ROLE OF NATURAL ANTISENSE TRANSCRIPTS PERTAINING TO TUMOR SUPPRESSOR GENES IN HUMAN CARCINOMAS

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Overlapping transcripts in opposite orientations can potentially form perfect sense-antisense duplex RNA. Recently, several studies have revealed the extent of natural antisense transcripts (NATs) and their role in important biological phenomena also in higher organisms.

In order to test the hypothesis that the function of NATs in man might represent an essential element in the regulation of gene expression, especially at transcriptional level, in this study we planned to look for, systematically examine, and characterize NATs belonging in the human genome to the tumour suppressor class of genes, so to identify physiological (and potentially pathological) modulators in this gene class.

Firstly, we created a database of 251 human tumour suppressor genes, identified through a manual bibliographic research. For each gene we collected from various public data banks all available information, which we considered essential for their characterization and description.

Then we carried out an *in silico* bioinformatics analysis in order to identify how many of the 251 genes had an antisense transcript. We found that 116 genes contained sequence data supporting this hypothesis; from these we selected a subset consisting of 46 genes (~20% of the original set), which appeared to be, compared to the remaining identified genes, more reliable in terms of predictive quality.

In order to define the expression patterns of tumour suppressor genes containing natural RNA antisense, we initially used *in situ* hybridization (ISH) on tissue microarray (TMA) in normal and neoplastic cells for six tumour suppressor genes (TFAP2A, SLC22A18, BCL-10,LIMD1,CUL5 e SMARCB1) and for the six corresponding natural antisense, chosen in the 46 gene subset. The following tissues were tested by TMA: both normal and tumoural samples from liver, larynx, kidney, colon, lung, breast and stomach; SNC tumours, lymphomas, melanomas, fibroadenomas, and normal linfonodes and tonsils.

The analysis of the *in situ* hybridization results showed a very low level of or absent expression of the tumour suppressor genes and antisense transcripts in the tested tissues, in both normal and neoplastic cells, with a moderate tissue-specificity for some of them.

The only exception to this general observation was the tumor suppressor SMARCB1 (SWI/SNF related gene) and its antisense transcript DERL3 (derlin member 3). However, expression of these two transcripts appeared limited to the linfoid tissue, where the histological diversity inside the tonsils seemed to correlate with the different expression of the sense and antisense transcripts.

We also carried out a second expression study by SYBR Green based real-time RT-PCR on eight pairs of tumour suppressor genes and their corresponding natural antisense.

In more detail: PCGF2 e CUL5 (Cullin 5) were tested on eight samples of mammary carcinoma and on four samples of normal mammary gland; SIAH1 (seven *in absentia* homolog

1, Drosophila) and PINX1 (PIN2-interacting protein 1) were tested on eight cases of hepatocellular carcinoma and on four samples of normal liver; PINX1 was also tested on four cases of gastric tumours and four samples of normal stomach. TFAP2A (Transcription Factor AP2 alpha) and NEURL (Neuralized-like, Drosophila) were tested on seven lines of glioblastomas, on one sample of normal brain tissue and on neuronal stem cells; finally, DRR1 (Down Regulated in Renal cancer 1) and ANKRD15 (ankyrin repeat domain 15) were tested on eight cases of renal carcinomas and eight samples of normal kidney.

Only one of the samples we examined was informative: PCGF2; all other matched observations generally revealed cases of: i) absent biological validation of the antisense transcript; ii) minimal basal expressions, approaching the limits of sensitivity of the technique; iii) absence of normal/tumoral variation in the expression of the onco-suppressor gene; iv) expression of the onco-suppressor gene in total disagreement with published data, i.e. expression levels higher in cancer samples than in normal samples. In contrast, the average expression level of the onco-suppressor PCGF2 in mammary tumours showed an 18% reduction compared to the average level of expression in normal tissues, concomitantly with a 34% increase in the average expression level of its antisense transcript. However, no variation was observed after testing the same gene and its antisense in the other tissues described above. Further studies will be necessary to better understand and evaluate the significance of the results obtained in the mammary tumours with the onco-suppressor PCGF2 and its antisense, and to determine a possible role of their respective expression, in normal and cancer tissues, in the pathogenesis or maintenance of cancer.

The description of possible *cis*-regulation pathways mediated by antisense transcripts depends on good and reliable conditions of expression of the studied transcripts and, therefore, on the availability of adequate biological systems in which carrying out the research. At the state of the art, we think that neither condition is present in the systems so far utilized: ultimately, it would be necessary to consider the use of a larger panel of samples, including several cancer cell lines or tumoral tissues, to screen for the expression of tumour suppressor genes involved in sense-antisense pairs.

Publications of the project

Engstrom PG, Suzuki H, Ninomiya N, Akalin A, Sessa L, Lavorgna G, Brozzi A, Luzi L, Tan SL Yang L, Kunarso G, Ng EL, Batalov S, Wahlestedt C, Kai C, Kawai J, Carninci P, Hayashizaki Y, Wells C, Bajic VB, Orlando V, Reid JF, Lenhard B, Lipovich L. Complex *Loci* in human and mouse genomes. PLoS Genet 2006;2(4):e47. Epub 2006 Apr 28.

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Expression and function of microRNAs in prostate cancer

In this study, we investigated the involvement of miR-205 in prostate carcinogenesis. Preliminary evidence had shown that the expression of miR-205 was reduced in prostate carcinoma cell lines compared to normal cells. In this context, we measured miR-205 levels in a series of 31 tumor specimens and matched normal tissues obtained from prostate cancer patients subjected to radical prostatectomy. Tumor specimens showed significantly lower miR-205 expression levels compared to their normal counterparts, with a particularly pronounced reduction being observed in carcinomas from patients with locoregionally disseminated disease.

Restoring the expression of miR-205 in DU145 prostate cancer cells resulted in cell rearrangements consistent with a mesenchymal-to-epithelial transition, such as up-regulation of E-cadherin and reduction of cell locomotion and invasion, and in the down-regulation of several oncogenes known to be involved in disease progression, suggesting a possible function of miR-205 in tumor suppression. The integration of gene expression profiles of cells ectopically expressing miR-205 with the results of computational target prediction led to the identification of genes putatively regulated by miR-205 and responsible for its tumor-suppressive role. Overall, our data suggest that miR-205 down-regulation can concur to prostate cancer progression, favoring epithelial-to-mesenchymal transition and supporting cell migration/invasion. Restoring miR-205 expression might reduce the aggressiveness of prostate cancer cells. These results have been submitted to Cancer Research as full research article and are currently under revision.

Parallely, the study focused on miR-21, widely endowed with oncogenic functions in different human tumors. The studies conducted thus far are controversial concerning the involvement of miR-21 in prostate carcinogenesis. In a series of 30 tumor specimens and matched normal tissues obtained from prostate cancer patients subjected to radical prostatectomy, miR-21 expression was not significantly different in carcinomas compared to their normal counterparts. This finding suggested that miR-21 may have a negligible role in sustaining prostate cancer development. Consistently, the down-regulation of miR-21 in two prostate cancer cell lines (DU145 and PC-3), obtained through the use of an antisense LNA, did not impair cell growth nor perturbate cell cycle progression. In addition, down-regulation of miR-21 did not increase the rate of spontaneous and drug-/radiation-induced apoptosis. Overall, our results suggest that miR-21 does not exert relevant oncogenic functions in human prostate.

Identification of gene expression markers in hereditary breast cancer

The aim of the present study is the identification of specific expression profiles of coding and non coding genes miRNAs) able to distinguish among breast cancers those with alterations in BRCA1 or BRCA2 genes, or defined as BRCAX (familial cases with no mutations in BRCA1 or BRCA2 genes). We also intend to verify if it is possible to find specific sub-groups in the BRCAX class.

We performed a transcriptome analysis of coding and non coding RNAs from a group of BRCA1, BRCA2, BRCAX and sporadic breast cancers using the microarray technology for identifying possible miRNA dependent mechanisms involved in this tumor type. The two expression profiles highlighted differentially expressed transcripts specific of the different classes and split the BRCAX class into two distinct sub-groups. To identify miRNA-target gene pairs deregulated in breast cancer, particularly in hereditary breast cancer, we integrated the two analyses. First we searched the public databases for the putative miRNA regulators (predicted to bind complementary sequences) of the genes we found differentially expressed among our breast cancer groups (miRNAbase, Targetscan, Pictar). We then confirmed their presence in the miRNA lists we generated from our expression profiling. We focused on one pair differentially expressed between Estrogen Receptor (ER)-positive and ER-negative cases and between a BRCAX sub-groups we identified and the other classes. This pair involved miR-342 and ID4 gene. ID4 expression is known to be inversely correlated to that of ER and it has also been proposed as a negative regulator of BRCA1. QRT-PCR analysis of miR-342 and ID4 gene our case series and of a panel of breast cancer cell lines confirmed the inverse expression of this putative pair. The putative interaction between ER or ID4 and miR-342 was also validated *in silico* using data retrieved from the Oncomine database.

We functionally validated the interaction observed in an experimental model. We cloned the 3' UTR sequence of the gene containing the predicted miRNA binding site in a luciferase reporter construct in 293T cell line, using different concentrations of the synthetic precursor of miR-342 (hsa-premiR-342) and of a scrambled miR, used as control. We observed a 35% reduction of the luciferase activity when cells were transfected with miR-342, thus confirming the interaction between miR-342 and ID4.

We measured the ability of miR-342 to silence expression of endogenous ID4 in breast cancer cell lines either ER- or ER+, by transfecting them with the synthetic precursor of miR-342. We also detected the levels of BRCA1 (negatively regulated by ID4) and of ESR1 (encoding for the estrogen receptor gene), whose expression is inversely correlated to that of ID4. We observed that miR-342 increases expression of BRCA1 (0.6 fold increase respect to not transfected cells) and ESR1 (3.5 fold increase respect to untrasfected cells). We did not find any significant expression difference for ID4 gene.

In conclusion, miR-342 seems to be involved in breast cancer by indirectly regulating the ESR1 expression. Our results highlight that miR-342 is also able to indirectly regulate the expression of BRCA1 gene and could contribute to predisposition to familial breast cancer in the BRCAX sub-group we identified by transcriptome analysis.

We are currently writing a paper on these data.