patients with epithelial circulating cells, which might indicate a capacity of these malignant cells to invade blood vessels.
Supported by Fapesp.

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Development of a measles virus vector targeting breast cancer cells by expression of single chain antibody against HER-2/neu

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Background: HER-2/neu is overexpressed in 25% of breast cancers, and is associated with poor prognosis. In order to develop effective therapies for breast cancer, we used a novel virus vector targeting the neu protein. We previously established a reverse genetics system for measles virus (MV) using the MV-HL strain. By using this system, we have previously constructed a recombinant MV (rMV) that expresses a single chain antibody (ScFv) against human alpha-fetoprotein (AFP). This recombinant virus inhibited colony formation of AFP-positive human hepatoma cells. In the present study, we constructed rMV expressing ScFv against activated rat HER-2/neu protein to be tested *in vivo* in a transgenic mouse model of spontaneous breast cancer.
Materials and Methods: We constructed a cDNA in which the ScFv against rat HER-2/neu is fused with the transmembrane domain (TMD) of vesicular stomatitis virus (VSV)-glyco (G) protein, and inserted it as an additional transcription unit between the N and P genes of the MV genome. We then rescued the virus (rMV-aneu), and investigated the ability to replicate in breast cancer cells and the potential as an antitumor agent.
Results: We succeeded in rescuing the rMV-aneu from the construct using the reverse genetics system. The rMV-aneu replicated as well as the parent MV in B95a cells, derived from marmoset B lymphoma. The rMV-aneu grew in N2C cells, a mammary carcinoma cell line established from a spontaneous BALBneuT tumor expressing rat HER-2/neu on their surface, whereas the parental MV did not show any infectivity. The protein produced by the inserted gene within the recombinant MV was properly expressed in the infected N2C cells. The rMV-aneu significantly reduced cell viability as measured by the metabolic activity of the infected cells. In contrast, the parental MV and mock infection did not cause any change in the activity. The effects of the rMV-aneu on the rat neu transfected human cells and on the transformed cells *in vivo* are currently under investigation.
Conclusions: As an approach to develop tumor-targeted, replication-competent viruses useful in breast cancer treatment, we constructed an rMV that express ScFv recognizing activated HER-2/neu. The insertion of the ScFv gene fused with G-TMD into the rMV genome induced its infectivity. The rMV-aneu significantly inhibited the HER-2/neu+ cell activity *in vitro*. These results suggest the possibility that the rMV-aneu may be useful in breast cancer therapy.

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Association between serum estrogen and androgen concentrations and tumour receptor status in postmenopausal breast cancer

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Elevated levels of endogenous sexual hormones; estrogen (E2), estrone (E1), testosterone (TE) and their precursors; androstenedione (AD), dehydroepiandrosterone (DHEA) and DHEA sulphate (DHEA-S), E1 sulphate (E1-S), have been associated with the risk of breast cancer in postmenopausal women. In this study we investigate the correlation between serum hormone concentrations and tumour receptor status. Besides the levels of sexual hormones and precursors, sex hormone-binding globulin (SHBG), insulin-like growth factor-1 (IGF-1) and IGF binding protein-3 (IGFBP-3) were also measured by fully automatized equipment using RIA and IRMA methods. The estrogen (ER) and progesterone receptors (PR) expression in tumour tissues were determined by ICH, and MedCalc Software was used for statistical analysis.
Our study involved 444 postmenopausal patients with primary breast cancer of Stage I, II prior to surgical intervention and 250 healthy controls [average age in both groups was 64 years]. 358 of cancer patients were diagnosed for invasive ductal carcinoma (DC), 55 for invasive lobular carcinoma (LC), 29 for DC in situ (DCIS), and 2 for LCIS. 297 were ER and PR-positive [ER+/PR+], while 78 were ER and PR-negative [ER−/PR−]. Significant increase of E1, E1-S, AD, TE, DHEA, DHEA-S levels and significant decreases of SHBG level in cancerous cases were found by Mann-Whitney statistical analysis.
The median value of serum E1, AD, E1-S, IGF-1, E2 and TE were higher in patients with [ER+/PR+] receptor status than in patients with [ER−/PR−] receptor status. In E1 ($p < 0.0003$), AD ($p < 0.0009$) and E1-S ($p < 0.0096$) levels the difference was highly significant. Patients were rank ordered according to the increasing serum E1, E1-S and AD concentrations. In the highest quintile of each series 74–80% of patients were [ER+/PR+], while 9–11% of them were [ER−/PR−]. Using logistic regression analysis, the probability of tumour receptor positivity can be predicted based on the knowledge of serum hormone (E1, E1-S, AD) levels. In addition, E1-S and TE levels tend to be markedly associated with invasive DC (DC vs. LC, $p < 0.0109$ for E1-S; $p < 0.019$ for TE).
Our study supports the hypothesis that the circulating sex steroid hormone levels are strongly correlated with risk of [ER+/PR+] breast tumours.
Some kits for IGFBP-3, E1-S, AD and MedCalc software were gratefully donated by Laborexpert Ltd., Budapest, Hungary.

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The prognostic influence of Plasminogen Activator Inhibitor-1 in early breast cancer is not related to estimates of angiogenesis

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Introduction: Plasminogen Activator Inhibitor type-1 (PAI-1) is one of the key proteases involved in tumour invasion and microenvironment remodelling. Indeed, high levels of PAI-1 have been associated with poor prognosis in several tumour types. In several experimental studies PAI-1 has been shown to play a role in angiogenic processes, and since estimates of tumour angiogenesis have been demonstrated as predictors of poor prognosis this study investigates the relationship between estimates of tumour angiogenesis and protein levels of PAI-1 in breast cancer.
Materials and methods: Tumour specimens from 438 patients diagnosed with primary unilateral non-metastatic breast cancer were used. Median follow-up was 9.5 years, and 168 patients (38%) had died from cancer. Angiogenesis scores were performed on paraffin-embedded tissue slides stained with anti-CD34, and vessels were counted using a Chalkley grid in hot spots. Protein levels of PAI-1 were measured in supernatants from frozen tumour tissue using a sandwich ELISA kit with monoclonal catching and detecting antibodies.
Results: Median Chalkley count was 5.00 (range, 2.67–12.00), and median PAI-1 level was 0.70 ng/mg protein (range, 0–90 ng/mg protein). Chalkley counts were not correlated with PAI-1. Both high Chalkley counts and high PAI-1 were significantly correlated with high malignancy grade and lack of estrogen receptor, and high Chalkley counts furthermore correlated with large T size. High Chalkley counts and PAI-1 in tertiles were both correlated with poor disease specific survival (DSS) ($P = 0.002$ and $P = 0.05$, respectively). Combining low/low versus high/high tertiles of Chalkley counts and PAI-1 showed actuarial survival rates of 75% versus 52%, respectively ($P = 0.0008$). In multivariate analysis high N-stage ($P' < 0.0001$), grade ($P < 0.0001$) and increasing levels of PAI-1 ($P = 0.004$) were identified as independent markers of cancer-death.
Conclusions: In univariate analysis both Chalkley counts and PAI-1 levels were associated with poor DSS. Combining lowest versus highest tertiles of both factors separated the patients into groups with significantly different survival. This study suggests that the prognostic impact of PAI-1 is independent of its supposed involvement in angiogenic processes.

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Gene expression associated to response to doxorubicin based primary chemotherapy in breast cancer

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Background: This study was undertaken to identify genes that could predict response to doxorubicin based primary chemotherapy in breast cancer patients.
Patients and methods: Patients (pt) with confirmed invasive breast cancer on samples obtained by core or incisional biopsy, clinical stages (CS) II or

III, were candidates to this study of gene profile, associated to response to primary chemotherapy. Thirty eight pt were included in a training set and another four, in an independent validation set. Median age of pt was 51 years, the majority of them presented large lesions with a mean diameter of primary breast tumor and axyllary lymph nodes of 91.1 mm. All pt (except two) received 4 courses of doxorubicin and cyclophosphamide (AC) therapy at 60 and 600 mg/m^2, respectively, in a routine basis protocol. Median duration of chemotherapy was 67 days and mean administered dose of doxorubicin was 96.1%. Response to chemotherapy was clinically evaluated and based on RECIST guidelines, 34 pt, who presented at least a 30% reduction in tumor dimension, were considered responsive, and eight, were considered resistant. Samples obtained from tumor biopsies before chemotherapy, were hand dissected and only samples composed of at least 80% malignant cells, were further processed. RNA was extracted, amplified and gene expression analysed using cDNA microarrays glass slides, containing 657 sequences, printed in three or six replicates. cDNA microarray platform, complying with MIAME format, was submitted to the Gene Expression Omnibus (GEO) data repository (GPL 1727).
Results: Seventeen genes were differentially expressed between responsive and resistant tumors, however, hierarchical clustering was not able to discriminate the groups. cDNA microarray gene expression data was confirmed by quantitative real time PCR measurements and Spearman rank correlation between these assays was significantly positive for five of seve genes analyzed. A classifier was designed using multiple transcripts and a trio was identified comprising: EMILIN1, FAM14B and PBEF. The classifier error was 5.41% and sensitivity to detect responsive tumors was 100%, both in the training set and in a small validation set, which means that all patients, who presented an objective response, were identified.
Conclusions: Our results suggest that a trio of genes might distinguish responsive tumors to doxorubicin based therapy.

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Down regulation of the DNA Double strand breaks repair genes in early stage breast cancer

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Of the many types of DNA damage, the Double-strand breaks (DSBs) are the most dangerous, because of the intrinsic difficulty of their repair as compared with other types of DNA damage. In this study we have analyzed genes expression of the main genes involved in DNA Double Strands repair pathways in 20 early stage (pT1cN0M0) breast cancers.
A total of 13 genes implicated in both homologous recombination (HR) and Non-homologous End Joining (NHEJ) mechanisms were analyzed on a 7700 Sequence Detection System (Applera Italia) by Quantitative Reverse Transcription Polymerase Chain Reaction, using MGB probes chemistry. For each case matched pathologically normal breast tissue and tumor were analyzed. Expression of the target gene was normalized by the expression of the housekeeping gene GAPDH and for each gene mRNA levels were determined as relative expression (RE) in the tumor as compared with matched normal tissue (RE in the normal equal to 1). Thus RE value < 1 indicate reduced expression and RE >1 indicate an increased expression. A substantial reduction in mRNA relative expression was found for the majority of the genes tested.
Approximately 70% of the tumors showed down regulation for MRE11, RAD52, BRCA1, G22P1, and XRCC5. ATR, NSB1, RAD50 and RAD54, and Artemis were down regulated in approximately 60% of the cases. The up-regulated genes were RAD51 (70% of the cases), BRCA2 (60%) and ATM (50%). The majority of the cases showed RE value from 0.5 to 1.5 corresponding to a 50% down or up-regulation in the tumor as compared with matched normal tissue. However, a subgroup of breast cancer (approximately 50% of the cases) showed a marked reduction of RE expression levels for ATR, NSB1, MRE11. For these genes, RE value were between 0.062 e 0.315 corresponding to reduction in mRNA levels from 94% to 70% as compared with matched normal breast tissue.
Overall our data suggest a substantial down-modulation of both mechanisms involved in DNA double strands break repair in a relative early stage of breast cancer progression. Moreover our data indicates a marked down-regulation of the MRE11 complex (MRE11/NSB1/RAD50) in at least half of the tumors. This complex plays a role in each of the aspects of chromosome break metabolism acting as a break sensor, in the activation and propagation of checkpoint signalling pathways and in promoting recombination between sister chromatides.

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Investigation of ICAM-1 Genetic Markers (+241G/A and 469 A/G) in patients with breast cancer

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Background: ICAM-1 is an adhesion molecule which plays a key role in leukocyte recruitment and migration. ICAM-1 also acts as a co-stimulatory molecule for T cell activation and effector function. In this investigation, the frequency of ICAM-1 genetic markers (+241 G/A and +469 A/G) were analyzed in breast cancer patients and healthy control group.
Method: 269 patients and 231 female healthy controls were genotyped for polymorphism in Exon 4 of ICAM-1 gene (+241 G/A) using an Allele Specific Polymerase Chain Reaction (AS-PCR). Exon 6 (**+**469 A/G) dimorphism in ICAM-1 gene was also investigated in 250 patients and 184 healthy controls using a PCR− Restriction Fragment Length Polymorphism (PCR-RFLP).
Results: There was a significant decrease of GG genotype at position +241 G/A, in patients in comparison to the healthy controls (85.5% vs. 93.5%, respectively; P = 0.003). The frequency of G allele at this position, among patients and controls were also found to be 92.8% and 96.7%, respectively. Accordingly, the decrease in the frequency of G allele in patients was statistically significant (P = 0.004). There were no significant differences in allele or genotype frequencies between patients and controls in the case of **+**469 A/G polymorphism.
Conclusion: Data of this investigation conclude that polymorphism at position +241 of ICAM-1 gene may be associated with the susceptibility to breast cancer. To confirm this finding, the protein expression and assessment of sICAM-1 in these patients are under investigation.

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Thomsen-Friedenreich antigen – prognostic factor in primary breast cancer tissue, expression on disseminated tumor cells and target for immunomagnetic enrichment

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Introduction: The Thomsen-Friedenreich (TF) antigen is a specific oncofetal carbohydrate epitope (Galb1−3GalNAca-O-epitope) expressed on the surface of various carcinomas. It mediates endothelium adhesion and tumor invasion. The presence of disseminated tumor cells in the bone marrow of breast cancer patients (DTC-BM) indicates worse prognosis at all stages of the disease. We therefore examined the expression of TF in primary breast cancer tissue and on DTC-BM. Additionally, TF was examined as a target for the immunomagnetic enrichment of DTC-BM.
Methods: TF expression was examined immunohistochemically on Tissue Micro Arrays (TMA) of 265 breast carcinomas from patients with known bone marrow status, using the semiquantitative immunoreactive score (IRS). The prognostic impact together with that of DTC-BM was calculated by Kaplan-Meier and Cox-regression analysis. Second, bone marrow of 25 patients screened positive for DTC-BM was double stained for cytokeratin (CK) / TF and TF / MUC1 by immunofluorescence. Third, immunomagnetic enrichment with anti-TF antibody and Cytokeratin (CK) staining was done on bone marrow samples of 48 patients.
Results: TF expression was demonstrated on 136 / 169 evaluable TMAs. Median IRS score was 2 (0−12). 68 of the 265 patients (25.7%) showed DTC-BM. TF positivity correlated with HER2 negativity (p = 0.048), but not with other histological parameters or DTC-BM. After a follow up of 60.5 months (7−255), the presence of DTC-BM showed prognostic significance for OS (p = 0.032), whereas TF negativity was significant for DFS (p = 0.032), DDFS (p = 0.021) and OS (p = 0.026). Double staining experiments on DTC-BM showed co-expression of TF and CK in 98% of the cells. After immunomagnetic enrichment, 31/48 pts showed DTC-BM, increasing positivity rate from 20.8% to 64.6%.
Discussion: TF seems to be a prognostic factor in breast cancer. It shows nearly complete expression on DTC-BM and is by this a suitable marker for immunomagnetic enrichment of those cells. Antibody based therapy against TF and vaccination studies showed efficacy *in vitro* and in animal models.