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Development, characterisation and gene expression profiling of a novel more invasive variant established from the human breast cancer cell line Hs 578T

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Background: The purpose of this study was to identify invasion-specific genes in breast cancer cell lines using Affymetrix microarray technology. Since an isogenic cell line model (i.e. different cell lines with the same genetic background) with different *in vitro* invasiveness was not available a new more invasive variant was generated from the Hs 578T breast cancer cell line.
Methods: To generate invasive variants from the Hs 578T, 1×10^6 cells were allowed to invade for five hours through six well matrigel invasion chambers, invading cells were removed from the bottom of the membrane and designated Hs 578Ts(i)$_1$ and this selection procedure was repeated eight times. Invasion and migration assays were done using matrigel 24 well invasion chambers and falcon 24 well inserts respectively, assays were stained using crystal violet. All cells were cultured under standard conditions in DMEM supplemented with 10% foetal calf serum, 10% L-glutamine and 0.1 unit/ml bovine insulin. Growth, detachment and adhesion assays were carried out in 24 well plates and quantified using CellTiter 96® AQueous One Solution Reagent (MTS), absorbance was read at 492 nm. Gene expression profiles were identified using the Affymetrix Human Genome U133A gene chip. Data analysis was done using GeneSpring 6.0 software. Real-time PCR analysis was completed using SYBR green.
Results: A distinct difference in invasiveness and other properties emerged between the Hs 578T and the new variant Hs 578Ts(i)$_8$. The new cell line was three fold more invasive than Hs 578T and grew significantly faster (up to four times faster). Hs 578Ts(i)$_8$ detached faster on trypsinisation (four minutes for s(i)$_8$ against sixteen minutes for Hs 578T) and there was a small increase (no more than two fold) in migratory ability. In soft agar, Hs 578Ts(i)$_8$ formed four times more colonies than the parental cell line. Microarray analysis of the two cell lines showed that 508 genes were differentially expressed. Twenty of these genes were chosen for validation and further studies. To date seven genes have been validated using Real-Time PCR analysis. Cathepsin Z was five fold up-regulated and Lumican was 14 fold down regulated, correlating with array data.
Conclusion: We have generated and extensively characterised a novel more invasive variant from the Hs 578T breast cancer cell line. We have also identified a panel of genes associated with invasive breast cancer.

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Plasma kinetics and uptake of a cholesterol-rich microemulsion (lde) associated to a derivative paclitaxel by neoplastic breast tissue

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Background: Previously we described the association of paclitaxel to a cholesterol-rich nanoemulsion (LDE) that binds to low-density lipoprotein (LDL) receptors and concentrates in neoplastic tissues. The association of the drug is stable, preserves the anti-proliferative activity of the drug and reduces the toxicity to animals. The present study was designed to determine the plasma kinetics of the association LDE: paclitaxel oleate and to verify whether the complex has the ability to concentrate in malignant breast cancer.
Material and methods: To facilitate the association to LDE, paclitaxel is derivatized with oleic acid. [^{3}H]paclitaxel oleate associated to LDE labeled with [^{14}C]-Cholesteryl oleate was intravenously injected into 3 patients with breast cancer (60±7 yr.) 24 h before surgery. Blood samples were collected over the 24 h period to determine the plasma decay curves of the radioactive labels. Radioactivity present in plasma aliquots was quantified in a scintillation solution and the pharmacokinetic parameters were calculated by compartmental analysis. Specimens of tumors and normal breast excised during the surgery were collected for lipid extraction, separation by thin layer chromatography and radioactive counting. The experimental protocol was approved by the Ethics Committee of the hospital and an informed consent was obtained from each participant.
Results: Fractional clearance rate (FCR) of LDE and of the drug were similar (0.030±0.026 e 0.018±0.018, respectively, P = 0.5742). The uptake of both [^{14}C]-LDE and [^{3}H]-paclitaxel oleate by breast malignant tissue was two and three fold greater than that of the normal breast tissue, respectively (LDE uptake = 680±481 and 290±247 and paclitaxel oleate uptake = 1134±1549 c.p.m./g and 469±695, respectively). Paclitaxel oleate has a $T_{1/2}$ = 19 h, AUC = 1.4 mg/h/L, VSS = 41.8 L and CL = 1.5 L/h.
Conclusions: Our results indicate that most of the drug is retained in the microemulsion particles until its removal from the circulation and internalization by the cells. In addition, we showed that when paclitaxel is associated with LDE, the drug can be concentrated in malignant breast tissues while deviating from the normal tissue. Therefore, LDE can be used to target paclitaxel against malignant breast cells.

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Leptin expression on breast cancer patients and the effects of leptin expression on survival in obese postmenopausal women with antiestrogen-treated breast cancer

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Background: Leptin is an adipocyte-derived cytokine that acts through its receptor and is related to obesity. Obesity in postmenopausal women is associated with an increased risk of breast cancer. Recent trials suggest that functional cross talk between leptin and estrogen systems exists. We tried to evaluate whether leptin & leptin receptor (ObR) effect on the prognosis of the obese postmenopausal breast cancer patients who were treated with antiestrogen, tamoxifen in adjuvant setting and to investigate leptin and ObR expression in breast cancer patients.
Material and methods: From 1994 to 2004, 91 patients who were diagnosed as stage I or II breast cancer after a curative resection were analyzed. The expression levels of leptin and ObR were measured using immunohistochemical (IHC) staining with rabbit polyclonal anti-human leptin and mouse monoclonal anti-mouse ObR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) from paraffin-embedded primary tumor specimens. Compared with the intensity of IHC staining of adipocyte, that of tumor cells was divided into two groups (negative, positive).
Results: Among a total of 91 patients (stage I/II = 23/68 pts), 82 pts were hormone receptor positive breast cancers and the median age was 48 years. 43 pts (52.4%) were menopause status and BMI (body mass index) were 23 or higher in sixty-one pts (67.0%). 69 pts received adjuvant chemotherapy. All pts with hormone receptor-positive tumors received tamoxifen. The median follow-up duration was 41 months. Leptin expression was observed in 77 pts (84.6%) of total pts. Among 82 pts with hormone receptor-positive tumors, 40 pts (48.8%) were menopausal status, 54 pts (65.9%) were 23 or higher BMI and 28 pts (70%) of 40 menopausal pts were obese. Leptin expression was positive in 70 pts (85.4%) of 82 pts, in 34 pts (85.0%) of 40 menopausal pts, in 46 pts (85.2%) of 54 obese pts and in 25 pts (89.3%) of 28 obese postmenopausal pts among 82 hormone receptor-positive pts. 14 pts (17.1%) of 82 pts experienced relapse. No significant difference of disease-free survival (DFS) was shown between leptin expression and obese postmenopausal breast cancer (p = 0.42). But DFS in leptin expression of obese postmenopausal patients was shown to tend to be higher than that of no expression. The result of ObR expression will be presented at the meeting.
Conclusions: This current study is the one among a few data to evaluate the relation of leptin with breast cancer patients and analyzed survival according to leptin expression of obese postmenopausal patients with tamoxifen-treated breast cancer. Present negative result must be continuously observed to overcome short median follow-up duration.

315 PUBLICATION

Cytochromes P450IID6 genotypes and its influence on the behavior of breast cancer in women under forty years

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Background: CYP2D6, a member of the Cytchromes P450 (CYP) family, is a phase I metabolic enzyme involved in the oxidative metabolism of numerous endogenous and exogenous molecules, including cytotoxic agents. The CYP2D6*4 polymorphism has been reported to be a major cause of CYP2D6 poor metaboliser phenotype, leading to the absence or decrease in the amount and activity of its protein. The aim of this study was to understand the role of *CYP2D6* genotypes on the behavior of breast cancer in women under forty years.
Methods: This study included 112 patients diagnosed with breast cancer under the age of forty in the Portuguese Institute of Oncology –

Porto, Portugal. DNA extracted from peripheral blood was submitted to Polymerase Chain Reaction (PCR) followed by Restriction Fragment Length Polymorphism (RFLP), in order to identify the *CYP2D6* genotypes. The mean disease free survival time was assessed using Kaplan-Meier methodology and the log rank test.
Results: From the global sample, the *CYP2D6* polymorphism was observable in 79 patients: CYP2D6 homozygotic (wt) was present in 59.5% of all cases, heterozygotic in 36.7% and homozygotic poor metabolizer (pm) in 3.8%. The mean disease free survival time (months) was significantly better in the patients that are carriers of the *CYP2D6* wt genotype (215 vs 46, $p = 0.028$). This was particularly evident in early stages (Stages I and II), with a mean disease free survival time of 247 ± 39 for homozygotic wt genotype carriers and 49 ± 6 for heterozygotic and pm homozygotic genotypes carriers ($p < 0.001$).
Conclusion: Our results suggest a role for *CYP2D6* polymorphisms in the clinical outcome of early onset breast cancer patients. The characterization of the drug metabolising genetic individual profile might lead to an individual chemotherapy approach, which would allow drug dosing on an individual's capacity to respond, thus leading to a more efficient and less toxic treatment.

316 PUBLICATION

Analysis of BRCA1 and BRCA2 mutations in high-risk patients from the Prague-area

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Background: About 5–10% of all breast cancer cases are due to inheritance of a susceptibility allele and a substantial proportion of these are due to germline mutations of the two major highly penetrant cancer susceptibility genes, *BRCA1* and *BRCA2*. The purpose of this study was to estimate the incidence, spectrum and possible clustering of disease phenotypes associated *BRCA1* and *BRCA2* mutations. The analysis was performed in breast/ovarian cancer families and in high-risk patients not selected on the basis of their family history of cancer.
Material and methods: 122 Czech families with recurrent breast and/or ovarian cancer and 69 patients considered to be at high-risk but with no reported family history of cancer were screened for mutations in the *BRCA1/2* genes. The entire coding region of each gene was divided into overlapping fragments with a size range of 880–1569 bp and amplified by the polymerase chain reaction. Mutational analysis was carried out by the protein truncation test and direct DNA sequencing.
Results: Within 191 analyzed individuals, 48 (25.1%) carried a *BRCA1* mutation and 10 (5.2%) a *BRCA2* mutation. One novel truncating mutation was found in *BRCA1* (c.1866 A>T) and two in *BRCA2* (c.4167delC and c.5991dupT). *BRCA1* mutations comprised 14 different alterations. Five recurrent mutations accounted for 81.2% of individuals with detected gene alterations. The *BRCA1* 5382insC detected in 56.2% of mutation positive women was the most prominent gene defect. A total of 8 different mutations were identified in *BRCA2*. The novel c.5763dupT mutation and c.5682C>G, which appeared in two unrelated families each, were the only recurrent alterations of the *BRCA2* gene. Pathogenic mutations were found in 24.0% of breast cancer families and in 62.8% of families with the occurrence of both breast and ovarian cancer. In addition, deleterious mutations were detected in 10.0% of women with early-onset breast cancer. A total of 4 hereditary mutations in *BRCA1* were identified among 17 (23.5%) women with a medullary breast carcinoma selected for examination regardless of the family history.
Conclusions: Mutational analysis of *BRCA1/2* genes characterized the spectrum of gene alterations in Czech population and demonstrated the dominant role of the *BRCA1* c.5382insC allele, which accounted for more than 46% of all identified gene alterations.
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317 PUBLICATION

Epidermal growth factor receptor levels in progesterone receptor positive breast tumors

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Introduction: Some breast tumors express epidermal growth factor receptor (EGFR) in different concentrations, and it has been related with poor prognosis. Higher levels of EGFR are related with hormonal receptors negative status. Our purpose is observing the behaviour of EGFR depending on different cut-off values of progesterone receptor (PR) positive breast cancer tissues.
Patients and methods: 472 patients aged between 27–88 years old were analyzed. 268 breast tissues with infiltrative ductal carcinoma (IDC) were used to measure progesterone receptor and EGFR. Hormonal receptor was determined with quantitative enzymatic immunoassay. EGFR was measured with radioligand assay. Statistic analysis was performed with Mann-Whitney U test.
Results: In the following PR cut-off points, the results were:
- >1 fmol/mg cytosol protein: median (p50) EGFR levels in PR positive tumors were 3.9 fmol/mg cytosol protein vs 4.25 in PR negative tumors (non signif.)
- >5 fmol/mg cytosol protein: median EGFR levels in PR positive tumors were 3.7 vs 4.75 in PR negative tumors ($p < 0.001$).
- >10 fmol/mg cytosol protein: median EGFR levels in PR positive tumors were 3.65 vs 4.45 in PR negative tumors ($p < 0.05$).
- >15 fmol/mg cytosol protein: median EGFR levels in PR positive tumors were 3.6 vs 4.5 in PR negative tumors ($p < 0.05$).
- >20 fmol/mg cytosol protein: median EGFR levels in PR positive tumors were 3.55 vs 4.45 in PR negative tumors ($p < 0.05$).

Conclusion: Higher values of EGFR were measured in PR negative samples of IDC of the breast using five different cut-off points of positivity, starting in 5 fmol/mg cytosol protein. We can conclude that EGFR levels show inverse relation with hormone dependent infiltrative ductal carcinoma of the breast.

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Antisense chemoradioimmunotherapy inhibit the endothelin axis with subsequent induction of type I, type II PCD and metastatization in advanced breast cancer characterised by hypermethylated oncosuppressor promoter CpG islands and overexpression of oncogenes

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Advanced breast cancer is resistant to almost all cytotoxic drugs and radiation making it one of the most aggressive malignancies in humans with the worst mortality. The failure of tumour cells to undergo apoptosis cause resistance to chemoradiological therapies due to overexpression of oncogenes and transcriptionally repressed apoptotic tumour suppressor genes due to aberrant methylation (CIMP+). Also, overexpression of endothelins enhances tumour proliferation.
We obtain tumour cells from a patient with metastatic breast cancer MS-PCR detected methylation of tumour suppressor genes p53, p16, RASSF1A, RAR-b2, BRCA2, PTEN, E-cadherin, hMLH1, ESR1, CDH1, TRbeta1, GSTP1 and CCND2. Quantitative IHC, WB, SB and PCR exhibited overexpression of COX-2, PGE2, bcl-2, ET-A, Raf-1, cdc25c, c-fos, c-myc, c-jun, EGFR and VEGF. We treated the tumour cells with antiET-A scFv attached onto high energy radioisotopes, vinorelbine tartrate and 21 nucleotide double standed siRNA segment generated against DNMT1. Post-treatment, we detected re-expression of oncosuppressor genes after inhibition of DNMT1mRNA. Downregulation of paracrine/autocrine factor ET-A due to targeted scFv inhibited the endothelin induced signal transduction pathways by blocking binding of ET-1 to the ET-AR in the plasma membrane.
This blocked the signal transduction pathway through G9 causing inactivation of PLC, PTKs such as FAK and RAS blocking the RAF/MEK/MAPK pathway. Inhibition of ET-1/ETAR caused downregulation of ILK, IOGAP1, a2, b3 and b1 integrins, N-cadherin, COX2, PGE2, VEGF and upregulation of connexin, E-cadherin and b-catenin. This inhibited intracellular Ca++, PKC, MAPK, p42/44MAPK kinase and p38 MAPK blocking transcription of EGFR, c-fos, c-myc and c-jun leading to inhibition of cell growth and mitogenesis. It also inhibited PIK3-mediated AKT activation. Vinorelbine caused inactivation of bcl-2, Raf-1 and cdc25c by phosphorylation. We detected upregulation of p21Waf1, p27Kip, E-cadherin and Bak. The high energy radioisotopes induced DNA double strand breaks in tumour cells arresting synergistically with MT depolymerizing VRL their growth at the G2/M transition according to flow cytometry. We detected externalisation of PS, depolarization of mitochondrial transmembrane potential, activation of caspase 3.9, bax and DNA fragmentation. TEM exhibited irreversible D2 apoptotic signs forming apoptotic bodies indicating typeI PCD after chromatin condensation and nuclear fragmentation. Overexpression of Beclin-1, PTEN, p70, DAPK and BNIP3 induced ceramide mediated autophagic cell death termed as typeII PCD where LC3 is localised in autophagosome membranes. BrdU and MTT exhibited inhibition of DNA synthesis and metabolic activity of treated tumour cells compared to untreated controls.
We have achieved to induce type I and type II PCD leading to eradication of advanced breast Ca cells which is correlated with inhibition