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ANALYSIS OF CHROMOSOMAL REARRANGEMENTS AND GENE COPY NUMBER CHANGES IN BREAST CANCER CELLS

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Äntligen!

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ABSTRACT

Genome changes in terms of numerical chromosomal aberrations and structural rearrangements, including deletions, amplifications and translocations, gradually accumulate in the genome during tumor development. These genomic changes are likely to play an important role in the process of tumor progression. A detailed high resolution study of genome rearrangements in breast cancer and their relation to tumor progression and risk for metastasis was performed.

A combination of the high resolution arrayCGH (aCGH) technique ROMA (Representational Oligonucleotide Microarray Analysis) and the molecular cytogenetic technique FISH (Fluorescence In Situ Hybridization) made it possible to identify genes and chromosomal regions that display aberrations as gains, losses and rearrangements. These chromosomal aberrations were analyzed quantitatively and related to the progression of the cancer disease.

Three characteristic patterns of genomic copy number variations in breast cancer were identified, simplex, complex I ("sawtooth") and complex II ("firestorm"). The simplex pattern has broad segments of duplication and deletion, usually comprising entire chromosomes or chromosome arms. The "sawtooth" pattern is characterized by many narrow segments of duplication and deletion, often alternating, more or less affecting all the chromosomes. The "firestorm" pattern resembles the simplex type except that the cancers contain at least one localized region of clustered, relatively narrow peaks of amplification, with each cluster limited to a single chromosome arm. The simplex pattern is associated with low malignant tumors whereas the complex patterns are associated with high malignant tumors. M-FISH enabled us to study the spatial organization between different chromosomal regions in the cell nucleus and validate the observed gene copy number changes quantitatively.

A method, named Sector-Ploidy-Profiling (SPP), was developed and used to compare different subpopulations from different areas in a tumor. Clonal genomic heterogeneity was found to be very common in breast cancers. The clonal subpopulations were found to be either anatomically separated or intermixed. By comparing the different subpopulations, a better understanding of the order of genomic events during tumor development could be obtained.

Inspired by the simplex and complex patterns we developed an objective estimate of genomic alterations in aCGH data. By using this method a clear relationship between genomic alterations (copy number changes and structural rearrangements) and gene expression subtypes was found.

By combining ROMA with M-FISH it became possible to study specific translocations in interphase chromatin in clinical samples. The specific translocation between chromosome 1 and chromosome 16, t(1;16), could now be visualized in both invasive breast carcinoma and in ductal carcinoma in situ (DCIS), indicating that the translocation t(1;16) is an early event during breast cancer development. In DCIS however, cells with and without translocations were found to co-exist in the same area of the tumor. This suggests that intraductal proliferation leading to DCIS precedes the translocation t(1;16).

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LIST OF ABBREVIATIONS

aCGH	Array CGH
BAC	Bacterial artificial chromosome
bp	Base pair(s)
BRCA1	breast cancer 1, early onset
BRCA2	breast cancer 2, early onset
CAAI	Complex arm aberration index
CCD	Charged coupled device
CGH	Comparative genomic hybridization
CNP	Copy number polymorphism
CNV	Copy number variants
COBRA	Combined binary ratio labeling
DCIS	Ductal carcinoma in situ
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ER	Estrogen receptor
FACS	Fluorescent-activated cell sorting
FISH	Fluorescence In Situ Hybridization
HER2	Human epidermal growth factor receptor 2
kb	Kilo base pairs
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
M-FISH	Multi-color FISH
N.A.	Numerical aperture
PCR	polymerase chain reaction
PgR	Progesterone receptor
pRB	Retinoblastoma protein
QM-FISH	Quantitative multigene FISH
Ras	Rat sarcoma
ROMA	Representational oligonucleotide microarray analysis
SKY	Spectral karyotyping
SPP	Sector ploidy profiling
TNM	Tumor, Node, Metastasis
TSG	Tumor suppressor gene
WAAI	Whole-arm aberration index

1 INTRODUCTION

Cancer is a genetic disease that develops from single cells that acquire mutations in the genome as the cells evolve from normal to malignant (Vogelstein and Kinzler 1993). Mutations can be a single nucleotide change, deletion or duplication of a DNA sequence (Wiedemann and Morgan 1992). Cancer represents a spectrum of biologically different diversities and can occur in most organs of the human body. Each cancer is unique with its own genetic defects. However, the characteristic phenotypes of cancer cells are similar; The hallmarks of cancer cell (Hanahan and Weinberg 2000) is self-sufficiency in growth signals, insensitivity to antigrowth signals, evading apoptosis, limitless potential for replication, sustained angiogenesis and tissue invasion and metastasis. Each one of these hallmarks will be briefly discussed below.

Self-sufficiency in growth signals. To initiate cell division, normal cells require mitogenic signals from the surrounding environment to divide and proliferate. Cancer cells, on the other hand, often produce their own growth signals and can proceed through the different cell cycle phases and divide without signals from its surroundings.

Insensitivity to antigrowth signals. Normal cells can arrest and differentiate in response to antiproliferative signals. Cancer cells on the other hand, are often insensitive to these antimitogenic signals and continue to proliferate unchecked

Evading apoptosis. Many pathways and signals, such as DNA damage, can activate the cellular suicide program. When the cell receives a cell death signal, it undergoes organized degradation of its cellular organelles following activation of proteolytic enzymes, known as caspases. Caspase activation leads to DNA fragmentation, shrinking of the cell membrane and eventually neighboring cells consume the cellular remains. Tumor cells avoid apoptosis even when the damaged DNA has not been repaired, which can result in genomic instability.

Limitless potential for replication. Normal cells undergo a limited number of cell divisions and then lose their ability to divide. However, tumor cells can replicate an infinite number of times. The process of aging is complex and how it is regulated is not exactly understood. Normally telomeres get shorter with every round of DNA replication but tumor cells can avoid this by, for example, activating a mechanism that maintains telomere length. One way to do this is by activation of telomerase (Greider and Blackburn 1985).

Sustained angiogenesis. In normal tissues the growth of new blood vessels is transient and tightly regulated. Cancer cells, however, induce and sustain blood vessel growth. A fundamental step of malignant progression is that the tumor expands and grows beyond a certain size. For this to happen, the cancer cells in the tumor secrete signals that stimulate formation of new blood vessels to support its growth.

Tissue invasion and metastasis. Normal cells do not have the ability to grow and survive outside their own niche. Cancer cells, on the other hand, have the potential to invade surrounding tissue and metastasize to distant locations. A whole series of

controls that normally keep cells in its true environment is altered when cancer cells escape from its originating tissue including the degradation of proteins that make up the surrounding extracellular matrix (ECM). When tumor cells metastasize, they are called secondary tumors, as compared to the primary tumor where the cancer originated from.

Acquisition or elimination of the individual properties mentioned above is enabled by mutations in genes regulating cellular homeostasis and DNA. These genes can be categorized into two gene groups that are responsible for tumorgenesis: oncogenes and tumor suppressor genes (TSG). Properties of these groups of genes will be discussed below.

2 BREAST CANCER

Breast cancer, is, like most other cancer types, a heterogeneous disease with respect to its clinical and biological behavior (Perou, Sorlie et al. 2000; Sorlie, Perou et al. 2001). Breast cancer is the most common cancer disease among women in the western world. In Sweden 7049 women and 39 men were diagnosed with the disease. The relative survival rate of the disease five years after diagnosis is about 90 percent, and after 10 years the corresponding number is 80 percent (Cancer Incidence in Sweden 2008 2009). The majority of breast cancers are sporadic, but there is a small group that is attributed to known hereditary factors. Two genes involved are BRCA1 and BRCA2. With mutations in these genes the risk of breast cancer is highly increased (Edlich, Winters et al. 2005).

Breast cancer classification includes histopathological type, stage, grade and receptor status and are described below.

2.1 HISTOPATHOLOGICAL TYPE

The main tissue of the breast are the mammary glands. Each mammary gland consists of multiple lobules connected to ducts and surrounding tissue, and includes blood vessels. Malignant breast tumors usually arise from one of the above mentioned structures. Ductal carcinomas are most common (75-80% of cases), followed by lobular carcinomas (10-15% of cases). Malignancies arising from other connective tissues of the breast are not as common.

2.2 STAGING

Tumor stage is a clinical evaluation of how far the disease has progressed at the time of diagnosis. In Sweden the TNM (Tumor, Node and Metastasis) classification is used for classifying the extent of cancer spread. TNM is based on primary tumor size, presence and distribution of regional lymph nodes and distant metastases.

2.3 GRADING

Grading of the disease is a microscopic estimation of the morphological abnormality and is an indicator of prognosis in breast cancer. Invasive breast carcinoma is histologically graded (grade 1-3) using the Elston-Ellis system, which is based on the status of tubule/gland formation, nuclear pleomorphism and mitotic count (Elston and Ellis 1991) (Elston and Ellis 2002). Each of these characteristics is scored from 1 to 3 and the scores are then summarized resulting in a value between 3 and 9. The grade is then assigned as follows: Grade 1 (3-5p) – well differentiated, Grade 2 (6-7p) – moderately differentially and Grade 3 (8-9p) – poorly differentiated.

2.4 RECEPTOR STATUS AND PROLIFERATION MARKERS

Hormone receptor status and proliferation markers of the breast tumor are also tested. These include the status of Estrogen (ER) and Progesterone receptors (PgR). Each of these receptor statuses are scored as either positive or negative. Cancers that are hormone-receptor-positive (ER+/PgR+) usually grow slowly, and have a much better chance of responding to hormone-suppression treatment than cancers that are hormone receptor negative (ER-/PgR-) (Key and Pike 1988). Another marker assessed is HER2-receptor status, since it effects the treatment the patient will receive. HER2 positive breast cancers are, for example treated with Herceptin which is a drug that targets the HER2-receptor protein on the cell surface (Albanell and Baselga 1999). Proliferation markers, such as Ki67 and Cyklin A, may also be used as prognostic factors.

In 2000, Sørlie and Perou et al. proposed that breast cancer could be divided into subsets that have different clinical outcome. The subsets are based on expression of a few hundred mRNA transcripts. Five molecular subtypes have been identified: basal like, normal like, HER2, Luminal A and Luminal B. The subgroups differ in clinical characteristics and prognosis. Luminal A has a good prognosis and Luminal B, also described as triple negative (ER-, pGR- and HER2-) has an unfavorable prognosis (Perou, Sorlie et al. 2000) (Sorlie, Perou et al. 2001). These patterns are further compared with the genomic pattern in Paper III.

2.5 TREATMENT

Breast cancer is usually treated by surgical removal of the primary tumor with breast-conserving surgery (partial mastectomy, segmental resection or lumpectomy) or removal of the whole breast (mastectomy) and axillary lymph node sampling, e.g. sentinel node procedure. Information about lymph node status has great impact on the decision of further treatment. Breast-conserving surgery is usually followed by radiotherapy. Adjuvant therapy includes chemotherapy, endocrine therapy and/or biological target therapies. Choice of therapy is based on staging, grading and receptor status.

3 CANCER GENOMICS

As mentioned above, mutations of two major types of genes are involved in the development of cancer. Some of these genes are positive regulators of cancer growth, oncogenes, and some are negative regulators of cancer growth, tumor suppressor genes (TSG).

3.1 ONCOGENES

The first indication of the existence of oncogenes was done by Peyton Rous, as he identified a retrovirus that caused tumor formation in chicken (Rous 1911). Michael Bishop and Harold Varmus later showed that oncogenes in retroviruses have a cellular origin, and this led to the idea that oncogenes represent incorrectly activated normal genes (Stehelin, Varmus et al. 1976). An oncogene is a mutated form of a proto-oncogene, a gene that normally drives cell division in a controlled manner when it is required (Todd and Wong 1999).

Oncogene activation can result from chromosomal translocations, gene amplifications or mutations leading to deregulation of the of the gene product (Vogelstein and Kinzler 2004). An example of an oncogene in breast cancer is erbB2 which is amplified and overexpressed in about 20% of primary breast cancers. Overexpression of the corresponding protein HER2, leads to aberrant cell growth regulation (Lane, Beuvink et al. 2000). Other common oncogenes are CCND1, c-MYC and Ras (Rat sarcoma) (Osborne, Wilson et al. 2004).

3.2 TUMOR-SUPRESSOR GENES

The tumor suppressors are in involved in the control of cell division and thereby have capacity to prevent tumor formation. Alfred Knudsen formulated a “two hit hypothesis” in 1971 (Knudson 1971) which states that both copies (maternal and paternal) of the particular TSG must be inactivated before any effect can be seen. As long as one allele is intact it can usually compensate for the loss of the other. Loss of function of both gene copies commonly arises through a loss of an entire chromosome or chromosome arm, coupled with a mutation of the other allele. Mutations that inactivate TSGs are deletions, point mutations and epigenetic silencing (Knudson 2002).

Tumor suppressor genes are involved in many pathways such as apoptosis, negative cell cycle control and DNA repair and can be divided into two major groups i.e. “gatekeepers” and “caretakers” (Kinzler and Vogelstein 1998). The “gatekeepers” includes all direct inhibitors of cell growth. The “caretakers” are genes that indirectly suppress tumor development by being involved in DNA repair and prevention of genomic instability. One of the most studied tumor suppressor gene is p53, which has been referred to as “the guardian of the genome” (Lane 1992) because of its importance in cancer protection. This is achieved by inducing cell cycle arrest and/or apoptosis as a response to, for example DNA damage or oncogene activation (Levine 1997). Mutations of p53 are estimated to occur in 20-30% of the breast cancers (Hollstein,

Sidransky et al. 1991). Other identified TSG in breast cancer are the Retinoblastoma gene (RB), BRCA1 and BRCA2 (Macleod 2000).

4 CHROMOSOMAL ABERRATIONS

Already in 1914 Theodore Boveri suggested that cancer might result from a disturbance in the chromosome balance (Holland and Cleveland 2009). This was only a theory until 1960 when the Philadelphia chromosome, identified as a minute chromosome, was discovered in patients with leukemia (Nowell 1962). The Philadelphia chromosome was later identified as a translocation between chromosome 9 and chromosome 22 (Rowley 1973).

Chromosomal aberrations can involve numerical chromosomal changes and/or structural chromosomal changes including deletions, amplifications and translocations. Numerical chromosomal abnormalities is a condition known as aneuploidy. Aneuploidy is a defect in chromosome number as a result of miss-segregation of chromosomes during cell division (Lengauer, Kinzler et al. 1997; Thompson and Compton 2008). Human tumors can be divided into two groups, A-tumors and D-tumors, based on their gross ploidy level as judged from their nuclear DNA content. A-tumors represent highly aneuploid tumors and D-tumors represent diploid or near-diploid tumors. It was shown in 1970s and early 1980s that A-tumors are associated with bad prognosis (Atkin 1972; Eneroth and Zetterberg 1975; Eneroth and Zetterberg 1976; Zetterberg and Esposti 1976; Auer, Caspersson et al. 1980).

Furthermore, it has been shown that A-tumors, besides from being aneuploidy, also are associated with regional structural changes on several chromosomal locations. Most D-tumors on the other hand, have fewer structural changes usually consisting of gain or loss of whole chromosome arms (Ried, Just et al. 1995). We performed a high resolution analysis of structural chromosomal rearrangements and its relation to gross ploidy level (A-type or D-type) in 2005 (Hicks, Muthuswamy et al. 2005). It was found that a small proportion of the D-tumors that metastasis early showed a high degree of rearrangements similar to that of A-tumors. These results were extended and related to survival data showing a clear association between number and type of structural chromosomal rearrangements and prognosis as described in detail in paper I.

Acquired knowledge of chromosome aberrations increases our understanding of the evolutionary pathways and factors that are important in the initiation, development and progression of cancer. Comparing aberrations in different tumors and assuming that mutations accumulate as the tumor progresses is one way to infer tumor progression. An alternative, and more direct approach, to study tumor progression is to compare multiple samples taken from different parts of a tumor, (See paper II)

Frequently observed genomic alterations in breast carcinomas include gain of 1q, 8q, 16p,17q and 20p, loss of 16q and 17p and amplification in 8q12-24, 11q11-13, 17q12-21, 17q22-24 and 20q13-ter reviewed in (Reis-Filho, Simpson et al. 2005)

4.1 DELETIONS/DUPLICATIONS

One common abnormality in cancer cells is gain or loss of whole chromosomes or chromosome arms or segments. The fundamental consequence of this is difficult to

establish because the gain/loss extends tens or thousands of mega bases and affect segments that contain hundreds of genes.

Deletions can be seen as 3 different types: (i) whole arm deletions, (ii) segmental deletions of partial chromosome arms or (iii) small regional deletions adjacent, and possible associated, to amplifications. These types are described in detail in paper I, III and IV (see further below).

The most frequent event in breast cancer tumors with few chromosomal alterations involves gain of the long arm of chromosome 1 (1q) and loss of the long arm of chromosome 16 (16q). In breast cancer loss of 16q is associated with lower grade tumors that do not metastasize (Buerger, Otterbach et al. 1999).

4.2 GENE AMPLIFICATIONS

Gene amplification means an increase of the number of genes or several genes in a chromosomal region. This phenomenon was first described by Schimke et al. (Schimke, Alt et al. 1978; Schimke, Sherwood et al. 1986). N-myc was the first gene described as amplified in tumor material (Kohl, Kanda et al. 1983; Schwab, Alitalo et al. 1983). Amplifications are the most studied chromosomal aberration in breast cancer (Santarius, Shipley et al. 2010) (Albertson 2006) and gene amplification is an important mechanism for oncogene overexpression in malignant tumors including breast cancer. Amplifications involving chromosomes 11q13.3 (CCND1) and 17q12 (ERBB2) are among the most common high level copy number aberrations in breast tumors (Bates and Peters 1995) (Slamon, Clark et al. 1987; Latham, Zhang et al. 2001) (Letessier et al., 2006).

Gene amplifications seem to be initiated by DNA double-strand break, and only occur in cells which are not able to detect DNA damage (Albertson 2006). There have been several suggestions as to what could promote amplifications, including error during DNA replication, telomere dysfunction and fragile sites, i.e. sites associated with an increase in chromosome instability. Studying amplifications in cell lines and tumors have revealed that amplifications can be organized in different ways: (i) as double minutes, which is extrachomosomal copies, (ii) in tandem arrays, which is series of copies of a gene arranged in tandem along a chromosome and (iii) within a chromosome or distributed at various locations throughout the genome (Albertson, Collins et al. 2003). The size of an amplicon can varies from kilobases to tens of megabases (Albertson 2006).

4.3 CHROMOSOME TRANSLOCATIONS IN BREAST CANCER

Chromosomal rearrangement in terms of translocations is a common property of malignant cells (Mitelman 2000). A translocation is a type of chromosomal rearrangement in which a chromosome breaks and a portion of it reattaches to a different chromosome. The translocation can be either reciprocal, which means that two chromosomes swap portions of arm with each other resulting in two translocation products, or non-reciprocal where one of the translocation products is lost (resulting in

only one translocation product) (Ferguson and Alt 2001). In balanced translocations, exchange of chromosomal material does not result in loss or gain of genetic information, in contrast, unbalanced translocations can result in both gain and loss of genetic material.

Balanced translocations can dysregulate critical cancer genes in different ways. A translocation can rearrange a gene to chromosomal regulatory regions that cause the gene to be deregulated (Look 1997). Translocations can also result in fusion of two separate genes resulting in a chimeric oncoprotein with transforming abilities (Rabbitts 1994). For example, hematological malignancies often display single balanced translocations, whereas the study of translocations in epithelial cancers has been limited mostly because of technical limitations in cytogenetics (Mitelman, 2000). For a long time it was thought that translocations in common epithelial cancers were unbalanced and that the consequence of that was mainly loss of a loss of tumor suppressor genes. (Dutrillaux 1995). Recent technical advances made it possible to search for fusion genes, findings in the past few years show that gene fusions caused by balanced translocations indeed exists in these cancer forms, and may play an important role in common epithelial cancers such as breast cancer (Edwards 2010).

Translocation studies on established breast cancer cell lines and cells from tumor samples indicated that chromosomes 1, 8, 16, 17 and 20 are most frequently involved in chromosome translocations of breast cancer (Dutrillaux, Gerbault-Seureau et al. 1990; Kokalj-Vokac, Alemeida et al. 1993; Tsuda, Takarabe et al. 1997; Kytola, Rummukainen et al. 2000). These chromosomes are often involved in rearrangements as gains and losses in the breast cancer genome (Ried, Just et al. 1995) (Jonsson, Staaf et al. 2010).

Loss of 16q is frequently found in breast tumors. In some tumors the loss of 16q takes place simultaneously with a gain of 16p. Gains and losses on chromosome 16 seem to occur at a very high frequency in tumors that also have a gain of 1q. Our study in paper number IV is based on the assumption that this gain on 1q together with a simultaneous loss of 16q might reflect a previously described chromosome translocation (Flagiello et al., 1998; Kokalj-Vokac et al., 1993; Pandis et al., 1992; Tsuda et al., 1997).

5 CYTOGENETIC AND MOLECULAR METHODS USED TO DETECT CHROMOSOMAL ABERRATIONS

Cytogenetics refers to the study of the chromosomes, description of their structure and identification of chromosomal aberrations. In 1970 Casperson et al. (Caspersson, Zech et al. 1970) invented the chromosome banding technique which made it possible to produce a visible karyotype by staining condensed chromosomes of metaphase plates with the fluorescent agent quinacrine. Later Giemsa banding was later introduced as an alternative technique (Evans, Buckton et al. 1971). This technique is still used to identify chromosomal aberrations, such as translocations.

5.1 CHROMOSOME NOMENCLATURE

In Paris in 1971 a group of 50 scientists engaged in cytogenetic diseases formulated a system of nomenclature for individual chromosome bands (Paris Nomenclature 1973). A short summary of the system is presented here:

Each chromosome consists of two arms, uneven in length, separated by the centromere. The short arm is labeled p (for petit) and the long q (for queue). On each chromosome arm regions and bands are numbered sequentially from the centromere and out on each arm. To describe a specific chromosomal region on an individual band the chromosome number, arm symbol, region number and band number are listed in order without space or punctuation. For example 11q13 is the long arm of 11, region 1 and band 3. To describe a sub-band a decimal is placed after the band followed by the sub-band number, for example 11q13.3 (Figure 1).

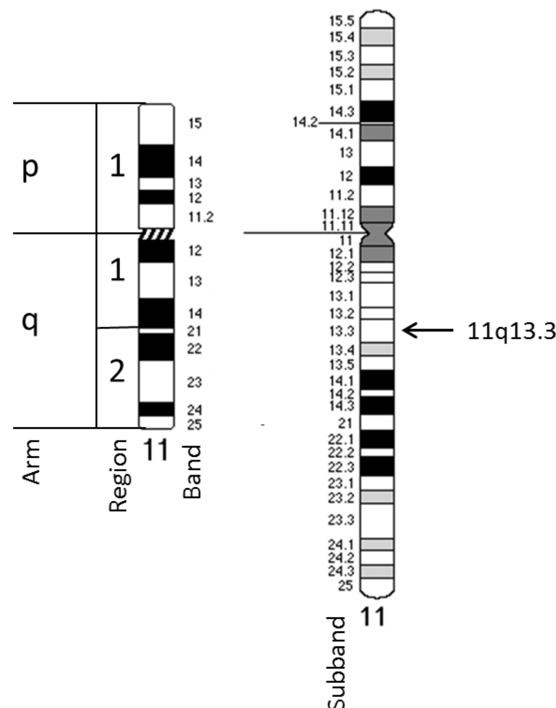

Figure 1. Schematic representation of chromosome 11.

5.2 METAPHASE SPREAD ANALYSIS

Advances in the metaphase spread analysis with the introduction of fluorescence in situ hybridization (FISH) methods using combinatorial labeling or ratio labeling techniques made it possible to study structural chromosomal aberrations and complex chromosomal rearrangements in a more detailed manner. These techniques made it possible to simultaneously visualize all 24 chromosomes in different colors in one experiment. Technologies that used this techniques are multi-color FISH (M-FISH) (Speicher, Gwyn Ballard et al. 1996), spectral karyotyping (SKY)(Schrock, du Manoir et al. 1996) and combined binary ratio labeling (COBRA)(Tanke, Wiegant et al. 1999).

5.3 COMPARATIVE GENOMIC HYBRIDIZATION (CGH).

One problem in using banding technique and spectral karyotyping is the need of metaphase spreads, which is especially problematic when analyzing solid tumors. To overcome this problem, Kallioniemi et al.(Kallioniemi, Kallioniemi et al. 1992) developed a new method, comparative genomic hybridization (CGH). CGH provides an overview of the aberrations across the genome, with the restriction that it is only sensitive to changes in the genomic content and cannot detect balanced translocations (Albertson, Collins et al. 2003). The principle of CGH is simple, tumor DNA and reference DNA are labeled differentially – for example, with the tumor DNA labeled with green fluorescent dye and normal DNA with red fluorescent dye. The labeled DNA is then mixed in a 1:1 ratio. The combined probes are then applied on metaphase chromosomes were they will compete for complementary hybridization sites. A fluorescence microscope is used to capture images of the chromosomes. If a region in the tumor DNA is amplified, the corresponding region on the metaphase chromosome will display more green dye and if a region is lost the corresponding region will appear redder. The ratios of tumor and normal are calculated by special image analysis software.

5.4 ARRAY CGH

The sensitivity and resolution of the CGH technique was further improved by the development of array CGH (aCGH). aCGH made it possible to identify chromosomal aberrations that were not previously detectable. The principles of aCGH are similar to those of CGH, but instead of having metaphase chromosome as a target, the platform is composed of a large number of mapped clones (for example bacterial artificial chromosomes [BAC] covering regions of interest) on a microarray. The resolution of the analysis is therefore limited to the clone size and space between the clones. The first platforms had large clones, such as BACs, as targets (Solinas-Toldo, Lampel et al. 1997) (Pinkel, Segraves et al. 1998). However, the use of oligonucleotide arrays increased the resolution even more additionally. To reduce probe complexity, and to optimize for fragments of a specific size, oligonucleotide arrays can use “representations” of the genome instead of using genomic DNA (Lucito, Healy et al. 2003). This will be elaborated on further under the part describing Representational Oligonucleotide Microarray Analysis (ROMA).

Array CGH is an excellent method for identifying copy number alterations and chromosomal breakpoints in unbalanced rearrangements, but cannot identify balanced rearrangements such as reciprocal translocations (Davies, Wilson et al. 2005).

5.5 INTERPHASE FISH

Interphase Fluorescence In Situ Hybridization (FISH) makes it possible to study the 3D organization of the genome. Structural rearrangements such as translocations have been visualized with FISH (Arnoldus, Wiegant et al. 1990; Tkachuk, Westbrook et al. 1990). Another advantage with interphase FISH is the possibility to study the distribution of cells in a sample when looking for different clones in a tumor population.

5.6 NEXT GENERATION SEQUENCING

During the last few years advances in technology for high-throughput, massively parallel sequencing (next generation sequencing) has made it possible to allow sequencing at single nucleotide resolution of the entire genome at a relatively low cost (Reis-Filho 2009). This technique has already been used in identifying somatic rearrangements in breast cancer (Stephens, McBride et al. 2009), and will probably be the main technique to study chromosomal rearrangements in the future.

5.7 ROMA - REPRESENTATIONAL OLIGONUCLEOTIDE MICROARRAY ANALYSIS

Representational Oligonucleotide Microarray Analysis (ROMA) was developed at Cold Spring Harbor laboratories (CSHL) (Lucito, Healy et al. 2003). ROMA is an array based hybridization method that uses genomic complexity reduction based representations, which in a single experiment can report genome wide copy number changes at a resolution of 30kb (kilo base pairs).

In the material presented in this thesis we performed comparative hybridization using Bgl-II representations (as described below), and arrays of 85 000 oligonucleotide (50 mer) probes with a mean interprobe distance of 35 kb. Differences in gene copy number between two genomes, for example normal DNA and tumor DNA, were compared on a microarray. The genome was digested with a restriction enzyme (Bgl-II, an enzyme with a typical 6-bp recognition site), ligated with adapters specific to the restriction fragment sticky ends and amplified by PCR. The representations of the different genomes were labeled with different fluorophores and hybridized to a microarray with probes specific to locations spread throughout the genome (Figure 2).

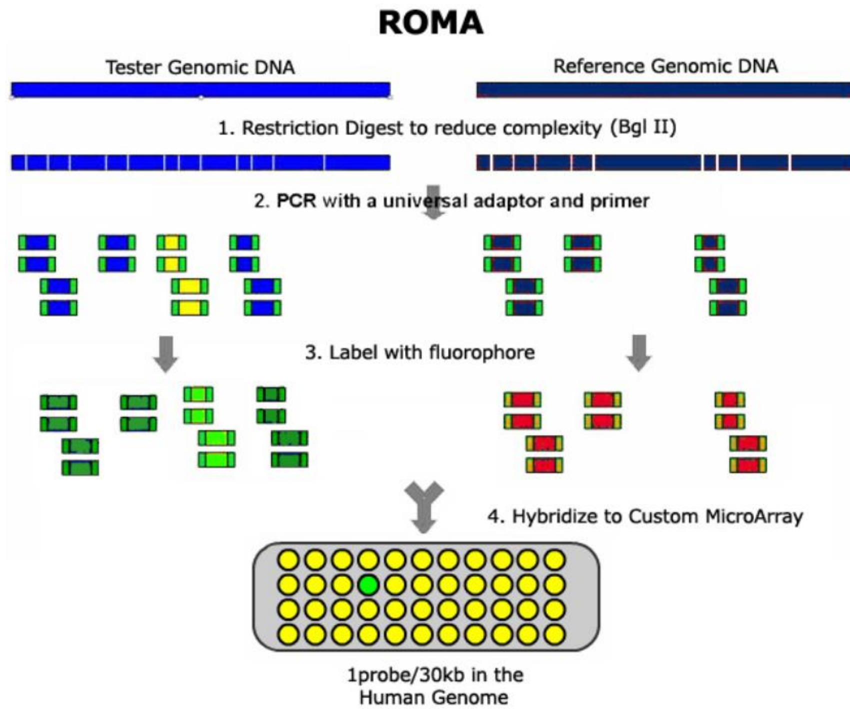

Figure 2. Representational Oligonucleotide Microarray Analysis (ROMA). (This figure is reproduced with the permission from Nicholas Navin)

Each experiment was performed twice with color reversal, and data was rendered as normalized ratios of probe hybridization intensity of test to normal. Each raw ROMA profile was segmented as described in Hicks et al. 2005 (Hicks, Muthuswamy et al. 2005)

Rare copy number variants (CNVs) and more prevalent copy number polymorphisms (CNPs) will be present in any high-resolution copy number scan, when comparing one person to another due to individual differences in genome content (Sebat, Lakshmi et al. 2004). A tumor profile is obtained by comparison to a standard normal male. Therefore normally occurring CNPs and CNVs are masked so the analysis does not mistake either for a cancer lesion. Figure 3 shows a typical raw ROMA profile and segmented profile. Ratios above 1 are considered as a gain and below 1 as a loss.

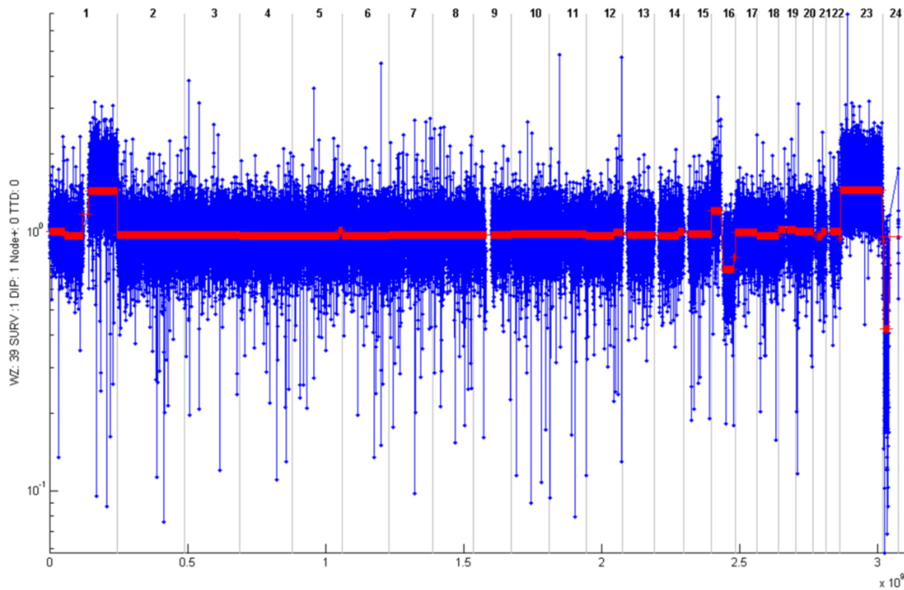

Figure 3. Raw ROMA-profile in blue and segmented ROMA-profile in red. Copy-number ratio is graphed on the y axis. Chromosomes 1-22 plus X and Y are displayed in order from left to right according to probe position on the x axis.

5.8 FLUORESCENCE IN SITU HYBRIDIZATION - FISH

Fluorescence In Situ Hybridization (FISH) is a cytogenetic method which, when it was introduced 25 years ago (Pinkel, Gray et al. 1986), opened new opportunities in the way chromosome structures could be studied. FISH is a useful tool for locating DNA sequences on fixed chromosomes or interphase nuclei. It is a very straightforward technique that consists of heat denaturation of the target DNA chromatin and hybridization of a fluorescently labeled DNA probe to its complementary sequence. Probes are generally labeled by nick translation (Rigby, Dieckmann et al. 1977) (Langer, Waldrop et al. 1981) either indirectly with a hapten, a molecule that can bind antibodies, or directly via the incorporation of nucleotides that has been modified to contain a fluorophore. The target is then visualized in a fluorescence microscope.

To locate a unique sequence, the probe must cover a chromosomal region large enough to give a detectable signal. A problem with this technique is that each probe will contain small or large portions of dispersed repetitive DNA sequences. During the hybridization process those sequences will anneal to complementary genomic DNAs distributed throughout the genome. This results in a background that can have almost the same intensity as the target genomic locus. In 1987 Landegent et al. (Landegent, Jansen in de Wal et al. 1987) presented a solution to this problem by efficiently reducing the background fluorescence by suppressing the nonspecific hybridization of probes derived from BAC clones by blocking it by Cot DNA, which contain DNA fragments that are 50 to 300 bp (base pairs) in size and is enriched for repetitive DNA sequences. Figure 4 shows an example of the influence cot DNA has on an FISH image.

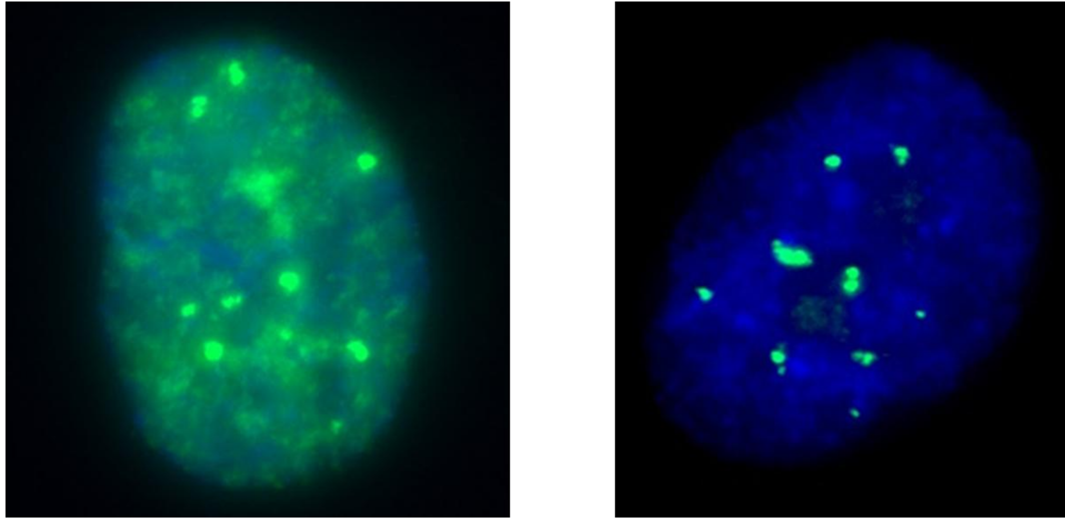

Figure 4. FISH performed on HDF cells with and without the presence of cot-DNA. BAC clones from several chromosomal areas were labeled with a green fluorescent dye. In the left image FISH was performed without cot and as one can see the nuclear background level is high. In the right image FISH was performed with cot DNA, and one can clearly see that the background has decreased, rendering a better signal to noise value. Both images were acquired with the same acquisition time. And the display settings for the images are the same.

Another approach to reduce background is to generate highly specific PCR probes that cover a region of a chromosome without any repetitive genomic sequences (Navin, Grubor et al. 2006). An example of a FISH showing an amplification of BAG4 and deletion of DBC1 can be seen in figure 5.

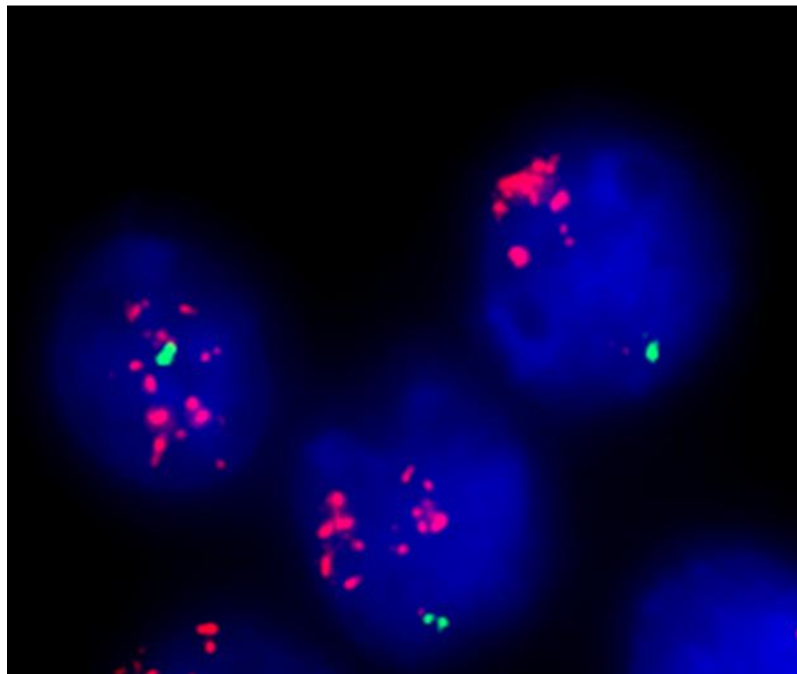

Figure 5. A FISH example. Amplification of BAG4 and deletion DBC1

5.9 ROMA COMBINED WITH FISH

Both FISH and ROMA are high resolution cytogenetic techniques that can detect small deletions, duplications and amplifications in the DNA of tissue samples. When studying chromosomal aberrations in tumors, ROMA and FISH techniques complement each other. Interphase FISH can reveal the number of specific chromosomal regions in each individual cell, and can therefore identify presence or absence of different subclones within a tumor sample. A limitation of FISH is that the genes or chromosomal regions of interest have to be known to generate specific probes. ROMA, on the other hand, can be used to identify chromosomal regions that contain copy number changes and/or chromosomal breaks involved in suspected translocations without any prior knowledge of the chromosomal regions involved. Information rendered from the ROMA profiles enables generation of FISH probes for further analysis on cell to cell basis. ROMA gives an average value of the tumor DNA and will not reflect clonal variability in a tumor. In paper II of this thesis this particular problem is addressed.

6 FLUORESCENCE MICROSCOPY

Fluorescence Microscopy is a powerful tool to study intracellular structures and dynamic processes with a high degree of accuracy. It is an essential technique in basic research when studying chromosome abnormalities, structure and behavior.

The basic function of a fluorescence microscope is to illuminate a fluorescent specimen with a specific wavelength and to separate the emitted light, which will be of a longer wavelength, from the excitation light. The resulting image should be generated only by the emitted light, which reaches the eye or the detector.

6.1 FLUORESCENCE

Fluorescent molecules absorb light at one wavelength and emit light at another, longer wavelength. It is the result of a three stage process that occurs in certain molecules called fluorophores or fluorescent dyes.

First a fluorescent molecule absorbs a photon of a specific energy, which causes the molecule to enter an excited state that exists for a finite time (1-10 nanoseconds). Thereafter a photon, with longer wavelength than the absorbed photon, is emitted. The difference between exciting and emitted wavelengths, known as Stokes shift (Lakowicz 2006), is a critical property in fluorescence microscopy. The process responsible for this can be explained by using a Jablonski diagram, see figure 6. Fluorescent molecules are normally found in their ground state (S0 in figure 6). The molecules can absorb energy from a photon by changing its vibrational state. If the absorbed energy is large enough and if it fits the energy gap of the fluorophore, an electron can move to an orbital of higher energy (S1 or S2 in figure 6). The transition to this excited state happens on a timescale in the order of femtoseconds. After that, the molecule loses energy by undergoing vibrational relaxation so that the electron ends up in the lowest level of the first excited state (S1 in figure 6). This process occurs on a timescale of picoseconds. From this state, the molecule can return to the ground state through the emission of a photon of lower energy and therefore longer wavelength than the exciting photon. This phenomenon is referred to as fluorescence.

Fluorescence emission is not the only way for a fluorophore in its excited state to lose its excess energy. Another pathway of energy loss occurs after intersystem crossing where the excited molecule ends up in a long lived triplet state. From this state the molecule can go to ground state without emission or by a light emission termed phosphorescence. The triplet-state molecules can also undergo photochemical reactions that can cause permanent bleaching which cause problems. [methods in cell biology vol 81]

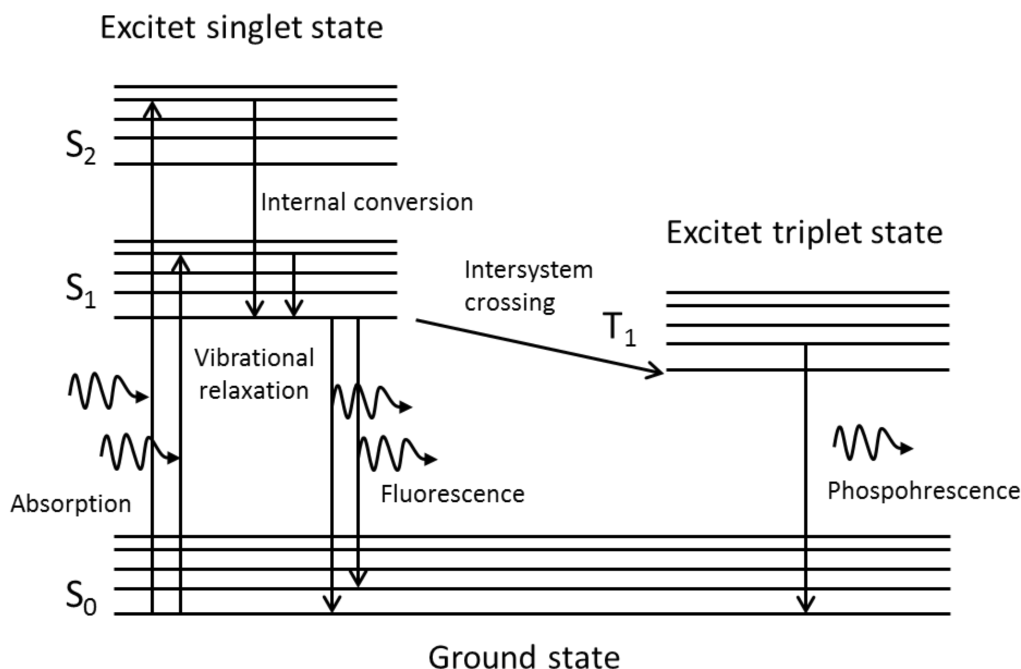

Figure 6. A Jablonski diagram.

6.1.1 Quantum Yield and fluorescence intensity.

The number of emitted photons that reach the detector is often very low in comparison to the number of exciting photons, giving an image that is not as bright as desired. This problem consists of several parts. The first part is related to quantum yield, which is defined as the ratio between the number of photons emitted and the number of photons absorbed. The quantum yield is an inherent property of a fluorophore. Generally the number of photons absorbed is greater than the number emitted resulting in a quantum yield below one. To maximize intensity of fluorescence it is important to choose a fluorophore with high quantum yield. The second part of this problem has to do with the fact that the fluorescent light is emitted in all directions, and only a small part emitted in the correct direction reaches the objective. A third part is due to the light having to pass through a setup of filters, which reduces light intensity. In order to maximize the number of photons reaching the detector, high energy mercury lamps are usually used to maximize the number of photons exciting the fluorophore.

A limited problem in fluorescence microscopy is photo bleaching or fading. Fading occurs when the fluorescent probes lose their ability to fluoresce. The number of fluorescence cycles a fluorophore can fluoresce is limited dependent on the molecule and the environment (Lakowicz 2006).

6.2 THE FLUORESCENCE MICROSCOPE

Figure 7 shows a schematic image of a fluorescence microscope. Fluorescent molecules in the stained sample absorb light of a certain wavelength and emit light of a longer wavelength. The dichroic mirror acts as a cut off and reflects light below a given

wavelength, while transmitting light of longer wavelengths. The transmitted light is then detected by the eye, a detector or a camera.

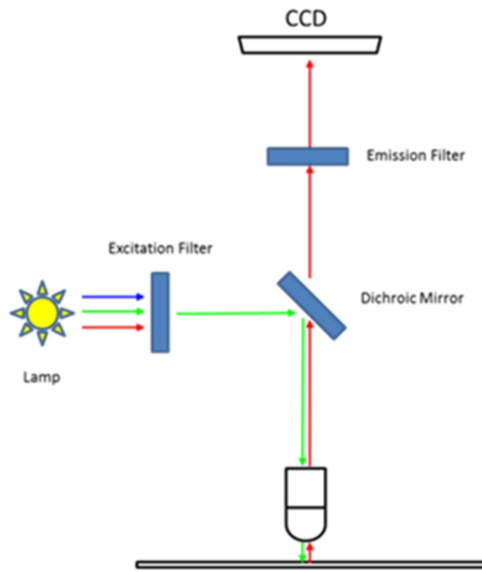

Figure 7. Schematic image over a fluorescence microscope.

6.2.1 Objectives

In fluorescence microscopy the objective is both a source of light to the specimen and the collector of emitted light and therefore it has large impact in the resulting image. To choose the correct objective is very important to get images as good as possible. The magnification of the objective used is important but even more important is the numerical aperture (N.A.) that determines the resolution of the lens. The magnification is only used to increase the apparent size of an object, whereas increased N.A. improves resolution. The numerical aperture (N.A.) of an objective is defined as

$$N.A. = n \sin \alpha_{max}$$

Where n is the refractive index of the medium between the front lens and the specimen and α_{max} is the half angular aperture of the objective. A high N.A. means that the objective collects more light and is also related to the resolution of the objective. The resolution of an optical microscope is defined as the shortest distance between two points on a specimen that can still be distinguished by the observer or camera system as separate objects. Using Rayleigh criterion the minimum resolved distance in the microscope specimen, D, is given by

$$D = \frac{0.61\lambda}{N.A.}$$

Many objectives used today are of immersion type, meaning that the medium between lens and specimen is filled with a fluid (oil or water) having a refractive index, $n > 1$. This will increase N.A., and therefore improve the resolution.

The resolving power of a microscope is the most important feature of the optical system and impacts the ability to discriminate between fine details of a specific specimen. Modern objectives have a complex lens system composed of numerous internal glass lens elements. This gives the objective the best possible performance for its application (Abramowitz, Spring et al. 2002).

6.2.2 Filter

The primary filter element in most fluorescence microscopes is a set of three filters housed in a filter cube: the excitation filter, emission filter and dichroic beamsplitter. The excitation filter should only transmit light that excites the fluorophore of interest, and these filters are usually of a bandpass type. The emission filter or barrier filter have to attenuate all light transmitted by the excitation filter but transmit the emitted light. The emission filter can be a bandpass filter or a longpass filter depending on the set up. The dichroic beamsplitter is a mirror placed 45 degrees to the optical path of the microscope. This mirror has the unique ability to reflect one wavelength (the exciting light) but transmit another (the emitted fluorescence). By completely filtering out the excitation light, but letting the emitted fluorescence through, it is possible to see only the objects that are fluorescent and are of interest.

In the beginning fluorescence filters were made of colored glass, also called absorption glass. Nowadays, interference filters are used for exciter filters to pass the correct wavelengths of light with great selectivity and high transmission. Dichroic beam splitters are specialized interference filters. Barrier filters may be either made of colored glass or interference filters.

6.2.2.1 Filter design

When designing a filter the most important parameter is the spectral characteristic of the dye. If this were the only parameter to be considered, one would illuminate the specimen using a shortpass excitation filter and observe the fluorescence using a longpass emission filter that transmits the entire emission spectrum filter. Pathological specimens, such as paraffin embedded tissue sections, however, have some autofluorescence. Furthermore, light of shorter wavelength such as UV light might increase the rate of photobleaching of the fluorophore. Therefore it is better to limit the band to a region where the fluorophores excitation is at its maximum, but that is still broad enough to get a bright signal. If a second fluorophore is introduced, spectrally close to the first there is a risk to detect some emitted light of that fluorophore, often referred to as cross-talk or bleed through. This is an undesirable effect and in this case a narrower bandpass filter should be used on the emission side. In many applications high numbers of fluorochromes are used, and filters with as narrow bands as possible have to be selected. The problem with narrow bands is that the light level will decrease and there is a risk that intensity contrast will be lost. But a narrow band-pass filter can also

maximize the collection of specific signal compared to autofluorescence, resulting in a better signal to noise value.

The number of probes that simultaneously can be monitored depends of the number of fluorochromes that can be separated based on wavelength. When a high number of fluorophores are used, special attention has to be paid to the spectras, especially the emission spectras, to avoid crosstalk between fluorophores. Below is an example how emission and excitation filters can be configured, with minimal cross-talk, when using four different fluorophores (figure 8).

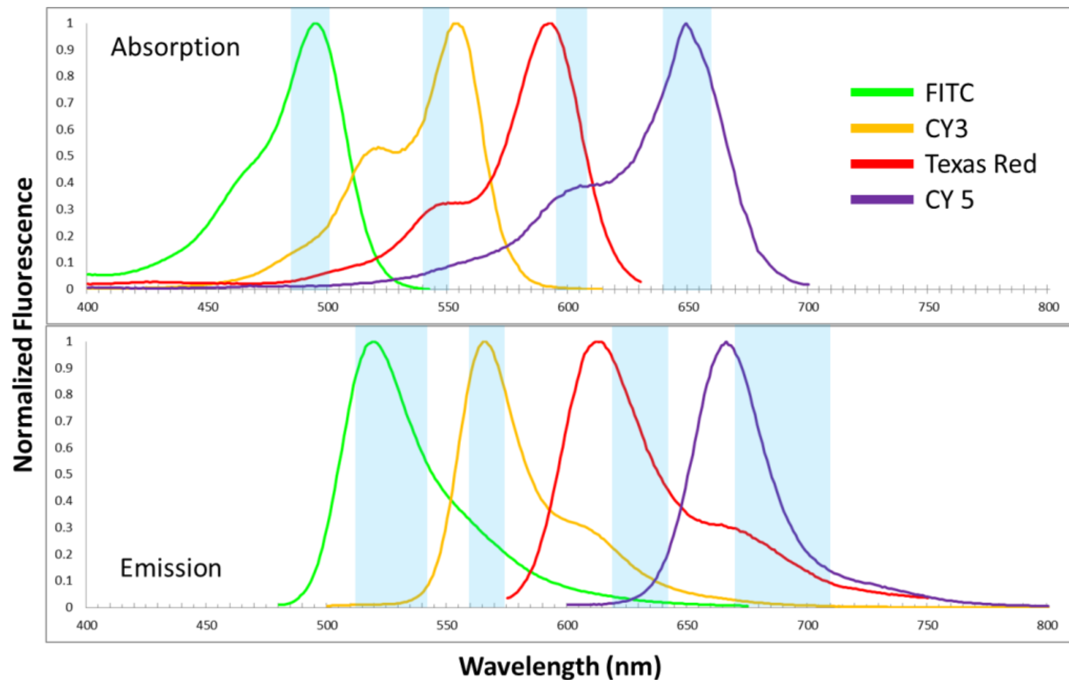

Figure 8. Absorption- and emission spectra from four different fluorophores (FITC, CY3, Texas Red and CY5). In multichannel systems, the excitation- and emission filters spectral bandwidths must be selected to optimize collection of the desired fluorescent signal, and at the same time avoid channel cross talk. The blue rectangles show optimal settings for excitation and emission filters.

Instead of separating the fluorophores with narrow filters multichannel unmixing is an approach. Spectral unmixing is a function that mathematically removes the crosstalk between fluorescent dyes in the digital image (Mansfield, Hoyt et al. 2008).

6.2.3 Illumination

Mercury lamps, chosen for their brightness, are the most common light sources used in fluorescence microscopy. The spectrum of this light is not continuous; the light output is concentrated to a few narrow bands. Choosing filters should be matched to get best light possible for each fluorophore. Another illumination source is the xenon arc lamp which has a more continuous spectra but is not as bright as a mercury lamp.

A common problem in the microscope setting is uneven illumination due to that the lamp is not aligned in a correct way. To avoid this liquid light guides can be used resulting in uniform illumination in the microscope field.

6.2.4 Cameras for Digital Microscopy

The images in fluorescence microscopy are usually acquired with a charged coupled device (CCD) camera. The CCD is composed of a large matrix of photosensitive elements, pixels, which simultaneously capture an image. The light intensity, i.e. the number of photons, for each pixel will be stored as an electronic charge and converted to an analogue voltage. This voltage is then converted to a numerical value and stored in the computer's image file. The most important property of the CCD is its sensitivity to detect photons. For weak fluorescence it is important that the CCD can generate electric charge from incident photons. The image resolution of the CCD is directly proportional to the number of pixel elements in the CCD.

6.3 THREE-DIMENSIONAL MICROSCOPY

The original image collected determines the extent of what you can analyze. Fluorescence images are often blurred with scattered light coming from fluorescent emission that have been diffracted, reflected and refracted by out of focus fluorescence from the specimen. As a consequence, the image can be difficult to analyze. To overcome this problem there are optical and computational methods that can be applied to enhance image quality.

Confocal microscopy (Carlsson and Aslund 1987) is currently the most widely used optical sectioning technique for fluorescence imaging. It is based on the principle that out of focus information is rejected by the presence of a pinhole in front of the detector and the light source. Only the part of the specimen that is in the focal plane of the objective is imaged and light collected from outside the focal plane is rejected by the pinhole. The size of the pinhole determines the thickness of one acquired plane. Small pinhole size gives better z-resolution, but as a consequence the signal intensity is decreased.

An alternative method for optical sectioning that has been developed for wide-field illumination is structured illumination microscopy (Wilson, Neil et al. 1998). In this method images in three spatial positions of a grid are acquired and an optically sectioned imaged is produced. The depth resolution is similar to that achievable with a confocal microscope (Weigel, Schild et al. 2009).

Deconvolution microscopy (Agard, Hiraoka et al. 1989), on the other hand, is a computational method for enhancement of image resolution. A deconvolution algorithm is used to restore images to an approximation of what the original object looks like.

All these methods improve the images by removing out of focus light by generating an "optical section" through the sample. Only the image information that lies entirely within the objective's depth of field is displayed.

Even if a fluorescence microscopy system was to be equipped with perfect lenses, has optimal alignment and large N.A. the theoretical optical resolution is still limited to half a wave length. However, in the last decade several new techniques have developed that break this limits. These have created new possibilities to investigate the structure of the cell. Examples of these so called super-resolution techniques are: Structured illumination microscopy (SIM), stimulation depletion depletion (STED) and Single molecule localization and composition (PALM/STORM). All these techniques are reviewed in (Schermelleh, Heintzmann et al. 2010).

6.4 DIGITAL IMAGE ACQUISITION, PROCESSING AND ANALYSIS

Manual classification and quantification of fluorescence labeled genes in a FISH image is a very tedious and time consuming task. If the probes are labeled in different color combinations, as in M-FISH, the task is almost impossible. We have therefore developed an image analysis system for detecting, classifying and quantifying FISH labeled genes in cell nuclei. A short version of the methodology used to create a complete and working FISH analysis program can be summarized in the following steps:

Sample preparation and molecular hybridization: Good sample preparations are essential in achieving good images. In order to generate a strong and specific gene signal in the FISH image all steps in sample preparation and molecular hybridization have to be optimized. Equally essential is reducing the background noise caused by unspecific probe binding and auto fluorescence.

Image acquisition: The image acquisition is a very important step in the process. Images of low quality will have a negative effect on the image analysis. There are several factors that influence the quality of the final image. One of these factors is the choice of objective. Another one is the choice of filters, to avoid cross-talk between different color channels. Furthermore, the image stack acquired has to be large enough to cover the entire cell nucleus, and the optical sections have to be thin enough not to miss signals.

Pre-processing: The image has to go through some pre-preparations before analysis can begin. This includes removal of dark current from the CCD camera, removal of noise and normalization of signal intensities.

Segmentation I: To able to study the number of signals in each individual cell nucleus, the nuclei of interest must be separated from the surrounding nuclei in the image. This type of cell segmentation is done semi-automatically in the DAPI (4',6-diamidino-2-phenylindole) channel. DAPI is a fluorescent stain that binds strongly to DNA and is used to counter stain the nuclei.

Segmentation II: Each labeled chromosomal region will appear as dots in the FISH image. Each dot in the nuclei will automatically be extracted.

Feature extraction: As discussed previously one common artifact in FISH images comes from binding of probe to repetitive sequences and this unspecific binding results as false dots in the FISH-image. The false dots have to be recognized and removed from the image.

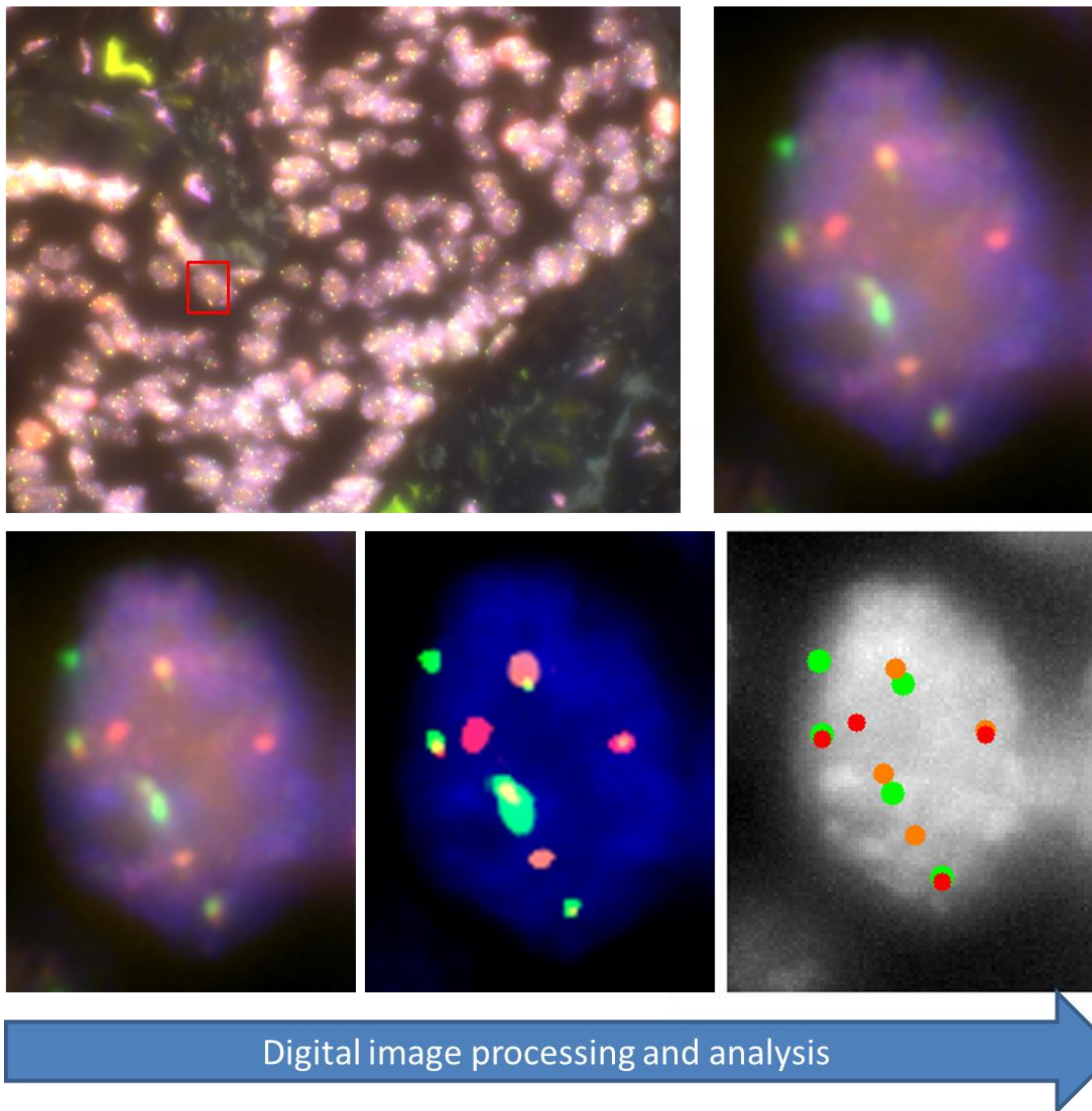

Figure 9. Schematic image of the image analysis, including segmentation of the nuclei, preprocessing and dot detection. In the analyzed M-FISH imaged six genes where labeled in different color combinations (Green, orange, red, green/orange, green/red and orange/red) and hybridized to a paraffin section.

7 AIMS OF THIS THESIS

The overall aim of this thesis was to study genome rearrangements in breast cancer and their relation to tumor progression and risk for metastasis.

More specifically the aims were:

- To identify chromosomal alterations and their spatial organization in the cell nucleus
- To study clonal evolution during tumor development and the genetic heterogeneity in tumor populations
- To develop a method to analyze chromosomal translocations in interphase chromatin in paraffin embedded tissues
- To study the translocation between chromosome 1 and 16 in invasive carcinoma and DCIS.
- To study the relationship between genomic alterations, gene expression subtypes and clinical data.

8 RESULTS AND DISCUSSION

Paper I

The introduction of the high resolution aCGH techniques, such as ROMA, has made it possible to study recurrent aberrations at a much higher resolution than before. In this study we used two methods to study genomic aberrations of breast cancer with high resolution: ROMA, which is an aCGH method and interphase M-FISH. Using a combination of these two methods we could identify genes and chromosomal regions that displayed aberrations as gains or losses and study the quantitative genetic imbalances in relation to the progression of the tumors. We especially focused on the metastatic process. This was done in detail by examining high resolution data from 243 breast tumors from two separate studies for which detailed clinical data is available.

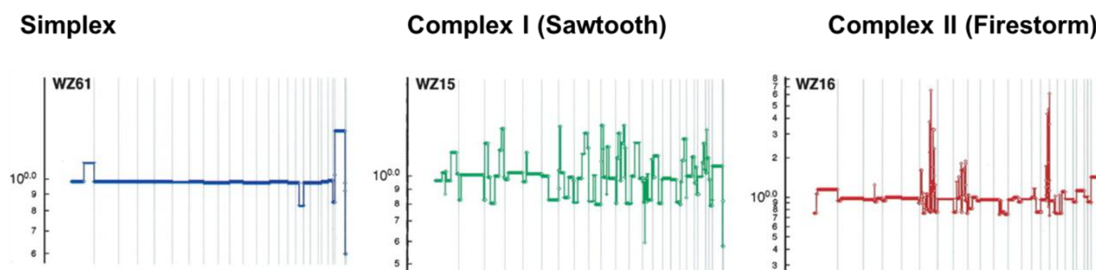

Figure 10. Major types of tumor genomic profiles. Segmentation profiles for individual tumors representing each category: (A) simplex; (B) complex type I or sawtooth; (C) complex type II or firestorm. The y-axis displays the geometric mean value of two experiments on a log scale Chromosomes 1-22 plus X and Y are displayed in order from left to right according to probe position.

By visual inspection of the ROMA profiles, which give information about genomic changes of the entire cell population of the tumor, we classified the profiles in to three different patterns (Figure 10): The first pattern has broad segments of duplications and deletions of whole chromosomes or chromosome arms, and was referred to as "simplex". The second pattern is characterized by many narrow segments of duplications and deletions, affecting more or less all of the chromosomes, and we called this pattern complex 1 or "sawtooth". The third pattern is similar to the simplex pattern, except that the ROMA profile has one or more localized region consisting of a collection of narrow peaks of amplifications. We call this pattern complex 2 or "firestorm".

The simplex group was shown to be associated with good outcome and is typical of low-grade cancers, frequently displaying coexisting 1q gain and 16q losses. On the other hand the complex patterns highly correlate with aggressive disease and poor survival. In the simplex tumors some specific chromosome arm gains and losses are more frequent than others, and a subset of these seems to appear in almost every simplex tumor. The aberrations in this subset are duplications of 1q, 8q, and 16p and deletions of 8p, 16q and 22q.

For a more detailed analysis on the individual cell level we used interphase FISH to validate the segmented ROMA profiles. For this BAC clones or Oligonucleotide Probes representing different loci, both peaks and valleys in the segmented ROMA profiles, were selected as probes for FISH. Studying tumors with the firestorm pattern clearly indicated that amplifications of neighboring peaks on a chromosome occur in the same cell and that they do not arise from clonal subpopulations (Figure 11B). This is something the population based ROMA method could not depict. Co-localization of amplifications from the same chromosome arm also indicates that the amplifications occur on the same chromosome arm (Figure 11C).

Firestorms regions could be seen at least once on most chromosomes in the tumors of the firestorm tumor set. Some chromosome arms, however, seem to undergo this process more often. Chromosome 6, 8, 11, 17 and 20 are frequently affected, and of these 11q and 17q are affected the most.

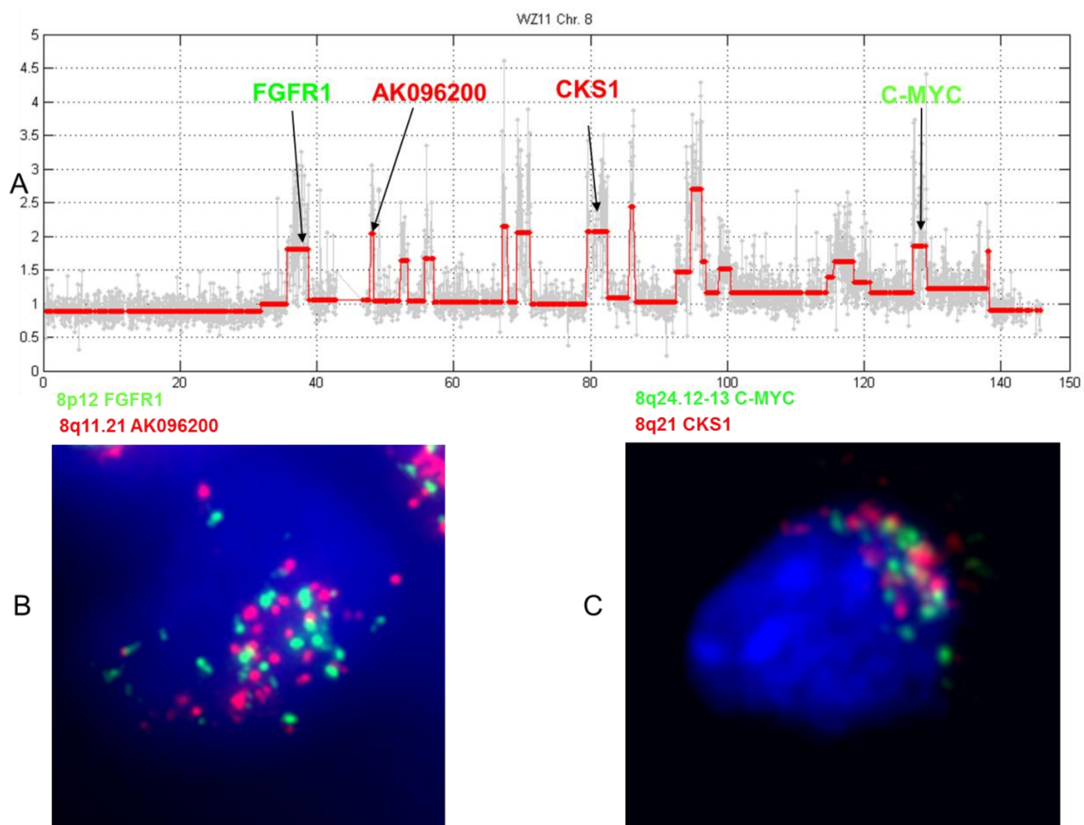

Figure 11. Validation of peaks and valleys in ROMA profiles by interphase FISH. (A) Expanded ROMA profile of a firestorm on chromosome 8 in a diploid tumor. The graph shows the normalized raw data (gray) and segmented profile (red) along with the genes for which the probes shown in the FISH images were constructed. Several distinct conditions are exemplified in the images. Tight clusters of multiple copies corresponding to ROMA peaks are also shown in the FISH images for CKS1A, MYC, TPD52, and the uncharacterized ORF AK096200. Note that the FISH signals corresponding to distinct loci cluster together irrespective of their distance on the same arm (CKS1A/MYC) or across the centromere (BAG4/AK09620)

Taken together, the profiles described in this study can be useful for evaluating the relationship between “firestorms” and the locations of candidate oncogenes and tumor suppressors in the genome. It can also help us identify genes that drive cancer

progression and solve the complex genetic pathway that underlies the process of tumor metastasis.

We have identified about between 50 and 100 chromosomal regions that have been found to be amplified or deleted in breast cancer. The fact that the number of chromosomal regions that are altered in cancer cells is relatively limited enables us to study them with FISH.

Paper II

Until recently, most tumor CGH-analyses have been done on samples on a large number of tumor cells, a method that hides individual variation among cells and can cause misclassification of cancer. We developed a new technique, sector ploidy profiling (SPP), which allows us to sort and segregate single cells and amplify portions of their genome for analysis. This technique made it possible to utilize the heterogeneity in breast cancers and to examine the genomic evolution of the tumors, by studying individual cells within a tumor.

In this paper we combined SPP with M-FISH to study the clonal composition of a tumor and tumor progression. Our hypothesis was that some solid tumors contain subpopulations with variations in their genomes. To test this assumption, four high grade tumors were dissected into four sectors, and ROMA profiles were rendered from DNA isolated from each sector. Two of the tumors contained minimal variation in their genomic copy number profiles in all four sectors, whereas the other two tumors had a large degree of genomic variation in the different sectors. The two tumors that displayed a stable genome profile are referred as 'monogenomic' because it consists of one single tumor profile. The other two tumors, that showed diverse genomic profile between the different sectors, are referred to as 'polygenomic', since there are multiple tumor subpopulations present. These results indicate that tumor heterogeneity is quite common. However, one problem with this approach is that the proportion of normal and tumor cells can vary greatly between different tumor sectors, and this may confound the interpretation of the copy number profiles. To avoid this problem a method to separate subpopulations within the tumor before ROMA analysis was developed. Using this novel method help us to further improve the genetic classification of tumors.

To be able to study the number of populations and to get a clearer picture of the subpopulations and their clonal relationship the method SPP was developed. This method separates tumor subpopulations by region and ploidy and mitigates the mixing effect of normal and tumor cells.

To perform SPP, a tumor is macro dissected into 12 sectors. From six of these nuclei are isolated and stained with DAPI for flow sorting. The sorting of cells by FACS is gated according to differences in total genomic DNA content. DNA from each peak is then prepared and analyzed using ROMA. The remaining 6 sections were saved for interphase M-FISH.

After performing SPP on 16 tumors we could still classify the tumors into two groups: (i) monogenomic (7 tumors) and (ii) polygenomic (9 tumors). However, using the SPP approach we were, in addition to the grouping, able to identify many subpopulation-specific chromosome markers. These markers are useful for studying tumor progression particularly when elucidating the genetic evolution in the tumors. By assuming that genomic complexity increase during tumor progression, we sorted the copy number profiles by increasing number of chromosomal breakpoints and identified genetic events that occurred during tumor evolution.

To explore the spatial organization of the subpopulations of these tumors, we used interphase FISH on tissue sections to study single cells. In one polygenomic tumor we identified a massive amplification in two of the six sectors by using SPP. The discovered area is a 3.6 mb amplicon containing the KRAS oncogene and several other genes. To study this area of the genome in detail we generated a BAC probe for FISH, and hybridized it to all tissue sections analogous to the sectors and as expected we identified the amplification in sectors showing amplification in the ROMA-profiles (Figure 12D). Furthermore, in one sector we observed isolated cells with amplification for KRAS, which was not detected by the ROMA (Figure 12C). This result shows that tumor subpopulations can be anatomically segregated into different regions of a tumor. We could also, by doing M-FISH, show that genetically different tumor subpopulations and normal diploid cells can be intermixed within tissues.

Comparing different subpopulations from different areas in a tumor enabled us to infer pathways of cancer progression and the organization of tumor growth.

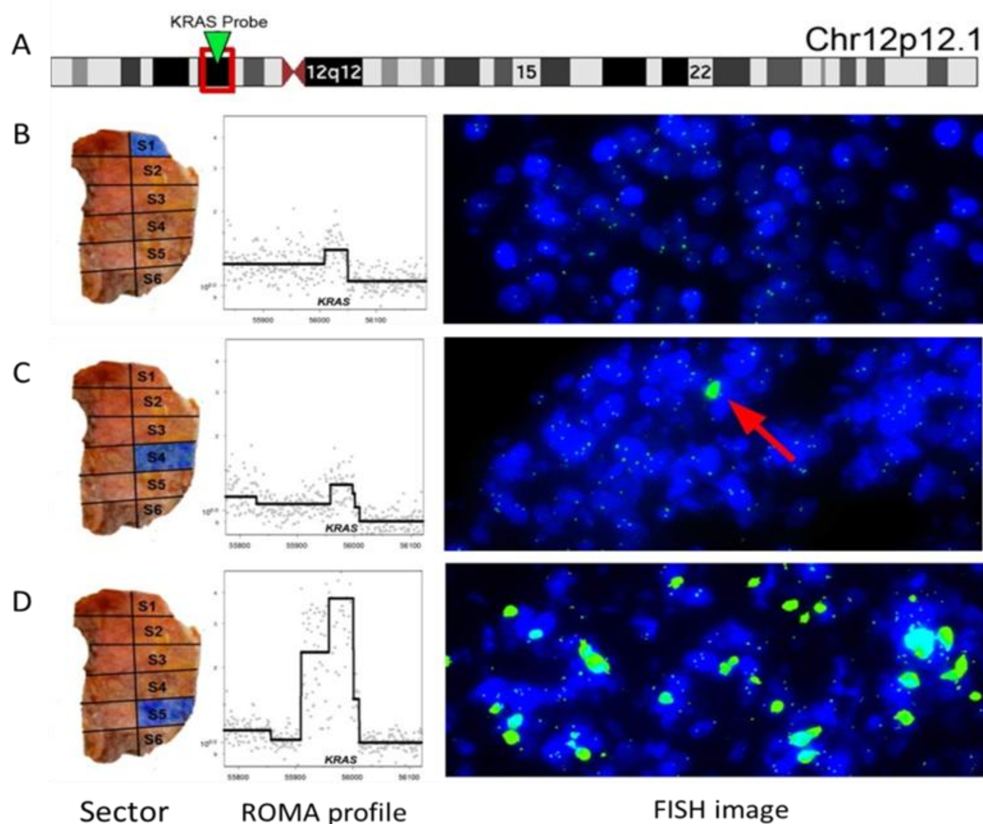

Figure 12. Regional amplification of the KRAS locus. (A) Ideogram showing the cytobands and location of the KRAS FISH probe. (B-D) Topography of each tumor sector (left), ROMA profile (middle) and FISH-image (right). (B) Sector 1 show two or three copies of the KRAS locus in the stromal and tumor cells. This corresponds well to the ROMA-profile. (C) In sector 4 a majority of the tumor and stromal cells has two or three copies, which corresponds well to the ROMA-profile. However one tumor cell shows a massive amplification. (D) Sector 5 show numerous tumor cells with massive amplification, this correspond well to the ROMA-profile.

Paper III

Inspired by the three different patterns of segmented genome profiles, "simplex", "firestorm" and "sawtooth" presented in paper I, we developed an objective estimate of genomic alterations in aCGH data. Further we investigate the relationship between genomic alterations, molecular expression subtypes, structural rearrangements and clinical data.

Two new algorithms to objectively estimate genomic complexity were developed using segmented aCGH data: 1) whole-arm aberration index (WAAI) and 2) complex arm aberration index (CAAI). The WAAI score was used to capture events on whole chromosome arms. CAAI on the other hand was used to recognize regions with structural complexity. We defined whole arm gains as WAAI $\geq$ 0.8 and whole arm losses as WAAI$\leq$-0.8. Areas of complex rearrangements were found by selecting chromosome arms with a CAAI $\geq$0.5.

Luminal A tumors, dominated by ER-positive tumors, have few chromosomal alterations, often with gain of 1q and loss of 16q. Basal-like tumors, on the other hand, have many alterations affecting most of the chromosomes. Loss on 5q and gain on 10p have been suggested as a specific Basal-like variation (Bergamaschi, Kim et al. 2006). On the basis of this, and by using the WAAI cutoffs described above, we defined 4 subgroups based on genomic architecture: (A) 1q gain and/or 16q loss, (B) regional loss of 5q and/or regional gain of 10p, (AB) both A and B and (C) those with neither. These subgroups showed distinct differences with respect to whole arm losses and gains. The groups were further characterized by dividing them into CAAI subgroups. CAAI<0.5 for all arms were denoted A1, B1, AB1 and C1 and CAAI $\geq$0.5 for at least one arm were denoted A2, B2, AB2 and C2.

Comparison of the expression based classification with this novel aCGH based grouping showed that the A group was enriched in Luminal A tumors and the B group in Basal-like tumors. The A and B groups displayed different types of chromosomal aberrations. In the A group, gains and losses of whole chromosomes and chromosome arms dominated, whereas B tumors had more genomic loss and more regional aberrations. The mortality in the B group was twice as high as it was in the type A group independent of lymph node status, tumor size, histological grade and treatment.

Tumors of type A were selected due to having gain of 1q and/or loss of 16q, and these events were frequently concurrent. An unbalanced translocation between chromosomes 1 and 16 has previously been reported (Tsarouha, Pandis et al. 1999). We believe that the gain of 1q and loss of 16q we observe in the A group is a consequence of this translocation. This hypothesis was confirmed when we showed this translocation by M-FISH in one of the tumors in group A. Tumors with this typical 1q gain, 16q loss pattern are further studied in paper IV.

Clinical follow up data were available for a majority of the patients, and the CAAI score was shown to be an independent prognostic factor. In patients with tumors with high CAAI score the risk of breast cancer related death was twice as high as in patients with tumors with a low CAAI score. This was independent of lymph node status, tumor size, histological grade and WAAI class.

Paper IV.

In papers I and III we concluded by observing ROMA profiles, that there is a group of low malignant tumors that often have a duplication of 1q simultaneously with a deletion of 16q. This aberration has previously been detected by others and is considered to be one of the earliest changes in carcinogenesis of the breast. Based on this, we assumed that a gain on 1q together with loss of 16q reflects the translocation t(1;16) which have previously been described as an common translocation in breast cancer.

By combining ROMA with M-FISH it is possibly to identify specific translocations in interphase chromatin in clinical samples. As a first approach we identified the suspected breakpoints in frequency plots generated from 140 ROMA profiles. In the frequency plots we could clearly see that most chromosomal breakpoints were close or within the centromere on both chromosome 1 (1p12-1q12) and chromosome 16 (16p11.2-16p11.1).

Chromosomal regions specific for each side of the breakpoints were defined and BACs were selected from each chromosomal region. To be able to visualize suspected translocations, which would show up as co-localization of signals deriving from 1q and 16p, hybridization probes from each side of the breakpoints were selected and labeled in different colors. By this approach we were able to identify translocations in tumor samples from paraffin sections (Figure 13). In paper I it was suggested that the gain of 1q together with loss of 16q is an early event in cancer development. We therefore used this method on tumor sections with ductal cancer in situ (DCIS) and could identify the tranlocation t(1;16).

In paraffin sections, some of the cells are cut as part of the sample preparation process. As a consequence of this parts of the cell nucleus can be lost and the M-FISH pattern specific for translocation cannot be seen in all cells. To determine if an area/subpopulation have cells with translocations or not we made an assumption that cells where 16p and 16q always co-exist are normal cells and where 16q have been lost have a translocation. We scored all signals from 16p and 16q in the area and calculated the 16p/16q ratio as an estimate of the frequency of translocation t(1q;16p). By analyzing the distribution of cells, with this approach, in a DCIS area we observed a diversity of cells, where some cells had translocation pattern and others that did not (Figure 14). This suggests that intraductal proliferation leading to DCIS precedes the translocation t(1;16).

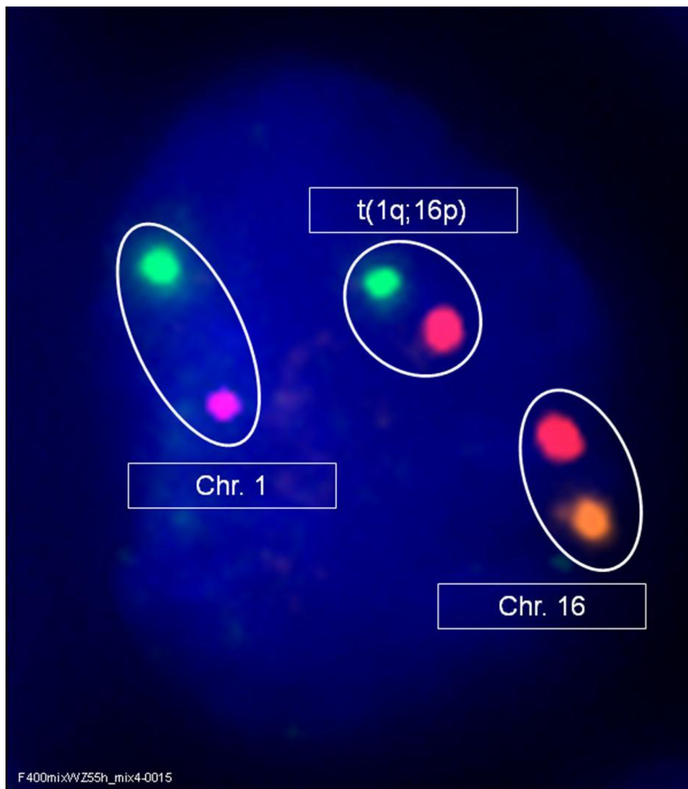

Figure 13. The image shows one normal copy of chromosome 1 (purple (1p) -green (1q)) and one normal copy of chromosome 16 (red (16p) – orange (16q)). In the translocation t(1q;16p) the green probe (1q) has been translocated close to the red probe (16p).

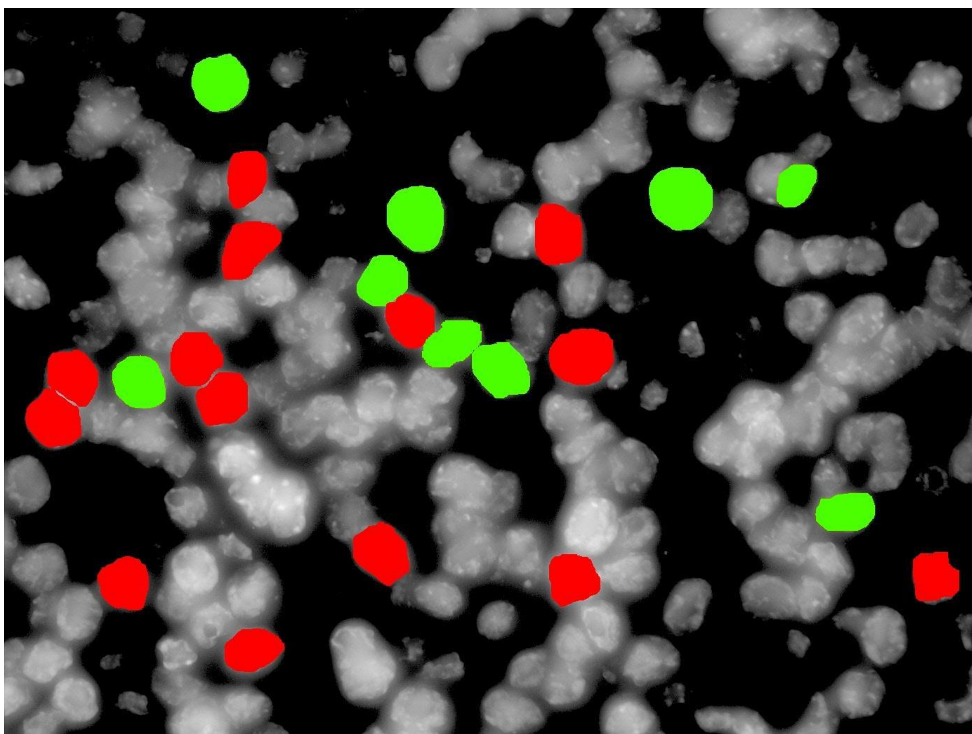

Figure 14. Cells in a DCIS area divided into two groups depending on the ratio 16p/16q. "Normal" cells with intact chromosome 16 were labeled green and cells with translocation pattern (loss of 16q) were labeled red. We can see that the two populations are fairly mixed in the area.

9 GENERAL CONCLUSIONS

Based on the findings in this thesis the following conclusions can be made:

- Three characteristic patterns of genomic rearrangements were identified, and found to be associated with tumor progression and risk for metastasis.
- Clonal genomic heterogeneity was found to be common in breast cancer. The clonal subpopulations may be anatomically separated or intermixed. A better understanding of the order of genomic events during tumor development could be obtained.
- By combining ROMA with M-FISH it became possible to study specific translocations in interphase chromatin in clinical samples. The specific translocation t(1;16) could be found in both invasive breast cancer and DCIS.
- An objective mathematical analysis of the genomic rearrangements can be used to define genome expression subtypes of breast cancer. Genomic alteration such as complex rearrangements measured objectively can be used as a prognostic tool in breast cancer.

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