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Basic Science/Translational Research

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Luminal A Breast Tumours Divided in Two Clusters by DNA Methylation

T. Fleischer[1], J. Jovanovic[2], H. Edvardsen[1], G.I.G. Alnæs[1], B. Naume[3], A.L. Børresen-Dale[1], J. Tost[4], V.N. Kristensen[5]. [1]*Norwegian Radium Hospital, Department of Genetics, Oslo,* [2]*University of Oslo, Institute of Clinical Medicine, Oslo,* [3]*Norwegian Radium Hospital, Cancer Clinic, Oslo, Norway;* [4]*CEA − Institute de Génomique, Centre National de Génotypage, Paris, France;* [5]*Department of Genetics, Norwegian Radium Hospital, Oslo, Norway*

Background: Gene expression profiles and DNA methylation profiles have been shown to be of importance for breast cancer development and survival of patients. Based on gene expression profiles five groups of tumours have been identified (Luminal A, Luminal B, ERBB2 enriched, basal-like and normal-like) and the groups show different survival. Luminal A is usually the largest group and has the best prognosis, while basal-like has the worst prognosis. Lately, analyses have identified three groups based on DNA methylation (called Cluster 1−3) that also show different survival. Cluster 3 has best prognosis, Cluster 1 has an intermediate prognosis, while Cluster 2 has worst prognosis. The concordance between gene expression groups and DNA methylation groups is strong, though, interestingly, the Luminal A tumours are split quite evenly between Cluster 1 and Cluster 3. Based on the split between Cluster 1 and 3, patients having Luminal A tumours show different survival. This study set out to further investigate the two groups of Luminal A tumours.
Material and Methods: DNA material from 80 breast tumours were analyzed by Illumina GoldenGate interrogating 1505 CpGs, and 102 breast tumours were analyzed by Illumina Infinium 27K methylation array interrogating more than 27 thousand CpGs. Whole genome expression profile was available for all samples. The samples were collected at hospitals in Oslo/Akershus and all patients have given informed consent and the projects are approved by the local ethical committee.
Results: Using SAM analysis on Luminal A tumours, 41 genes were found differentially methylated between Cluster 1 and Cluster 3 (FDR < 5%), and these included *BIRC4*, *CD40*, *CDKN1C*, *EGFR*, *ESR2*, *ICAM1*, *KIT*, *MAS1*, *SFRP1*, *TERT*, *WNT1 and WT1*. Further, the gene list was used to do hierarchical clustering on Luminal A breast tumours analyzed by Illumina 27K methylation array, and also these tumours was split in two groups. When applying Kaplan–Meier survival analysis on these two groups, a significant difference was observed. Further results will also include biological analysis of the genes involved in separation of the two clusters.
Conclusions: Earlier work has shown that Luminal A tumours are split between two DNA methylation clusters and that these show different survival. Here we show which genes drive the separation of the Luminal A tumours into two groups by DNA methylation, and we show that the difference in survival is apparent on multiple dataset and analyzed on different platforms. We will also show a biological interpretation of the genes involved.

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Anti-cancer Therapy Mediated by a Histone Deacytelase Inhibitor Engages the Immune System

A.C. West[1], A.J. Christiansen[2], S. Mattarollo[1], K.M. Banks[1], N.M. McLaughlin[1], H. Duret[1], N.M. Haynes[1], M.J. Smyth[1], R.W. Johnstone[1]. [1]*Peter MacCallum Cancer Institute, Cancer Immunology Program, Melbourne, Australia;* [2]*Swiss Federal Institute of Technology Zurich, Pharmacogenetics, Zurich, Switzerland*

Histone deacetylase inhibitors (HDACi) are novel anti-cancer agents, that potently induce tumour cell-specific apoptosis. HDACi are thought to enhance tumour cell immunogenicity and promote engagement of immune effector mechanisms that can contribute to the anti-tumour activities of the compounds. We therefore hypothesised that combining HDACi with immunotherapy could provide enhanced therapeutic efficacy when tested in preclinical murine models of carcinoma and lymphoma. Indeed, while the HDACi vorinostat could inhibit the growth of established colon, breast and renal carcinomas in vivo, anti-tumour activity was significantly enhanced when vorinostat was co-administered with immunomodulatory monoclonal antibodies to 4−1BB and CD40 (56%, 25% and 25% complete regressions respectively). Further investigation determined vorinostat induced an immunogenic form of tumour cell-specific apoptosis and enhanced phagocytosis of tumour cells by antigen presenting cells in vitro, together suggesting an inherent capacity of vorinostat to enagage the immune system. We have also shown vorinostat alone significantly prolongs survival of mice bearing Eμ-Myc B cell lymphoma compared to controls (20 days). However, this survival advantage was not observed in immunocompromised Rag-$2^{-/-}$common-$\gamma^{-/-}$, RAG1 and CD8-depleted mice (7.5, 8 and 12.5 days). Equivalent survival was observed when immunocompetent and Rag-$2^{-/-}$common-$\gamma^{-/-}$ mice were treated with etoposide (29 and 32 days), demonstrating the specificity of vorinostat-mediated immune engagement during anti-cancer treatment. Finally, treatment of immunocompetent mice with vorinostat bearing Eμ-Myc B cell lymphoma resulted in altered numbers of immune cell subsets at sights of primary tumour burden (spleen and lymph nodes), suggesting a further mechanism by which HDACi mediate anti-cancer activity. These novel data demonstrate an important requirement for adaptive and possibly innate arms of the immune system in HDACi-mediated anti-cancer therapy. Future studies will characterise the mechanism of HDACi-mediated effector cell engagement and activation in mouse models of cancer, and this information aid in the design of more potent combination strategies utilizing HDACi's.

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Senescence Induction by BRAFV600E is Associated With the Induction of Autophagy and Targeted Degradation of BRAF and CRAF

M. Aguilar Hernandez[1], B. Patel[1], N. Bate[1], A. Bottrill[1], H. Jin[1], C. Pritchard[1]. [1]*University of Leicester, Biochemistry, Leicester, United Kingdom*

More than 90% of *BRAF* mutations in human cancer are represented by a valine to glutamate mutation at residue 600 known as the ^{V600E}*BRAF* mutation. Our laboratory has generated mouse models expressing a conditional knock-in allele of $BRaf^{V600E}$ and in previous work we have shown that expression of the oncogene in a range of tissues including embryonic fibroblasts (MEFs), lung tissue, melanocytes and gastrointestinal crypts induces ~10 population doublings after which senescence ensues. At senescent time points we have found that the expression level of both BRAF and CRAF proteins is significantly reduced and this occurs concomitantly with a drop in ERK1/2 phosphorylation. We have further investigated the mechanisms underpinning this drop and, through qRT-PCR experiments, have found that it cannot be accounted for by down-regulation of the corresponding mRNAs. Furthermore, inhibition of the proteasome does not lead to a rescue in the expression level of the proteins. Our studies do, however, show that BRAF and CRAF accumulate in the insoluble fraction and, using LC-MS/MS, we have found that this accumulation is associated with phosphorylation of a novel serine residue at position 675 of BRAF. This unusual processing of the RAF proteins is associated with the induction of hallmarks of autophagy in the senescent cells as assessed by electron microscopy, GFP-LC3 aggregation and LC3II turnover. Thus, we propose that selective degradation of BRAF and CRAF proteins by autophagy can occur as a protective cellular response in the early stages of cancer development induced by ^{V600E}BRAF.

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Crosstalk Between Homeobox Proteins and Polycomb Complexes in P16INK4a Regulation

J. Gil[1], J. Popov[1], N. Martin[1], S. Raguz[1], S. Li[2], M. Walsh[2]. [1]*MRC Clinical Sciences Centre, Imperial College London, London, United Kingdom;* [2]*Mount Sinai School of Medicine, Department of Paediatrics, New York, USA*

Background: The *Ink4a*/*Arf* locus is the chromosomic region most frequently altered in human cancer. It encodes for two gene products ($p16^{INK4a}$ and ARF) that regulate the Rb and p53 pathways and which are key senescence effectors. Controlling the expression of the locus is critical to cell homeostasis. In normal circumstances, the locus is repressed by Polycomb repressive complexes (PRC), and its expression induced in response to stresses such as oncogenic signalling. How PRCs are recruited to their target genes, and specifically to the *Ink4a*/*Arf* locus is not fully understood, as there is no PRC core component with a DNA binding domain.
Material and Methods: We cloned 12 candidate transcription factors in retroviral vectors and expressed them in IMR90 human fibroblast to screen for their ability to regulate senescence by performing growth curves, BrdU incorporation and SA-β-Gal assays.
Results: Amongst the factors tested, we observed that expression of the homeobox-containing protein HLX1 extended cellular lifespan and delayed replicative senescence on human fibroblasts. Expression of HLX1 also blunted the growth arrest induced by Ras expression in an in vitro model of Oncogene-induced senescence. Expression of HLX1 did not significantly affect p53 or p21 levels, but when HLX1 was expressed we consistently

observed decreased $p16^{INK4a}$. Detailed analysis showed that HLX1 affects *INK4a* expression at the mRNA level and HLX1 binds to the *INK4a* promoter as observed by ChIP. We also observed increased levels of the repressive H3K27me3 mark and recruitment of PRC2 components at the *Ink4a*/*Arf* locus correlating with HLX1 expression. Co-immunoprecipitation studies showed that HLX1 associates with the PRC2, in particular with Suz12. RNAi studies showed that the repression of $p16^{Ink4a}$ by HLX1 is dependent of PRCs. In an attempt to understand if the repression of HLX1 was a property shared by other homeobox genes, we tested 20 homeobox-containing genes and identified that multiple homeobox can also repress $p16^{INK4a}$.

Conclusions: We identified the homeobox protein HLX1 as a novel $p16^{INK4a}$ repressor. HLX1 binds to the *INK4a* promoter region and recruits Polycomb repressive complexes. Multiple homeobox proteins can also regulate $p16^{INK4a}$ expression, which implies a conserved role for this family oftranscription factors in regulating the *Ink4a*/*Arf* locus, highlighting its potential physiological relevance for both senescence and carcinogenesis.

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Lactate Influx and Efflux Through Monocarboxylate Transporters Bridge Cancer Cell Metabolism and Angiogenesis

F. Vegran[1], R. Boidot[1], P. Sonveaux[1], O. Feron[1]. *[1]UCL Institute of Experimental and Clinical Research (IREC), Angiogenesis and Cancer Research Lab, Brussels, Belgium*

Background: Tumour cells fuel their metabolism with a variety of nutrients to meet the bioenergetic and biosynthetic demands of proliferation. In particular, conversion of pyruvate into lactate allows the production of intracellular NAD^+ to maintain high glycolytic flux in tumours. Here, we investigated whether the consecutive lactate accumulation in the tumour microenvironment could indirectly modulate the endothelial cell phenotype and thereby promote angiogenesis.

Materials and Methods: Microfluidic low-density arrays were used to examine the influence of lactate on the endothelial expression profile of angiogenesis-related genes. The consecutive identification of IL-8/CXCL8 mRNA as the major upregulated transcript in response to lactate led us to study the signaling pathway bridging lactate and IL-8-driven angiogenesis using dedicated gene silencing and pharmacological strategies. We also addressed the *in vivo* relevance of this pathway in different mouse tumour models combining the injection of shRNA-transduced tumour and endothelial cells into extracellular matrix plugs.

Results: We found that lactate could enter endothelial cells through the monocarboxylate transporter MCT-1 and then stimulate an autocrine NFkB/IL-8 (CXCL8) pathway driving endothelial cell migration and tube formation. We further identified the capacity of lactate to activate NFkB through the phosphorylation and consecutive degradation of IkBa. These effects were prevented by 2-oxoglutarate and reactive oxygen species (ROS) inhibitors, pointing to a role for prolyl-hydroxylase and ROS in the integration of lactate signaling in endothelial cells. Prolyl-hydroxylase PHD2 silencing in glucose-fuelled endothelial cells recapitulated the pro-angiogenic effects of lactate, whereas a blocking IL-8 antibody or IL-8-targeting siRNA prevented them. Finally, we documented in mouse xenograft models of human colorectal and breast cancers that lactate release from tumour cells through the MCT4 (and not MCT1) transporter was sufficient to stimulate IL-8-dependent angiogenesis and tumour growth.

Conclusions: Our findings establish the existence of a lactate-driven feed-forward IL-8 autocrine loop driving angiogenesis in tumours and the key roles of monocarboxylate transporters MCT1 and MCT4 in this lactate-based dialog between cancer cells and endothelial cells. More generally, our study provides a new rationale for associating elevated lactate concentrations in tumours and negative outcomes for patients, and further supports the current enthusiasm for new cancer treatments targeting metabolic pathways.

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HER-3, IGF-1, NF K-B and EGFR Gene Copy Number in the Prediction of Clinical Outcome for Colorectal Cancer Patients Receiving Irinotecan-cetuximab

M. Scartozzi[1], R. Giampieri[1], E. Maccaroni[1], A. Mandolesi[2], A. Zaniboni[3], L. Fotios[4], R. Berardi[1], A. Falcone[4], I. Bearzi[2], S. Cascinu[1]. *[1]AO Ospedali Riuniti, Oncologia Medica, Ancona, [2]AO Ospedali Riuniti, Anatomia Patologica, Ancona, [3]Casa di Cura Poliambulanza, Oncologia Medica, Brescia, [4]AO Pisa, Oncologia Medica, Pisa, Italy*

Background: A large proportion of colorectal cancer patients does not benefit from the use of anti-EGFR treatment although in the absence of a mutation of the K-RAS gene. Preliminary observations suggested that HER-3, IGF-1, NF-kB and EGFR GCN might identify patients not likely to benefit from anti-EGFR therapy. We tested the interaction between HER-3, IGF-1, NF-KB, EGFR GCN and K-RAS mutational analysis to verify the relative ability of these variables to identify a sub-group of patients more likely to benefit from EGFR-targeted treatment among those harbouring a K-RAS wild type status.

Materials and Methods: We retrospectively collected tumours from 168 patients with metastatic colorectal cancer patients treated with irinotecan-cetuximab. KRAS was assessed with direct sequencing, EGFR amplification was assessed by chromogenic in situ hybridization and HER-3, IGF-1 and NF-kB were assessed by immunoistochemistry.

Results: In patients with K-RAS wild type tumours, the following molecular factors resulted independently associated with response rate: HER-3 (OR = 4.6, 95% CI: 1.8–13.6, p = 0.02), IGF-1 (OR = 4.2, 95% CI: 2–10.2, p = 0.003) and EGFR GCN (OR = 4.1, 95% CI: 1.9–26.2, p = 0.04). These factors also independently correlated with overall survival as follows: HER-3 (HR = 0.4, 95% CI: 0.28–0.85, p = 0.008), IGF-1 (HR = 0.47, 95% CI: 0.24–0.76, p < 0.0001) and EGFR GCN (HR = 0.59, 95% CI: 0.22–0.89, p = 0.04) (table 1).

Conclusions: We believe that our data may help further composing the molecular mosaic of EGFR resistant tumours. HER-3, IGF-1 and CISH EGFR GCN proved to possess a relevant role in defining subgroups of colorectal cancer patients more likely to benefit from anti-EGFR treatment. Interestingly HER-3 and the IGF-1 driven pathway have also been demonstrated to be possible molecular targets as part of a treatment protocol focused on control of either HER receptors or the PI3K/AKT pathway. The possibility to use HER-3 and IGF-1 inhibitors in biologically-selected anti-EGFR resistant tumours promise then to be a crucial challenge for the future development of targeted therapy in colorectal cancer patients.

Table 1

	HER-3		IGF-1		EGFR	
	Positive (n = 46)	Negative (n = 44)	Positive (n = 59)	Negative (n = 31)	<2.12 (n = 47)	⩾2.12 (n = 43)
Response Rate (%)	25%	50%	22%	65%	6%	37%
Multivariate OR (95% CI	4.6 (1.8–13.6)		4.2 (2–10.2)		4.1 (1.9–26.2)	
Logistic regression *p* value	0.02		0.003		0.04	
Median Overall Survival (months)	11.3	25	8.3	25	10.4	18
Multivariate HR (95% CI)	0.4 (0.28–0.85)		0.47 (0.24–0.76)		0.59 (0.22–0.89)	
Cox regression *p* value	0.008		<0.0001		0.04	

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Prognostic Factors for Progression-free Survival (PFS), Overall Survival (OS), and Long-term OS (LT-OS) With Sunitinib in 1,059 Patients, Treated on Clinical Trials, With Metastatic Renal Cell Carcinoma (mRCC)

R.J. Motzer[1], B. Escudier[2], R. Bukowski[3], B.I. Rini[3], T.E. Hutson[4], C.H. Barrios[5], X. Lin[6], K. Fly[7], E. Matczak[8], M.E. Gore[9]. *[1]Memorial Sloan Kettering Cancer Center, Department of Medicine, New York, USA; [2]Institut Gustave Roussy, Immunotherapy Unit, Villejuif, France; [3]Cleveland Clinic Taussig Cancer Institute, Solid Tumour Oncology, Cleveland, [4]Baylor Sammons Cancer Center-Texas Oncology, Internal Medicine, Dallas, USA; [5]PUCRS School of Medicine, Hematology and Oncology, Porto Alegre, Brazil; [6]Pfizer Oncology, Clinical Statistics, La Jolla, [7]Pfizer Oncology, Oncology, New London, [8]Pfizer Oncology, Oncology, New York, USA; [9]Royal Marsden Hospital NHS Trust, Medicine, London, United Kingdom*

Background: With the advent of multiple targeted therapies for mRCC, further information on factors affecting prognosis facilitates both clinical decision making and trial design for evaluation of new therapies. Here, we report a retrospective analysis of prognostic factors for PFS, OS and LT-OS (⩾30 months) in patients (pts) with mRCC treated with sunitinib in 6 clinical trials (NCT00054886, NCT00077974, NCT00083889, NCT00338884, NCT00137423, NCT00267748; Pfizer).

Methods: Analyses used pooled data from 1,059 pts treated with single-agent sunitinib on the approved 50 mg/day 4-week-on/2-week-off schedule (n = 689; 65%) or 37.5 mg continuous once-daily dosing (n = 370; 35%), in the first- (n = 783; 74%) or second-line (n = 276; 26%) setting. Baseline variables were analyzed for prognostic significance using a Cox proportional hazards model, with each factor investigated in univariate and then multivariate analyses using a stepwise algorithm.

Results: Multivariate analysis of PFS and OS identified 9 and 10 independent predictors, respectively (Table). Overall, 215 pts (20%) survived at least 30 months. An analysis of baseline characteristics of these long-term survivors showed characteristics differed between these pts and non-long-term survivors, including risk status based on the published Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic criteria (Motzer, 2002; P < 0.0001). For example, 70% of the long-term survivors had favorable risk features compared with 31% of non-long-term