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

cell-cycle inhibitory protein p27, uncontrolled cell proliferation and poor overall survival in breast cancer. The mammalian target of rapamycin (mTOR) is a downstream effector of the PI3K/Akt signaling pathway that mediates cell proliferation. By targeting mTOR, rapamycin induces cell-cycle arrest in the G_1 phase and upregulates p27 expression. The effects of rapamycin on Skp2 expression and the mechanism of p27 proteolysis, however, are unknown.

Materials and Methods: Rapamycin-treated and untreated T47D and MDA-MB-231 breast cancer cell lines were subjected to real time RT-PCR and western blot analysis to determine p27 and Skp2 mRNA and protein levels, respectively. Cells were also transiently transfected with a plasmid containing a Skp2 insert to determine the effect of rapamycin on translational regulation. Skp2 degradation rate was assessed in cycloheximide-treated cells and by pulse-chase analysis.

Results: Skp2 levels were downregulated in rapamycin-treated cells in a dose and time-dependent fashion, which was more prominent in cells expressing higher basal Skp2 levels. The decrease in Skp2 levels was followed by a reciprocal decrease in p27 ubiquitination, an increase in nuclear p27 levels and cell-cycle arrest at G_1. Rapamycin significantly inhibited Skp2mRNA transcription and rate of protein degradation, but not protein translation.

Conclusions: Rapamycin inhibits Skp2 expression and ubiquitin-mediated degradation of p27, resulting in upregulation of p27 and cell-cycle arrest. These results suggest that mTOR inhibition may be a novel target for the treatment of Skp2 overexpressing breast cancers.

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Tumour aromatase as measured by immunohistochemistry in patients treated neoadjuvantly with either letrozole or tamoxifen in the P024 randomised trial – correlations with other biomarkers

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Aromatase, the key enzyme responsible for oestrogen biosynthesis, is a major therapeutic target of endocrine therapy in patients with breast cancer and is present in about 70% of tumours.

The aim of this study was to measure aromatase in tumours before and after 4 months of treatment with letrozole or tamoxifen by immunohistochemistry using a novel monoclonal antibody (677), which has recently been developed to detect aromatase in archival material. IHC staining was performed in 185 cases from the P024 neoadjuvant trial randomising patients to tamoxifen or letrozole. Scoring was measured by assessing the proportion of immuno-positive cells and their intensity of reactivity in malignant epithelial, stromal, adipose and normal/benign compartments in each case.

Of 185 patients, sufficient tumour material was available for meaningful analysis in paired pre- and post-treatment samples from 171 cases (81 on letrozole and 90 on tamoxifen). Staining was detected in all tumour compartments but the highest scores were generally observed in malignant epithelial cells. Although there were highly significant correlations in scores between the malignant, stromal, adipose and normal compartments, patterns of staining differed between individual cases. Aromatase staining was not related to oestrogen receptor score, progesterone receptor status, Ki67 score or tumour size. Whilst median tumour aromatase values did not change significantly with treatment, marked changes in score (either increases or decreases) did occur in individual cases; effects were more noticeable for patients treated with letrozole. Correlation of these changes with clinical response is ongoing.

It is concluded that the 677 monoclonal antibody is a useful tool by which to assess and immunolocalise aromatase protein in breast cancers. Staining occurs in both malignant and non-malignant compartments and expression may be influenced by treatment with endocrine therapy.

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c-kit: identification of coregulated genes in breast cancer patients by gene expression analysis

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Expression of the proto-oncogene c-kit has been found in malignant tissue including a subset of breast cancers. c-Kit is also expressed in normal breast tissue and several authors found a loss of c-kit expression in breast carcinoma suggesting it might be involved in the growth control of mammary epithelium. Until now, only a few markers were described to be co-regulated with c-kit as e.g. CK-5/-17, Her-1 and PDGFR. To elucidate the possible role of c-kit in malignant transformation, we analyzed gene expression data of breast cancer patients.

Experimental procedures: Tumor tissue of n = 171 breast cancer patients were analyzed by gene expression profiling using Affymetrix Hg U133 Arrays (22,500 genes) and bioinformatic analyses. Tumor samples with high stromal and low epithelial cell content by gene expression profiling were excluded for further analysis.

Summary: A total of 10.5% of the tumors showed strong c-kit expression (2.5 fold above median). Comparing gene expression profiles of 10 samples with highest c-kit vs. those 10 with lowest expression did not reveal genes with a good correlation to c-kit. However, when samples were first subdivided into molecular tumor subtypes using the intrinsic gene set according to Sorlie et al., most of the tumors with highest c-kit expression clustered in the normal like subgroup. Interestingly, analyzing samples from the different molecular tumor subtypes separately revealed strong correlations of c-kit with the expression of a large cluster of genes containing several for whom c-kit coexpression was already described (HER1, CK-5/-17, PDGFR) as well as several members of the wnt signalling pathway, providing a possible novel link to mammary epithelial differentiation.

Conclusions: Gene expression analysis of mammary tumor subgroups according to Sorlie et al. provides new insight in the biological function of c-kit. This may help to identify patients who could have a benefit from the treatment with STI571 as a new therapeutic option.

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Insulin-like growth factor-1 expression and its prognostic significance in breast cancer

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Introduction: It is established that Insulin-like Growth Factor-1 (IGF-1) has pro-mitogenic, anti-apoptotic and growth stimulatory properties on breast cancer cells. Within the area of breast cancer, the main source of IGF-1 production, whether it is by tumour or normal breast tissue has yet to be confirmed. Also, the prognostic significance of high levels of local IGF-1 production and IGF-1 receptor (IGF-1R) expression in breast cancers remains to be validated. We examine the expression IGF-1 and IGF-1receptor expression in breast cancer and adjacent normal tissue (ANT) and the effects of IGF-1 on established prognostic factors used in breast cancer management.

Methods: Using real-time quantitative PCR, we measured the IGF-1 and IGF-1R expression in 88 oestrogen receptor (ER) positive and 40 ER negative pairs of breast cancer tissue samples and their corresponding ANT. CK-19 was used as a housekeeping gene for comparison of mRNA levels. The level expressed in these tissues were correlated with patient's age at surgery and prognostic factors: grade, lymph node status, tumour size and presence of lymphovascular invasion.

Results: In ER negative breast tissue, there was a significantly higher expression of IGF-1 in ANT compared to tumour tissue ($p = 0.000$) but this was not the case for ER positive breast cancers ($p = 0.887$). There was no difference in mean IGF-1 expression in ER positive and negative breast cancers. In general, there was also no difference in IGF-1R expression in tumour or ANT.

In ER positive cancers, there was a positive correlation between the number of positive metastatic lymph nodes and IGF-1 expression in normal tissue ($p = 0.034$) but not by IGF-1 expression in tumour tissue ($p = 0.564$). There was a marginally significant higher occurrence of lymphvascular invasion in samples with high tumour IGF-1 expression in ER positive tumours($p = 0.080$). In ER negative tissues, IGF-1 expression in tumour and ANT did not have any correlation with age or any of the prognostic factors.

In all cases, IGF-1R expression did not have any effects on prognostic factors.

Conclusion: We conclude that IGF-1 expression is higher in ANT which supports a paracrine production IGF-1. This data suggests that this relationship is mainly present in ER negative breast cancers. Increased IGF-1 expression by ANT and tumour tissue is associated with an increase in some prognostic factors but not IGF-1R. Future treatments of breast cancers involving a reduction IGF-1 production in normal and cancerous breast tissue should be considered.