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Molecular Genetic Studies of Chromosome 8 and Oncogene c-*myc* in Breast Cancer

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ACADEMIC DISSERTATION

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following communications, referred in the text by their Roman numerals:

I. Kytölä S, Rummukainen J, Nordgren A, Karhu R, Farnebo F, Isola J and Larsson C (2000): Chromosomal alterations in 15 breast cancer cell lines by comparative genomic hybridization and spectral karyotyping. Genes Chromosomes Cancer 28:308-17.

II. Rummukainen J, Kytölä S, Karhu R, Farnebo F, Larsson C and Isola JJ (2001): Aberrations of chromosome 8 in 16 breast cancer cell lines by comparative genomic hybridization, fluorescence *in situ* hybridization, and spectral karyotyping. Cancer Genet Cytogenet 126:1-7.

III. Rummukainen JK, Salminen T, Lundin J, Kytölä S, Joensuu H and Isola JJ (2001): Amplification of c-*myc* by fluorescence *in situ* hybridization in a population-based breast cancer tissue array. Mod Pathol 14:1030-5.

IV. Rummukainen JK, Salminen T, Lundin J, Joensuu H and Isola JJ (2001): Amplification of c-*myc* oncogene by chromogenic and fluorescence *in situ* hybridization in archival breast cancer tissue array samples. Lab Invest 81:1545-51.

ABBREVIATIONS

BRCA1 and 2	breast cancer-associated gene 1 and 2
CCD	cooled charged coupled device (camera)
CDK	cyclin dependent kinase
CGH	comparative genomic hybridization
CISH	chromogenic *in situ* hybridization
DAB	diaminobenzidine
DAPI	4', 6-diamino-2-phenylindole
DCIS	intraductal breast cancer (=ductal carcinoma *in situ*)
DDFS	distant disease-free survival
DNA	deoxyribonucleic acid
dUTP	deoxyuridinetriphosphate
ER	estrogen receptor
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
HRP	horseradish peroxidase
IDC	invasive ductal breast cancer
LOH	loss of heterozygosity
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PR	progesterone receptor
RT	room temperature
SKY	spectral karyotyping
SPF	S-phase fraction
SSC	standard saline citrate

ABSTRACT

Comparative genomic hybridization (CGH) studies have shown that chromosome 8 is a frequent target for chromosomal imbalances in sporadic breast cancer. The oncogene c-*myc*, which is located at 8q24.1, has been shown to be amplified, but the proportion of tumors with *myc* amplification seems to vary extensively. Similarly, there is an unresolved debate whether the clinical features (e.g. tumour size, grade, histology etc.) correlate with c-*myc* amplification. Although fluorescence *in situ* hybridization (FISH) is considered to be the most accurate method for the detection of oncogene amplification in human tumors, it has been applied to the analysis of c-*myc* only in three studies. Due to the difficulty of growing carcinoma cells in culture, many karyotyping studies on oncogenes have used breast cancer cell lines as models for primary breast tumors. However, the chromosomal aberrations of the publicly available cell lines have only recently been characterized. The impetus for the more technical considerations described here was the fact that the microscopy of FISH is demanding, especially in histologically complex clinical tumor samples. In these studies, new modifications to chromogenic *in situ* hybridization (CISH) are developed.

The aim of this study was to characterize the chromosomal alterations in breast cancer cell lines, with the focus on chromosome 8q and c-*myc* at 8q24.1. Our aim was also to characterize the clinical correlations of c-*myc* in primary breast tumors. Furthermore, we wanted to develop a straightforward but also specific detection method, CISH, to replace the laborious FISH method.

In the first study, CGH and spectral karyotyping (SKY) were used to characterize chromosomal alterations in 15 commonly used breast cancer cell lines. These, and one

additional cell line, were used in study II, which focused on the structural aberrations of chromosome 8. In this study, we confirmed the results gained from CGH and SKY analyses by arm- and locus-specific FISH. In the third study, we analyzed the amplification and clinico-pathologic correlation of the c-*myc* oncogene in 261 primary breast cancers diagnosed in Tampere University Hospital district in 1991 and 1992. The fourth study was designed to compare the sensitivity and specificity of CISH with FISH, in order to introduce a more convenient method for oncogene amplification analysis. CISH and FISH methods use basically the same hybridization procedure, but CISH analysis is technically easier and less time consuming, allowing also for evaluation of the tissue architecture with an ordinary transmission light microscope.

Our results revealed the wide variation of chromosomal aberrations in breast cancer cell lines. CGH indicated that the most frequent gains were detected at 1q, 8q, 20q, 7, 11q13, 17q, 9q, and 16p, whereas losses were most common at 8p, 11q14-qter, 18q, and Xq. The high level amplification of c-*myc* was less common than previously thought, being found only in SK-BR-3. Since there are frequent aberrations of chromosome arm 8q, it is argued that there must be other oncogenes in this genome region involved in breast cancer pathogenesis. SKY revealed a multitude of numerical and structural chromosomal aberrations, the most common being the simple translocations of the entire chromosome arms. When each chromosome was analyzed separately, chromosomes 8, 1, 17, 16, and 20 were involved more frequently in the translocation events, but the resulting derivative chromosomes and their breakpoints were strikingly dissimilar.

In the clinico-pathologic analysis of the c-*myc* oncogene in primary breast tumors, 14.6 % of the tumors showed amplification. A statistically significant association was found between c-*myc* amplification and DNA aneuploidy, progesterone receptor negativity, high histological grade, and a high S-phase fraction. A tendency of association

was seen with positive axillary nodal status. In contrast, c-*myc* amplification showed a non-significant trend with overall survival of patients with invasive cancer. These data suggest that c-*myc* amplification is associated with those breast cancers which possess more malignant features, but that it is unlikely to become a clinically useful prognostic factor. The methodological study (IV), comparing CISH and FISH methods, showed that the CISH method was highly promising in terms of sensitivity and specificity. CISH analysis is technically easier and less time consuming than FISH, though these benefits are not achieved at the expense of sensitivity or specificity.

In conclusion, the c-*myc* oncogene was found to be highly amplified in 31.3 % of the cell lines, and in 14.6 % of our primary breast tumor material. Thus, c-*myc* cannot explain all of the frequent cytogenetic aberrations in chromosome 8, especially those in the q-arm. The c-*myc* amplification is associated with more aggressive primary breast tumors, but it is not likely to be a useful prognostic marker for clinical purposes. Although further comparative studies on other clinically important oncogenes are needed, the present results suggest that CISH may eventually replace FISH in oncogene screening studies on large tumor materials, tissue arrays, and ultimately, in clinical diagnostics since it is a more simple and economical technique that also allows direct identification of the cells showing gene amplification.

INTRODUCTION

Breast carcinoma is and has been the most common malignancy among women in the western countries for the last few decades. It has been estimated that every tenth woman will be diagnosed with breast cancer during her lifetime (Pukkala et al. 1997).

Heterogeneity is in many respects the most characteristic phenomenon in breast cancer. The age of onset, clinical course, hormone sensitivity and genomic instability all vary greatly from one breast cancer patient to the next. Although there seems to be at least some epidemiological evidence for certain risk factors (e.g. old age, residence in western countries, obesity, nulliparity, early menarche, exogenous estrogens), a family history of breast cancer seems to remain the strongest risk factor in breast cancer. The vast majority (90-95 %) of breast cancers are sporadic. In the cases of hereditary cancer, the dominant risk factors include constitutional mutations of certain susceptibility genes (e.g. *BRCA1* and *BRCA2*), and these are not present in the sporadic counterparts. The hereditary tumors also show an earlier onset (reviewed in van de Vijver 2000).

Primary prevention is almost impossible since we have only sketchy knowledge of the biological mechanisms underlying the etiologic factors, much effort is being devoted to secondary prevention. The early detection methods, and general counseling of women about breast cancer and its self-detection have resulted in the diagnosis and treatment of patients at an earlier stage. As a result of these efforts, tumors are found earlier and smaller-sized, which may improve the prognosis of the patients (McPherson et al. 1997).

It is now known that the multiple successive DNA alterations are responsible for transforming normal cells into malignant cells. However, very little is still known about the importance of the many frequently detected chromosomal aberrations on the tumor

progression. As our knowledge expands about the biology and the cellular steps behind the malignant behavior of tumor cells, more precise and effective therapies will be developed. A good example of these innovations in breast cancer therapy is the introduction of the specific anti-cancer drug, trastuzumab. This monoclonal antibody is targeted against the specific particles on the surface of breast cancer cells, in tumors presenting amplification of the *HER-2/neu* oncogene.

There are numerous genetic alterations waiting to be analyzed further and possibly transformed into knowledge leading to more efficient treatment of the patients. One frequent change in breast cancer has been the amplification of chromosome arm 8q (Courjal et al. 1997). There is only one well-characterized oncogene, c-*myc*, located (at 8q24.1) in this region. The amplification of the c-*myc* oncogene has been reported in both breast cancer and in other solid tumors in a large number of studies (Escot et al. 1986, Visscher et al. 1997, Nesbit et al. 1999).

The present study focusses on clarifying the role of c-*myc* in breast cancer by studying the chromosomal changes in primary breast tumors. The emphasis was on chromosome 8 and c-*myc*, but genomic aberrations in breast cancer cell lines were also characterized. One aim was to improve the methods for detecting gene amplifications, and in that way to develop a convenient, but still specific and sensitive method.

REVIEW OF THE LITERATURE

1. Breast cancer

1.1. Epidemiology

The number of female breast cancer patients in Finland - as in many other Western countries - has been increasing over the past decade. It has been estimated that every tenth woman will be diagnosed with breast cancer at some point in her life (Pukkala et al. 1997). During the years 1994-98, the mean annual number of new breast cancer cases was 3236, rising by one hundred per year, reaching a peak of 3426 in 1998 (Finnish Cancer Registry 2000). The latest preliminary results on new cancer cases show a steady increase, with 3554 new breast cancer cases diagnosed in 1999. In 1998 about half of the breast cancers were diagnosed in women between the ages of 45 to 64 years (1744 cases out of the 3426 cases, 50.9 %), but there still remains marked variation of the age of onset. The highest peak incidence was in the age group of 50-54 years old women (579, 16.9 % of all the cases) (Figure 1).

1.2. Pathogenesis of breast cancer

The studies on colorectal cancer have constructed a pioneering model for the multi-step nature of human carcinogenesis (Fearon and Vogelstein 1990). This has occurred because it is possible to conduct histopathological definition of the different stages of the disease. There is some evidence also for the sequential progression in breast cancer; the transformation of normal cells via the steps of adenoma, premalignant changes and *in situ*

carcinoma to invasive carcinoma (Micale et al. 1994, Beckman et al. 1997). In spite of extensive efforts, the evolutionary steps of breast carcinoma are still not well understood.

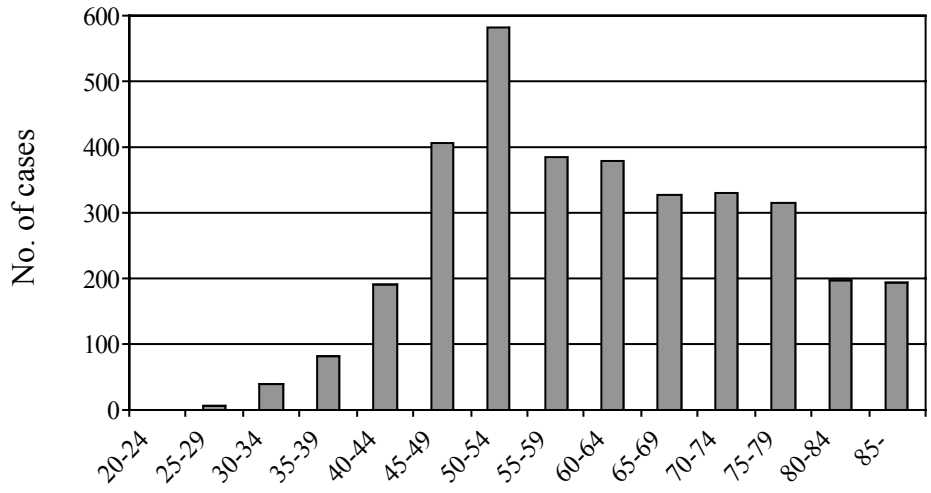


Figure 1. The number of new cases of female breast cancer in 1998 by 5-year age groups (statistics from the Finnish Cancer Registry, 2000).

Various risk factors for breast cancer have been proposed and tested over the years, factors like diet, oral contraception, postmenopausal substituent treatment with estrogen, breast irradiation, geography, occupation, etc. Epidemiologic studies have found statistical significance of estrogen stimuli, and chemical and physical carcinogenic stimuli (carcinogenic agents in food or the environment, mechanical stimuli such as irradiation, etc.). However, the underlying cytobiological processes of how these candidate factors cause carcinogenicity have remained unknown. A family history of breast cancer remains the main risk factor in breast cancer (reviewed in Biéche and Lidereau 1995).

Heterogeneity and complexity are in many respects the most characteristic phenomena in breast cancer (e.g. Escot et al. 1986). The age of onset, clinical course, hormone sensitivity and genomic instability vary greatly from one breast cancer patient to

the next. Histopathologic variance is also seen, although almost 80 % of all cases are histologically classified as ductal carcinoma. Hereditary breast cancers (about 5-10 % of all breast carcinomas) manifest with earlier onset: the mean age at diagnosis is less than 45 years (reviewed in van de Vijver 2000).

2. Methods used in the genomic studies of breast cancer

Oncogenes manifest themselves at the cellular level either by increasing the protein production through amplification of the gene or by altering the gene transcription e.g. by translocating the gene to an active transcribing position, resulting in overactive or otherwise abnormal protein function. **Immunohistochemistry** (IHC) has been used to detect overexpressed proteins in tumor tissues, giving indirectly a gross evaluation of the gene amplification through the transcription activity. The widespread use of IHC is partly due to the fact that the IHC technique itself is widely available in pathology laboratories, and that the analysis using a brightfield microscope is easily performed. However, IHC is a relatively insensitive method, due to background signaling. In the recent years there has been an intense search for ways to improve the pretreatment methods for recovering the antigenicity of tissues and gaining optimal results (Shi et al. 2001).

The genomic alterations may be studied directly using methods such as Southern blotting and karyotyping. The more recent studies have utilized techniques like loss of heterozygosity (LOH), fluorescence *in situ* hybridization (FISH) and its new multicolor variants, including spectral karyotyping (SKY), polymerase chain reaction (PCR) comparative genomic hybridization (CGH), mutation detection methods, etc.

2.1. Studies on genomic aberrations in breast cancer

The conventional cytogenetics has been applied to the identification of chromosomal abnormalities. **Karyotyping** is based on short-term cultured cells arrested in metaphase, and Giemsa stain to make the chromosomal banding visible. For many years, this was the only method to characterize chromosomal abnormalities. The complexity of the abnormal karyotypes in breast tumors makes it difficult to characterize the whole genetic "profile" of any given tumor, and to reliably define the frequency of the genomic changes found in the samples. Most of the studies revealing the karyotypes of primary breast tumors have been case reports with only one to three patients, but a few larger studies involving up to a hundred cases or more have been conducted, focusing on slightly different aspects.

In one of the earliest modern studies on chromosomal abnormalities in breast cancer (Dutrillaux et al. 1990), gain of chromosome 1q and loss of 16q were found most frequently in the less rearranged tumors. In cases with more anomalies, gains of 1q and 8q were the most frequent, whereas deletions involving many chromosomes were detected. It was concluded that the trisomy 1q and monosomy 16q were early changes, whereas other deletions and gain of 8q represented secondary changes. Later on, the group of Dutrillaux (1991) analyzed cytogenetically 113 breast carcinomas. They found cytogenetic evidence on the steps of genetic evolution from diploid to hyperploid, and in general, towards LOH at multiple sites in the genome. Rohen et al. (1995) found clonal or non-clonal chromosomal abnormalities in 118 out of 185 (64 %) cases of breast cancers. Pandis et al. (1995) found also polyclonality, 2-8 clones in 79 out of 97 cases of breast cancers (81 %).

Mertens et al. (1997) surveyed the karyotypic data on 3185 neoplasms, including 508 breast carcinomas. In that material, overall losses were more frequent than gains, and

the only chromosomal areas more often gained than deleted were 1q, 7, 12q and 20. Adeyinka et al. (1999) studied the associations of chromosomal aberrations with metastatic phenotype, identifying differences among the genetic lesions present in node positive (66 cases studied) and node negative (63 cases) breast cancers.

The majority of the oncogene studies on breast cancer have applied **Southern blotting**, which was one of the first direct methods to detect gene amplification (Southern 1975). However, its relatively low sensitivity has resulted in widely variable results in oncogene amplification studies. There are several weaknesses in this technique: first, normal cells "contaminate" the tumor sample, i.e. DNA from non-malignant cells dilutes the DNA of malignant cells, thereby decreasing its sensitivity to detect amplified gene copies (Nesbit et al. 1999). Second, the specificity and sensitivity of Southern blotting on formalin-fixed tissue samples has been questioned. After the initiation of use of the PCR techniques, there are now very few investigators performing Southern blotting on formalin-fixed tissue (Reinartz et al. 2000).

Northern blot hybridization is a variant of Southern blotting, detecting mRNA from the sample instead of DNA. The target RNA is extracted from freshly frozen tissue, digested and size-fractionated before hybridization. The result gives a gross evaluation of the transcription level of a specific gene.

The analysis of chromosomal aberrations was boosted with the **CGH** method, which was developed in 1992 (Kallioniemi et al. 1992). CGH is applicable for analyzing the numerical changes in the whole genome at the same time, and it may be used as a screening method for novel chromosomal areas either showing copy number gains or losses. CGH can also work with genomic DNA from either formalin fixed and paraffin embedded tissue, or freshly frozen tissue, and it can efficiently reveal chromosomal copy number changes (gains and losses) with a minimum size of 10 Mb in one hybridization

(Bentz et al. 1998), when the chromosomal aberration is present in at least 60 % of the cells studied (Kallioniemi OP. et al. 1994). After screening by CGH, the newly identified chromosomal areas may be studied by other methods, such as locus-specific FISH and sequence analysis, for more specifically restricted amplifications or deletions, i.e. for new oncogenes and tumor suppressor genes.

To detect balanced changes, such as reciprocal translocations - which are also important pathways of oncogene activation - newer methods, such as **SKY** and different variations of **FISH** have been developed. SKY is a method that detects genetic material from each chromosome by individual chromosome labeling, thus being able to detect both translocation partners at the same hybridization. For SKY, metaphase spreads from short term cultured cells are needed. The specific probes used in FISH give even more detailed information about which subregions of chromosomes are involved. FISH is accessible for formalin fixed tissue samples or cells from short-term cultures, arrested either in interphase or in metaphase, depending on the aim of the study. The multitissue arrays have brought new possibilities to utilize FISH, while large tumor selections, either on the same type of tumor or completely different types and tissues, may be gathered for screening for specific probes for oncogenes.

2.2. Comparative studies using multiple techniques

Before a novel technique is accepted for research, confirmation of its reproducibility is required, preferably along with comparative studies with the known methods as reference on the same samples. The comparison of the different methods is not always straightforward, because of the fact that they are focusing on the chromosomes from different perspects. For example in the same tumor, LOH can indicate loss of one allele, while CGH and FISH may show normal results. The reason for this discrepancy is that

loss of one allele may be combined with duplication of the other and this will result in a diploid gene copy number. CGH is not capable of detecting small allelic losses, while FISH can be used for the detection, with a locus-specific probe.

Persson et al. (1999) evaluated the frequencies of gains and losses found by G-banding cytogenetics and CGH by studying 29 invasive breast cancers with three different methods. The comparisons between karyotyping (after converting the karyotypes to net gains and losses) and CGH, and DNA flow cytometry and CGH were only partly concordant. Only 56 % of the cases (15/29) were in agreement by both methods. Ten out of 12 discordant cases showed a higher DNA index in flow cytometry compared to CGH, and 9 had a "simple" abnormal karyotype. Ploidy discordance was also detected in 12 cases; all the cases were cytogenetically diploid, but nondiploid by flow cytometry, showing disagreement in DNA and chromosome indices. Possible explanation for the CGH discrepancies given by the authors was that different cell populations were used in the various studies. CGH detected the predominant, often aneuploid cell clone, whereas the near-diploid minor cell clones had a growth advantage in vitro, and were used for G-banding cytogenetics. This is in line with earlier findings on the detection of chromosomal abnormalities: e.g. in the study of Steinarsdottir et al. (1995), complex chromosomal changes were yielded in 87 % of the cases by direct harvesting, compared to 44 % after culture of digested tissue ($P < 0.01$). Also polyploidy was more common in direct-harvested samples.

3. Molecular pathology of breast cancer

The multistep nature of carcinogenesis in general, as well as in breast carcinoma, has been widely accepted. Similarly to other solid tumors, the development of breast cancer is thought to be initiated after multiple successive changes in the genome of the

cells in the "target" tissue (reviewed in Biéche and Lidereau 1995, Courjal and Theillet 1997). Early studies of the age-dependence of cancer suggested that on average 6-7 successive somatic mutations are needed to convert a normal cell into an invasive carcinoma cell (Renan et al. 1993, Strachan and Read 1999).

Clinically, the genomic changes manifest as pathologically aggressive growth, invasion and metastatic behavior of the cells. The main genomic changes in breast carcinogenesis are activation of proto-oncogenes mainly through amplification, and co-ordinated inactivation of tumor suppressor genes (reviewed in Biéche and Lidereau 1995, Beckman et al. 1997, reviewed in Mertens et al. 1997). When CGH is used, the most common chromosomal imbalances detected in sporadic breast cancers are gains of 1q, 8q, 16p, 17q, and losses involving 8p, 13q, 18q, and 16q (Isola et al. 1995, Ried et al. 1995, Rohen et al. 1995, Tirkkonen et al. 1998, reviewed in Knuutila et al. 1998 and 1999). In sporadic cancer, when near-normal breast cells evolve to malignancy, the genetic abnormalities developed are so diverse that it has been suggested that no two tumors or tumor cells in any one tumor are likely to be genetically identical (Lengauer et al. 1998).

Many of the somatic genetic changes seen in sporadic cancers are present more frequently in *BRCA1* and *BRCA2* epigenetically mutated cells, confirming the nature of these breast cancer susceptibility genes as DNA stability conserving genes (reviewed in Ingvarsson 1999). Epigenetic lesions have been shown to drive genetic lesions in cancer (Esteller 2000). This may also be seen in the more malignant nature of the hereditary breast cancers as a whole.

The development of breast cancer is known to involve many types of activated or inactivated genes in order to promote malignancy. The sequential steps, however, are far less understood than what is known of the best example of tumor progression, colorectal carcinoma (reviewed in Ingvarsson 1999, Fearon and Vogelstein 1990). Studies on LOH

in synchronous benign and malignant lesions, intraductal (DCIS) and invasive breast cancer (IDC) mostly favor the theory of progression from DCIS to IDC (O'Connell et al. 1994, Munn et al. 1995, Radford et al. 1995, Fujii et al. 1996a,b), as well as there being a common clonal origin of DCIS and IDC. However, some studies have not found any common mutations in DCIS and IDC cells (Munn et al. 1996). There are also recent studies suggesting more complex pathways to breast carcinoma than the straightforward linear progression model (Kuukasjärvi et al. 1997). Nevertheless, it has been shown that both the structural and numerical aberrations tend to be more complex in more malignant and more aggressive tumors than in intraductal or less aggressive types of invasive carcinomas (Kuukasjärvi et al. 1997, Adeyinka et al. 1998, Aubele et al. 2000, Cuny et al. 2000).

4. Gene amplifications in breast cancer

To date, there are at least ten known oncogenes found frequently amplified in breast cancer (Table 1): *EGFR* (at 7p13) in 3 % of breast cancers, *FGFR1* (at 8p12) in 10 %, c-*myc* (at 8q24) in 10 %, *FGFR2* (at 10q26) in 12 %, *CCND1* and *EMS* (at 11q13) in 15 %, *HER-2/neu* (also known as *ERBB2*, at 17q12) in 20 %, *PS6K* (at 17q22-24) in 10 %, and *AIB1* and *CAS* (at 20q11-13) in 15 % of breast cancers (reviewed in van de Vijver 2000).

In addition, there are various chromosomal regions, which are frequently amplified, but no specific oncogene driving these amplifications has yet been identified. The arm amplification of entire 1q (in up to 40-60 % of breast cancers) has long been detected for, but no oncogene involved in breast carcinogenesis in this region has been identified. In addition to c-*myc* at 8q24, 8q12-22 is amplified in 10 % of breast tumors. Region 12q13 is found amplified in 6 % of breast cancers, including proto-oncogene *MDM2*, a downstream regulator of p53. However, *MDM2* expression (using

immunohistochemistry) has shown clinical relevance in breast cancer (Bankfalvi et al. 2000), which suggests that there may be another oncogene in the same region. Region 16p11-12 is found amplified by CGH in 20 % of breast cancers (Courjal and Theillet 1997). The possible oncogenes responsible for these amplifications are still not known.

Table 1. Breast cancer linked oncogenes and amplified chromosomal regions (according to van de Vijver 2000).

Amplified region	Breast cancer linked oncogenes	Incidence in breast cancer (%)
7p13	*EGFR*	3
8p12	*FGFR1*	10
8q24	c-*myc*	10
10q26	*FGFR2*	12
11q13	*CCND1*	15
	EMS	
17q12	*HER-2/neu,*	20
	also known as *ERBB2*	
17q22-24	*PS6K*	10
20q13	*AIB1 and CAS,*	15
	(others?)	
1q	?	40
8q12-22	?	10
16p11-12	?	20
20q11	?	6

Like *HER-2/neu* and c-*myc*, also many other oncogenes are more active in the early stages of the development of human embryo and fetus (Downs et al. 1989). Their proteins act in the early development and in adults in many crucial steps in the differentiation and growth of the cells, including apoptosis (e.g. c-*myc*), cell cycle regulation (*CCND1*, c-*myc*, *MDM2*), promoting cells to move from G1 to S-phase, etc. In tumorigenesis, amplification and activation of a quiet proto-oncogene results in overexpression of its protein(s), which leads to malignant features like cell cycle excitation, pathological growth and invasion of the tumor cells, lower stage of apoptosis and reduced cell differentiation.

After the introduction of a novel anti-cancer drug trastuzumab (Herceptin®), analysis of the *HER-2/neu* oncogene amplification by FISH has become an integral part of breast cancer diagnostics (Tubbs et al. 2001). Although no other oncogene has yet been employed in the therapy of breast cancer, many research groups are focusing on the prognostic significance and clinical relevance of many of the oncogenes and also in finding new candidate oncogenes in the amplified regions.

5. Tumor suppressor genes

The other main line of genetic changes in tumorigenesis is the inactivation of the cell "protector" genes, tumor suppressor genes. These genes function mainly to repair damaged DNA. One group is called the mismatch repair genes (e.g. *MSH2*, *MLH1* in colorectal carcinoma, Caluseriu et al. 2001). Replication errors involving the defects in DNA mismatch repair genes (RERs) are key features in hereditary non-polyposis colorectal cancer (HNPCC), whereas in breast cancer they have been reported only at a low frequency (reviewed in Ingvarsson et al. 1999).

Table 2. Tumor suppressor genes known to be linked to sporadic breast cancer (according to van de Vijver 2000).

Rearranged region	Tumor suppressor genes	Incidence in breast cancer (%)
6q26-27	*IGF-II*	<10
9p21	*p16INK4a* (*p16*)	(rare)
10q23	*PTEN*	1
17p12	*p53*	20
16q22.1	E-cadherin gene	?

There are to date five known tumor suppressor genes affected in sporadic breast cancer, two of which are more frequent: *TP53* (at 17p12) and E-cadherin gene, *CDH1* (at 16q22.1) especially in lobular carcinomas (reviewed in van de Vijver 2000). In hereditary breast cancer, there are germline mutations in the susceptibility genes, such as *BRCA1* (at 17q21) and *BRCA2* (at 13q12) (Miki et al. 1994, Wooster et al. 1994, Zheng et al. 2000). These genes function as tumor suppressor genes in the maintenance of genetic stability through participating in the cellular response to DNA damage (Zheng et al. 2000). No mutations in *BRCA1* and *BRCA2* have been reported in sporadic late onset breast cancers.

In tumor progression, the first mutation is critical: it should provide an otherwise normal cell with a growth advantage over the adjacent cells. The gatekeeper hypothesis of Kinzler and Vogelstein (1996, 1997) suggests that some certain gene is responsible in any given tissue for maintaining a constant cell number. A mutation of a gatekeeper leads to a permanent imbalance of cell division over cell death, offering a growth advantage, while mutations in other genes have no long-term effects. No gatekeeper gene has been proposed so far for breast cancer (reviewed in Ingvarsson et al. 1999).

In many cases, one tumor suppressor gene allele is mutated by a relatively subtle mutation (point mutation, small insertion or deletion), while the other allele is completely lost. LOH studies have been the main source of information on tumor suppressor gene changes; in addition to the known genes, a relatively large number of other regions have been detected by LOH, where efforts are being dedicated to identify the tumor suppressor genes (reviewed in van de Vijver 2000). Other means to identify tumor suppressor genes are further elucidation of the lost regions seen by CGH, or studying the rare familial cancers - as in the case of aggressive retinoblastoma (tumor suppressor gene *RB1* at 13q14) - following the Knudson's two-hit mechanism (Knudson 1971). According to this theory, two successive mutations are needed to turn a normal cell into a tumor cell. This has been confirmed to be a valid mechanism in tumor progression in the studies of tumor suppressor genes (Strachan and Ried 1999), but also contradictory results have been reported, e.g. in a study on E-cadherin (*CDH1*) (Cheng et al. 2001).

Inactivation of tumor suppressor gene is possible through a few mechanisms, e.g. point mutations and deletions. Recently, also gene inactivation by promoter region hypermethylation leading to transcriptional silencing has been demonstrated (Jones and - Laird 1999, Krop et al. 2001). Examples of abnormal methylation in the promoter region of genes include the first tumor suppressor gene described, the retinoblastoma (*RB1*) gene (Jones and Laird 1999). Abnormal methylation has been detected also in some genes involved in breast cancer: the p16 (*INK4a*), the estrogen and progesterone receptors, and E-cadherin (Esteller 2000), as well as a putative tumor suppressor gene *HIN-1*, which is frequently inactivated in the early stages of tumorigenesis (Krop et al. 2001). Methylation of DNA may play an important role in cancer pathogenesis, because this change is epigenetically transferred to the next generation. Furthermore, the frequency of changes

in methylation has probably been underestimated, because methylated bases can not be detected by the standard techniques for mutation screening (Strachan and Ried 1999).

6. Chromosome 8 in breast cancer

A gain of the chromosome arm 8q is one of the most common alterations in primary breast cancer found by CGH (Isola et al. 1995, Ried et al. 1995, Nishizaki et al. 1997, Tirkkonen et al. 1998, reviewed in Knuutila et al. 1998, Roylance et al. 1999). Amplifications of proto-oncogene c-*myc* at 8q24.1 and the more proximal subregion of 8q12-q22 are frequently present. No oncogenes mapping to the region of 8q12-q23 have yet been identified as being involved in breast cancer, but it has been suggested that there is one oncogene which may be deregulated by copy number increases in some tumors and by translocation in others (Fejzo et al. 1998).

6.1. The central role of c-*myc* in cellular functions

Proto-oncogene c-*myc* has been extensively studied since it was first discovered nearly 20 years ago (Bishop 1982, reviewed in Liao and Dickson 2000). The c-*myc* gene is a nuclear transcription factor, which encodes three c-Myc proteins, c-Myc1, c-Myc2 and c-MycS, which arise from alternative initiation codons (Batsché and Crémisi 1999). At the C-terminus, there is a helix-loop-helix and leucine zipper region (HLH-LZ), which is the binding site for the Max protein, the dimerization partner protein needed for transactivation by Myc (reviewed in Liao and Dickson 2000). It is now known that the c-*myc* gene participates in most aspects of cellular function, including replication, growth,

metabolism, differentiation, and apoptosis (reviewed in Dang 1999, and in Liao and Dickson 2000).

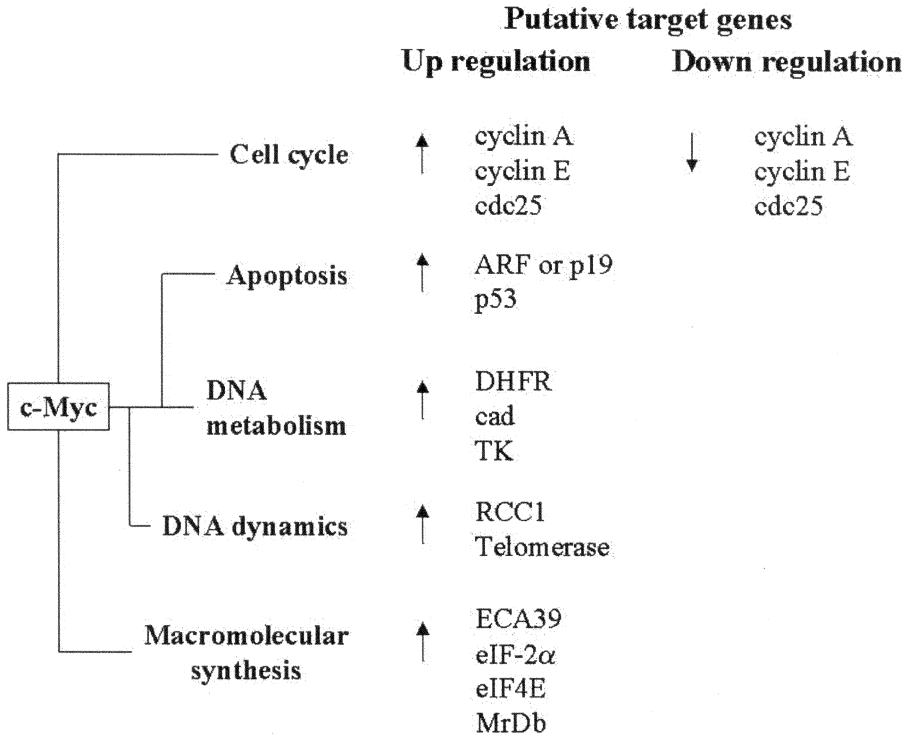


Figure 2. Cellular functions, which are influenced by c-Myc has influence, and the putative target genes that c-Myc proteins regulates. Modified from Dang 1999 (Fig. 4 and Table 1).

Since the c-*myc* oncogene is so crucially involved in the malignant outcome of various different tissue types, the complex mechanisms of its function have been intensely studied. Many target genes for c-*myc* have recently been identified, but the specific pathways have remained obscure (Dang 1999, Coller et al. 2000). For example, the c-Myc protein activates several positive regulators of the cell cycle (Fig. 2), such as *cyclins D1*, *E* and *A*, G1-specific cyclin dependent kinases (CDKs), in particular cyclin

E/CDK2 complex, and apparently also cyclin D/CD4 and CDK6 complexes (reviewed in Amati 1998, Obaya et al. 1999). The promotion of cell cycle by c-*myc* gene was originally thought to be elicited via activation of those target genes (reviewed in Amati 1998), but further studies have revealed more complex patterns of c-*myc* gene function.

Only a few of these putative target genes (e.g. cyclin D1 and CDK4, reviewed in Liao and Dickson 2000) include a Myc E-box element in their regulatory region which is the necessary binding site for the transactivating/trans-suppressing Myc-Max dimer. On the other hand, apoptosis driven by Myc has been detected also in the absence of Max (Wert et al. 2001). Furthermore, *in vitro* studies show that instead of transactivation, c-*myc* suppresses the transcription of the *cyclin D1* gene (Jansen-Durr 1993, Philipp 1994).

These paradoxes have led to different explanations, such as indirect mechanisms of c-*myc* gene activating functions (reviewed in Liao and Dickson 2000), and the involvement of other concurrent c-Myc binding proteins, in addition to Max, which may change the c-Myc-Max functions (Sakamuro and Prendergast 1999). It is also known that the ratio of the two Myc proteins is disrupted *in vivo* in many tumor types, and this has been suggested as an alternative pathway to amplification in the malignant transformation of cells (Batsché and Crémisi 1999).

6.2. The proto-oncogene c-*myc* in breast cancer

Amplification is considered as the main pathway of c-*myc* activation in breast cancer (Dang 1999, reviewed in Deming et al. 2000). In some other tumor types, c-*myc* is known to be activated by translocation to an active chromatin domain, e.g. in Burkitt's lymphoma, where c-*myc* is translocated to 14q32, 2p12 or 22q11, i.e. loci for the immunoglobulins IGH, IGK and IGL, respectively (Strachan and Read 1999). In these cases, the noncoding exon 1 is usually not included in the translocated sequence, and

being sited at an actively transcribed region, c-*myc* is expressed at an inappropriately high level (Strachan and Read 1999).

The proportion of breast tumors with c-*myc* amplification ranges widely in different studies, from 1 to 94 %, with an average of 15.5 % (reviewed in Deming et al. 2000). Also the relationships between amplification and c-*myc* overexpression have not yet been described, and overexpression without coexisting gene amplification has also been proposed to occur (reviewed in Deming et al. 2000, and Liao and Dickson 2000). Until recently, Southern blotting has been the most widely utilized method for evaluating the amplification of c-*myc*, the other two methods used being PCR and slot blotting (Visscher et al. 1997, reviewed in Deming et al. 2000). The inconsistency found in the results is suggested to arise from the differences in the methodologies used, and normal cell contamination of the tumor samples (Nesbit et al. 1999). FISH is currently considered as the most accurate method for analyzing oncogene amplification in human tumors (Persons et al. 1997, Press et al. 1997, Tubbs et al. 2001), but it has been applied to c-*myc* in breast cancer only in three studies. The proportion of tumors with amplification of c-*myc* seen in these studies has varied from 12 % (Schraml et al. 1999), 21 % (Persons et al. 1997) up to 86 % (Visscher et al. 1997).

In breast cancer, the amplification of c-*myc* may correlate either positively or negatively with alterations in other genes (reviewed in Liao and Dickson 2000). Positively correlating genes include *HER-2/neu*, where co-amplification results in a significant reduction in the survival of the patients, indicative of cooperative effects (Cuny et al. 2000). One of the Myc downregulating proteins is the product of the putative tumor suppressor gene *BRCA1* (breast cancer-associated gene 1); this can physically bind to the c-Myc protein and repress c-*myc*-mediated transcription (Wang et al. 1998). This indicates that the tumor suppressor function of BRCA1 may at least partly be related to its

repression of the transcriptional activity of c-*myc*. Another tumor suppressor gene also inactivated in breast cancer is PTEN, which has been shown to repress the transcription of c-*myc* in MCF-7 and MDA-MB-486 breast cancer cell lines (Ghosh et al. 1999). In the tumors developing after administration of these cells to nude mice, the PTEN induced repression of c-*myc* is followed by increased apoptosis and growth inhibition (Ghosh et al. 1999).

AIMS OF THE STUDY

The aims of the present study were:

I To characterize chromosomal alterations in breast cancer cell lines.

II To further characterize structural aberrations affecting chromosome 8.

III To clarify the role of c-*myc* amplification as a prognostic marker in breast cancer.

IV To compare CISH and FISH techniques in the analysis of c-myc oncogene status in formalin-fixed paraffin-embedded tumor samples.

MATERIALS AND METHODS

1. Breast cancer cell lines (I, II)

Fifteen established breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD): BT-474, BT-549, CAMA-1, DU4475, MCF7, MDA-MB134, MDA-MB-157, MDA-MB-361, MDA-MB-436, SK-BR-3, T-47D, UACC-812, UACC-893, ZR-75-1 (I, II), and MDA-MB-415 (II). The MPE600 breast cancer cell line (I, II) was a generous gift from Prof. Helen Smith (Geraldine Brush Cancer Research Institute, San Francisco, CA).

The cells were cultured in L15, MEM with Earle's salt, M199, or RPMI 1640 (all from GIBCO, New York, NY), with 10-20 % fetal calf serum, and with additives recommended by the ATCC. The harvesting of cells, fixation, and preparation of metaphase spreads for FISH and SKY were prepared according to standard protocols. The slides were stored at -20°C until they were balanced at room temperature for 1-10 days and hybridized. Genomic DNAs of the cell lines and the reference DNAs from peripheral blood lymphocytes were isolated using proteinase-K digestion and phenol-chloroform-isoamylalcohol extraction according to standard protocols (Sambrook et al. 1989).

2. Primary breast cancer patients and tumor samples (III, IV)

A total of 351 archival paraffin blocks from primary invasive breast cancers were collected. According to the data files of the Finnish Cancer Registry, these tumors represent 84.2 % of all primary invasive cancers operated during 1991-1992 in the Tampere University Hospital district (population ~400,000). The mean age of the patients

was 61.6 years. The histologic types were available for 241 tumors including 198 (82 %) ductal, 21 (9 %) lobular and 22 (9 %) tumors of special histologic types. The previously collected clinico-pathologic data (Lundin et al. 2001) included patient age at the time of diagnosis, postoperative tumor size, axillary nodal status and presence of distant metastases at the time of the diagnosis (pTNM), histologic grade (G), estrogen and progesterone receptor status (ER and PR, respectively), DNA ploidy and the S-phase fraction (SPF). The median follow-up time for the patients still alive was 6.8 years (range 5.1 to 7.8 years), which enabled analysis of distant disease-free survival data (DDFS) five years from the diagnosis.

The tumors were routinely fixed (overnight in 10 % buffered formalin) and processed into paraffin blocks according to the established protocols. Representative tumor regions were selected from hematoxylin and eosin–stained sections. Tumor tissue array blocks were made as follows: one tissue cylinder with a diameter of 0.6 mm was punched through the selected tumor areas from each “donor” tissue block. One tissue core from each donor block was then inserted into four ”recipient” tissue array paraffin blocks using a specific custom-made instrument as described elsewhere (Kononen et al.1998). The 351 tumors formed a set of four tissue array blocks. Sections of the resulting multitumor tissue array blocks were then cut at 5 µm, collected on SuperFrost Plus glass slides, and baked in a 60ºC oven for 2 to 4 hours before starting the FISH and CISH procedures.

3. Comparative genomic hybridization (I, II)

CGH was carried out and the results were analyzed as described, using the recently developed four-color modification of the basic technique with minor modifications. This technique includes two reference DNAs, thereby providing an internal standard in each

experiment (Karhu et al. 1999). Briefly, DNA samples from the cell lines were labeled with FITC-12-dUTP (DuPont, Boston, MA) by nick translation, and normal female and male DNAs were labeled with Texas Red-5-dUTP (DuPont, Boston, MA) and Cy5-dUTP (Amersham Life Science, Arlington Heights, IL), respectively. The denatured sample DNA and the two reference DNAs were hybridized together with Cot-1 blocking DNA onto denatured metaphases of normal lymphocytes (Vysis, Downers Grove, IL). After hybridization and washing, the slides were counterstained with DAPI (4', 6-diamino-2-phenylindole, Boehringer Mannheim) in ProLong mounting medium (ProLong® Antifade Kit, Molecular Probes, Eugene, OR, USA) and analyzed as described by Karhu et al. (1999). DNA sequence copy number changes were detected by analysis of the hybridization intensities of tumor and reference DNAs along the length of all chromosomes in each metaphase cell as described (Karhu et al. 1999). Eight to twelve ratio profiles were averaged for each chromosome, and green-to-red (g/r) fluorescence ratios less than 0.80 were scored as losses, those exceeding 1.20 as gains, and regional aberrations with a g/r ratio >2.0 as high-level amplifications adopted for the four-color CGH.

4. Spectral karyotyping (SKY) (I, II)

The commercially available probe cocktail containing 24 differentially labeled chromosome-specific painting probes and Cot-1 blocking DNA (SKY kit, ASI Applied Spectral Imaging, Migdal Ha'Emek, Israel), was denatured and hybridized to denatured tumor metaphase chromosomes according to the protocol recommended by the manufacturer. After hybridization and washing, the chromosomes were counterstained with DAPI. Image acquisitions were performed using a SD200 Spectracube system (ASI) mounted on a Zeiss Axioskop microscope with a custom-designed optical filter (SKY-1,

Chroma Technology, Brattleboro, VT). The conversion of emission spectra to the display colors was achieved by assigning blue, green, and red colors to specific sections of the emission spectrum. For each cell line, at least seven metaphase cells were analyzed. Abnormal chromosomes denoted as SKY derivative chromosomes were defined as containing material from two or more chromosomes detected by SKY.

4.1. Comparison of CGH and SKY data (I)

For comparison of CGH and SKY, the SKY data were transformed to copy number imbalances (reviewed in Mertens et al. 1997). The sum of chromosomal material (indicated by each SKY color) was estimated and was transformed to an average copy number by dividing the 'sum' of each chromosome by the average ploidy (= number of chromosomes divided by 23). DAPI banding was used to reveal large deletions, duplications, isochromosome formation etc., which were also transformed to copy number imbalances.

5. Fluorescence *in situ* hybridization (I-IV)

5.1. Probes

In Studies I and II, conventional FISH on metaphase spreads was performed using standard protocols. This was to confirm the SKY results and to further characterize chromosome 8. The whole chromosome painting probes for chromosomes 4, 6, 8, 13, 15, 16, 17, and 20, the arm-specific probes for 8p and 8q (provided by Prof. Paul Meltzer, Cancer Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD) were labeled by nick translation using FITC-12-dUTP and Texas Red-5-dUTP (DuPont, Boston, MA). The locus-specific probe for chromosome 8

centromere (pJM128, ATCC, Rockville, MD) was labeled by nick translation using FITC, and the probe for c-*myc* (RMC08p001) was labeled with either Texas Red or digoxigenin (II-IV). To improve the success rate of FISH in Study III, a second set of tissue array slides was hybridized with a commercial c-*myc* probe labeled with Spectrum Orange (LSI *c-myc*, Vysis, Inc, Downers Grove, IL), using the same centromere 8 probe as the reference.

5.2. Procedure

The FISH procedure was conducted according to standard protocols (Sambrook et al. 1989). The probe cocktail containing the appropriate probes (the whole chromosome painting probes in Study I, chromosome arms 8p and 8q in Study II, and c-*myc* gene and the centromere of chromosome 8 in Studies II-IV) with human placental DNA and Cot-1 DNA (Roche Molecular Biochemicals, Mannheim, Germany) was applied onto the slides of metaphase spreads. The slides were then coverslipped and sealed with rubber cement.

Tumor array tissue sections (III,IV) were first de-paraffinized before hybridization, and pre-treated in a microwave oven for 10 min at 92°C in Tissue Pretreatment Buffer, (Zymed Inc, South San Francisco, CA) followed by digestion with proteinase K (0.25mg/ml in 2x standard saline citrate solution, SSC) for 20min. The slides were rinsed with 2 x SSC, dehydrated with graded ethanols and air-dried, prior to hybridization. Ten µl of the same probe cocktail (probes for c-*myc* gene and the centromere of chromosome 8 with human placental DNA and Cot-1 DNA) was applied onto tumor array slides. The slides were then coverslipped and sealed with rubber cement, and denaturation was carried out at 94°C for 3 min on a thermal plate.

After an overnight hybridization at 37°C, the slides were stringency washed with two successive incubations in 0.5 x SSC and 4 x SSC. The digoxigenin-labeled c-*myc*

probe was detected with anti-digoxigenin rhodamine (0.7 µg/ml, Roche Molecular Biochemicals). The slides were counterstained with 0.4 µM DAPI in antifade solution (Vectashield, Vector Laboratories, Burlingame, CA), and embedded.

5.3. FISH microscopy

The microscopic analysis of FISH was performed using an Olympus BX50 epifluorescence microscope (with 60x oil immersion objective), equipped with a CCD camera (Photometrics, Tucson, AZ). At least ten metaphases were analysed for the c-*myc* oncogene copy number. For the tumor tissue arrays, at least 50 nonoverlapping nuclei in every tumor sample were scored for the analysis.

The c-*myc* scoring results were expressed as the actual copy numbers per cell in the majority of the cells in each sample. The analysis was performed in two different ways: as two-color FISH (c-*myc* and centromere 8) (II-IV), and single-color FISH (c-*myc* only) (IV). In two-color FISH, the average c-*myc* copy number was compared with the centromere 8 copy number count. Amplification was defined to be present when at least two extra copies of c-*myc* were seen in at least 20 % of the analyzed cells. In single-color FISH, the presence of six or more copies of c-*myc* was considered as amplification. Copy number counts with two c-*myc* probes (RMC08p001 and the Vysis' probe) were virtually identical, and the FISH result was recorded if either probe set yielded a satisfactory hybridization result. All c-*myc* analyses were carried out blinded to the clinical outcome of the patients.

6. Chromogenic *in situ* hybridization (CISH) (IV)

The protocol used has been modified and improved from Tanner et al. (2000, 2001). Prior to hybridization, the tissue sections were deparaffinized in xylene, washed in 100 % ethanol, and air-dried. Slides were then incubated in 0.1 M Tris-saline (pH 7.3) at 121°C in an autoclave for two minutes. After cooling at room temperature (RT) for 15 minutes and rinsing twice in phosphate-buffered saline (PBS) for 3 minutes, the tissue sections were covered with 100 µl of pepsin solution (Digest-All 3, Zymed) at 37°C for 6-8 minutes. The slides were then washed in PBS three times at RT for 2 minutes, dehydrated in graded ethanols and air-dried. Ten µl of probe cocktail (2 µl of digoxigenin-labeled P1 probe to c-*myc* oncogene RMC08p001, 1 µl of 9.9 µg/µl of human placental DNA and 1 µl of 1µg/µl of Cot-1 DNA, Roche Molecular Biochemicals, and 6 µl of master mix) was applied on the tissue sections. Slides were coverslipped and sealed with rubber cement to prevent evaporation of the probe solution. Slides and the probe mixture were co-denatured at 94°C for 3 minutes on a thermal plate, and hybridization was carried out in a humid chamber at 37°C for 36-40 hours (over two nights). After hybridization, the slides were washed with 0.5 x SSC for 5 minutes in 75°C, followed by three washes in PBS for 2 minutes in RT. The c-*myc* probe was detected with sequential incubations with 0.3 µg/ml mouse anti-digoxigenin (Roche Molecular Biochemicals, in PowerVision Blocking solution), goat anti-mouse HRP polymer (PowervisionR, ImmunoVision Technologies Co, Daly City, CA), and diaminobenzidine (DAB) (ImmunoVision Technologies Co) as chromogen. Incubation in DAB Enhancer (Zymed) for 3 min was used to further enhance the signal intensity. After light counterstaining with hematoxylin, the slides were dehydrated and embedded.

CISH hybridizations were analyzed using a Nicon Labophot transmission light microscope with a 40x objective. At least 50 non-overlapping nuclei in every tumor sample were scored to determine the number of c-*myc* signals. The results were expressed as the actual copy numbers per cell in each sample. Amplification was defined to be present when six or more copies of c-*myc* were detected in at least 20 % of the screened malignant cells.

7. Statistical methods (III, IV)

Contingency tables of c-*myc* gene status by FISH (III, IV) and CISH (IV), and clinico-pathologic variables were analyzed with Fisher's exact test. The χ^2 test for trend was used to compare c-*myc* with histologic grade. The analysis of disease-free survival was performed using Kaplan-Maier survival analysis and survival of different groups was compared with log-rank test. All p-values were two-tailed.

RESULTS

1. Chromosomal alterations in breast cancer

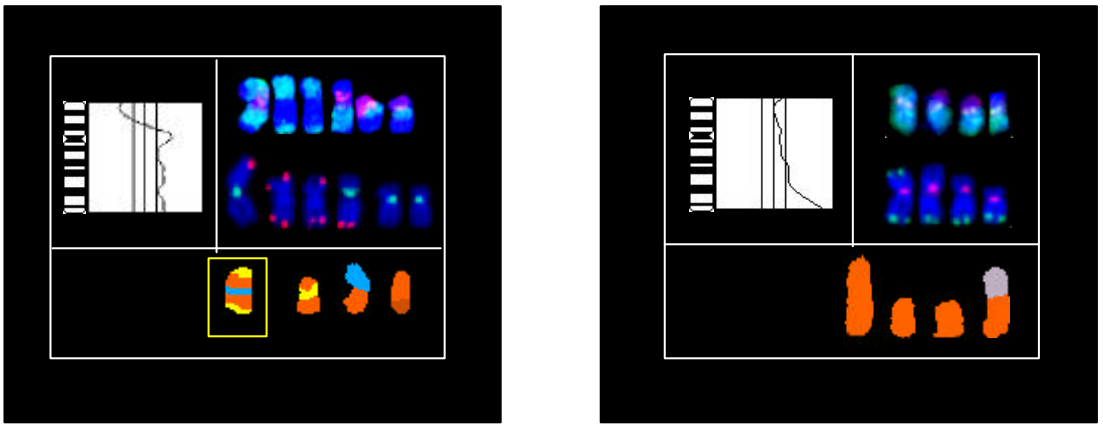
1.1. Comparative genomic hybridization

The mean number of CGH alterations in the fifteen breast cancer cell lines was 19.6 ± 9.4, mean ± SD (range 7-39) per cell line, and in general, gains were more common than losses (12.4 ± 6.3 and 7.2 ± 4.2, respectively). Gains were detected in all lines and most frequently involved 1q (87 %), 8q (87 %), 20q (80 %), 7 (73 %), 11q13 (73 %), 17q (73 %), 9q (60 %), and 16p (47 %), with high-level amplifications within 1q, 8p10-p12, 8q12-qter, 11q13, 16p, 17q, and 20q. Losses were identified in all cell lines except UACC-812 and were seen most frequently at 8p (53 %), 11q14-qter (53 %), 18q (47 %), and Xq (47 %).

1.2. Spectral karyotyping

The chromosome numbers in 15 breast cancer cell lines ranged from 35 to 144 (modal number 39-103). Hypotriploid to hypertetraploid chromosome contents predominated and were seen in ten out of the fifteen cell lines. MDA-MB-361 and MDA-MB-157 were found to be hyperdiploid, MPE600 was diploid, and MDA-MB-134 and MDA-MB-436 were hypodiploid. In two cell lines (MDA-MB-157 and MDA-MB-436), two clones with different chromosome numbers were identified; in addition to near-diploid clones, hypertetraploid or hypertriploid clones with several of the markers present in duplicate were also seen.

Most of the SKY-derivative chromosomes (i.e. those having translocations or insertions), were simple including material from two chromosomes (232 out of 303 derivative chromosomes, 76.6 %). Based on inverted DAPI banding, many of these translocations were shown to consist of entire chromosome arms. Complex translocations including chromosomal material from up to four chromosomes were also found on several occasions. In SK-BR-3, complex derivative chromosomes were detected, two of which consisted of 5 and 7 different chromosomes.



A **B**

Figure 3. The cell lines CAMA-1 (A) and UACC-812 (B) are shown as examples of the usefulness of different methods. Figure of CGH-ratio of chromosome 8 (in the left box), chromosomes including material from chr. 8 from metaphase spreads of arm-specific and locus-specific FISH (upper row on the right side), and SKY chromosomes (in the box at the bottom). Chr. 8 is shown in orange, and the translocation partner chromosomes are shown in yellow (chr. 1), turquoise (11), brown (5) and lilac (3) (see chapter 1.3 in text).

Reciprocal translocations were identified in five cell lines, BT-474 t(3;20)x2, BT-549 t(X;10), DU4475 t(X;10), UACC-812 t(5;12), and ZR-75-1 t(4;7). Isochromosomes of lq, 5p, 8q, and 20q were found in the DU4475, MDA-MB-361, MCF7, and ZR-75-1 cell lines, respectively. Rearranged chromosomes that are compatible with the definition

of an isoderivative chromosome were found in two cell lines (BT-474, CAMA-1; boxed in Figure 3A). In these chromosomes, the normal p or q arm had been lost, whereas the other arm was rearranged and duplicated thus forming a symmetric isoderivative chromosome. No double minutes or ring chromosomes were found.

1	**53**																					
2		**24**																				
3	2	4	**38**																			
4	2			**15**																		
5	2	2	2		**19**																	
6	1	4		3	1	**30**																
7	2	3	2	2		4	**38**															
8	6	4	6	2	3	2	3	**79**														
9	1		2					2	**18**													
10	3	1	1			2	2			**24**												
11		2	4	1	1		1	**13**	1		**37**											
12	3	1	2		3	3	2	4				**33**										
13	2		5			1	2	2	1		1	2	**32**									
14	4		1	1		2		2	4	2			1	**23**								
15	3	1	2	1	2	2	3	2	2	1		2		1	**30**							
16	5					1		9			3	6	1	1	3	**43**						
17	4		1			2	3	8	3		3	1	2		3	5	**45**					
18	2		1					1		1	1			2		1	1	**13**				
19	2	1	1	1	1		2	3		2	2	1		1			5		**26**			
20	5	1	2		2		4	3	1	3			9			4	2	2	3	**42**		
21	1			1		1	1			1	2	1	1			1					**10**	
22							1	1		1	2	1	1	1	1	1	2		1	1		**14**
X	3			1		1	1	3	1	4		1	1		1	2		1					**20**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**X**

Figure 4. Translocation events between chromosomes. Events on chromosome 8 are framed by a bold line. Translocation frequencies of five or more are highlighted with a gray background color, the highest frequency with dark gray (8;11). The total numbers of translocation events per chromosome are shown in bold on the right side, diagonally.

A quantitative analysis of the data revealed 3-46 (23.7 ± 13.4) SKY-derivative chromosomes per cell line. In 11/15 cell lines, two identical derivative chromosomes were found. In addition, translocations between the same two chromosomes occurred more than once in the same cell line; for example, material from chromosomes 8 and 11 (n = 13), 13 and 20 (n = 9), 8 and 16 (n = 9), and 8 and 17 (n = 8) was frequently translocated together. (Figure 4). The most common translocations in the different cell lines, however, were t(8;11), t(12;16), t(1;16), and t(15; 17), which were seen in 6, 5, 5, and 4 cell lines, respectively. When each chromosome was analyzed separately, chromosomes 8, 1, 17, 16, and 20 were most frequently involved in translocation events. When the number of translocation events was adjusted to the size of the chromosome (Morton, 1991), chromosomes 20, 17, 8, 16, and 19 were most frequently involved.

1.3. Combination of CGH, SKY and FISH

The parallel use of SKY, CGH, and inverted DAPI banding pattern was found to be most powerful in the characterization of the chromosomal rearrangements. An example of the parallel use is given in Figure 3; which shows the analysis of chromosome 8 and its translocation partners in cell lines CAMA-1 and UACC-812.

We assessed the concordance between CGH and SKY by analyzing copy number imbalances of the 23 chromosomes in all 15 cell lines. Altogether, 345 chromosomes were analyzed, in which CGH and SKY (including inverted DAPI banding information) were matching in 329 (95 %) and not matching in 16 instances (5 %). A positive correlation between an increasing number of CGH alterations and an increasing number of translocation events in the individual cell lines was demonstrated.

Quantitatively, the number of copy number gains and losses by CGH correlated well with the number of translocation events in each cell line ($r = 0.70$, $P < 0.0001$).

2. Structural aberrations of chromosome 8 in breast cancer

2.1. Imbalances of chromosome 8 by CGH

Fourteen out of sixteen (87.5 %) breast cancer cell lines showed copy number changes by CGH, most frequently gains on q-arm and losses on p-arm. No imbalances were detected in DU4475 and MPE600.

The minimal common region of loss on p-arm was defined to be 8p22-pter, and was detected in eight of the cell lines (BT-474, CAMA-1, MCF7, MDA-MB-134, MDA-MB-157, MDA-MB-361, MDA-MB-415, and UACC-893). A gain of p-arm was seen in four cell lines BT-549 (8p), MDA-MB-134 (amplification of 8p10-p12), MDA-MB-436 (at 8p21-pter), and ZR-75-1 (at 8p10-p21).

A gain of q-arm was detected in 14 cell lines. The gain involved the entire q-arm in six cell lines (BT-549, CAMA-1, MDA-MB-134, MDA-MB-157, UACC-812 and UACC-893), and the minimal common region of 8q23-qter in seven cell lines (BT-474, MCF7, MDA-MB-361, MDA-MB-415, MDA-MB-436, SK-BR-3 and T-47D). In ZR-75-1, the gain was located juxtacentromeric on 8q10-q21. High-level gains (suggesting amplification) were seen in only four cell lines: BT-474, MDA-MB-436 and T-47D on 8q23-qter and SK-BR-3 on 8q12-q22 and 8q23-q24.

2.2. Translocations of chromosome 8 by arm-specific painting FISH and SKY

The cell lines BT-549, DU4475 and MPE600 contained five, four and two copies of normal chromosome 8, respectively, while the remaining 13 cell lines had one or more derivative chromosomes with material from chromosome 8. Two structurally normal-like copies of chromosome 8 and one or two chromosomes showing translocations were present in four cell lines: MDA-MB-415, UACC-812, T-47D and ZR-75-1. Numerous

complex derivative chromosomes containing material from chromosome 8 were seen in nine cell lines: BT-474, CAMA-1, MDA-MB-134, MDA-MB-157, MDA-MB-361, MDA-MB-436, MCF7, SK-BR-3 and UACC-893. Three cell lines showed only derivative chromosomes: CAMA-1, MDA-MB-436, and UACC-893.

The number of derivative chromosomes containing material from chromosome 8 varied from one to seven, with some of the chromosomes being present in duplicate. Chromosome 11 was the most frequent translocation partner (Fig. 4) participating in one derivative chromosome in five cell lines (MCF7, MDA-MB-134, MDA-MB-436, SK-BR-3, ZR-75-1) and in two different translocated chromosomes in two cell lines (BT-474 and CAMA-1, Fig. 3A). Translocations with chromosomes 5 and 6 were also common, being seen in three and four cell lines, respectively. Using the inverted DAPI bands the breakpoints appeared to be strikingly dissimilar. Translocation counterparts excluded only four chromosomes: 10, 18, 19, and 21. One copy of isochromosome 8q was present in MCF7 and UACC-812. One copy of isoderivative 8q was found in CAMA-1, and two different copies in MDA-MB-157.

3. The role of c-*myc* amplification in breast cancer

3.1. Copy numbers of c-*myc* in breast cancer cell lines by CGH and FISH

Ten cell lines showing gain of the c-*myc* gene region 8q24 by CGH (BT-474, CAMA-1, MCF7, MDA-MB-134, MDA-MB-361, MDA-MB-415, MDA-MB-436, SK-BR-3, UACC-812, and UACC-893), contained 1-11 extra copies of c-*myc* by FISH. A high-level amplification of c-*myc* was found only in SK-BR-3.

The region of the c-*myc* gene showed gain by CGH in MDA-MB-157 and T-47D, but by locus-specific FISH, no copy number changes of c-*myc*, relative to the number of

centromere 8 were seen. In the remaining three cell lines (BT-549, DU4475 and MPE600), the copy number of chromosome 8 was assessed as normal by both methods.

3.2. Amplifications of c-*myc* by FISH in primary breast tumors

In FISH analysis, 261 out of 351 primary tumor samples were interpretable. c-*myc* was amplified in 38 (14.6 %) out of the 261 informative cases. The distribution of extra copy numbers varied from 2 (12 cases) to over 10 (3 cases) with the median being 4 additional copies relative to chromosome 8 centromere. The proportion of cells showing amplification varied from 20 to 100 %.

3.3. Association with the clinico-pathologic data and patient survival

The statistically most significant association of c-*myc* amplification was found with aneuploidy (p = 0.0011, odds ratio 4.8). The negative correlation of c-*myc* amplification with progesterone receptor expression was also very significant (P = 0.0071, odds ratio 0.34), PR-negativity being associated with amplifications. The high S-phase fraction tended to be associated with c-*myc* amplification with a p-value of 0.052 (odds ratio 2.4), and the presence of axillary nodal metastases with a p-value of 0.080 (odds ratio 2.0). The other parameters investigated (age, tumor size, presence of metastases, estrogen receptor expression and distant disease free survival) were not significantly associated with c-*myc* amplification.

4. Comparison of CISH and FISH methods using the c-*myc* oncogene status

CISH and FISH results were both available for 177 from the total of 351 primary breast tumors. In this material, c-*myc* oncogene was found amplified in 14.1 % of tumors by CISH, 14.0 % by two-color FISH, and in 10.8 % by single-color FISH. Amplification detected by CISH was highly concordant with both two- and single-color FISH. The kappa coefficients measuring agreement between the assays were 0.76 and 0.67, respectively.

The associations between the clinico-pathologic factors and c-*myc* amplification as detected by single-color FISH and CISH were essentially the same. Thus, c-*myc* amplification detected by both CISH and two-color FISH was significantly associated with high histologic grade, a negative progesterone receptor status, DNA aneuploidy, and a high S-phase fraction. Furthermore, c-*myc* FISH was associated also with a positive lymph node status, but the association between c-*myc* amplification detected by CISH and the lymph node status did not reach statistical significance ($P = 0.11$).

Amplification of c-*myc* as detected by CISH was associated with poor distant metastasis free survival in a univariate survival analysis ($P = 0.0013$), but this association was weaker when amplification was detected by FISH ($P = 0.16$ for two-color FISH and 0.065 for single-color FISH). In addition, it is noteworthy that a non-amplified but aneuploid copy number of c-*myc* by CISH (4 to 5 copies/cell) was associated with poorer outcome than the presence of 1-3 copies/cell, whereas when the c-*myc* copy number was determined by one-color FISH, patients with low-degree aneuploidy had a similar outcome as those with no c-*myc* amplification. Quite analogously with the CISH analysis, when c-*myc* was determined by two-color FISH, patients with low-degree aneuploidy had a similar prognosis as patients with a high c-*myc* copy number.

DISCUSSION

1. Chromosomal alterations in primary breast cancer and breast cancer cell lines

Heterogeneity and complexity are characteristic phenomena in sporadic breast cancer. This is also true on a genome-wide scale: genetically identical tumors have not been described (Lengauer et al. 1998). Genetic aberrations are responsible for transforming the normal cells into malignant cells, but no "gatekeeper" gene has been shown to be responsible for the ignition of tumorigenesis in sporadic breast cancer. Cytogenetic analyses have shown that out of the wide variation of genomic alterations, there are a few chromosomal regions predominantly involved in breast carcinoma.

The most commonly amplified regions in primary breast cancer are 1q, 8q, 17q, and 20q (Isola et al. 1995, Ried et al. 1995, Rohen et al. 1995, Tirkkonen et al. 1998, reviewed in Knuutila et al. 1998 and 1999). Our CGH analysis performed on fifteen established breast cancer cell lines agreed well with the previous studies on primary tumors. The CGH analysis revealed a wide quantitative and qualitative variation of 7 to 39 genetic alterations per cell line. In general, gains were more common than losses, suggesting that there is a predominance of oncogenes over tumor suppressor genes. The most frequent gains were detected in concordance with the earlier studies. In our material, there were also high-level amplifications detected within the region of the oncogene *FGFR1* (8p10-p12), *CCND1* and *EMS* (11q13). In two highly amplified regions, 8q12-qter and 16p, there are still no known oncogenes.

While CGH gives a valid overall picture of the numerical changes, i.e. gains and losses, of DNA material, SKY offers a more qualitative analysis of the nature of the

changes. A quantitative analysis of our data from the SKY analyses revealed 3 to 46 (23.7±13.4) chromosomes per cell line including material from two or more chromosomes. The most common translocation events in the different cell lines were t(8;11), t(12;16), t(1;16), and t(15;17). When each chromosome was analyzed separately, chromosomes 8, 1, 17, 16, and 20 were most frequently involved in translocation events.

Interestingly, there was a positive correlation between the numeric alterations seen in CGH and the number of translocation events detected by SKY in each cell line. It may be concluded that the aberrations are most often the result of unbalanced chromosomal changes. The parallel use of SKY, CGH, and inverted DAPI banding pattern was found to be most powerful in the characterization of the chromosomal rearrangements; CGH is optimal for detecting the gross numerical changes, whereas SKY with DAPI banding reveals the fine structure of the translocation partners and the breakpoints.

The overall impression of the genetic alterations of breast cancer is that of chaos: most of the aberrations are not recurrent, resulting in highly individual genetic profiles in the cell lines – as well as of the primary tumors. The aberrations include mostly unbalanced translocations, with varying breakpoints. Furthermore it seems, that along with the change from primary tumors to the cell lines, also the complexity of the genomic changes is increased. This is a reflection of the sequential multiplication of genomic changes included in the progression of the tumorigenesis in breast carcinoma.

1.1. The cell lines as models for primary tumors

Since solid tumors are technically difficult to analyse by cytogenetic methods, other means for cancer studies have been developed. Established cell lines are generally considered as powerful tools in the analysis of cytogenetic and molecular genetic changes related to tumorigenesis (Larramendy et al. 2000).

A few studies have compared genetic alterations of the cell lines with the primary tumors (Kallioniemi A. et al. 1994, Davidson et al. 2000, Forozan et al. 2000, Larramendy et al. 2000). In the study of Kallioniemi A. et al. (1994), the most common copy number changes were remarkably similar both in their set of primary tumors and the established cell lines: gains of whole chromosome arm at 1q and 8q, and regional amplifications at 17q22-q24 and 20q13.

Davidson et al. (2000) compared the CGH results of 19 breast cancer cell lines and 106 primary breast carcinomas, and performed 24-color karyotyping on the cell lines. The variation of alterations was from perfectly diploid to highly aneuploid. Almost all translocations (98 %) were unbalanced. Also this group found the t(8;11) to be the most frequent change, and chromosome 8 to be the most frequently translocated chromosome.

In the large study of Forozan et al. (2000), 38 established cell lines were analyzed by CGH. In the comparative study of the CGH from the cell lines and the DNA copy number changes collected from 17 published studies, including 698 primary breast tumors, several genes were found that were recurrently highly overexpressed; e.g. RCH1 (at 17q23), TOPO II at (17q21-q22), CAS and MYBL2 (20q13).

Larramendy and his coworkers (2000) used 18 breast cancer cell lines and five primary tumors, from which also five of the cell lines were derived. In their analyses, the cell lines showed more DNA copy number changes by CGH than the primary tumors, but all aberrations in the primary tumors, with one exception, were also found in the corresponding cell lines.

These previously published data along with our recent results, suggest strongly that the cell lines broadly represent the fresh primary tumors, and that they may well be used as a tool for research in breast cancer tumorigenesis.

2. The role of chromosome 8 and c-*myc* in breast cancer

Since the c-*myc* oncogene is so markedly involved in the malignant outcome of various different tissue types, its amplification as well as the complex mechanisms of its function have been intensively studied.

In our studies, 10 cell lines (62.5 %) showed gain of the c-*myc* gene region 8q24 by CGH, five cell lines (31.3 %) showed 2-11 extra copies of c-*myc* by FISH, when referenced to the centromere count. A high-level amplification of c-*myc* was found only in SK-BR-3. In the SK-BR-3 cell line, chromosome 8 material was also seen as a part of several SKY-derivative chromosomes: c-*myc* was detected on one normal-appearing chromosome 8 and on six complexly translocated chromosomes. The c-*myc* signal always co-localized with the presence of 8q material, suggesting other co-amplified meaningful regions at 8q. As seems to be the tendency in previous studies, the primary tumors showed lower amplification rates also in our studies: in 14.6 % of the tumors c-*myc* was amplified, but only 3 cases (1.1 %) showed high amplification with 10 or more extra copies of c-*myc*.

The previous studies on c-*myc* have been mostly performed by Southern blotting, the other two commonly used methods are PCR and slot blotting. Only a few studies have used FISH. The proportion of breast tumors with c-*myc* amplification ranges widely in different studies, from 1 to 94 %, with the average being 15.5 % (reviewed in Deming et al. 2000). Since also recent FISH studies are inconsistent, only part of this variation can be explained by differences in the assay methods. Another possible reason is that the tumors used belong to varying clinical stages; c-*myc* has been associated mostly with the more aggressive tumors. However, most of the recent studies have used tumors smaller than 2 cm in diameter, thus the amplification incidences of c-*myc* could be expected to be lower. Another possible source of bias is the fact that the histologic diagnoses of lobular

and ductal carcinoma are usually not analyzed separately, but mixed as one group of tumors. One recent study has found differences in the amplification of c-*myc* between lobular and ductal carcinomas (Janocko et al. 2001). The conclusion was that the amplification of c-*myc* was a characteristic feature in non-lobular breast cancers along with *HER-2/neu* amplification and *p53* dysfunction. The majority of breast tumors are ductal carcinomas (approximately 80 %). Since there is a small percentage of lobular carcinomas, and because lobular carcinomas are usually found among grade I tumors, the grading has been considered as an adequate clinical parameter. It might still be of clinical and academic interest to further analyze the differences between the two main subgroups of breast tumors.

Due to the low amplification frequencies, the activation of c-*myc* through amplification-independent pathways has been proposed. The promotion of the cell cycle by the c-*myc* gene was originally thought to be elicited via activation of some of the positive regulators of the cell cycle, such as *cyclin D1* (reviewed in Amati et al. 1998). However, only few of the putative target genes include a Myc E-box element in their regulatory region which is the necessary binding site for the c-myc-max dimer. Different theories have been proposed, such as indirect mechanisms of c-*myc* gene activating functions (Liao and Dickson 2000), and the involvement of other concurrent c-Myc binding proteins, in addition to max, which may change the c-myc-max functions (Sakamuro and Prendergast 1999). A tumor suppressor gene PTEN (phosphate and tensin homolog deleted on chromosome ten) inactivated in breast cancer, has been shown to repress transcription of c-*myc* in MCF-7 and MDA-MB-486 breast cancer cell lines (Ghosh et al. 1999).

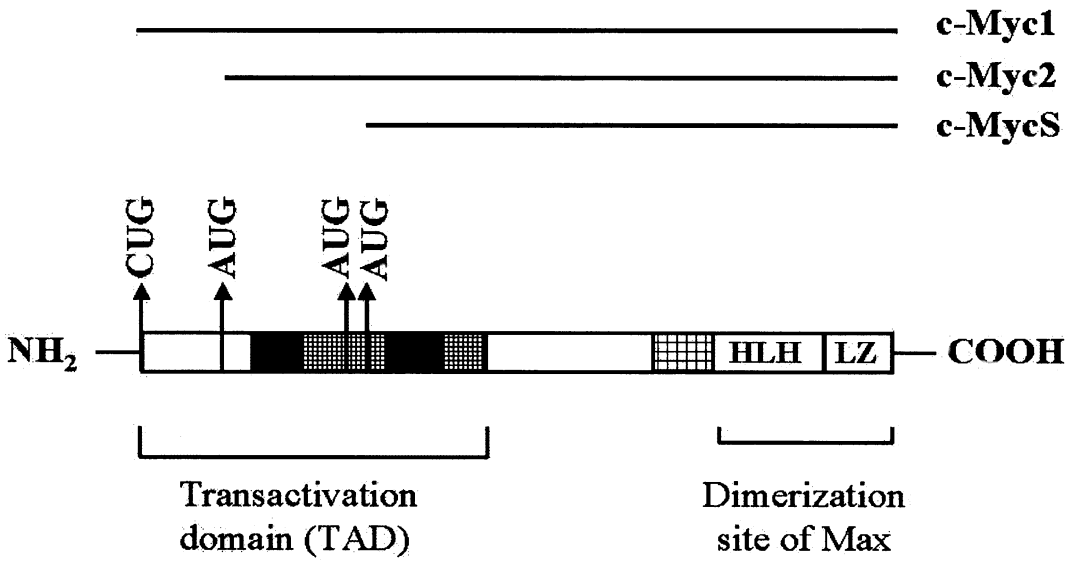


Figure 5. Schematic diagram of the structure of c-*myc*. The initiation sites of the three proteins are marked with arrows (CUG and AUGs). The helix-loop-helix and leucine-zipper –region of the max dimerization site are shown at the 3' end. Modified from Figure 1 in Liao and Dickson 2000.

Also the significance of the alternative c-Myc products, c-Myc1, c-Myc2 and c-MycS, has been studied (reviewed in Liao and Dickson 2000). The major form, c-Myc2 (usually referred to as "c-Myc"), is approximately 62 kDa, while c-Myc1 is 2-4 kDa larger (Fig. 5). c-MycS initiates downstream, resulting in a protein lacking about 100 amino acids at the N-terminus. Myc1 and Myc2 harbor in their amino terminus a transactivation domain (TAD), within which are two highly conserved regions among the members of Myc family; the Myc homology boxes I and II (MBI and MBII). The MBI has been shown to be required for the transactivation activities of c-Myc, whereas the MBII is needed for the trans-suppression activities (Sakamuro and Prendergast 1999).

While both MBI and MBII are included in the full-length c-Myc proteins, the c-MycS lacks MBI, and thus is deficient for transactivation but still retains trans-suppression. It would be interesting to resolve further the regulation of the transcription of the different proteins of c-Myc, and to determine to what extent this prospect reveals the many complex roles of the c-*myc* gene in cellular life.

3. CISH as a new method to detect oncogene amplification

Studies on genomic alterations in sporadic breast cancer have employed different techniques, such as G-banding and karyotyping, CGH, SKY, and chromosome painting- and arm-specific FISH. CGH is a powerful tool for screening the whole genome in only one hybridization, giving a gross evaluation of the genomic regions of interest. However, only after the additional analysis with a more specific method, such as SKY or FISH, can the minute changes, e.g. translocations, be characterized (II, Nielsen et al. 1997, Teixeira et al. 2001).

When one wishes to perform a genome analysis on amplified chromosomal subregions, FISH is considered to be the most specific and sensitive method. The main advantage of gene-specific FISH is its high sensitivity and specificity, as well as the possibility to analyze archival formalin-fixed tumor samples. However, it is a demanding method, requiring an epifluorescence microscope - and instant analysis of the fast fading signals. One clear disadvantage of FISH is the inability to ensure the malignant nature of the analyzed cells.

Since the introduction of a novel anti-cancer drug trastuzumab (Herceptin®), analysis of the *HER-2/neu* oncogene amplification by FISH has become an integral part of breast cancer diagnostics (Tubbs et al. 2001). Due to the inherent technical disadvantages of FISH, a new modification, CISH, has been developed (Tanner et al.

2000, 2001). In CISH, the probe detection is based on the use of a peroxidase reaction, the end product of which is visible in conventional light microscopy. FISH and CISH use similar tissue section pretreatments and probe hybridization protocols, and differ only in probe detection. According to our hypothesis, the specificity and sensitivity of the *in situ* hybridization procedure can also be achieved with a more convenient detection system. Compared with FISH, CISH offers simple and straightforward microscopic evaluation, potential for concomitant morphological examination of the target tissue, and simple identification of those cell types showing gene amplification.

In the FISH analyses, the reasons for unsuccessful analyses included tissue damage, and a weak hybridization signal and high background. According to our experience, weak hybridizations occur in 10-15 % of FISH with ordinary tumor tissue sections as well. The tissue array technique itself has a few additional technical problems: the small samples are sometimes detached from the slides during the procedure, and the pretreatments needed for each sample vary greatly. The same pretreatment can give perfect signals on one sample, whereas a second sample may be either totally damaged or totally intact showing no signals at all.

In our CISH analyses, 207 out of 345 (60 %) of the tissue array samples were interpretable. Logically, with the similar pretreatment and hybridization protocol, the same reasons for drop-outs as in FISH analysis were also valid with CISH. In CISH, we used no differentially labeled reference probe. Instead, we determined the cut-off level as six copies of c-*myc*, since the polyclonality studies have not shown six-fold polysomies, suggesting that copy numbers exceeding six cannot be due to DNA aneuploidy (Tanner et al. 2000). This may lead us to overlook the meaningful low-level amplifications of 4 to 5 copies. However, in FISH analysis, the average oncogene copy numbers detected may be diluted by the copy numbers of non-malignant cells, which, in turn, would decrease the

average copy number counts. We would argue that the high sensitivity of the probe detection by CISH, and in particular the possibility to restrict the copy number count to the truly malignant cells on a morphologic basis, means that we are bound to end up with more accurate results.

4. The future prospects on genomic aberrations in breast cancer

The sequence and the meaning of many alterations in breast cancer still remains unknown. There are a variety of oncogenes known to be mutated to a varying extent in any given tumor type, also in breast cancer. The involvements of chromosome 8 and c-*myc* in breast cancer tumorigenesis have been proven. However, characterization of the additional pathways of activation of c-*myc* would be a major step forward, because of the discrepancies between oncogene copy numbers detected and expression and functions of its proteins. The oncogene c-*myc* and all its networks are not yet fully understood.

To date, only one gene has proved to be of therapeutic importance in breast cancer, the *HER-2/neu* oncogene, whereas many other oncogenes have prognostic potential. It is a challenging task to profile the meaningful genomic changes in breast cancer, and eventually not only to link the changes to the clinical features to gain prognostic information, but also to generate more efficient treatments to the profiled breast tumors, perhaps including also c-*myc*.

It is my conclusion that the chromogenic detection applied to the *in situ* hybridization technique will become a widely used method due to its inherent advantages compared with the accurate FISH method.

SUMMARY AND CONCLUSIONS

The genomic changes present in the established breast cancer cell lines have been poorly characterized. We analyzed the chromosomal alterations of fifteen breast cancer cell lines by CGH, FISH and SKY. Since chromosome 8 has frequently been demonstrated as aberrant both in primary tumors and in cell lines, it was selected for detailed analysis. The cell lines appeared to be almost chaotic in their variety of genetic alterations. The copy number gains by CGH correlated well with the frequency of translocation events by SKY, suggesting that the majority of the translocations were unbalanced. Also the break points in the derivative chromosomes varied remarkably. The genomic alterations were usually non-recurrent, although some chromosomal regions predominated, for example the terminal end of the chromosome arm 8q. The results were concordant with the previous data on primary breast tumors and cell lines, confirming the validity of using cell lines as models for primary breast tumors.

Amplification of the oncogene c-*myc* (at 8q24) has been frequently detected. However, only a few studies have been performed using FISH, which is considered as the most accurate method for detecting oncogene amplification. It has been proposed that c-*myc* might have a central role in the pathogenesis of breast cancer, and that it may be clinically useful. In our series, the oncogene c-*myc* was found to be amplified in 31.3 % of cell lines and in 14.6 % of the primary breast tumors. The frequency of amplification of c-*myc* was lower than previously thought. Although the copy number of c-*myc* seems to correlate to the more advanced and aggressive forms of breast cancer, it is present at such a low frequency, that it does not provide a clinically useful prognostic marker. On the other hand, the protein c-Myc is seen overexpressed more frequently, posing the

question, what are the other pathways of c-Myc activation in addition to gene amplification.

After the finding of therapeutic significance for one amplified oncogene (*HER-2/neu*), a method as specific and as sensitive as FISH, but also less complicated, is needed for routine laboratory use. Chromogenic *in situ* hybridization was evaluated in comparison with the results of locus-specific FISH using the c-*myc* as the target gene. Our results confirm that the CISH method is as sensitive and specific as FISH. Before the wider use of CISH, evaluation with a more highly amplified oncogene, such as *HER-2/neu*, is recommended.

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