648 Molecular characterization of breast cancer subtypes derived from joint analysis of high throughput miRNA and mRNA data

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Background: Deregulation of micro-RNAs (miRNAs) has been increasingly implicated in cancer. Several miRNAs have aberrant expression profiles in breast cancer and the expression of some has been correlated to specific clinical features of breast cancer. miRNA dependent regulation is mediated through changes in mRNA levels and function, and miRNA/mRNA interaction in the context of breast cancer highlights clinically relevant pathways. To search for such interactions we performed a comprehensive joint analysis of whole genome miRNA and mRNA data.
Material and Methods: We have performed expression profiling of 799 miRNAs along with genome-wide matched mRNA profiling in a cohort of 101 human primary breast tumour samples with extensive clinical information. The miRNA/mRNA interdependencies were examined using correlation and statistical enrichment methods. Profiling was performed using microarrays and validated by qPCR. The effect of miRNAs on proliferation was validated using high-throughput transfection arrays in breast cancer cell lines.
Results: Statistically significant differentially expressed miRNAs were found in examining different molecular subtypes, extracellular matrix (ECM) classes, TP53 mutation status, proliferation status, and survival. Taking a systems biology approach to study the relationship between miRNA and mRNAs, we identified several cellular processes, such as proliferation, cell adhesion and immune response, which were significantly enriched among genes with strong negative or positive correlation to the expression pattern of certain miRNAs or groups of miRNAs (miRNA-GO association networks). Functional validation assays identified 13 miRNAs that affect proliferation in both tumours and cell lines. Furthermore a group of miRNAs associated to disease free survival was identified.
Conclusions: We introduce a dataset of mRNA and miRNA expression profiles measured in a well studied patient cohort. We show that miRNAs can distinctly differentiate between tumour subtypes, various clinical subclassifications and survival characteristics. In addition, we present functional validation linking some miRNAs to proliferation. Finally, we show that miRNAs can act as reliable proxies to the activity of known biological processes related to breast cancer progression such as cell-cycle, immune response and cell adhesion.

649 Regulation of p53 and cell proliferation by the nucleolar factors

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Background: The nucleolus, one of the subnuclear organelles, has been regarded as a factory of ribosome biogenesis. But, recent works revealed that the nucleolus also plays roles in various cellular events, including regulation of the cell cycle, DNA repair, apoptosis and so on. It is also reported that some nucleolar proteins are involved in the regulation of tumour suppressor, p53 that is a key factor of checkpoint control and apoptosis.
We focused on the regulation of the cell cycle and apoptosis by nucleolar proteins in cancer cells. To identify the proteins involved in these processes, we treated human breast cancer cells with an siRNA library for nucleolar proteins.
Materials and Methods: We screened for nucleolar proteins involved in cell cycle and cell death using a siRNA library. Briefly, MCF-7 cells, the breast cancer cell line, were treated with siRNA (stealth RNA, invitrogen) for about 400 kinds of nucleolar proteins. Then we examined cell proliferation by MTT assay, cell cycle distribution by flow cytometry (Guava cell cycle Reagent, Millipore), and apoptosis by TUNEL assay (DeadEnd Fluorometric TUNEL system, Promega).
Result and Conclusion: We identified several nucleolar proteins whose knockdown suppressed cell growth and induced cell death. Among them, we focused on NOL1/NOP2/Sun domain family, member 1 (NOL1) that is known as a marker of cellular proliferation. We found that NOL1 knockdown induced G1 arrest and apoptosis. Given that p53 regulates G1 checkpoint and apoptosis, we examined the p53 expression level. As expected, we found that NOL1 knockdown resulted in the increase of p53 protein, followed by the enhancement of its target gene products, Cdk-inhibitor, p21 and proapoptotic factor, PUMA. These phenomena were not observed in p53 deficient cells, indicating that the effect of NOL1 knockdown depends on the p53 pathway.
In addition, we found that there were many nucleolar factors, knockdown of which resulted in the accumulation of p53. We have also analyzed the p53 modification, expression level of p53 downstream genes, and cellular phenotypes by the knockdown. Furthermore, we are trying to classify these factors into several categories according to the modification status of p53 and/or cellular phenotypes, which are then compared with the reported functions of the nucleolar factors. We believe that this study will provide a new insight into the relationship between nucleolar functions and p53 regulation mechanisms.

650 Use of the blood based, 96-assay set for breast cancer detection

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Background: Increased survival from breast cancer is achieved by increasing the number of cancers detected at an early stage. Approximately one third of breast cancers in the US are detected at stage 2 or later. Detection of breast cancer at stage 0 or 1 would allow more treatment options and increased survival. Mammography is the primary modality for detecting cancer but has shortcomings, particularly in younger women where breast density is more common. Breast density interferes with mammograms reducing the sensitivity of the test. In addition, mammography has low sensitivity for the detection of lobular cancer which has a diffuse growth pattern and often lacks microcalcifications. We have developed a blood-based gene expression test for the detection of breast cancer. Whole genome array analysis was performed for assay selection and through a series of studies a commercially available 96-assay set has been verified and validated (BCtect®). Gene expression was measured using reverse-transcriptase real-time PCR to determine mRNA levels in whole blood. An algorithm was developed in a calibration study to distinguish between BC and non-BC patients. The current study reports the results from an independent validation of the gene expression test (BCtect®).
Materials and Methods: In a multicentre-study, blood samples were collected from women from 3 groups (1) Stage 0−III BC, (2) benign breast lesions, or (3) negative mammograms. Blood samples were collected in PAXgene™ tubes and shipped on dry ice to a central laboratory for RNA extraction. Quality control of RNA was performed using the Agilent 2100 BioAnalyzer and Nanodrop ND-1000. Gene expression analysis was performed using real time RT-PCR (AB7900 HT) with a microfluidic card containing the BC-specific 96 assay signature. The test software provided a test score for each subject in the independent validation cohort. A positive test score classified a subject as positive for BC, whilst a negative score classified a subject as negative for BC.
Results: The model correctly predicted the class of 78 of the 109 validation samples (overall accuracy 72%). Performance was similar for early and late stage cancer with a sensitivity of 74% for stage 0/1 breast cancer (stage 0 = in situ cancer, and stage 1 = T1N0M0; staging defined by AJCC 2002). The test performed equally well in pre- and post-menopausal women. Lobular cancers were predicted with 72% sensitivity. The test gave a positive result in a cohort of 20 pregnant women suggesting the test measures a biological feature common to both breast cancer and pregnancy. The assays code for proteins that have a biological function in immune response, signal transduction, and cellular metabolism amongst others.
Conclusions: The blood-based gene expression test showed efficacy for the detection of early breast cancer in both pre- and post-menopausal women suggesting the test may be a valuable tool for young women, a group in which mammography has poor performance. Additional validation is ongoing.

651 Trop-2 is a general cancer growth stimulator through ubiquitous tetraspanin platforms

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Background: Trop-2 is a transmembrane calcium signal transducer, first identified in trophoblast and subsequently found in diverse transformed cells. The expression of Trop-2 has been associated with biological aggressiveness and poor prognosis of pancreatic, gastric, oral, ovarian and colorectal cancers, suggesting a potential role in tumour progression. However, a comprehensive scenario of Trop-2 expression is still missing and the function of Trop 2 is as yet unknown.
Material and Methods: Human tumours and normal tissues were analysed for the expression of the *TROP2* gene by DNA microarray, EST, SAGE, RT-PCR, Northern blot analysis. Expression of the Trop-2 protein and of downstream signal transducers was investigated by immunohistochemistry, flow cytometry, high-throughput Western blotting, proteomic array, 2D gels/mass spectrometry and dynamic multi-color imaging. Induction of expression and somatic knockdown of relevant targets was utilized for function analysis, together with protein-protein, protein-gene network interaction analysis.

Results: Trop-2 was demonstrated to be overexpressed by most human cancers, but not on non-epithelial malignancies, suggesting strong selective pressure for a conserved function. Trop-2 was then demonstrated to be necessary and sufficient to stimulate cancer growth, with a linear relationship between growth rates and Trop-2 expression levels. Cell growth stimulation was shown to be conserved across cell-types and species. These findings indicated impingement on a ubiquitous downstream signal-transduction module. Trop-2 was demonstrated to bind multiple tetraspanins, triggering their growth-promoting ability *via* a feed-forward activation loop of CD9-recruited PKCa and phosphorylation of the Trop-2 cytoplasmic tail. We demonstrated that both CD9 and PKCa stimulate growth in a Trop-2-restricted manner and that these signaling structures are coordinately transported in recurrent waves to membrane ruffles and podosomes. Trop-2 induction was shown to activate the ERK pathway, to up-regulate NF-kB, and to modulate apoptotic factors, including p53 and Rb. Key members of the Trop-2 signaling pathway were shown to be coordinately upregulated in large human cancer case series, indicating functional relevance of this growth stimulatory mechanism in tumours in man.
Conclusions: These findings reveal the existence of a unique, strikingly widespread mechanism of stimulation of cancer growth. This is quantitatively driven by overexpressed, but otherwise wild-type, Trop-2 and acts upon ready-to-signal, but otherwise silent, ubiquitous signal-transduction platforms.

652 ADAM23 splicing isoforms: distinct roles on the modulation of avb3 integrin activity

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The ADAMs (a disintegrin and metalloprotease domain) constitute a family of type I transmembrane glycoproteins with a common structural organization, which includes a metalloprotease and a disintegrin domain. Because of their proteolytic and cell adhesion activity, the ADAMs are involved in both the remodelling of the extracellular matrix and the changes in cell adhesion that characterize many biological and pathological processes, such as tumour development and progression. ADAM23 exhibits the typical structure of ADAM family members; however its metalloprotease domain is inactive, suggesting that it is exclusively involved in cell adhesion [1]. More than 12 ADAMs (including ADAM23) have been shown to interact with integrins *in vitro*, modulating integrin-mediated cell migration, adhesion and proliferation [2]. The ADAM23 protein was demonstrated to interact specifically with avb3 integrin by its disintegrin domain [3]. The ADAM23 gene is frequently silenced by promoter hypermethylation in breast, gastric, pancreatic, colorectal and head and neck tumours. In breast tumours, silencing of ADAM23 gene is associated with the development of distant metastasis and a worse disease outcome [4–5]. Recently, we demonstrated that ADAM23 negatively modulates avb3 integrin activation during metastasis [5]. Knockdown of ADAM23 expression using shRNA enhanced integrin activation by 2–4 fold and increased cell migration and adhesion to classical avb3 integrin ligands. Three ADAM23 splicing isoforms have been described so far, two of them *(alpha* and *beta)* encode transmembrane domains that share 54% similarity in their aminoacid sequence, and the third one *(gama)* does not encode a transmembrane domain, suggesting to be a secreted or cytoplasmic protein [6]. Here we show that ADAM23 splicing isoforms are differentially expressed in a panel of 12 tumour cell lines derived from several tissues. Moreover, using siRNA to specifically knockdown the expression of each splicing isoform, we found that they play different roles on the modulation of avb3 activity, affecting migration and adhesion to classic avb3 ligands.

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653 CK2 phosphorylation controls PRH/HHEX dependent transcriptional repression of multiple VEGF signalling genes and cell survival

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Background: The Proline Rich Homeodomain protein (PRH) is a repressor of transcription that regulates haematopoietic and vascular development. Protein kinase CK2 phosphorylates PRH and blocks its DNA binding activity and its ability to regulate transcription. CK2 is up-regulated in many tumours including Acute Myeloid Leukaemia (AML) and its up-regulation correlates with a poor prognosis. Vascular Endothelial Growth Factor (VEGF) is a mitogen that stimulates proliferation and survival of endothelial and haematopoietic cells, via its cell surface receptors VEGFR-1 and VEGFR-2. VEGF and the VEGF receptors are elevated in many tumours and haematopoietic malignancies. Loss of PRH expression has been shown to correlate with abnormal vascular development and elevated VEGF expression.
Material and Methods: VEGF and the VEGF receptor gene expression was studied using qPCR in K562 cells after knockdown and over-expression of PRH. Chromatin Immunoprecipitation (ChIP) and promoter reporter assays were used to analyse gene specific PRH binding. Cell growth and apoptosis were analysed using trypan blue cell staining, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, and Annexin V staining analysed by flow cytometry.
Results: We show using that the genes encoding Vegf, Vegfr-1, and Vegfr-2 are all repressed by PRH. ChIP and reporter assay data reveals that PRH binds to the promoter regions of all three of these genes. Thus we demonstrate that PRH is a direct repressor of multiple genes within a single signalling pathway. We also demonstrate that the manipulation of PRH levels directly impinges on the survival of haematopoietic cells and breast cancer cells. Moreover we show that VEGF and VEGF receptor signalling mediates the effects of PRH on cell growth. Importantly we demonstrate that CK2 can antagonise both PRH-induced cell death and transcriptional repression of these genes.
Conclusions: These findings suggest that PRH is a key regulator of multiple genes in the VEGF signalling pathway and loss of PRH transcriptional activity, through elevated CK2 activity, could play a role in tumourigenesis and leukaemogenesis.

654 The effect of p53 isoforms on p73 activity in tumour cells

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Background: The p53 tumour suppressor protein is critical in the cell growth control and the maintenance of genomic stability. These activities are due, at least in part, to its ability to form homooligomers that bind to specific DNA sequences and activate transcription. Recently discovered, p73, a homologue of p53, can transcriptionally activate p53 target genes *in vivo*. It generates transactivating forms (TAp73) as well as a number of N-terminally truncated transactivation-deficient transdominant isoforms (called ΔTAp73). Recently was discovered that *p53*, like *p73*, has a second promoter P2 and undergoes alternative splicing to generate multiple isoforms that might play important roles in carcinogenesis. Since some mutant p53 form complexes with TAp73α or TAp73β it was important to find out whether p53 isoforms can do the same and potencially act as dominant-negative inhibitors of TAp73.
Materials ans methods: Human lung cancer p53 null cells H1299 were transfected using Lipofectamine 2000®. Proteins were extracted and western blot was preformed by standard methods. Coimmunoprecipitation assay was used to detect the protein complex. Apoptosis was detected using annexin-V assay by flow cytometry and fluorescent microscope, and to analyze transcriptional activity, we performed reporter assays using promoters with the p73/p53 binding site driving the luciferase reporter.
Results: All six p53 isoforms can form complex with TAp73β, while only isoforms D133p53, D133p53β and D133p53γ can form complex with TAp73α. Inhibitory interactions of two proteins in complex often lead to their stabilization. Our results have shown that only three isoforms (Δ133p53, Δ133p53β i Δ40p53) stabilize TAp73β. Furthermore, we have shown that all isoforms of p53 inhibit transcriptional activity but with different efficiency. The apoptotic acitivity of TAp73β was augmented by coexpression of p53b, but Δ133p53 and Δ133p53β inhibit its apoptotic activity most efficiently. We have determined the half lives of different p53 isoforms and have shown that p53γ isoform has the shortest while Δ133p53γ has the longest half life.
Conclusions: Defining the interactions between p53/p73 would gain insight into how the p53 isoforms modulate the functions of p73. The discovery of p53/p73 network could have a major clinical impact in prognostic use and p53 targeted drug design.

655 Functional analysis of CDKN2A/p16INK4a 5′UTR variants predisposing to melanoma

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The CDKN2A gene, located on 9p21, is the most common high penetrance susceptibility gene identified to date in melanoma families. Germline *CDKN2A* mutations are observed in 20–50% of melanoma-prone families. We identified melanoma patients that were heterozygous for non-coding germline variants in the 5′UTR of *CDKN2A* (c.–21C>T; c.–25C>T & c.–180G>A; c.–56G>T;