7.2 to 8.5 years, p = 0.0001–0.0008). In patients with CHEK2 mutations, breast cancer occurred 2.1 to 3.8 years earlier than in patients without mutations in CHEK2 gene. However, the identified differences were found not significant (p = 0.4802 and 0.2060). Among remaining 125 patients with bilateral breast cancer, who had not germline mutations in BRCA1, BRCA2 and CHEK2 genes, 85 had family history of neoplasm and 40 had not. The women with bilateral breast cancer and family history of breast cancer only were not diagnosed for earlier occurrence of bilateral breast cancer. However, the age of women with metachronous breast cancer and with family history of breast and ovarian cancer or ovarian cancer only was different from the age of women with no such family history (a difference of 7.7 years, p = 0.0608).

The age of women with family history of neoplasm other than breast and ovarian, at which bilateral breast cancer was diagnosed, was significantly lower than the age of patients with no such family history (a difference of 5.2 years, p = 0.0169).

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The role of steroid sulfatase (STS) and organic anion transporter polypeptide B(OATP B) mRNA expression in predicting the clinical outcome in human breast cancer

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Introduction: Steroid sulfatase (STS) is the enzyme responsible for hydrolysing biologically inactive estrogen sulfates to active estrogens. Therefore it plays a significant role in supporting the growth of hormone-dependent tumours of the breast, endometrium, and prostate. There is little evidence as to what controls its expression in vivo. OATP-B is a member of family of membrane transporter proteins that regulates the uptake of steroid sulfates through cell membranes.

This study examines mRNA expression of STS and OATP-B genes located on chromosome X (location Xp22.32) and chromosome 11 (location 11q13) respectively. Our objective was to determine, using quantitative PCR, whether the mRNA expression levels of these genes were positively correlated with clinical outcome in human breast cancer.

Methods: A total of 153 samples (120 tumour tissues and 33 normal tissues) were analysed. The levels of transcription of STS and OATP-B were determined using real-time quantitative PCR. The mRNA expression was normalized against CK19. The levels of expression were analyzed against tumour's stage; grade, nodal status, local relapse, distant metastasis and survival over a 10 year follow up period. The levels were also analysed against hormone receptors status including ERα, ERβ, and HER1–4.

Results: The levels of STS mRNA were significantly higher in malignant samples compared with normal breast tissue samples (p = 0.031). They were also higher in node positive disease (p = 0.0222). STS mRNA expression increased with increasing tumour grade but this did not reach statistical significance. We also noted an increase in levels correlating with tumour stage using TNM classification. This became statistically significant when we compared stages TNM1 and TNM2, TNM2 and TNM3, and TNM3 and TNM4 ($p \leqslant 0.00001$, 0.0017, and 0.02 respectively). Furthermore, STS expression levels positively correlated with progression of disease as levels were significantly higher in samples of patients who developed metastasis, local recurrence, or died of breast cancer when comparing to those who were disease free for > 10 years (p = 0.0036).

We found no significant correlation between levels of STS expression and ERα/ERβ status. The levels positively correlated with HER1 and HER3 receptors.

The levels of mRNA expression of OATP-B were higher in malignant tissue compared to normal tissue, this however did not reach statistical significance (p = 0.4045). Levels were also higher in node positive disease (p = 0.0672). Expression levels increased with increasing tumour grade, this became statistically significant when comparing grade 1 to 2, and grade 2 to 3 (p = 0.0271, 0.0289 respectively). We also observed an increase in levels correlating with TNM tumour staging, this however did not reach statistical significance. There was no significant correlation between OATP-B expression levels and clinical progression of breast cancer. We found no correlation between STS and OATP-B expression levels.

Discussion: This study demonstrates a compelling trend for STS transcription levels to be higher in cancerous tissues. These levels were even higher in patients who developed progressive disease. OATP-B expression levels correlated with the grade and stage of the disease but not with clinical outcome. These results suggest that STS mRNA has a significant potential as an independent predictor of clinical outcome in patients with breast cancer.

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p21 as a target for breast cancer therapy: the role of p53 status in its efficacy

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$p21^{waf1/cip1}$ has emerged as an important but pleiotropic regulator of differentiation, cell cycle progression, senescence, and apoptosis, and this molecule has been suggested by us and others as a molecular target in breast cancer treatment. Recent reports have shown that doxorubicin differentially activates Akt in some breast cancer cell lines, including p53-mutant T47D cells, and that activated Akt increases cytosolic expression of p21. We undertook this study to investigate whether p21 is conveying anti-apoptotic effects in breast cancer as a function of p53 status. We find that doxorubicin treatment of T47D, a p53-mutant human breast cancer cell line, results in a dose-dependent decrease in both p21 levels and the anti-apoptotic protein, XIAP, with a concomitant increase in PARP cleavage (indicating apoptosis). No changes in the apoptosis-related proteins CAS (cell apoptosis susceptibility) and Apaf-1 were seen with doxorubicin treatment. Similar results were found in a p53 mutant renal cell carcinoma cell line, 786-O. To determine whether p21 is conferring the anti-apoptotic effect seen with lower dosage of doxorubicin treatment, we used an RNAi approach. Down regulation of p21 with siRNA did not change PARP cleavage or expression levels of XIAP, CAS, and Apaf-1 as compared to cells treated with doxorubicin alone. However, p21 down regulation enhanced apoptosis induced by lower dosage of doxorubicin in the p53-wt renal cell carcinoma cell line, ACHN. We are currently investigating this effect in p53-wt breast cancer cell lines. Due to the fact that p21 is a downstream target of p53 in the DNA repair pathway, our results suggest the anti-apoptotic function of p21 is dependent on p53 status and are consistent with previously published data showing that p21 accumulation after doxorubicin treatment only occurs in p53 wild-type breast cancers. More importantly, these results suggest caution when choosing p21 as a therapeutic target in breast cancer therapy. The genetic composition of tumors, such as p53 status, should be carefully considered in selecting the therapeutic regimen.

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Analysis of genetic alterations in plasma DNA from breast cancer patients: a possible molecular biomarker in early detection and prognosis of breast cancer

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Introduction: Genetic alterations are associated with the development of breast cancers, which are the most common malignancy in women. Tumor metastasis is the major cause of death in cancer patients. In the process of metastasis, tumor cells disseminate from the original site through the circulatory system and establish the secondary tumors in distant organs. Therefore, increasing levels of circulating tumor DNA was found in the bloodstream of cancer patients and apoptotic and necrotic cells are a major source for plasma DNA. Plasma DNA may be an indicator for cancer. Early diagnosis and identification of molecular tumor markers are the main topics of clinical cancer research. Molecular tumor markers described in plasma include oncogene amplifications, and microsatellite alterations, such as loss of heterozygosity (LOH) and microsatellite instability (MSI). It was postulated that identifying the genetic alterations in plasma may play an important role in the cancer diagnosis and prediction of cancers.

Patients and Methods: A total of 116 cases were analyzed in our study, including 34 non-metastatic patients with breast cancer, 41 metastatic patients with breast cancer originally, and 41 anonymous individuals without tumor, with previously identified breast cancer-specific microsatellite grade I markers, such as LPL, TP53, and D16S413, and grade II marker, D17S855, which located in the intron of the BRCA1 gene, using ABI 3100 capillary genetic analyzer. Additional 42 plasma samples, including 13 non-metastatic patients with breast cancer, 16 metastatic patients with breast cancer originally, and 13 anonymous individuals without tumor, were also used to analyze MYCN oncogene amplification with real time quantitative PCR using LightCycler instrument.

Results: LOH/MSI was detected in 6 of 41(14.6%) anonymous individuals with non-tumor disease, in 14 of 34(41.2%) non-metastatic patients, and in 18 of 41 (43.9%) metastatic patients. The frequency of LOH/MSI of plasma DNA was significantly lower in anonymous individuals

without tumor than that of non-metastatic patients and metastatic patients ($p = 0.009$ and $p = 0.003$, respectively; with c2 test). However, no significant associations were found between the presence of LOH/MSI in plasma DNA and clinicopathologic parameters except poor prognosis. As a result, it was suggested that grade I markers could be an early marker for carcinogenesis and predicting outcome of breast cancer. In addition, the results of real time quantitative PCR showed that MYCN amplification was detected in plasma DNA from in 2 of 16 (12.5%) metastatic patients, but deletions were observed in 10 of 16 metastatic patients (62.5%). However, 2 of 13 (15.4%) non-metastatic patients revealed MYCN deletion, and 7 of 13(53.8%) non-metastatic patients showed MYCN amplification. We also assessed plasma from non-metastatic, metastatic patients, and anonymous individuals without tumor to evaluate the quantity of plasma DNA by generating a standard curve for reference gene, b-actin. Low amounts of plasma DNA were found in the plasma samples from anonymous individuals without tumor.

Conclusion: As a conclusion, increasing tumor DNA was detectable in plasma, especially in breast cancer patients. Here we provided plasma DNA-based noninvasive methods, which, we hope, could be applied for clinical detection for breast cancer in the future.

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An ERbeta isoform that lacks exon 5: correlation with ERalpha, PR and classical prognostic indicators in breast cancer

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In this study, the relationship of ERbeta1 (wt) mRNA and ERbeta-delta5 mRNA expression to ER and PR levels was investigated in breast carcinomas. Samples and RNA Isolation: 60 samples of operable breast carcinoma were analyzed. All samples were examined histologically and only regions of tumor with more than 60% of malignant epithelial cells were used for RNA isolation. Levels of ERalpha and PR were measured by biochemical assay as recommended by EORTC. Total RNA was isolated with Trizol reagent and reverse transcribed with random hexamer. Quantitative real-time PCR: All PCR reactions were performed on a 7000 Sequence Detection System. Real time PCR was carried out with TaqMan Pre-Designed Gene Expression Assay specific for target transcript sequence. The target ERbeta1 (wt) mRNA and ERbeta-delta5 mRNA relative expression levels was normalized on beta-actin as endogenous control. Level of expression is expressed in relative units as N-fold differences in target gene expression normalized to the beta-actin gene and the relative to calibrator (sample with smallest amount of target gene mRNA). Regarding ERbeta1 (wt) mRNA and ERbeta-delta5 mRNA, samples were considered positive where the Ct value was above 32 (Ct – cycle threshold value). Analysis of correlation between ERbeta1 (wt) mRNA or ERbeta-delta5 mRNA and ERalpha or PR levels showed:

- absence of any correlation between ERbeta1 (wt) mRNA and ERalpha or PR expression;
- statistically significant negative correlation between ERbeta-delta5 mRNA and ERalpha expression;
- statistically significant negative correlation between ERbeta-delta5 mRNA and PR expression.

We also examined the relationship between ERbeta-delta5 mRNA expression status and classical clinical (age and menopausal status) and pathological parameters (nodal status, tumor size and type and histologic grade of tumor). There were no statistically significant correlations. Our findings indicate that ERbeta-delta5 mRNA expression, opposite to ERbeta1 (wt) mRNA expression, blocks ERalpha signaling pathways. In addition, ERbeta-delta5 mRNA expression could be useful biomarker, in its own right, of progression and endocrine response of breast cancer.

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Large genomic alterations of the BRCA1 gene in Iranian breast cancer patients

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Breast cancer (BC) is the most frequent carcinoma in women. Some 5–10% of all cases of BC under the age of 35 years have a hereditary origin. BRCA1/BRCA2 mutations are responsible for 3–8% of all cases of BC and 30–40% of familial cases.

The lifetime risk of breast cancer in female carriers of a *BRCA1* mutation is 60–80% *BRCA1* is a large gene with 22 coding exons encoding a 220 kD protein that functions in maintaining genomic integrity and in transcriptional regulation.

In countries with mixed populations, such as Iran, point mutations of *BRCA1* are scattered throughout the coding sequence without any hot spots or prominent founder mutations. Conventional methods used to screen for mutations focus on genomic DNA and are usually PCR-based, enabling the detection of sequence alterations such as point mutations, and small deletions and insertions. However, during recent years an increasing number of large deletions and amplifications have been described in the *BRCA1* gene.

In this study we used multiplex ligation-dependent probe amplification (MLPA) method for detecting gross copy number changes and large deletion/amplification in *BRCA1* gene. MLPA is a method developed especially for detecting gross copy number changes in genomic sequences. Remarkably, many of the known genomic alterations of *BRCA1* have been found by this screening method.

Briefly, 100 ng of target DNA in a total volume of 5 µL TE was used for the ligation reaction. After the ligation step, multiplex polymerase chain reaction (PCR) amplification (33 cycles) was performed by adding 5 µL of the ligation mixture to 2 µL 10* SALSA PCR buffer and 13 µL aqua. Five microliters of polymerase mix containing 1 µL SALSA PCR primers, 1 µL SALSA enzyme dilution buffer, 0.25 µL SALSA polymerase, and 2.8 µL aqua was added to the tubes on ice, and then the amplification step was started in a PCR machine at the denaturation temperature of 95ºC. After PCR amplification, the DNA fragments were analyzed on a ALFexpress DNA sequencer (Pharmacia Biotech) using AlleliLink software. The SALSA BRCA1-MLPA kit contains probes for each of the 24 exons of the *BRCA1* gene, including two probes for the large exon 11. For quality control, we used samples from known *BRCA1* gene mutation carriers.

Using MLPA, we have screened 20 families and detected 5 families with aberrant exon copy numbers. Three deletions and two duplications were identified.

Our results show that MLPA is a rapid, reliable, and sensitive technique, which allows high-throughput screening.

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Preclinical studies of the combination of RAD001 with tamoxifen or letrozole in breast cancer

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Resistance to endocrine therapies remains a major problem in breast cancer. Improved understanding of mechanisms underlying this is important for the development of new clinical strategies. We have characterized two MCF7 cell lines modeling relapse after long-term oestrogen deprivation (LTED) and resistance to tamoxifen (TamR). Cross-talk between HER-2 and ER pathways appears to contribute to endocrine-resistance in both settings leading to elevations in pMAPK and AKT, respectively. One strategy to improve the efficacy of current endocrine agents as well as delaying the onset of resistance is to target concomitantly ER and pertinent signal transduction pathways. One target is mTOR, a downstream member of the PI-kinase related kinase family, which regulates cell cycle progression, by enhancing translation initiation. We investigated the use of RAD001 (everolimus), a specific inhibitor of mTOR in combination with tamoxifen or letrozole on human breast cancer cell lines modelling endocrine-sensitive (MCF7 cells, wild-type (wt) and stably transfected with aromatase, Arom-1) and -resistant disease. The LTED and TamR cell lines revealed enhanced expression of proteins downstream of mTOR. Treatment of the cell lines with increasing concentrations of RAD001 resulted in a dose-dependent decrease in both proliferation and ER alpha-mediated transcription together with a concomitant decrease in phosphorylated p70S6 and S6. The TamR cells were highly sensitive to the anti-proliferative effects of RAD001: no additive effect was noted with the addition of tamoxifen. However, treatment of the 2% FBS MCF7 cells (parental line for TamR) with RAD001 in combination with 1uM tamoxifen resulted in an increase in sensitivity to RAD001. Similarly treatment of the Arom-1 cells with letrozole (0.1uM) in combination with a sub-optimal dose of RAD001 resulted in a further 50% reduction in proliferation compared to letrozole alone.

These data provide evidence that combinations of RAD001 with tamoxifen or letrozole may provide enhanced anti-tumour activity in endocrine-resistant disease and may delay the onset of resistance in the treatment of primary disease.

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The mTOR inhibitor rapamycin stabilizes p27 by downregulating the expression of its ubiquitin ligase Skp2 in breast cancer

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Introduction: Recent studies have shown that overexpression of the ubiquitin ligase Skp2 results in rapid ubiquitin-mediated degradation of the