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Chapter 8

Discussion

Discussion

Materials and methods

Sample material: formalin-fixed, paraffin-embedded

In most pathology laboratories worldwide, formalin-fixed, paraffin-embedded (FFPE) samples are the only tissue specimens available for routine diagnostics. To have access to a vast amount of material, and to be able to implement our results in routine diagnostics in the future, we have limited ourselves to the use of FFPE material in the studies described in this thesis. However, it was the experience in our research laboratory from earlier studies that DNA extracted from FFPE tissue is not always suitable for array CGH analysis. Time and money could be used more efficiently if the sample quality can be assessed prior to array CGH experiments. This has motivated us to develop a multiplex PCR to determine the maximum length of DNA able to be amplified as a measure of DNA quality. Short DNA fragments due to degradation, or DNA strands that are unable to form single strands due to crosslinks, may cause unspecific binding and thus hamper hybridization efficiency which results in unreliable array data. **Chapter 2** describes the development of a multiplex PCR that is able to test the possibility of producing DNA fragments of 100, 200, 300, or 400 bp long. We postulated that the longer the fragments are that can be produced, the better the sample quality is (1). For this test, we have chosen the gene *GAPDH* as genomic target because of its importance in cancer cell survival. GAPDH plays a central role in glycolysis-dependent energy supply, and because cancer cells metabolize glucose mainly through the glycolytic pathway and depend far less on oxidative phosphorylation, tumor cells are highly dependent on GAPDH for survival and proliferation. Therefore, it is very unlikely that *GAPDH* will be lost in breast tumors, making it a reliable target for investigation. Indeed, we have not observed a successful aCGH experiment using DNA from which no PCR product could be produced *post hoc*.

The multiplex PCR has been very helpful during our studies, especially when investigating breast cancer families (see for example **Chapter 7**). Registration of families carrying a germline predisposition for breast cancer already covers several generations on many occasions in our hospital. In such cases where tumor tissue has been archived, the material can be very old and the DNA degredated beyond usage. Here, our multiplex PCR has given us insight in which samples could still be investigated by array CGH. In the past decades, fixation time, usage of buffered formalin, and storage conditions have been improved and standardized. We have seen in our studies that this has led to better conservation of sample material as DNA isolated from newer material (after 1990) is of better quality compared to older material (before 1990) in our hospital. Also other laboratories have been using our DNA quality test (2-5). And although array CGH technology has improved much in the latest couple of years to even combined SNP genotyping and copy number alteration analysis, sample quality is hard to control, which underscores selection of samples prior to analysis for even these more advanced microarray platforms (4, 5).

Array CGH: hybridization

In cancer research, array CGH has become a valuable tool for the detection of chromosome copy number alterations. Challenges of this technique were to obtain high signal-to-noise ratios to detect single copy number changes, and to obtain highly reproducible results with a minimal variance for FFPE material. In the past, we performed the hybridization procedure and washing of the arrays manually, which often led to capricious results. **Chapter 3** describes the standardization of an array CGH protocol for FFPE material, making use of a hybridization station. Deployment of a hybridization station allowed us to hybridize with a more viscose hybridization mixture, because we were no longer limited in the force of mixing by a rocking table as with the manual procedure described in **chapter 2**. A higher viscosity facilitated by doubling dextran sulphate concentration increased the effective concentration of labeled DNA and thereby increased the signal-intensities. Additionally, automation of the hybridization and washing steps of the arrays resulted in highly reproducible results and less overall variance in the CGH profiles. Another major improvement in our protocol was the elimination of formamide, which is commonly used in large amounts in most microarray wash protocols but is very toxic. Last, although DNA from FFPE tissue is generally more problematic than fresh material, our automated protocol has proven to be very successful in the use of FFPE material (6).

Further improvements in the technology could be made. In an unpublished study, we have looked at the dispersion of labeled DNA over the microarray during hybridization in a hybridization station. For this, we have injected hybridization buffer into the hybridization chamber with only the last few microliters containing labeled DNA. After hybridization and scanning, labeled DNA was found to have hybridized over the first half of the slide only (Figure 1). These results suggested that not every DNA fragment will meet its target and signal-intensities can theoretically be increased even more by applying a different mixing mechanism. The hybridization station described in this thesis uses agitating membranes, pushing the hybridization buffer back and forward, to facilitate mixing. Improvement in hybridization efficiency can be made by applying a continuous flow of the hybridization buffer over the whole slide. Nonetheless, our protocol has proven to be very efficient as it has been applied in many successful studies in our hospital as in studies by others [this thesis and *e.g.*, (7-15)].

Array CGH: the platform

When performing CGH studies, one should be aware that this technique is unable to detect copy-neutral abnormalities in the genome. Only by using more complex techniques (*e.g.*, paired-end mapping and next generation sequencing), DNA translocations, inversions and loss of heterozygosity (LOH) can be visualized on a genome wide scale in high throughput fashion (16, 17). Compared to the platform described in this thesis, other techniques and platforms with higher resolution plus the ability of genotyping (*i.e.*, SNP arrays) were available at the time we performed our experiments but were proven not to be suited for FFPE tissue. Advances in the field of high-resolution copy number analysis now allow for the use of FFPE material, but were made after the initiation of our studies (18). SNP arrays make use of single nucleotide polymorphisms (SNP) that allow for the detection of copy numbers and LOH.

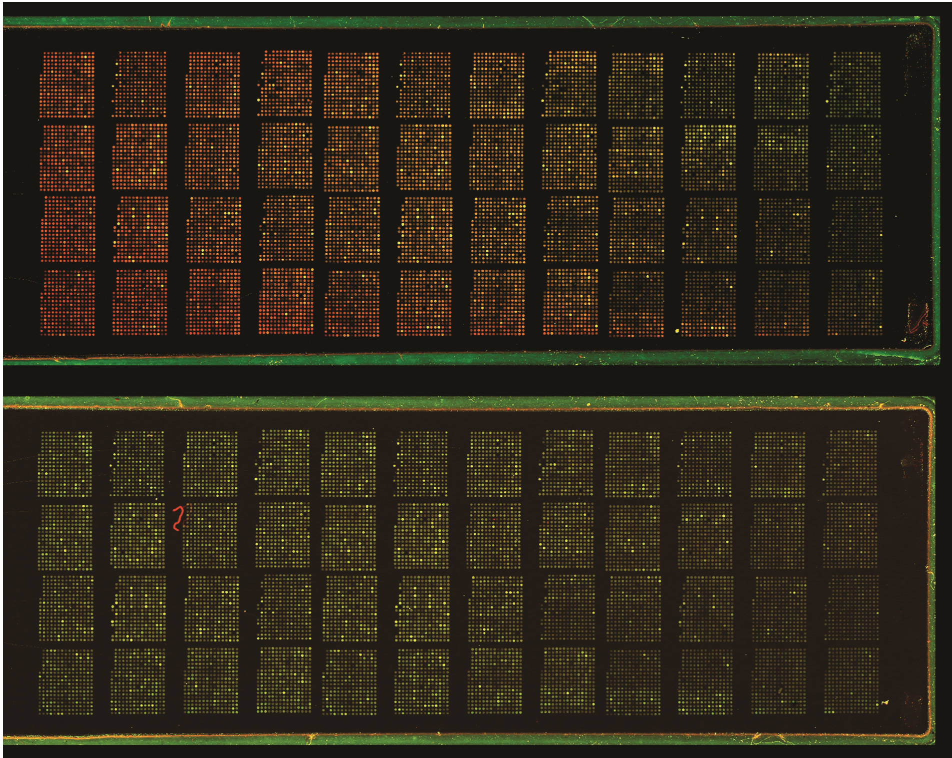

Figure 1 - Scans of two microarrays, displaying the dispersion of labeled material during the hybridization procedure. Material was injected into the hybridization chamber on the left side.

The BAC (bacterial artificial chromosome) array platform we used had several advantages over the other existing platforms. First, our arrays were printed "in-house", which was an enormous financial advantage. Second, the probes called BAC clones cover several hundreds of kbp each, allowing the binding of sufficient labeled DNA to produce clear signals for detection (high signal-to-noise ratio). Lastly, every probe was printed three times on a single array, thereby increasing the accuracy of every genomic measurement. The increased signal and triplicate measurements were required to produce very reliable results from FFPE material, which is a difficult source of DNA. Still, the relatively low resolution of the BAC array compared to other CGH platforms and the difficulty in array CGH to detect low copy number changes raised the question whether small, but also low, copy number aberrations are correctly called by the BAC array platform. To test this, we investigated whether we could properly detect a 2-fold gain of just 600 kbp (*ESR1* amplification) in a related study (19). For this, an *in silico* experiment was performed using array CGH data generated from a subject with four X chromosomes versus reference DNA with two X chromosomes (4X/2X) and array CGH data generated from reference versus reference DNA (2X/2X). Next, log2ratio values from

random segments of the X chromosomes, corresponding to the same number of probes spanning the 600 kbp region, were swapped from the 4X/2X data into the 2X/2X data. By doing so, we modeled a 2-fold gain of 600 kbp in the 2X/2X data. This procedure was repeated 100 times and the fraction that a gain was correctly called was calculated. We could identify the segment as gain in 71 out of 100 times, showing that the BAC array can detect 2-fold gains of small regions most of the time. Nonetheless, the genomic coverage of the BAC array is low and small aberrations between BAC arrays will be missed. Whether hereditary breast cancer shows such small aberrations and the detection of them would be of prognostic value are still unknown and have to be tested.

Other molecular techniques

A widely used microarray technique to study the behavior of cancer cells is gene expression profiling (GEP) (20). This technique has been proven to be very useful in the classification of molecular breast cancer subtypes (21) and prognostic signatures (22-24). However, the biggest disadvantage of this approach to study cancer is the requirement of messenger RNA, which is an unstable molecule and quickly degrades. Freshly frozen tumor material is needed to extract intact mRNA, which is not available in such large amounts as FFPE material. Although mRNA can be extracted from selected FFPE samples, the overall success rate of obtaining informative mRNA is much lower as for DNA (25). Because the studies in this thesis describe the investigations of hereditary breast cancer covering several generations of patients from breast cancer families, obtaining informative mRNA of a statistically sufficient number of samples would be extremely difficult. Therefore, DNA is a very suitable platform to study in our situation. Although DNA copy numbers carry a different form of information compared to mRNA gene expression patterns, it has been shown for classification problems that breast cancer can also be subtyped with array CGH as well as with GEP (26, 27).

Currently, the most elegant technique to determine chromosomal aberrations and simultaneously detect mutations and loss of heterozygosity (LOH) on a genome wide scale is next generation sequencing (NGS). It outperforms array CGH in resolution to the single nucleotide and is not limited by predefined probes. Compared with array CGH, the disadvantages of NGS are still the high costs and the computer power it requires to map sequence reads and calculate copy number variations. For diagnostic purposes, NGS will not replace CGH where such detailed knowledge about the complete genome is not required. For example, in pre-implantation genetic diagnostics only specific genomic aberrations are being investigated like monosomy for chromosome 18 and 21 or trisomy for chromosome 22. I anticipate that also for the diagnostic application for which we have used array CGH, finally not NGS but array CGH or a PCR based technique will still be used. For exploratory studies, NGS will most likely develop to become the standard of investigating chromosomal aberrations.

BRCA status prediction in breast cancer

BRCA1 status prediction in hereditary breast cancer

Routine diagnostics uses a diversity of techniques to identify as many *BRCA1* muta-

tions as possible in women in whom the suspicion of breast cancer susceptibility is high. Still, novel defects are being found and it is not known to what extent *BRCA1*-associated breast cancer patients are missed with current diagnostics (see **Chapter 1**). Therefore, we developed an array CGH based test that can discriminate between *BRCA1*-associated and sporadic breast tumors with high accuracy (28). **Chapter 4** describes the construction and validation of an array CGH based BRCA1 classifier. Using this classifier, we tested a group of 48 non-BRCA1/2 patients of whom two cases were found to be BRCA1-like. Additional analysis did not reveal a novel *BRCA1* mutation within theses cases; however, we could identify LOH of the *BRCA1* locus in both tumors (suggesting possible loss of function) and hypermethylation of the promoter of *BRCA1* in one case. Additional analysis of the latter patient also revealed *BRCA1* promoter methylation in the patent's ovarian tumor. Although recent studies have shown that hypermethylation can be inherited (29), it is questionable whether hypermethylation in this case is a germline epimutation. Although we did not investigate mRNA transcription, DNA extracted from blood did not show methylation of the *BRCA1* promoter.

Several points need to be addressed regarding the methodology of this study. First of all, the control group was a group of randomly picked sporadic tumors, only stratified on p53 status by immunohistochemistry. It has been proposed that, similar to a previous study by our group (30), breast tumors from non-BRCA1/2 patients should be used as a control group, because that will be the group on which the classifier will be employed most. However, this latter study reported on many false positive cases in their validation group that prove to be real *BRCA1*-mutated cases later on. Therefore, such control group could contain many unidentified *BRCA1*-mutated cases, weakening the classifier's power. Another possibility as control group would have been breast tumors, matched for hormone receptors, age and grade. Such a group would mainly consist of triple-negative (basal-like) breast tumors and would most likely result in a poor classifier, because approximately half of the basal-like breast tumors do not express *BRCA1* and harbor a CGH profile that is similar to *BRCA1*-mutated breast tumors (**Chapter 6**). Therefore, training of a BRCA1 classifier using basal-like tumors as control group requires at least twice as many cases as initially calculated to achieve similar power. Nevertheless, we have shown that by enriching for IHC status and comparing with our initial results, the BRCA1-specific genomic aberrations were not correlated to IHC status (**Chapter 4**).

Second, instead of using fixed thresholds for the identification of gains and losses, it would have been more appropriate to have used CGH profile and cell percentage dependent thresholds to compensate for technical variation as described elsewhere (35). The fixed thresholds were too stringent and lowered the frequency of all aberrations. Although re-analyses of the *BRCA1*-mutated breast tumors using variable thresholds showed higher frequencies of the aberrations, no differences were observed in the results in respect to the comparison of *BRCA1*-mutated and sporadic breast tumors (36, 37). Compared to other studies, many of the prominent aberrations in *BRCA1*-mutated breast tumors have also been identified by others (Table 1). Aberrations commonly identified to be specific in *BRCA1*-mutated breast cancer are gain on chromosome 3q and 10p and loss on chromosome 5q. Differences between studies can be explained by the different techniques used to detect and call aberrations and the

Table 1 *- Prominent chromosomal aberrations in BRCA1-mutated breast tumors from five different studies (28, 31-34), summarized per chromosome arm (Chr. arm).*

Chr. arm	**Tirkkonen (n=21)**	**Van Beers (n=36)**	**Jönsson (n=14)**	**Stefansson (n=11)**	**Joosse (n=34)**
2q	2q loss (40%)				
3p		3p gain (33%)			
3q		3q gain (67%)	3q gain (>75%)		3q gain
4p	4p loss (64%)		4p loss (>75%)	4p loss	
4q	4q loss (81%)			4q loss	
5p					5p loss
5q	5q loss (86%)	5q loss (72%)	5q loss (>75%)	5q loss	5q loss
6p	6p gain (40%)				6p gain
7p					7p loss
7q			7q gain (>75%)		7q gain
8p		*8p no gain*			
9p		9p gain (33%)			
10p	10p gain (30%)	10p gain (50%)	10p gain (>75%)	10p gain	10p gain
12p					12p gain
12q	12q loss (40%)				12q loss
13q	13q loss (55%)	13q gain (25%)			
15q			15q loss (>75%)		
16p		*16p no gain*			
16q				16q gain	*16q no loss*
17p			17p loss (>75%)		
17q	17q gain (45%)				
18p		18p gain (28%)			
20q					20q loss
Xp				Xp loss	
Xq				Xq loss	

choice of control samples to which the *BRCA1*-mutated cases were compared.

Although the classifier requires validation in a larger cohort, its usefulness was demonstrated in several subsequent studies. Van den Ouweland and colleagues showed that a novel rearrangement in *BRCA1*, deletion of exon 1a-2, was a pathogenic *BRCA1* mutation (38). In this study, breast tumors from several families were investigated by array CGH and tested for similarity to our *BRCA1* specific CGH profile. Tumors from exon 1a-2 deletion carriers were classified as being associated with deficient *BRCA1*, whereas one tumor from a family member that was diagnosed not to carry the deletion was classified as sporadic breast cancer. This study shows that deletion of exon 1a-2 in the *BRCA1* gene is not uncommon in the Dutch population, indicating that techniques such as MLPA are necessary to detect these mutations.

In a study by Tischkowitz and colleagues, the pathogenicity of the missense variant M1775K was assessed by a combination of functional, crystallographic, biophysical, molecular and evolutionary techniques, and classical genetic segregation analysis (39). These techniques included array CGH profiling of breast tumors from carriers of the M1775K variant. Our BRCA1 classifier classified the breast tumors from M1755K carriers as BRCA1-like and thus provided another line of evidence of the pathogenic effect of the missense variant. Furthermore, in one of our following publications we studied the CGH profile of the breast tumor from a subject carrying the intronic BRCA1 UV c.81-9-C>G (36). The tumor was classified as *BRCA1*-associated and as further analyses indicated, this variant results in a frame shift, thus having a pathogenic effect.

The above described results demonstrate that the BRCA1 classifier can be helpful to clinical diagnostics in providing indications of *BRCA1*-association in non-BRCA1/2 breast cancer patients, but can also aid the diagnosis of unclassified variants in providing additional indications for the pathogenicity of the variant.

BRCA2 status prediction in hereditary breast cancer

Similar to *BRCA1* mutation screening, it is unknown how many *BRCA2*-associated breast cancer patients are missed in current routine diagnostics. Furthermore, identification of unclassified variants of *BRCA2* can complicate clinical management. An additional tool, indicating the involvement of mutated *BRCA2* in cancer formation could therefore be very helpful in hereditary breast cancer diagnostics. Inspired by the success of detecting a CGH profile specific for *BRCA1*-mutated breast tumors, we investigated the chromosomal aberrations of *BRCA2*-mutated breast tumors. **Chapter 5** describes the identification of chromosomal aberrations specific for *BRCA2*-mutated breast tumors when compared to sporadic and *BRCA1*-mutated breast tumors. Table 2 describes the most prominent aberrations present in *BRCA2*-mutated breast tumors found in our and four other studies. Commonly identified were loss on chromosome 13q and gain on chromosome 17q, but also many discrepancies are present between the different studies. Based on the array CGH data, we developed and validated an array CGH BRCA2 classifier using breast tumors from proven *BRCA2*-mutation carriers and sporadic breast tumors from women without any family history for cancer. By testing a large group of 89 breast cases from non-BRCA1/2 patients, we could identify 17 cases to have a BRCA2-like CGH profile (36). Additional evidence for pathogenic mutations and non-functional BRCA2 protein was found in several cases; however, many of the BRCA2-like samples remained unexplained. During validation, we observed that the BRCA2 classifier is not very accurate in discriminating between *BRCA2*-mutated and sporadic breast tumors; this could be due to several reasons. First, the control group of the BRCA2 classifier might contain *BRCA2*-associated breast tumors and thereby diluting the aberrations of interest; however, this is highly unlikely given the prevalence of *BRCA2*-mutations in the population (see **Chapter 1**). Second, *BRCA2*-associated breast tumors might exhibit a similar pattern of chromosomal aberrations as sporadic breast cancer. Dysfunctional *BRCA2* might not lead to unique chromosomal aberrations or *BRCA2* might also become dysfunctional in sporadic breast cancer leading to similar aberrations. Third, technical limitations might be responsible

Table 2 *- Prominent chromosomal aberrations in BRCA2-mutated breast tumors from five different studies (31-34, 36), summarized per chromosome arm (Chr. arm).*

Chr. arm	Tirkkonen (n=15)	Van Beers (n=25)	Jönsson (n=12)	Stefansson (n=18)	Joosse (n=47)
1p	1p loss (45%)			1p loss	
3p	3p loss (55%)			3p loss/gain	
3q		3q gain (56%)			
6q	6q loss (60%)			6q loss	
8p				8p loss	
8q				8q gain	
11q	11q loss (65%)		11q loss (>75%)	11q loss	
13q	13q loss (73%)			13q loss	13q loss (78%)
14q				14q loss	14q loss (62%)
16p					*16p no gain*
16q	*16q no loss*			16q loss	*16q no loss*
17p				17p loss	
17q	17q gain (85%)		17q gain (>75%)	17q gain	17q gain (36%)
20q	20q gain (60%)		20q gain (>75%)		
Xp				Xp loss	

for not identifying BRCA2 specific aberrations, *i.e.*, the microarray resolution was too low or the studied cohorts were too small. Last and probably the most likely reason, because *BRCA2*-related breast tumors are also pathologically a heterogeneous group (40), the diversity among the CGH profiles might be biologically driven. It is unclear at which time point a BRCA2 deficient cell transforms to a tumor cell and what its cell of origin is. The heterogeneity among the differentiation of *BRCA2*-mutated tumors suggests different cells of origin, *i.e.*, luminal progenitor cells in diverse stages of differentiation (41). As such, *BRCA2*-mutated tumors follow different paths of development, leading to different genomic profiles (42). To be able to find BRCA2 specific characteristics in such a heterogeneous group, larger cohorts are required and subgroups might have to be defined.

BRCA1 status prediction in sporadic breast cancer

We have seen that *BRCA1*-mutated tumors harbor chromosomal aberrations that distinguish them from sporadic tumors (28). The result of classifying a tumor with *BRCA1* promoter hypermethylation as BRCA1-like suggested that the classifier selects for *BRCA1* deficiency, instead of *BRCA1* mutation status (**Chapter 4**). *BRCA* deficiency in sporadic breast cancer is also referred to as "BRCAness", and holds important implications for the clinical management of these cancers (42). The increasing evidence following our initial study that sporadic basal-like breast tumors are similar to hereditary *BRCA1*-mutated breast tumors due to *BRCA1* deficiency (43), motivated us to investigate these two breast tumor subgroups in more

detail for similarities by array CGH. As suspected, BRCA1 related aberrations were not unique to hereditary *BRCA1*-mutated breast cancer but could also be found in a subset of sporadic basal-like tumors where *BRCA1* expression has been lost (37). **Chapter 6** describes that *BRCA1* deficiency in hereditary as well as in sporadic breast cancer exhibits a common genomic profile. To define *BRCA1* deficiency, we assessed *BRCA1* mRNA expression by qRT-PCR in tumor cases with methylated *BRCA1* promoter. Subsequent cases with *BRCA1* qRT-PCR values within the 95% confidence intervals of the methylated cases were considered also to be *BRCA1* deficient. More appropriate would have been to calculate the 95 percentile using bootstrapping on the *BRCA1* qRT-PCR values of the methylated samples and use that as a cutoff for *BRCA1* deficiency and proficiency (44). Bootstrapping 1000 iterations resulted in a median 95 percentile of 0.3475 (unpublished results), and would have been more appropriate to report in the original manuscript. Nevertheless, this cutoff approaches the cutoff described in **Chapter 6** and all the subsequent results would remain identical. The results of this study show that *BRCA1* deficient sporadic basal-like tumors harbor a similar CGH profile as hereditary *BRCA1*-mutated and display the characteristic gain on chromosome 3q and 10p and the loss on chromosome 5q. These findings might allow for the identification of *BRCA1* deficiency in (sporadic) breast cancer, leading to targeted therapy in the future for such cases.

Testing the sporadic basal-like breast cancer cases with our BRCA1 classifier did not result in a clear classification of *BRCA1* deficient and proficient tumor cases as would have been expected (unpublished results). If our BRCA1 classifier would be accurate in identifying true *BRCA1*-related cases, it should be able to distinguish tumors that are not expressing *BRCA1* from those that are. Instead, many of the *BRCA1* proficient cases as defined by qRT-PCR were classified as BRCA1-like breast tumors. These results indicate that our BRCA1 classifier is suffering from underlying biologically driven noise and that the specificity is different than reported. Due to the choice of unmatched control samples, the classification might be based on triple-negative/basal-like characteristics rather than BRCA1 deficiency. A more appropriate control group for the construction of a BRCA1 classifier might had been triple-negative, basal-like breast tumors, expressing *BRCA1*.

BRCAness and homologous recombination deficiency

Both BRCA1 and BRCA2 are involved in homologous recombination to maintain chromosomal integrity. Lack of one of these genes would sensitize tumors to both DNA double-strand breaks (DSB) inducing chemotherapy and PARP inhibitors (45). Therefore, it was proposed to use the BRCA1 and BRCA2 specific CGH profiles described in this thesis as markers for homologous recombination deficiency (HRD) and to study BRCAness in sporadic breast cancer. Lips and colleagues have studied the response on neoadjuvant chemotherapy in 163 breast cancer patients in relation to the outcome of the classifiers (10). Although no association was detected between BRCA1 classification and therapy response in triple negative tumors, luminal BRCA2-like samples seem to respond significantly better to neoadjuvant chemotherapy than those classified as sporadic like. The results of this study indicate that our BRCA2 classifier has strong predictive value for neoad-

juvant chemotherapy in ER positive breast tumors. In another study, Vollebergh and colleagues investigated a cohort of tumors from breast cancer patients that were randomly assigned to adjuvant high-dose platinum-based chemotherapy or conventional anthracycline-based chemotherapy (46). The tumors were tested with our BRCA1 classifier as a synonym for HRD. Patients that received high-dose chemotherapy and of which the tumors were related to BRCA1 by array CGH lived longer than all other patients, suggesting more benefit from DSB-inducing agents. These results indicate that an interesting window of opportunity lies in the identification of *BRCA* status to guide therapy in the future.

Clinical application

The classifiers described in this thesis might function as additional diagnostic tools to screen for *BRCA* related breast cancer. It can serve as indicator for the association with *BRCA* in unclassified variants but also suggest *BRCA* deficiency in sporadic breast cancer cases (28, 36). However, the application of array CGH requires a specialized laboratory, expensive equipment, and is labor-intensive. Therefore, we have developed a PCR based method to test for *BRCA1*-association which can be performed in most diagnostic laboratories. From our array CGH based BRCA1 classifier, the most important chromosomal aberrations have been selected for which a multiplex ligation-dependent probe amplification (MLPA) assay was designed (47). This assay is able to classify *BRCA1*-mutated and sporadic breast cancer with a sensitivity and specificity of 85% and 87%, respectively, and could successfully identify BRCA1 deficient breast tumors with *BRCA1* hypermethylated promoter. Next, we studied 46 breast cancer patients with triple-negative disease that received high-dose platinum-based (HDPB) chemotherapy or conventional chemotherapy. Classifying these 46 cases with our MLPA or array CGH BRCA1 classifier clearly showed that patients with BRCA1 like breast cancer had more benefit from HDPB than conventional chemotherapy (Figure 2). These data suggest that BRCA1 deficient breast tumors can be identified by their chromosomal aberrations and that these patients might benefit from other therapy than conventional chemotherapy. It might therefore be suggested to screen every triple negative hereditary and sporadic breast tumor for BRCA1 deficiency using our BRCA1 classifier.

BRCA status unknown

BRCAX

A major part of the breast (and ovarian) cancer families, which are tested for mutations in the *BRCA1* and *BRCA2* genes, are presented with a negative test result; the genetics behind their predisposition for breast and ovarian cancer remains unknown (**Chapter 1**). Although familial breast cancer is still similarly treated as sporadic breast cancer, targeted therapy might become available in the future and identifying the genetic defects responsible for cancer formation would then be of vital importance. Therefore, much effort has been undertaken to search for undiscovered high-risk breast cancer susceptibility genes. Because linkage analysis has so far not been able to identify new high-risk genes, it is suggested that more than one risk-conferring locus is involved. To be able to identify these loci, genetically homogeneous groups have to be selected first. Using array CGH, we have seen that certain genetic mutations are correlated with specific chromosomal

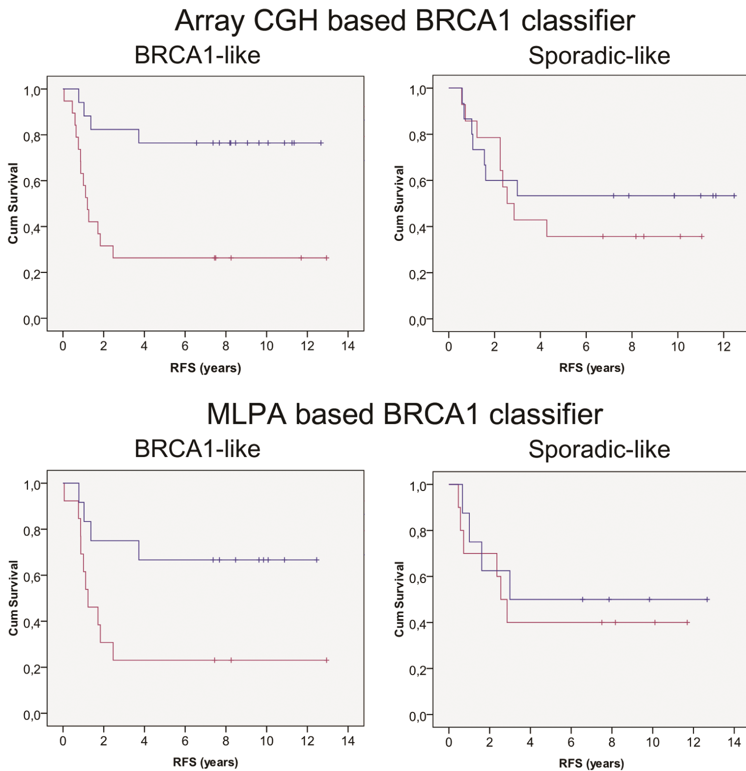

Figure 2 *- Survival curves of patients with triple-negative breast cancer, treated with high-dose platinum-based chemotherapy (blue) or conventional chemotherapy (red), separated based on BRCA1 classification using our original array CGH (upper two panels, n=64) or MLPA classifier (lower two panels, n=40) (47). RFS: relapse free survival. Cum Survival: cumulative survival.*

aberrations (**Chapter 4 and 5**). It might therefore be possible that non-BRCA1/2 families that have the same genetic defect also present similar chromosomal aberrations. **Chapter 7** describes the array CGH study performed on tumors of non-BRCA1/2 breast cancer families (referred to as BRCAX tumors) in order to find clusters of families with similar genetic profiles and to identify their key genetic characteristics.

First, the array CGH profiles of the BRCAX tumors were compared with those of sporadic breast tumors that were taken from an earlier study (28). Although several clear differences could be distinguished between the two cohorts in respect to aberration frequency, it should be noted that the particular control group was used because of its accessibility and was not matched in any way with the BRCAX tumors. This might explain why the differences that have been found between the groups and those found in a similar study by Gronwald and colleagues (48) are not alike. In addition, because the studied groups are not matched, the results are not independent from other factors and those aberrations found to be prominent in BRCAX tumors but not in the control tumors may not be called "BRCAX-specific" and can not solely be correlated to a single hereditary factor.

Next, several of the CGH profiles of *BRCA1*- and *BRCA2*-mutated breast tumors from two of our earlier studies (28, 36) were compared with those of the BRCAX tumors. Here, as also true for the control tumor comparison, several issues of the methodology have to be dealt with. To be

able to call gains and losses in the CGH profiles, fixed threshold of 0.2 and -0.2 on the copy number alteration segment values are applied (**Chapter 7**). By this, variance between samples due to tumor cell percentage is not taken into account. Furthermore, no differentiation has been made between gain and amplification of genomic regions. It might have been more appropriate to have used CGH profile and tumor cell percentage dependent thresholds as described elsewhere (35). Subsequently, the correlations of the number of gain/no-gain and loss/no-loss between the different cohorts were studied using 2 x 2 Fisher's exact tests. By investigating only two possible observations per measurement (*e.g.*, gain versus no-gain), one assumes that a different state of the measurement is non existent; however, this is not true in respect to chromosomal aberrations. From a biological point of few, a more adequate model would have been to transfer the continuous data of copy number measurements into the following discrete parts: lost (< 2 copies), neutral (2 copies), gained (> 2 copies), and amplified (high-level gain) (35). By this, a 4x2 table containing categorical data (a contingency table) is formed and should be analyzed appropriately (*i.e.*, 4 x 2 Fisher's exact test or 4 x 2 Chi^2 test).

Another point that should be addressed is the comparison between the BRCAX and sporadic breast cancer groups by the average numbers of 'aberrant clones' (log2ratio measurements exceeding the fixed gain/loss thresholds). Such a comparison does not provide any biological information and might only reflect the level of technical noise (although other and better analyses are available for such comparisons). More relevant comparisons would have been the number of chromosomal aberrations, the sizes, and the type of CGH profiles (see **Chapter 1**) which are all missing in the study described in **Chapter 7**.

The more relevant analyses of this study were the comparisons of the BRCAX cases among each other. The main finding was that a part of the BRCAX samples showed gain of chromosome 22 while another part showed loss of chromosome 22, which was also commonly seen in sporadic breast cancer. This suggests that the studied BRCAX samples consisted of at least two subgroups. Interestingly, the chromosome 22 aberrations were consistent among the family members within the same BRCAX families, indicating that gain of chromosome 22 is a BRCAX subgroup specific aberration. Following on this finding, a classifier was built based on the samples that had gain of chromosome 22 (n = 10) and those with loss of chromosome 22 (n = 15). The classifier was built using the nearest shrunken centroid algorithm (49); however, it lacked power for reliable classification. For an error tolerance of < 0.10, more than 15 samples of each class would have been needed (50). This could explain why in subsequent testing of other BRCAX cases, samples with loss of chromosome 22 were classified within the "22-gain" class. An alternative explanation is that gain of chromosome 22 might not be a specific aberration for a subgroup of BRCAX. Therefore, it should be investigated in larger series of BRCAX cases, whether gain of chromosome 22 is a relevant marker and is not related to other factors such as *TP53* mutation status. Because of the limited linkage information of each of the "22-gain" families, a meaningful linkage analysis could not be performed.

In conclusion, because of the high heterogeneity among BRCAX tumors, much larger cohorts are needed in order to identify genetically similar subgroups to perform linkage analyses on.

BRCA status related biology

Co-occurrence of mutated *TP53* and *BRCA1*

It has been shown in mouse models that the development of mammary tumors is highly accelerated when both *Brca1* and *Trp53* have been knocked out. Because *Brca1*$^{-/-}$ mice are embryonic lethal but lethality can be partially rescued by *Trp53* knockout, it is suggested that loss of *Trp53* is required to alleviate the cell-lethal effects of loss of *Brca1*. This has raised the question whether *TP53* loss of function is also required in *BRCA1*-mutated human breast cancer. We therefore have sequenced *TP53* of *BRCA1*-mutated and sporadic breast tumors from our study described in **Chapter 4**, and found that indeed most *BRCA1*-mutated tumors have a pathogenic *TP53* mutation (51). In respect to immunohistochemistry (IHC), it has been shown that *BRCA1*-associated breast tumors are more frequently p53 positive compared to sporadic breast tumors. However, our study shows that *BRCA1*-mutated tumors, negative for p53, are carriers of *TP53* truncation mutations. These results indicate that loss of *TP53* is required in *BRCA1*-mutated tumors. Although it has been shown that *TP53* mutations can be found more often in basal-like breast cancer compared to luminal breast cancer (15), it would be interesting to investigate whether sporadic basal-like breast tumors deficient in *BRCA1* expression harbor a *TP53* mutation more frequently compared to *BRCA1* proficient basal-like breast tumors.

The above findings also open new therapeutic possibilities by targeting the BRCA1 and p53 deficiency combination. Recent research has proposed inhibitors against Cdc7 kinase as a highly specific anti-cancer drug in triple-negative breast cancers (52). Inhibition of Cdc7 activates a p53-dependent checkpoint, resulting in cell cycle arrest to avoid lethal S phase entry in normal cells. Lack of p53 results in abrogation of the Cdc7-inhibion checkpoint, which is followed by lethal S-G2-M phase progression (52).

For the training of our BRCA1 classifier described in **Chapter 4**, we have compared *BRCA1*-mutated with sporadic breast tumors, stratified for IHC based p53 status. As mentioned, mutated *TP53* is common in *BRCA1*-mutated breast tumors, even among the p53 IHC negative cases, making IHC a difficult indicator to interpret for *TP53* status. If we assume that the IHC based p53 status reflects the *TP53* mutation status in sporadic breast tumors well, it might be possible that our study was not stratified for *TP53* status after all because only 43.5% was positive for p53. Specifically, it is known that *TP53* status is associated with loss of chromosome 5q (53, 54); indeed, loss of chromosome 5q was abundantly present in *BRCA1*-mutated breast tumors and therefore part of the BRCA1 classifier (**Chapter 4**). Although *TP53* mutation is a characteristic of *BRCA1*-mutated tumors, and thus also loss of chromosome 5q is, it has to be taken into account that a higher BRCA1-like score might be given to any *TP53* mutated breast tumor *per se*. Although further investigation has to be performed, these results suggest that the CGH profile described specifically for *BRCA1*-mutated breast cancer in **Chapter 4**, probably includes several chromosomal aberrations that are not only specific for *BRCA1*-mutated status but also for other characteristics like ER negative status or *TP53* mutation that are both correlated with loss on chromosome 5q (53).

Secondary BRCA2 mutation

Tumors that are deficient in *BRCA1/2* have a decreased capability to repair DNA and are therefore sensitive to chemotherapeutic agents causing DNA cross-link such as cisplatin or carboplatin and are susceptible to synthetic lethality from PARP inhibitors (55, 56). However, *BRCA1/2* mutation carriers frequently develop recurrent disease that is resistant to platinum agents. It has been shown in *BRCA*-mutated ovarian cancer that *BRCA* function can be restored by a secondary somatic mutation in the tumor, leading to chemotherapy resistance (57-60). So far, little is known about the occurrence of secondary mutations in *BRCA* in breast cancer. To date, cell line HCC1428 is the only cell line providing evidence of secondary mutations occurring *in vivo* in breast cancer (57). HCC1428 was isolated from a woman heterozygous for 6174delT mutation in *BRCA2* after she had undergone chemotherapy. The

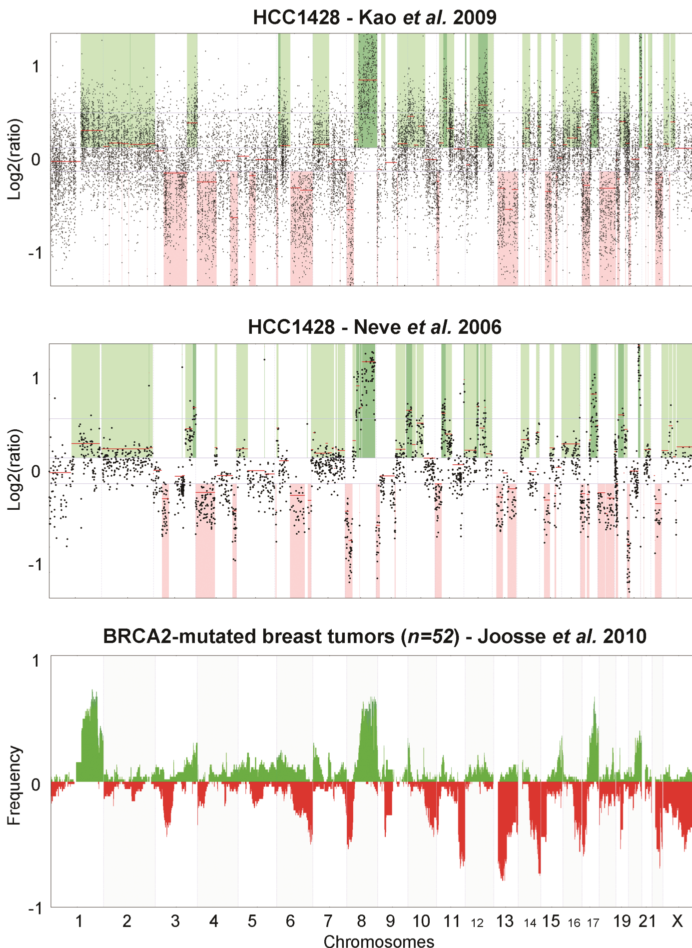

Figure 3 *- Array CGH profiles of breast cancer cell line HCC1428 by Kao et al. and Neve et al. (61 6,2) (upper two panels). Frequency plot of array CGH profiles of 52 proven BRCA2-mutated breast tumors (36). Gain of genomic material is depicted in green; loss of genomic material is depicted in red.*

BRCA2 gene in HCC1428 has a 2135 bp internal deletion that spans the original 6174delT mutation, leading to expression of functional *BRCA2* transcripts. Consequently, HCC1428 is resistant to cisplatin but could be sensitized again by BRCA2 depletion.

From a biological perspective, it would be most interesting to investigate the genomic profile of *BRCA*-mutated breast tumors that have developed a second mutation to restore *BRCA* function. Because of natural selection during the development of a tumor, certain chromosomal aberrations in breast cancer are recurrently found that (indirectly) provide growth advantage. As gain on chromosome 3q and 10p and loss on chromosome 5q are characteristic for *BRCA1*-mutated breast tumors and probably necessary for tumor survival, it can be speculated that upon restoration of *BRCA1* function, some of these aberrations might become redundant to even obnoxious. Further selection on the tumor could then change its genomic make-up. Studying *BRCA*-associated breast tumors with secondary mutations might give further insight on how selection based development of chromosomal aberrations takes place and which role *BRCA1/2* plays in these selection processes.

To test the hypothesis that the genomic makeup changes after a second mutation, I have investigated the CGH profile of the *BRCA2* double mutated cell line HCC1428, published by Kao and colleagues and Neve and colleagues (61, 62). Figure 3 shows the CGH profiles of cell line HCC1428 of both publications (upper two panels) and the frequency plot the CGH profiles of 52 *BRCA2*-mutated breast tumor specimens from our earlier study (bottom panel) (36). Although the cell line shows many similarities with *BRCA2*-mutated tumors in respect to chromosomal aberrations, our CGH BRCA2 classifier classifies the cell line as sporadic-like. This suggests that HCC1428 is not similar to *BRCA2*-mutated tumors as expected. In this specific case however, it is not certain whether the difference is due to the secondary mutation, the preservation of the cells in culture for a long period of time, or that the primary tumor never resembled the general BRCA2-associated CGH profile in the first place.

Conclusion and future perspectives

Several methods exist to predict the association of *BRCA1* or *BRCA2* in the development of cancer. In this aspect, microarray technology is a useful technique that is able to characterize breast cancer at the molecular level, linking these characteristics to *BRCA1* or *BRCA2*. The results of our classification studies and the results found by others indicate that 4-12% *BRCA* related cases, which have not been found by routine BRCA mutation screening, can be identified due to the investigation of the tumor's chromosomal aberrations (28, 36, 63). Although further validation in larger cohorts is required, prediction of *BRCA*-association based on chromosomal aberrations shows to be a promising technique. Using the predictive markers described in this thesis to develop a MLPA based assay is a logical next step to assist mutation screening in high-risk breast cancer patients, or to provide another link between *BRCA* unclassified variants and breast cancer. In addition to hereditary breast cancer diagnostics, linking *BRCA* status to sporadic breast cancer might lead to targeted therapeutic options for these patients in the future. Because BRCA1 and BRCA2 are required for DNA repair by homologous recombination, our BRCA classifiers might also be useful in the

identification of homologous recombination deficient tumors and help to guide anti-breast cancer therapy.

Future perspectives regarding the investigation of *BRCA* status specific chromosomal aberrations would include optimization of a control group for *BRCA1* deficient breast cancer. Such a control group would consist of *BRCA1* proficient basal-like breast cancer. Furthermore, because of the heterogeneity in *BRCA2*-mutated breast cancer, larger cohorts are required for the identification of possible subgroups and their specific aberrations. The same is applicable in the search for BRCAX subgroups where larger cohorts are required. For the detection of chromosomal aberrations, array CGH has been a valuable tool but newer tools such as SNP arrays and next generation sequencing are currently available and provide more detailed and accurate data.

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