Molecular biology of breast cancer

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FHIT and breast cancer. The conference was opened by the chairman, Anne-Lise Børresen-Dale, and after lectures on the importance of breast cancer research from the patient’s and clinician’s view by Susan Leigh (National Coalition for Cancer Survivors, USA) and Nancy Davidson (Johns Hopkins University, USA) a Ruth Sager’s Honorary Lecture was given by Carlo M. Croce (Kimmel Cancer Center, Philadelphia, USA) on the genetics of breast cancer, mainly focusing on the FHIT gene. In addition to summarising the symposium and addressing questions regarding the future of breast cancer research at the end of the meeting, George Klein (Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm) presented an approach in the search of TSG, the so-called elimination test, developed by Dr Stephan Imreh at the same institute. This test is based on microcell fusion where human chromosome 3 is introduced to a malignant mouse cell line, resulting in reduced tumorigenicity. Upon further passage of the cells (up to 4 passages) into new mice, these cells become more malignant as genetic material from human chromosome 3 is lost. Mapping these deletions indicated a TSG location at 3p21 and some of the data pointed towards the FHIT gene at 3p14.2.

Croce reviewed findings concerning frequent gene amplifications (and corresponding overexpression) in breast tumors, MYC (10%), Cyclin D1 (10-20%) and HER2 (20-30%); gene mutations of TP53 (20-30%), BRCA1 (3%) and BRCA2 (3%), and gene deletions, FHIT (60%). Accordingly, Croce concluded that the deletions of FHIT are the most frequent gene alteration in breast tumors. The FHIT gene is located at the most common fragile site in the genome, FRA3B, which is aphidicolin sensitive and is frequently deleted in human tumors. The FHIT gene is the second largest gene in the human genome (500 kb), still the mRNA is only 1.1 kb, with 5 non-coding and 5 coding exons. The protein product has a diadenosine triphosphate hydrolase activity. Little is known about the biochemical pathways in which the Fhit protein participates with respect to cell growth; diadenosine triphosphate possibly acts as a messenger of growth and is inhibited by binding to Fhit and inactivated by its hydrolase activity. Further knowledge on these pathways will add evidence to the role of Fhit in growth control. Loss of the FHIT gene as well as abnormal transcripts have been extensively studied in several tumors and tumor derived cell lines. Abnormal transcripts were detected in 30% of breast tumors. The majority of lung cancer show FHIT alterations, particularly in smokers. This is the earliest gene alteration detected in lung cancer while loss of FHIT occurs later in the progression of breast cancer. It is surprising that an important TSG is located at such a fragile site in the genome and the involvement of the gene loss in tumorigenesis can be questioned because frequent aberrations may simply reflect the unstable chromosome region. By transfection experiments Croce has added evidence for the role of FHIT as a tumor suppressor and concluded that Fhit is involved in cell cycle arrest and apoptosis signaling. Decreased tumorigenicity by Fhit was detected by scoring for growth of tumor derived lines in immunosuppressed mice. To further support the conclusion that FHIT is a TSG,

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*The Second International Symposium on the Molecular Biology of Breast Cancer, held in Lillehammer, Norway, March 12-16, 2000, was organized by Anne-Lise Børresen-Dale (Symposium Chairman, Department of Genetics, The Norwegian Radium Hospital, University of Oslo, Norway), Per Eystein Lanning, Tone Ildahl Andersen, Gry Aarum Geitvik, Ragnhild A. Lothe and Manuel R. Teixeira. Co-organizers of the meeting were: the American Association for Cancer Research, The European Association for Cancer Research and The Norwegian Cancer Society. The approximately 240 participants heard 36 formal lectures and 70 poster presentations and the symposium provided an optimal setting in which European and American breast cancer researchers could meet and exchange information. A great amount of progress has been made since the first symposium, 5 years ago. The focus was on new molecular findings in association with breast cancer progression and clinical implications were discussed.

Abbreviations: AT, ataxia telangiectasia; ATM, AT mutated gene; CDK, cyclin dependent kinase; CGH, comparative genomic hybridization; CHK2, checkpoint kinase 2; DCIS, ductal carcinoma in situ; EGFR, epidermal growth factor receptor; ER, estrogen receptor; EST, expressed sequence tag; FAK, focal adhesion kinase; FHIT, fragile histidine triad gene; FISH, fluorescence in situ hybridization; GF, growth factor; HAT, histone acetylase; HIF, hypoxia inducible factor; IHC, immunohistochemistry; MAPK, MAP kinase; LCL, lymphoblastoid cell line; LFS, Li-Fraumeni syndrome; PR, progesterone receptor; RTK, receptor tyrosine kinase; TSG, tumor suppressor gene; VEGF, vascular endothelial growth factor

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Croce created knockout mice. Even though the fragile region in the mouse is smaller and does not break as frequently as in humans, the homozygous knockout mouse develops tumors with high incidence at 9 months of age, particularly leukemias and lymphomas but also some solid tumors. The heterozygous mice were treated with a carcinogen known to induce gastric cancer and after 10 weeks these mice developed tumors, both gastric and skin tumors that have similarity to the sebaceous tumors detected in the Muir-Torre syndrome of humans. It would be of interest to analyze somatic alterations of the FHIT gene in the sebaceous tumors from Muir-Torre patients carrying germine mutation in one of the mismatch repair genes and germline mutations of the FHIT in those patients not carrying a mismatch repair gene mutation.

Receptor tyrosine kinases and gene amplifications. Breast tumors express high levels of type I receptor tyrosine kinases and their ligands. This receptor family is composed of 4 related receptors where the Egfr and Her2 are best studied in breast cancer. Jose Baselga (Hospital General Vall D’Hebron, Spain), Dennis Slamon (UCLA, USA), Charles Vogel (Miami University, USA), Olli-Pekka Kallioniemi (NIH, Bethesda, USA), Jean-Marc Nabholz (University of Alberta, Canada) and Jan G.M. Klijn (Dr Daniel Den Hoed Kliniek, The Netherlands), reviewed these receptors, their activation in breast tumors and clinical implications. These receptors are composed of an extracellular binding domain, a transmembrane domain and an intracellular protein tyrosine kinase domain, with a regulatory carboxy terminal segment. The HER2 gene is amplified in 20-30% of breast tumors with corresponding overexpression. The expression of Her2 in tumors with HER2 gene amplification is generally detected in all cells, and is not heterogeneously expressed, as frequently seen for other breast cancer association proteins, such as p53 and ER. In amplified breast tumors the number of Her2 receptors increases from 40,000 to 80,000 to several millions per tumor cell. Kallioniemi showed that many genes are overexpressed in the Her2 amplicon by using chromosome 17q specific cDNA micro-array. In the 17q12-13 amplon the HER2 is the most highly overexpressed. The amplification and overexpression of HER2 is associated with reduced survival, particularly in node negative patients. According to Klijn the MYC amplification also affects survival, but does not influence response rate, as HER2 amplifications do.

It has been suggested that RTK’s are optimal targets for breast cancer therapy and a series of monoclonal antibodies are currently being evaluated for use in therapy in addition to specific Egfr tyrosine kinase inhibitors (ZD1839). Baselga described Egfr as a target for cancer therapy by analysing growth of the MB468 breast cancer line in nude mice and showed that treatment with an antibody against Egfr (528ab) in combination with taxol had synergistic effects. Another antibody, Mab225, prevents binding of the ligands to the Egfr and inhibits growth of cancer cells, both in tissue culture and in human tumor xenografts. This antibody also enhances the antitumor effects of chemotherapeutic agents active against breast cancer. Application of Mab225 also results in synergistic effects when given with doxorubicin, taxol or herceptin, which is an antibody directed against Her2. Phase I clinical trials are in progress. It can be concluded that pre-clinical studies indicate that the antibodies can be effective in suppressing growth of human tumor cells in vitro as well as breast cancer.

Slamon, Vogel and Nabholz showed that similar to the effect of the Egfr antibodies, antibodies against the Her2 in combination with other therapeutic modalities can have additive and occasionally synergistic effects with chemotherapeutic agents both in vitro and in vivo. The pivotal phase III study comparing standard therapy versus addition of herceptin demonstrates that this new biological agent improves objective response rates by 54%, response duration by 58% and increases time to progression by 65%. In addition, initial use of herceptin as a part of the combination therapy leads to a decrease in relative risk of death by approximately 25% after 2.5 years. Vogel’s clinical trial of herceptin suggested synergistic effects with doxorubicin in the treatment of breast cancer. Herceptin plus chemotherapy is superior to chemotherapy alone in all parameters of effectiveness, including a 5-month survival advantage after two years of follow-up. The putative risk of cardiac dysfunctions by herceptin were also discussed.

Helen Hurst (Imperial Cancer Research Fund Molecular Oncology Unit, ICSM Hammersmith Hospital, London, UK) described a transcriptional targeting approach to trick the cancer cells into suicide. The general idea is that normal cells do not express an enzyme that converts a prodrug into an active cytotoxic agent, while in tumor cells a toxic metabolite is created (GPAT, genetic prodrug activation therapy). Tumor-specific promoter/enhancer can be used. Only transient transcription is required and expression in all cells is not needed since the enzyme and drug can travel between cells. The proximal promoter of the HER2 is the prototype system. Transfection with suicide genes has been performed and phase I clinical trial of direct intratumoral injection of a cytosine deaminase under the control of the HER2 promoter to patients with advanced breast cancer has been reported. Eleven out of twelve patients received successful gene transfer and cytosine deaminase activity is restricted to Her2 positive cells. Combination of MUC1 and HER2 elements has also been used for dual-specificity targeting.

Joe Gray (UCSF, USA) has analysed increased and decreased DNA copy numbers by CGH, and concluded that aberrations are similar in paired DCIS and invasive breast cancers. At the 20q13 amplicon in breast tumors two genes, ZABC1 (encoding a zinc finger protein) and AIBC1 (encoding a steroid receptor co-regulator) were shown to be amplified and overexpressed. Of interest is also that the gene encoding the Snail transcriptional repressor of the E-cadherin promoter is located at chromosome 20q13.2. This transcriptional factor has recently been shown to regulate the epithelial mesenchymal transition in mouse embryos and has a transcriptional pattern reversed to that of E-cadherin in several tumor lines and tumors. It would be of interest to see if the Snail transcription factor is upregulated in E-cadherin negative breast tumors.

Cell cycle, apoptosis, immortalization and metastasis. As Jiri Bartek (Institute of Cancer Biology, Danish Cancer Society, Copenhagen) pointed out, breast cancer, like other cancer types, is a disease of the cell cycle, but cell cycle disturbances are not the only causes of cancer growth. Bartek
reviewed the cell cycle control and its deregulation in cancer. He concluded that three steps in the cell cycle control were important in oncogenesis: i) the restriction point control is frequently disrupted in cancer and this is a direct effect. Examples are pRB, p16, cyclin D, Myc, Ras. Cyclin D and Myc are located within an amplified region in a subset of breast cancer. An increase of cyclin D leads to binding and activation of CDK4 and subsequent phosphorylation of pRB triggers the release of the transcription factor E2F, resulting in progression throughout G1- and S-phases of the cell cycle. Similarly, overexpression of the Myc transcription factor runs the cell through the cell cycle. Myc has a broader control spectrum of the cell cycle progression than E2F. In addition to inducing S-phase genes similar to E2F, Myc controls the transcription of cde25 phosphatase, which activates the CDK with subsequent cell cycle progression. Myc also controls the transcription of factors involved in protein synthesis. ii) Disruption of S-G2-M machinery is less frequently involved in oncogenesis. iii) Disruption of checkpoint control is common and affects genome stability. Examples are p53, Atm, Arf, Brca1, Brca2, Chk2. Some of these proteins, like p53, Atm, Brca1 and Brca2 have been implicated in breast cancer progression (see later).

Charles Streuli (School of Biological Sciences, University of Manchester, UK) reviewed the control of apoptosis in the mammary gland. Apoptosis occurs naturally at several stages of normal breast development; during the formation of intraductal lumina, at the end of each menstrual/oestrus cycle and during involution that follows lactation. Apoptosis is of clinical and therapeutic significance since one effect of chemotherapy is cell death by apoptosis. Apoptosis of mammary epithelial cells was studied in relation to adhesion, i.e., to basement membrane network (collagen IV and laminin) in comparison with adhesion to collagen I. Apoptosis occurred on collagen I while basement membrane suppressed apoptosis, indicating that adhesion survival signals are specific in their responses. The apoptotic inducer Bax is upregulated when the cells do not adhere to the basement membrane. When conformational changes of Bax are triggered by adhesion (inhibited by vanadate), cytochrome C is released from the mitochondria and apoptosis occurs due to protein complex formation and protease cleavage of latent caspase proenzymes resulting in activation of caspases. This can possibly be explained by difference in integrin binding to basement membrane and collagen I. FAK was shown to be involved; by using a dominant negative mutant that resulted in increased apoptosis and Bax expression. It was concluded that both conformational changes and transfer of Bax to mitochondrial membrane are essential for apoptosis. Cross-talk was shown to occur between FAK and RTK with the corresponding phosphorylation cascade. Expression studies of Bcl2 family members in virgin, pregnant, lactating and involuting mice show that part of the apoptosis control comes from the developmental regulation of genes in this family. The maximum apoptosis is in pregnant and lactating mice. Bcl2 and Bclw expression was high in virgin, down-regulated in pregnant and lactating and upregulated again in involuting mice. Bak and Bad are upregulated in pregnant and down-regulated in involuting mice. The commitment to mammary apoptosis in vivo is probably also regulated by Bclx.

The mechanism of breast tumor vascularization was discussed by Adrian L. Harris (ICRF Medical Oncology Unit, Churchill Hospital, Oxford, UK). Without suitable angiogenesis, the tumor cannot grow and metastasize. The number of blood vessels in breast cancer is related to prognosis and angiogenesis provides a site of therapeutic intervention. Multiple GF pathways control angiogenesis, VEGF is a key player in breast angiogenesis and thymidine phosphorylase also plays a role. The carbonic anhydrase IX promoter is regulated by Hif1a and upregulation of this enzyme could serve as a new marker of hypoxia in tumors. It was concluded that hypoxia influences regulation of many genes via the Hif1 or Hif2 transcription factors, including the VEGF gene, and that some of these genes could be potential targets for therapy. An induction of Hif2 expression was observed in stromal macrophages within the breast tumors and Hif1 was expressed in the epithelium around the areas of necrosis. Thus, two different hypoxia-regulated pathways are activated in breast tumors.

Jerry Shay (The University of Texas Southwestern Medical Center, Dallas, TX, USA) gave a lecture on telomerase and its role in cell immortalization. Somatic cells have usually low and cancer cells high levels of telomerase. Cells lacking telomerase can only undergo a certain number of cell divisions and cells become senescent before they can grow to become cancer. Telomerase activity is enhanced in more than 85% of cancers. The data on increased telomerase activity presented by Shay included; normal breast (0 out of 14 tissue samples), fibrocystic disease (0/40), in situ breast cancer (19/23), stage I breast cancer (50/63), advanced carcinoma (233/257) and adjacent carcinoma (2/84). The therapeutic agent 2-O-mRNA, an antisense RNA, was used to inhibit telomerase. The telomere shortening in various tumor cells (from breast, colon, cervical, and renal cancer) results in enhanced apoptosis. Spontaneous immortalization of LFS-derived breast epithelial cells with heterozygous mutation of TP53 can be prevented by expressing a dominant-negative mutant of the telomerase catalytic subunit. Shay's results not only validate telomerase as a target for breast cancer prevention and therapy, but also provide insights into the properties that successful anti-telomerase agents will require. Perhaps anti-telomerase activity could be applied after surgery in combination with chemotherapy or radiotherapy.

In breast cancer as in other cancer types the optimal would be to detect metastases at single cell level and to detect their viability. These cells are targets for therapeutics. Klaus Pantel (University of Hamburg, Germany) reviewed the problems in the detection of micrometastases with standard methods. One of the primary metastatic sites for breast cancer is the bone and bone marrow. Density centrifugation and IHC of cytokeratin was performed and served as specific markers for epithelial cancer cells in bone marrow. Of 552 breast cancer patients, 36% were found with cytokeratin-positive cells in the bone marrow compared to 1% of the 191 controls. The presence of micrometastases in bone marrow was associated with the occurrence of clinically overt distant metastasis and death from cancer-related causes. It was concluded that presence of occult cytokeratin positive metastases in bone marrow increases the risk of relapse in patients with stage I, II or III breast cancer and can serve as an independent estimator.
of the risk of dying from cancer after adjustments were made for the use of systemic adjuvant chemotherapy.

p53, Atm and Chk2. The activity of the transcriptional factor p53, a key molecule in down-regulating cell division (where the pathways involving transcriptional activation of p21 in the G1 checkpoint and 14-3-3ε in the G2 checkpoint by p53 are of importance) and stimulating apoptosis (where the role of transcriptional activation of the BAX gene by p53 is well documented) is controlled by proteolysis. Upon stress stimuli like hypoxia, DNA damage and oncoprotein action, the p53 degradation pathway is inhibited, the p53 levels rise, and cell-cycle arrest and apoptosis is induced. Additional data on the p53 function from Curtis C. Harris (Laboratory of Human Carcinogenesis, National Cancer Institute, NIH, Bethesda, MD, USA) suggested that the p53 may modulate either DNA repair or apoptosis by binding to and regulating the activity of the DNA helicases of dysfunction in the cancer-prone disorders like Xeroderma pigmentosum and Bloom syndrome. Since p53 is at the crossroads of these pathways, it provides a biological basis for being a prime target of somatic mutations in human cancers, including a subset of breast cancers, as reviewed by Scott Lowe (Cold Spring Harbor Laboratory, NY, USA). The mutation prevalence of TP53 in breast tumors is relatively low, or 20-30%, but p53 protein levels are elevated in more than 50% of breast cancers, suggesting that p53 function may be deregulated by mechanism other than mutation, and in some cases this p53 accumulation may be due to genomic alterations and rearrangements within the breast tumor cell. Pierre Hainaut (International Agency for Research on Cancer, Lyon, France) reviewed the TP53 mutation spectrum, but over 10,000 mutations have been described in different tumors. Diverse missense mutations are detected in the p53 gene, to a higher level than several other TSG, and may in some instances function as dominant negative mutations. In the IARC TP53 mutation database there are about 1,000 mutations listed in breast tumors. In breast cancer the pattern of TP53 mutations shows a relatively high prevalence of insertions, deletions and non-sense mutations, or 25% of mutations. The TP53 mutations are associated with aggressive tumor types and risk of poor prognosis and outcome, in both node-negative and node-positive tumors. Per E. Lønning (Haukeland Hospital, Norway) concluded that the use of IHC to detect p53 is of limited value to predict outcome of chemotherapy but p53 mutations are predictive for chemoresistance and a difference in patient survival has been observed, depending on TP53 mutation type. Tumors expressing p53 with mutations in the DNA-binding surface have a poorer response to some forms of treatment than tumors with mutations at other sites. The proportion of certain TP53 mutations in cancer at different organ sites varies. GC→TA changes are low in colon cancer, high in lung cancer, and thyroid, breast and liver cancers have intermediate frequency, while CpG alterations are high in colon and low in lung cancers. The most frequent mutation type in breast cancer is GC→AT transitions (40%), equally affecting CpG and non-CpG sites. Cohort comparisons have shown differences in the nature, localization and frequency of mutations. The frequency of hot spots for p53 mutations is the same for breast cancer as in other cancers. In breast cancer the mutation spectrum is different in different geographic areas, for example specific mutation frequency is reported in the Japanese population. Breast cancer frequently arises in Li-Fraumeni families, where germline mutations can be in the TP53 gene or the CHK2 gene. The Chk2 is a protein kinase that is activated in response to DNA damage and may regulate cell cycle arrest. Germline mutation of CHK2 gives similar Li-Fraumeni phenotype as germline TP53 mutation. It would be of interest to analyse whether CHK2 is mutated at somatic level in breast tumors. According to Tak Mak (Ontario Cancer Institute, Canada) there is no induction of p53 in Chk2 knockout cells after γ-irradiation and therefore it can be concluded that Chk2 acts upstream of p53. Similarly to p53 negative cells there is no induction of p21 and Bax in Chk2 negative cells. The Chk-/- cells failed to maintain γ-irradiation induced arrest in the G2 phase of the cell cycle and were resistant to DNA damage induced apoptosis. This is specific for irradiation since this is not seen when cells are treated with UV. This phenotype is reversible when Chk2 is reintroduced to the system. Mak also concluded that the Chk2 is a major effector of the Atm kinase and carries out several of its functions. It is known that Atm phosphorylates p53 at ser15 and it was shown that Chk2 phosphorylates p53 at ser20. This phosphorylation inhibits the binding of the Mdm2 ubiquitin ligase to p53, providing a mechanism for increased stability of p53 by preventing ubiquitination in response to DNA damage. The LFS TP53 mutations may be considered as representative of spontaneous mutations arising in breast cancer but comparison with sporadic cancer shows that two transversions, G→T and G→C, are not found in LFS breast cancer patients, but represent 18% of somatic breast cancer mutations. The mutations show a strong strand bias and occur at sites often mutated in lung cancers from smokers (codon 157, 248, 249 and 273) or in bladder cancers from smokers and/or dye-exposed workers (codons 158 and 280). Overall, these data indicate that although most of breast cancer mutations probably have a spontaneous origin, a small proportion of mutations show signatures that suggest the involvement of exogenous carcinogens.

Many DNA tumor virus-derived oncogenes affect the function of the p53 pathway. Two cellular proteins, Mdm2 and Arf, play a critical role in the regulation of specific p53 stability. Mdm2, which is a specific E3 ubiquitin ligase, binds to a specific motif at the amino terminal of p53, and targets it to the proteasome for degradation and probably also for nuclear export. Arf binds to Mdm2 and inhibits its ubiquitin ligase activity. David Lane (CRC Laboratories, University of Dundee, UK) and co-workers have recently discovered that p53 is also modified by the small ubiquitin-like protein Sumol at amino acid K386, and this modification may inhibit the degradation of p53. Modification in vitro requires Sumol, a Sumol activation enzyme and Ubc9. Sumol and ubiquitin modification do not compete for the same lysin acceptor sites in p53. Overexpression of Sumol activates the transcriptional activity of wild-type p53, but not K386R p53, where the Sumol acceptor site has been mutated. The Sumol modification pathway therefore acts as a potential regulator of the p53 response and may represent a novel target for the development of therapeutically useful modulators of the p53 response. Experiments were performed on peptides that block the
Mdm2-p53 interaction and consequently activate p53. Down-regulation of Mdm2 is observed by UVC but not with X-rays. Mdm2 is post-translationally modified in response to ionizing radiation. N-terminal Arf peptide 3 binds to Mdm2 and blocks in vitro ubiquitination of p53. The ability to induce the p53 response with non-genotoxic agents combined with the recognition that p53 mutant human tumors lack the Mdm2 dependent degradation pathway opens up many exciting new approaches to drug discovery in the p53 pathway.

The Atm kinase is activated upon DNA damage with corresponding phosphorylation and stabilization of p53. As reviewed by Richard A. Gatti (UCLA School of Medicine, Department of Pathology, Los Angeles, CA, USA) and Janet Hall (International Agency for Research on Cancer, Lyon, France) the phenotype of AT patients involves radiosensitivity and cancer risk, including lymphomas/leukemias, leiomyosarcoma and breast cancer. Hall pointed out that normal tissue damage has been observed in a subset (10%) of breast cancer patients following radiotherapy and 16% of LCL from breast cancer patients show enhanced chromosome radiation sensitivity. Carriers of one ATM mutated allele have been reported with sensitivity for ionizing radiation in in vitro studies. LCL from radiosensitive and non-radiosensitive breast cancer patients were studied. No mutations of the ATM were detected in the non-radiosensitive, while elevation of nucleotide changes (truncation mutations in a minority) were found in the radiosensitive LCLs. The ATM is a large gene, the ATM protein is 370 kDa and 70% of mutations are truncating mutations. It is not known if both alleles are expressed equally. There is an elevated risk of breast cancer in heterozygous individuals and it is possible that the missense mutations are overrepresented, and could create dominant negative effects. The milder AT phenotype shows increased risk of breast cancer and Hall concluded that 8% of breast cancer could be due to AT heterozygosity. It was concluded that ATM is involved in breast cancer; two groups of ATM heterozygous mutations exist, truncation and missense, and the latter are of higher relevance for breast cancer, but the function remains to be studied.

**Hereditary breast cancer.** As concluded by Barbara Weber (University of Pennsylvania, USA) the last several years have yielded advance in all areas pertaining to genetic susceptibility testing, and the promise of cancer prevention associated with the isolation of BRCA1 and BRCA2 is becoming a reality. Weber addressed the need for accurate and predictive tests of BRCA1 and BRCA2 mutations, standardized clinical interventions, organized education program for clinicians, costs of mutation analysis and the psychological and medical risks. An example of methodological problems in analysing mutations in the BRCA1 and BRCA2 genes is that many of the currently used methods in the laboratory do not score for large genomic rearrangements. Also some of the variant sequences are of uncertain significance, although truncating mutations are clearly associated with increased risk of breast and ovarian cancer.

Even though the Brca1 and Brca2 proteins share a common function in homologous DNA repair, they differ in penetrance, histology of tumors and somatic changes of the genome, suggesting that the two proteins have a specific role. Various evidence indicates that the transformation of BRCA1 or BRCA2 deficient cells follows abrogation of specific cell-cycle control and apoptosis mechanisms, and results in genetic instability and tumor progression along distinguishable pathways. It is widely accepted that germline mutations in BRCA1 or BRCA2 enhance the lifetime risk of breast cancer. Still, the penetrance varies; different mutations within the genes may give different penetrance or phenotype, and even the same mutation can give variable penetrance in different families, presumably due to additional factors like modifying genes. In younger women, the BRCA1 gives higher risk of breast cancer than BRCA2, but similar risk is observed later in lifetime for mutations in either of the genes. In families with germline mutation in either of the genes there is also a higher risk of ovarian cancer, particularly in BRCA1 mutated families. Male breast cancer is elevated in BRCA2 families, and cancer at additional sites, such as prostate and pancreas. Bruce Ponder (CRC Department of Oncology, University of Cambridge, UK) reviewed the data from the Breast Cancer Linkage Consortium on penetrance of other cancer types than breast cancer in BRCA1 and BRCA2 families. In BRCA1 carriers the overall risk of ovarian cancer was estimated as 30% by age 60, and 3- and 4-fold increases in risk of prostate and colorectal cancer, respectively. The estimated risk of ovarian cancer in BRCA2 carriers is 27% by age 70. An elevated risk in BRCA2 carriers was detected for prostate (RR, 3.64), pancreas (RR, 2.93), melanoma (RR, 2.43), stomach (RR, 2.06), and gall bladder (RR, 4.14) cancers. Ponder concluded that these overall risks will differ in individual cases according to the specific BRCA1 or BRCA2 mutation and genetic and non-genetic modifiers.

Mike Stratton (Institute of Cancer Research, UK) and Åke Borg (Department of Oncology, Lund University, Sweden) presented the pathology of familial breast cancer. Several publications, although not all consistent, have reported that the pathology of BRCA1 and BRCA2 tumors differs from each other and particularly, from sporadic breast tumors. Different reports have suggested excess of lobular breast cancer and elevated medullary and absence of tubular breast cancer in familial cases. BRCA1 and BRCA2 tumors are of higher grade than sporadic tumors, particularly BRCA1 tumors. An increase of medullary and atypical medullary was observed in BRCA1 carriers. As reported by Stratton higher grade of nuclear pleomorphism was detected in BRCA1 patients. In a multivariate analysis; mitotic count, lymphocyte infiltration and continuous pushing margins are associated with BRCA1 germline mutation and tubule formation and continuous pushing margins are associated with BRCA2 tumors. BRCA1 tumors are more frequently negative for ER, PR and Her2, but are p53 positive and carry TP53 mutations at higher frequency in comparison to sporadic tumors. Tumors in BRCA2 carriers are less frequently Her2 positive than sporadic tumors. Stratton concluded that in a population of breast cancer patients diagnosed at a given age where 7% are mutation positive, 45% would be positive if the specific pathology is used as a criterion for familial breast cancer. The BRCAX tumors, or tumors from breast cancer families without BRCA1 or BRCA2 germline mutations, seem to be more heterogeneous, but exhibit, in general, a less aggressive phenotype than BRCA1 and BRCA2 tumors, and are of steroid receptor positive
status. The BRCAx tumors possibly have a subgroup of tumors with high levels of PR. According to Borg the BRCAx tumors are also more heterogeneous with respect to chromosome aberrations, but chromosomes 6, 9, 11, 13 and 18 could be involved.

CGH analysis may give hints to the location of genes involved in the pathogenesis of BRCA1 and BRCA2 tumors, by showing frequent loss of chromosome 4, 5q, 12q, 13q, and Xq in BRCA1 tumors, and of 1p, 3p, 6q, 8p, 9p, 11q, 13q and Xq in BRCA2 tumors. Frequent copy number gains are seen at 1q, 6p, 8q, 10p, 16p and 17q in BRCA1 tumors, and at 1q, 8q, 16p, 17q, 19 and 20q in BRCA2 tumors. The analysis was extended to the level of gene expression by Borg, using cDNA microarrays containing 6,526 human genes or ESTs. Sporadic, BRCA1 and BRCA2 breast cancer can be separated into distinct clusters of gene expression. This expression profile presumably reflects the distinct biological features of the tumors. Of special interest is the cyclin D1 upregulation and Her2 down-regulation in BRCA2 tumors.

Manfred Schwab (Division of Cytogenetics, German Cancer Research Center, Heidelberg, Germany) described several male breast cancer BRCA2 families with abnormalities at chromosome 9p detected by classical G-banding and FISH, in lymphocytes and fibroblasts from the breast cancer patients. In a breast tumor from an individual in a family with a BRCA2 2041insA mutation, an interstitial tandem duplication of chromosome 9p23-p24, distal to the CDKN2A (p16) gene and proximal to DMT1, was detected in fibroblasts. In this family 3 brothers and their father developed breast cancer. In another breast cancer patient from the same family a 10-cM inversion of 9p23-p24 was observed and the 3rd brother also carried an inversion at the same chromosome region. Therefore, it was concluded that a germline transmission of chromosome 9p instability had occurred, but the architecture of the somatic rearrangements is different in different individuals, and either chromosome of the pair could be involved. In another family with a BRCA2 7789delC germline mutation an amplification and inversion was observed in the 9p23-p24 region. In a third family, with a BRCA2 999del5 mutation, a deletion in a 12-cM region was detected at the 9p23-p24 region, that differs in all the lymphocytes from the 5 patients tested. In a fourth family a 7-cM deletion was detected at the 9p region. It remains unanswered whether 9p is the only chromosome region that is unstable in lymphocytes and fibroblasts of BRCA2 patients, but several other chromosomes have been found unstable in tumors of BRCA2 (and BRCA1) patients. The nature of this putative fragile site at 9p23-p24 is not defined and it may be questioned if this is a specific target for the BRCA2 protein and how a putative impairment of DNA repair is involved. Presumably one copy of the BRCA2 gene is intact in the lymphocytes and fibroblasts from these BRCA2 carriers, and therefore a dominant negative mutation should be considered.

Mak reviewed the Pten, Brcax1, Brcax2 and Chk2 (see earlier) knockout mice. Germine mutations in the PTEN gene are found in patients with Cowden's disease, and breast cancer is one of the phenotypic characteristics. To add further evidence towards the PTEN gene as a TSG the Pten knockout mice develop endometrial and mammary cancer. This supports the relevance of Pten function for maintaining the mammary tissue at non-malignant stage. To study the biological function of breast cancer genes and to create animal models for these cancer susceptibility genes, several strains of mice mutated in the Brcax1 and Brcax2 genes have been generated by gene targeting. The Brcax1 and Brcax2 knockout mice die at embryo level, there is no gastrulation and no mesoderm development. There is an elevated expression of p53 and p21 in both the Brcax1 and Brcax2 knockout mice and prolonged death occurs in a p53- or p21- background. Presumably the p53 is activated when Brcax1 or Brcax2 repair is non-functional, with subsequent cell arrest and apoptosis. The p53 pathway is probably also activated in early stages of breast tumor progression in human BRCA1 or BRCA2 mutation carriers and can be deactivated by mutation or other mechanism at later stages. However, the pathways of p53, Brcax1 and Brcax2 are probably more complicated than this, e.g. a synergistic transcriptional control of Brcax1 or Brcax2 together with p53 has been described. p53 knockout mice develop various tumors at 3 months of age and when also heterozygous for a knockout of Brcax1, the mice tend to develop mammary cancer. In addition, Mak created conditional recombinants by using Cre and showed that Brcax1 deletion in thymus results in reduction of cells by 15-20 times. Leaky Brcax2 knockout develop tumors, mainly lymphomas. Conditional Brcax1/- knockouts, specific for the mammary tissue, develop preferably mammary carcinoma. Therefore, it seems that intact Brcax1 and Brcax2 are needed to prevent mammary tumorigenesis of both mice and humans. It can be concluded that Brcax1-/- and Brcax2-/- mice have been of great value to determine the role of these genes in DNA repair and to analyse several pathways, such as the pathways involving p53, with effects on cell proliferation and apoptosis.

P. Hohenstein (Department of Pathology, LUMC, Leiden) reported new knockout mice, the Brcax1-1707T knockouts, where exon 20 is disrupted and the 2nd BCTR domain is truncated from the Brcax1 protein. Since this mutated Brcax1 protein is mainly intact, only lacking the carboxyl part of the protein, and contains among other domains, the p53 binding domain, nuclear localization signal and Rad51 interaction domain, it is a candidate for a dominant negative function. Although stable expression of the mutant protein has not been shown, the expression was confirmed at RNA level. The heterozygous mice are normal and fertile and are without tumors at 2 years of age. Tumors do not increase after ionizing irradiation or in Apc1638, Msh2 or p53 knockout crosses. The homozygous mutation is lethal but the embryos show headfolds at stage E9.5 days, impaired somitogenesis, BrdU incorporation and the mesoderm is present. At E10.5 day there is no turning, there is an increased cell mass compared to E9.5, no BrdU incorporation, massive apoptosis and the resorption has begun. These mice look similar to earlier reported Brcax1-p53 double knockouts. According to these findings it was speculated that the delay in embryonic death could possibly be due to blocking of p53 in a Brcax1-1707T complex with reduced function, resulting in delay of proliferation block due to checkpoint activation of p53 upon DNA damage.

Steroid receptors. In recent years there has been substantial progress in our understanding of the molecular mechanism of estrogen action; an additional ER has been discovered, the role of co-repressors and co-activators, and the importance of
conformational changes of ER has been clarified. The role of estrogen in growth, development, and function of the mammary gland is well established, but the roles of the two estrogen receptors remain unclear. The hormone resistance of ER positive breast cancer cells has been a mystery, but recently experiments have been performed to clarify this. These new findings were discussed in a session of steroid receptors by Myles Brown (Dana Farber Cancer Institute, USA), Kathryn B. Horwitz (University of Colorado, USA), Suzanne Fuqua (Baylor College of Medicine, USA), Jan Åke Gustafsson (Karolinska Institute, Sweden) and Mitchell Dowsett (Royal Marsden Hospital, London, UK). A significant proportion of ER positive tumors does not respond to tamoxifen and responding tumors can relapse. Tamoxifen can have different effects in various organs, for example anti-estrogen effects in the breast and estrogen effects in the uterus. There are several alternative explanations for the estrogen insensitivity of cells despite the presence of ER; such as limited concentration of co-activators, excessive amount of co-repressors, rapid inactivation of the hormone through metabolism or sequence variants of the receptor. Brown hypothesised that the different cell type responses to estrogens are governed by the complement of the expressed ER co-regulators. Of interest is that in 20% of breast tumors, preferentially ER\(^+\) tumors, there is an amplification of the 20q chromosome region carrying the AIB1 co-regulator, of the SCF-1 family that has HAT activity. Presumably the HAT activity is responsible for transferring an acetyl group to the histone complex, resulting in opening of the chromatin structure for active transcription. It is known that GF stimulate the activity of ER through the activation of MAPK and the direct phosphorylation of ER. The activity of the AIB1 phosphoprotein is also enhanced by MAPK, and it is phosphorylated in vitro by MAPK. This phosphorylation stimulates the recruitment of p300 and associated HAT. These results suggest that the ability of GF to mediate estrogen action can be mediated through MAPK activation of AIB1, suggesting a cross-talk between GF and estrogen signaling in ER positive breast cancer.

According to Horwitz there is a cross-talk between progesterone and EGF, both in inhibitory and stimulatory pathways. A synergistic upregulation of p21 by R5020 and EGF was detected. The PR\(^{+}\) has highly phosphorylated forms and phosphorylation of PR, at ser294, stimulates protein degradation through the ubiquitin/proteasome pathway. Progestin treatment promotes nuclear translocation of the transcription factor Stat5. While the PR\(^{+}\) is most often strongly activated, dominant negative forms are only seen for the PR\(^{-}\). Both forms are expressed in target tissues. Cell lines expressing either \(\alpha\) or \(\beta\) forms have been constructed in order to study the differential gene regulation by the PR\(^{+}\) and PR\(^{-}\). While several genes are regulated by both PR\(^{+}\) and PR\(^{-}\) the list for upregulation of genes is longer for PR\(^{+}\) (12 vs. 2), and the list for down-regulation of genes is longer for PR\(^{+}\) (11 vs. 2). Eight genes are regulated by only \(\alpha\) or \(\beta\) receptors, respectively.

The role of angiogenesis in tamoxifen resistance was discussed by Fuqua, and data was presented on introduced MCF-7 cells and estradiol pellet into ovarietomized mice. Expression array experiment was performed on estrogen-sensitive, tamoxifen-sensitive, tamoxifen-resistant and foslodex-resistant tumors. Changes were observed in the expression pattern of genes important for angiogenesis and seven genes, including genes for angiogenesis like VEGF, were down-regulated in tamoxifen-sensitive compared to estrogen-sensitive tumors, and upregulated again in the tamoxifen-resistant tumors. Nineteen genes, including endothelial receptor-related protein, were upregulated in tamoxifen-resistant tumors.

The biological actions of ER\(^{a}\) and ER\(^{b}\) were reviewed by Gustafsson. Both forms of ER bind estrogen, but the ER\(^{a}\) binds it with much higher affinity than the ER\(^{b}\) form. By using specific antibodies for both \(\alpha\) and \(\beta\) form of ER, both forms were shown to be expressed in the rat mammary gland but the presence and cellular distribution of the two receptors are distinct. The ER\(^{a}\) and ER\(^{b}\) act in different complexes, e.g. ER\(^{b}\) does not bind Hsp90. ER\(^{a}\) and ER\(^{b}\) are co-expressed in rat mammary gland and ER\(^{b}\) is more stable in expression in pregnancy and lactation, compared to the variable expression of ER\(^{a}\). In prepubertal rats, ER\(^{a}\) was detected in 40% of epithelial nuclei, 30% at puberty and continued to decrease throughout pregnancy to 5% at day 14. Induction of ER\(^{a}\) up to 70% is observed at day 21 of lactation. During pregnancy, co-expression of both receptors was rare, but 60% of epithelial nuclei show expression of both receptors during lactation in the post-proliferation phase. While earlier studies, using RT-PCR and in situ hybridization of mRNA, have shown the existence of ER\(^{b}\) in mammary gland, this study confirms the expression of the ER\(^{b}\) protein, but the precise role of the ER\(^{b}\) in the breast remains to be defined. The majority of ER\(^{b}\) positive cells does not proliferate and a high proportion of dividing cells is negative for both forms of ER and therefore, it can be concluded that the presence of these receptors in epithelial cells is not a prerequisite for estrogen mediated proliferation.

Dowsett demonstrated that the proliferation of the estrogen-sensitive breast cancer cell line MCF7 is reduced upon estrogen starvation, but after several weeks an estrogen-independent growth was observed. For putative maximal tumor control, a stepwise estrogen suppression can be used. A 4-fold apoptotic index was observed in estrogen-deprived cells. Apoptosis was increased upon tamoxifen and decreased with varozole, an aromatase inhibitor. On the contrary, IGFI expression is decreased by tamoxifen and increased by varozole. HER2 transfected MCF7 cells do not respond to tamoxifen.

Microarray methods and biological classification of breast tumors. Microarray methods in the breast cancer field were addressed by David Botstein (Stanford University School of Medicine, USA), Olli-Pekka Kallioniemi (NIH, Bethesda, USA), Åke Borg and Joe Gray. Botstein was able to group the breast tumors with respect to expression patterns of certain cell types, Kallioniemi studied specific amplics at chromosome 17q, Gray a specific amplisc at chromosome 20q and Borg different expression pattern of sporadic, BRCA1 and BRCA2 tumors (see earlier). The strength of Botstein's data is partly due to the material studied, since samples were from a clinical trial (collaboration with Anne-Lise Børresen-Dale); 62 surgical specimens from 40 patients: the 22 tumor pairs were sampled before and after 16 weeks doxorubicin treatment.
and two tumors were paired with lymph node metastases. Botstein presented a cDNA microarray of 8,102 genes. As a control 3 normal breast tissues were used and the expression pattern was closely similar. The cluster of co-expressed genes allowed classification of tumors into subtypes, depending on difference in gene expression patterns. Two types of epithelial cell clusters were observed: basal (expressing keratin 5) and luminal (expressing keratin 8). Furthermore, a specific expression pattern of normal cells within the tumors, such as B- and T-cells, and fibroblasts/stromal cells that turned out to be specific fibroblasts. Therefore, it can be concluded that this is a basis for a new molecular classification of breast cancers and the normal cell expression pattern shows the normal cell types within the tumors. The basal epithelial subgroup is associated with worse survival, to a similar level as Her2 positive tumors, and the luminal epithelial subtypes exhibit better prognosis. The p53 status in the different subgroups was as expected, except that luminal subtype A showed no mutations.

Kallioniemi discussed the benefits of CGH, tissue microarray and cDNA microarray in breast cancer research. Up to 1,000 tissue samples can be analysed in one experiment by tissue microarray and in situ analysis of DNA can be done in addition to RNA and protein analysis, and 300 sections can be cut from each block. These methods are useful in detecting target genes involved in tumor progression, like gene activation by chromosome translocation or gene amplifications, that is of significant relevance in breast cancer. By CGH, 34 DNA amplification sites have been detected in breast cancer. Kallioniemi analysed the 17q12-q21 (see earlier) and 17q23-q24 amplicons by CGH and followed the results up by tissue microarrays and chromosome 17q specific cDNA array (699 transcripts). Even though the ribosomal protein S-6 kinase is in the second place (after MB1) of genes with elevated expression on the 17q23-q24 amplicon it is 4 times amplified and 15 times overexpressed. A high level of S6 kinase activity was also detected. In 668 breast cancer cases, 8.8% show amplification of the S6 kinase and 15% overexpression. This overexpression was associated with worse prognosis and patients with tumors having elevation of both Her2 and S6 kinase have the worst prognosis. Therefore, it was concluded that the S6 kinase is a candidate oncoprotein, relevant for breast tumor progression.

Conclusion. In this intense meeting many exciting new molecular approaches and findings in breast cancer were presented. Although much remains to be done it was obvious that a remarkable progress has been achieved and the field of molecular biology of cancer will continue to provide new information and understanding regarding the complex nature of neoplastic disease in the breast. Some of these findings will possibly lead to better diagnosis and treatment of breast cancer. Abstracts presented at the Lillehammer meeting have been published in Breast Cancer Research 2, 2000, and can be found at http://breast-cancer-research.com.