cancer xenografts grown in nude mice.

Cyclic RGD peptide was synthesized at a purity of 95% and was labeled with Texas Red. The peptide was linked via a sulfhydryl group at the cysteine residue to the MLs formulation. The liposome suspension was incubated with cyclic RGD peptide at a molar ratio of 10:1 overnight. Unbound cyclic RGD peptide was separated by high gradient magnetophoresis. Tumor growth was be induced by the engraftment of 10^7 SKOV-3 cells in Swiss nude mice. All MR measurements were performed using a 9.4 T Bruker Biospec small animal MR scanner. RGD-MLs uptake in the tumor site was evaluated using FLASH and RARE sequences and T2 maps. *In vivo* FLI was performed using IVIS Spectrum (Perkin Elmer). After the animals were sacrificed, the tumors were harvested and *ex vivo* FLI was performed. To quantify the signal, regions of interest were drawn around the tumors.

Signal intensities were measured before administration and 2, 4, 24 and 48h after the intravenous administration of the particles via the tail vein. Preliminary results show the lowest T2 values and highest average radiance after 2h with respectively MRI and FLI indicating highest RGD-MLs accumulation in the tumor site after 2h. After 24h the signals had decreased back to baseline on FLI and T2 values were increasing. *Ex vivo* FLI after 48h showed a perceptibly higher signal in the tumors of animals injected with RGD-MLs compared to control animals.

MLs were successfully functionalized with a cyclic RGD peptide and previously we have shown that they can be used for cell labeling (Garcia Ribeiro *et al.*, EMIM, 2016). In this study, our preliminary results indicate that RGD-MLs can be used for to target SKOV-3 xenografts in nude mice and that we can visualize their tumor uptake *in vivo* by both MRI and FLI. Therefore, the RGD peptide is a good ligand for liposome surface-modification with a potentially interesting application in cell culture and for targeted imaging. More experiments will be performed to confirm these results.

Acknowledgements
The research leading to these results received funding from the People Program (Marie Curie Actions) of the European Union's Seventh Framework Program FP7/2007-2013/ under REA grant agreement n°289932 and the IWT Strategic Basic Research Grant n°140061 from the Flemish Government.

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Nr 36. Evaluation of HER2 expression and amplification on CTCs using DEPArray analysis and sorting followed by FISH

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Introduction

In metastatic breast cancer (MBC), discordant expression levels of the human epidermal growth factor receptor 2 (HER2) have been noted between primary tumors (PT) and matched metastatic lesions. Reassessment of HER2 status during treatment decisions in patients with advanced disease might help to optimize outcome. Circulating tumor cells (CTCs) offer the potential to provide a repeatedly accessible source of tumor cells for the real-time assessment of actual tumor characteristics. However, little is known on the concordance of HER2 expression on CTCs measured by immunofluorescence and the amplification status.
Here we report on a preclinical study, using five spiked breast cancer cell lines, comparing semi-quantitative HER2 scoring on CellSearch (Riethdorf 2010) with objective DEPArray analysis, and subsequent FISH analysis on DEPArray-sorted tumor cells. At the moment these data are also being generated for 10 patient CTC samples. Expression and amplification status of CTCs will be compared with primary tumor tissue.

Materials and methods

MDA-MB-436, MCF-7, BT-20, KPL-4, and SKBR3 cells (increasing HER2 status) were spiked into donor blood and subjected to CellSearch enrichment. HER2/FITC intensity was scored manually on the CellSearch analyzer. All cell lines were injected into the DEPArray and exposure settings were optimized (FITC: exposure time 800 ms, gain 5%). These settings are further used for all preclinical and clinical samples. HER2 scoring was based on relative fluorescent units (rfu) of the HER2/FITC signal with background subtraction. Cells were sorted into pure batches of HER2 positive (DAPI+/CK+/HER2+/CD45-) and negative (DAPI+/CK+/HER2-/CD45-) tumor cells. Cytospins were formalin fixed and subjected to DAKO IQFISH.

Results

HER2 expression on CellSearch turned out to be very heterogeneous within the same cell line. DEPArray data was highly reproducible for all cell lines ($p<0.001$) and also showed a broad range of FITC rfu within the HER2 positive cell lines. Significant differences were observed between every cell line ($p<0.001$). The SKBR3 cell line sample also harbored a minor population of HER2- cells while this was the most positive cell line. However with FISH analysis, both HER2- and HER2+ SKBR3 cells were highly amplified (absolute HER2 count of 12-20 and HER2/CEN17 ratio of >4). MDA-MB-436 and MCF-7 cells showed no gene amplification on FISH, while in KPL-4 there was a HER2/CEN17 ratio of >2.
Four patient samples with HER2 positive status on CellSearch have been run on the DEPArray. For patient 1, 1005 CTC were analyzed, 32.4% were HER2+. This was 53 (69.8% HER2+), 352 (5.7% HER2+), and 622 (6.7% HER2+) for patient 2-4 respectively. These numbers are comparable with CellSearch analysis.

Discussion

HER2 expression analysis by immunofluorescence is comparable between CellSearch and DEPArray, however DEPArray has the advantage that it is user-independent and highly reproducible. Furthermore, CTCs can be sorted into pure batches for downstream analysis. The FISH technique on DEPArray sorted cells is now optimized and will be used to determine the correlation between the immunofluorescent HER2 scoring and the actual amplification status of the CTCs. These data will be incorporated prior to upcoming BACR.

Nr 37. Senescence (SA-β-Gal) is induced in melanoma cells that are sensitive or intrinsically resistant to the ^{mut}BRAF inhibitor Vemurafenib, while it is reversed in those made resistant and addicted to the drug

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Abstract

Background: Targeting MAPK pathway in melanoma with the specific BRAF inhibitor, vemurafenib, showed robust initial responses of variable duration in the majority of patients with oncogenic BRAF tumors followed by relapses due to acquired resistance to the drug. In ^{mut}BRAF melanoma cells, senescence-associated β-galactosidase activity (SA-β-Gal) is often encountered in a constitutive manner or induced after MAPK inhibition. This senescence-like process in in the context of resistance to BRAF inhibition is not fully understood yet.

Methods: We aimed to evaluate the effect of the presence of ^{V600}BRAF and its specific inhibition on SA-β-Gal in melanoma cells by comparing MAPK/AKT and pRB/Cyclin D1 pathways, cell proliferation, cell morphology and β-galactosidase activity using a large panel of ^{V600}BRAF melanoma lines vemurafenib-sensitive, or with -intrinsic and -acquired resistance to the drug.

Results: We found that hyperactivation of MAPK pathway in ^{V600}BRAF cells is associated with low proliferation, high β-galactosidase activity and high cell volume in comparison with ^{WT}BRAF melanoma cells. Interestingly, vemurafenib exacerbated SA-β-Gal activity in 4 out of the 5 sensitive lines and in 2 out of the 5 lines with intrinsic resistance. Interestingly, 2 out of 3 lines with acquired resistance became addicted to vemurafenib. In the absence of the drug, they showed a substantial increase in SA-β-Gal together with a lower proliferation both in vitro and in vivo. The observed senescence (SA-β-Gal) upregulation was associated with pAKT and Cyclin D1/ pRB inhibition.

Conclusion: SA-β-Gal is promoted after BRAF inhibition in sensitive and intrinsically resistant melanoma cells while it is inhibited in those with acquired resistance showing drug addiction in vitro and in vivo.

Nr 40. Evaluation and optimization of single cell RNA-sequencing methodologies in circulating tumour cells from patients with advanced prostate cancer

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Background:
Androgen deprivation therapy (ADT) remains the standard-of-care for patients with advanced prostate cancer. However, *de novo* or acquired therapy resistance is an ever recurrent theme in the treatment of these patients, which ultimately leads to lethal castration-resistant disease (CRPC). Recently, the presence of androgen receptor (AR) splice variant 7 (AR-V7) was associated with resistance to novel endocrine therapies, i.e. abiraterone acetate and enzalutamide. However, not all AR-V7 negative patients respond to therapy, which emphasizes the need for novel predictive biomarkers. Biopsies of metastatic sites as a means for real-time tumor biomarker analysis is often impossible, mainly due to bone-limited disease. Hence, the molecular analysis of purified circulating tumour cells (CTCs) would allow for safe, real-time and repeatable biomarker assessment. Here, we want to develop a single cell RNA-sequencing (scRNA-seq) workflow, which allows expression profiling of CTCs from patients with CRPC. In this proof of concept study we assessed the performance of Ampli1™ whole transcriptome amplification (WTA) (Silicon Biosystems (SB), Bologna , IT) by quantitative real-time polymerase chain reaction (qPCR).
Methods:
Live and unfixed LNCaP and PC-3 prostate cancer cells were stained with Hoechst and subsequently isolated as single cells or small pools of cells using the DEPArray system (SB, Bologna, IT). Upon cell isolation, RNA from 23 single LNCaP and 22 single PC-3 cells was extracted and subjected to WTA. Quality of purified cDNA libraries was assessed by a multiplex PCR using the Ampli1™ WTA QC Kit (SB). Additionally, single cell cDNA libraries were pre-amplified (TaqMan PreAmp, Applied Biosystems, Foster City, CA, USA) for five marker (*EPCAM*, *AR*, *AR-V7*, *AR-V3* and *CD44*) and three reference genes (*HMBS, GUSB, HPRT1*). Pre-amplified samples were subjected to qPCR to evaluate RNA integrity after WTA.
Results
Preliminary analysis at moment of submission suggests that single cell expression profiling by qPCR upon Ampli1^{TM} WTA is feasible, with amplification and identification of various RNA transcripts. LNCaP cells, known to be an AR-positive cell line, demonstrated abundant expression of full-length AR transcripts. As expected, in contrast to LNCaP cells, PC-3 cells demonstrated CD44 expression. Final analysis results will be presented at the conference in January 2017.
Conclusions:
Our single cell workflow allows downstream transcriptional analysis, which will be further evaluated by scRNA-seq.

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Nr 44. Inflammatory breast cancer cells have a specific SMAD3-independent transcriptional program associated with MYC overexpression after TGF-β exposure.

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Background: Inflammatory breast cancer (IBC) is a rare form of locally advanced breast cancer with a high mortality rate. It is perceived as a very aggressive cancer subtype with an increased metastatic potential. Histologically, IBC is characterized by the presence of numerous tumor emboli (TE) in the dermal and parenchymal lymph vessels. These TE are thought to be functionally involved in the biology of IBC. We have previously demonstrated the role of an altered TGF-β signaling both in IBC preclinical models and in clinical IBC samples. In addition, we have evidence indicating that the mechanisms potentially involved in deregulating TGF-β signaling in IBC is particularly active in TE.
Goal: Our aim was to unravel the underlying mechanism of altered TGF-β signaling and its role in tissue invasion in the metastatic progression of IBC.
Methods: We performed RNA sequencing on a panel of IBC and non-IBC cell lines after TGF-β1 exposure at either 0hrs, 1hr, 4hrs or 14hrs. Next, immunohistochemistry was done for Smad3 in IBC and non-IBC patient samples. Protein data were then integrated with gene expression data from the same patient samples.
Results: RNA sequencing revealed a SMAD3-dependent, transcriptional program in non-IBC cells, while in IBC cells, target genes of MYC were found to be overexpressed. These results were confirmed using patient samples, in which a significant attenuation of the SMAD3 nuclear expression was observed in IBC compared to non-IBC samples. Integration of SMAD3 protein and gene expression data then showed that the absence of SMAD3 activity in samples from patients with IBC correlates with MYC transcriptional activity, whereas SMAD3 activity in samples from patients without IBC is associated with non-classical TGF-β signaling, mediated by p38MAPK and ATF2. Further analysis of TGF-β- induced genes in IBC cells revealed an enrichment of YY1 target genes.
Discussion: In this study we identified several genes that might potentially contribute to TGF-β deregulation in IBC, i.e. SMAD2, SMAD3, MYC, ATF2 and YY1. To further confirm previous observations we are currently performing follow-up experiments. Therefore we again treated nine cell lines (i.e. 4 IBC, 4 non-IBC and 1 normal) with TGF-β for 0hrs, 1hr, 4 hrs and 14hrs. Each of these samples were then subjected to RNA, DNA and whole cell lysate extraction and subsequently all samples were prepared for signal transduction network analysis. First we plan to perform RT-PCR on the genes possibly driving altered TGF-β signaling. Second, protein expression of the same genes will be assessed using Western Blot and last, the activity of tyrosine or serine/threonine kinases will be measured using the PamGene system. By combining all these techniques, we hope to have a better global understanding concerning signal transduction in IBC.

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ABSTRACT BACR

Nr 46. Mutational Landscaping of Liver Metastases with Desmoplastic and Replacement Growth Patterns

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Abstract *(500 words maximum)*

Objective – To characterize the mutational landscape of colorectal liver metastases with distinct growth patterns.

Background – Different histological growth patterns (HGP) have been described and reflect the biological heterogeneity of liver metastases (LM)[1]. In the desmoplastic HGP tumor cells are separated from the liver parenchyma by a layer of desmoplastic stroma containing new blood vessels resulting from sprouting angiogenesis. In the replacement HGP, the tumor cells replace the hepatocytes in the liver cell plates thereby co-opting sinusoidal blood vessels as a means of blood supply. Recent research has confirmed that vessel co-option mediates resistance to anti-angiogenic therapy in LM[2]. To get an insight in the differences between the two distinct HGP, molecular characterization is needed.

Methods – Twenty-two colorectal liver metastases (ten desmoplastic and twelve replacement) and adjacent normal liver tissue were sampled at the tumour-liver interface. Extracted RNA was converted in cDNA libraries using the Truseq stranded mRNA kit and sequenced on a Hiseq 1500 (CMG Antwerp) using 2x100bp paired-end sequencing. After quality trimming, sequencing reads were aligned with the STAR aligner. Variant calling was performed using Varscan2 and annotated using Annovar and the dbSNP144, COSMIC v77, 1000 Genomes and ClinVar databases. Multiple filters were used: only exonic, non-synonymous mutations that were not present in 1000Genomes, that were present in COSMIC and that were pathogenic were included. Variants with a total depth of ≥ 20 and a variant read support ≥ 3 were kept.

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Results – A mean of 40020 (range 9573-53150) variants were picked up in the twenty-two metastatic samples with no significant differences between the desmoplastic and replacement HGP liver metastases. A total of 20 pathogenic significant mutations were picked up in 14 of 22 samples (range 0 – 3). These SNV's occurred in the APC, KRAS, PIK3CA, SMAD4 and TP53 genes and are in line with current published literature of metastatic colorectal cancer. KRAS status of the primary tumor or metastasis was known in 7 LM and results were concordant in 6/7 (85%). None of the mutations are shared between LM with distinct HGP at the moment. Gene expression data of the same LM was used to infer the consensus molecular subtype (CMS) subtype[3]: 7 LM were CMS4, 6 CMS2 and 1 CMS1. KRAS mutations were observed more in CMS4 group, whereas TP53 and APC mutations were observed more in the CMS2 group. These results concur with the CMS paper.
Further integration of gene expression data will be performed. Additional samples are currently being analysed and results will be presented at the BACR meeting in January 2017.

Conclusion – Mutational profiling of colorectal LM with distinct HGP showed a total of 20 relevant mutations in the APC, KRAS, PIK3CA, SMAD4 and TP53 genes. These results are concordant with current literature and accentuate the feasibilty of variant calling on RNA-seq samples.

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Nr 48.

GATA3 expression in ductal carcinoma in situ of the breast: a potential prognostic marker for recurrence in DCIS?

Introduction: Ductal carcinoma in situ (DCIS) is a breast lesion with uncertain malignant potential. Myxoid stroma has been proposed as a prognostic marker for local ~~invasive~~ recurrence. A microarray showed reduced expression of GATA3 in DCIS with myxoid stroma. GATA3 is a growth factor that induces growth and differentiation of luminal epithelial cells in normal breast tissue. In this study, myxoid stroma was used as a surrogate prognostic marker. The aim of this study was to validate this association at the protein level. Therefore, expression of GATA3 was assessed immunohistochemically and its association with stromal architecture was investigated.
Materials and methods: Eighty-one patients, surgically treated for DCIS at Ghent University Hospital between 2012 and 2015, were included in the study. Patients with microinvasive tumors or a history of ipsilateral invasive breast cancer were excluded. All available hematoxylin & eosin, as well as immunohistochemically stained slides were evaluated by two observers. Statistical analysis was performed using SPSS. Chi square test and logistic regression analysis were applied to assess the association between stromal architecture and GATA3 expression in DCIS as well as the following morphological characteristics; nuclear grade, periductal inflammation, comedonecrosis, growth pattern, the presence of calcifications and tumor size.
Results: Univariate analysis showed that attenuated GATA-3 expression was more frequently observed in DCIS with surrounding myxoid stroma (73%) compared to sclerotic stroma (38%) (P=0,006). DCIS with myxoid stroma presented with larger tumor size, high nuclear grade, moderate to extensive periductal inflammation and extensive comedonecrosis.
Conclusion: GATA-3 loss was more frequently seen in association with myxoid stromal architecture, which enabled us to translate the previous findings from the RNA level to the protein level. A decrease in GATA3 expression has been observed in invasive breast tumors with poor differentiation and high invasive and metastatic potential. It also seems to play a role in the immune response of the tumor micro-environment. GATA-3 expression has not yet been studied in depth in DCIS. Further studies are needed to elucidate the role of GATA-3 in breast cancer progression, and to explore its role as a prognostic marker for DCIS recurrence.

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BACR 2017 ABSTRACT

Nr 50.

EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research

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Introduction

While the first observations of cell-derived vesicles were reported almost 50 years ago, only in the last decade has there been a rapid increase in the number of studies on physiological and pathophysiological functions of extracellular vesicles (EVs). The large number of functional studies on EVs is contrasted by a minority of publications that focus on defining the characteristics of different subtypes of EVs and optimizing EV purification and analysis strategies. Paradoxically, despite limited basic knowledge on EV biology, a vast array of functional effects have been described as EV-specific. Standardized and transparent reporting is needed to improve the rigor and facilitate interpretation of EV research.

Methods

We performed a PubMed search for research articles with keywords “exosomes” or “extracellular vesicles” published in 2010-2015. After manual inspection, 1226 articles were withheld for inclusion. Publications that included multiple sample types and/or isolation methods were separated into multiple entries, which resulted in a total of 1742 experiments. Experiments were analysed based on a matrix containing 115 parameters comprising article content, aim, and EV isolation and characterization procedures.

Results

To assess current practice in EV experiments, we performed an in-depth analysis of the recorded data in the EV-TRACK knowledgebase. This revealed widespread heterogeneity in EV isolation methods and inconsistent implementation and reporting of experimental parameters. Isolation methods increasingly comprise commercial kits (15% in 2014) at the expense of density gradients (10% in 2014). Differential ultracentrifugation is by far the most used method (>50%), but with a large heterogeneity in centrifugation steps. Quality controls are often omitted, with more than 2 protein markers being checked in 40% and non-EV enriched proteins in less than 15% of experiments (dependent on sample type). From these analyses, we extracted a set of 9 relevant but frequently unreported experimental parameters that we condensed into a single metric, called the EV-METRIC (to MEasure Transparent Reporting of Isolation and Characterization methods). It represents a checklist to assess the completeness of reporting of generic and method-specific information necessary to interpret and reproduce the experiment. Less than 6% of experiments have a EV-METRIC of 50% or more. We established the crowd-sourcing EV-TRACK platform as a next step towards increasing experimental rigor, enhancing biological knowledge, and creating timely and mature minimal information checklists in EV research. It is available at http://evtrack.org.

Summary/conclusion

We are confident that the EV-TRACK platform will contribute to the maturation of EV research by guiding and informing EV researchers as well as tracking the evolution of the field.

Nr 51. Clinical drug auranofin enhances tumor radioresponse through targeting redox system

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Background and purpose:

Auranofin (AF) is an anti-arthritic drug considered for combined chemotherapy due to its ability to impair the redox homeostasis in tumor cells. In this study, we want to explore whether AF may in addition radiosensitize tumor cells by targeting thioredoxin reductase (TrxR), a critical enzyme in the antioxidant defense system. Our principal findings in murine 4T1 and EMT6 tumor cells as well as human colon rectal cancer cell HCT116 are that AF at 3-10 μM is a potent radiosensitizer in both aerobic and hypoxic conditions. At least two mechanisms are involved. The first one is linked to an oxidative stress, as scavenging of reactive oxygen species (ROS) by N-acetyl cysteine counteracted radiosensitization. We also observed a decrease in mitochondrial oxygen consumption. The combination of AF and buthionine sulfoximine (BSO) was shown to significantly improve tumor radioresponse in tumor-bearing mice.

Methods:

Murine mammary carcinoma cancer cell lines 4T1 and EMT6, and human colon rectal cell line HCT116 were exposed to AF and/or BSO for 30 min and 16 hours respectively at various concentrations. To scavenge ROS production, n-acetyl cysteine (NAC) was applied for 1 hour before AF and/or BSO. To induce hypoxia, murine cells were placed in a tissue-mimetic culture system with diffusion-limited oxygenation; HCT116 cells were trypsinized and centrifuged into micro-pellet. Tumor cell survival after radiation was measured by colony formation assay, and an enhancement ratio was calculated at a surviving fraction of 0.1. BALB/c mice were inoculated intramuscularly into the left hind limb with 4T1 cells (0.5×10^6) and 4 days later randomized with 6 mice/group, AF (3 mg/kg) and/or BSO (25 mg/kg) were administrated subcutaneously from day 4 to 8 and day 11 to 15.

Results:

In this study we examined the hypothesis that the anti-arthritic drug AF may be repurposed for radiotherapeutic applications and undertook the first step to evaluate its radiosensitizing potential in different tumor cells. Our fundamental findings are that AF at 3-10 μM increases tumor cell radiosensitivity *in vitro* over 2-fold. Besides that, BSO at 1 μM could considerably potentiate the radiosensitizing effect of AF at 2.5 μM, a

sub-optimal dose that did not induce radiosensitization. Finally in the 4T1 tumor bearing mice, radiation alone (15Gy) delayed tumor growth by 6 days measured at a tumor volume of 1000mm^3. AF combined with BSO enhanced tumor radioresponse resulting in a tumor growth delay of 13 days, while neither of these pharmaceuticals was effective when administered in their own. These experiments pointed to the necessity of dual targeting of the TrxR/GSH systems by the combination of AF and BSO.

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Nr 53. Targeting mitotic-exit phosphatases for cancer therapy

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Anti-mitotic drugs of the taxane, epothilone and vinca-alkaloid families are widely used for the treatment of various types of cancer. These molecules kill cancer cells by interfering with microtubule dynamics, resulting in the chronic activation of the mitotic spindle assembly checkpoint (SAC) and cell death. Unfortunately, the clinical use of these drugs is hampered by serious side effects and the development of resistance. The development of new drugs against novel mitotic signaling pathways has the potential to alleviate these problems through synergistic combination therapies. An extremely important signaling mechanism in mitosis involves the reversible (de-)phosphorylation of proteins by protein kinases and phosphatases. Small molecule inhibitors of important mitotic kinases have already been developed and several of them are under clinical investigation. The major mitotic phosphatases are Cdc25, PP1 and PP2A. However, these phosphatases are still largely neglected in drug discovery efforts, mainly due to real or expected difficulties in developing specific inhibitors. The catalytic subunits of PP1 and PP2A form many different holoenzymes with a diverse array of regulatory subunits that determine when and where these phosphatases act. Each of these holoenzymes has a limited set of substrates and can be specifically targeted by interference with substrate recruitment or holoenzyme assembly. This can be achieved with small molecules that inhibit protein-protein interactions, and has been shown recently for PP1-GADD34. We aim to inhibit the PP1-Sds22 holoenzyme, which has an essential role in the progression of mitosis. Interference with the function of PP1-Sds22 prevents accurate chromosome segregation and induces a chronic activation of the SAC, which possibly works synergistically with taxane, epothilone or vinca alkaloid drugs to kill cancer cells. Our efforts are directed towards the screening for small molecules that prevent PP1-Sds22 holoenzyme formation. On the one hand, we have developed a cellular target-based screening approach that has been used to successfully identify inhibitors in small compound collections. The assay has been optimized up to 384-well format and will allow us to do large-scale screenings. On the other hand, we have recently solved the crystal structure of Sds22 and have successfully built and validated a PP1-Sds22 holoenzyme model. This will allow us to use innovative approaches for the development of protein-protein interaction inhibitors, such as fragment-based drug design.

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Nr 55.

TEMOZOLOMIDE-RESISTANT GLIOBLASTOMA CELLS ARE GLYCOLYTIC AND ESCAPE CELL CYCLE CHECKPOINTS

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Gliomas are characterized by dismal prognosis, with glioblastoma (GBM) representing the ultimate grade of malignancy. The current standardized clinical protocol includes maximal surgical resection followed by radiotherapy and concomitant and adjuvant temozolomide (TMZ) chemotherapy. TMZ is an orally available alkylating agent that was initially developed for brain cancer therapy based on its ability to freely cross the blood-brain barrier. It is a prodrug, the conversion/activation of which is entirely pH-dependent and does not require enzymatic activation. Indeed, TMZ is stable/inactive at acidic pH but decomposes to monomethyl-triazeno-imidazole-carboxamide (MTIC) at pH > 7. MTIC is stable/inactive at alkaline pH but fragments to produce methyldiazonium ion, the active compound, at pH < 7. Metabolism influences both intra- and extracellular pH. Most aggressive cancer cells are usually glycolytic, and a glycolytic metabolism generally accounts for a slighly alkaline intracellular pH and an acidic extracellular pH. Therefore, we aimed to understand whether metabolic changes could provide resistance to TMZ in GBM. We generated isogenic TMZ-resistant and TMZ-sensitive T98G (T98G-R and T98G-S) and U373 (U373-R and U373-S) human glioblastoma cell lines. Their comparison revealed that TMZ-resistant cells have higher aerobic glycolytic and acidification rates and display a high normoxic activity of transcription factor hypoxia-inducible factor-1 (HIF-1) compared to TMZ-sensitive cells. TMZ-resistant cells did also overexpress MGMT, a methyltransferase that can induce resistance to TMZ by specifically demethylating O6-MeG adducts on DNA. However, we excluded a major involvement of glycolysis and MGMT in the acquired resistance of the two cell lines to TMZ. Compared to sensitive cells, resistant cells showed a significantly higher basal level of DNA damage and the capability to overcome cell cycle checkpoints even in the absence of TMZ, which we believe could account for resistance as the cells did not undergo a block in proliferation following TMZ treatment. Altogether, our data thus indicate that acquired resistance to TMZ in glioblastoma is independent of aerobic glycolysis and MGMT expression. TMZ-resistant cells are capable to escape cell cycle checkpoint blockage and therefore proliferate even when they undergo DNA damage.

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Submitted for an oral presentation

Nr 57. Title: "Characterization of a novel liquid fiducial marker for organ motion monitoring in prostate SBRT"

Robin De Roover[1*], Wouter Crijns[2], Kenneth Poels[2], Ronald Peeters[3], Karin Haustermans[1,2], Tom Depuydt[1,2]

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Purpose

Stereotactic body radiotherapy (SBRT) for prostate is a cost-effective treatment option with improved patient comfort and maintained excellent clinical outcomes. However, to ensure low levels of toxicity very accurate delivery is imperative, especially when combined with integrated focal boosts as in the Hypo-FLAME clinical trial methodology. Within this context, intra-fraction organ motion management becomes even more relevant. The novel BioXmark® (Nanovi A/S) biodegradable radio-opaque liquid fiducial marker was studied as alternative for current markers used in prostate motion management. The marker can be injected with very thin needles (down to 25G) and the injection procedure allows to vary the marker-size by altering the injected volume. In this study the automatic detectability of BioXmark® in 2D kV X-ray imaging was determined. Additionally, as Hypo-FLAME involves a multi-modality delineation of the boost foci, visibility/artefacts in different types of volumetric imaging was investigated.

Materials and Methods

BioXmark® consists of sucrose acetate isobutyrate (SAIB), iodinated-SAIB and ethanol solution. Upon injection, ethanol diffusion out of the solution causes a viscosity increase and formation of a gel-like marker. A total of 8 markers (size 5-300 µL) organized in a rectangular grid were injected into a gelatin phantom.

X-ray projection images using the Varian TrueBeam STx OBI were obtained by putting the gelatin phantom on top of an anthropomorphic pelvic phantom. A total of 120 images of each marker were acquired varying the positions of the marker relative to pelvic bony structures and using 24 clinically relevant X-ray kVp/mAs settings. Volumetric imaging was performed with CT, CBCT and MRI using a CIRS pelvic phantom.

Automated marker detection was based on the normalized cross-correlation (NCC) of the projection image with a marker template retrieved from the CT image. Prior to detection, single markers were artificially isolated to minimize interference between detection of the different markers. Reference marker positions were manually determined on the image with highest exposure settings. A detection was successful if the optimal NCC value lied within a 1 mm (3 pixels) tolerance of the reference position. The tolerance was extended to 4 pixels to deal with the uncertainty of manual delineation.

Results

Detection success rates augmented with increasing marker-size obtaining a maximum for intermediate size (25-75 µL) markers (Figure 1). Larger marker sizes (>75 µL) had decreased detection success rates due to higher susceptibility for interference with the bony structure edges. Volumetric image artefacts are minimal whilst the markers itself are clearly visible (Figure 2).

Conclusion

Intermediate size (25-75 µL) BioXmark® liquid fiducial markers show high detectability and minimal image artefacts making them a patient friendly alternative (thin needles) for the current markers used in fiducial-marker-based intra-fraction organ motion monitoring in prostate SBRT.

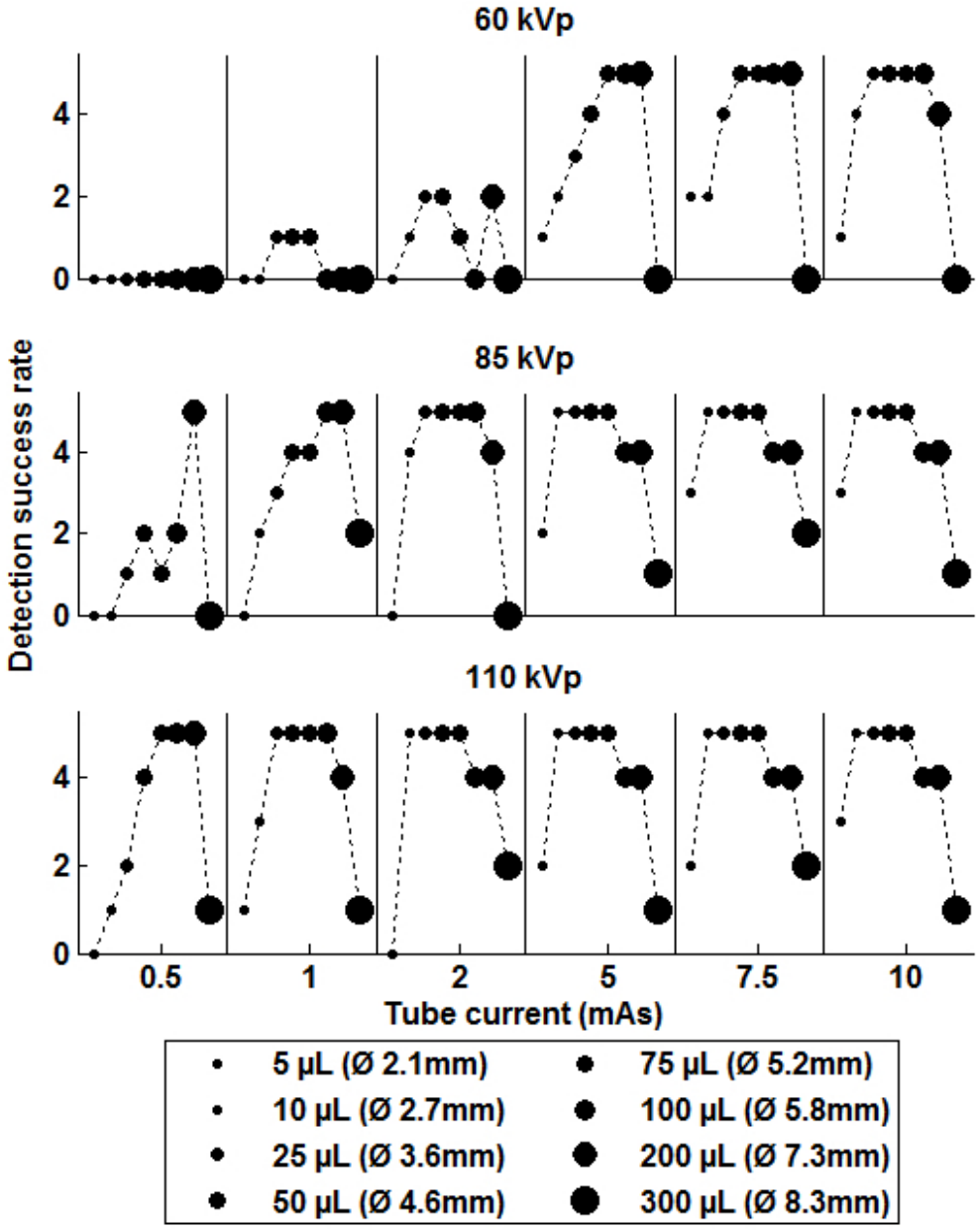

Figure 1: detection success rate (range 0 – 5) of the different marker sizes ordered in terms of ascending volume for the different kVp/mAs image settings. One can clearly observe an increase in detection success rate with increasing marker size until a maximum value is reached for markers of intermediate size (25-75 µL). Larger markers (> 75µL) are more prone to interference with bony structure edges resulting in lower detection success rates. The results for 15 mAs and 20 mAs have been excluded as they are identical to the results for 10 mAs and offer no new information.

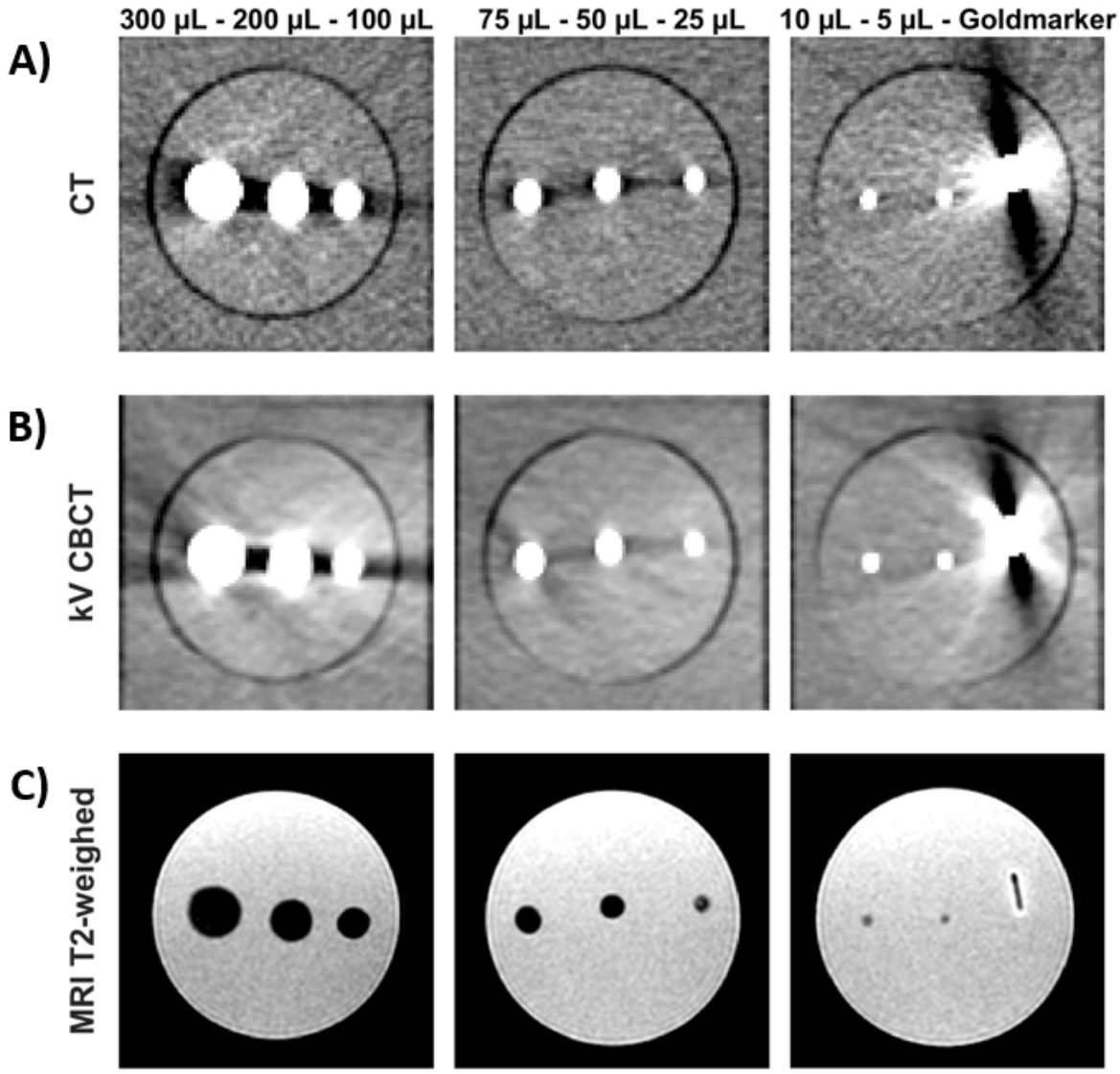

Figure 2: overview of the visibility and volumetric imaging artefacts for the different imaging modalities employed in this study. A to C: shows transversal slices along the center of the markers for CT (120 kVp, AEC), CBCT (125 kVp, 1080 mAs) and MRI (1.5 T, T2-weighed) ordered in terms of declining marker volume. A cylindrical gold marker (7 mm length, 1 mm diameter) is shown as a qualitative reference. Volumetric imaging artefacts on CT and CBCT are minimal for small and intermediate size markers (< 75 µL) whereas no imaging artefacts are visible on T2-weighed MRI.

Nr 58. Evaluation of Z-VAD-FMK as an anti-apoptotic drug to prevent granulosa cells apoptosis and follicles death after human ovarian tissue transplantation

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Removal and cryopreservation of ovarian strips before gonadotoxic treatments, followed by their autotransplantation after cancer remission, have been successfully used to re-establish female fertility. However, the pregnancy rate after the complete procedure is about 30%. Indeed, the main problem after grafting is follicular loss due to ischemic reperfusion injury. Our study was designed to evaluate the use of the pan-caspase inhibitor Z-VAD-FMK to prevent follicle loss.

In vitro experiments were performed with human granulosa cells (HGL5 cell line and primary cells isolated from follicular fluids). Cells were cultured with Z-VAD-FMK (50µM) with or without $CoCl_2$ (500µM) inducing chemical hypoxia. Metabolic activity tests (WST1) and flow cytometry analyses indicated that Z-VAD-FMK protects HGL5 cells against chemically induced hypoxia. Metabolic activity and viability of primary granulosa cells were not affected by Z-VAD-FMK. However, western blot revealed that Z-VAD-FMK prevents $CoCl_2$ induced PARP cleavage, which is a major substrate of caspase and therefore a valuable apoptotic marker.

For the *in vivo* part of the study, human ovarian cortex fragments encapsulated in collagen gel containing Z-VAD-FMK or not were stitched onto SCID mice ovaries. Mice were sacrificed 3 days or 3 weeks after transplantation. Morphology, apoptosis, proliferation, hypoxic areas, revascularisation and follicular density were evaluated after recovery of the transplanted fragments. When ovarian cortex was grafted in the presence of Z-VAD-FMK, no improvement of follicular pool and global tissue preservation was observed in fragments recovered three days post-grafting. Conversely, after three weeks of transplantation, the mean number of primary follicles was increased in fragments treated with Z-VAD-FMK. This improvement was associated with a decreased percentage of apoptosis in the tissue. We also observed that proliferation was always detected in areas without hypoxia, as revealed by pimonidazole staining.

In summary, our *in vitro* results clearly indicate that Z-VAD-FMK is able to limit HGL5 cell death and partially reverse the PARP cleavage induced by $CoCl_2$ treatment. Inclusion of this caspase inhibitor in the collagen matrix during *in vivo* transplantation of ovarian cortex slightly improves primary follicular preservation and reduces global apoptosis only after three weeks of transplantation.

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Nr 60. Function of Granzyme B in myeloid-derived suppressor cells

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For a long time, only NK cells and cytotoxic T lymphocytes (CTLs) were considered to express granzyme B (GzmB). However, in recent years, several publications reported the expression of GzmB in other cell types as well, for example in mast cells and neutrophils, both in mice and human. A large variety of myeloid-derived suppressor cells (MDSCs)-mediated immune suppressive functions are already described, however there could still remain several unrecognized mechanisms by which MDSCs can counteract the immune system and generate pro-tumoral microenvironment. In our culture system that exists of *in vitro*-generated MDSCs, we have found a high GzmB expression and an increase in perforin. Perforin together with GzmB is a key component of the lytic machinery of CTLs, leading to activation of caspase-3, which results in apoptosis of the target cell. However evidence of perforin-independent functions of GzmB also exist, such as inflammation and involvement in extracellular matrix degrading by the cleaving of extracellular proteins. Similar observations with regard to GzmB and perforin expression, were made in MDSCs isolated from spleen and tumor from tumor-bearing mice (in breast, lung and colon cancer models). *In vitro* functional assays did not yet reveal any contributions of GzmB to MDSCs immunosuppressive function, possibly because of the lack of environmental perforin, however GzmB activity assays show functional GzmB expression in MDSCs. There is no evidence yet about the exact mechanisms in which GzmB contributes to the MDSC biology and consequent immune suppression, but we might have discovered a new therapeutic target to dampen MDSC responses and thereby facilitate the introduction of immunotherapy.

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Nr 62. Cold atmospheric plasma treatment of melanoma and glioblastoma cancer cells

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Cancer is the second leading cause of death worldwide and treatments are in many cases still harsh for patients or ineffective. In this regard, plasma oncology is emerging as a possible new treatment modality. Plasmas are ionized gases which can be seen as a reactive chemical cocktail consisting of electrons, ions, neutral species (including radicals or excited species), and photons. It is believed that the reactive oxygen and nitrogen species (RONS) are key players in the interaction between plasma and cells. In this work, two types of melanoma and glioblastoma (GBM) cancer cell lines are treated with cold atmospheric plasma (CAP) to assess the effect of several parameters on the cell viability.

Two melanoma (Malme-3M, SK-MEL-28) and two GBM (LN229, U87) cell lines were used as target cells. The COST-action reference micro-atmospheric pressure plasma jet (μ-APPJ) was used as plasma source. Plasma was generated using 230V and 13.56MHz for two different gas mixtures at a flow rate of 1.4L/min. The μ-APPJ was placed 8mm above the surface of the treated sample. Direct plasma treatment was compared with an indirect treatment method using plasma-activated medium (PAM), which was transferred onto the cells. Plasma treatment duration ranged between 1-11min and samples were analysed after 24h and 72h. Cell viability was flow cytometrically assessed using AnnexinV (AnnV) and propidium iodide (PI).

The cell viability decreases with treatment duration and time until analysis in all cell lines with varying sensitivity. The majority of dead cells stains positive for both AnnV and PI, indicating that the plasma-treated non-viable cells are mostly late apoptotic or necrotic. Genetic mutations might be involved in the response to plasma. Comparing the effects of two gas mixtures, as well as indirect PAM versus direct treatment, gives different results per cell line.

In conclusion, this study confirms the potential of plasma for cancer therapy and emphasizes the influence of experimental parameters on therapeutic outcome. In our recent paper, we further elaborate on experimental parameters to take into account when designing plasma oncology experiments.

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Nr 63. ABSTRACT BACR 2017

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Expression of the small GTPase Rab27B is associated with stromal inflammation in ductal carcinoma in situ of the breast.

Aims. Ductal carcinoma in situ (DCIS) is considered to be a non-obligate pre-invasive precursor of invasive ductal carcinoma. Some DCIS present with a periductal inflammatory infiltrate in the stroma, but the etiology of this stromal inflammatory response is currently unclear. Rab27B is a small GTPase that is involved in the release of exosomes, i.e. small intraluminal vesicles that are released upon fusion of multivesicular endosomes with the plasma membrane. Rab27B is upregulated in invasive breast cancer, but its role in early breast cancer progression and the tumor immune microenvironment is still relatively unexplored. The aim of this study was to investigate Rab27B expression in DCIS, as well as its relation with stromal inflammation.

Materials and methods. Histopathological features were analyzed by two pathologists in a cohort of 71 DCIS patients. Immunohistochemistry was performed on whole mount slides to assess hormone receptor status and Rab27B expression. HER2 amplification status was determined by dual-probe fluorescence in situ hybridization (FISH) analysis. Multivariate logistic regression analysis was performed in SPSS to analyze which histological characteristics were associated with stromal inflammation.

Results. Thirty-five (49%) DCIS presented no or mild stromal inflammation, and 36 (51%) showed moderate to extensive inflammation in the periductal stroma. In multivariate analysis, high nuclear grade (p=0.005), HER2 amplification (p=0.002) and high Rab27B expression (p=0.007) were independently associated with the presence of moderate to extensive stromal inflammation in DCIS.

Conclusions. DCIS with moderate to extensive stromal inflammation present more often with high Rab27B expression, independently of HER2 amplification status and nuclear grade. Since Rab27B is involved in vesicle trafficking and exocytosis, secretory products of malignant epithelial cells might evoke a host inflammatory response in the tumor microenvironment. Additional studies are required to investigate this hypothesis, and to explore the prognostic potential of Rab27B protein expression for the biological behavior of DCIS. Future research should focus on the precise characterization of the stromal inflammatory infiltrate in DCIS, and explore the mechanisms by which this immune response is evoked.

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Nr 64

Title: HPV-related innate peptide inhibition promotes the acquisition of bacterial/fungal infections within the genital tract

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- Introduction : most anogenital (pre)cancerous lesions are etiologically linked to the infection with one or several carcinogenic (high-risk) HPV genotypes. Despite HPV E6/E7 oncoproteins are sufficient to immortalize infected cells, several exogenous and/or endogenous factors are presumed to positively influence HPV-related carcinogenesis. Among these latter, co-infection with other microorganisms (C. *trachomatis, N. gonorrhoeae,...)* have been extensively investigated and a higher prevalence of other sexually transmitted diseases (STDs) in case of HPV infection has generally been reported.

- Aims : In the present study, we tested the following hypothesis: by altering innate molecule secretion, HPV could also promote the acquisition of bacterial/fungal infections within the genital tract. Therefore, a crosstalk (positive association) between HPV and other STDs could exists.

- Methods and results : we first analyzed the expression of numerous antimicrobial peptides in both microdissected healthy and HPV-positive cervical biopsies as well as in several cell lines. Collectively, our results highlighted a significant down-regulation of several cationic peptides of the defensin superfamily (i.e. HβD2-4 and elafin) in HPV-infected cells. In order to investigated the implication of each viral oncoprotein on this alteration of the innate defense, primary keratinocytes were stably transfected with HPV16 E6 or E7 genes before being stimulated with TNFa or LPS. Interestingly, we noticed no increased expression of elafin and other antimicrobial peptides as well as a lack of IκB degradation in E7-transfected cells. These results were further confirmed by CHIP assay and *in situ* (anti-p65 immunostainings on a large cohort of tissue specimens).

- Conclusions : altogether, our preliminary data suggest that the down-regulation of catinonic peptides observed in HPV-infected cells would be linked to a E7-related inhibition of the NF-κB pathway.

Nr 66. ADJUVANT ADDITION TO CIDOFOVIR TREATMENT: EFFECTS ON TREATED PRIMARY AND UNTREATED SECONDARY HUMAN PAPILLOMAVIRUS POSITIVE TUMOR

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Introduction: Cidofovir (CDV) reduces the size of a human papillomavirus (HPV) positive tumor and of a secondary, untreated HPV positive tumor. However, the effect on the latter was less pronounced. Therefore, adjuvant (aluminum 0.5% + MPLA 0.05%) was added to CDV treatment to prolong and/or enhance the effects on the secondary tumor.

Methods: Nu NMRI mice were injected with SiHa cells, tumor 1 (T1), and tumor 2 (T2) was injected after three weeks of intratumoral treatment of T1 with CDV, PBS or no treatment, in combination or not with adjuvant. Flow cytometry analysis of immune cells (neutrophils, macrophages, B-cells, γδ-T-cells and NK-cells) in spleen, tumors and lymph nodes (LN) was performed. Levels of IgG and IgM in serum were evaluated by ELISA.

Results: Addition of adjuvant to CDV treatment did not reduce T1 size compared to CDV treatment alone. However, a slightly (but not significant) smaller tumor size was observed in the untreated T2 when adjuvant was added to CDV treatment compared to CDV treatment alone. Conform to the fact that adjuvant did not influence the size of T1, immune cells in T1 and in the spleen were not altered by adjuvant addition except for a higher percentage of macrophages in mice that received adjuvant. However, high percentages of macrophages, B-cells, γδ-T-cells and NK-cells in T2 of mice injected with adjuvant were observed compared to T2 of mice that did not receive adjuvant. In the LN of mice injected with adjuvant, higher percentages of γδ-T-cells were observed. Levels of IgG were increased in the serum of mice that received adjuvant while levels of IgM were not changed by adjuvant addition. IgG levels were increased in mice that had T2 and adjuvant injections in T1.

Conclusions: These results suggest that adjuvant inoculation in T1 prior to T2 injection functions like a vaccine. Adjuvant addition causes an increase in γδ-T-cells in LN and T2, where levels of NK-cells, B-cells and macrophages were also elevated.

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Nr 68. Immunohistochemical expression study of ATRX and DAXX, ALT suppressor proteins, in small cell lung cancer

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Introduction: Small cell lung cancer (SCLC) is a dismal disease, with high proliferative capacity suggesting the presence of, hopefully drugable, immortalization mechanisms. 15% of cancers use telomerase (TA) -independent Alternative Lengthening of Telomeres (ALT) immortalization mechanisms. ALT activation has been found to be robustly correlated with ATRX and/or DAXX inactivating mutations and consequent loss of nuclear protein expression. In this study we looked for the presence or absence of ALT in SCLC using ATRX and DAXX immunohistochemistry as surrogate marker.

Materials and Methods: FFPE material of 28 SCLC (16 primary tumors, 6 nodal end 6 visceral metastases) cases was stained using an anti-ATRX monoclonal antibody (Sigma, clone CL0537, dil. 1/200) and anti-DAXX polyclonal antibody (Sigma, product number HPA008736, dil. 1/200) on the Ventana BenchMark XT automated immunostainer (OptiView detection system). Nuclei of normal endothelium and immune cells served as positive controls for ARTX and DAXX. Nuclei of the U-2 OS ALT cell line served as negative controls in the ATRX group. At present no DAXX-negative cell lines are available. Staining results were categorized in 'ATRX/DAXX loss', 'ATRX/DAXX indeterminate' and 'ATRX/DAXX retained', corresponding to signal loss in >90% , 10-90% or <10% of tumor nuclei, respectively.

Results: 25/28 cases (89%), including 5 visceral metastases (liver, adrenal, spine) and 6 nodal metastases, were classified 'ATRX retained'. 1/28 cases (4%) were classified 'ATRX indeterminate' with 15% of nuclei entirely negative and variable signal intensity in positive nuclei. 2/28 (7%) cases showed 0% signal in tumor nuclei ('ATRX loss'), with 3+ signal in intratumoral normal endothelial nuclei. 21/28 cases (75%), were classified 'DAXX retained'. 8/28 cases (29%) were classified as 'DAXX intermediate', of those 1 case was also classified as 'ATRX indeterminate'. No complete loss of DAXX was seen. One 'ATRX loss' case showed concomitant loss of DAXX in extreme pleiomorphic nuclei of intratumoral giant cells, additionally serving as internal negative control.

Discussion and conclusions: Unlimited replicative potential is a hallmark of cancer. Tumor cell immortalization occurs either through re-activation of TA or by activation of ALT. Both processes can be neutralized using TA- (Jäger, Genes 2016, 7, 39) or ALT-inhibitors (Flynn, Science 2015, 347 (6219):273). Since ALT is not detected in normal cells, targeting ALT+ cancer cells would be of great interest as a potential new targeted therapy strategy, overcoming the toxicity issues met in TA-inhibitor administration. In order to predict tumor response of SCLC it is worthwhile dissecting immortalization processes in (mostly) small SCLC biopsies. Activity of TA can only be measured in fresh protein lysates, requiring large tumor volumes. ALT activation on the other hand can be detected

indirectly by (loss of) ATRX/DAXX expression studies. Solely based on ATRX-IHC this study suggests that the majority of SCLC (25/28 or 89%) might be TA-driven, also being concordant with its epithelial nature. 2 cases (7%) showed complete ATRX loss and will be screened for inactivating ATRX mutations. Interestingly, these two patients' age was well below the reported median age at time of diagnosis (56 years compared to 68 years as regularly reported). ALT-independency was further confirmed by positive DAXX immunohistochemistry, another ALT suppressor gene product. The occurrence of an ATRX-/DAXX- phenotype for the pleiomorphic nuclei deserves further biologic study. In 4/28 (14%) cases, as well for ATRX as for DAXX discernible nuclear signal intensity variation was present. It needs further study to find out which process gives rise to this heterogeneous and variable pattern (genetic or epigenetic changes?), and whether the observed variation equals tumor ALT-TA mosaicism necessitating the administration of a cocktail of ALT- and TA-inhibitors.

Nr 70. Biological model of cancer associated fibroblasts in colorectal cancer for therapies assessment

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The tumor microenvironment has impaired the successful outcome of several therapeutic approaches, including chemotherapy and immunotherapy. Particularly in colorectal cancer, cancer associated fibroblasts (CAF) have shown several adverse effects as an increase of the extra-cellular matrix density, a paracrine secretion of factors improving tumor growth and metastasis, and immune actors recruiting associated to a immunosuppressive environment[1].

Our purpose is to develop new nanomedicines which would specifically target and kill CAF population. It could impair tumor microenvironment and improve sensitivity to further immuno- or chemotherapies. To assess those experimental treatments, we develop a biological model allowing *in vivo* and *in vitro* evaluation of CAF. This model will help us to select the best drug to encapsulate in our nanomedicines, to test different ligands for targeting and entrapment, and to assess their *in vivo* effect.

The mouse colon carcinoma cell line CT26 was grafted subcutaneously on syngeneic BalbC mice. After the tumors reached a size >20mm³, mice were divided into three groups: the first group was treated with intratumoral tranilast, an anti-fibrotic agent, at the dosage of 20nmol every day[2]; the second group was treated with intratumoral NaCl as control; and the third group was treated by intraperitoneal injection of oxaliplatin, at 5mg/kg 3 times weekly[3]. After 2 weeks of treatment, mice were sacrificed and tumor were extracted for Immunohistochemistry (IHC) or Immunofluorescence (IF).

Additionally, we have performed co-cultures of CT26 with fibroblasts. Extracted tumors from non-treated mice were cultured after mechanical and enzymatic dissociation. We seek hereafter on infiltrated CAF presence by FACS or Immunocytochemistry (ICC). Other co-cultures were obtained by direct contact between CT26 and 3T3, a fibroblastic mouse cell line, with or without inserts[4].

We have highlighted the presence of 2 specific markers of CAF by IHC and IF: (i) the Fibroblast Activation Protein (FAP) and (ii) the Smooth Muscle Actin (α-SMA), with a different sensitivity on the three mice groups. We demonstrated that α-SMA quantification can be used to highlight expression differences. In the co-cultures model, FACS has not shown any results, and ICC protocols are being optimized.

To conclude, our model to assess CAF in colon cancer should allow us to highlight their presence, and to quantify their expression. The next step would be the isolation of CAF population from co-cultures for *in vitro* evaluation of treatments.

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Nr 71. **Title :** Modulation of cancer cell response to etoposide by M2 polarized macrophages

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Tumor associated macrophages (TAMs) are an important part of the tumor microenvironment in most cancers. It has been shown that TAMs are mostly anti-inflammatory M2-like macrophages. It is now clearly known that M2 macrophages in tumor can inhibit immune responses but the direct effect of these M2 macrophages on cancer cell response to chemotherapeutic drugs is still unknown. The aim on this work is to characterize for the first time the direct effect of TAMs on cancer cells chemoresistance. Here, by using THP-1 monocytes cell line first differentiated into macrophages (M0) with phorbol 12-mystrate 13-acetate (PMA) then polarized in M2 macrophages with IL-4 and IL-13, in co-culture with cancer cells, we investigated the effect of these polarized macrophages on the cancer cell resistance to etoposide. The co-culture of macrophages and cancer cells reveals that M2 macrophages decreased the etoposide-induced cell death. These results have been observed by western blot for cleaved Caspase-3 and cleaved PARP-1 and Caspase-3 activity assay. Other results suggest that these effects occur by an increase in PI3k/Akt/mTOR and MAPK pathways and are due to diffusible molecules, which are not proteins. Moreover, co-culture between M2 macrophages and HepG2 cancer cells leads to the expression of cancer stem cell markers by HepG2 cells. Taken together, these results show a direct protective effect of M2 macrophages on etoposide induced cell death of HepG2 cancer cells.

Nr 72. SREBP-1 inhibition re-sensitises therapy resistant BRAF mutant malignant melanoma to BRAF inhibition

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Abstract

One of the major challenges associated with molecularly targeted cancer treatment is the development of therapy resistance. This is dramatically illustrated in the case of malignant melanoma of the skin, which often carries activating mutations in BRAF. Whilst the majority of BRAF-mutant tumours regress in response to targeted agents such as vemurafenib, in nearly all cases, therapy resistance ensues with fatal consequences.

Here, we pinpoint the cancer cell's aberrant and sustained activation of lipogenesis through the master regulator of lipogenesis, Sterol Regulator Element Binding Protein-1 (SREBP-1), as a key mediator of resistance to BRAF-targeted therapy. We show that SREBP-1 inhibition both re-sensitises therapy resistant melanoma to BRAF inhibition and works synergistically with BRAF inhibitors in inhibiting tumour growth.

Establishment of this novel concept may find important applications for a more stable management of cancer using a novel combinatorial approach involving anti-lipogenic and targeted agents.

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