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Loss of Heterozygosity and Mutation Analysis of DNA Repair Genes Rad51, XRCC2 and XRCC3 in Sporadic Breast Cancer Patients

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CONTENT

MY FAMILY

1. Introduction

Breast cancer is the most frequent malignancy in women in the Western world, with a cumulative lifetime risk estimated to be 10 to 20% (Claus, 1991; Eeles, 1994). As the major affliction of women, breast cancer affects as many as one in eight and is responsible for as many as one in five cancer-related deaths of women in western countries (Beckmann, 1997). The incidence of this disease is still increasing. Cytogenetic and molecular genetic analysis of breast cancer samples suggest that the development of human breast cancer is based on the accumulation of various genetic alterations, including activation of oncogenes as well as inactivation of tumor-suppressor genes (Black, 1994; El-Ashry, 1994).

The human genome, like other genomes, encodes information to protect its own integrity (Lindahl, 1999). To ensure the high-fidelity transmission of genetic information, cells have evolved mechanisms to monitor genome integrity. Cells respond to DNA damage by activating a complex DNA-damage-response pathway that includes cell-cycle arrest, the transcriptional and post-transcriptional activation of a subset of genes including those associated with DNA repair, and, under some circumstances, the triggering of programmed cell death. An inability to respond to properly, or to repair DNA damage leads to genetic instability, which in turn may enhance the rate of cancer development. Indeed, it is becoming increasingly clear that deficiencies in DNA-damage signalling and repair pathways are fundamental to the aetiology of most, if not all, human cancers (Khanna, 2001).

1.1 Genetic instability and Tumorigenesis

There is now evidence that most cancers may indeed be genetically unstable, but that the instability exists at two distinct levels. In a small subset of tumors, the instability is observed at the nucleotide level and results in base substitutions or deletions or insertions of a few nucleotides. In most other cancers, the instability

is observed at the chromosome level, resulting in losses and gains of whole chromosomes or large portions thereof.

Numerous genetic alterations that affect growth-controlling genes have been identified in neoplastic cells over the past 15 years, providing persuasive evidence for the genetic basis of human cancer. The study of how genomic integrity is regulated is important not only in the formation and progression of neoplasia, but also in how a response to tumor therapy. Genomic instability has been hypothesized to be a driving force behind multistep carcinogenesis (Nowell, 1976). A number of genetic changes are required for a normal cell to become tumorigenic (Fearon and Vogelstein, 1990). If genomic instability increase the rate at which genetic alterations occur, then the accumulation of changes and subsequent selection for growth and motility advantage may lead to the formation of a neoplasia. Thus, the new variants generated during tumor progression may be fueled by an underlying genomic instability. Once a cell becomes neoplastic, its evolution may continue to malignancy. Further genetic changes are required to confer metastatic properties on the tumor cell. These properties include the ability to invade surrounding tissues, enter the vasculature, extravasate, and colonize a secondary site. Proficiency at each step is necessary for a tumor cell to become fully metastatic. Genetic alterations are the basis for this acquired variation (Rubin, 1987; Liotta, 1987). The emergence of drug-resistant or radiation-resistant variants is one of the most disappointing aspects of treating a neoplasia. These variants are generated by the same forces that allow the tumor to become established and progress.

The genetic alterations in tumors can be divided into four major categories. First, subtle DNA sequence changes. These changes involve base substitutions or deletions or insertions of a few nucleotides. Second, alterations in chromosome number. Alterations in chromosome number involve losses or gains of whole chromosomes (aneuploidy). Such changes are found in nearly all major human tumor types. Third, chromosome translocation. At the molecular level, such translocations can give rise to fusions between two different genes, endowing the

fused transcript with tumorigenic properties. The fourth is gene amplification (Lengauer, 1998).

1.2 DNA double strand break and DNA Repair

DNA double-strand breaks (DSBs) are a common form of DNA damage and DSB rejoining is a fundamental mechanism in genome protection. Breaks can arise in a number of ways, by ionizing radiation, by spontaneous chromosome breaks during DNA replication, or by the programmed action of endonucleases, such as in meiosis. Broken chromosomes can be repaired either by one of several homologous recombination (HR) mechanisms, or by a number of nonhomologous repair processes. The participating proteins are listed in Figure 1. The pathways are conserved between *Saccharomyces cerevisiae* and mammalian cells.

There are two distinct and complementary mechanisms for DNA DSB repair: homologous recombination (HR) and non-homologous end-joining (NHEJ) (Fig. 2). Homologous recombination repair (HRR) entails the invasion of an undamaged DNA molecule by a damaged molecule of identical or very similar sequence, followed by resynthesis of the damaged region using the undamaged molecule as a template. A sister chromatid may be used as the template for repair, or less frequently the paternal and maternal copies of chromosomes provide the required homology. HRR allows the replacement of damaged regions without loss or alteration of base sequence. In HR, the DNA ends are first resected in the 5´ to 3´ direction by nucleases. The resulting 3´ single-stranded tails then invade the DNA double helix of a homologous, undamaged partner molecule, and are extended by the action of DNA polymerase, which copies information from the partner. Following branch migration, the resulting DNA crossovers (Holliday junctions) are resolved to yield two intact DNA molecules (Fig. 2). Although this is a widely accepted paradigm for DSB repair in meiotic cells, recent studies indicate that this model may not be applicable to mitotic HR, as mitotic gene

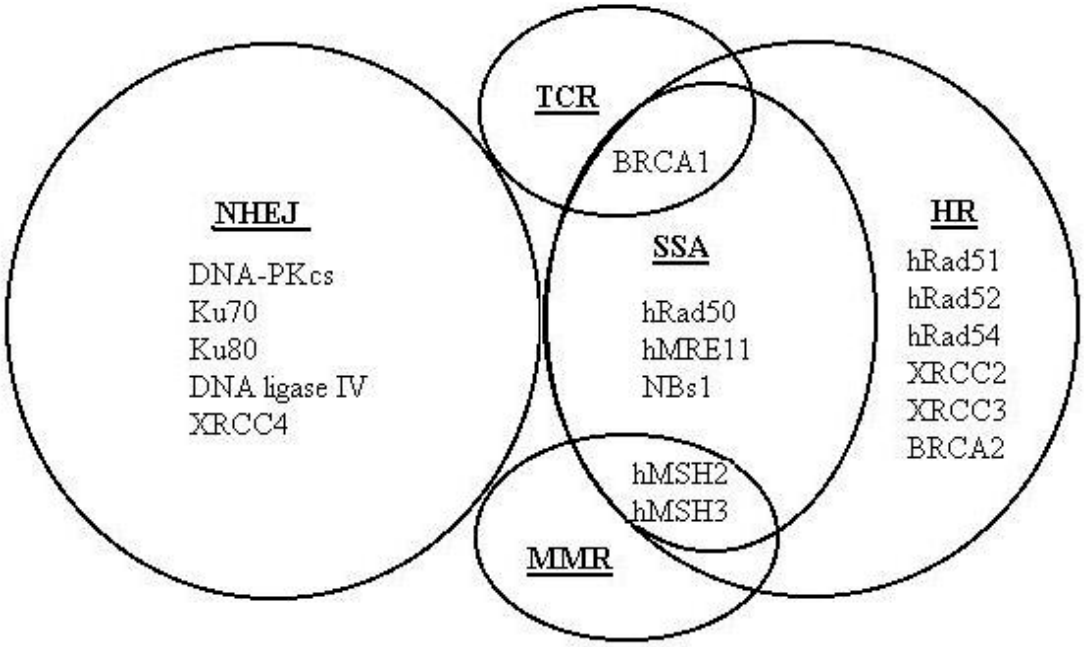

Figure1 Proteins involved in DSB repair. *The circle on the left contains a list of the known participants in NHEJ. Gene products involved in DSB repair by HR are indicated in the large circle to the right. The SSA pathway which requires some degree of homology between joined termini is depicted as a subpathway of HR. Possible overlaps with the human excision (TCR) and mismatch repair (MMR) pathways are indicated although it should be noted that there is at present no direct indication of involvement of hMSH2 or hMSH3 in the human SSA pathway.*

conversions, unlike meiotic events, are not usually associated with crossing over, whether in yeast nor in mammalian cells (Johnson, 2000). There are several types of homologous repair: gene conversion, break-induced replication and single-strand annealing (Paques and Haber, 1999). Therefore, it seems that mitotic recombination may not involve the resolution of Holliday junctions, but instead may be coupled intimately with DNA replication. A variant of HR — the single strand annealing (SSA) pathway — takes place when direct repeat sequences flank the two DNA ends and leads to loss of one of the two direct repeats and the intervening DNA.In contrast, NHEJ of two DNA ends does not

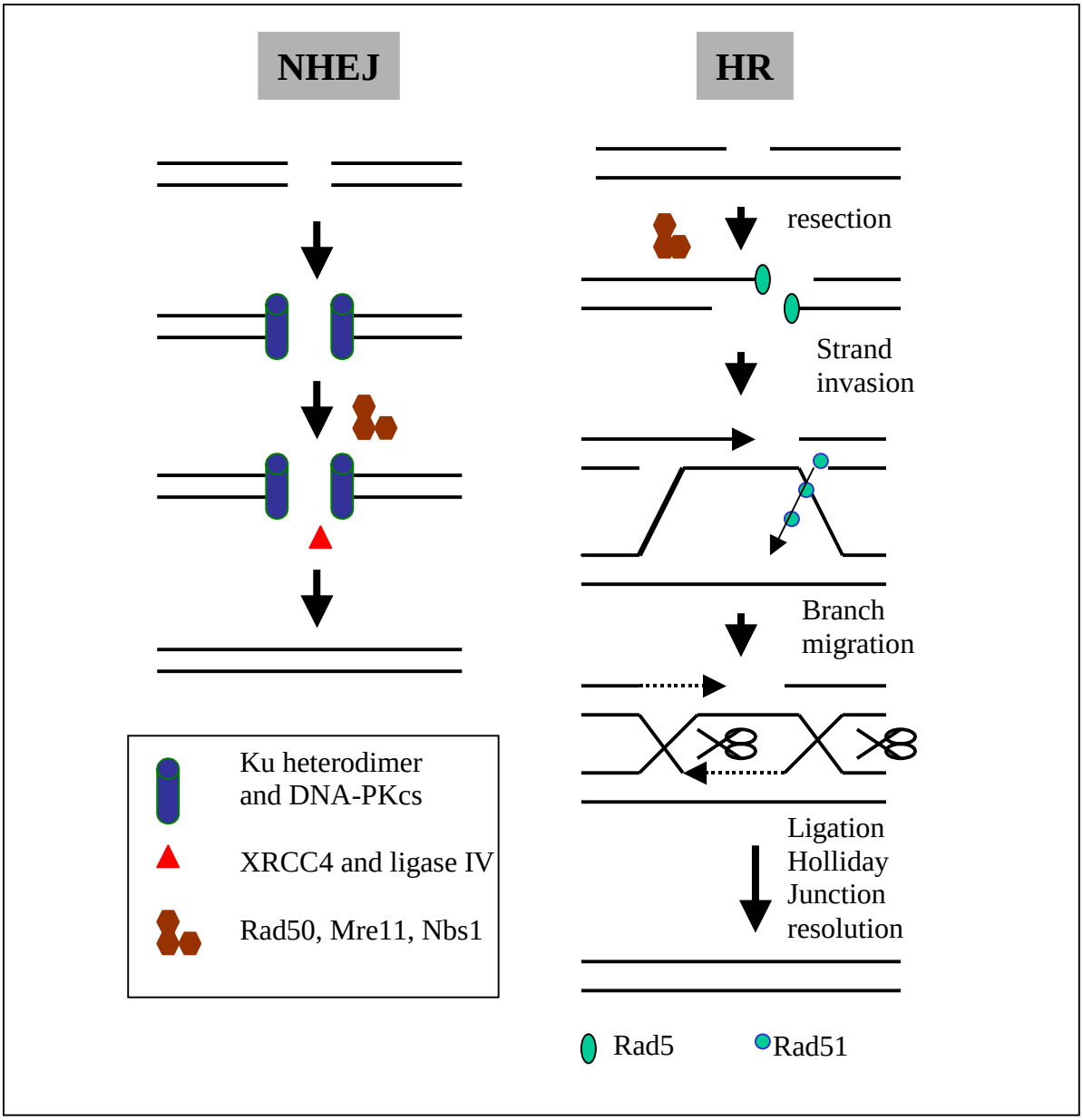

Figure 2 Pathways of DSB repair. *The termini of a DNA DSB introduced by ionising radiation or other means are bound either by the Ku heterodimer/DNA-PKcs complex or by hRad52. In the NHEJ rejoining pathway, repair is completed by DNA ligase IV and XRCC4. DNA strand invasion of the intact sister chromatid, facilitated by hRad51, initiates repair by homologous recombination. Resection and annealing of short regions of complementary sequence initiates repair by the SSA pathway in which ligation is preceded by the trimming of noncomplementary single-stranded DNA tails. The scheme is based on (Rijkers, 1998).*

require an undamaged partner and does not rely on extensive homologies between the two recombining ends. In this process, sometimes after limited degradation at the termini, the two ends are ligated together. Consequently, NHEJ is often prone to error, and small sequence deletions are usually introduced. Initial studies suggested that NHEJ was the predominant mechanism of DSB repair in higher eukaryotes, but it is now established that HR also has a crucial role. Conversely, despite the fact that the NHEJ pathway was not identified through classical genetic approaches in the yeasts Schizosaccharomyces *pombe* and *Saccharomyces cerevisiae*, it is now known that these organisms possess an NHEJ apparatus that is evolutionary conserved with that of higher eukaryotes. Recent research has begun to clarify the enzymology of DNA DSB repair pathways and has indicated key roles for these pathways in preventing mutations, chromosomal instability and cancer.

DNA repair systems are responsible for maintaining the integrity of genome and have a critical role in protecting against mutations that can lead to cancer (Bohr, 1995; Mohrenweiser, 1998). Absent or incorrect repair can initiate carcinogenesis through the activation of oncogenes, the inactivation of tumor-suppressor genes, or the loss of the heterozygosity. Repair of damaged DNA involved many proteins performing functions directly at damaged DNA as well as the interaction and interplay with proteins involved in regulation of DNA replication and progression through the cell cycle (Lehmenn, 1998). Studies have shown that genes directly involved in DNA repair and the maintenance of genome integrity, or genes indirectly involved in DNA repair through the regulation of the cell cycle, are critical for protecting against the mutations that lead to cancer (Bohr, 1995; Mohrenweiser, 1998).

1.3 Loss of heterozygosity

Loss of heterozygosity (LOH) of tumor suppressor genes (TSG) is a crucial step in the development of sporadic and hereditary cancer (Wijnhoven, 2001). LOH

screening identifies locations of TSGs. Somatic cells contain two copies of each autosomal chromosome, one inherited from each parent. Probes detecting DNA sequence polymorphisms allow investigators to distinguish between the maternally and paternally derived copies of particular DNA sequences in both normal and neoplastic cells. By screening paired blood and tumor samples with markers spaced across the genome, we can discover candidate locations for TSG. For meaningful results, a large panel of tumors must be screened with closely spaced markers. Highly polymorphic microsatellite markers are used in order to minimize the number of uninformative cases where constitutional DNA is homozygous for the marker. Not all tumors will show the pattern of one small and one large abnormality that is needed to produce visible LOH. Advanced cancer cells often show LOH at as many as one quarter of all loci, so large samples are needed to tease out the specific changes from the general background chromosomal instability. Finally, most pathological tumor samples contain a mixture of intergrowing tumor and non-tumor (stromal) tissue, so that LOH shows as a decreased relative intensity (allelic imbalance) rather than total loss of the band from one allele. According to the classical two-hit model for inactivation of TSGs described first by Knudson (Knudson, 1971) the recessive mutation in the TSG is uncovered by loss of the second copy of the gene. Therefore, LOH at polymorphic markers located in or close to a tumor suppressor gene is an indirect hint for the somatic inactivation of the remaining copy of this suppressor gene and consequent loss of the tumor-suppressing function of its gene product. Because of its theoretical feasibility and technical simplicity, LOH has been widely used to identify chromosome regions which contain putative TSGs involved in the tumorigenesis (Beckmann, 1996b; Niederacher, 1997).

1.4 Rad51 family

Rad51 is a homologue of bacterial RecA, which is required for meiotic and mitotic recombination and for recombinational repair of double-strand DNA

breaks. Recently a number of novel RAD51-like genes, including XRCC2 and XRCC3, have been found (Thacker, 1999b). XRCC2 and XRCC3 proteins have a low level of similarity with HsRad51 (or Rad51) and other mammalian Rad51-family members (Liu, 2000). Four additional Rad51-like human proteins were identified recently: Rad51B (Albala, 1997; Rice, 1997), Rad51C (Dosanjh, 1998), and Rad51D (Pittman, 1998), as well as the meiosis-specific homolog HsDmc1 (Habu, 1996). Searching databases for ORFs that are similar to Rad51 first identified Rad51B, Rad51C, and Rad51D. The Walker nucleotide binding motifs A and B are present in all of these proteins, suggesting that they can hydrolyze ATP. Whereas HsDmc1 has ~50% identity to HsRad51, the other human Rad51-family members have 20~30% identity with HsRad51 and show comparable similarity to each other.

1.4.1 Rad51

The RAD51 gene belongs to the RAD52 epistasis group (RAD50–57, -59, MRE11, XRS2) of Saccharomyces cerevisiae (Petrini, 1997). Mutations in RAD51 cause reduced meiotic and mitotic recombination, hypersensitivity to ionizing radiation and methyl methanesulfonate, and a deficiency in repairing DNA DSBs) (Game and Mortimer, 1974; Shinohara, 1992; Game, 1993). Rad51 protein (Shinohara, 1992) is an analog of RecA, a central player in recombination in *Escherichia coli* (Roca and Cox, 1997). Biochemical studies show that Rad51 forms filaments on single-stranded DNA and has ATP-dependent and strand transferase activity (Ogawa, 1993; Sung, 1994, 1997). HsRad51, the human counterpart of the yeast Rad51, shares many biochemical properties with Rad51. It binds to single- and double-stranded DNA, exhibits DNA-dependent ATPase activity, forms helical nucleoprotein filaments, and mediates homologous pairing and strand exchange between DNA molecules (Benson, 1994; Baumman, 1996; Gupta, 1997). During the S phase of the mitotic cell cycle, HsRad51 forms nuclear foci (Haaf, 1995) that are not seen in G0/G1 phase (Tashiro, 1996; Scully, 1997), suggesting a role in DNA replication.

Recombination is a fundamental process essential to all living cells, as is the repair of DNA damage. Therefore, it was highly probable that similar recombination proteins are present in various organisms. Shinohara (1993) cloned genes from human, mouse and Schizosaccharomyces pombe (fission yeast) that are homologous to Rad51. The 339-amino acid proteins predicted for the 2 mammalian species were also identical and were highly homologous (83%) with the yeast proteins. The mouse gene was transcribed at a high level in thymus, spleen, testis, and ovary and at a lower level in brain. The mouse gene was localized to the F1 region of chromosome 2 by fluorescence in situ hybridization (FISH); the human gene was mapped to chromosome 15 by analysis of a somatic cell hybrid panel. By FISH, Takahashi et al. (1994) assigned the RecA gene to 15q15.1 and to mouse 2F1. Disruption of the Rad51 gene in mice was shown to give early embryonic lethality (Lim and Hasty, 1996; Tsuzuki, 1996), perhaps because of its key role in HRR and the need for high-fidelity repair in replicating cells of the developing embryo. However, other important HRR genes, such as Rad52 and Rad54, do not give embryonic lethality when disrupted (Essers, 1997; Rijkers, 1998). The homozygous Rad51 null mutation can be characterized as a preimplantational lethal mutation that disrupts basic molecular functions of cells. RAD51 functions in recombination and in DNA repair. The BRCA1 and BRCA2 proteins, implicated in familial breast cancer, form a complex with RAD51, and these genes are thought to participate in a common DNA damage response pathway associated with the activation of HR and DSB repair (Scully, 1997).

1.4.2 X-ray repair, complementing defective, in chinese hamster, 2; XRCC2

Thacker et al. (1995) fused the V79 hamster cell line irs1, which is a repair-deficient mutant that shows hypersensitivity to a number of different DNA-damaging agents (Jones, 1987), to normal human cells, resulting in complementation of the defect. The hybrid cells showed correction of sensitivity to both x-rays and mitomycin C and contained human chromosome 7. Hybrids showing unstable retention of human chromosomes were subcloned to show that

loss of chromosome 7 and loss of resistance to mitomycin C occurred concordantly. Two separate hybrids were found to have a smaller piece of chromosome 7, and specific DNA probes and microsatellite markers defined this as a contiguous region at 7q35-q36. Hybrid irradiation-fusion methods were used to reduce further the size of the complementing genomic region and to localize the gene to an approximately 3- to 5-Mb region at 7q36.1 (Jones, 1995). They formed somatic cell hybrids by fusing irs1 cells with human lymphocytes and selecting for complementation in medium containing concentrations of mitomycin C that are toxic to irs1 cells but not their human fusion partners. Retention of chromosome 7 or of the region 7q36 resulted in cells that were resistant to mitomycin C. Tambini et al. (1997) took the radiation reduction of human/hamster hybrids further to locate the XRCC2 gene to a small genomic region defined by a single microsatellite marker D7S483. Yeast artificial chromosomes (YACs) carrying that marker were then fused to the irs1 hamster cell line and a YAC that carried the complementing gene was identified. This YAC was used for direct cDNA selection experiments to identify the XRCC2 gene.

The gene was found to share homology with the yeast Rad51 gene and its human homolog, which are involved in the recombinational repair of DNA damage. Strong support for the candidacy of this gene as XRCC2 was obtained from its refined map position and by the full complementation of irs1 sensitivity with a 40-kb cosmid carrying the gene (Tambini, 1997).

XRCC2 is essential for the efficient repair of DNA DSB by HR between sister chromatids (Johnson, 1999). Hamster cells deficient in XRCC2 showed a more than 100-fold decrease in HR induced by DSBs compared with the parental cell line. This defect was corrected to almost wildtype levels by transient transfection with a plasmid expressing XRCC2. The repair defect in XRCC2 mutant cells appeared to be restricted to recombinational repair because NHEJ was normal. This suggests that XRCC2 is involved in the repair of DNA DSBs by HR (Johnson, 1999).

1.4.3 X-ray repair, complementing defective, in chinese hamster, 3; XRCC3

The mutagen-sensitive CHO line irs1SF was first isolated on the basis of hypersensitivity to ionizing radiation and was found to be chromosomally unstable as well as cross-sensitive to diverse DNA-damaging agents: UV radiation, ethyl methanesulfonate, camptothecin, and the cross-linking agents mitomycin C, cisplatin, nitrogen mustard, and melphalan. A human cDNA sequence (XRCC3) that corrected X-ray and cross-linking sensitivities, as well as spontaneous chromosomal aberrations, of irs1SF was cloned (Tebbs, 1995). With genomic DNA from 2 independent hybrid clone panels, FISH and Southern blot hybridization mapped XRCC3 to human 14q32.3. XRCC3 and Rad51B have been observed to interact with Rad51C in the yeast two-hybrid system (Dosanjh, 1998). XRCC3 interacts directly with RAD51 and may cooperate with RAD51 during recombinational repair (Liu, 1998).

1.5 Single nucleotide polymorphism

Polymorphism arises as a result of mutation. The type of mutation that created them typically refers to the different types of polymorphism. The simplest type of polymorphism results from a single base mutation which substitutes one nucleotide for another. The polymorphism at the site harboring such changes has recently been termed a ‘single nucleotide polymorphism (SNP)’, although previously, in some instances, such variation was referred to by the particular methods used to detect it. For example, the first systematic studies of single base variants were pursued through the identification of restriction enzyme sites, where a single base pair change could result in the loss or gain of a restriction site. Digestion of a piece of DNA containing the relevant site with an appropriate restriction enzyme could then distinguish alleles or variants based on resulting fragment sizes via electrophoresis, and this type of polymorphism was thus referred to as ‘restriction fragment length polymorphism (RFLP)’ (Botstein, 1980). Other SNPs, which do not directly create or destroy a restriction site, have

been identified, often by creating restriction sites via PCR primer design, by oligonucleotide probing, or by direct sequencing.

Other types of genetic polymorphism result from the insertion or deletion of a section of DNA. The most common type is the existence of variable numbers of repeated base or nucleotide patterns in a genetic region (Cooper, 1999). Repeated base patterns range in size from several hundreds of base pairs, known as 'variable number of tandem repeats' (VNTRs or 'minisatellites'), to the more common 'microsatellites' consisting of two, three or four nucleotides repeated some variable number of times. Another type of insertion:deletion polymorphism involves the presence or absence of Alu segments at a genetic location.

The high frequency with which SNPs are found on the genome gives them definite utility for trait or disease gene discovery purposes. Thus, one can use SNPs as markers for very dense gene mapping studies in positional cloning efforts, or, more importantly, as candidate polymorphisms to be tested directly as the functional or causal mutations for a trait or disease.

SNPs are found throughout the genome, e.g. in exons, introns, intergenic regions, in promoters or enhancers, etc. Hence, they are more likely to yield, upon collection, a functional or physiologically relevant allele than other sorts of polymorphism. What is of extreme interest in this regard is the nature of the effect that a simple base pair substitution can have on a trait or disease. Thus, a SNP in coding region may directly impact a relevant protein, an intronic SNP can influence splicing (Krawczak, 1992), a SNP in a promoter can influence gene expression (Drazen, 1999), etc. The degree to which each kind of SNP influences phenotypic expression is likely to receive a great deal of attention as more and more SNPs are identified and studied.

New SNP alleles arise as mutations at different loci and at different points in time, and they occur with such great abundance over the genome, groups of neighboring SNPs may have alleles that show distinctive patterns of linkage disequilibrium (LD, i.e. LD is the phenomenon whereby the presence of one allele on a chromosome may suggest a high probability that a particular allele will

be present at a neighboring site on the same chromosome) and as such may create a haplotypic diversity that can be exploited in both genetic linkage and direct association studies (Nickerson, 1992). Since SNPs can occur very close to one another, study of the patterns of LD they show may reveal sites for recurrent mutation, gene conversion, or recombination ‘hot-spots’. Such information may be very useful when assessing a genomic region for linkage or association with a particular trait or disease (Clark, 1998; Chakravarti, 1998).

2 Material and Methods

2.1 Breast tumor and normal breast tissues

Tumor and normal tissues of breast cancer women with sporadic breast cancer [invasive ductal (IDC) and invasive lobular (ILC)] used in this study derived from patients of the Department of Obstetrics & Gynecology, Heinrich-Heine University, Duesseldorf, Germany from 1993 to 1999. Surgically removed tissues were snap frozen (liquid nitrogen, -80°C) for later extraction of DNA. Haematoxylin-eosin (H&E) staining was used for routine pathological evaluation. The content of tumour cells in all the tumour samples used was assessed to be more than 70%. Peripheral blood leukocytes were used as a source of normal DNA, which were obtained from patients at the time of surgery. The histopathological status contained: TNM classification (according to the UICC classification): T = tumour size, N = status of region lymph node, M = metastasis. Histological grad (classification used Scarff-Bloom-Richardson); Hormone receptor status: estrogen and progesterone receptor; the clinical data of the patients are shown in Appendix.

2.2 DNA isolation

2.2.1 DNA isolation from peripheral blood leukocytes

To isolate lymphocytes 3 ml patients' blood (in 0.1 mM EDTA) was mixed with 9 ml erythrocyte lysis buffer (155 mM ammoniumchlorid, 10 mM $KHCO_3$, 0,1 mM EDTA, pH 7,4) and centrifuged (1000 rpm, 10 minutes, 4°C). Sediment was washed with 5 ml PBS once, centrifuged (1000 rpm, 10 minutes, 4°C), and resuspended in 3 ml SE buffer(75 mM NaCl, 25 mM EDTA, pH 8,0) with 10% SDS. 100 µg/ml proteinase K (Sigma, MO, USA) was added to digest at room temperature (R.T.) over night followed by extractions twice with phenol: chloroform: isoamylalcohol (25:24: 1) and once with chloroform: isoamylalcohol (24: 1). 0.5 volume 7.5M ammoniumacetat and 2.5 volume prechilled absolute

ethanol were added to the supernatant to precipitate DNA at –20°C for 30 minutes. After centrifugation (3500 rpm for 10 minutes) the DNA precipitate was washed with 70% ethanol followed by drying in a thermoblock at 37°C. Thereafter DNA was dissolved in bidistilled water and stored at –20°C pending analysis after DNA quantitation. DNA samples were quantitated on a Lambda Bio Spectrophotometer (Perkin Elmer, Oberlingen, Germany). DNA concentration was determined by the absorption value at 260 nm. The ratio of absorption value at 260 nm and 280 nm was used as index of DNA purity.

For DNA isolation exclusive we used a QIAGEN extraction kit (QIAamp DNA blood midi kit). In detail, to isolate genomic DNA, 2 ml patients' whole blood (in 0.1 mM EDTA) was mixed at room temperature (R.T.) thoroughly with 200µl QIAGEN protease stock solution and 2.4 ml Buffer AL, then incubate at 70°C for 10 minutes, for optimal binding with 2 ml of ethanol (100%) to the sample and mix again by vortex, carefully transfer half of the solution (3.3 ml) onto the membrane of QIAamp Midi column placed in a 15 ml centrifugation tube, and centrifuge at 1850×g (3000 rpm) for 3 minutes, DNA is adsorbed onto the QIA amp silica membrane, discard the filtrate, load the remainder of the solution onto the QIAamp Midi column and recentrifuge at 1850×g (3000 rpm) for 3 minutes, then QIAamp Midi column was washed with 2 ml of Buffer AW1 and centrifuge at 4000 rpm for 1 minutes, then washed with 2 ml of Buffer AW2 and centrifuge at 4000 rpm for 15 minutes, followed incubate the QIAamp Midi column for 10 minutes at 70°C in an incubator to evaporate residual ethanol, thereafter DNA was dissolved with 300 µl of elution Buffer AE onto the membrane of the QIAamp Midi column, incubate at room temperature for 5 minutes and centrifuge at 4000 rpm for 5 minutes, reload the 300 µl of elute containing the DNA onto the membrane of the QIAamp Midi column for maximum concentration, incubate at room temperature for 5 minutes, centrifuge at 4000 rpm for 5 minutes, DNA stored at –20°C pending analysis after DNA quantitation.

2.2.2 DNA isolation from tissues

Tissues taken from liquid nitrogen were sliced into pieces, immersed into liquid nitrogen immediately and then pulverized by using a microdismembrator (Braun Melsungen, Germany) at 2000 rpm for 1 minute. Then, 3—4 ml digestion buffer (10mM Tris/HCl, pH 8.0; 25mM EDTA, pH 8.0; 100mM NaCl, 0.5% SDS) with proteinase K (0.1 mg proteinase K/ml digestion buffer) was added. After incubation for 12 -16 h at 50°C the sample was subjected to DNA extraction as described above (3.2.1.1).

2.3 LOH analysis for Rad51, XRCC2 and XRCC3

2.3.1 PCR for LOH analysis for Rad51, XRCC2 and XRCC3

DNA from tumor tissues and matched normal blood were used for LOH analysis. Four polymorphic microsatellite markers were choosen for the LOH analysis of Rad51, XRCC2 and XRCC3. Microsatellite marker D15S118 in the vicinity of the Rad51 located at 15q15.1 was selected for Rad51, D7S483 and one intragenic XRCC2MS marker were used for the XRCC2, one intragenic marker XRCC3MS at 14q32.3 was choosen for the XRCC3. Primer-Sequences and length of each fragment were indicated in table 2.1. These four microsatellite markers were dinucleotide repeats.

Table 2.1*: Primers for LOH analysis.*

Marker	Sequence	Length	Reference
D15S118	5-*TCAAAGACCCATATCAACCA-3 5-GTGCTGAAAAGCGACACTTA-3	218-230 bp	GDB: 187991
D7S483	5-*AGTGGTCATTAGCCTTGGCAAAATC-3 5-AACCAGAGTTGTAAGCCATGAAAGT-3	166-168 bp	GDB: 187927
XRCC2MS	5-*GGGTGGAGTGAGGATGGTTA-3 5-GCTGAGATGGCACCATTGTA-3	207 bp	GenBank: AC003109
XRCC3MS	5*-GACAATATGCATGTATTACTTTG-3 5-GTGTGCAGTTTATATAAGGCAGG-3	204 bp	Price EA et al., 1997

"" means that primer is marked with Cy5 so that the PCR products can be detected in an A.L.F.-Sequencer. The four microsatellite markers were dinucleotide repeats.*

PCR reaction was pipetted in a final volume of 50 µl contained 50ng genomic DNA, 1 x PCR reaction buffer, each 125 µM dNTPs (dATP, dCTP, dGTP and dTTP), 25 mM 5´- and 3´- primers, 2 U Taq DNA polymerase (Amersham Pharmacia Biotech) and bidistilled water. The Cy5 labeled primers were used for the detection with A.L.F. or A.L.F. Express sequencer. These PCR reactions were amplified in Onmigene Thermal Cycler (Hybaid) overlaid with two drops of mineral oil. Firstly, the double-stranded DNA was denatured at 95°C for 10 minutes. After 5 minutes 2.0 U Taq polymerase was added to each cup, this process was called "hot-start" to minimize unspecific annealing and to maximize Taq polymerase activity. A final extension at 72°C for 8 minutes was performed. The PCR conditions for each fragment were summarized in table 2.2.

Table 2.2*: primer conditions for LOH analysis*

Marker	Reagents		PCR condition
D15S118	10x reaction buffer	5.0 µl (1x)	94 °C 30 sec
	dNTP	2.5 µl (125 µM)	55 °C 30 sec
	Primer each	2.5 µl (25 pmol)	72 °C 2 min
	DNA	5.0 µl (50 ng)	30 cycles
	Taq	0.4 µl (2 U)	
	Add aqua dest. to	50 µl	
D7S483	10x reaction buffer	5.0 µl (1x)	94 °C 30 sec
	dNTP	2.5 µl (125 µM)	58 °C 30 sec
	Primer each	2.5 µl (25 pmol)	72 °C 1 min
	DNA	5.0 µl (50 ng)	30 cycles
	Taq	0.4 µl (2 U)	
	Add aqua dest. to	50 µl	
XRCC2MS	10x reaction buffer	5.0 µl (1x)	94 °C 1 min
	dNTP	2.5 µl (125 µM)	57 °C 1 min
	Primer each	2.5 µl (25 pmol)	72 °C 1 min
	DNA	5.0 µl (50 ng)	30 cycles
	Taq	0.4 µl (2 U)	
	Add aqua dest. to	50 µl	
XRCC3MS	10x reaction buffer	5.0 µl (1x)	94 °C 1 min
	dNTP	2.5 µl (125 µM)	52 °C 1 min
	Primer each	2.5 µl (25 pmol)	72 °C 1 min
	DNA	5.0 µl (50 ng)	30 cycles
	Taq	0.4 µl (2 U)	
	Add aqua dest. to	50 µl	

2.3.2 Gel electrophoresis with the A.L.F. and A.L.F. Express to detect LOH

Material:

-1 x TBE

0,2 M Tris

0,17 M Boric acid

2 mM EDTA, pH 8,0

-Bind-silane

4 ml ethanol absolute

15 µl Bind-Silane (Pharmacia)

1 ml 10% acidic acid

-Acrylamide/bis-acrylamide mixture (29:1)

29% Acrylamide

1% N,N-Methylen-bisacrylamide

-Gel solution for ALF-Gel

25,2 g Urea

9 ml Acrylamide/bis-acrylamide mixture (40 %)

25,5 ml Aqua dest.

6 ml 6 x TBE

0,2 ml 10% APS (ammonium persulphate)

40 µl TEMED (NNN`N`-tetramethylethylene diamine)

-Loading-buffer for A. L. F. sequencer

100 ml Formalid (deionised with amberlite)

600 µg Dextranblue

4 ml 20 mM EDTA, pH 8.3

-Amberlite (Pharmacia)

-TEMED (N, N, N, N, -Tetramethylethylendiamin)

-10% APS (Amoniumpersulfate) in Aqua dest.

Small amounts of fluorescence-labeled (Cy5) PCR-products can be detected using an automatic DNA sequencer (A.L.F. and A.L.F. Express of Pharmacia). The

intensity of the emitted fluorescent was measured through the optic system of A.L.F. or A.L.F. Express and then converted automatically into electronic signals. Using the corresponding software Fragment Manager the data were quantified in terms of peak size, height and area under the curve.

Glass plates were rinsed completely with distilled water and absolute ethanol, polished dry with lint-free tissues, and assembled together with gel clamps ensuring a 0.5 mm space between the plates. 25.2 g urea and 9ml 29:1acrylamide/bis-acrylamide mixture was dissolved in 25.4 ml distilled water. The Mixture solution was stirred 10 minutes with 5 g amberlite. The stirred solution filtered under vacuum after 6 ml 6 x TBE through a 0.2 µm cellulose acetate filter. The solution was degassed for 5 minutes, and 400 µl 10% APS and 40 µl TEMED were added. The gel solution was mixed well and poured into the space between the glass plates. After polymerization of 2 hours the gel cassette was fitted into the A.L.F. or A.L.F. Express system.

For fragment analysis the PCR products were diluted properly according to the yield of the PCR reaction (diluted between 1:5 and 1: 25). 5.5 µl diluted PCR product was mixed with 5.5 µl ALF loading buffer followed by denaturing 5 minutes at 95°C and cooling 3 minutes on ice. Thereafter, the samples were loaded on the gel and electrophoresis was processed under the following conditions.

Running condition	**Fragment analysis**
Voltage:	1500 V
Current:	34 mA
Power:	38 W
Temperature:	40°C
Run buffer:	0,6 x TBE
Run time	150~300 minutes

For calculation of product quantities the Fragment Manager Software (Pharmacia) was used.

LOH analysis was carried out with normal and tumour DNA of each patient. Normal DNA indicated whether or not a patient is informative (heterozygote) for markers analysed. In heterozygous individuals, two PCR products of different size for both alleles can be detected. In “not-informative” (homozygote) individuals both alleles shared the same number of dinucleotide repeats and only one PCR product can be detected. In heterozygote individuals the loss of one allele was indicated through the reduction at tumour PCR products compared to normal DNA. Because PCR fragments of different sizes are amplified with different efficiencies, the ratios of allele peak areas were calculated in matched normal and tumor DNA samples. The peak areas of the shorter length allele divided by peak areas of the longer length allele. First proportion of both alleles in normal and tumour PCR products [Ratio (normal) und Ratio (Tumor)] were calculated. Then the allele proportion Q can be calculate through both ratios as follows.

$$\textbf{Ratio (Normal)} = \frac{\textbf{Area (1. Allele)}}{\textbf{Area (2. Allele)}}, \qquad \textbf{Ratio (Tumor)} = \frac{\textbf{Area (1. Allele)}}{\textbf{Area (2. Allele)}}$$

$$\textbf{Allele proportion Q} = \frac{\textbf{Ratio (Tumor)}}{\textbf{Ratio (Normal)}}$$

The ratio obtained in tumor DNA divided by that of paired normal DNA gives a result range of 0.00 - 1.00. Theoretically, a complete allele loss results in a value of 0.00 and both alleles retained in the tumor DNA gives a ratio of 1.00. In case the shorter length allele was lost in the tumor DNA, which results in ratios greater than 1.00, the ratio was converted (1/x) to obtain values below 1.00. Considering contaminating normal DNA in the tumor DNA samples, ratio below 0.65, was considered to be indicative of allele loss (Niederacher et al., 1997).

2.4 DHPLC analysis

2.4.1 PCR for DHPLC analysis of XRCC2 and XRCC3

All 3 coding exons of XRCC2 and 7 coding exons of XRCC3, as well as intron sequences adjacent to coding exons, were PCR amplified from genomic DNA through use of the 12 primer pairs (Metabion GmbH, Munich Gemany) listed in table 2.3 and 2.4. All primers were complementary to intron sequences near the exon boundaries. Primers for the remaining exon regions were selected from the published genomic sequence for XRCC2 (GenBank Accession number: AC003109) and *XRCC3* (GenBank Accession number: AF037222).

PCR amplification was performed in 30--50µl reactions using Expand High Fidelity *Taq* Polymerase (Roche), in a hot lid PCR cycler (Thermocycler Firma Biometra) without mineral oil. The samples were then taken directly from PCR to a denature/reanneal protocol, which involves heating to 95°C then slowly cooling to 65°C over 30 minutes. PCR conditions displayed in table 2.5 and 2.6 (1 cycle of 95°C for 5 minutes; followed by 35 cycles of 94°C for 30 sec, different annealing temperature of each primer pair for 30 sec, 72°C for 2 minutes; and 1 cycle of 72°C for 8 minutes. To monitor for the presence of contamination, every plate had a negative control containing no DNA template.

Table 2.3*: Primers for DHPLC analysis of XRCC2 (GenBank Accession number: AC003109)*

Exon	Sequence	Length (bp)	Position
Exon1	5-GAAAGTTGAGTCTCTCCTGCG-3 5-AGAGGGTGCCAGCATCGCGG-3	236	4285--4520
Exon2	5-TCTTACAGACTTTCGGAAAATGG-3 5-GTGAGGAGTATGTGTATACATGT-3	261	15928--16188
Exon3.1	5-CTTTCACATTCCAGTAAGTGTCT-3 5-TAAGTGGGTGCTACTACTGCAG-3	364	30931--31294
Exon3.2	5-GCACAGACTATCCCAAAGCT-3 5-TTCTTCTGATGAGCTCGAGG-3	334	31206--31539
Exon3.3	5-ATGTTCTCAGTGCTTAGAGAAG-3 5-AAGGCTTGCGTAGTACCCTGC-3	363	31440--31802

***Table 2.4**: Primers for DHPLC analysis of XRCC3 (GenBank Accession number: AF037222)*

Exon	Sequence	Length (bp)	Position
Exon2	5-TCTACAGTGAAGTCTCCTCCA-3 5-CCACCCACACCCTTTATGTAA-3	278	6210--6487
Exon3	5-CTGCGTTGTGACAGTCTGACA-3 5-CACCCCTGGCAGAGATGCCA-3	192	8773--8964
Exon4	5-AGGCCTGACACTATCCCTGC-3 5-AAGCTGTCCCACACAAAGCAG-3	306	10241--10546
Exon5	5-CACATCAGGCACTCTTGCTTC-3 5-AATGGTCCTGAATAGCTTGCC-3	234	14131--14364
Exon6	5-CACAGGACACCTTGTTGGAG-3 5-CTCACCTGGTTGATGCACAG-3	223	17897--18119
Exon7	5-TGTGCCTAACCATCGAGAAGA-3 5-TGAGAAACAGGAAGCAGGCAA-3	221	18243--18463
Exon8	5-TGTGCACCTCTGTGCAGGTG-3 5-CTTCTCAGGCAGGGCTGTTGT-3	350	18370--18719

***Table 2.5**: PCR conditions for DHPLC analysis of XRCC2*

Exon	Reagents		PCR condition
1 ; 3.1	10x reaction buffer	5.0 µl (1x)	94°C 30 sec
	dNTP	2.5 µl (125 µM)	62°C 30 sec
	Primer each	2.5 µl (25 pmol)	72°C 2 min
	DNA	5.0 µl (50 ng)	35 cycles
	Taq	0.6 µl (2 U)	
	Add aqua dest. to	50 µl	
2 ; 3.2	10x reaction buffer	5.0 µl (1x)	94°C 30 sec
	dNTP	2.5 µl (125 µM)	58°C 30 sec
	Primer each	2.5 µl (25 pmol)	72°C 2 min
	DNA	5.0 µl (50 ng)	35 cycles
	Taq	0.6 µl (2 U)	
	Add aqua dest. to	50 µl	
3.3	10x reaction buffer	5.0 µl (1x)	94°C 30 sec 61°C 30 sec 72°C 2 min 35 cycles
	dNTP	2.5 µl (125 µM)	
	Primer each	2.5 µl (25 pmol)	
	DNA	5.0 µl (50 ng)	
	Taq	0.6 µl (2 U)	
	Add aqua dest. to	50 µl	

***Table 2.6**: PCR conditions for DHPLC analysis of XRCC3*

Exon	Reagents		PCR condition
2 ; 5 ; 7	10x reaction buffer	5.0 µl (1x)	94°C 30 sec
	dNTP	2.5 µl (125 µM)	57°C 30 sec
	Primer each	2.5 µl (25 pmol)	72°C 2 min
	DNA	5.0 µl (50 ng)	35 cycles
	Taq	0.6 µl (2 U)	
	Add aqua dest. to	50 µl	
3	10x reaction buffer	5.0 µl (1x)	94°C 30 sec
	dNTP	2.5 µl (125 µM)	60°C 30 sec
	Primer each	2.5 µl (25 pmol)	72°C 2 min
	DNA	5.0 µl (50 ng)	35 cycles
	Taq	0.6 µl (2 U)	
	Add aqua dest. to	50 µl	
4	10x reaction buffer	5.0 µl (1x)	94°C 30 sec
	dNTP	2.5 µl (125 µM)	58°C 30 sec
	Primer each	2.5 µl (25 pmol)	72°C 2 min
	DNA	5.0 µl (50 ng)	35 cycles
	Taq	0.6 µl (2 U)	
	Add aqua dest. to	50 µl	
6 ; 8	10x reaction buffer	5.0 µl (1x)	94 °C 30 sec
	dNTP	2.5 µl (125 µM)	59°C 30 sec
	Primer each	2.5 µl (25 pmol)	72°C 2 min
	DNA	5.0 µl (50 ng)	35 cycles
	Taq	0.6 µl (2 U)	
	Add aqua dest. to	50 µl	

2.4.2 Agarose gel electrophoresis

The aim of agarose gel electrophoresis was to determine the yield and to know the effect of PCR amplification. For separating of DNA fragments of different length 1.5~2.0% agarose gel was used. The agarose was made up in 1 x TBE, heated in a microwave oven to melt the agarose. After coagulation (minimum 30 minutes) 10 µl samples (5 µl PCR products and 5 µl sample buffer) were loaded on the gel. Electrophoresis was processed at 100 V with 1 x TBE as running buffer and 1 Kb DNA ladder as length standard for 40—60 minutes. Thereafter the gel was immersed in the ethidium bromide staining solution for about 40 minutes followed by visulization under UV light (λ=312 nm).

2.4.3 DHPLC analysis

Material: 2M Triethylammonium acetate (TEAA) buffer (Transgenomic)
acetonitrile (J. T. Baker)
LiChrosolv® water for the chromatography (Merck)

-Puffer A: 50 ml 2M TEAA
250 µl HPLC grade acetonitril
ad 1 l LiChrosolv® water

-Puffer B: 50 ml 2M TEAA
250 ml HPLC grade acetonitrile
ad 1 l LiChrosolv® water

-Puffer C: 750 ml HPLC grade acetonitrile
ad 1 l LiChrosolv® water

-Syringe (wash solution): 80 ml HPLC grade acetonitrile
ad 1 l LiChrosolv® water

DHPLC analysis was performed on an automated DHPLC instrument (Transgenomic Inc., San Jose, CA). The stationary phase consisted of a DNASep® column, which binds DNA during analysis. The mobile phase consisted of two eluents (pH7.0). Buffer A contained TEAA, which interacts with the negatively charged phosphate groups on the DNA as well as with the surface of the column (http://www.transgenomic.com/Pages/Applicationnotes.html#101). Buffer B contained TEAA with 25% of the denaturing agent acetonitrile. Fragments were eluted with a linear acetonitrile gradient of 2% per minutes at a flow rate of 0.9 ml/min. Increasing the concentration of acetonitrile at a fixed temperature will denature the fragments. Temperatures for successful resolution of heteroduplexes were both calculated by the DHPLC Melt program (http://insertion.stanford.edu/cgi-bin/melt.pl) and experimentally determined for the fragments. For detection of mutations samples were analyzed at increasing column temperatures, until a significant decrease in retention time occurred.

PCR amplicons of each XRCC2 and XRCC3 exons were analyzed by DHPLC by using

***Table 2.7**: General DHPLC methods for purification of DNAsep column (Column Clean Up and Instrument Clean Up) and for stand-by (Sleep)*

Method	Temp	Gradient [%B]	Gradient [%C]	Time [min]	Flow rate [ml/min]
Sleep	50°C	50		5.0	0.05
Column Clean Up	56°C	5-100; 100-100		30.0; 3.0	0.5
Instrument Clean Up	60°C	100%C - 50	100	30.0; 5.0	0.9

***Table 2.8:** DHPLC conditions to analyse XRCC2*

	Temp. I	Temp. II	Gradient [%B]	Time [min]
Exon1	65 °C	67 °C	45-50, 50-59, 59-100	0.5; 4.5; 0.1
Exon2	55 °C		45-51, 51-60, 60-100	0.5; 4.5; 0.1
Exon3.1	58 °C	60 °C	49-54, 54-63, 63-100	0.5; 4.5; 0.1
Exon3.2	59 °C		45-50, 50-59, 59-100	0.5; 4.5; 0.1
Exon3.3	59 °C		49-54, 54-63, 63-100	0.5; 4.5; 0.1

***Table 2.9**: DHPLC conditions to analyse XRCC3*

	Temp. I	Temp. II	Temp. III	Gradient [%B]	Time [min]
Exon2	56 °C	59 °C	64 °C	47-52, 52-61, 61-100	0.5; 4.5; 0.1
Exon3	59 °C	62 °C		44-49, 49-58, 58-100	0.5; 4.5; 0.1
Exon4	64 °C	67 °C		47-52, 52-61, 61-100	0.5; 4.5; 0.1
Exon5	64 °C			45-50, 50-59, 59-100	0.5; 4.5; 0.1
Exon6	65 °C	67 °C		44-49, 49-58, 58-100	0.5; 4.5; 0.1
Exon7	65 °C	68 °C		43-48, 48-57, 57-100	0.5; 4.5; 0.1
Exon8	66 °C	67 °C		49-54, 54-63, 63-100	0.5; 4.5; 0.1

the Transgenomic WAVE system (Transgenomic, Omaha, Neb.). Aliqots of 5-10 µl PCR product in according with the PCR yield were automatically loaded on the DNAsep column (Transgenomic) and eluted on a linear acetonitrile gradient in a 0.1 M triethylamine acetate buffer (pH 7) with a constant flow rate of 0.9 ml/min. The gradient start and end points were adjusted according to the size of the PCR amplicon. Temperatures for successful resolution of heteroduplexes were both calculated by using the WAVEmaker software, Version 3.3.3. (Transgenomic). For this latter purpose samples were analyzed at increasing column temperatures, until a significant decrease in retention time occurred.

The conditions of the general methods (purification of DNAsep column and stand-by status) and the conditions (gradient and temperature) for DHPLC analysis of XRCC2 and XRCC3 genes are shown in table 2.7, 2.8 and 2.9.

2.5 DNA Sequencing

Material:

- Thermo Sequenase fluorescent labelled primer cycle sequencing kit (Amersham Pharmacia Biotech)
- Sequence primer (sequence see Table 2.10)
- MicroSpin S-300 (for PCR fragment <200 bp)
- MicroSpin S-400 (for PCR fragment ≤200 bp)
- Mineral oil
- 19:1Acrylamide/bis-acrylamide mixture (19% Acrylamide, 1% N,N-Methylen-bisacrylamide)
- Amberlite (Pharmacia)
- TEMED (N,N,N,N,-Tetramethylethylendiamin)
- 10% APS (Amoniumpersulfate) in Aqua dest.
- 1 x TBE

2.5.1 PCR for DNA sequencing

DHPLC positive DNA sequences were amplified using primer pairs as shown in table 2.11. PCR reactions of a final volume of 50 µl contained 50 ng genomic

DNA as described in 2.3.1. The Cy5 labelled primers were used for the detection with an A.L.F. Express sequencer system. This PCR reaction were amplificated in Onmigene Thermal Cycler (Hybaid) overlaid with two drops of mineral oil. 5 µl PCR products were electrophoresed in 1.5% agarose gel. For the sequencing only clearly viewable bandswere used without unspecific side bands under ethidiumbromide staining.

2.5.2 Dye-Primers sequencing with Themo sequenase

The sequencing reaction was made with the "Thermo Sequenase fluorescent labelled primer cycle sequencing kit" (Amersham Pharmacia Biotech). This Kit was based on the principle of the Sanger sequencing and deployed the fluorescent marked primers to detect the sequencing products with automatic laser sequencer. The PCR products were cleaned with the "MicroSpin S-300 or S-400 HR Columns" (Amersham Pharmacia Biotech) to avoid the interfering signals through the reactions of unused primer and dNTPs. The columns are supplied pre-equilibrated in TE buffer. The columns were centrifuged at 3000 rpm for 1 minute in an Eppendorf centrifuge, the 50 µl PCR products were applied to the top-center of the resin and recentrifuged for 3 minutes at 3000 rpm. The eluentscontain purified PCR products which could be used directly in the sequencing reactions. Each of the four sequencing reactions contains:

***Table 2.10**: Sequencing primer and conditions for the sequencing with Thermosequenase (*:Cy5-tagged)*

Primer name	Sequencing primer 5'--> 3'	Conditions
		60°C 30 sec
		95°C 30 sec
Universal	* CGACGTTGTAAAACGACGGCCAG	30 cycles
		55°C 30 sec
		95°C 30 sec
Reversal	*CAGGAAACAGCTATGAC	30 cycles

Table 2.11 *Primers for the PCR of DNA sequencing*

Gen Exon	Primer sequence	Condition
XRCC2 Exon2	5´-CGACGTTGTAAAACGACGGCCAGTCTTACAGACTTTCGGAAAATGG-3´ 5´-CAGGAAACAGCTATGACGTGAGGAGTATGTGTATACATGT-3´	94°C 1 min 58°C 1 min 72°C 1 min
XRCC2 Exon3.2	5´-CGACGTTGTAAAACGACGGCCAGGCACAGACTATCCCAAAGCT-3´ 5´-CAGGAAACAGCTATGACTTCTTCTGATGAGCTCGAGG-3´	94°C 1 min 58°C 1 min 72°C 1 min
XRCC2 Exon3.3	5´-CGACGTTGTAAAACGACGGCCAGATGTTCTCAGTGCTTAGAGAAG-3´ 5´-CAGGAAACAGCTATGACAAGGCTTGCGTAGTACCCTGC-3´	94°C 1 min 61°C 1 min 72°C 1 min
XRCC3 Exon3	5´-CGACGTTGTAAAACGACGGCCAGCACCCCTGGCAGAGATGCCA-3´ 5´-CAGGAAACAGCTATGACCTGCGTTGTGACAGTCTGACA-3´	94°C 1 min 60°C 1 min 72°C 1 min

5 µl purified PCR products

1 µl sequencing primer (1pmol/µl)

2 µl reactions mixture (ddATP, ddCTP, ddGTP or ddTTP)

8 µl total volume

As sequencing primer Cy5-tagged M13 universal or reverse primer were developed . The reaction took place with 1 drops of mineral oil for 5 minutes denaturing at 95°C and 30 cycles (see table 2.10).

To stop the sequencing reaction 5 µl stop solution was added and sequencing product was separated from mineral oil, and separated by gel electrophoresis.

2.5.3 Sequencing with the A.L.F. Express

The gel electrophoresissetting was the same as in 2.3.2, except the acrylamide/bis-acrylamide mixture was 19:1 instead of 29:1. After denaturing 5 minutes at 95°C and cooling 3 minutes on ice, the sequencing products were loaded onto the A.L.F. gel and electrophoresis was processed under the following conditions:

Running condition	**Fragment analysis**
Voltage:	1500 V
Current:	34 mA

Power:	30 W
Temperature:	50°C
Run buffer:	0,6 x TBE
Run time	200~350 min

For analyses of the sequencing results the “Sequence Analyser Software” (Pharmacia) was used.

2.6 Statistical methods

Association of LOH with other clinicopathological factors and correlation between different microsatellite markers were calculated by the chi-square test. The statistical analyses were performed using the SPSS 10.0 for Windows statistical packages.

3 Results

3.1 LOH analysis with microsatellite markers

Tumor suppressor genes are often inactivated by loss of heterozygosity. Somatic cells contain two copies of each autosomal chromosome, one inherited from each parent. Probes detecting DNA sequence polymorphisms allow to distinguish between the maternally and paternally derived copies of particular DNA sequences in both normal and neoplastic cells.

In heterozygous individuals two alleles, i.e., two PCR products of different size, can be detected in normal DNA. In general, the sizes of the two alleles were assigned to the peaks of greatest height; smaller peaks were interpreted as polymerase artifacts, so called stutter bands. Because PCR fragments of different sizes are amplified with different efficiencies, the ratio of allele peak areas was calculated in matched normal and tumor DNA samples. Peak areas of the longer length allele were divided through the peak areas of the shorter length allele. The ratio obtained in tumor DNA divided by the allele peak ratio of paired normal DNA gives a result range of 0.00-1.00. A complete allele loss results theoretically in a value of 0.00; both alleles retained in the tumor DNA give a ratio of 1.00. In case the shorter length allele was lost in the tumor DNA—which results in ratios greater than 1.00—the ratio was converted (1/x) to obtain values below 1.00. A ratio below 0.6, which means an allele signal reduction of 40%, was considered to be indicative of allele loss. This limit was chosen because the tumor cell content was assessed to be greater than 70% and inter assay variations of the detection system were below 5%.

To define the cutoff to discriminate between specific allele loss and cases with both alleles retained, the ranges of allele ratios were determined for all informative cases and markers (Fig. 3.2). The graph showed a bimodal distribution of allele ratios with ratios ranging from 0.8 to 1.0 (both allele present) and a broader range of allele ratios between 0.0 and 0.65, indicating allele losses.

3.1.1 LOH at loci of Rad51, XRCC2 and XRCC3

219 paired normal and tumor DNA were analyzed for microsatellite marker D15S118. XRCC2MS was an intragenic microsatellite marker designed with help of DNA sequences from GenBank (accession number: AC003109). XRCC2MS amplified a 22(AC) repeats fragment in 201 paired DNA. This repeat is 36.3 kb distant from exon3 of the XRCC3 gene. The microsatellite marker D7S483 is located at 7q36.1 near the XRCC2 gene. The distance from XRCC2 gene was smaller than 850kb (Tambini, 1997). 144 paired DNA samples were analyzed for D7S483. The XRCC3MS marker was designed around an $(AC)_{15}$ repeat identified within the XRCC3 plasmid 16D8-PO to amplify a product of 204 bp (Price, 1997). 217 paired DNA samples have been analyzed for XRCC3MS. The PIC (percentage of informative cases) values of D15S118, D7S483, XRCC2MS and XRCC3MS were 0.740, 0.729, 0.692 and 0.608, respectively (Table3.1). In 162 informative cases of D15S118, 92 were IDC, 33 ILC and 37 were other tumors and unknown. In 144 D7S483 informatic cases: 63 IDC, 21 ILC and 21 other type tumors. In 139 XRCC2MS cases: 78 IDC, 31 ILC and 30 other type tumors. In 132 XRCC3MS cases, there were 73 IDC, 29 ILC and 30 other type tumors. The LOH of D15S118 and XRCC2MS between IDC and ILC have significant differences (p=0.008 and p=0.006, respectively. Table 3.1).

3.1.2 Correlation of LOH analysis with the histopathological and clinical parameters

The histological classification of tumor and clinical parameters of patients is based on the TNM status (T = tumor size; N = lymphoid node status; M= metastasis), histological grade and steroid hormone receptors (progesterone and estrogen receptor). LOH of Rad51 (D15S118), XRCC2 (D7S483 and XRCC2MS) and XRCC3 (XRCC3MS) were correlated with the histopathological/clinical parameters (Table 3.2). The statistical analyses of the data sets were performed by using chi-square test to determine the statistical significance. ($P<0,05$).

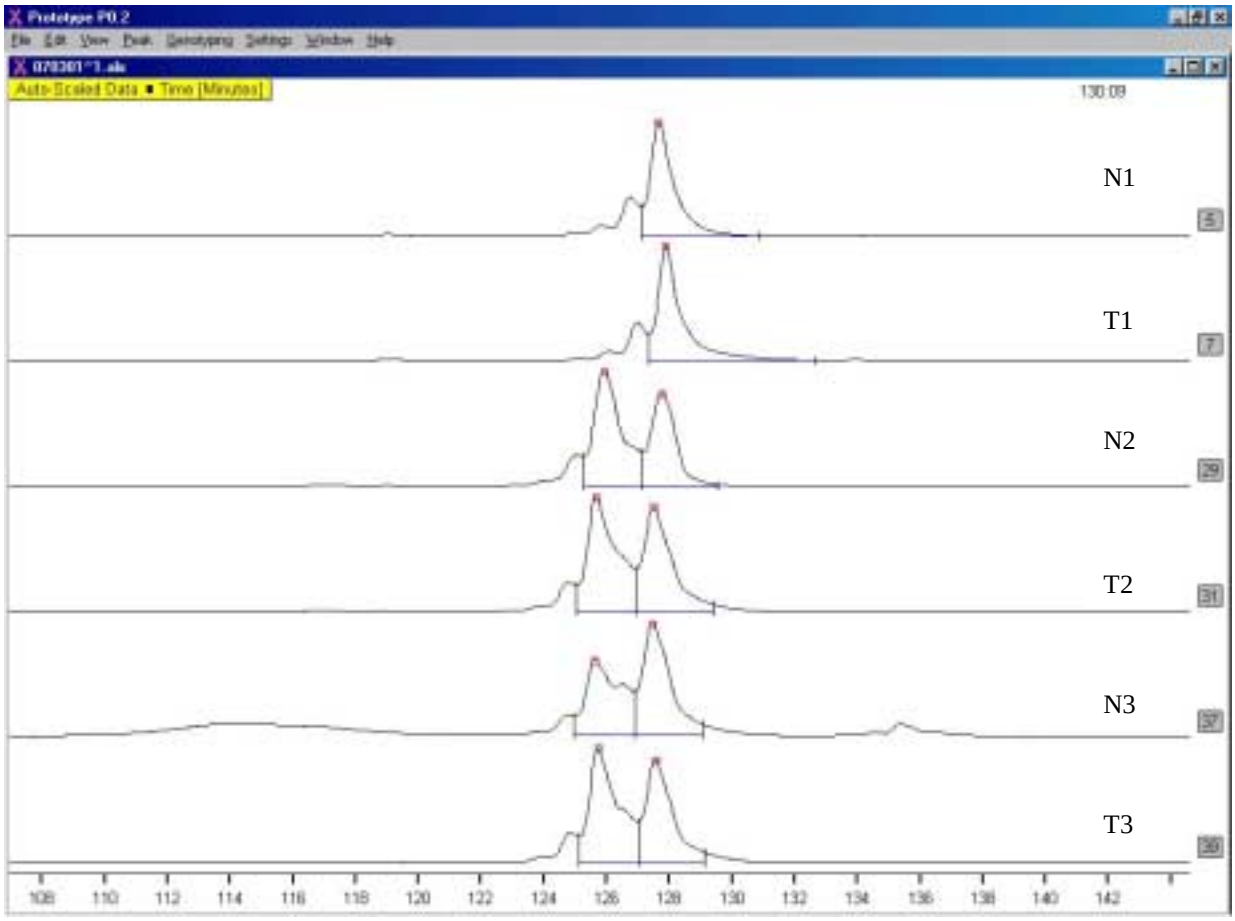

Figure 3.1: *LOH analysis of microsatellite marker D7S483 [N: normal DNA; T: tumor DNA; N1/T1: homozygosite, not informative; N2/T2: heterozygosite, no LOH; N3/T3: heterozygosity, loss of heterozygosity (LOH)]*

Table 3.1 *The PIC values and the number of checked samples for IDC, ILC and other breast cancer.*

		IDC			ILC				Others and unknown		
Marker	PIC	$N_{n.inf}$	$N_{inf.}$	LOH n (%)	$N_{n.inf}$	N_{inf}	LOH n (%)	P *(IDC/ILC)*	$N_{n.inf}$	N_{inf}	LOH N (%)
D15S118	0,740(162/219)	33	92	34(37,0)	9	33	4(12,1)	***0,008***	15	37	9(24,3)
D7S483	0,729(104/144)	18	63	15(23,8)	11	21	1(4,8)	*0,061*	10	21	3(14,3)
XRCC2MS	0,692(139/201)	41	78	24(30,8)	6	31	2(6,5)	***0,006***	15	30	8(26,7)
XRCC3MS	0,608(132/217)	56	73	24(32,9)	11	29	4(13,8)	*0,083*	18	30	12(40,0)

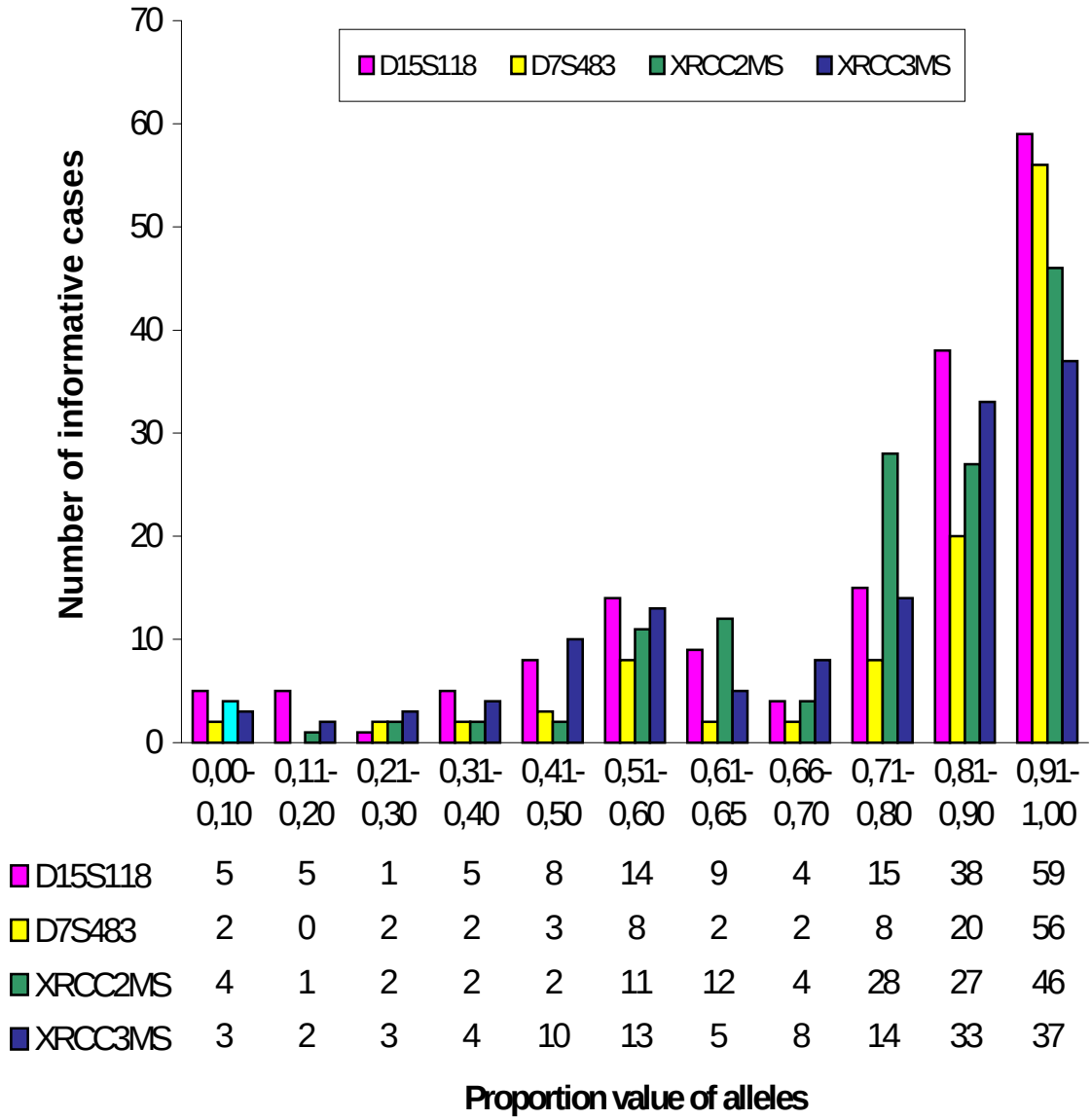

Figure 3.2 *the summary of the proportions of alleles (X-axe: proportions of microsatellite markers of Rad51, XRCC2 and XRCC3 genes; Y-axe: numbers of the informative cases)*

In 154 IDC breast cancers LOH of D15S118, XRCC2 and XRCC3 correlated with negative Progesterone receptor (P=0.048, 0.008 and 0.023, respectively. Table 3.2). LOH of XRCC2 correlates with older age (p=0.036. Table 3.2). There are no further statistical significant association of the LOH and pathologic/clinical parameters.

Table 3.2 *LOH of in three Rad51 paralogs in invasive ductal carcinoma (n=154) correlated with patient's age and histopathological findings*

Criteria	D15S118			XRCC2			XRCC3		
	LOH	No LOH	P	LOH	No LOH	P	LOH	No LOH	P
Age									
<50	7	19	0,211	7	32	**0.036**	10	17	0.562
≥50	27	39		24	40		14	32	
Tumor									
T1	17	25		16	32		7	20	
T2	12	19	0,628	9	24	0,844	11	17	0,336
T3/4	4	11		6	13		5	7	
Nodes									
N0	21	29	0,276	17	38	0,642	11	23	0,741
N1/2	12	27		12	33		13	23	
Grade									
I	6	11		6	13		2	10	
II	15	30	0,631	11	38	0,525	11	22	0,405
III	12	15		12	33		10	16	
ER									
Negativ	10	16	0,888	13	21	0,233	9	11	0,180
Positiv	21	36		15	42		12	31	
PgR									
Negativ	15	16	**0,048**	15	17	**0,008**	11	10	**0,023**
Positiv	14	38		12	47		10	32	

LOH, loss of heterozygosity; P, overall P-value.

3.1.3 Correlation of LOH between three Rad52 paralogs

Using the chi square test analyzed the correlation of LOH between the three Rad52 paralogs in all breast cancer (IDC and ILC). There were significant correlations between LOH of the three Rad52 paralogs. LOH of RAD51

(D15S118) correlated with LOH of XRCC2 and XRCC3 (<0.0001 and p=0.002, respectively. Table 3.3 and table 3.4), LOH of XRCC2 correlated with LOH of XRCC3 (p<0.001. Table 3.5).

Table 3.3 *Correlation between between Rad51 and XRCC2 in breast cancer*

		XRCC2			P
		LOH	No LOH	Total	
Rad51	LOH	22	15	37	<0,0001
	No LOH	11	81	92	
	Total	33	96	129	

Table 3.4 *Correlation between between Rad51 and XRCC2 in breast cancer*

		XRCC3			P
		LOH	No LOH	Total	
Rad51	LOH	13	13	26	0,002
	No LOH	12	55	67	
	Total	25	68	93	

Table 3.5 *Correlation between between Rad51 and XRCC2 in breast cancer*

		XRCC3			P
		LOH	No LOH	Total	
XRCC2	LOH	15	9	24	<0,001
	No LOH	16	60	76	
	Total	31	69	100	

3.2 DHPLC

Denaturing high-performance liquid chromatography (DHPLC) is a novel, non–gel-based method that is very sensitive for detection of DNA sequence variations. The heteroduplex-analysis by DHPLC is based on differences in retention of perfectly matched homo- and heteroduplexices containing one or more

mismatched base pairs (Xiao, 2001). The choice of temperature during DHPLC is critical for achieving maximum detection sensitivity, which is 96%–100%. Blinded analyses have shown that DHPLC is superior to conventional methods (O'Donovan, 1998; Jones, 1999; Wagner, 1999*b*).

DHPLC is a new technology that can replace gel electrophoresis for analysis of PCR fragments and techniques such as SSCP for detection of point mutations. This technology can accommodate high-throughput analyses for quantitative PCR, genotyping, and LOH determinations and for detection of DNA mutations and polymorphisms.

The feasibility of using DHPLC for the analysis of double-stranded DNA was demonstrated by Oefner and Underhill (Oefner P, Underhill P. 1995). The use of ion-pairing high-performance liquid chromatography (HPLC) on alkylated nonporous poly (styrene-divinylbenzene) particles has since been adapted to rapid, automated examination of PCR-amplified DNA for both quantitative (changes in gene expression, LOH) and qualitative (mutation detection and polymorphism detection) analyses (Marino, 1998; Hayward-Lester, 1995; Choy, 1999). PCR fragments subjected to DHPLC in a column of alkylated nonporous particles under conditions of partial heat denaturation or buffer gradients are eluted off the column in an acetonitrile gradient and detected by either ultraviolet (UV) absorbance or fluorescence. This technique is sensitive enough to analyze PCR products in the picomole-to-femtomole range with as little as 1 µl of a PCR mixture and has been shown to be linear over at least three orders of magnitude (Choy, 1999).

The 96-well format autosampler allows for automatic sample injection and processing of 96-well plates directly from a PCR thermocycler. Sample separation occurs on the column, which is located in the oven component of the system. The highly stable temperature of the column is maintained during sample separation, but automatic temperature changes can be made from sample to sample if necessary. An in-line UV detector registers separation results as fragments elute from the column, and these results can be viewed in real time on a

computer screen. Sample run times are 15 min, allowing analysis of about 100 samples in 25 h.

Individuals who are heterozygous in a mutation or polymorphisms have a 1:1 ratio of wild-type and mutant DNA. A mixture of hetero- and homoduplexes is formed when the PCR products is hybridized by heating to 95°C and cooling slowly. The DNA from individuals who have two mutant alleles (homozygous mutation) must be mixed with wild-type DNA and hybridized. After this treatment, a sample will contain a mixture of hetero- and homoduplexes (Figure 3.3).

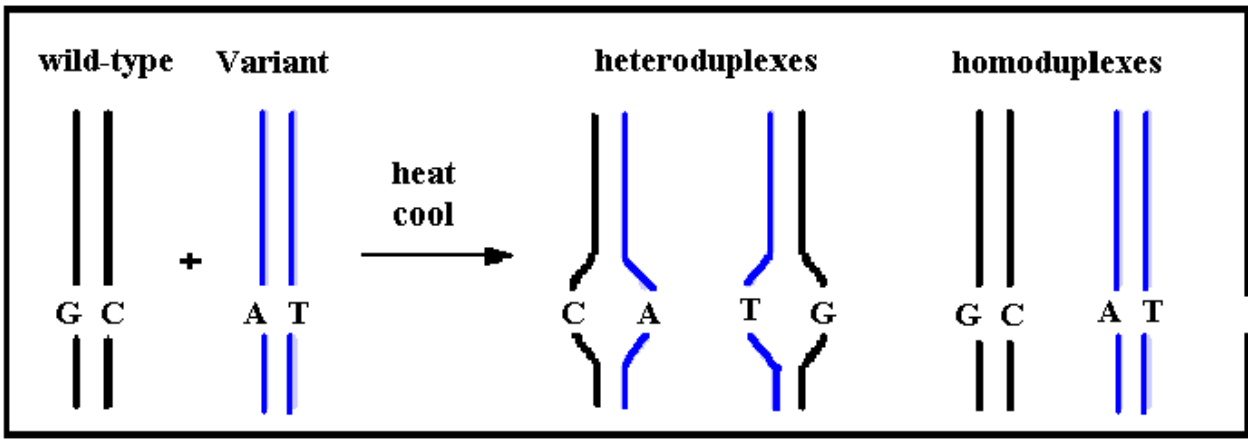

Figure 3.3 *Creation of a mixture of hetero- and homoduplxes through Hybridization*

3.2.1 DHPLC results of XRCC2

Five fragments were created which included the entire coding region of XRCC2 as well as its exon/intron boundaries. Thirty-four breast cancer tissues that showed LOH or not informative by LOH analysis were selected and screened for genetic alterations in exon 1 through 3. DNA isolated from blood lymphocytes was used to get information about the germline status of the respective patients. Each PCR fragment was analyzed with DHPLC under different oven temperatures and gradients. In exon 2 and in both fragment of exon 3 DHPLC positive results were found (Table 3.6 and figure 3.4). For the positive DHPLC fragments, the normal DNA were then amplified and analyzed using DHPLC to

compare the results with tumors. DHPLC results of normal DNAs were the same as in corresponding tumor DNA.

Table 3.6 *DHPLC results of XRCC2 gene (N: normal; T: tumor)*

Pat. Nr.	LOH	Exon1	Exon 2	exon3.1	exon3.2	exn3.3
2T	n.i.	-	-	-	-	-
5T	0,57	-	-	-	-	-
14T	0,62	-	-		-	-
17T	n.i.	-	-	-	-	-
22T	0,59	-	-	-	-	-
30T	n.i.	-	-(+ in 30N)	-	-	-
31T	0,72	-	-	-	-	-
32T	n.i.	-	-	-	-	-
39T	0,45	-	-	-	-	-
40T	0,57	-	-	-	-	-
48T	n.i.	-	-	-	-	-
54T	n.i.	-	-	-	-	-
56T	n.i.	-	-	-	-	-
60T	0,26	-	-	-	-	-
70T	0,73	-	-	-	-	-
71T	n.i.	-	-	-	+	+
76T	0,74	-	-	-	+	+
82T	0,52	-	-	-	-	-
84T	n.i.	-	-	-	-	-
86T	0,54	-	-	-	-	-
91T	n.i.	-	-	-	+	+
96T	0,41	-	-	-	+	+
218T	0,01	-	-	-	-	-
223T	0,01	-	-	-	-	-
226T	0,67	-	-	-	-	-
239T	0,54	-	-	-	-	-
241T	0,36	-	-	-	-	-
259T	0,38	-	-	-	+	+
331T	0,6	-	-	-	-	-
335T	0,7	-	-	-	-	-
340T	0,26	-	-	-	-	-
364T	0,44	-	-	-	-	-
373T	0,34	-	-	-	-	-
37T	n.i.	-	-	-	-	-

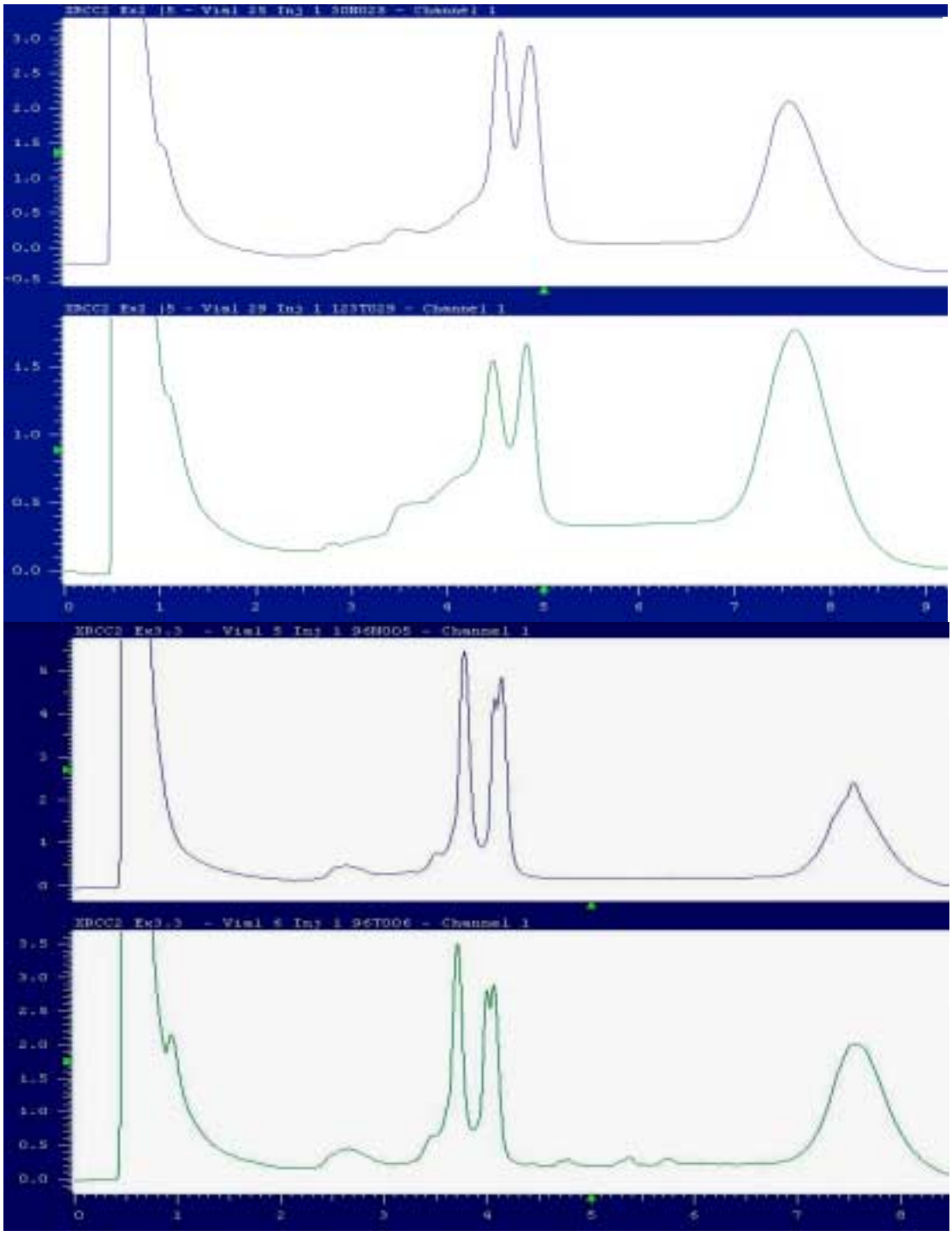

Figure 3.4 *the positive DHPLC results of XRCC2 exon 2 and exon 3.3*

A: normal DNA of patient 30 (Exon2)

B: tumor DNA of patient 123 (Exon2)

C: normal DNA of patient 96 (Exon 3,3)

D: tumor DNA of patient 96 (Exon 3,3)

3.2.2 DHPLC results of XRCC3

As analysis of XRCC2, for XRCC3 28 breast cancers DNA (LOH or not informative) were selected to check the genetic variants with DHPLC. Seven PCR fragments of exon 2 to exon 8 were synthesized from the collective DNA to detect genetic variants. Each PCR fragment analyzed with DHPLC under different oven temperature and gradient. All DHPLC results showed in table 3.7. In exon 3, we found a positive result in the tumor DNA of patient 66. The positive DHPLC results of exon 6 were identified as the polymorphism reported by (Shen, 1998). In patient 66, the DHPLC results in tumor DNA showed questionable heterozygous peaks, while normal DNA showed a clear heterozygous result (Figure 3.5).

Table 3.7 *DHPLC results of XRCC3 gene (T: tumor).*

Pat. Nr.	LOH	Exon2	exon3	Exon4	Exon5	Exon6	Exon7	Exon8
5T	0,29	-	-	-	-	+	-	-
9T	0,69	-	-	-	-	+	-	-
15T	0,69	-	-	-	-	+	-	-
16T	0,59	-	-	-	-	+	-	-
24T	0,7	-	-	-	-	+	-	-
26T	0,58	-	-	-	-	-	-	-
29T	0,5	-	-	-	-	+	-	-
30T	n.i.	-	-	-	-	-	-	-
32T	n.i.	-	-	-	-	-	-	-
34T	n.i.	-	-	-	-	-	-	-
36T	n.i.	-	-	-	-	-	-	-
39T	0,59	-	-	-	-	+	-	-
49T	0,64	-	-	-	-	-	-	-
52T	0,68	-	-	-	-	+	-	-
59T	0,48	-	-	-	-	+	-	-
66T	n.i.	-	+	-	-	-	-	-

82T	0,5	-	-	+	-	-	-	-
85T	0,46	-	-	-	-	+	-	-
96T	0,01	-	-	-	-	-	-	-
201T	0,24	-	-	-	-	+	-	-
212T	0,54	-	-	-	-	+	-	-
223T	0,01	-	-	-	-	-	-	-
224T	0,54	-	-	-	-	-	-	-
243T	0,35	-	-	-	-	+	-	-
244T	0,63	-	-	-	-	+	-	-
248T	0,42	-	-	-	-	-	-	-
256T	0,53	-	-	-	-	-	-	-
259T	0,65	-	-	-	-	+	-	-

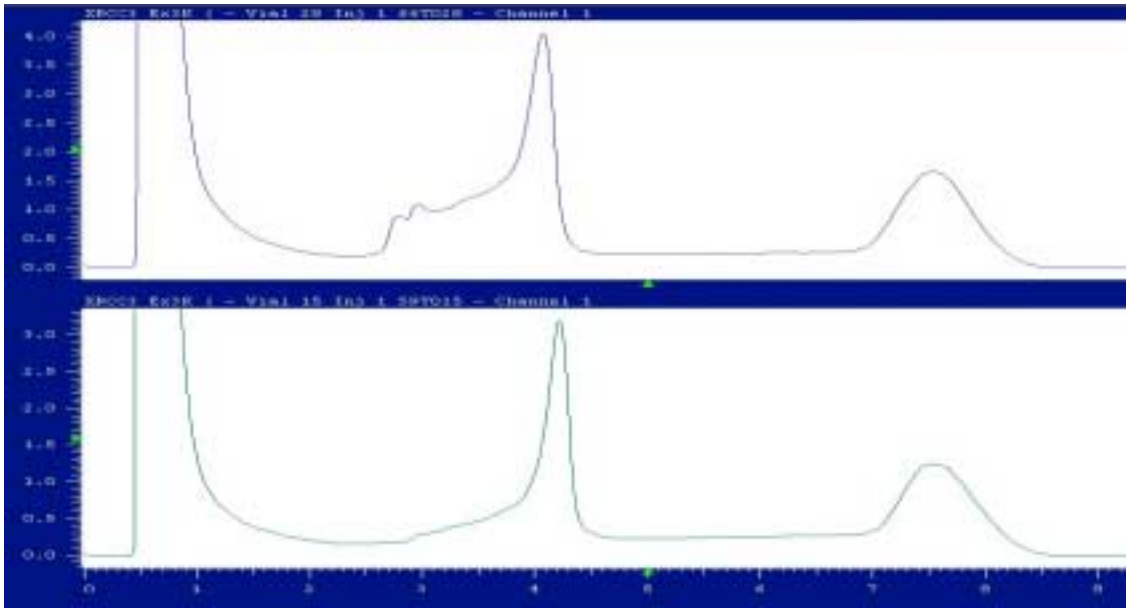

Figure 3.5 *the positive DHPLC results of XRCC3 exon 3*
A: positive tumor DNA result of patient 66;
B: negative tumor DNA result of patient 59.

3.3 DNA sequencing of DHPLC positive samples in the XRCC2 and XRCC3 fragments

After DHPLC analysis of tumor DNA, positive results were reanalysed with matched normal of the same patient. In all cases normal DNA were hetrozygous

as well, indicating any likely a polymorphism. Used M13 tagged primers all DHPLC positive fragments exclusive XRCC3 exon 6 were sequenced with ALF Express. Three genetic variants were confirmed, there was a C to T variant in intron 1 at nucleotide 19609 of XRCC2, a G to A at codon 188 of XRCC2, a C to T at codon 56 of XRCC3 (Table 3,8; Figure 3.6). Due to allele loss nucleotide 8834 of XRCC3 exon 3 in tumor DNA showed the only the T allele, while in normal DNA a C and a T allele was seen (Figure 3.6 D and E). These results matched with the DHPLC results.

Table 3.8 *DNA Sequencing of DHPLC positive cases in the XRCC2 and XRCC3 fragments*

Gene, Exon/intron	Position	Codon	Genetic variant	Amino acid substitution
XRCC2, intron 1	19609 (10bp before Exon 2)	-	C>T	-
XRCC2, exon 3	31479	188	G>A	Arg -->His (CGC-->CAC)
XRCC3, exon 3	8834	56	C>T	His-->Tyr (CAC->TAC)

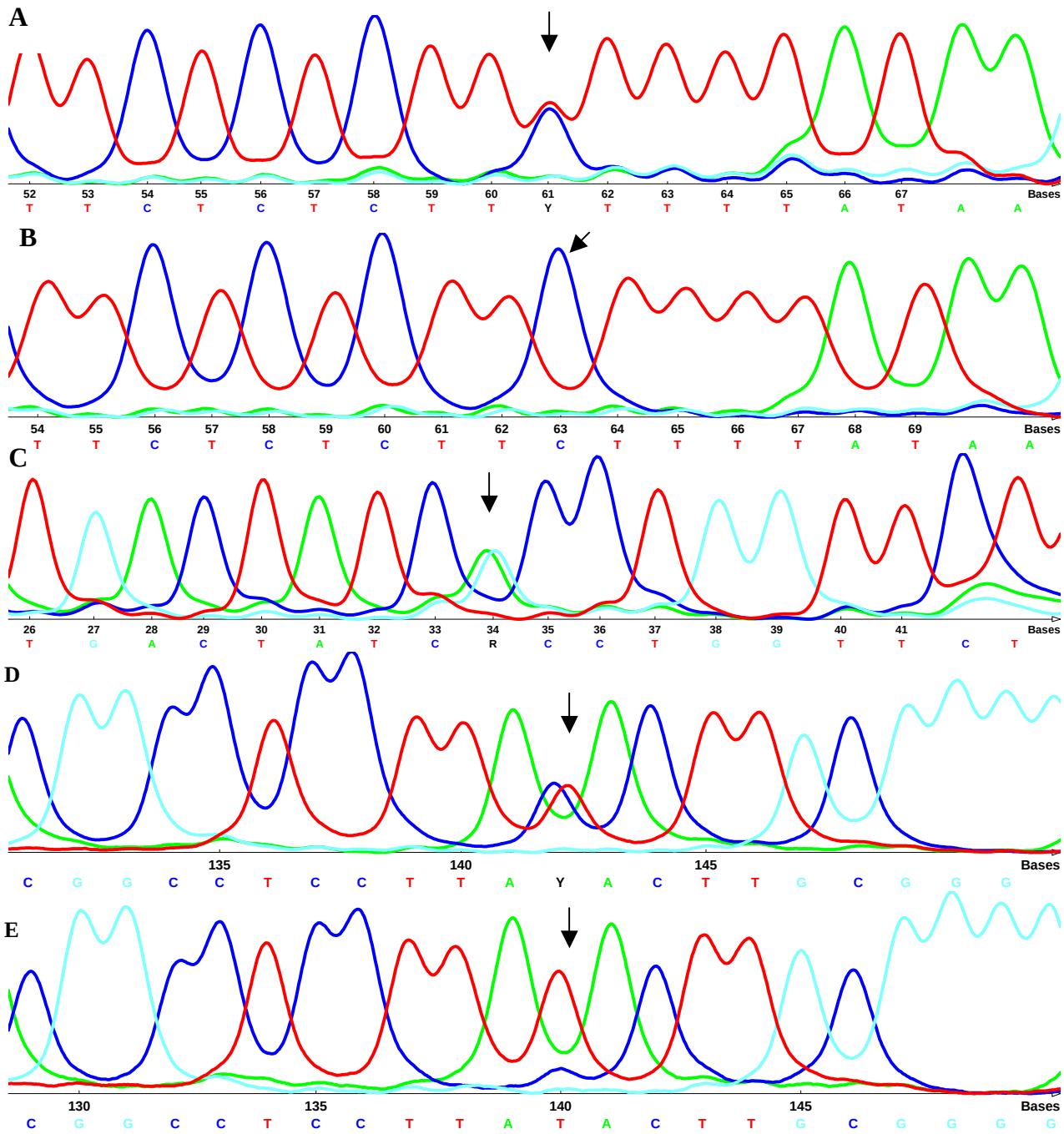

Figure 3.6 genetic variants in XRCC2 and XRCC3

A/B: genetic variant in the intron 1 of XRCC2

C: polymorphism in XRCC2 exon 3 G to A in normal DNA;

D/E: genetic variant in exon 3 of XRCC3. D was the normal DNA and E was the tumor DNA of same patient;

Discussion

4.1 Breast cancer and LOH analysis at chromosome region at 7q36, 14q32 and 15q15.1

Cancer is believed to result from a series of genetic alterations leading to the progressive disordering of the normal mechanisms controlling growth, differentiation, cell death, or genomic instability. The complete understanding of the etiology of breast cancer or any other tumors will require studies that comprehensively evaluate both the genes participating in tumorigenesis and tumors of different pathological and clinical stages to reflect the sequential steps occurring during tumorigenic progression. This understanding is likely to emerge slowly, because research is only beginning to move from single-gene studies to multigenic or genome-wide studies (Fearon, 1990). The functional effect of the loss of one allele of a specific gene is hypothetical because the loss of the gene function will depend on the inactivation of the other allele by homozygous deletion, promoter methylation or point mutation. The identification of chromosomal regions with allele losses is a useful method for screening genes implicated in the pathogenesis of human malignant tumors.

Human Rad51 has been found to be associated with BRCA1, BRCA2, and p53 either directly or indirectly. While inactivation of DNA MMR (mismatch repair) clearly leads to instability of repeated sequences and to an increased risk for tumorigenesis. Recently, a high frequency of loss of heterozygosity at chromosome 15q14–15, near the genomic region containing Rad51, has been reported in human tumors (Wick, 1996). In breast cancer there were high frequencies of LOH at chromosome 15q14-15 (Schmutte, 1999). Gonzalez et al. (1999) reported 31 LOH in 98 breast carcinomas (32%). They found that LOH of Rad51 correlated with positive progesterone receptor in contrast to our study where Rad51 loss is associated with a negative progesterone receptor status. LOH

of XRCC2 and XRCC3 was correlated with negative progesterone receptor, too. In IDC LOH of Rad51 and XRCC2 was more frequent than in ILC.

In the 12 cases of myeloid leukemia material with loss of 7q, a commonly deleted region of approximately 4 to 5 megabasepairs in size encompassing the distal part of 7q35 and the proximal part of 7q36 was identified. The identification and delineation of translocation and deletion breakpoints provides the first step towards the identification of the gene(s) involved in the pathogenesis of 7q35-q36 aberrations in myeloid disorders (Dohner, 1998). Overall, 103 of 169 (61%) acute lymphoblastic leukemia patients had an abnormal karyotype, including nine with 14q32 translocations, and eight with 7q32-q36 breakpoints (Heerema, 1998). To assess the distribution of gains and losses of genetic material in malignant solid neoplasms, 11 tumor types for which at least 100 short- term cultured cases with clonal chromosome aberrations had been reported in the literature were selected. The study was based on cytogenetic information from different cancers. Deletions were more common than gains in all tumor types. The relative distribution of losses indicated that different bands/regions are affected in different tumor types and that, often, several distinct candidate tumor suppressor gene loci can be discerned within the same chromosome arm, e.g., 1p12-13, 1p22, 1p34, and 1p36 on the short arm of chromosome 1 and 7q22, 7q32, and 7q36 on the long arm of chromosome 7. (Mertens, 1997).

The LOH analysis of chromosome 14q showed, 49% loss of heterozygosity in 76 ovarian carcinomas. One region was defined with microsatellite markers D14S65 and D14S267 at 14q32 (Bandera, 1997). 285 primary human carcinomas of the urinary bladder were examined for allelic loss on chromosome 14q using 17 highly polymorphic dinucleotides. Loss of hetrozygosity for at least one marker was observed in 72 (25.3%) tumors. Thirty-four of these 72 tumors (47.2%) lost the entire long arm (monosomy), as suggested by loss of heterozygosity at all informative sites. One region (approximately 3 cM) was bounded by markers D14S51 and D14S267. The results demonstrate that 14q loss is common in invasive bladder cancer and suggest that one potential suppressor loci at 14q32.1-

32.2 may contribute to the genetic progression of this common cancer (Chang, 1995). Cytogenetic analysis was performed on 363 diffuse large B-cell lymphomas. Among 248 samples successfully karyotyped, clonal chromosomal abnormalities were noted in 215 (87%) cases with most breakpoint clustered at 14q32 (Cigudosa, 1999). RFLP and microsatellite analysis with 23 polymorphic markers spanning the entire long arm of chromosome 14 in 108 neuroblastomas showed allelic loss in 19 out of 107 informative tumors. One minimal deletion region could be determined within band 14q32. These results suggest the presence of a putative tumor suppressor gene loci on chromosome 14 (Theobald, 1999).

There were no reports about allele loss of XRCC2 and XRCC3 in cancers. Here we used intragenic microsatellite markers of XRCC2 and XRCC3 to investigate the LOH in 219 sporadic breast cancer patients. The LOH of Rad51 and XRCC2 between IDC and ILC have statistical significances. This means that the DNA repair genes take part more in the development of IDC than ILC. The three genes belong to the DNA repair gene and participate together HR. Interesting correlated the LOH of three Rad51- related genes with each other. The correlation between the three genes and the progesterone receptor need further investigations.

Biochemical analysis of human Rad51 shows that it possesses strand-exchange activity, similar to yeast Rad51 and bacterial RecA (Baumann 1996). Moreover, disruption of the mouse Rad51 gene leads to an early embryonic lethality (Lim, 1996; Tsuzuki, 1996), and short-term cultures of cells recovered from dying *Rad51*$^{-/-}$ embryos are radiation-sensitive and have sharply reduced chromosome numbers (Lim, 1996). Two hamster cell lines that contain defects in XRCC2 and XRCC3 have also showed large decrease in recombination, of 100- and 25-fold respectively (Jonson 1999; Pierce 1999). A striking connection between HR and tumorigenesis has recently been suggested by the observation that the gene products disrupted in the hereditary breast cancer syndromes, BRCA1 and BRCA2, interact with the Rad51 protein. The interaction between BRCA2 and Rad51 was found to be direct, being detected by both two-hybrid and

co-immunoprecipitation assays (Chen, 1998; Sharan, 1997). For BRCA1, the interaction with Rad51 may be indirect, possibly mediated by BRCA2 (Chen, 1998; Scully, 1997). Recently, a mouse cell line that expresses a hypomorphic allele of BRCA1 was tested for defects in HR. Results from these experiments show that the BRCA1-defective cells had an approx. 6-fold decrease in HR compared with wild-type cell lines (Moynahan, 1999). These results suggest that altered recombination mechanisms may be important in the pathogenesis of hereditary breast cancer and ovarian cancer.

In light of the clues provided by familial breast cancer syndromes, this genome-wide study was carried out to examine whether breast cancer progression is manifested as a mutator phenotype and whether a particular form of genomic instability, i.e., DSB-initiated chromosome instability (CIN), drives breast tumorigenesis. Unlike previous genome-wide studies (Rooney, 1999), in which CGH (comparative genomic hybridisation) was usually used to detect genomic alterations at the chromosomal level, the present study, based on LOH of intragenic microsatellite markers, was able to address this hypothesis more comprehensively, providing a more concise insight into breast tumorigenesis driven by genomic instability.

Genomic instability exists at two distinct levels, at the nucleotide sequence level, resulting in base substitutions (nucleotide instability: NIN) or deletions or insertions of a few nucleotides (microsatellite instability: MIN), and at the chromosome level, resulting in the loss or gain of whole chromosomes or large portions thereof (Lengauer, 1998). In breast cancer, MIN is seen in only a small subset (<10%) of tumors (Ingvarsson, 1999), and there is little evidence of mutation hotspots to support a significant etiological role of NIN. In contrast, aneuploidy, an abnormal number of chromosomes representing the prototype of CIN, is relatively common in breast cancer.

One cautionary note should be raised. We were aware that LOH at a few markers cannot be taken as sufficient evidence for the involvement of the genes in tumorigenesis, and the demonstration of specific mutation in putative genes

would be required. However, the causal link between some of these suggested genes, such as p53, ATM, and BRCA1, and breast cancer has been confirmed (Rahman, 1998; Rotman, 1998; Bertwistle, 1998). The increased risk of developing breast cancer in familial cancer syndromes, such as xeroderma pigmentosum and Muir-Torre syndrome (Lindor, 1998), leads additional support to the contribution to breast cancer development of certain other genes, such as XPA and hMLH1. On the other hand, to the best of our knowledge, with the exception of p53, the probability of finding somatic mutation in these checkpoint/repair (caretaker) genes (e.g., RAD51, RAD52, FA-A, FA-D, XPA, and hMLH1) has been shown to be extremely low. Because LOH only represents the specific indicator of the "one-hit" needed to inactivate TSGs, these genes do not seem to fulfil Knudson's "two-hit" criterion for a TSG in cancer formation. However, recent evidence, which suggests two nonmutually exclusive possibilities, provides support for a tumorigenic role of these genes defined solely by LOH in breast cancer. The first is that a growing number of common TSGs, including p53 and ATM, have been found to exhibit the haplo-insufficiency phenotype (Venkatachalam, 1998; Barlow, 1999), which implies that homozygous inactivating mutations and complete loss of function are not necessary to cause defective tumor suppressor function. Thus, a half normal level of the gene product, resulting from allelic loss or LOH per se, would be able to produce the phenotypic manifestation (Macleod, 2000). It is still possible that other (epigenetic) mechanisms of inactivation abrogating the function of these genes are implicated in breast tumorigenesis. For example, hypermethylation of the BRCA1 promoter region has been found in breast cancer (Catterau, 1999), supporting the role of BRCA1 in sporadic breast cancer development (Wilson, 1999). Therefore, our identification of LOH loci possibly targeting DSB-related genes should not be considered to merely reflect the sites of putative TSGs. Instead, these findings can be further regarded as clues suggesting that breast cancer progression is driven by the accumulation of multiple genetic alterations to inactivate the genes participating in the common defensive mechanism against

DSB. In accordance with our findings, a recent breast cancer study involving the detection of LOH at the loci of five DSB-repairing genes (BRCA1, BRCA2, RAD51, RAD52, and RAD54) showed that the probability of simultaneous LOH at more than two of these loci was much higher than expected (Gonzalez, 1999) and underlines the additional importance of finding LOH at these DSB-related loci. More importantly, these findings, including our own, suggest that these proteins associated with DSB-related checkpoint/repair work together to maintain chromosomal stability. As a result, a decrease in the amount of any one of these DSB-related genes as a result of LOH may alter the stoichiometric relationship between them. This inference can be supported by molecular findings that certain of these proteins are found in the same DNA-repairing complex or pathway (Kanaar, 1998; Venkitataman, 1999; Chen, 1999). Consequently, disruption of any one component of this DNA-repairing complex or pathway may hinder DNA repair and result in the accumulation of unrepaired DSB, subsequently leading to CIN without cell cycle checkpoint and to tumor formation.

4.2 Polymorphisms in Rad51, XRCC2 and XRCC3

Polymorphism arises as a result of mutation. The type of mutation that created them typically refers to the different types of polymorphism. The simplest type of polymorphism results from a single base mutation which substitutes one nucleotide for another. The polymorphism at the site harboring such changes has recently been termed a ‘single nucleotide polymorphism (SNP)’, although previously, in some instances, such variation was referred to by the particular methods used to detect it. For example, the first systematic studies of single base variants were pursued through the identification of restriction enzyme sites, where a single base pair change could result in the loss or gain of a restriction site. Digestion of a piece of DNA containing the relevant site with an appropriate restriction enzyme could then distinguish alleles or variants based on resulting

fragment sizes via electrophoresis, and this type of polymorphism was thus referred to as ‘restriction fragment length polymorphism (RFLP)’ (Liu, 1992).

Current evidence supports the hypothesis that mutations are early events in carcinogenesis, so defects in DNA repair probably represent a risk factor for many types of cancer (Yao, 1999; Rajewsky, 1998). Recent evidence that some DNA repair functions are haploinsufficient (Lai, 1999; Drotschmann, 1999) adds weight to the notion that sequence variants in DNA repair genes constitute part of the spectrum of defects contributing to cancer risk (Shcherbakova, 1999). DNA repair pathways are among the most critical components that mediate individual response on exposure to environmental carcinogens. SNPs represent an important class of genetic variation, and SNPs within and outside coding sequences are under intense examination for possible associations or mechanistic links in diseases (Wang, 1998; Cargill, 1999; Halushka, 1999).

An important component of differences among individuals is variation in gene coding sequence (Mohrenweiser, 1998). Preston (1996) defines two classes of genes amounts predisposition to disease, including cancer: susceptibility variants confer increased cancer risk without obvious contribution form environmental factors, whereas sensitivity variants confer increased cancer risk only in conjunction with environmental exposure. Susceptibility variants (e.g. RRCA1 and MSH2) are relatively infrequent but highly penetrant. Sensitivity variants are relatively frequent, but less penetrant than susceptibility variants. As a result, sensitivity variants are difficult to detect using standard methods for genetic epidemiology (family studies). DNA repair acts in response to exposure to environmental carcinogens, and hence repair variants are reasonable candidates for sensitivity genes.

DHPLC is based on temperature-modulated liquid chromatog-raphy (Kuklin, 1997), and uses a high-resolution matrix as a hydrophobic stationary phase that binds negatively charged DNA via triethylammonium acetate (TEAA) present in the liquid phase. Separation of heteroduplexes from wild-type and mutant homoduplexes is dependent on two key features (Hecker, 1999). First,

heteroduplexes melt at the mismatch site and generate a partially denatured *(e.*g., single-stranded) region at a temperature below the melting temperature of the wild-type and mutant homoduplexes (Oefner and Underhill, 1995). Second, the column retention time of single-stranded DNA is less than double-stranded DNA.

The advent of highly sensitive fluorescent sequencing technology with wide dynamic ranges for signal detection used laser makes it possible to detect readily sequence bands that are at much lower intensity them the main band. This capability is the foundation of technique for identifying sequence variants. Comparing multiple sequences at the same gene, single nucleotide polymorphisms are identified easily by visual inspection of the peaks that got through the equipment and software, where variant and reference are both visualized in a single DNA sequencing run.

There were some studies to report the correlations between the XRCC3-T241M and different cancers. Exposure to UV radiation is a major risk factor for the development of malignant melanoma. DNA damage caused by UV radiation is thought to play a major role in carcinogenesis. In an investigation of the association between polymorphisms in DNA repair genes and the development of malignant melanoma. The presence of an XRCC3-241 Met variants allele was significantly associated with melanoma development (Samantha and Winsey 2000). Matullo (2001) found a statistically significant association between the Thr241Met polymorphism of the XRCC3 gene and the risk of bladder cancer.

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Abbreviation

A. L. F.	automated laser-activated fluorescent dna sequencer
APS	ammonium persulfate
BC	breast cancer
bp	base pair
BRCA1	breast cancer gene I
BRCA2	breast cancer gene II
BSA	bovine serum albumen
C	cytosine
CIN	chromosome instability
ca.	carcinoma
CGH	comparative genomic hybridization
DCC	deleted in colorectal carcinoma
DCIS	ductual carcinoma in situ
DHPLC	denature high-performance liquid chromatography
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-PK catalytic subunit
DNA	desoxyribonucleic acid
DSB	double-strand breaks
dATP	2'-deoxyadenosine triphosphate
dCTP	2'-deoxycytidin triphosphate
dGTP	2'-deoxyguanosine triphosphate
dNTP	desoxyribonucleoside triphosphate
dTTP	2'-deoxythamidine Triphosphate
DSB	double-strand break
EDTA	ethylenediaminetetraacetic acid
FISH	fluorescence in situ hybridization
HR	homologous recombination
HRR	homologous recombination repair
het.	heterozygote
IDC	invasive ductal carcinoma
ILC	invasive lobular carcinoma
LD	linkage disequilibrium
LOH	loss of heterozygosity
min	minute
MIN	microsatellite instability

MMR	mismatch repair
MSH2	mismatch repair gene 2
MTS1	multiple tumor suppressor I
NHEJ	non-homologous end joining
NIN	nucleotide instability
NM23	nonmetastatic protein 23
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate balanced saline
PCR	polymerase chain reaction
PIC	percentage of informative cases
RFLP	restriction fragment length polymorphism
rpm	round per minute
R. T.	room temperature
RT-PCR	reverse-transcription polymerase chain reaction
RNA	ribonucleic acid
SD	standard deviation
SDS	Natrium Dodecyl Sulfate
SNP	single nucleotide polymorphism
SRO	smallest region of overlap
SSA	single-trand annealing
T	thymidin
Taq	Taq DNA polymerase
TBE	Tris/Boric/EDTA
TCR	transcription-coupled repair
TE	Tris/EDTA
TEMED	N, N, N'N'-tetramethylethylenediamine
TNM	tumor size, lymph node status, metastasis status
Tris	tris(hydroxymethyl)aminomethane
TSG	tumor suppressor gene
U	unit
UICC	union internatinale contre le cancer
VNTR	variable number of tandem repeats
vs	versus
XRCC1	x-ray repair cross-complementing protein 1
XRCC2	x-ray repair cross-complementing protein 2
XRCC3	x-ray repair cross-complementing protein 3
XRCC4	x-ray repair cross-complementing protein 4

XRCC5	x-ray repair cross-complementing protein 5
yr.	year

Appendix: LOH Dokumentation of four microsatellite markers

Pat.ID	D15S118	XRCC2	D7S483	XRCC3
1	n.i.		0.83	
2	0.85	0.64	n.i.	n.i.
3	0.96	ni	0.90	n.i.
4	0.95		0.84	0.73
5	0.37	ni	0.57	0.29
6	0.7	ni		0.68
8			0.97	
9	n.i.	ni	0.93	0.69
10	0.95	0.82	n.i.	0.97
11	n.i.	0.92	0.94	n.i.
12	0.94	0.98	0.92	0.86
13	0.99	ni	0.98	n.i.
14	0.12	0.5	0.62	n.i.
15	0.52	0.95	0.90	0.69
16	0.97	0.78		0.59
17	n.i.	0.91	n.i.	0.99
21	n.i.	0.93	0.97	n.i.
22	0.60	0.66	0.59	n.i.
23	0.64	0.9	0.96	0.84
24	0.74	0.93	0.94	0.70
25	0.70	0.92	0.92	0.99
26	0.92	0.98	0.93	0.58
28	0.75	ni	n.i.	0.88
29	0.55	0.99	0.87	0.50
30	0.34	ni	n.i.	n.i.
31	n.i.	0.66	0.72	0.99
32	0.76	0.8	n.i.	n.i.
33	0.98	0.96	0.98	0.89
34	0.76	ni		n.i.
35	0.54	ni	0.89	0.98
36	0.98	ni	0.93	n.i.
37	0.98	0.92	n.i.	0.96
38	0.83	ni	0.86	0.86
39	n.i.	0.3	0.45	0.59
40	0.96	0.6	0.57	n.i.
44	0.93	ni	0.91	0.91
45	0.93	ni	0.93	n.i.
46	0.93	0.94	0.99	n.i.
47	0.83		0.84	0.75
48		ni	n.i.	0.89
49	0.98	ni	0.97	0.64
50	0.83		0.98	

Pat.ID	D15S118	XRCC2	D7S483	XRCC3
56	0.81	ni	n.i.	0.81
57	0.88			
59	0.63	0.6	0.99	0.48
60	0.56	0.73	0.26	n.i.
61	0.81	ni	0.84	n.i.
62	0.71	ni	0.94	0.83
63		0.96		
66	0.05	0.86	0.94	n.i.
67	0.99		0.83	n.i.
68		ni		0.60
69	0.82	0.78	0.97	0.97
70	n.i.	0.84	0.73	n.i.
71	0.66	0.94	n.i.	n.i.
72	n.i.		0.97	n.i.
73	0.95	0.65	0.87	0.99
74	n.i.	ni	0.85	0.71
75	0.53	0.63	0.92	n.i.
76	0.70	0.04	0.74	0.95
77	0.40	ni	-	0.85
78	0.94	0.99	0.96	n.i.
79	0.01	ni	0.91	n.i.
80	0.97	0.76	0.83	0.85
82	0.88	0.74	0.52	0.50
83	0.88			
84	0.88	0.63	n.i.	n.i.
85	0.13	0.96	0.99	0.46
86	0.43	0.61	0.54	n.i.
87	0.94	ni	0.96	0.86
88	0.97	0.74	0.97	n.i.
89	0.60	0.8	0.83	n.i.
90	0.97	0.92	0.91	0.81
91	n.i.	0.95	n.i.	0.99
92	0.87	0.8	0.92	0.82
93	0.71	ni	0.94	n.i.
95	0.94	0.95	0.94	0.93
96			0.41	0.01
98	0.96	0.88	0.98	n.i.
99	0.98	0.91	0.99	0.86
100	ni	0.51		ni
101	0.48	ni		0.79
102	0.76	0.74		ni
103	0.61	0.54		ni

Pat.ID	D15S118	XRCC2	D7S483	XRCC3
52		ni		0.68
54	0.99	ni	n.i.	n.i.
55	0.91	ni	0.98	0.96
111	0.4			ni
118	0.73	0.77		0.82
119	ni	0.8		ni
120	0.93	0.88		0.97
123	0.56	0.10		0.01
128	0.64	0.95		ni
130	ni			0.10
131	0,00	0.9		ni
132	0.9	ni		0.92
133	0.54	ni		0.60
134	0.89	0.87		ni
143	ni	ni		ni
150	ni	0.74		0.90
152	ni	0.77		0.78
153	ni	0.88		0.36
156	ni	ni		0.40
157	ni	ni		0.46
158				0.80
160	0.99	0.61		0.66
161		0.54		
162	ni	0.81		0.90
163	0.87	0.87		
164	0.81	0.66		0.90
165	0.94	ni		0.51
167	0.4	0.2		0.50
168	0.3	0.6		0.90
169	0.9	ni		ni
171	0.77	0.65		ni
173	0.65	0.54		0.50
174	0.98	0.87		0.96
175	0.45	ni		0.78
176	ni	0.8		0.60
179	0.95	0.93		ni
180	0.86	0.76		ni
181	0.95	ni		0.85
183	0.56	0.8		ni
185	0.42	0.75		ni
186	ni	ni		0.94
187	0.82	0.81		0.78
189	ni	0.6		ni
190	0.19	ni		0.5
191	0.15	ni		0.42
192	0.89	0.71		0.98
193	0.61	ni		ni

Pat.ID	D15S118	XRCC2	D7S483	XRCC3
104	0.85	0.9		0.97
105	ni	ni		0.96
107	ni	0.74		ni
202	0.97	0.91	0.88	n.i.
204	n.i.	0.85	0.93	0.93
205	0.96	0.87	0.81	0.98
212	0.97	0.67	0.77	0.54
213	n.i.	0.95	n.i.	0.84
214	0.99	0.99	n.i.	n.i.
215	n.i.	0.63	0.65	0.39
216	0.97	0.92	n.i.	n.i.
218	0.01	0.1	0.01	n.i.
219	0.90	0.91	0.96	0.89
220	0.85	0.95	n.i.	0.97
221	0.88	ni		0.90
222	0.86	ni	n.i.	0.99
223	0.01	0.63	0.01	0.93
224	n.i.	0.98	n.i.	0.54
226	n.i.	0.98	0.67	n.i.
227	0.97		n.i.	n.i.
228	0.58	0.49	n.i.	
229	0.81	0.75	0.95	n.i.
231	ni	0.02	n.i.	n.i.
232	0.90	0.99	0.86	n.i.
233	0.93	0.96	n.i.	0.89
234	0.97	0.87	n.i.	0.90
235	n.i.	ni	n.i.	n.i.
236	0.55	0.29	0.76	0.73
237	0.86	0.75	0.96	0.78
239	n.i.		0.54	n.i.
240	0.94	0.99	n.i.	0.98
241	ni	0.37	0.36	0.58
242	ni	0.95	0.99	0.94
243	0.94		0.99	0.35
244	0.98	ni	0.99	0.63
245	n.i.	ni	0.99	0.74
246	0.93	0.95	0.96	n.i.
247	0.98	0.93	0.92	0.95
248	0.99	0.88	0.95	0.42
249	0.84	0.94	n.i.	0.98
250	0.62	0.89	n.i.	n.i.
253	0.86		n.i.	0.95
254	n.i.	ni	n.i.	0.82
255	n.i.		n.i.	n.i.
256	n.i.	0.83	0.95	0.53
258	0.43	ni	n.i.	0.96
262	0.96		0.86	

Pat.ID	D15S118	XRCC2	D7S483	XRCC3
194	0.82	ni		0.97
196	0.91	ni		0.86
197	ni	0.34		0.11
198	ni	0.88		0.92
200	n.i.		0.82	0.86
201	0.50	ni	0.76	0.24
277			0.59	
286	0.9	0.73		ni
287	0.94	0.98	0.96	ni
301	0.63	0.61		0.53
322	0.99	0.84	0.91	ni
327	0.93	0.84	0.99	ni
329			0.92	
331	0.92	0.8	0.60	ni
332	0.94	0.95	0.796	0.81
333	ni		0.93	ni
335			0.70	
336	0.64	0.61	n.i.	0.69
340	0.18	ni	0.26	0.19
342	0.8			
345	0.45	0.9		0.73
347	0.95	0.96		0.84
349			0.96	
351	0.76	ni	0.96	0.87
352	0.76	0.77		0.87
353	0.6	0.61		0.78

Pat.ID	D15S118	XRCC2	D7S483	XRCC3
264	0.98			
265	0.86			
266	n.i.		0.97	
267	0.74		n.i.	
268				0.89
273	ni	0.97	0.77	ni
354	ni	0.77		ni
359	0.42	0.56		ni
360	0.8	0.89		0.99
361	ni	0.93		0.78
364	0.93	0.9	0.44	0.65
366	ni	0.53		0.93
368	0.92	0.88		ni
370	0.74	0.8		ni
372	0.89	ni		ni
373	0.87	0.96	0.34	0.3
375	0.82	0.97	n.i.	0.91
376			0.96	
377	0.58	0.6		ni
380	0.94	0.77		0.64
381			0.89	
384				0.68
385	ni	0.99	n.i.	
387				0.62
388	0.84	0.94		0.58
402	0.88			0.95

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Erklärung

Hiermit erkläre ich, dass die vorliegende Dissertation von mir selbstständig angefertigt wurde. Es wurden ausschließlich die von mir angegebenen Hilfsmittel verwendet.

Ferner erkläre ich, dass ich diese Dissertation in der vorliegenden oder einer ähnlichen Form an keiner anderen Institution eingereicht habe.

Lebenslauf

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Ming Du

Molekulargenetische Analyse der DNA-Reparaturgene *Rad51, XRCC2* und *XRCC3* in sporadischen Mammakarzinomen

Zusammenfassung

Fragestellung: Die Inaktivierung von Tumorsuppressorgenen (TSG) sind wichtige Schritte in der Karzinogenese des Mammakarzinoms. Der Nachweis von LOH („loss of heterozygosity“) deutet auf die Inaktivierung und somit auf den Funktionverlust eines TSGs hin. Die auf den Chromosomen 15q, 7q and 14q lokalisierten DNA-Reparaturgene *Rad51, XRCC2* und *XRCC3* sind aufgrund ihrer Funktion innerhalb der DNA Reparatur potentielle Tumorsuppressor Gene („care taker“). Die Bedeutung von *Rad51, XRCC2* und *XRCC3* innerhalb der Karzinogenese von Mammakarzinomen sollte durch LOH- und Mutationsanalyse von 200 sporadischen Mammakarzinomen untersucht werden.

Methodik: Die LOH-Analyse beinhaltet die PCR-Amplifikation von polymorphen Markersequenzen (Mikrosatelliten) mit fluoreszenz-markierten Primern gefolgt von der DNA-Fragmentanalyse der PCR-Produkte auf einem automatischen DNA Sequenzierautomaten. Als Vorscreeningmethode für die Analyse der Gene XRCC2 und XRCC3 wurde die denaturierende Hochdruck-Flüssigkeitschromatographie (DHPLC) etabliert. Die DHPLC-positiven Proben wurden mittels direkter DNA-Sequenzierung (A.L.F. express™) sequenziert.

Ergebnisse: Im Gegensatz zu der bekannten physiologischen Funktion der drei Gene innerhalb von DNA-Reparaturprozessen ist die Bedeutung der drei Genen bei der Entstehung und Progression des sporadischen Mammakarzinoms noch weitgehend ungeklärt. Für die LOH-Analyse konnten polymorphe Marker innerhalb der zu untersuchenden Gene identifiziert und die Frequenz der Heterozygotie (PIC-Wert, „percentage of informative cases“) wie folgt bestimmt werden: (*Rad51*) D15S118: 0,74; (*XRCC2*) D7S483: 073, XRCC2MS: 0,69 und (*XRCC3*) XRCC3MS: 0,61. Durch LOH Analysen mittels dieser intragenischen Mikrosatelliten-Marker konnte ein häufiger Allelverlust in den Regionen der Gene *Rad51* (D15S118: 37%), *XRCC2* (D7S483: 23,8%; XRCC2MS: 30,8%) und *XRCC3* (XRCC3MS: 32,9%) nachgewiesen werden. Statistisch signifikante Unterschiede ergaben sich in der Häufigkeit des LOH *Rad51* (p=0.008) und *XRCC2* (p=0.006) in IDC (invasive ductal carcinoma) und ILC (invasive lobular carcinoma). In IDC (n=154) korrelierte ein Allelverlust der Gene *Rad51* (p=0.048), *XRCC2* (p=0.008) und *XRCC3* (p=0.023) jeweils mit negativem Progesteron-Rezeptorstatus. LOH *XRCC2* korrelierte mit dem Alter der Patientinnen (p=0.036). Positive Korrelationen fanden sich für Allelverluste der jeweiligen Regionen untereinander: LOH *Rad51* und LOH *XRCC2* (p<0.0001) bzw. LOH *XRCC3* (p=0.002) sowie LOH *XRCC2* und LOH *XRCC3* (p<0.0019).

Zur Mutationsanalyse wurden die codierenden Sequenzen der Gene *XRCC2* und *XRCC3* in fünf bzw. sieben PCR-Fragmenten amplifiziert. Unter den jeweils etablierten DHPLC Analysebedingungen wurden vorwiegend LOH positive Tumoren untersucht (*XRCC2*: n=34; *XRCC3*: n=28). In der *XRCC2* Sequenz wurden zwei Sequenzveränderungen (Intron1: C19609T; exon3: G31479A) und in der *XRCC3* Sequenz eine Punkt-Mutation (exon3: C8834T) nachgewiesen werden. Der Nachweis dieser drei Sequenzvarianten sowohl in Normal-DNA der jeweiligen Mammakarzinom-Patientin als auch in DNA-Proben eines untersuchten Kontrollkollektivs zeigten, dass es sich bei diesen Sequenzvarianten um Polymorphismen handelt. Inaktivierende Strukturgenmutationen konnten nicht nachgewiesen werden.

Schlußfolgerung: Die in sporadischen Mammakarzinomen häufig auftretenden Alleverluste in den untersuchten Genregionen können nicht durch entsprechend inaktivierende Mutationen in den Kandidatengenen *Rad51, XRCC2* und *XRCC3* erklärt werden, sodass einerseits alternative Inaktivierungsmechanismen wie Promoterhyper-methylierung oder cis-regulatorische Mutationen von Bedeutung sein könnten, oder aber andere Kandidatengene in diesen chromosmalen Regionen lokalisiert sind. Ob den hier entdeckten „missense“ –Mutationen ein prädisponierender Effekt in der Karzinogenese von Mammakarzinomen zugeschrieben werden kann, müsste in weitergehenden Assoziationsstudien untersucht werden.