

## WHOLE SLIDE IMAGING SYSTEM TO CHARACTERIZED LYMPHATIC VASCULATURE THROUGH CERVICAL CANCER PROGRESSION AND ASSESS PREDICTION OF LYMPH NODE EXTENSION

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Uterine cervical cancer is the third most frequent cancer and the fourth leading cause of cancer death in female worldwide. Lymph node extension is the main prognostic factor for overall and disease free survival. Recently, research has focused on lymphangiogenesis and its potential role in promoting this metastatic process. Especially, high peritumoral lymphatic vessel density (LVD), resulting from early lymphangiogenic activation and progressive increase through the initial step of cervical neoplasia, appears to correlate with nodal metastases. However, when conventional optical microscopy is used, as is the case in all previous works, interobserver variability is significant and, despite promising predictive impact of LVD quantification, inconsistent conclusions are currently obtained. Therefore, we have developed a new original method based on computerized image analysis applied on whole slide scanned tissue sections following immunohistochemical staining in order to better characterize tumoral lymphatic vasculature. In this work, we first aimed at evaluating lymphatic vessel modifications through cancer progression. Secondly, lymphatic vessel parameters have been correlated to a risk of nodal extension.

Seventy-nine cases of cervical neoplasia (12 CIN3, 10 FIGO stage 1A1 and 57 FIGO stage 1A2 to 1B1) and 10 cases of normal tissues were reacted with p16<sup>INK4a</sup> and D2-40 antibodies, specific to tumor cells and lymphatic endothelial cells respectively. Immunostained structures were automatically detected on the whole slide using a colorimetric method with further computer assisted mathematical morphology post-processing. Finally, images of detected structures were binarized and decimated. Whole field of the cervical tissue was assessed from the normal squamous tissue through to transformation zone and normal glandular tissue. LVD, lymphatic vessel area and distribution were measured in the intra and peritumoral areas of invasive neoplastic lesions and correlated with risk of nodal extension.

Our imaging system allows to perform a detailed characterization of lymphatic vasculature through cancer progression and to study its significance in promoting lymph node dissemination. Particularly, an objective LVD quantification, morphological parameters as well as stromal lymphatic vessel distribution can be assessed. We described for the first time the presence of numerous lymphatic vessels under the transformation zone in normal cervical tissue which preexists before neoplastic events. Further modification in lymphatic vessel distribution is then observed near the tumor through tumor progression.

## **RATIONAL SELECTION OF ANTIGENS FOR TARGETED THERAPY OF LIVER METASTASES**

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Patients suffering from liver metastases are diagnosed late and have a poor outcome. Targeted therapies are gaining a major stake in current and future treatment options. However, the malignant lesions are heterogeneous in nature offering niches for cancer cells causing treatment resistance and relapse. Therefore, a rational strategy is needed to select targetable antigens that would overcome this intra-tumoral heterogeneity.

Here we generated a quantitative picture of the proteome heterogeneity in colorectal carcinoma liver metastases. We focus on membrane bound and extracellular proteins and show the differential distribution of novel targets and antigens against which the antibodies are already involved in clinical trials or treatment of liver metastases. Extensive clustering and validation experiments highlight novel markers that offer the potential to homogeneously cover the metastatic lesion and become better targets.

Two such antigens, LTBP2 and TGFBI are selected for functional analysis in colorectal carcinoma cells. In vitro and in vivo experiments show that both proteins are relevant for migration and proliferation capacities of cancer cells. Their depletion leads to significant inhibition of tumor growth, crystalizing them as bona fide targets for development of anti-metastases therapies.

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## FREQUENCY OF EGFR AND K-RAS MUTATIONS AND HPV INFECTION IN HNSCC PATIENTS.

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**Background:** Head and neck cancer is a heterogeneous and complex disease, affecting mainly the oral cavity, pharynx and larynx. More than 90% of these cancers originate from the squamous epithelium. Worldwide, Head and Neck Squamous Cell Carcinoma (HNSCC) is the sixth most common cancer. In more than 90% of these tumors, overexpression of the epidermal growth factor receptor (EGFR) is observed. Activation of this pathway leads to cell proliferation, anti-apoptosis, angiogenesis and metastasis. Therefore, anti-EGFR biotherapeutics were developed in order to prevent activation of EGFR by ligand binding or dimerization. However, activating mutations in EGFR or downstream in the EGFR pathway can also lead to activation of this pathway. Therefore, the mutation status of several genes in the EGFR pathway was investigated in a HNSCC patient population, as was the human papillomavirus (HPV) status.

**Methods:** 52 newly diagnosed HNSCC patients (27 oropharynx, 25 larynx) were screened for mutations in codon 12 and 13 of exon 2 of the *K-Ras* gene by high-resolution melting analysis (HRMA), after manual microdissection. Abnormal melting patterns will be sequenced for further analysis. The HPV status was determined by GP5+/6+ PCR. Currently, the EGFR gene is being screened for EGFRvIII mutations and mutations in the tyrosine kinase domain (exons 19-21).

**Results:** Out of 52 HNSCC patient samples, 47 could be used for HRMA analysis. Six (12.8%) had an abnormal melting pattern, indicating mutated *K-Ras*. No correlation between K-Ras mutation and clinicopathological parameters was found. All 52 samples were tested for HPV infection, 12/52 (23.1%) were found HPV positive, from which 9/28 (33.3%) were located in oropharynx and 3/25 (12.0%) in larynx. Preliminary results from EGFR-TK HRMA indicate for exon 19: 0 – 5 – 28; exon 20p: 10 – 9 – 15; exon 20d: 1 – 7 – 29 and exon 21: 3 – 4 – 22 abnormal, border (on the detection limit) or normal melting patterns respectively.

**Discussion:** Mutations in K-Ras have been described previously at a frequency around 5%. We found a frequency of 12.8%. This might be explained by a higher sensitivity of HRMA compared to sequencing. HPV infection in oropharyngeal tumors is frequent, whereas in larynx patients HPV was present in 3/25 (12.0%).

## TARGETING THE EPIDERMAL GROWTH FACTOR RECEPTOR IN NON-SMALL CELL LUNG CANCER CELLS: THE EFFECT OF COMBINING RNA INTERFERENCE WITH TYROSINE KINASE INHIBITORS OR CETUXIMAB

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**Key words:** EGFR; RNA interference; Tyrosine kinase inhibitors (TKIs); Anti-EGFR monoclonal antibodies (mAbs); Proliferation; Apoptosis; Lung cancer

**BACKGROUND/AIM** The epidermal growth factor receptor (EGFR) is a validated therapeutic target in non-small cell lung cancer (NSCLC). However, current single agent receptor targeting does not achieve a maximal therapeutic effect, and some mutations are resistant to current available agents. In the current study we have examined, in different NSCLC cell lines, the combined effect of RNA interference targeting the EGFR mRNA, and inactivation of EGFR signaling using different receptor tyrosine kinase inhibitors (TKIs) or a monoclonal antibody cetuximab .

**MATERIALS AND METHODS** NSCLC cells (cell lines HCC827, H292, H358, H1650 and H1975) were transfected with EGFR siRNA and/or treated with the TKIs gefitinib, erlotinib, and afatinib, and/or with the monoclonal antibody cetuximab. The reduction of EGFR mRNA expression was measured by real-time quantitative PCR. The down-regulation of EGFR protein expression was measured by western blot, and the cell proliferation, viability, caspase3/7 activity and apoptotic morphology were monitored by spectrophotometry, fluorimetry and fluorescence microscopy. The combined effect of EGFR siRNA and different drugs was evaluated using a combination index.

**RESULTS** EGFR-specific siRNA inhibited strongly EGFR protein expression almost equally in all cell lines and inhibited cell growth and induced cell apoptosis in all NSCLC cell lines studied, albeit with a different magnitude. The effects on cell growth inhibition obtained with siRNA was strikingly different from the effects obtained with TKIs. The effects of siRNA probably correlate with the overall oncogenic significance of the receptor, which is only partly inhibited by the TKIs. The cells which showed weak response to TKIs, such as the H1975 cell line containing the resistance T790M resistant mutation, were found to be responsive to siRNA knockdown of EGFR, as were cell lines with downstream TKI resistance mutations. The cell line HCC827 harboring an exon 19 deletion mutation was more than tenfold more sensitive to TKIs proliferation inhibition and apoptosis induction than any of the other cell lines. Cetuximab alone had no relevant *in vitro* activity at concentration obtained in the clinic. The addition of EGFR siRNA to either TKIs or cetuximab additively enhanced growth inhibition and induction of apoptosis in all five cell lines, independent of the EGFR mutation status (wild-type or sensitizing mutation or resistant mutation). The strongest biological effect was observed when afatinib was combined with an EGFR-specific siRNA.

**CONCLUSIONS** EGFR knockdown by siRNA further decreases the cell growth of lung cancer cells that are treated with TKIs or cetuximab alone, confirming that single agent drug targeting does not achieve a maximal biological effect. The combined treatment of siRNA and EGFR inhibitory agents is additive. The combination of a potent, irreversible kinase inhibitor such as afatinib, with EGFR-specific siRNAs should be further investigated as a new strategy in the treatment of lung cancer and other EGFR dependent cancers, including those with downstream resistance mutations.

## COMBINED TARGETING OF THE EGFR AND c-MET PATHWAYS IN NON-SMALL CELL LUNG CANCER

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**Key words:** EGFR; c-MET; RNA interference; Tyrosine kinase inhibitors (TKIs); Anti-EGFR monoclonal antibodies (mAbs); Su11274; Lung cancer

**BACKGROUND/AIM** The epidermal growth factor receptor (EGFR) and c-MET receptors are widely expressed on non-small cell lung cancer (NSCLC) cells. They are implicated in the development and progression of cancer through a plethora of effects on cell cycle progression, apoptosis, motility and metastasis. However, current single agent receptor targeting does not achieve a maximal therapeutic effect and some EGFR mutations are resistant to current available EGFR receptor tyrosine kinase inhibitors (TKIs). In the current study, we have examined the combined effect of targeting EGFR and c-MET pathways by RNA interference, EGFR and c-MET TKIs, and anti-EGFR antibody.

**MATERIALS AND METHODS** NSCLC cells (cell lines HCC827, H292, H358, H1650 and H1975) were transfected with different EGFR-specific-siRNAs. H1975 cells containing the T790M resistance mutation were transfected with several T790M-specific siRNAs. The most potent EGFR or T790M siRNAs were further selected to combine with c-MET siRNA. NSCLC cells were also treated with the TKIs gefitinib, erlotinib and afatinib, or the monoclonal antibody cetuximab, and combined with su11274, a c-MET specific TKI. The reduction of mRNA expression was measured by real-time quantitative PCR and protein expression was assessed by western blot. The cell proliferation, viability, caspase3/7 activity and apoptotic morphology were monitored by spectrophotometry, fluorimetry and fluorescence microscopy, respectively. The combined effect was evaluated by applying a combination index.

**RESULTS** EGFR-specific siRNAs inhibited cell growth and induced cell apoptosis in all NSCLC cell lines studied. The cells which showed weak responses to TKIs, especially the H1975 cell line containing the resistance T790M mutation, were found to be sensitive to siRNAs induced knockdown of EGFR. Compared to EGFR-specific siRNAs, the T790M-specific siRNAs had a weaker effect on cell growth inhibition and apoptosis induction in H1975 cells. The combination of EGFR siRNA plus c-MET siRNA enhanced growth inhibition, induction of apoptosis and inhibition of the downstream signaling in H358, H1650 and H1975 cells. EGFR TKIs or cetuximab plus su11274 was also consistently superior to either agent alone on cell growth suppression, apoptosis and downstream signaling inhibition in H358, H1650 and H1975. The strongest biological effect was observed when afatinib was combined with su11274, which had a synergistic effect.

**CONCLUSIONS** EGFR-specific siRNAs could become a potential tool to treat NSCLC. There was a synergistic effect on cell growth with dual EGFR/HER2 and c-MET inhibition in H1975 cells. EGFR/HER and c-MET combination based targeted inhibition can be a promising treatment strategy for NSCLC, especially for those T790M-mediated resistance.

## MiR-146A INHIBITS CELL GROWTH AND INDUCES APOPTOSIS IN NON SMALL CELL LUNG CANCER CELLS

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**Keywords:** MiR-146a; Lung cancer; EGFR; Proliferation; Apoptosis; Mobility

**BACKGROUND/AIM** Aberrant expression of microRNA-146a (miR-146a) has been reported to be involved in development and progression in various types of cancers, but its role in non small cell lung cancer (NSCLC) has not been fully elucidated. The aim of this study was to investigate the effect of miR-146a on human NSCLC.

**MATERIALS AND METHODS** Cell growth, apoptosis, cell caspase-3/7 activity and migration ability of NSCLC cells transfected with miR-146a inhibitor or mimic were detected by spectrophotometry, fluorimetry, fluorescence microscopy and wound-healing assay. The combination effect of miR-146a mimic together with different EGFR pathway targeting drugs was evaluated using a combination index. The expression and significance of miR-146a were further explored in clinical FFPE tissues.

**RESULTS** Functionally, miR-146a mimic suppressed cell growth, induced cell apoptosis and inhibited EGFR downstream signaling in NSCLC (cell lines H358, H1650 and H1975). Moreover, miR-146a inhibited migration of NSCLC cells. The EGFR and NF- $\kappa$ B signaling were down-regulated by miR-146a. Furthermore, miR-146a enhanced the cell proliferation inhibitory effect by drugs targeting EGFR, including TKIs (gefitinib, erlotinib and afatinib) and monoclonal antibody (cetuximab). These effects were independent of the EGFR mutation status (wild-type or sensitizing mutation or resistant mutation) and were weaker compared to the effect by the siRNA targeting of EGFR alone. We also found in clinical FFPE samples that low expression of miR-146a was correlated with late clinical TNM stages and distal metastasis in NSCLC ( $P < 0.05$ ). The patients with high miR-146a expression showed longer progression-free survival (25.6 weeks in miR-146a high patients vs 4.8 weeks in miR-146a low patients,  $P < 0.05$ ).

**CONCLUSIONS** MiR-146a could be useful to suppress proliferation and induce apoptosis in NSCLC by partly targeting EGFR and NF- $\kappa$ B signaling. Thus increasing or inducing miR-146a level might be a critical targeted therapy strategy for NSCLC.

**BONE MARROW- AND TUMOR-DERIVED MESENCHYMAL CELLS PROMOTE COLORECTAL CANCER PROGRESSION THROUGH PARACRINE NEUREGULIN 1/HER3 SIGNALING**

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**Objective:** Bone marrow-derived mesenchymal stem cell (BM-MSC) migrate to primary tumors and drive tumor progression. This study aimed to identify the molecular mechanisms associated with these heterotypic cellular interactions and analyse their relevance in colorectal cancer (CRC).

**Design:** Paracrine interactions of BM-MSC and tumor-derived mesenchymal cells (T-MC) with CRC cells were studied using collagen invasion assays, cell counts, flow cytometric cell-cycle analysis and tumor xenograft models. The role of neuregulin 1 (NRG1) and the human epidermal growth factor receptor (HER) family pathways were investigated using tyrosine kinase assays, mass spectrometry, pharmacological inhibition, antibody-mediated neutralisation and RNA interference. Transmembrane NRG1 (tNRG1) expression was analysed in primary CRCs (n = 54), adjacent normal colorectal tissues (n = 4), liver metastases (n = 3) and adjacent normal liver tissues (n = 3) by immunohistochemistry.

**Results:** BM-MSC and T-MC stimulate invasion, survival and tumorigenesis of CRCs through release of soluble NRG1, activating the HER2/HER3-dependent PI3K/AKT signaling cascade in CRC cells. Mesenchymal cells in primary CRCs demonstrate high tNRG1 expression, which is significantly associated with advanced UICC stage (P = .005) and invasion depth (P = .04) and decreased 5-year progression-free-survival (PFS) (P = .01).

**Conclusions:** Paracrine NRG1/HER3 signals initiated by BM-MSC and T-MC promote CRC cell progression, and high tNRG1 expression is associated with poor prognosis.

## GEOMETRIC ACCURACY OF A NOVEL GIMBALS BASED RADIATION THERAPY TUMOR TRACKING SYSTEM

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**Objective:** To determine the geometric accuracy of the real-time moving tumor tracking in pan and tilt direction of a novel gimbaled linac system.

**Methods and Materials:** The VERO system, a novel platform for image guided stereotactic body radiotherapy, is a joint product of BrainLAB and MHI. A new type of 6 MV linac with attached MLC is mounted on an O-ring gantry. Orthogonal gimbals hold the linac-MLC assembly, which allows pan and tilt motions of the therapeutic beam enabling tracking of moving tumors. Beside an ExacTrac IR camera system, the VERO system is equipped with a dual kV imaging systems attached to the O-ring at 45° from the MV beam, allowing simultaneous acquisition of orthogonal X-rays images and fluoroscopy for guidance of tumor tracking.

A tracking system prototype currently installed in our hospital can actively track an IR marker with the beam using the gimbals system. A video camera based detection unit was developed to simultaneously acquire the position of the tracked object and of the tracking beam using the light field of the linac system, at a 30 fps frame rate. An assessment was made of the tracking capabilities in terms of tracking errors, system lag and the equivalence of pan and tilt motion performance. To determine the tracking error, an IR marker was placed on a 1D moving phantom. A sinusoidal motion was produced by the phantom with different frequencies from 0.083 Hz to 0.5 Hz, and a fixed amplitude of 20 mm. The VERO tracking forward prediction, a 2nd order polynomial prediction function, in the prototype software was set to 20 ms, 35 ms and 50 ms for all frequencies. To determine system lag, the forward prediction time was set to 0ms. The tracking error was quantified in terms of systematic error, root mean square error (RMSE) and 90% percentile of the absolute tracking error (E90%). Additionally, representative patient signals were applied to the phantom and a 2D Lego robot, and measured with the video camera system.

**Results:** Systematic tracking errors were below 0.21 mm. The system lag was experimentally determined to be 47.7 ms (2.3 ms) and 47.6 ms (2.0 ms) for the pan and tilt motion respectively. This was in agreement with the sum of the loop sub-system latencies determined by the vendor: IR marker position acquisition of 25 ms, gimbals position calculation 2 ms, gimbals control cycle time of 20 ms. With a forward prediction of 50 ms, the system can track the IR marker sinusoidal motion with an E90% < 0.82 mm, up to frequencies of 0.5 Hz with similar performance for pan and tilt direction. The 2D tumor trajectories were tracked with an average E90% of 0.54 mm, and tracking error standard deviations of 0.20 mm for pan and 0.22 mm for tilt.

**Conclusion:** The gimbals geometric accuracy and system lag are considered adequate for real-time tracking of tumors. The tracking performance for pan and tilt direction is equivalent.

## OPTIMIZATION AND VALIDATION OF HIGH RESOLUTION MELTING ANALYSIS FOR THE DETECTION OF MDM2 PROMOTOR SNP309

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**Background:** Murine double minutes-2 (MDM2) protein plays a key role in the regulation of the cell cycle and apoptosis. The MDM2 protein acts as a p53 antagonist and is linked to p53 in a tight auto-regulatory feedback loop by which the two proteins mutually control their protein levels. P53 enhances MDM2 transcription in response to genotoxic stress, whereas MDM2 binds to p53 and directs it for proteosomal degradation through ubiquitinylation.

About half of all human tumors retain wild-type p53 and in these tumors, the normal regulation of p53 might be disrupted through direct overexpression of MDM2 caused by e.g. MDM2 gene amplification or a T to G substitution (SNP309) in the promotor region of MDM2.

High resolution melting analysis (HRMA) provides a valid approach to efficiently detect DNA mutations. The current study aimed at validating and implementing HRMA for screening of colorectal cancer patients to detect MDM2 promotor SNP309. Pyrosequencing was used to confirm and characterize HRMA results.

**Materials:** First, HRMA sensitivity was established using a cell line and a FFPE (formalin-fixed paraffin embedded) dilution model. In addition, the same dilution models were used to evaluate the sensitivity of pyrosequencing to detect MDM2 promotor SNP309. Next, HRMA was validated on 10 cell lines. Since the MDM2 SNP309 status for these cell lines was unavailable, all HRMA results were confirmed and characterized by pyrosequencing.

**Results:** The cell line dilution model revealed a detection limit of 6% while the detection limit in a background of FFPE wild-type DNA was found to be 3%. The detection limit of pyrosequencing to analyze SNP309 in a background of wild-type cell line DNA seems to be between 20% and 15%. In a background of FFPE wild-type DNA, the detection limit seems to be 3%.

HRMA revealed abnormal melting patterns in 5/10 cell lines and pyrosequencing confirmed the presence of 2 homozygous (G/G) and 3 heterozygous (G/T) genotypes.

Preliminary results on FFPE material from colorectal cancer patient samples showed abnormal melting patterns in 6/13 samples. These are currently being confirmed using pyrosequencing.

**Discussion:** In conclusion, HRMA was found to be a fast, efficient, sensitive and reproducible screening method for MDM2 promotor SNP309 detection with a detection limit between 3% and 6%, which is higher than that of conventional sequencing methods. Preliminary results indicate that HRMA can be used as a screening method for MDM2 SNP309 by which DNA from FFPE tissues can be tested. Currently, a cohort of colorectal cancer patients is being tested for the presence of SNP309 using HRMA and pyrosequencing.

## COMPUTER-ASSISTED CORNEAL NEOVASCULARIZATION QUANTIFICATION

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Cornea engraftment is the most common organ transplantation practiced around the world. The cornea is totally devoid of blood or lymphatic vessels, except in a peripheral zone called the limbus. This property, named “corneal angiogenic privilege”, is conserved among all mammals to maintain cornea transparency and optimal visual acuity. In pathological conditions such as trauma, infections or hypoxia, blood and lymphatic vessels can grow into the avascular cornea, reducing visual acuity. In case of keratoplasty, it also considerably increases the risk of cornea graft rejection and is so considered as a high-risk keratoplasty. Treatments improving cornea survival after transplantation need to be developed, notably aiming at blocking corneal neovascularization. In this context, corneal neovascularization mice models allow the study of potential anti-lymph/angiogenic molecules but lack precise quantification method.

Here, we developed a new quantification method to finely characterize blood and lymphatic networks. Cornea vascularization was induced by thermal cauterization applied in the center of C57BL/6 mice cornea. Double CD31–LYVE-1 immunolabeling was performed on cornea whole mounts to evidence blood and lymphatic vessels respectively. Image processing algorithms were first developed in order to extract blood and lymphatic vessels from the background. This resulted in two independent binary images in which lymphatic and blood vessels were represented by white pixels (pixels value equal to 1) on a black background (pixels value equal to 0). After binarization, the following automatic measurements were performed: 1) area density, defined as the surface covered by vessels reported to total cornea area; 2) end-point density, defined as the vessel network extremity number per corneal area unit. This parameter indicates the increase/decrease of the first order vessels; 3) branching density, defined as the vessel branching number per corneal area unit which indicates the complexity degree of the structure; 4) length density which represents the cumulative length of vessels; 5) maximal length of vessels which indicates the largest migration distance; and 6) spatial distribution of vessels in relation to the limbal vessel. This distribution gives local information about the number of vessels at each given position in relation to the limbal vessel.

The proposed methodology was used to evaluate the efficacy of Sunitinib, a broad-spectrum tyrosine kinase receptor inhibitor, to reduce experimental corneal neovascularization. We observed an inhibition of angiogenesis after 17 days in Sunitinib-treated mice, where blood vessel relative surface, end-point density, branching density and length density were 1.8-fold decreased. Maximum length of blood vessels was also significantly reduced in the Sunitinib treated group at days 11 and 17. Lymphangiogenesis was strongly inhibited from day 6 to day 17 after cauterization where all parameters, except maximum length of lymphatic vessels, were significantly decreased.

In conclusion, we developed a new neovascularization quantification method allowing extended concomitant characterization of blood and lymphatic networks developing in mice cornea. The use of Sunitinib strongly reduced corneal neovascularisation and could potentially enter in early treatment of such eye lesions to avoid vision loss and risk of cornea graft rejection.

## INCREASED EPIDERMAL $\Delta Np63\alpha$ EXPRESSION PROMOTES TUMOR DEVELOPMENT IN A CHEMICAL CARCINOGENESIS MODEL

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P63 is a transcription factor crucial for ectodermal development and the formation of stratifying epithelia. In accordance, p63-deficient mice have no epidermis or epithelial appendages, on top with truncated limbs and abnormal craniofacial development. Unlike its family member p53, the p63 gene is rarely mutated in human cancers; nevertheless it is often deregulated or overexpressed. This suggests an important role for p63 in cancer development, which is distinct from p53. Squamous Cell Carcinoma (SCC) is a common and treatment-refractory form of human cancer in epithelial tissues where the p63 locus is frequently targeted for genomic amplification and/or overexpression, leading to increased levels of  $\Delta Np63\alpha$ . To investigate the contribution of  $\Delta Np63\alpha$  in the promotion and progression of SCC, we developed K5-Cre/LoxP-controlled ROSA26 promoter-driven  $\Delta Np63\alpha$  conditional transgenic mice. These mice develop mild epidermal hyperplasia and hair abnormalities, eventually leading to alopecia. So far (age of 1 year), no spontaneous tumors have been observed. However, when subjected to the chemical carcinogenesis model DMBA/TPA, we find that epidermal  $\Delta Np63\alpha$  overexpression results in a dramatic increase in papilloma formation. In contrast to control mice, K5- $\Delta Np63\alpha$  transgenic mice develop papillomas already 6 weeks after initiation with DMBA. At week 15 the average tumor incidence is 48 papillomas per mouse in K5- $\Delta Np63\alpha$  transgenic mice, while 33% of the control mice are still tumor-free and the average tumor incidence in tumor-bearing mice is 9 papillomas per mouse. Also the average papilloma size is significantly higher in the transgenes compared to controls at this point. These data strongly suggest a tumor-promoting function of  $\Delta Np63\alpha$ . Currently we are checking whether these papillomas progress to carcinomas.

**PHASE II STUDY OF HELICAL TOMOTHERAPY IN THE MULTIDISCIPLINARY TREATMENT OF OLIGOMETASTATIC COLORECTAL CANCER.**

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Background: Complete metastasectomy provides a real chance for long-term survival in patients with oligometastatic colorectal cancer (CRC). For patients who are not amenable for metastasectomy, we demonstrated in a previous study the feasibility of moderately hypofractionated intensity-modulated and image-guided radiotherapy

(RT) by helical tomotherapy (1). Aiming at higher response rates, we evaluated in this study helical tomotherapy delivering 50 Gy in daily fractions of 5 Gy.

Methods: Inoperable CRC patients with  $\leq 5$  metastases were enrolled. No limitations concerning dimension or localization of the metastases were imposed. Whole body PET-CT was performed at baseline and 3 months after the initiation of RT to evaluate the metabolic response rate according to PERCIST v 1.0. Side effects were scored using the NCI CTC AE v 3.0 scale.

Results: We report the results of the first 22 patients. Thirteen patients (59%) received previous chemotherapy for metastatic disease, displaying residual (n=7) or progressive (n=6) metabolic active metastatic disease at time of inclusion. A total of 51 metastases were treated. Most common sites were the lung, liver and lymphnodes. One patient (5%) experienced grade 3 dysphagia; 2 patients (9%) and 1 patient (5%) grade 2 dysphagia and diarrhea, respectively. Twenty patients were evaluated by post-treatment PET-CT. Five and 6 patients achieved a complete and partial metabolic response, resulting in an overall metabolic response rate of 55%. At a median follow-up of 11 months, 17 patients (77%) developed progressive disease, of which 3 isolated local progression. Five patients (23%) are in remission in all irradiated areas without evidence of distant recurrence.

Interestingly, those 5 patients received previous chemotherapy with residual oligometastatic disease at time of inclusion.

Conclusions: Ten fractions of 5 Gy resulted in a promising 55% metabolic response rate with limited toxicity. Helical tomotherapy is an attractive modality in the multidisciplinary approach of oligometastatic CRC, more specifically in the consolidation of residual and inoperable oligometastatic disease in patients previously treated with chemotherapy.

*(1) Phase II study of helical tomotherapy for oligometastatic colorectal cancer - B. Engels, H. Everaert, T. Gevaert, M. Duchateau, B. Neyns, A. Sermeus, K. Tournel, D. Verellen, G. Storme & M. De Ridder - Annals of Oncology 2011 Feb;22(2):362-8. Epub 2010 Aug 4)*

## SPHEROIDS, A 3D MODEL OF CELL SPROUTING: IMAGING AND QUANTIFICATION

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**INTRODUCTION:** Lymphangiogenesis, the formation of new lymphatic vessels from preexisting ones, is an important biological process associated with metastatic dissemination. The lymph node status is a major determinant for cancer staging and prognosis, guiding finally the therapeutic decisions. Although the development of lymphatic vascular network occurs within the interstitium of most tissues, the exploration of the interactions occurring between extracellular matrix (ECM) components and lymphatic endothelial cells (LEC) is poorly documented.

**AIMS:** The present work aims at investigating the interactions occurring between LEC and type I collagen, the main ECM component surrounding lymphatics.

**METHODS:** To unravel the mechanisms underlying LEC migration and differentiation into capillaries in an interstitial environment, we have setup a model of multicellular spheroids of lymphatic endothelial cells (HTERT-HDLEC) embedded in a 3D-type collagen matrix. To address the importance of matrix structure, gels of pepsin-extracted type I collagen that does not contain the non helical telopeptides at the N- and C-termini (pepsinized collagen) and intact telopeptide containing collagen (native collagen) were used. Quantification of the lymphangiogenic response was performed through original computerized methods allowing the study and quantification of two cell behaviors: primary cell expansion in the spheroid bulk and cell migration from the spheroid surface. To achieve this goal, image processing was applied on original grey level images to obtain a binary image. From binary images the following parameters were measured: 1) spatial cell distribution in the spheroid bulk, as a reflect of changes in spheroid structure and expansion preceding cell detachment; 2) spatial distribution of migrating cells including the maximal distance of cell migration.

For real-time analysis of matrix degradation in live cells, collagen fiber remodeling was evaluated through reflectance in an inverted confocal laser-scanning microscope. Fluorescent dequenching of DQ-collagen (FITC-labeled collagen fluorescing upon cleavage) was assessed concomitantly with collagen-degradation visualization. Collagen remodeling was also assessed by using electron microscopy transmission.

**RESULTS:** By comparing with classical quantification methods, we provide evidence for the suitability of our novel method of quantification that allows avoiding misinterpretation related to spheroid expansion preceding cell migration. LEC migration was increased when cells were embedded in pepsinized collagen than in native collagen. We next evaluated the impact of metalloprotease blockage through the use of inhibitors or by down-regulating their expression with siRNA. Our results demonstrate the implication of an interstitial collagenase during LEC migration in a collagen gel. The utilization of DQ collagen, microscopy reflectance in real-time movies, as well as electron microscopy transmission, confirmed the importance of the collagenolytic activity for lymphangiogenesis.

**CONCLUSION:** We developed a new in vitro model for studying sprouting lymphangiogenesis that has proven suitable to investigate the importance of collagen remodeling during this complex process associated with cancer dissemination. The proposed computerized method of quantification can be also applied to spheroids formed by other cell lines as well as spheroid tumors.

**DENTIN MATRIX PROTEIN 1 (DMP1) IS DIFFERENTIALLY EXPRESSED IN NORMAL AND MALIGNANT HUMAN BREAST TISSUES.**

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Dentin Matrix Protein 1 (DMP1), a member of the Small Integrin Binding Ligand N-linked Glycoprotein (SIBLING) family, has been mainly studied in the field of dentin and bone biomineralization. Previous immunohistochemical studies conducted in our laboratory demonstrated that DMP1 is expressed in breast and lung cancers. We showed that a high expression of this protein in breast tumors is correlated with a small tumor size and a better prognosis for the patients. Consistent with these observations, we have recently demonstrated an anti-angiogenic function of DMP1 via the inhibition of the Vascular Endothelial Growth Factor Receptor 2 (VEGFR-2) pathway in human umbilical vein endothelial cells. To explore further the role of DMP1 during cancer progression, we analyzed the expression of DMP1 in a series of matched normal and tumoral human breast tissues at both the protein and mRNA levels using western blotting and RT-QPCR analysis, respectively. Preliminary results consistently showed that DMP1 is weakly detectable in tumors when compared to normal tissues where it is highly expressed. MDA-MB-231 human breast cancer cellular clones overexpressing DMP1 will be generated and tested in proliferation, adhesion and invasion assays. We will also examine human recombinant DMP1 ability to engage integrin-type cell-surface receptors and subsequently activate intracellular signaling pathways in breast cancer cells. Finally, the chicken tumor chorioallantoic membrane (tumor-CAM) assay will be used as a model to test the growth of DMP1-overexpressing breast cancer cells grafted on the CAM. The volume of the tumors will be measured and angiogenesis will be evaluated on histological sections specifically stained to reveal blood vessels. This ongoing study will help understanding the possible mechanisms by which loss of DMP1 expression may contribute functionally to malignancy, particularly in breast cancer.

## DEVELOPMENT OF AN IMPROVED CHICK CHORIOALLANTOIC MEMBRANE MODEL FOR HUMAN PANCREAS ADENOCARCINOMA

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Pancreatic carcinoma is the fourth most common cause of cancer death in developed countries. The late diagnosis and the poor efficacy of classical chemotherapeutic agents are responsible for the rapid death of pancreatic carcinoma patients. Clearly, new approaches to therapy and models that can be used in place of human tissue are needed. The chick chorioallantoic membrane CAM is a commonly used method to study angiogenesis and more recently tumor growth. Two studies using the CAM assay with pancreatic cancer cells were published, but tumors remain small (100  $\mu\text{m}$ ).

The aims of our study were: first, to improve the size of tumors; second, to characterize the tumor histology; third, to validate the usefulness of this model by impairing the tumor growth.

We grafted BxPC-3 cells in a matrigel matrix at day 11 after fertilization. Cells grew on CAM during 7 days. Tumors reached a volume of  $43 \pm 10 \text{ mm}^3$ . HE staining showed typical cohesive clusters of cells. These structures exhibited a tubulo-glandular appearance with a central lumen. Cytokeratin 7 (CK7) and CK19 were immunodetected in all pancreatic cells. Tumor cells were negative for CK20 and CD56. This profile corresponds to 41% of human pancreatic adenocarcinoma. Moreover, most of cells were positive for the proliferation marker KI67 and for Carcinoma Embryonic Antigen. FITC-conjugated *Sambucus nigra* (SNA) lectin showed many blood vessels surrounding cell clusters. These vessels contained erythrocytes. Fluorescent probe injection in CAM vasculature demonstrated the functionality of tumor vessels as fluorescence was observed between all cell clusters.

Our results showed macroscopic angiogenic BxPC3 tumors. The functional perfusion of the tumors allows us to expect the possibility to use this model to detect new circulating biomarkers and to test new drugs.

**INTEGRATING HYPOXIA AND NATIVE CONDITIONS FOR IMMUNE COMPLEX FORMATION IN THE SEROLOGICAL PROTEOME ANALYSIS (SERPA) TO IMPROVE THE DETECTION OF AUTOANTIBODIES AS CANCER BIOMARKERS**

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The expression by tumor cells of proteins with aberrant structure, expression or distribution accounts for the development of a humoral immune response. Autoantibodies (AAbs) to tumor-associated antigens (TAAs) may thus be particularly relevant for early detection of cancer. Several proteomic approaches have been developed to identify circulating AAbs. One approach called SERPA is based on the immunoblotting with cancer patient serum, of 2DE-separated tumor cell proteins and the consecutive MS identification of reactive spots. This method has the advantage to use post-translationally modified proteins (contrary to methods using phage peptides or bacteria-produced proteins). Limitations are however the use of poorly relevant plastic-cultured tumor cells and the detection of AAbs reaction against denatured proteins.

Here, we propose an optimization of the SERPA method based on (i) the pre-exposure of tumor cells to hypoxia to allow the expression of a pattern of proteins closer to the *in vivo* conditions and/or (ii) the incubation of tumor cell extracts directly with purified seric IgG to allow interaction with TAAs in native conditions. Resulting immune complexes are consecutively purified via affinity chromatography before MS identification of the antigens. This modality also allows to deplete lysates of tumor-unspecific antigens by rounds of pre-incubation with IgG isolated from control sera.

We used human breast cancer cells MDA-MB231 and human colorectal cancer cells HCT116 that we exposed for 48 hours to 1% O<sub>2</sub>. With the mammary cell line, only spots positive after immunoblotting of hypoxic cell lysates with the sera of tumor-bearing mice, were collected and identified by MS analysis. Specific ELISA were developed for 6 proteins and confirmed the presence of corresponding AAbs in the serum of tumor-bearing mice (*vs* healthy mice) ( $P < 0.01$ ), the titer of which increasing with tumor growth. With the colorectal cancer cell line, we combined the strategy of hypoxia exposure to provide a more relevant repertoire of TAAs with LC-based isolation of IgG from patients with colorectal cancer (*vs* healthy volunteers). This led us to document the formation in native conditions, of immune complexes not detected by conventional SERPA.

In conclusion, this study provides evidence that integrating the hypoxia criteria and the interaction in native conditions between TAAs and AAbs may considerably increase the efficacy of SERPA method to identify relevant cancer biomarkers.

## HEPATOCTES EITHER RADIOPROTECT OR SENSITIZE COLORECTAL CANCER CELLS THROUGH NITRIC OXIDE-INDUCED OXYGEN SPARING IN HYPOXIC MICROENVIRONMENT

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**Purpose:** Impaired tumor oxygenation remains a major obstacle to curative radiotherapy, and a number of radiosensitizing strategies focused on re-enforced oxygen supply were suggested in the past. In this study, tailored to metastatic colorectal cancer (CRC), we explored an alternative approach to re-oxygenate tumor cells through inhibition of oxygen consumption in host cells (hepatocytes and macrophages) that form the liver parenchyma.

**Methods and Materials:** Primary mouse hepatocytes, macrophages and mouse CT26 or human DLD-1, HCT116, HT29 and SW480 CRC cells were exposed to a cytokine mixture (IFN- $\gamma$ , TNF- $\alpha$  IL-1 $\beta$  and LPS). Afterwards, iNOS expression and activity was examined by RT-PCR, western blotting, and production of nitrite. To model metabolic hypoxia, hepatocytes and tumor cells were co-cultured in a tissue-mimetic system with restricted oxygen diffusion, and oxygen consumption was measured by a fluorescence-based assay. Co-cultures were irradiated at doses of 0, 4, 8 and 12 Gy, and cell survival was measured by an 8-day colony formation assay.

**Results:** In the tissue-mimetic culture system, hepatocytes consumed 10 to 40-times more oxygen than mouse CT26 and human DLD-1, HT29, HCT116 and SW480 CRC cells, suggesting that hepatocytes are the major effectors of hypoxic conditioning. Co-culture of tumor cells and hepatocytes at  $0.6 \times 10^6$ /ml (1:1) displayed similar hypoxia profiles compared with hepatocytes alone, while tumor cells alone were not able to induce hypoxia below  $3 \times 10^6$ /ml. At cell densities of  $0.6-1.2 \times 10^6$ /ml, hepatocytes induced radiobiologically-relevant hypoxia within 20 min, and thereby radioprotected tumor cells by 2.0 to 2.5-times. Following exposure to cytokines, hepatocytes revealed significant activation of iNOS-mediated NO production. The molecular targets of NO appeared to be mitochondrial aconitase and complexes II, as their activities were inhibited by 81% and 30% respectively, and oxygen consumption was drastically blocked up to 90 min. The spared oxygen caused efficient re-oxygenation of hypoxic CRC cells, which in turn resulted in a 1.9 to 2.7-fold radiosensitization. Both oxygen sparing and radiosensitization could be counteracted by aminoguanidine, a metabolic iNOS inhibitor. Contrasting, all CRC cell lines showed little if any iNOS expression and did not contribute to the oxygen-sparing and radiosensitizing effects of NO-producing hepatocytes. NO-producing macrophages were able to reverse hypoxia-induced radioresistance as well but required up to 20-times higher cell densities compared with hepatocytes. Finally, both the NO donor SNAP and the mitochondrial inhibitors rotenone, antimycin A and sodium azide were able to block the mitochondrial respiratory chain and reverse hypoxia induction, thus linking a respiratory self-arrest and oxygen sparing in hepatocytes.

### Conclusions:

Hepatocytes may profoundly modulate the hypoxic microenvironment under limited oxygenation and reveal either radioprotecting or sensitizing properties at physiological cell densities. Our study for the first time indicated the possibility to re-oxygenate and radiosensitize hypoxic CRC cells through NO-induced oxygen sparing in normal liver cells.

**CKIT OVEREXPRESSION AND WILD-TYPE NRAS/BRAF PREDICT HIGH SENSITIVITY TO THE TYROSINE KINASE INHIBITOR DASATINIB IN MELANOMA CELLS**

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Patients with advanced metastatic melanoma have limited effective therapy. Thus, there is an urgent need to evaluate new targeted drugs and to identify predictive markers. We evaluated the effects of dasatinib (BMS-354825, Sprycel) on melanoma cell proliferation in relation with NRAS and BRAF mutation status, drug targets and key proteins involved in melanoma signalling pathways. We examined 21 melanoma cell lines and found that 6 lines were highly sensitive to dasatinib ( $IC_{50}s \leq 10^{-9}$  M), 7 were moderately sensitive ( $IC_{50}s$  from  $10^{-8}$  to  $10^{-6}$  M) and 8 were resistant ( $IC_{50} \geq 10^{-5}$  M). All highly sensitive lines expressed high cKIT, whereas the others had lower levels. Importantly, all highly sensitive lines had no mutation on BRAF or NRAS, while 58% of the moderately sensitive and 75% of the resistant cell lines had activating mutations. Moreover, dasatinib dramatically inhibited the phosphorylation of cKIT, SRC, ERK and AKT in sensitive cells, while it had no effect on ERK and AKT phosphorylation in BRAF-mutant cells. This suggests a selective effect on proliferation/survival of cells with cKIT expression not harbouring BRAF mutations, that may render melanoma cells much less dependent on cKIT signalling for survival. In conclusion, we found that a large range of very low dasatinib concentrations ( $10^{-10}$  -  $10^{-8}$  M) were highly effective to induce cytotoxicity in a subgroup of melanoma lines characterized by high cKIT expression and wild-type NRAS/BRAF. Consequently, some metastatic melanoma patients would benefit from dasatinib treatment considering the expected wide therapeutic window of the drug.

## FUNCTIONAL CHARACTERIZATION OF THE CANDIDATE TUMOR SUPPRESSOR PROTEIN PROTOCADHERIN-10

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Protocadherins are transmembrane proteins that differ in various aspects from classic cadherins, and whose functions are largely unexplored. We are especially interested in two smaller protocadherin subfamilies (delta1- and delta2-protocadherins) featuring two or three conserved motifs (CM) in their cytoplasmic domains. These CMs are only present in long isoforms, generated by alternative splicing. In this study we are focusing on protocadherin-10 (PCDH10), which was proposed to act as a tumor suppressor. Indeed, the human *PCDH10* gene is frequently silenced in several carcinomas, and its ectopic expression in an esophageal tumor cell line strongly suppresses tumor cell growth, migration and invasion. Previously, a germline *Pcdh10* knockout mouse has been reported on. This mouse has a severe brain abnormality leading to death within three weeks after birth. To avoid this lethality problem we have generated conditional *Pcdh10* knockout mouse models to delete *Pcdh10* in a tissue- and time-specific manner. We have been following two approaches: The first is to knock out all isoforms of *Pcdh10* in order to investigate the role of *Pcdh10* in specific organs and cellular processes. In the second approach we have been targeting only the long isoforms of *Pcdh10*, and the latter mice will be used to study the role of the conserved cytoplasmic domains CM1 and CM2 in intracellular signaling pathways, including oncogenic ones. Our preliminary results show that knocking out only the long isoforms of *Pcdh10* does not lead to brain defects or early death in these mice.

In view of the wide spread silencing of *PCDH10* in many human cancers, we are going to breed our mice with floxed *Pcdh10* alleles with a selection of tissue-specific Cre lines, including the K5-Cre (stratified epithelium), the PB-Cre4 (prostate) and the GFAP-Cre (astrocytes).

In addition to extensively analyzing 'spontaneous' tumor formation in these tissue-specific *Pcdh10*-KO mice, we will investigate the possible synergy of *Pcdh10* inactivation with the activity of known oncogenes by additional breeding of our KO mice with well-studied transgenic tumor model mice, in particular the p53 conditional KO mouse and the Hi-Myc prostate cancer model.

These *in vivo* experiments will surely provide better insight into the putative tumor suppressor role of PCDH10 and into the possibly differing roles of long and short PCDH10 isoforms.

**V600E BRAF MUTATION LEADS TO A SENESCENCE-LIKE PHENOTYPE ASSOCIATED WITH A LOW PROLIFERATION IN MELANOMA CELLS BUT WITH A HIGHER DEPENDENCE ON MAPK PATHWAY FOR SURVIVAL**

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**Background**

The mutations of BRAF are described in about 50% of melanoma tumors with 90% of these mutations occurring at a single site, leading to the V600E substitution. This mutation leads to the constitutive activation of the MAPK pathway which is critical for cancer cell survival.

**Objectives**

We evaluated the impact of V600E BRAF mutation on melanoma cell proliferation and response to various MAPK inhibitors.

**Methods**

We compared three V600E BRAF (MM032, MM043, MM074) with three wildtype (WT) BRAF melanoma cell lines (HBL, LND1, MM079) addressing (i) cell morphology (ii) protein expression/ phosphorylation (Western blotting), (iii) cell proliferation/survival (crystal violet staining/MTT), (iv) apoptosis (annexin V-positive cell detection), (v) senescence ( $\beta$ -galactosidase activity), (vi) cell cycle (propidium iodide flow cytometric staining), and (vii) response to MAPK inhibitors (U0126, PD98059 and Vemurafenib).

**Results**

The morphology of the V600E BRAF cells showed a 3.1-fold higher volume than WT BRAF cells. The V600E BRAF cells have a lower proliferation index and survival rate than the WT BRAF cells, suggesting a stronger control of the proliferation in mutated cells. However, mutated cells were only a little bit more apoptotic than WT cells, and then apoptosis was not supporting such difference in cell growth. Importantly,  $\beta$ -galactosidase activity was extremely high in mutated cells indicating that constitutive MAPK hyper-activation induces a senescence-like phenotype. Furthermore, we confirm that mutated cells exhibited significantly higher levels of ERK1/2 phosphorylation and marked decreases of AKT phosphorylation and of the specific phosphatase PTEN expression. Interestingly, we found a high expression of the inhibitor of cyclin-dependent kinases p21<sup>Waf1</sup> in MM032 cells, indicating that the lower growth rate of these cells was rather due to a reduced cell cycling. Indeed, we observed an accumulation of MM032 cells in the G0/G1 phase of the cell cycle. In addition, we found that MEK inhibitors (U0126 and PD98059) and V600E BRAF inhibitor (Vemurafenib) decreased cell proliferation with IC50s by 10-fold and 3-fold lower in mutated cells than in WT ones, respectively. Importantly, both mutated and WT cells were sensitive to MAPK inhibition, undergoing cell cycle arrest in G2/S and G0/G1, respectively, and with evidence of high apoptosis in both groups.

**Conclusions**

We found that the V600E BRAF mutation leading to the hyper-activation of ERK in melanoma cells is associated with a senescence-like phenotype and a low proliferation index as compared to WT BRAF cells. Mutated cells are more dependent on MAPK signaling for their survival than WT cells as MEK inhibitors were more effective to inhibit cell proliferation of V600E BRAF cells, by inducing cell cycle arrest and apoptosis.

## DYNAMIC QUANTITATION OF CELL VIABILITY AND MOTILITY KINETICS BY NOVEL *REAL-TIME* TECHNOLOGY IN COMPARISON WITH CLASSIC CELL-BASED ENDPOINT ASSAYS.

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### Background

A state of sustained proliferation as well as increased motility and invasiveness are hallmarks featured by cancer cells, eventually leading to tumor progression and metastasis. Elucidation of biological mechanisms underlying these derailed processes require accurate cell-based monitoring. The xCELLigence Real Time Cell Analysis (RTCA) technology (Roche Applied Science) comprises a non-invasive and label-free approach to assess cell viability and motility in *real time*, hereby countering key features of classic label-based endpoint detection methods. Here we correlate results of *in vitro* time resolution detection of cell viability and migration on 2 malignant cell lines using xCELLigence RTCA DP with data obtained from parallel experiments carried out using established assessment methods for each process.

### Methods

All experiments have been performed using the MDA-MB-231 (breast cancer) and A549 (lung cancer) cell lines. Real-time viability and migration measurements were carried out using the xCELLigence RTCA DP instrument. Cell viability was assessed during 10 days of incubation using modified 16-well plates (E-plate) containing microelectrodes at the well bottoms for impedance-based detection of attachment, spreading and proliferation, expressed as a Cell Index (CI) value. Cell migration was measured during 38 hours using 16-well plates (CIM16) consisting of an upper and a lower chamber separated by a microporous membrane equipped with a similar detection system at the bottom side. The Sulforhodamine B (SRB) assay and a 24-well Transwell set-up served as reference tools to assess viability and migratory kinetics. Viability was estimated by optical density (OD) reading (540 nm) of solubilised cells that were fixed and stained with SRB at a rate of 1 plate daily. The experimental Transwell design allowed dynamic quantitation of tumor cell migration by fixing and staining of duplicate insert membranes in methanol and crystal violet at 10 time points during a 24 hour-incubation.

### Results

SRB and RTCA CI correlated fairly for MDA-MB-231 cell concentrations of 5x10<sup>3</sup> and 10<sup>4</sup> cells/mL (Spearman's  $\rho = 0.79$  and  $0.84$  resp) resulting in similar doubling times ( $p = 0.459$ ). Pixel area quantitation of migrated cells showed strong correlation with xCELLigence CI (Spearman's  $\rho = 0.90$  for both cell lines). However, OD measurements (590 nm) correlated even stronger with CI (Spearman's  $\rho = 0.96$  and  $1.00$  for MDA-MB-231 and A549). Moreover, analysis of random migration indicated a significant difference between RTCA CI and area/OD ( $p < 0.001$ ) implying reduced detection limits of the xCELLigence system, which is also reflected by the increase with time of intra-experimental replicate variance.

### Discussion

Based on our findings, the similarity between observations as performed with conventional approaches and xCELLigence makes both methods interchangeable. Added with results indicating reduced detection limits, xCELLigence provides an accurate detection platform for high-throughput kinetic screenings and for determination of time-dependent cell proliferation and motility dynamics. However, considering reduced detection limits and the variability inherent to cell culture handling, we tend to advise to perform experiments at least in triplicates.

## A TRANSCRIPTION FACTOR ANALYSIS REVEALS REDUCED EMT-PROFILES IN SAMPLES FROM PATIENTS WITH IBC

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**Introduction** – IBC is an aggressive form of locally advanced breast cancer with an elevated invasive and metastatic potential. Recent studies have shown both increased and decreased activation of potent inducers of Epithelial-To-Mesenchymal Transition (EMT). For example, NFκB and LYN are activated in IBC samples whereas TGF-beta is inhibited. As EMT is a biological process governed by a plethora of transcription factors, the purpose of this study is to investigate which EMT-related transcription factors contribute to the phenotype of IBC.

**Methodology** – First we developed an algorithm to study transcription factor (TF) activation using gene expression data sets by focusing on the expression of TF target genes. Relevant gene lists were downloaded from the TFACTS database ([www.tfacts.org](http://www.tfacts.org)). A TF activation score was calculated by subtracting the median expression level of the TF non-targets from the median expression level of the TF-targets. To validate our algorithm, 27 publicly available gene expression data sets from cell lines treated for perturbation of in total 40 different TFs were tested. Next the algorithm was applied onto a gene expression data set of 137 IBC and 252 nIBC samples. Using a thorough literature search we composed a list of 38 TFs involved in EMT, that would become the focus of the current study.

**Results** – For the 40 comparisons done, we failed to correctly predict the TF activation in only 2 cases (error rate: 5%). For those experiments for which the TF activation was predicted correctly (N=38), we observed significance in 75% of the times at a P-value of 0.1, which was considered significant given the small samples sizes involved in these analyses. Application of the algorithm onto our IBC and nIBC gene expression data set resulted in the identification of two EMT-related TFs: SMAD (FDR=0.049; P<0.001) and TCF3/TCF4 (FDR=0.049; P=0.002). The activation profiles of both TFs were significantly reduced in IBC at an FDR less than 0.1. Other EMT-related TFs with significant nominal P-values were STAT3 (FDR=0.072; P=0.003) and NFκB (FDR=0.161; P=0.017). Probe-level analysis of the involved TFs revealed a higher expression of SMAD2 (Z-score = 2,97) and SMAD4 (Z-score = 6,55), in samples from patients with nIBC corroborating our data.

**Conclusion** – In this study we have developed a TF-algorithm that allows for the analysis of TF activation profiles in gene expression data sets. When applied to IBC and nIBC expression data and focusing on EMT-associated TFs, we were not able to discern a clear picture with respect to the involvement of EMT in the biology of IBC. Of note, not all EMT-related TFs identified in our literature search were investigated with our algorithm because their targets were not defined in the TFACTS database. Part of the ongoing research is to define target gene list for all EMT-related TFs and validate our findings using alternative techniques.

**PROLIFERATIVE PHENOTYPE OF MT4-MMP EXPRESSING TUMORS IS DEPENDENT ON TYROSINE KINASE ACTIVITY AND RB SIGNALING.**

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We have previously demonstrated that MT4-MMP (MMP17), a membrane-anchored MMP essentially expressed by breast tumor cells, promotes (i) tumor growth, (ii) pericyte detachment from tumor vessels and, (iii) metastatic dissemination to the lung, while no effect was observed *in vitro* in cells expressing the enzyme. Despite increasing evidences on the role of MT4-MMP in tumor progression, the precise molecular mechanism is not defined yet. Herein, we describe MT4-MMP as new cell cycle regulator enzyme through its regulation of Rb signaling and cell proliferation control *in vivo*. We performed immunohistochemistry method to quantify proliferative cells *in vivo* and demonstrate a significant increase in proliferation of MT4-MMP expressing tumors compared to their control tumors. In order to determine the molecular mechanism regulated by MT4-MMP in promoting tumor cell proliferation, we assessed the downstream signaling pathways activated by MT4-MMP through a global proteomics phosphoantibody array approach. This allows us to identify an increase in Retinoblastoma (Rb) protein phosphorylation level on the sites [S807-811] only in MT4-MMP expressing cells. By immunoblot we confirmed Rb phosphorylation at [S807-811] in MT4-MMP tumors while no other Rb phosphorylation sites was modulated. As hyperphosphorylation of Rb leads to inhibition of its activity and release of the transcription factor E2F which transcribes factors essential for cell proliferation, we investigated whether MT4-MMP regulates factors that affect Rb phosphorylation status. We demonstrated that several targets involved in Rb signaling are regulated by MT4-MMP, strengthening the implication of this pathway in MT4-MMP proliferative effect. Whereas no difference in proliferation of cells expressing or not MT4-MMP was observed on cell monolayer culture *in vitro*, a significant increase in proliferative ratio was observed in MT4-MMP cells cultured in or on top Matrigel. Moreover, the 3D culture model was used to test different signaling pathway inhibitors and revealed a tyrosine kinase (TK) receptor as potential intermediate of MT4-MMP mediated Rb hyperphosphorylation. In conclusion, our data demonstrate for the first time a new mechanism of a membrane anchored MMP MT4-MMP/TKs/Rb axis in promoting tumor cell proliferation *in vivo* and open up a new avenue for cancer research to target MT4-MMP as new tumor cell proliferation regulator enzyme.

## HIGH NUMBER OF CIRCULATING TUMOUR CELLS IS PREDICTIVE FOR INTRAVASCULAR TUMOREMBOLI IN THE LUNG IN END-STAGE METASTATIC CANCER PATIENTS

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**Background:** We have shown that in patients with metastatic breast cancer (MBC) significantly higher numbers of circulating tumour cells (CTC) can be found in central venous blood (CVB) samples as compared to peripheral venous blood samples, suggesting that the lung microvasculature acts as a filter on the circulation of these cells in the blood stream. Especially in patients with high numbers of CTC, cumulative entrapment of CTC in the lung could contribute to respiratory distress. The aim of this study was to investigate the relation between high numbers of CTC in CVB, the presence of intravascular tumour cell emboli (TCE) in the lung and respiratory distress in end-stage cancer patients.

**Methods:** Four patients with MBC and one patient with a metastatic cervical carcinoma were included in this study. All patients suffered from end-stage disease. CTC were measured using the CellSearch CTC test (Veridex, Raritan, NJ, USA) in 7.5 ml CVB obtained from the subcutaneous port catheter. The presence of TCE was studied in lung tissue samples obtained from a limited autopsy. All patients and families gave informed consent.

**Results:** All blood samples were obtained within five days before death. On autopsy, multiple intravascular or perivascular TCE were observed in two and one patients, respectively. Corresponding CTC counts were 404, 164 and 122/7.5 ml CVB. All three patients had a history of rapidly evolving respiratory distress in the last week of life. Although one of the three patients had known pre-existing lung metastases and pleural effusions, radiological examination did not show significant interval changes in any of these patients. In one patient with 4 CTC in the CVB sample, only one solitary TCE could be demonstrated in the lung. In the fifth patient, a very high number of 1296 CTC was found in the CVB sample. However, histological examination of the lung showed diffuse bronchopneumonia in which no TCE could be identified.

**Conclusions:** Multiple TCE were observed in 3 out of 4 patients with substantially elevated numbers of CTC in CVB. In these patients, high CTC counts were more informative than radiological examination for explaining the clinical picture of rapidly evolving respiratory distress.

**Acknowledgement:** This project was supported by a grant from the Belgian Foundation against Cancer.

## HDAC5 DEPLETION MODULATES HETEROCHROMATIN PLASTICITY AND TRIGGERS PROGRAMMED CELL DEATH OF HUMAN CANCER CELLS

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Histone deacetylases (HDAC) is a family of enzymes which play fundamental roles in the epigenetic regulation of gene expression and contribute to the growth, differentiation, and apoptosis of cancer cells. In this study, we further investigated the biological function of HDAC5 in cancer cells. We found HDAC5 is associated with actively replicating pericentric heterochromatin during late-S phase. We demonstrated that specific depletion of HDAC5 by RNAi resulted in profound changes in the heterochromatin structure and slowed down ongoing replication forks. This defect in heterochromatin maintenance and assembly are sensed by DNA damages checkpoint pathways which triggered cancer cells to autophagy, apoptosis and arrested their growth both *in vitro* and *in vivo*. Finally, we also demonstrated that silencing of HDAC5 led to enhanced sensitivity of DNA to DNA-damaging agents, suggesting that chromatin decondensation induced by HDAC5 silencing may enhance the efficacy of cytotoxic agents that act by targeting DNA. Together, these results highlighted for the first time an unrecognized link between HDAC5 and the maintenance/assembly of chromatin structure and demonstrated that its specific inhibition might contribute to increase the efficacy of DNA alteration-based cancer therapies in clinic.

## VERIFICATION OF DYNAMIC INFRARED TRACKING BY GIMBALS POSITION LOGGING AND 3D X-RAY TARGET RECONSTRUCTION ON THE VERO™ SBRT SYSTEM.

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### Introduction

Patient breathing causes lung tumors to move periodically during radiation therapy. The breathing motion is normally taken into account by applying additional treatment margins.

Instead of a larger treatment margin, we could actively compensated tumor motion by MLC, treatment couch or linear accelerator movement also known as tumor tracking radiation therapy (TTRT). Vero™ SBRT is a new IGRT system developed by MHI and BrainLab AG for TTRT using a gimballed linear accelerator.

In this work we verified the performance of Vero™ SBRT tumor tracking on a moving phantom for both static fields and arc delivery.

### Methods and Materials

The Quasar phantom (Modus Medical Devices, London, Canada) was used to evaluate infrared (IR) dynamic tracking performance on a moving hidden target with 4 inserted spherical gold markers. Orthogonal X-ray images acquired every second imaged the moving marker geometry during tracking.

Multiple motion 1D motion patterns were programmed to the quasar phantom. Here for we applied both periodic functions (see Figure 1) with increasing frequency and external breathing motion patterns to the quasar phantom.

A correlation model between internal fiducial markers and external IR markers was used to steer the gimbals based on vertical IR marker motion. In this study tracking was performed by both a general correlation model at standard gantry angle of 0° and gantry angle specific correlation model to investigate robustness of the standard correlation model. IR dynamic tracking performance was evaluated on the gantry angles 0°, 90°, 180° and 270° and during a 350° dynamic arc.

Tracking errors were calculated by assigning a logged gimbals pan and tilt position to every reconstructed X-ray target position after identification of the X-ray acquisition timestamps in the gimbals log file. We expressed the tracking error in the beams-eye view plane at 100 cm from the source.

### Results

A summarization of all measured values of  $E_{90\%}$  in our experiments were given in Table 1. The average of differences in  $E_{90\%}$  between gantry angle pairs 0°-90°, 0°-180° and 0°-270° were small:  $0.03 \pm 0.2$ mm,  $0.002 \pm 0.4$ mm,  $-0.005 \pm 0.2$ mm when using a beam specific correlation model. For tracking experiments using the single 0° gantry angle correlation model for the entire treatment, the average of differences in  $E_{90\%}$  between gantry angle pairs 0°-90°, 0°-180° and 0°-270° showed a slight increase compared to the beam specific correlation model measurements:  $0.15 \pm 0.3$ mm,  $-0.25 \pm 0.3$ mm,  $0.07 \pm 0.4$ mm.

Patient breathing motion showed a maximum  $E_{90\%}$  of 0.78 mm for beam specific correlation model and maximum  $E_{90\%}$  of 1.2 mm for standard 0° gantry angle correlation model.

Tracking performance during dynamic arc showed maximum  $E_{90\%}$  of 1.1 mm for patient breathing. No statistical significant difference was found between overall measured  $E_{90\%}$  of dynamic tracking arc and  $E_{90\%}$  dynamic tracking static beams ( $p > 0.05$ ).

**Conclusion:** Submillimetric accuracy was achievable with Vero SBRT IR tracking for 1D patient breathing motion. No considerable gravity effects on gimbals motion were seen with our setup. Dynamic arc and static beams gave similar tracking accuracy results due to the decoupled gimbals motion.

**Conflict of interest:** Research supported by Hercules foundation and BrainLab AG.

$$\text{motion 1: } 20 \cdot \sin \left[ 2\pi\Delta t \left( \frac{\text{bpm}}{60} \right) \right]$$

$$\text{motion 2: } 15 \cdot \left( 1 - 2 \cos \left[ 2\pi\Delta t \left( \frac{\text{bpm}}{60} \right) \right]^6 \right)$$

$$\text{motion 3: } 15 \cdot \left( 1 - 2 \cos \left[ 2\pi\Delta t \left( \frac{\text{bpm}}{60} \right) + \sigma \right]^6 + \sigma C \sin(\pi\Delta t f) \right)$$

Figure 1: expressions of the periodic motions with bpm: breaths per minute,  $\sigma$  a normal distributed random variable, constant C and  $f \ll \text{bpm}$ .

	bpm	Beam specific correlation model				Standard 0° gantry angle correlation model				
		E <sub>90%</sub> 0° [mm]	E <sub>90%</sub> 90° [mm]	E <sub>90%</sub> 180° [mm]	E <sub>90%</sub> 270° [mm]	E <sub>90%</sub> 0° [mm]	E <sub>90%</sub> 90° [mm]	E <sub>90%</sub> 180° [mm]	E <sub>90%</sub> 270° [mm]	E <sub>90%</sub> Arc [mm]
motion 1	10	0.59	0.61	0.73	0.71	1.03	0.62	1.19	1.09	0.90
	15	0.94	1.19	0.70	1.17	1.13	0.87	1.23	1.16	1.45
	20	1.13	1.44	1.31	1.17	1.30	1.25	1.58	1.78	1.36
	25	1.83	2.15	2.08	1.73	1.79	2.11	2.06	1.92	1.90
motion 2	10	0.50	0.32	0.43	0.51	0.54	0.34	1.08	0.60	0.69
	15	0.73	0.60	0.45	0.65	0.91	0.80	1.28	0.89	1.41
	20	1.12	1.11	1.73	1.64	1.68	1.19	1.44	0.81	0.89
	25	2.25	2.19	1.69	2.13	2.39	2.63	2.52	1.55	2.81
motion 3	10	0.66	0.32	0.47	0.41	0.58	0.45	1.06	0.65	0.81
	15	0.90	0.61	0.58	0.77	0.80	0.87	1.47	1.41	0.92
	20	0.98	1.33	1.58	1.31	1.43	0.64	1.02	0.86	0.97
	25	1.71	1.50	1.99	1.47	1.84	1.79	2.02	1.61	2.10
Patient 1		0.86	0.78	0.71	0.72	0.90	0.65	1.20	0.79	1.09
Patient 2		0.58	0.24	0.30	0.42	0.46	0.48	1.09	0.61	0.99

Table 1: Tracking error E<sub>90%</sub> for different motions at 0°, 90°, 180° and 270° gantry angle for both a beam specific correlation model and a standard 0° gantry angle correlation model.

## PREDICTION OF THE RESPONSIVENESS OF THE DIFFERENT MOLECULAR BREAST CANCER SUBTYPES BY USING 6 ENDOCRINE-RELATED GENES

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*Background:* Estrogen receptor (ER)-positive breast cancers are generally treated with endocrine therapy, but in some cases resistance occur. Previous research showed that NFκB may play a role in the development of resistance to hormonal therapy. In this study, we investigate the role of NFκB, and its interaction with ER, on the responsiveness to endocrine therapy. We use resistant and responsive cell lines selected by using several markers for endocrine resistance selected from a previous study: ADAMDEC1, ITK, ABAT, CLEC7A, ETS1 and STC2.

*Material and methods:* Cell lines of each molecular subtype were selected using publically available gene expression profiles of cell lines (GSE12777 Hoefflich et al, 2009; GSE16795 Hollestelle et al, 2009). Next, the expression of the 6 genes, that can predict the responsiveness to endocrine therapy is evaluated in the MCF7, BT20, CAMA1, MDA-MB-361, MDA-MB-231, MDA-MB-134, MDA-MB-468 and MDA-MB-436 cell lines. RNA was isolated from the cell lines and used for quantitative RT-PCR for ADAMDEC1, ITK, ABAT, CLEC7A, ETS1 and STC2, and for 18S and beta-actin as housekeeping genes.

*Results:* There was a significant higher expression of ABAT and STC2 in the ER-positive cell lines: MCF7, MDA-MB-134 MDA-MB-361 and the CAMA1 cell line. The ER-negative cell lines, MDA-MB-468 and MDA-MB-231, showed respectively a significant higher expression of CLEC7A and ETS1 compared to the luminal subtypes. There is a significant difference in the expression of the resistant markers between ER-positive tumors with and without NFκB-expression. Also NFκB-negative tumors with or without ER expression, double negative and double positive, and ER-negative breast cancers with or without NFκB-expression showed a significant difference in the expression of the 6 markers. Although these differences in expression between the different expression patterns of ER and NFκB, these 6 markers are not allowed to distinguish tumors based on the expression of ER and NFκB, as some cell lines with the same expression pattern, for example MCF7 and MDA-MB-361, also possesses a significant difference.

*Conclusion:* The expression of the 6 endocrine responsive genes differ between cell lines with a different expression of ER and NFκB, which indicate a difference in prognosis between these cell lines. Cell lines of the same subtype also showed a difference in expression of the marker genes, indicating that tumors with the same expression pattern for ER and NFκB can have a different response to endocrine therapy. In the future we will evaluate the change in expression of these response predictive genes after stimulation of ER and NFκB and evaluate their predictive value for response to hormonal therapy. Also ELISA and EMSA will be performed to evaluate the activity of ER and NFκB in the different cell lines before and after stimulation.

These are preliminary data and will be upgraded.

## MACROPHAGE MANNOSE RECEPTOR-SPECIFIC NANOBODY-BASED TARGETING AND IN VIVO IMAGING OF TUMOR-ASSOCIATED MACROPHAGES

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Tumor-associated macrophages (TAMs) are an important component of the tumor stroma and can exert several tumor-promoting activities. We have previously documented that TAMs in various pre-clinical tumor models contain molecularly and phenotypically distinct subsets differing in angiogenic properties and intra-tumoral localization, with Macrophage Mannose Receptor (MMR) being highly expressed on strongly pro-angiogenic, MHC II<sup>low</sup> TAMs residing in hypoxic tumor areas. In the current study, we set out to specifically target MMR-positive cells using nanobodies: single-domain antigen-binding fragments derived from *Camelidae* heavy-chain antibodies.

Nanobodies generated against MMR were found to stain TAMs, and more intensely the MHC II<sup>low</sup> subset, on ex vivo single-cell suspensions of subcutaneous tumors. Upon intravenous injection of <sup>99m</sup>Tc-labeled nanobodies in wild-type and MMR-deficient mice, total-body scans acquired using Pinhole SPECT and micro-CT revealed that these probes specifically targeted several tissues. The nanobodies were detected predominantly in liver and spleen and to a lesser extent in cardiac muscle, lymph nodes and bone marrow. In subcutaneous tumor-bearing mice, receptor-specific uptake of anti-MMR nanobodies was observed in the tumor. Interestingly, coinjection of excess unlabeled anti-MMR nanobodies strongly reduced the accumulation of <sup>99m</sup>Tc-labeled anti-MMR nanobodies in extra-tumoral organs such as spleen and liver, but not in the tumor. Coinjection of unlabeled bivalent anti-MMR nanobodies further reduced the signal in extra-tumoral organs to background levels, while uptake of <sup>99m</sup>Tc-labeled anti-MMR nanobodies in the tumor was only slightly diminished, rendering the tumor the site with the highest MMR-specific nanobody uptake.

These results offer diagnostic and therapeutic perspectives of using anti-MMR nanobodies for selective in vivo targeting of TAM subpopulations.

**ANGIOGENESIS-RELATED CYTOKINES AND PTEN EXPRESSION AS POTENTIAL PREDICTIVE BIOMARKERS IN A PHASE II TRIAL EVALUATING EVEROLIMUS EFFICACY IN LOCALLY ADVANCED OR METASTATIC BLADDER TRANSITIONAL CARCINOMA CELL.**

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**Background:** Dysregulation of mammalian Target of Rapamycin (mTOR) pathway and subsequent angiogenesis activation play a role in Transitional Carcinoma Cell (TCC). Despite growing interest for mTOR inhibitors in cancer, there is up to now no potential biomarker able to predict disease control. Here, we evaluated in a phase II trial the efficacy of the mTOR inhibitor everolimus in patients with palliative bladder TCC after failure of platinum-based therapy. Moreover, we explored as potential predictive biomarkers, the angiogenesis-related proteins in plasma and the tumoral expression of proteins implicated in mTOR pathway dysregulations.

**Materials and Methods:** Patients with locally advanced or metastatic bladder TCC received everolimus 10 mg/day continuously until progressive disease (PD) or unacceptable toxicity. Primary endpoints were control disease rate, including complete response (CR), partial response (PR) or stable disease (SD) at 8 weeks. Plasma samples were collected on day 1 (baseline before treatment), day 28 (during treatment), and at PD. A screening of 55 angiogenesis-related proteins was performed in plasma by cytokine arrays and most significant results were confirmed with dedicated ELISA. PTEN expression and *PIK3CA* mutations, detected on pre-treated tumor samples with immunohistochemistry and RT-PCR respectively, were correlated with disease control.

**Results:** 37 patients were included. Confirmed PR was observed in 2 patients and SD in 8 patients, resulting in a disease control rate of 27% at 8 wks. Analyzing changes in plasma cytokine concentrations between baseline and day 28, we found that everolimus induces globally an increase in the angiogenesis inhibitor angiostatin (+ 114%;  $p < 0.0001$ ). In patients with controlled disease, we observed an early decrease in two markers of tumor vessel maturation, namely angiopoietin-1 (- 80%;  $p = 0.010$ ) and PDGF-AB (-54%;  $p = 0.006$ ) compared to patients with non-controlled disease. The tumor endothelial cell marker endoglin showed an increase in patients with non-controlled disease (+ 19%;  $p = 0.06$ ) compared to patients with controlled disease (-18%;  $p = 0.010$ ). Moreover, baseline levels of angiopoietin-1 were much higher in patients with controlled disease than in patients with non-controlled disease (+ 242.5%;  $p = 0.011$ ). Pre-treated tumor samples were available for 20 patients (6 with controlled disease and 14 with non-controlled disease). Absence of PTEN expression was observed in 57% of patients with non-controlled disease and in 0% of patients with controlled disease. *PIK3CA* mutations were detected in only 15% of tumors.

**Conclusion:** Everolimus exerts antitumor activity in advanced bladder TCC, through a likely anti-angiogenic activity. Angiopoietin-1 appears as a potential biomarker to predict and track such response and should be considered in further clinical trials while PTEN loss might be associated with everolimus resistance.

**THE FUNCTIONAL ROLES OF OSTEOPONTIN SPLICING ISOFORMS IN GLIOBLASTOMA PROGRESSION.**

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Glioblastomas (GBM) are aggressive intrinsic brain tumors and are often fast growing associated with a poor prognosis for the patient. A number of reports have demonstrated that these tumors contain a subpopulation of stem cell-like tumor cells (glioma stem cells, GSCs) implicated in glioma progression, therapeutic resistance and recurrence. Osteopontin (OPN), a glycoprotein associated with tumorigenesis and metastasis, is overexpressed in GBM. Alternative splicing of the OPN gene generates three protein splicing isoforms (OPN-SI), designated as OPNa, OPNb and OPNc, which have demonstrated specific roles in different tumor models. This work aims to evaluate the expression profiling of OPN-SI in glioma stem cells isolated from GBM primary tumors and their putative roles in glioblastoma biology. The expression levels of OPN-SI in GSCs were analyzed using isoform specific qRT-PCR. Preliminary results demonstrated that the three OPN-SI were detectable at the mRNA level in all the GSCs analyzed in this study. OPN-SI expression profiles of GSCs indicated that OPNa and OPNb isoforms were upregulated when compared to OPNc. To investigate further the potential role of OPN-SI in GSCs, we used a well-characterized human GBM cell line (U87-MG), which was stably transfected with each OPN-SI expression vectors, as well as empty vector controls. The generation of stable clones will help us to test the hypothesis according to which OPNa and OPNb might be the OPN-SI that are critical for the maintenance of GSCs phenotype and the development of glioma tumors.

## DETECTION OF PIK3CA AND KRAS MUTATIONS IN BREAST CANCER CELL LINES: OPTIMIZATION AND COMPARISON OF NEW GENERATION SEQUENCING AND MUTATION DETECTION PLATE TECHNIQUES.

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**Introduction.** Breast cancer is the most frequent carcinoma and the second most common cause of cancer-related mortality in women. Detection of (new) mutations in a large number of samples is a considerable tool for disease prognosis and selection of treatment options. Gene expression profiles and genetic mutations are key parameters used for the characterization of tumors. An existing technique of mutation detection comprises new-generation sequencing technology, developed by Roche/454. This technique, specialized in high-throughput DNA sequencing, uses a large-scale parallel pyrosequencing system capable of in-depth sequencing of multiple amplicons. In the current study we sought to optimize this sequencing technique for two genes which have been reported to be mutated in some breast cancer cases, namely KRAS and PI3KCA, and compared the obtained results with another mutation detection technique (TaqMan® Mutation Detection Plates, Applied Biosystems).

**Materials and methods.** We determined K-RAS and PI3KCA mutations in breast cancer cell lines using next-generation sequencing and TaqMan Mutation Detection Assays (BRAF-KRAS plate). DNA was extracted from MDA-MB-361, MDA-MB-231 and SKBR3 cell lines using the QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA). For the procurement of special primers for use in the 454 Sequencing System we consulted the IDT website. Multiplex identifiers (MIDs) are included to serve as DNA barcodes to identify the specific cell line the amplicon is generated in. Optimization and quality control of the generated amplicons was performed using the Agilent 2100 Bioanalyzer. Simultaneous pyrosequencing of different amplicons was carried out with the GS Junior System. Thirteen distinct KRAS mutations were investigated by use of a BRAF-KRAS assay, powered by castPCR technology. To assess the assay sensitivity with which specific DNA mutations are measured against a background of wild type genomic DNA, an equimolar dilution series was performed in which DNA, carrying mutant alleles, is spiked into a quantity of normal cell line gDNA.

**Results\*.** The TaqMan Mutation Detection method is shown to be sensitive to detect mutations in a WT gDNA background with an assay sensitivity of 6,25%. Mutational detection by this method revealed KRAS mutations in the MDA-MB-231 cell line. We identified a G13D mutation for KRAS for the positive control (1ng/μl MDA-MB-231 whole genomic DNA). The mutational analysis by 454 sequencing methodology is still in progress and will be adjusted.

**\*The results consist of preliminary data and will be updated by February 2012**

**Conclusion.** As the clinical importance of mutation detection in (breast) cancer is defined, there will be an increasing need to be able to assess the mutation status of a patients tumor. The deep sequencing method, which allow mutations to be detected at extremely low levels in cancer samples, enables us to detect the mutational status of both well-known cancer genes and different breast cancer specific genes. An interesting example are the microRNA processing genes (e.g. *Dicer1*), which code for enzymes responsible for the biogenesis of microRNAs. These small non-coding RNAs have been shown to be involved in several biological processes and their deregulation plays an important role in the development of (breast) cancer.

**A FIRST INSIGHT INTO BREAST CANCER CELL RESISTANCE TO TRASTUZUMAB: MUC4<sup>EXPRESSION</sup> ON THE CELL MEMBRANE INTERFERES WITH THE DRUG BINDING TO HER2 RECEPTORS.**

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Human Epidermal growth factor Receptor (HER2)-overexpression occurs in 25%-30% of breast cancers. It is constitutively activated to promote cell proliferation and invasion. Trastuzumab (Herceptin), an anti-HER2 monoclonal antibody, has significantly improved treatment outcomes in patients with HER2-overexpressing breast cancer (1-2). Despite trastuzumab clinical usefulness, there is a need for more accurate prediction of trastuzumab's response and for overcoming both primary and acquired resistance. One of the postulated resistance mechanisms is the interference of a membrane mucin called MUC4 with the binding of trastuzumab to its target receptor (3-4).

Method: Breast cancer cell lines HER2- negative T47D cells, HER2-positive cell lines BT474, SKBr3 and JIMT1, with the latter overexpressing MUC4 and trastuzumab insensitive, were incubated with different mucolytic agents: N-Acetylcysteine (NAC) and Carboxymethylcysteine (CMC), MESNA and Bromelain in order to breakdown that mucin network that may mask HER2. Radiobinding assays were performed using trastuzumab labeled with Zirconium 89, a positron emitter, following the method of Verel et al.

Results: Trastuzumab was efficiently radiolabeled with (<sup>89</sup>Zr) with a high radiochemical purity (>98%), volumic activity (>15MBq/mL) and specific activity (> 14MBq/mg). MUC4 overexpressing JIMT1 cells exhibited a significant increase in binding ability of <sup>89</sup>Zr-Trastuzumab (up to 27%, p<0,001) when treated with mucolytic agents NAC or CMC, with NAC being the most potent effector at lower concentrations. MESNA had no effect. The EC50 was only affected in NAC treated JIMT cells, namely a 4 fold decrease in EC50. None of the other cell lines displayed any significant changes in either their trastuzumab binding ability or in their EC50 values.

Conclusion: N-Acetylcysteine may be an interesting drug to counteract one of trastuzumab's resistance mechanism by affecting mucin masking and improving the accessibility of trastuzumab to its receptors, HER2.