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PROFFERED PAPERS

Biology, immunology and pathology

51 ORAL

Interaction between HER2/neu and topoisomerase II-a amplification in preoperative doxorubicin-based chemotherapy for locally advanced breast cancer: integration of novel chromogenic in situ hybridization and tissue microarray technology

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Purpose: This study was designed to investigate the interaction of HER2/neu and topoisomerase II-a (topoII) gene amplification in doxorubicin-based preoperative chemotherapy of locally advanced breast cancer patients.

Patients and Methods: Fifty-one patients with primary breast cancer underwent preoperative chemotherapy with doxorubicin 50 mg/m2 and vinorelbine 25 mg/m2 every 3 weeks for 4 cycles. HER2/neu and topoII amplification was assayed by novel chromogenic in situ hybridization (CISH) using tissue microarray (TMA) technology. Therapeutic efficacy was correlated with CISH assay results.

Results: HER2/neu was amplified in 24 (47.1%) and topoII was amplified in 15 cases (29.4%). Responses were observed in 33 (64.7%) of 51 patients. Eighteen patients had partial response (PR: >50% reduction of tumor volume) and 15 patients had minor response (MR: 25-50% reduction). HER2/neu was amplified in 24 cases showing response whereas only MR was observed in 9 (33.3%) of 27 cases without HER2/neu amplification. All 15 patients with topoII amplification had coamplification of HER2/neu and had PR to chemotherapy whereas 18 of 36 patients without topoII amplification responded to chemotherapy (PR: 3, MR: 15). Differences in response rates between HER2/neu-amplified tumors and those with normal HER2/neu were statistically significant. Degree and rates of response were significantly different according to topoII amplification status in HER2/neu-amplified tumors.

Conclusions: Doxorubicin-based chemotherapy was effective in HER2/neu-amplified breast cancers and the therapeutic efficacy was more evident in topoII-coamplified tumors. The finding of the current study indicates that topoII amplification has a role in chemosensitivity of breast cancer to doxorubicin-based regimens. CISH appeared as a tempting novel technology to identify gene amplification in clinical cancers.

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Predicting HER2 status of breast cancer from basic pathology features: HER2 status of 1500 breast cancers determined by immunohistochemistry and fluorescence in situ hybridisation with pathology correlation

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Neither immunohistochemistry (IHC) nor fluorescence in situ hybridisation (FISH)is the ideal test for the routine assessment of HER2 status. To assess whether HER2 status could be predicted based on pathology features prior to HER2 testing, 13 Australian laboratories collected pathology data from 1500 cancers as part of the HER2000 International Study.

Aims: 1. Assess the frequency of HER2 overexpression in invasive breast cancer using HercepTest.

2. Assess interobserver variation in scoring slides stained by HercepTest.

3. Use FISH to determine whether a score of 3+ or 2+ is associated with HER2 gene amplification.

4. Correlate HER2 status with pathology features.

5. Identify specific phenotypes that may predict HER2 status.

Methods: Each laboratory tested 120 cancers using HercepTest and scoring the tumours 0-3+. Lymph node status, size, grade and type were known. FISH testing was performed using the HER2 DNA Probe Kit (PathVysion, Vysis). A set of slides from 5 breast cancers stained by HercepTest was circulated for scoring by each of the 13 pathologists.

Results: 1537 cancers were assessed. There was a statistically significant relationship between tumour type, grade, size and HER2 status determined by HercepTest ($p<0.05$,n=1214).

82% of the cancers were infiltrating NST, 10% infiltrating lobular, 5% special types and 3% rare types. 185 cancers (12%) were scored as 3+ by HercepTest and 206 (13%) were 2+. 94% of the 3+ cancers were NST and 95% were grade 2 or 3 (29% grade 2; 66% grade 3). Only 1 of the 156 infiltrating lobular carcinomas scored 3+ (a pleomorphic variant), and 16 scored 2+. No case of tubular or cribriform carcinoma scored 3+, 2 tubular carcinomas (4%) scored 2+. 2 cases of mucinous carcinoma (6%) scored 2 or 3+. 127 of 206 cancers scored as 2+ were retested by FISH, 28% showed HER2 gene amplification. 48 of 185 cancers scored as 3+ were retested by FISH and 96% showed amplification of the HER2 gene. There was moderate agreement between pathologists scoring HercepTest slides (Kappa 0.44).

Conclusions: The typical HER2 phenotype is a histological grade 2 or 3 infiltrating carcinoma NST.The frequency of HER2 overexpression is significantly rarer in infiltrating lobular, tubular or cribriform carcinomas. A HercepTest score of 2+ is an equivocal result requiring confirmation by FISH; in this study 28% showed gene amplification. Pathologists show moderate interobserver agreement in applying the HercepTest scoring system.

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Distinct responses to modified HER-2 vaccines in two different HER-2 transgenic mouse models lessons for the clinic

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The HER-2 growth factor receptor is overexpressed in many human tumors and is an attractive target for immunotherapy. Since HER-2 is a self protein, tolerance may limit the effectiveness of vaccination. To overcome this, we constructed DNA and protein vaccines (AutoVac®) which utilize potent helper T cell epitopes derived from tetanus toxin. DNA vaccines encoding modified rat HER-2, or purified protein vaccines containing the helper epitopes were tested in two transgenic mouse models of rat HER-2 tolerance. DNA vaccination induced HER-2 specific antibody and CTL responses and significant protection to a transplantable HER-2 positive tumor line in one model, but had little effect in the other. In contrast, protein vaccination induced antibody mediated tumor protection in both transgenic models. Thus, AutoVac® HER-2 vaccines can induce effective immune responses against tumors with potentially different susceptibilities to immune effector mechanisms. The susceptibility of tumors to immune effector mechanisms is likely to differ in the human patient population as well, therefore, we are pursuing both DNA and protein vaccinations as complementary strategies in a clinical setting. A human HER-2 DNA vaccine has been successfully evaluated in a primate toxicity study and is currently being evaluated in a Phase I clinical trial, to be followed shortly by toxicity and clinical trials with a protein vaccine.

54 ORAL

Concordant LOH at BRCA gene loci in paired tumour samples: a predictive tool for the identification of germline mutations

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Loss of heterozygosity (LOH) can arise randomly in sporadic breast cancer due to genetic instability. However, concordant LOH in tumours from related individuals may point to an underlying germline mutation in a tumour suppressor gene. To evaluate this, we tested tumour pairs of at-risk families for LOH at BRCA gene markers, and compared the findings with mutational analysis results.

67 tumour pairs and 52 sporadic breast cancer controls were studied, employing 5 microsatellite markers flanking and/or intragenic to each of the BRCA1 and BRCA2 genes, plus one control marker. Laser capture microdissection technology enabled the procurement of pure cancerous and normal cellular sub-populations. A validated PCR-based LOH assay analysed fluorescent-labelled products in an automated sequencer. Mutational analysis was carried out with techniques yielding a 50-70% mutation detection rate.

In familial cancers, the LOH/Informative ratio at BRCA markers was higher than in sporadic controls (54.7% vs. 38.3%, p=0.001), and higher than at the control marker (54.7% vs. 29.3%, p=0.004). 7/17 informative

tumour pairs showed concordant homoallelic LOH for 2 or more markers at BRCA1 (O/E=57.7, p<0.001) and, similarly, 2/7 did so for markers at BRCA2 (O/E=62.2, p<0.001). 8 of the 9 pairs showing LOH concordance underwent mutational analysis and 50% of these carried a mutation (3/6 BRCA1, 1/2 BRCA2). No mutations were found in 9 tested sporadic cancer controls.

In genetically related tumour pairs, concordant homoallelic LOH at two or more markers can unmask a mutated breast cancer predisposition gene. This easy and affordable procedure may be useful as a screening tool in at-risk families, and may also find an application in the search of other putative breast cancer predisposition genes.

55 ORAL

YB-1 expression (mRNA and protein) in sporadic breast cancer

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The Y-box binding protein, YB-1, is a highly conserved multi-function protein shown to have activity as a transcription factor. Clinical studies suggest that YB-1 has significance in cancer. We examined the expression (mRNA and protein) of YB-1 in one hundred cases of breast cancer.

Methods: One hundred breast cancer samples and surrounding matched normal breast tissue were removed at the time of surgery from women undergoing surgery for primary breast cancer and the samples were frozen at *70oC. Messenger RNA was isolated from the samples and the expression of YB-1 and 6 other genes (BRCA1, BRCA2, ER, PR, Relaxin and Clusterin) was assessed with quantitative real-time RT-PCR. Protein expression of these genes was assessed by preparing a tissue array and staining with both C- and N- terminal specific monoclonal antibodies for the respective proteins. Correlation of mRNA and protein expression was made with clinical-pathologic parameters (age, menopausal status, T-stage, N-stage, presence or absence of lymphatic and vascular invasion, tumour grade, and histologic ER staining) using univariate and multivariate analyses.

Results: The ratio of YB-1 mRNA expression in tumour compared to matched benign breast tissue was significantly correlated with ER status and tumour grade. In fact, there was a stepwise increase of YB-1 mRNA expression in tumour:normal ratio from ER strong positive, ER moderate positive, ER weak positive to ER negative ($p < 0.01$). ER negative tumours had a three-fold higher expression of YB-1 when normalised to their matched normal sample compared with strongly ER positive tumours. A similar pattern was seen in progressing from Grade I to Grade III tumours ($p < 0.01$). Correlation of YB-1 expression with the other 6 genes will be presented at the meeting.

YB-1 protein expression was increased in the tumours. Ninety percent of the tumours had moderately or strongly intense YB-1 staining, whereas 80% of the benign breast samples had absent or mildly intense YB-1 staining.

Conclusions: We have shown that mRNA expression of YB-1 a multifunctional transcription factor is associated with ER negative and high grade breast cancers, and that YB-1 protein expression is enhanced in breast cancers compared with benign breast tissue. In future studies we propose to search for YB-1 associated genes using high-density cDNA arrays and we plan to assess the effect of YB-1 antisense oligonucleotides on breast tumour growth.

56 ORAL

Immunohistochemical profile of high risk subsets of hyperplasia of usual type

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Hyperplasia of usual type (HUT) is associated with an increased risk for subsequent breast cancer development. However, this level of risk is not considered sufficiently high to justify chemoprevention. Further progress is likely to depend on delineating biological markers of risk. We designed a case-control study on 502 patients including 120 cases who had benign breast biopsies that progressed to breast cancer and 382 matched controls that did not develop breast cancer during a 20-year follow-up period. Foci of HUT (n= 163) and adjacent morphologically normal lobules (n=93) were identified in cases (n=21) and controls (n=29) then stained with antibodies for ERalpha, ER beta, Ki67 and the oestrogen related protein heat shock protein 27 (hsp27). The percentage of positively stained cells was assessed using image analysis. Oestrogen receptor proliferating cells were assessed using dual-label immunofluorescence. Following a biopsy containing HUT, the relative risk was 1.53 (CI= 1.1-2.13). A significantly higher median expression of ER alpha+, Ki67+ and hsp27+ cells was found in HUT foci from cases that progressed to breast cancer when compared with controls (P= 0.008, 0.001 and 0.018). However, there was no significant difference in the expression of either ER beta or dual-labelled cells. The median expression of all studied antibodies in morphologically normal lobules was not significantly different between cases and controls. In the cases, HUT contained higher percentages of median ER alpha and hsp27 expression (P=0.02 and <0.001) and lower median ER beta expression P= 0.01) than normal lobules. Our data highlight unequivocal differences in the expression of biological markers in high-risk subsets of HUT, which could be suitable candidates for prophylactic anti-oestrogens. This approach might have important implications in the assessment of breast cancer risk and in refining screening strategies.

57 POSTER

Lymphangiogenesis and its relationship to lymph node metastasis in breast cancer

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Introduction: Breast cancer spreads primarily via the lymphatic system, yet research into lymphangiogenesis has been restricted mainly by the lack of specific markers for lymphatics. Using two novel specific lymphatic markers that have never been previously studied in human cancer, and a more traditional marker, VEGFR-3, we quantified lymphangiogenesis using quantitative polymerase chain reaction (QPCR) methods and examined the relationship between lymphangiogenesis and axillary lymph node status.

Methods: RNA was extracted and equal concentrations of cDNA synthesised from 120 archival breast cancers and 33 background breast samples. Plasmids containing sequences from the following markers were synthesised, cloned and used as internal standards: LYVE-1, podoplanin and VEGFR-3. LYVE-1 is highly lymphatic specific. Podoplanin and VEGFR-3 are mainly found on lymphatic endothelium, but are also found in much lower levels on blood vessels. Real-time QPCR was performed on the breast cDNA and plasmids, using primers specific for the each of the three markers. The original concentration of each marker, and hence the level of lymphangiogenesis, was deduced by reference to the known concentrations of the plasmid standards. Results between the nodal groups were compared using the students t-test.

Results: Mean levels of LYVE-1 were significantly higher in the lymph node positive tumours than node negative, which were significantly higher than in the background tissue: 128.0 vs 12.1 vs 0.3 copies/μl, P<0.05. The differences using podoplanin were similar but not significant (23306 vs 7764 vs 4973 copies/μl,). Values for VEGFR-3 were: 129.4 vs 117.0 vs 118.4.

Conclusions: This is the first time lymphangiogenesis has been quantified in cancer. It is also the first time these markers have been measured in human cancer. Using the most specific lymphatic marker, LYVE-1, a significant correlation between the level of lymphangiogenesis and the presence of axillary lymph node metastasis was demonstrated.

58 POSTER

Dephospholylation of PKCalpha and MAPK/ERK by retinoic acid(ATRA) in breast cancer cell lines SKBR-3

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ATRA significantly inhibits the tumor growth in experimental systems and recently completed randomized clinical trial started in breast cancer patients. However, the mechanisms of cell growth suppression in breast cancer cell by ATRA is unclear. The aim of this study was to explore the role of PKCalpha and MAPK/ERK in breast cancer cell lines SKBR-3 after treated with ATRA. Treatment of SKBR-3 for 1h with 1microM ATRA, expression of phopholylated PKCalpha protein decreased significantly. PKC inhibitor GF109203X and specific PKCalpha inhibitor Go6976 inhibited the SKBR-

3 cell growth dose-dependently. ATRA could not inhibit the cell growth in SKBR-3 that overexpressed the PKCalpha by infection of replication-deficient adenovirus for PKCalpha. Treatment of SKBR-3 for 3h with 1microM ATRA, expression of phopholylated MAPK/ERK protein decreased significantly. Specific MAPK/ERK inhibitor PD98059 inhibited the SKBR-3 cell growth dose-dependently. Our results suggested that ATRA dephospholylated the PKCalpha and dephospholylated PKCalpha also inhibited the phospholylation of MAPK/ERK. The presently demonstrated specific mechanisms of inhibition of PKCalpha and MAPK by ATRA may help us to understand the effects of chemopreventive and chemotherapeutic activity of ATRA in breast cancer.

59 POSTER

IGF-1 and IGF-2 serum concentrations in patients with benign and malignant breast cancer: free IGF-2 is correlated with breast tumor size

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Insulin-like growth factors are potent mitogens for breast cancer cells in vitro and have been implicated in the promotion of breast cancer growth in vivo. We have analyzed serum levels of total IGF-1 and -2, and of their biologically active fraction free IGF-1 and -2 in 65 patients with benign and malignant breast lesions, and compared them to 38 women without breast disease. Total IGF-1 serum concentrations were significantly lower in healthy women than in breast cancer patients ($p<0.001$) or patients with benign breast lesions ($p=0.010$), but no differences were observed in serum levels of the corresponding free and presumably biologically active IGF-1. Conversely, healthy women had higher serum levels of free IGF-2 than women with breast neoplasms ($p=0.003$), and the ratio free IGF-2 / total IGF-2 was significantly higher in healthy women than in patients with benign or malignant breast tumors ($p=0.001$). Although IGF-1 or -2 serum concentrations of malignant breast tumors were not different from those of benign lesions, the size of a breast tumor was significantly correlated to the ratio free IGF-2 / total IGF-2 ($r= 0.469$, p-value 0.002). Breast cancer patients with a free IGF-2 / total IGF-2 ratio value at the 75 percentile had a 3.2 times higher chance of harboring a tumor larger than 20 mm than patients with a value corresponding to the 25 percentile. Furthermore, an increase of free IGF-2 from percentile 25 to percentile 75 would raise the tumor size by 5.4 mm. Taken together, our data strongly suggest that in contrast to IGF-1, IGF-2 is actively involved in the regulation of tumor growth. Pharmacological modulation of free IGF-2 concentrations in breast cancer patients might eventually turn out to be an interesting therapeutical approach in controlling the size of malignant breast tumors.

60 POSTER

Large scale BRCA1/2-mutation analysis to evaluate clinical outcome of breast cancer in gene-carriers

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The aim of this project is to assess the outcome of breast cancer (overall, breast cancer specific and disease free survival)) in BRCA1 and BRCA2 germline mutation carriers compared to non-carriers in a large and unselected sample of breast cancer patients. Other endpoints of interest are the prevalence of germline mutations (age- and tumor stage- specific) and the incidence of contralateral breast cancer and ovarian cancer. For an unselected cohort of 6500 women, diagnosed with breast cancer before age 50 between 1970 and 1994 in several Dutch hospitals, clinical data and paraffin blocks are being collected.

We have developed a method that enables us to evaluate 80% of all possible germline mutations present in Dutch hereditary breast cancer families. Both protein truncating- (frame shift mutations) as well as amino acid changes (missense mutations) can de detected using the DSDI-method (Detection of Small Insertions and Deletions) and the AD-method (Allelic Discrimination). For the first method several multiplex PCR's, each encompassing 2-4 different mutations, are combined in 1 lane per patient on the ABI 3700 and thus 40 mutations are evaluated by genescan concomittantly. For the second method, base substitutions are detected using 2 different fluorescent color labeled probes binding either to the wildtype or mutated allele, detected in a Taqman screen. Each deviant result will be confirmed with sequencing. With the use of robotics, large numbers of samples can be processed.

We expect to reach a complete analysis in at least 70% of the subjects (loss of paraffin blocks, degenerated DNA). The expected prevalence of BRCA1/2 carriers in this group is about 6%. We have estimated to detect a total of 230 carriers, reaching a 90% power to detect a survival difference of 12%. The evaluation of the first 500 samples reveals that BRCA1/BRCA2 mutation analysis on DNA extracted from paraffin blocks is feasible and that offers new prospects for large scale genetic studies on archival material.

61 POSTER

Tumour histological grade may progress between primary and recurrent invasive mammary carcinoma

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The worsening of histological grade of breast carcinomas during progression of the disease is debated. The constancy of the histological grade of invasive breast carcinomas was tested by comparing primary tumours with their axillary metastases and local or regional recurrences.

84 recurrent invasive breast carcinomas with a primary tumour or a previous recurrence were available for histological review from the period between 1980 and 2000. These baseline tumours and any further recurrences were graded by one observer.

There were 9, 24 and 51 tumours with grades 1, 2 and 3, respectively, that recurred. Grade 1, 2 and 3 tumours recurred within a median time of 88, 42 and 23 months, respectively. The intraobserver reproducibility of the histological grade was good (kappa=0.66), and the grades of the primary tumours and their axillary metastases or next recurrence also exhibited good agreement; however, when further (second to sixth) recurrences were included in the analysis, it was found that the agreement between the grade of the tumours and their last recurrence was only moderate (kappa=0.48). Only 2 (22%) of the grade 1 and 15 (63%) of the grade 2 tumours retained their grade in their last recurrence.

Low-grade carcinomas require a longer follow-up. These long-term data support the possibility of a transition from low-grade invasive breast carcinomas to higher-grade tumours. It is suggested that low-grade (well-differentiated) breast carcinomas are not a single entity: some do not, whereas others may progress to higher-grade tumours.

62 POSTER

Expression of estrogen receptors alpha and beta, progesterone receptor, co-activators and co-repressors in breast cancer

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Aims: The expression of four steroid receptor coactivators [steroid receptor coactivator 1 (SRC 1), transcriptional intermediary factor 2 (TIF 2), p300/CREB binding protein (p300/CBP), amplified in breast cancer 1 (AIB1)] and one corepressor [nuclear receptor corepressor (NCoR)], along with the ERalpha and ERβstatus was examined in normal and malignant breast tissues to provide insight into the formation of estrogen-mediated transcription complexes in human breast cancer.

Methods: Twenty-five samples, obtained from normal mammary glands and invasive ductal carcinomas of the same patients were subjected to immunohistochemistry (semiquantitative scoring) with antihuman SRC 1, TIF 2, p300/CREB, p300CBP, NCoR, ERalpha and ERβ antibodies. MCF-7 and T47D cell lines were used as a positive controls.

Results: The expression levels not only of ERalpha PgR ($r=0.61$, $p=0.001$) and NCoR ($r=0.4$, $p=0.043$) but also of ERβ and SRC 1 ($r=0.68$, $p<0.001$), TIF 2 ($r=0.64$, $p<0.001$) and NCoR ($r=0.48$, $p=0.014$) showed a significant correlation in malignant tissue samples. ERalpha was detected in 72% and ERβ was detected in 78% of malignant breast tumors, and in 20% of the ERβ positive cases no ERalpha was detected. Expression levels of ERβ (76% vs. 48%), p300/CBP (28% vs. 8%) and AIB1 (28% vs. 0%) were higher in invasive ductal carcinomas than in normal mammary tissue samples, whereas the expression levels of SCR 1, TIF 2 and NCoR did not differ significantly between malignant tumors and normal breast tissue. Furthermore, AIB1 and p300/CBP were found to be co-expressed in malignant

tissue (r=0.54, p=0.006), which indicates some kind of co-operation. Furthermore, significant correlation was also observed between node-positive tumors and the expression of TIF 2 (r=0.49, p=0.025).

Conclusion: Estrogen action, as the major stimulus for growth of hormone-dependent breast cancer, is clearly regulated by a differential expression pattern of ER and co-activators in malignant breast tissue. By confining clinical ER positivity to ERa protein expression, a substantial part of ERβ expresssing tumors is neglected, thus potentially withholding antihormonal treatment from these patients. Furthermore, we have identified two coactivators that are either expressed in malignant cells exclusively (AIB1), or in preferentially in tumors with a more malignant phenotype (TIF 2). These proteins could potentially be used as novel tumor markers or prognostic factors in vivo.

63 POSTER

Analysis of dendritic cells in the peripheral blood of patients with breast cancer

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Dendritic cells (DCs) are professional antigen-presenting cells that play a central role in antitumor immunity, related to their ability to process tumor antigens and present them on HLA class II and class I molecules, to their unique ability to stimulate naive T lymphocytes initiating tumor-specific cytotoxic immune responses, and to the production of immunoregulatory cytokines. Aim of the present study was to enumerate and functionally characterize DCs in the peripheral blood of patients with breast cancer. To avoid isolation or culture procedures for the enrichment of DCs that may induce the selection of particular cell subsets, our analyses were directly performed on whole blood samples by flow cytometry. We studied 48 breast cancer patients in different stages of disease before any surgical, chemotherapic and radiant treatment. Thirty-two healthy age- and sex-matched controls were also studied. Circulating myeloid DCs, identified as lin-/HLA-DR+/CD11c+ cells, were significantly fewer in breast cancer patients than in control subjects (10.2 $\pm$ 0.8 milions/L vs 17.8 $\pm$ 1.3,p<0.001). Significant decreases compared with controls were found only in patients with invasive cancer, without differences observed between patients assigned to stage I, IIA and IIB. DCs from cancer patients were found to be more mature, as the percentage of DCs expressing the maturation marker CD83 resulted significantly higher in patients than in controls (20.3 $\pm$ 5.9% vs 1.2 $\pm$ 0.4,p<0.02). DC production of the regulatory cytokines IL-12 and IL-10 was assessed in blood samples incubated with LPS, and intracellular accumulation of cytokines in lin-/HLA-DR+ cells was examined by flow cytometry. The percentage of IL-12 producing DCs was significantly lower in breast cancer patients than in healthy controls (25.3 $\pm$ 1.6% vs 35.7 $\pm$ 2.2, p < 0.001), with significant decreases compared with controls observed only in patients affected by invasive cancer. To evaluate whether the defects of circulating DCs observed in breast cancer patients were able to affect the pattern of cytokine production by T lymphocytes, the secretion of type 1 and 2 cytokines by CD3+ cells was evaluated, and significantly lower IFN-g to IL-4 ratio was observed in patients compared to control subjects. Taken together, our results seem to indicate that in breast cancer patients peripheral blood DCs present quantitative and functional alterations that may partly contribute to a defective function of cell-mediated immunity.

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Immunohistochemical investigation of ER and PR expression in primary breast cancer and their axillary lymph node metastases

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Objectives: Axillary and distant metastases from invasive breast cancer may represent a clonal outgrowth with a different hormone receptor status than the primary tumor and this may explain why metastatic ER positive breast cancer may not respond to anti-oestrogens. We examined the ER and PR expression in the primary breast cancer and its metastatic axillary lymph nodes.

Methods: We randomly selected 21 patients with node positive invasive ductal adenocarcinoma of the breast for which tissue blocks were available from both the primary cancer and the axillary lymph node metastases. Single samples (n = 84) of formalin-fixed tumour tissue from the primary breast cancer and the involved lymph node were IHC examined using the monoclonal antibodies NCL-ER-6S11 for ER and NCL-PR-1Q6 for PR. The IHC-method is a semiquantitative subjective way of expressing the ER and PR. We opted for the IRS scoring system [0–12]. Three independent readers (IVH, CM, LDB) gave a staining score from 0–12 for each tissue sample with a calculated mean being the sum of each divided by three.

Results: Seven primary tumours were ER negative (0/12). In 5 women, the involved nodes were also ER negative (0/12) but in two, ER expression in the axillary lymph node was discordant: ER was present in one with a score of 2/12 and in the other with a score of 6/12. Fourteen patients had ER positive breast cancer (2–12/12). All had ER expression in tumour tissue from the axillary node. Staining intensity was identical in 4, less in 5 but more intense in 5 others. Eleven primary tumours were PR negative (0/12). Three women had PR-expression in the involved axillary node with low expression intensities of 3/12, 3/12 and 2/12.

Ten women had PR positive breast cancers (2–9/12). One such patient didn't express PR in the metastatic lymph node, 6 stained positive for PR with an identical intensity and 3 had a lesser intense staining in the metastatic axillary node.

Conclusion: In most women with invasive breast cancer, hormone receptor expression in the primary tumor is concordant with hormone expression in the metastatic axillary lymph node. Staining intensity may be different (more or less) between the primary lesion and tumour tissue in the axillary node. Two and 3 of the 21 women expressed respectively the ER or PR in the axillary lymph node whereas the primary tumor was hormone receptor negative. Only one patient had hormone receptor negative tumour tissue in a lymph node while the primary tumour was positive; this was only for the PR and not for the ER.

There is no need for systematic measurement of hormone receptor expression of tumour tissue in metastatic axillary lymph nodes from breast cancer.

65 POSTER

Stem cell characteristics of transplanted rat mammary clonogens

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Rat mammary glands contain a subpopulation of clonogenic epithelial cells with large proliferation and differentiation potentials. Therefore, rat mammary epithelial clonogens (RMEC) were isolated and characterized in vitro and in vivo. By flow cytometry of RMEC stained with fluorescein isothiocyanate-peanut agglutinin (PNA-FITC) and phycoerythrin-anti-Thy-1.1 monoclonal antibody (Thy-1.1-PE), we could distinguish four cell subpopulations from 7-8 week old F344 female rat mammary glands: cells negative to both PNA-FITC and Thy-1.1-PE (B-), PNA-positive cells (PNA+), Thy-1.1-PE-positive cells (Thy-1.1+), and cells positive to both reagents (B+). When single PNA+ cells were isolated and cultured in Matrigel with irradiated (50 Gray) 3T3 fibroblast feeder cell layers, they gave rise to multicellular clonal structures of three types: alveolar, foamy alveolar, and squamous colonies. We grafted sorted single PNA+ cells into the fat pad of hyperprolactinemic recipient rats, about 2.93% of the injection sites showed the growth of alveolar units. When multicellular colones developed from sorted PNA+ cells in culture were transplanted into the fat pad of hyperprolactinemic, glucocorticoid-deficient recipient rats, about 8.33% of the sites showed ductal units. We conclude that the PNA+ cell subpopulation includes most of the stem-like clonogenic cells.

66 POSTER

Analysis of estrogen receptor polymorphism in codon 325 by PCR-SSCP in breast cancer: association with lymph node metastasis

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Breast cancer is the most frequent neoplasia in women. Expression of the estrogen receptor (ER), has a key role in breast cancer; the ER gene is

located at chromosome 6q24-q27 and is made up of 8 exons with a total of 140 kilobases. The Polymorphism in codon 325 of the exon 4 (ER325) is a transition CCC-CCG. The objective of this study is to analyse the frequency of this polymorphism in breast cancer using the Polymerase Chain Reaction - Single Strand Conformation Polymorphism technology.

DNA was extracted from tumour cells of 70 breast cancer patients and from peripheral blood of 69 individuals without any known pathology (control group). Amplification products of the ER gene were analysed by Single Strand Conformation Polymorphism.

In breast cancer patients the ER325 polymorphism was detected in 42.8% of the cases. In contrast, in the control group, the frequency obtained of the same polymorphism was 24.6. Statistical comparison of the frequency distributions revealed that they are significantly different (p=0.023). There was also an association between ER325 polymorphism and absence of lymph node metastases (p=0.038).

Our data suggests that there is a relationship between the ER325 polymorphism and susceptibility to breast cancer (OR= 2.3; 1.10<OR<5.1) and that it can also be related with the metastisation process.

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The group of ER-negative, PR-positive early breast cancer patients represents the unfavorable, but endocrine sensitive subgroup of receptor-positive patients

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Introduction: The Consensus Conference in St. Gallen stated that ER+ and/or PR+ early breast cancers should be considered as "receptor-positive". Consequently, they should be treated with endocrine adjuvant treatments. However, the possible differencies between these phenotypes (ER+/PR+, ER-/PR+, ER+/PR-) has been given less attention. An unusual status of ER-/PR+ phenotype of early breast cancer has been analyzed in this paper.

Patients and Methods: Previously, we have found that the group of ER-negative, PR-positive early breast cancer patients, within node-negative setting, untreated with any adjuvant therapy, represents the unfavorable subgroup of receptor-positive patients, according to the DFS. Thereafter, the analysis of 224 node-positive low-risk subgroup (N1-3, grade 1-2, T1-2) of receptor-positive patients, being treated with endocrine adjuvant therapies alone, was analyzed. All patients were radically operated. Premenopausal patients were treated with ovarian irradiation, and peri-/postmenopausal with tamoxifen alone. Steroid receptors were determined by biochemical DCC method.

Results: Within the receptor-positive group 32 (14%) ER-/PR+ early breast cancer patients were found. The DFS was better in ER-/PR+ subgroup, compared to ER+/PR+. When separated according to menopausal status, the DFS of postmenopausal ER-/PR+ patients was comparable to the ER+/PR+ group. On the contrary, premenopausal ER-/PR+ patients had significantly better DFS, in comparison to the ER+/PR+ ones.

Conclusion: It has been shown that tamoxifen improves the DFS in the unfavorable subgroup of receptor-positive early breast cancer patients. The effect of ovarian irradiation alone, which turns the most unfavorable group to the most beneficial, is unexpected, and not easy to explain. At least, according to these results, a further sub-analysis of the clinical significance of different steroid receptor phenotypes is warranted.

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Experimental studies on the effects of the combined use of N-(4-hydroxyphenyl)retinamide (4-HPR) and Tamoxifen (TAM) for Estrogen Receptor (ER)-negative breast cancer cell line MDA-MB-231

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We investigated the effects of combination therapy with retinoid (4-HPR) and TAM on ER-negative breast cancer cell line MDA-MB-231. TAM or 4-HPR alone had no effect, but the combined use of TAM and 4-HPR had a cell growth inhibitory effect. Cell cycle analysis showed an increased of the G2/M phases in the 4-HPR-TAM combination group. Measurement of 3H-TAM incorporation in the cell showed that, compared with the TAM group, the 4-HPR-TAM combination group incorporated about 1.45 times more TAM into the cell. Thin-layer chromatographic analysis of changes in the cell membrane ganglioside GM3 showed a marked increase in the 4-HPR-TAM combination group. We speculate that the administration of TAM in the presence of 4-HPR changes the membrane glycolipid GM3, increasing intracellular TAM concentrations, thus exerting antitumor activity. Thus, the combined use of TAM and 4-HPR inhibited the growth of the ER-negative breast cancer cell line MDA-MB-231. These results suggest that combination therapy with TAM and 4-HPR can be a potent supplementary therapy also for ER-negative patients in clinical practice.

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Antigens of peptidic and carbohydrate nature expressed by breast cancer tissues and benign counterparts

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MUC1 mucin expresses different epitopes at the protein core as well as their carbohydrate associated chains. The purposes of this research were 1- to study the expression of MUC1 peptidic and carbohydrate epitopes in normal, benign and malignant breast epithelia and 2- to correlate this expression with breast cancer disease stage. An immunohistochemical approach was performed in 81 tissues obtained from normal, benign and malignant sections; studies were performed following standard procedures and the immune reaction was graded according to positive staining, intensity and distribution. Three anti-MUC1 peptide core monoclonal antibodies (MAbs), C595, HMFG1 and SM3 were used and 4 anti carbohydrate antigens MAbs: sLewis x (KM93), Lewis x (KM380), Lewis y (C14) and Tn (Dakopatts, Denmark). Statistical analysis was performed employing a Principal Component Analysis with Kendall correlations. It was observed that malignant tissues expressed peptidic and carbohydrate MUC1 epitopes with high intensity and elevated percentages of positivity; MUC1 protein core was reactive in 89% in stage I, 87% in stage II, 75% in stage III and 83% in stage IV. SM3 MAb showed its highest staining (83%) in tumors belonging to stage IV patients. Lewis x and sLewis x were found coexpressed with a correlation value of 0.5487; Lewis y diminished its expression from 67% in stage I tumors to 50% in stage IV while Tn showed the lowest expression respect to the other antigens in any of the stages. Malignant cells reacted at membranes, cytoplasm and lumen debris. Benign samples exhibit a restricted reaction mainly at apical cell surface; a similar pattern was found in some normal breast sections. Statistical analysis showed a separation between normal and dysplasia versus cancer samples ($p < 0.05$).

Conclusion: Carbohydrate antigens are expressed in breast tissue under different patterns, intensity and extension being predominant in cancer cells. These results may encourage the use of these carbohydrate antigens for the preparation of vaccines for therapy in breast cancer.

70 POSTER

Cell cycle regulation by retinoic acid (ATRA) in breast cancer cells SKBR-3

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All-trans retinoic acid(ATRA) is a one of the synthetic retinoic acid and it was reported that ATRA inhibited the breast cancer cell growth. However, the mechanism of the cell growth inhibition by ATRA is still unclear. In this study we analysed the growth suppressive effect of ATRA on human breast cancer cell line SKBR-3. Compared to untreated cells, treated with ATRA (10nM-1mM) showed significantly dose-dependent growth inhibition. The effect of ATRA on cell cycle progression, ATRA treated cells showed a increased of G1 phase. Treatment of SKBR-3 for 48h with 1mM ATRA, expression of cyclin A, cyclin D1, cyclin E protein decreased and marked dephosphorylation of pRb involving Ser780 and Ser 807/811. Our data suggested that the antiproliferative activity of ATRA growth-supressive atatus in G1 phase, possibly through cyclin downregulation and inhibition of pRb phosphorylation. These results emphasize the potential role of the cell cycle regulation in the chemopreventive and chemotherapeutic activity of ATRA in breast cancer cells.

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NOEY2 (ARHI) mRNA expression in human breast cancer tissue

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Introduction: NOEY2 (ARHI) is a putative maternally imprinted tumor suppressor gene, which encodes a new member of the Ras superfamily of small G protein. NOEY2 is expressed consistently in normal ovarian and breast epitherial cells but is down-regulated frequently in ovarian and breast cancers. In this study, we have examined NOEY2 mRNA expression in human breast cancer tissue and compared with clinicopathological facters.

Material and Method: Breast cancer tissue and non cancerous tissue in surgical specimens were evaluated from 110 patients with breast cancer. Relative mRNA expression of NOEY2 in each samples was assessed by real-time quantitive RT-PCR analysis.

Result: Reduction of copy number of NOEY2 mRNA in cancer tissue was found in 61/ 110 (55%) patients. NOEY2 expression was more reduced in high nucleal grade tumors and lymph node positive patients, but this difference was not significant. There was no correlation between NOEY2 mRNA expression and other clinicopathological factors (tumor size, age, hormon receptor status). Conclusion: NOEY2 is down-regulated frequently in human breast cancer tissue and independent of standard clinic

72 POSTER

Adoptive immunotherapy of malignant effusions

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The data obtained from 21 breast cancer patiens with chemoresistant malignant pleural effusions were submitted to a pilot study with intrapleural (ip) recombinant interleukin-2 (IL-2) and lymphokine activated killer (LAK) cells administration. All patients were subjected to two or four courses of IL-2 and LAK. The patients were given 0.5–1.0 mln IL-2 (Proleukin) per day ip for 5 days course and twice 100–300 mln LAK ip every week. The LAK generation by IL-2 have been obtained from malignant effusion mononuclear cells with presence of IL-2. In this study, patients response (CR+PR) was 91%. IL-2/LAK-therapy were well tolerated and no interruption occurred. After ip immunotherapy main supopulations of blood lymphocytes was increased< aspeciolly actyvated T-cells (CD3+, CD25+) and NK-cells (CD16+). The tumor cells were disappeard from malignant effusions usually after 1–2 courses of IL-2/LAK-therapy. These data suggested the opportunity to initiated large prospective randomized trail using IL-2/LAK-therapy in patients with malignant effusion.

73 POSTER

Immune disorders induced by anaesthesia and surgery in ovarian hormone suppression for breast cancer. Comparison between laparatomy and laparoscopy

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Surgery and general anesthesia determine modifications of the immune system. As laparoscopy is still considered an issue for evaluation in the case of patients with cancer, the purpose of this research was to compare post-surgical immunological alterations determined by anesthesia and surgery for ooforectomy in open vs. laparoscopic surgery. It is a prospective, randomized study performed on patients with breast cancer and indication for surgical hormone suppression: 60 patients have been submitted to open (classical) surgery; 58 patients have had laparoscopic surgery. The two groups were similar from the point of view of: -age, -stage of the disease, -associated morbidity, -breast cancer treatment, -type of general anesthesia and surgical time length. Evaluation was performed before and 24 hrs, respectively 72 hrs after surgery. The immunological parameters studied were: -Lymphocyte blastic transformation test (LBTT); -Determination of immunoglobulins, Interleukin-6 and Interleukin-2, and C reactive protein; absolute number of lymphocytes; serum protein electrophoresis has been performed

Results: Before surgery, it was found in both groups a decrease in the number of lymphocytes, decrease of the LBT index to Phytohemaglutinin and increase of that index to Concanavalin A; IL-2 production in lymphocyte cultures of 32pg/0,1ml, and a serum level of 4.1 pg/ml IL6. In the group submitted to classical surgery, an increase in the leukocyte and a decrease in the lymphocyte count were found after surgery. In the group with laparoscopic surgery, there was an increase in the number of leukocytes, while the lymphocyte proportion remained constant, i.e. at the pre-surgery level; LBT index decreased from 7.3 to 6.1 in the classical interventions and from 7.3 to 6.9 in the laparoscopic ones. In the patients with classical surgery, IL-2 concentration in lymphocyte cultures was undetectable, while in the group with laparoscopic surgery IL-2 levels were 30pg/0,1ml. IL-6 in the classical group rose to 7.1 pg/ml, while in the laparoscopic group it rose to 5.9 pg/ml. This is correlated with the increase of the C reactive protein from 6 mg/l to 18 mg/l - in the classical group, and to 10 mg/l - in the laparoscopic one.

The analysis of the results leads to the conclusion that laparoscopic surgery induces fewer immunological disorders than open surgery, an element of importance in the case of a disease with immunological implications in its pathogenesis and evolution.

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Crude catechin regulated the cdc2 phospholylation in breast cancer cells MDA-MB-231

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Crude catechin (catechin) included 60% of epigallocathechin-3-gallate (EGCG). It was reported that EGCG inhibited the breast cancer cell growth and prevented the breast cancer. However, the mechanisms of the cell growth inhibition or prevention of the cancer by catechin is still unclear. In this study we analysed the growth suppressive effect of catechin on human breast cancer cell line MDA-MB-231. Compared to untreated cells, treated with catechin showed significantly dose-dependent growth inhibition. The effect of catechin on cell cycle progression, catechin treated cells showed a 8.6% increased of G2/M phase. Treatment of MDA-MB-231 for 48h with 100mocroM catechin, expression of phospholylated cdc2 protein decreased significantly. Our data suggested that the antiproliferative activity of cathechin growth-suppressive status in G2/M phase, possibly through regulation of cdc2 phospholylation. The presently demonstrated specific mechanisms of cell cycle is potential epigenetic molecular targets for breast cancer treatment or prevention by catechin.

Wednesday, 20 March 2002 16:30–18:00

PROFFERED PAPERS

Imaging the sentinel node

75 ORAL

Lymph node metastases detection by FDG-PET and sentinel node biopsy in breast cancer patients: comparison of these different approaches

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Background: Axillary dissection (ALND) for detection of metastatic involvement is used to plan adjuvant treatments for breast cancer (BC) patients. ALND is a costly procedure with various side effects.80% or more of T1 patients are node negative and ALND is useless. Recently, sentinel node (SN) biopsy has been suggested as method of reference for the evaluation of regional nodal metastases and for the decision on the need of a ALND. SN biopsy is an invasive approach, with a not negligible risk of false negative results. Conversely, Positron Emission Tomography (FDG-PET) is a non-invasive repeatable method able to evaluate all the regional nodes in BC: our PET experience on nodal involvement in BC has given interesting data of sensitivity and negative predictive value, comparable with SN