375 The role of A to I RNA editing enzymes in melanoma

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The roles of Adenosine Deaminase Acting on RNA (ADAR) enzymes in carcinogenesis are still mostly unknown, yet aberrant RNA editing patterns and lower ADARs expression have been observed in several human tumours. Here we study for the first time the role of ADAR1 in the biology of melanoma.
ADAR1 knockdown and subsequent rescue cell systems were constructed in the human melanoma cell line 624mel. The effect of altered ADAR1 expression was examined both *in vitro* (proliferation, invasion and cell cycle) and *in vivo* (human xenografts in SCID-NOD mice). In addition, we compared global gene expression of control and ADAR1-knockdown cells. Based on bioinformatic analysis and qRT-PCR of microRNA of two melanoma subclones, the highly aggressive C8161-HAG ($ADAR1^{low}$) and the poorly aggressive C8161-PAG ($ADAR1^{high}$), specific miRs (miR-432*, -17, -20a and -106a) targeting ADAR1−3′UTR were selected and further examined.
Our results demonstrate significant decrease of ADAR1 expression in more than 60% of 30 primary melanoma cultures tested, as compared to normal melanocytes. Importantly, specific knockdown of ADAR1 expression *in vitro* altered cell morphology and revealed a substantial and consistent enhancement in cell proliferation and invasion capabilities. Introducing ADAR1 into the knockdown cells successfully increased ADAR1 expression levels and reversed the effects on proliferation and invasion rates. *In vivo*, ADAR1 knockdown dramatically increased the growth rate of melanoma and caused intense black pigmentation. Moreover, the comparative global gene expression analysis confirmed that the most prominently altered gene clusters were cell growth, proliferation and motility. Over-expression of miR-432* and miR-17 in C8161-PAG melanoma subline significantly decreased ADAR1 expression and increased its net proliferation rate. These combined results strongly support the role of ADAR1 as a central regulator of cancer features in melanoma and imply that microRNAs may regulate its expression.
ADAR1 is ubiquitously expressed and downregulated in many tumours, therefore these findings may represent a general cellular mechanism, not confined to melanoma. Further unraveling of the function and regulation of ADAR1 may lead to the identification of novel diagnostic, prognostic and therapeutic interventions.

376 Regulation of tumour cell invadopodia by hypoxia-induced NHE-1 activation

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Invasive tumours are characterized by an acidic and hypoxic microenvironment that promotes metastasis. The Na+/H+ exchanger (NHE-1) plays an important role in the regulation of pH homeostasis. In tumour cells, it has been demonstrated that NHE-1 is constitutively active, promoting cell invasion, but the mechanisms are not defined. One of the ways that tumour cells are able to promote invasion is by forming invadopodia. Invadopodia are actin-rich structures that secrete matrix-metalloproteases (MMP) and are able to degrade the extracellular matrix. Our lab has shown that hypoxia significantly increases invadopodia formation in human fibrosarcoma cells (HT-1080). Also, it has been demonstrated that MMP matrix-degrading activity is dependent on extracellular acidic pH. Therefore, the aim of our study was to define whether NHE-1 participates in hypoxia-induced invadopodia production.
First, we observed that hypoxic stimulation increased NHE-1 RNA and protein expression. Intracellular pH monitoring by live-cell imaging revealed that NHE-1 activity was also increased under hypoxic conditions. Results using pharmacological inhibitors and shRNA-mediated depletion indicated that NHE-1 participates in invadopodia formation in HT-1080. NHE-1 inhibition also abolished invadopodia-mediated matrix degradation and this correlated with an inhibition of hypoxia-induced MMP-2 activation. Moreover, the ECM degradation areas induced by hypoxia clearly correlated with the presence of discrete puncta of NHE-1 at invadopodial structures.
Alltogether, our results suggest indicated that NHE-1 is necessary for both the formation of the invadopodium structure and the activity of proteinases involed in invadopodia-induced matrix degradation under hypoxia.

377 Oncogenic role of neuropilin-2

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Background: Neuropilins (NRPs) are transmembrane non-tyrosine kinase glycoproteins originally described in the nervous system. Initially characterized as neuronal receptors, NRPs were also found to be expressed in endothelial cells and subsequently were shown to play a role in the development of the vascular system. The multiple functions of NRPs were recently highlighted by the identification of NRP role in oncogenesis. In this study, we first confirmed the role of NRP2 in tumour progession in vitro and in vivo. We also extended the understanding of NRP2 oncogenic functions by investigating the ability of NRP2 to orchestrate epithelial-mesenchymal transition (EMT) in colorectal cancer cells.
Methods: We first sought to examine the expression of NRP2 glycoprotein in various cancer cell lines and tumoural tissues by immunofluorescence analysis and immunohistochemical staining. Using specific siRNA to target NRP2 expression, or NRP2 gene transfer we studied the influence of NRP2 expression on proliferation and tumour formation in vitro by MTT and soft agar assays and then in vivo using xenografts experiments. NRP2 induced-EMT was then investigated by flow cytometry, immunohistochemical (IHC) staining and quantitative real-time PCR.
Results: Immunofluorescence analysis confirmed that NRP2 is expressed at the membrane of several human cancer cell lines. Moreover, IHC staining showed that NRP2 is expressed at the membrane of human colon carcinoma and breast carcinoma while it is not expressed in non malignant tissues. Our results first confirmed the role of NRP2 in cancer proliferation *in vitro* and xenograft formation *in vivo*. Analysis of NRP2 transfected cell lines and NRP2 expressing xenografts established that NRP2-expressing tumour cells displayed an immunohistochemical phenotype of EMT characterized by the loss of E-Cadherin and an increase of vimentin expression. Moreover, the expression of NRP2 on colon cancer cell lines was shown to promote transforming-growth factor-β1 (TGF-β1) signaling, leading to an increased phosphorylation of the Smad2/3 complex in colorectal cancer cell lines. Specific NRP2 inhibition using siRNA prevented the promoter effect of TGF-β1 on colony formation.
Conclusions: Our results suggest a direct role of NRP2 in EMT and highlight a cross-talk between NRP2 and TGF-β1 signaling to promote cancer progression. These results suggest that NRP2 fulfill all the criteria of a therapeutic target to disrupt multiple oncogenic functions in solid tumours.

378 Aberrant phosphatidylcholine metabolism as source of biomarkers and therapeutic targets in human ovarian cancer

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Background: The detection and characterization by MRS of aberrant phosphatidylcholine (PC) metabolism in tumours fostered new areas of investigation in cancer cell biology and allowed identification of indicators of in vivo tumour progression by of choline-based MRS and PET.. We showed a 2- to 5-fold increases in total choline containing metabolites (tCho), with parallel 3- to 8-fold increases in phosphocholine (PCho), in human epithelial ovarian cancer (EOC)cells compared to non-tumoural cells (EONT) (Iorio et al, Cancer Res 2005).
Purposes of this study was to investigate the molecular mechanisms underlying aberrant choline metabolism in preclinical models of ovary cancer.
Methods: Human EOC cell lines were established from ascitic fluid or from primary tumours; EONT cells were either isolated from normal ovary surface epithelium or its immortalized variants. MRS analyses were performed on cell extracts at 16.4 or 9.4 T. Microarray-based gene expression was evaluated by Gene Set Enrichment Analysis on EOC and EONT data sets. Western blot experiments were performed using polyclonal rabbit anti-human Chok antibody [Glunde et al, Cancer Res 2005] and polyclonal rabbit anti-B. cereus antibodies (Spadaro et al, Cancer Res 2008). MRS/MRI were performed in human EOC xenografts at 4.7T.
Results: The increase in PCho content in cancer cells was associated with activation of enzymes involved in both biosynthetic and catabolic PC pathways. Choline Kinase (ChoK) was over-expressed at protein level (about 3×) and activated (9−25 ×) in EOC cells. Moreover, the mRNA level of ChoKα isoform was constitutively over-expressed in cancer cells, in presence of unaltered levels of the other Kennedy pathway enzymes. PC-specific phospholipase C (PC-plc) protein was also over-expressed (ca 3×) and activated (up to 17-fold) in EOC cells (Iorio et al, Cancer Res March 2010). Exposure of EOC cells to a D609 (inhibitor of PC-plc) led a drop in PCho content and reduced cell proliferation, in the absence of apoptosis. Furthermore, evidence in our laboratory also showed that: (a) activation of PC-plc was associated in EOC cells with accumulation of this enzyme on the external plasma membrane; and (b) D609 induced long-lasting block in the recovery of both PC-plc activity and S-phase fraction in receptor re-stimulated cancer cells (Spadaro et al, Cancer res 2008).
The availability of intra-peritoneal and sub-cutanous EOC xenotransplant models, allowed the identification of the tCho signal as the highest resonance in the in vivo spectra of xenografts, opening the possibility of preliminary clinical

examinations using 1H MRS together with MRI for in vivo metabolic evaluation of EOC.
Conclusions: Abnormal PC metabolism has implications in cancer biology and provide an avenue to the development of non-invasive clinical methods for diagnosis and treatment follow-up. PC-plc and ChoK may represent a novel target for the design of therapeutic strategies in ovary cancer.

379 The role of translesion DNA polymerase eta in p53 activation and DNA damage response

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Background: DNA polymerase eta (PolH), a Y-family translesion polymerase, is required for repairing UV-induced DNA damage, and loss of PolH is responsible for early onset of malignant skin cancers in patients with Xeroderma Pigmentosum Variant, an autosomal recessive disorder.
Materials and Methods: Both molecular and Cellular techniques, including transcriptional studies, gene expression, and protein stability and degradation, were used to define how PolH is regulated in vitro and in vivo.
Results: We found that PolH is induced by DNA damage and necessary for DNA damage-induced apoptosis in a p53-dependent manner. Interestingly, we also found that PolH is necessary for DNA damage-induced activation of p53. Due to the critical role of PolH in maintaining genome stability, PolH expression is subject to regulation by multiple mechanisms. Here, we found that PolH is degraded by proteasome, which is enhanced upon UV irradiation. We also found that PolH interacts with Pirh2 E3 ligase, a target of p53, via the polymerase associated domain in PolH and the RING finger domain in Pirh2. In addition, we showed that overexpression of Pirh2 decreases, whereas knockdown of Pirh2 increases, PolH protein stability. Interestingly, we found that PolH is recruited by Pirh2 to, and degraded by, 20S proteasome in an ubiquitin-independent manner. Finally, we observed that Pirh2 knockdown leads to accumulation of PolH and subsequently enhances the survival of UV-irradiated cells.
Conclusion: We uncovered a novel function for PolH: modulating the DNA damage checkpoint and p53 activation. In addition, we postulate that UV irradiation promotes cancer formation in part by destabilizing PolH via Pirh2-mediated 20S proteasomal degradation.

380 Combined analysis of SNPs in IL-6/IL-6R pathway predict prostate cancer aggressiveness and development of resistance to hormonal castration

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The IL-6/IL-6R pathway is involved in Prostate Cancer (PCa) progression and in the development of resistance to hormonal castration (RHC). We hypothesized that functional variants in *IL6*, *IL6R* and *gp130* genes, which influence transcriptional rate and protein function, can influence susceptibility, aggressiveness and response to treatment in PCa.
We conducted a case-control study in biopsy-proven PCa (n = 1263) and benign prostatic disease (n = 472) patients. Genotyping was performed in *IL6*, *IL6R* and *gp130* genes, through PCR-RFLP and Real Time-PCR allelic discrimination. Genotypes from *IL6*, *IL6R* and *gp130* polymorphisms were functionally combined according to the cell signalling activation potential attributed to genetic profile.
When analysed individually, results showed an increased risk for TT carriers in the *IL6* polymorphism at locus −6331 to present a PSA level $\geqslant$20 ng.mL-1 at the time of diagnosis ($P = 0.004$). Furthermore, in the polymorphism at locus −174 of *IL6* gene, C carriers had increased risk for aggressive disease (PSA $\geqslant$ 20 ng.mL^{-1}, $P = 0.012$; distant metastasis, $P = 0.089$ and poorer overall survival, $P = 0.033$). Univariate Kaplan–Meier function plots analysis evidenced shorter time to the development of resistance to hormonal castration in TT carriers of *IL6* −6331 T>C polymorphism ($P = 0.027$).
Moreover, when polymorphisms in the receptor were functionally combined according to the cell signaling genetic profile, an increased risk for developing PCa was observed for the high signallers ($P = 0.010$). Combined analysis of polymorphisms in the *IL6* promoter region showed association of a high/intermediate *IL6* expression genetic profile with aggressive disease (advanced stage, $P = 0.040$; distant metastasis, $P = 0.023$ and PSA $\geqslant$ 20 ng.mL^{-1}, $P = 0.001$). The functional combination between polymorphisms in *IL6*, *IL6R* and *gp130* genes, evidenced that IL-6/IL-6R high activation genetic profile carriers are overrepresented in the group with distant metastasis ($P = 0.017$) and earlier development of RHC ($P = 0.021$).
Combination of *IL6*, *IL6R* and *gp130* genetic polymorphisms according to the functional profile revealed its relevance in susceptibility, aggressiveness and in progression-free interval in patients submitted to hormonal castration. Accordingly, these results support IL-6/IL-6R pathway as a targetable mechanism in PCa. The *IL6*, *IL6R* and *gp130* functional polymorphism might be useful molecular markers for PCa aggressiveness and as a predictive factor for the RHC.

381 Functional studies of gene products and signal transduction mechanisms involved in cancer progression and metastasis – influence of extracellular S100A4

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The S100A4 protein is a small, multifunctional protein located in the cytoplasm, the nucleus and the extracellular space. S100A4 has been linked to the invasive and metastatic behavior of cancer cells, and its metastasis-promoting properties are mediated through both intracellular and extracellular functions, however the exact molecular mechanisms by which S100A4 exerts these effects are incompletely understood. Importantly, an association between exposure to, or expression of S100A4 and members of the matrix metalloproteinase (MMP) system is shown, and S100A4 is suggested to promote cancer progression by regulating remodeling of the extracellular matrix.
In a previous study we demonstrated that extracellular S100A4 induces phosphorylation of IkBa and activation of the classical NF-kB pathway in a subset of human cancer cell lines. In search for downstream effect molecules, we have demonstrated that S100A4 induces ephrin-A1 and the multifunctional chemokine osteopontin (OPN) through an NF-kB dependent mechanism. Interestingly, both ephrin-A1 and OPN are previously shown to influence cell migration, angiogenesis and metastasis.
In the present work we have investigated the S100A4-induced signal transduction pathways upstream of IkBa through the use of specific inhibitors and kinase activity assays with special focus on the involvement of the serine kinases MEKK1, AKT and NIK. Moreover, S100A4-induced effect of identified downstream target molecules on invasion and metastatic capacity is examined.
We have established that common signal transduction pathways (e.g.: PLC, PI3K and GPCR) are not involved in transmitting the signals from the plasma membrane to the ultimate activation of NF-kB, while Ser/Thr kinases seem to play a significant role. Importantly, in a transwell invasion chamber assay a significant increase in cell migration and invasion was observed upon exposure to S100A4 and induction of OPN. Furthermore, strong indications indicated that the proteases uPA and MMP-13 are responsible for these observed phenotypic effects. These novel findings are an important contribution to understand the molecular mechanisms underlying the biological effects induced by S100A4.
In conclusion, these results indicate that S100A4 through induction of Ser/Thr kinase-dependent NF-kB-activation of OPN initiates a metastatic cascade, which subsequently may enhance cellular dissemination and facilitate metastasis.

382 A simple and robust assay to study endothelial cell function in vivo

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Tumour growth depends on the ability to recruit blood vessels from the host tissue. Correspondingly, angiogenesis has become a major target for antitumour therapy. However, the complexity of the angiogenic cascade limits cellular approaches to studying angiogenic endothelial cells (EC). To overcome this limitation, we developed a reliable and robust *in vivo* angiogenesis assay that is based on the xenotransplantation of human EC in immunocompromized mice. Implantation of spheroidal endothelial aggregates in a Matrigel/fibrin matrix resulted in the formation of a complex three-dimensional network of human neovessels. Implants were dissected after 20 days and analyzed for microvessel density (MVD), mural cell recruitment and perfusion. The assay provides unique opportunities to perform studies in the field of vascular research. First, EC can be manipulated *ex vivo* prior to implantation for GOF and LOF studies to investigate vascular function. Downregulation of Ang-2 or PDGF-BB in HUVEC led to a significant reduction of MVD. Loss of Ang-2 or PDGF-BB in HUVEC resulted in the reduction of α-smooth muscle actin (α-SMA) coverage. However, Desmin coverage was only increased in Ang-2 silenced HUVECs. Second, EC can be manipulated to mimic pathological conditions. Towards this end we generated HUVECs lentivirally silenced for CCM1, one of the three genes causally involved in the formation of Cerebral Cavernous Malformations. CCM1 LOF led to the formation of hypervascularized vascular networks mimicking human CCM lesions. Third, HUVEC can