681 Characterization of a ubiquitin specific protease in the 17q23 amplicon

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Background: Previous studies identified chromosomal band 17q23 as a frequent site of gene amplification in breast cancer. *USP32* is one of the oncogene candidates in this region. Presence of Cys-His domains suggest that USP32 functions as a deubiquitinating enzyme. We hypothesized that amplification of *USP32* may contribute to breast tumourigenesis as a protein taking part in protein degradation pathways.
Materials and Methods: Real-time RT-PCR was performed to detect *USP32* overexpression in breast cancer cell lines and primary tumours. I*n vitro* deubiquitination assay was used to test the deubiquitination function of USP32. RNAi silencing of *USP32* was achieved by pSUPER shRNA (anti-USP32 oligo) and confirmed by real time RT-PCR. Proliferation rate of *USP32* silenced cells were determined by the MTT Assay. *In vitro* wound healing assay was performed to detect motility rate changes of the cells. GFP-fused USP32 was transfected to HeLa cells for sub-cellular localization and FPP (Fluorescence Protease Protection) studies.
Results: Real time RT-PCR analysis showed that *USP32* was expressed more than 5-folds in 30% of 10 human breast cancer cell lines and more than 2-folds in 22% of 41 human primary breast tumours compared to normal breast. We then confirmed USP32 as an active deubiquitinating enzyme by an *in vivo* deubiquitination assay. Stable silencing of *USP32* in HeLa cells caused a decrease in the proliferation rate up to 35% compared to control oligo and empty vector transfected cells. We didn't detect a significant difference in the motility of *USP32* silenced cells in an *in vitro* wound healing assay. In addition, FPP revealed that USP32 was a membrane bound protein and co-localization studies BODIPY® TR suggested that this protein resided in Golgi.
Conclusion: Our results showed that, *USP32* an active and membrane bound deubiquitinating enzyme, was expressed more than 5-folds in 30% of human breast cancer cell lines (3 out of 10) and 2-folds in 22% of human primary breast tumours (9 out of 41). Golgi localization of USP32 and its effect on proliferation might indicate vital roles for cellular processes. We are conducting further experiments to delineate its function in cells and its contribution to tumourigenesis.

682 Regulation of c-MYC expression by the calcineurin-NFAT1 pathway

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Background: The Nuclear Factor of Activated T Cells (NFAT) family of transcription factors encompasses four proteins (NFAT1−4) regulated by the Ca^{2+}/calcineurin signaling. NFAT proteins regulate genes involved in cell cycle, apoptosis and angiogenesis. NFATs are also implicated in human malignancies, being overexpressed in lymphoma, breast, pancreatic and colon cancers. Furthermore, the *c-myc* proto-oncogene, that is pivotal to the same functions, is also deregulated in these types of cancer. We found that lymphocytes from NFAT1−/− mice had a deregulated expression of c-MYC when compared to the NFAT1+/+, suggesting a role for NFAT1 in *c-myc* regulation. Recently, it was demonstrated that NFAT2 binds to a site located at the minimal *c-myc* promoter. Nevertheless, those studies did not evaluate NFAT2 binding to distal 5′ regions of the transcription start site, where essential elements to *c-myc* regulation are located. This study investigates if NFAT1 transcription factor directly regulates *c-myc* expression.
Material and Methods: By qRT-PCR, we analyzed c-MYC expression after TCR engagement. Hence, a bioinformatic analysis was performed to find putative NFAT-binding sites in *c-myc* promoter. NFAT1-binding to these sites was evaluated by EMSA and ChIP assays. At last, the responsiveness of the human *c-myc* promoter to NFAT1 was analyzed by luciferase reporter plasmids.
Results: We demonstrated that c-MYC mRNA is regulated by the calcineurin signaling in mouse lymphocytes. Seven putative NFAT binding sites conserved between human and mice were found in a region containing 2.5 kb of *c-myc* promoter. NFAT1 has bound to three of the seven putative sites *in vitro*, including the site upregulated by NFAT2. Besides, NFAT1 directly binds to two 5′ distant regions of the *c-myc* promoter *in vivo*. Deletions of *c-myc* promoter and site directed mutagenesis of NFAT binding sites showed that the distal NFAT elements are negative regulators, while the proximal site is as a positive one. In the context of full promoter, mutation analysis of the distal NFAT elements also demonstrated that the negative sites are dominant over the positive.
Conclusions: Our results demonstrated that the NFAT1 transcription factor contributes to the tight regulation of *c-myc* expression. We suggest that the outcome of regulation providing by NFAT1 depends of the balance among the two negative distal and the positive proximal elements, which may vary according to the cellular and/or microenvironment context.

683 MicroRNAs underexpressed in hereditary breast cancer target pathways involved in cell motility and proliferation

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Background: Over the last few years miRNA have emerged as an important new class of posttranscriptional gene regulators. Their aberrant expression has been associated with many tumours, including breast cancer. Given the fact that a single miRNA can target a large number of mRNA transcripts, miss-expression of a set of miRNAs could have significant effect on cellular function by affecting multiple signaling pathways. The goal of this study was to perform global computational analysis of biological processes and pathways altered in response to differential miRNA expression in hereditary breast tumours, and to identify genes regulated by these miRNAs.
Material and Methods: Global miRNA expression profiling was performed on a series of 19 fresh-frozen hereditary breast tumours and 15 normal breast tissues, using Locked Nucleic Acid (LNA) miRNA microarray platform covering >1000 human miRNAs. Quantitative Real Time-PCR (qRT-PCR) performed on selected miRNAs in an independent series of 18 sporadic breast tumours and their normal breast tissue counterparts confirmed the pattern of differential expression shown by microarray analysis. Furthermore, miRNA targets were predicted based on Diana MicroT 4.0 algorithm, and subsequently enrichment analysis of KEGG pathways was performed using DIANA miRPath program. Functional analysis of miRNA target genes is underway.
Results: We have identified 30 miRNAs differentially expressed between tumour and normal breast tissue, most of them being down-regulated in hereditary breast tumours. Under-expression of some of them was subsequently established by qRT-PCR in an independent series of sporadic breast tumours. This set of miRNAs collectively targets number of genes belonging to signaling pathways regulating cell motility, namely, reorganization of actin cytoskeleton, regulation of focal adhesion and control of adherence junction membrane proteins, as well as MAPK and ErbB signaling pathway.
Conclusions: Here we show that relatively small number of miRNAs could have a systemic effect by altering signaling pathways important for cell motility and proliferation, hallmarks of cancer progression. Our data shows miRNAs commonly miss-expressed in both hereditary and sporadic breast cancer suggesting common underlying mechanisms of tumourigenesis.

684 A novel large-scale screen to identify modulators of oncomir miR-21

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Alterations in miRNA expression significantly contribute to tumour growth by modulating critical genes. For example, miR-21 is upregulated in breast cancer, prostate cancer, glioblastoma and other cancers. This microRNA has been defined to play a role in several cancer-relevant physiological processes such as apoptosis as well as cell growth and cell invasion. The targets of miR-21 include well studied tumour suppressors such as phosphatase and tensin homologue (PTEN), programmed cell death 4 (PDCD4) and components of p53 pathway. Its differential expression also correlates with tumour stage and disease-free survival.
To identify genes involved in the regulation of miR-21 we have screened an siRNA-library of 4400 selected siRNAs, chosen based on their potential to modulate microRNA biogenesis including miRNA processing factors, nucleases, transcription factors, kinases, phosphatases and other signalling molecules. For a primary screen, target sites for miR-21 have been cloned into the 3′-UTR of a luciferase reporter-gene. In this way, changes in miR-21 level after transfection of siRNAs result in changes of luciferase concentration.
We have identified 100 genes that significantly regulate miR-21 activity in this screen. Hits in the primary screen were then analyzed in further screens. Firstly, changes in miR-21 concentration were validated by using quantitative real time PCR. Moreover, screens were carried out to analyze whether the candidate genes regulate the miR-21 promoter or miRNA maturation, so whether they function at the transcriptional or post-transcriptional level. Genes that have an influence on miR-21 processing were also analyzed for their ability to modulate other miRs to potentially identify general regulators of miRNA biogenesis and stability. Individual candidates are currently analyzed for their specific mode of miR-21-activity modulation.
Thus, by these screens we have identified general regulators of miR-processing as well as miR-21 specific regulators. The alteration of miR-21 expression by modulating expression of the identified genes might also change different steps of tumour development. Therefore, these genes may also serve as therapeutic targets.