70 years or more). It was lower in larger tumors (88.8%, 68.0% and 74.1% for tumors 1 cm or less, 1 to 2 cm and over 2 cm) and among estrogen receptor (ER) negative ones (61.5% versus 80.8% for ER-positive tumors). It also decreased with years in practice of the attending physician (83.5% and 74.6% for those having less versus 10 or more years of experience). Participation of a hospital in multicenter clinical trials had little or no impact on the proportion of patients treated according to the consensus statement. Systemic adjuvant therapy of node-negative breast cancer remains underutilized, especially among high-risk women. Better understanding of the clinical decision process and alternative strategies for the dissemination of practice guidelines are needed.

P6 The breast-cancer susceptibility genes association to epidermal growth factor receptor (EGFR) & to oncogenes

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The breast-Cancer Susceptibility genes BRCA1 & BRCA2 are biochemicals with biological functions that are relevant to tumorigenesis with many functional domains. The present studies examined the relation between the susceptibility genes and the Oncogenes. Breast cancer cells (BCC) were obtained from fresh biopsies of tumors from patients with benign (10), primary (25) and metastatic (18) and from breast cancer established cell lines MCF-&, T4TD & MDR-MB-231. Normal Breast Cells (NBC) were obtained from normal breast tissue biopsies. The cells were cultured in standard & estradiol supplemented media. The cell lysates were used for BRCA1, BRCA2, c-Ha-Ras, HER/Neu & EGFR. Monoclonal anti-BRCA1, Ab-2 antibody clone MS13;c-Ha-Ras (Ab-1) clone F235-1.7.1 with P21-RasGly-12 as Western Blott standard; c-erbB-2/c-Neu monoclonal 40 mer Prob, Human; EGF-Receptor 40 mer Ab-2 monoclonal clone 455; were used in Western Blott analysis (Hakim, J. Surg. Oncology 40: 21–31, 1989; ibid Diagnostic & Clinical Testing 2: 30–39, 1989). When cultured in standard media, cell lysates of normal breast tissue biopsies, & benign tumors were negative for BRCA1, BRCA2, c-H-Ras & for c-ErbB-2/Neu, but when cultured in estradiol supplemented media, cells of benign tumors showed the presence of c-H-Ras followed by BRCA1/BRCA2 & c-ErbB-2/Neu after 4, 8 & 16 weeks of in vitro culture in presence of estradiol, respectively. Lysates of NBC remained negative to the appearance of the oncogenes during this period. RasP21 and c-ErbB-2/Neu & BRCA1/BRCA2 were undetectable in cells from normal and benign tissues, but significantly elevated (overexpressed) in 21/25 & 15/18 primary & metastatic biopsies. Cells from metastatic breast tumors were ER negative & had c-ErbB-2/Neu amplified in 15/18, while cells from primary tumors were ER^+ and had c-ErbB-2/Neu amplified in 19/25 tumors. The presence of estradiol in the culture medium increased c-ErbB-2/Neu and decreased responsiveness to estrogen in the ER^- established BC cell lines. The results suggest that appearance of BRCA1/BRCA2 genes point to an already elevated levels of mutated RasP21 with activated Protein Tyrosine Kinases & require aggressive treatment with inhibitors of PTK as adjuvant.

P7 BRCA1 and BRCA2 germline mutations in early-onset breast carcinoma patients

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Two different cancer susceptibility genes, *BRCA1* and *BRCA2*, have been found to be responsible for approximately 70% of site-specific breast cancer families. However, only a small proportion of all breast cancers (about 6–10%) appears to be linked to *BRCA1* and *BRCA2*. Recent reports have demonstrated the presence of germline mutations in the latter genes in early-onset breast cancer cases regardless of a family history of breast cancer (*BRCA1*: 12–13%, *BRCA2*: 2.7%). In order to estimate the frequency of germline mutations in breast cancer predisposing genes associated with early-onset breast carcinoma in Italian women, we analyzed 57 patients with breast cancer diagnosed before 36 years old, unselected for family history of cancer. All cases were examined in *BRCA1* exon 11 and *BRCA2* exons 10 and 11 by Protein Truncation Test (PTT). In addition, 25 cases, which were wild-type in the above exons, were analyzed by sequencing of all coding exons and flanking intronic regions, whereas the remaining 32 patients were selectively screened for the presence of the common 5382insC mutation in *BRCA1* exon 20. Germline truncating alterations in the *BRCA1–2* genes were identified in 9 (15.7%) and 4 (7.0%) cases, respectively. These frequencies are higher than those previously reported by similar studies in other populations. Family history of cancer, age at onset of breast carcinoma, clinical and pathological features and follow-up of patiens with *BRCA1–2* germline mutations were the follow. In 3 cases the family history was negative for cancer in first degree relatives while in 1 case the family history was negative also in second degree relatives. On the contrary, 9 cases were positive for family history of breast and/or ovarian cancer in first and/or second degree relatives. The age at onset of breast cancer in mutation carriers was ranging between 23 and 34 years. All tumors were infiltrating carcinomas of the following types: 9 ductal, 1 lobular, 1 medullary and 2 non otherwise specified. During follow-up, in 6 cases a second cancer arised: 2 contro-lateral breast cancers, 2 ipsi-lateral breast cancers, 1 ovarian cancer, 1 case with an ipsi-lateral relapse followed by a contro-lateral breast cancer. Our results suggest that *BRCA1–2* genetic test should be recommended to women with early-onset breast carcinoma, independently of family history of cancer.

P8 High rate of one specific haplotype in the 13q12–13 region in breast cancer

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Aim: It is uncommon to find descriptions of the differences in germline rates of homozygosity and heterozygosity, or in the allelic frequencies for a specific marker between patients suffering breast cancer and the general population. The possible implication of these factors in the pathogenesis of the tumor remains unknown. The present study was undertaken to compare the rates of homozygosity and heterozygosity in patients with breast cancer and controls.

Methods: We investigated these parameters at loci of the 13q12–13 in 89 breast cancer patients and 62 controls. Two markers (D13S260 and D13S310) were used to assess the allelic status, and β-globin primers were employed for multiplex PCR to detect hemizygous deletions. The amplified products were electrophoresed on non denaturing 6%–12% polyacrilamide gels. The allelic bands were detected by a commercially available silver staining method.

Results: At locus D13S260, we found homozygosity in 30% of patiens *versus* 22% of controls, and heterozygosity in 70% *versus* 78%, repectively. At locus D13S310, the homozygosity rate was 49% in patients *versus* 32% in controls, and the rates of heterozygosity were 51% *versus* 68%, respectively. These differences were statistically significant at marker D13S310 and close to the significance in D13S260 marker. Double homozygosity was found in 16% of patiens and in 6% of expected cases; double heterozygosity in 39% and 54% respectively. In multiplex PCR analysis, no hemizygous deletions were observed in doubly homozygous patients. This allelic selection showed an abnormal distribution of the alleles that consequently offer one haplotype 4/4, the prevalence of which was statistically significant in our breast cancer patients.

Conclusions: These data reveal that the high rate of homozygosity observed at the 13q12–13 region is not related to hemizygous deletions and suggest that an abnormal allelic distribution could explain this homozygosity, as well as the presence of specific haplotypes associated with the disease.

P9 High risk breast/ovarian cancer families: Genetic counselling, testing and early cancer detection program

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Goals: Germline mutations of the cancer susceptibility genes BRCA1 and BRCA2 seem to be a major part of the hereditary breast/ovarian cancer syndrome. Genetic counselling and identification of high-risk families may be essential (1) to offer the opportunity to participate at a specific early cancer detection program, (2) to inform about prophylactic medication or surgery and (3) to provide individualized psychological support. An interdisciplinary counselling approach (gynecological oncology, human genetics, molecular biology, psychotherapy) was established.

Methods: From August 1994 until August 1997 305 consultees presented at the cancer genetics clinic, who were all couselled prospectivly applying the proposed approach. In case of positive inclusion criteria prospective predictive testing for BRCA1/2 was offered. Participation at the established early cancer detection program [palpation, ultrasound (US), mammography (MG), magnetic resonance tomography (MRT)], (prophylactic) medication or surgical procedures were discussed with all consultees. 141 consultees (families) met the inclusion criteria for genetic testing. For diagnostic genetic testing for BRCA1/2 mutations direct DNA sequencing is used.

Results: Detailed data about participation at the early cancer detection program, prophylactic medication or the surgery are available 70 consultees without and 101 with inclusion criteria remained in the recommended early cancer detection program and under surveillance. 9 prophylactic and 21 indicative operations were performed. Genetic testing of 32 families is completed. For BRCA1, 6 mutations and 15 polymorphisms, for BRCA2 no mutations and 4 polymorphisms could be detected.

Conclusions: Genetic testing for BRCA1/2 is technically challenging. In this study group an interdisciplinary approach proved helpfull for counselling, surveillance and individualized support for consulting women. Women with a family history of multiple sporadic breast/ovarian cancers and those with a hereditary BRCA1/2 defect may be distinguished, but incividual fear is a common phe-

nomenon in both groups. For the first group of patients the support may ease their concern, for the second group preventive measures may be discussed.

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Thursday, February 26, 1998 9.00–18.00

Biology/Metabolism

P10 Multistep carcinogenesis of sporadic breast cancer

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Introduction: Breast cancer emerges as a multistep process with transformation of normal cells via steps of proliferation, atypia and in situ carcinoma. Cytogenetic and molecular genetic analysis of breast cancer samples indicate that tumor development involves the accumulation of various genetic alterations including amplification of oncogenes and mutation or loss of tumour suppressor genes. Microdissection of histological sections is needed to correlate the specific histological change and the genetic alteration. Up to date, no studies with prove of a direct sequential genetic motif are published, but the concomitant analyses of various genetic alterations in early lesions may correlate the histological finding with a biological function.

Methods: In a prospective study, after microdissection (1) DNA from 127 breast cancers with matched normal tissue was isolated and (2) in 20 cases comparative analyses with benign and precursor lesions (DCIS, CLIS) of the same breast were performed. Oncogene amplification (erbB2, int-2, c-myc, cdk4) was measured by quantitative differential PCR. Allele loss of tumor suppressor genes (p53, BRCA1, BRCA2, HIC1, MTS1/p16, NME1) was analysed by PCR-based microsatellite polymorphisms detecting differences in short tandem repeat sequences, which are informative for assessment of loss of heterozygosity (LOH). Fluorescent labelled PCR products were analysed and quantitated by polyacrylamid gel electrophoresis in an automated DNA sequencer (A.L.F.™Pharmacia, Freiburg, Germany). Results were analysed with Fragment Manager™ software.

Results: Prior to the study, DNA extraction from microdissected sections stained with different methods (hematoxylin, toluidin) as well as the linearity of the PCR reactions had to be validated. Oncogene amplification was found in 24% for erbB2, 19% for int2, 18% for c-myc, and 12% for cdk4. LOH could be detected in 57% for TP53, 48% for HIC1, in 38% for BRCA1, in 35% for BRCA2, in 8% for MTS1/p16. The sequential analyses shows LOH of TP53, LOH HIC1 followed by LOH of BRCA1 and c-myc amplification. In microdissected in situ lesions of the same breasts oncogene amplification and LOH could be demonstrated. These findings show identical alteration as seen in invasive samples.

Discussion: Quantitative differential PCR and microsatellite analyses combined with detection of fluorescent labelled PCR products by an automated laser DNA sequencer are powerful tools in determination of genetic alterations. Especially in combination with microdissection where small lesions are analysed, they proved to be useful as analytical methods. The accumulation and the combination of different genetic alterations may lead to a hint to the time frame of multistep carcinogenesis. The simultaneous analysis of histology and genetics of precursor lesions offers the opportunity for a biological description of the histological picture. The results of this pilot study support the concept of multistep carcinogenesis in breast cancer. LOH of TP53 in precursor and invasive lesions point to a key function of TP53 in breast cancer development.
(DFG Be 1215/6-2)

P11 Loss of retinoic acid receptor β expression in breast cancer and morphologically normal adjacent tissue but not in the normal breast tissue distant from the cancer

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Retinoids and their receptors (RARs, retinoic acid receptors; RXRs, retinoid X receptors) play an important role in maintaining the balance between proliferation and apoptosis. Recently, Deng and coworkers (*Science* 274: 2057–2059, 1996) reported a loss of heterozygosity on chromosome 3p24 in breast cancer specimens and the morphologically normal appearing adjacent tissue. The 3p24 locus includes, among other genes, the region coding for RAR-β. This study was designed to determine whether there are abnormalities in the expression of retinoid receptors in surgical specimens of patients with breast cancer.

In fourteen patients, transcripts of nuclear retinoid receptors were detected by *in situ* hybridization in formalin-fixed, paraffin-embedded specimens by means of digoxigenin-labeled riboprobes specific for RAR-α, -β and -γ.

We found RAR-α expressed in all specimens, whereas RAR-γ was expressed in 100% of normal breast tissue, but only in 11 out of 14 tumorous lesions. RAR-β was found in all cases of normal breast tissue localized distant from the tumor, but in 13 out of 14 cases it was completely absent in the tumor and the morphologically normal appearing tissue adjacent to the tumor. One possibility to explain the suppression of RAR-β is a mutation in the promoter region. Sequencing the DNA extracted from paraffin-embedded tumor tissue of the corresponding breast cancer specimens, we were not able to detect any mutation in the retinoic acid responsive element (RARE).

Our results clearly indicate a crucial role of RAR-β in the carcinogenesis of breast cancer.

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P12 In vitro cultivation of human mammary epithelial cancer cells. Study of their phenotypic characteristics and biologic behavior

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The successful long-term growth of tumor cells from primary breast tumor explants is a rare event. To define the characteristics of tumor cells which govern their ability to grow in vitro as primary culture as well as continuous or established cell lineage, human mammary epithelial cancer (HMEC) cells from 18 cases of unselected primary breast cancer were propagated in culture. Propagation of HMEC cells in vitro as monolayer in primary culture was successful in 10 out of 18 (55.5%) cases, which showed continuous proliferation of tumor cells only up to 6–8 passages before they reached senescence. An investigation of the effects of phenotypic expression of estrogen receptors (ER), the progesterone receptors (PgR), C-erb B_2 oncoprotein and epidermal growth factor receptors (EGFR) on the capacity of HMEC cells to grow in vitro as monolayers showed that expression of ER and EGFR is required for controlling tumor proliferative activity in vitro. Expression of ER made the growth of HMEC cells more difficult, while expression of EGFR protein made their growth in vitro easier. Phenotypic characteristics of floating HMEC cells were found to be different from those grown on coverslip as adherent cultures, suggesting a selective growth of HMEC cells of a specific phenotype in culture. This suggests that cell lines are not appropriate tool for chemosensitivity and radiosensitivity studies because neither the primary cells nor passaged cells represent the heterogeneous population of original tumor. Cultured HMEC cells in subsequent passages showed a decrease in their proliferative capacity, alterations in phenotypic characteristics and development of morphological features of terminal differentiation, resulting in senescence.

P13 Involvement of plasminogen activator inhibitor PAI-1 in *in vitro* growth of human breast cancer cell lines

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Introduction: Effective proteolysis of extracellular matrix is a critical factor for tumor growth and metastasis. The integrity of extracellular matrix is affected by the activity of several different classes of proteinases, like serine proteases such as plasmin, generated by the urokinase pathway of plasminogen activation. The later is regulated by a specific cell-surface uPA receptor (uPAR) and by two inhibitors (PAI-1, PAI-2). It has been proposed that high levels of PAI-1 may protect the tumor against degrading itself and in this way promoting tumor growth. We have addressed the question whether human breast cancer cell lines express in a different manner components of the plasminogen activation system and whether this expression is correlated with their *in vitro* growth rate.

Material and Methods: 1×10^4/ml cells of 2 estrogen receptor (ER) as well as progesterone receptor (PR) negative (BT-20, MX-1) and 1 ER, PR positive (MCF-7) human breast cancer cell lines were cultivated in DMEM/F12 serum-free medium up to 6 days. suPAR, uPA and PAI-1 immunoreactivity were assayed by Elisa. MTT-assay as described elsewhere (Mossmann, 1986) was applied to estimate the *in vitro* growth capacity.

Results: In all media, uPA- and uPAR immunoreactivity was detected. MCF-7 cells did not express any PAI-1, whereas BT-20 tumor cells secreted a 7 fold higher PAI-1 amount (0.57 ng/ml) than the MX-1 cell line (0.08 ng/ml). Comparable levels of urokinase plasminogen activator were measured by all three cell lines. Again, BT-20 cells exhibited higher levels of uPAR concentration than MX-1 or MCF-7 cells. Interestingly BT-20 and MCF-7 tumor cells had similar in