Molecular analysis of early onset Indonesian breast cancer

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Moleculaire analyse van borstkanker bij jonge Indonesische vrouwen
(met een samenvatting in het Nederlands)

Analisis molekuler penderita kanker payudara usia muda di Indonesia
(dengan ringkasan dalam bahasa Indonesia)

Proefschrift

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I dedicate this thesis to my late Beloved Father, the living inspiration ever in my journey, Poernomo Sidhi

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I. CLINICAL ASPECTS OF BREAST CANCER

1.1. Epidemiology of breast cancer

Breast cancer is the second leading cause of cancer deaths in women today (after lung cancer) and is the most common cancer among women, excluding non-melanoma skin cancers. According to the World Health Organization, more than 1.2 million people will be diagnosed with breast cancer this year worldwide (http://www.who.int/whosis/whostat2006.pdf.). The American Cancer Society estimates that in 2005, approximately 211,240 women in the United States will have been diagnosed with invasive breast cancer (http://www.cancer.org/downloads/STT/CAFF2005BrF.pdf). The chance of developing invasive breast cancer during a woman's lifetime is approximately 1 in 8 (13.4%). Another 58,490 women will be diagnosed with in situ breast cancer, an early form of the disease. Though much less common, breast cancer also occurs in men. An estimated 1,690 cases will have been diagnosed in men in 2005.

There are no nationwide data on the incidence of breast cancer in Indonesia, but locally published data [1] on a pathology based cancer registry in Jogjakarta in 1982 showed that breast cancer incidence ranked second (18.9%) after cervical cancer (20%). Ghozali [2] showed in 1995 that breast cancer was even the most common cancer among women in Jogjakarta (24.58%), followed by cervical cancer (17.28%). Unpublished data on early onset ($\leq$ 40 years) breast cancer patients between 1998 to 2004 in Sardjito Hospital Jogjakarta, showed that the proportion of these patients was 26% of total breast cancer cases. This number is higher compared to data from The National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program that reveals that 75% of breast tumors occur in women aged >50 years and only 6.5% in women aged <40 years. *This indicates that breast cancer is also a major health care problem in Indonesian women, especially among young women.*

1.2. Etiology of breast cancer

The etiology of breast cancer is still poorly understood. Several known risk factors can only explain a small proportion of breast cancer cases [3]. At first, age is associated with increasing breast cancer risk. However, remarkably, most risk increase occurs during the reproductive years as breast cancer incidence is very low before age 25, and increases up to 100 fold by age 45 [4]. This pattern suggests the involvement of reproductive hormones in breast cancer etiology. Other known risk factors involve life style factors, environmental factors, a history of benign proliferative breast lesions, and genetic factors.

Reproductive factors such as nulliparity, early menarche and older age at first pregnancy have been associated with an increased breast cancer risk [4, 5]. In addition, exogenous hormonal influences like using oral contraceptives may increase breast cancer risk. However, the opposite was demonstrated in many epidemiologic studies in which no association between the use of oral contraceptive and the risk of breast cancer was shown. Recently, however, a large meta-analysis calculated a small but significant increase in relative risk of breast cancer (RR = 1.24) in current oral contraceptive users [6]. The use of hormone replacement therapy (HRT) by postmenopausal women was also shown to be associated with enhanced breast cancer risks, predominantly affecting the chance of development of a hormone receptor-positive breast cancer [7].

Life style factors, like increased height and weight have been associated with a higher breast cancer risk in a number of studies [8, 9]. Dietary factors such as high fat intake, low vegetables/fruit and low fibre intake may also increase risk [10]. Furthermore, alcohol consumption was significantly associated with a higher risk of breast cancer [11, 12]. Some studies related socio-economic status to breast cancer risk, but these findings can probably be explained by differential life styles such as alcohol, diet and reproductive patterns [13].

Besides these life style factors, other medical history factors are involved with increased breast cancer risk. Mammography density >75% is another well-established risk factor for breast cancer in both pre-and postmenopausal women [14]. Increased mammography density is seen predominantly in nulliparous women and thin women. Furthermore, a history of a benign proliferative breast conditions, especially atypical ductal hyperplasia (ADH) and fibroadenoma, is associated with an increased risk for breast cancer [15-17]. Other exogenous risk factors include exposure of the mammary gland to high dose ionizing radiation during childhood [4]. This latter relation is dose dependent and decreases gradually over time.

Finally, a positive family history of breast cancer is most significantly associated with increased risk of breast cancer. This is most strongly seen in families with a germline mutation in a breast cancer susceptibility gene such as BRCA1 and BRCA2 which were identified in 1990 and 1994, respectively [18, 19]. Such germline mutations are associated with a 50-80% life time breast cancer risk. In addition to the BRCA genes, several other high susceptibility genes have been identified, such as *p53*, *ATM*, *CHEK2* and *PTEN* [3]. However, because of their low allele frequencies in the general population, it is believed that only 5 to 10 percent of all breast cancers are associated with the presence a specific germline mutation.

Besides the above mentioned well known genetic alterations, epigenetic alterations are among the most common molecular alterations in human neoplasia [20-22]. Epigenetic changes differ from genetic changes mainly in that they occur at a higher frequency than genetic changes, are reversible upon treatment with pharmacological agents and occur at defined regions in a gene [21].

1.3. The multistep progression model of breast cancer

Breast cancer tumorigenesis can be described as a multi-step process [23] in which each step is thought to correlate with one or more distinct mutations in major or minor regulatory genes.

Breast development begins in the embryonic period. Ductal morphogenesis starts from a bud-like structure with branching, elongation and then canalization. Basal cells, expressing both smooth muscle actin, as well as high molecular weight cytokeratins appear at the end of the second trimester. In the adult breast two major cell types can be distinguished: the myoepithelial cell and the luminal secretory cell, that derive from a pluripotent CK5/6 positive stem cell that shows no compartimentalization. Clinically and histopathologically, various morphologically definable steps can be identified during progression to malignancy [24].

Ductal hyperplasia, characterized by proliferation of unevenly distributed polyclonal epithelial cells with overlapping nuclei of varying shape and chromatin pattern and haphazard lumina, is often a first sign of tendency towards malignancy. The cells have relatively little cytoplasm and no clear cell borders. Cytologically the cells are benign. The transition from hyperplasia to (clonal) atypical hyperplasia

with cells with more distinct cytoplasm, more regular nuclei with less overlap and more regular lumina, is clinically associated with an increased risk of breast cancer. The next step is development of carcinoma *in situ*, either ductal or lobular, which is defined as a proliferation of cells with cytological characteristics of malignancy, but without stromal invasion across the basement membrane. Lobular carcinoma *in situ* derives from the lobuli, and is usually diffusely spread throughout the breast (and even often bilateral), and is typically not palpable and unvisible by imaging. In contrast, ductal carcinoma *in situ* is a segmental ductal lesion that may reveal itself through microcalcifications and may be palpable.

As cells detach from the basement membrane and invade the stroma, the tumour becomes invasive. Through dissemination via blood and lymph vessels, invasive cells can give rise to metastases, either to loco regional lymph nodes or to distant organs. In this process, tumor cells need to escape the immune system to prevent clearance. At each of these steps, genetic events occur that give the cell new properties with a resulting clonal selective advantage for that cell. These genetic events range from small point mutations, via chromosomal deletions, translocations and amplifications to large-scale changes as whole chromosome losses or duplications. The result of these alterations could be modification of gene expression or functional alteration of gene products that are relevant for tumour progression. The accumulation of these genetic events is best believed to follow the “bingo principle” (in random order) than a fixed stepwise order, although some events seem to occur more often earlier than others.

It has become clear that the genetic events that play a role in carcinogenesis of sporadic and hereditary breast cancer show overlap as well as differences. It is beyond the scope to discuss of this thesis and introduction to review the panoply of different genetic events that have been described in sporadic breast cancer. Rather, we will focus on some important genetic aspects of hereditary breast carcinogenesis.

1.4. Treatment of breast cancer based on prognostic and predictive factors

Because adjuvant systemic therapy has associated risks, it should only be given to high risk patients. Therefore, it is essential to be able to estimate an individual patient’s risk to develop clinically manifest metastatic disease using prognostic factors [25-27]. With predictive factors, patients that most likely to benefit from a certain therapy can be identified.

The most significant prognostic indicator for breast cancer is the presence or absence of lymph node involvement as mentioned earlier. There is also a relationship between the number of involved axillary nodes and the risk of distance recurrence [28]. Therefore, axillary node status is the most important prognostic factor used in adjuvant therapy decision making. Secondly, tumor size is also an independent prognostic factor, with distant recurrence rates increasing with larger tumor size [29, 30].

Histologically, tumor grade scored according to the widely accepted Bloom-Richardson classification [31] has also prognostic significance. Of the three features encompassing grade, mitotic index is known to have overriding prognostic value. Furthermore, lymphovascular invasion is associated with recurrence and overall survival [32].

The estrogen (ER) and progesterone receptors (PgR) are useful for predicting the clinical response to endocrine therapy [33]. HER-2/*neu* (c-*erb*B-2) has recently been added as a routine immunohistochemical assessment, as protein overexpression of HER-2/*neu* predicts both higher chance to respond to trastuzumab treatment as well as taxane chemotherapy [34]. Further, such patients respond better to aromatase inhibitors than to tamoxifen [35-39]. Trastuzumab is a recombinant DNA-derived humanized monoclonal antibody that selectively binds to the extracellular domain of HER2. This specific biological drug has been approved in the USA since 1998 for *HER2*-positive patients with metastatic breast cancer. It is most effective in combination with chemotherapy. Recent clinical studies have shown that trastuzumab may also be useful as adjuvant therapy in breast cancer patients who overexpress HER2. The drug is generally well tolerated but cardiotoxicity has been reported in up to 27% of cases when used in combination with anthracycline and cyclophosphamide [40, 41]. This, and the significant costs of the therapy underline the need to optimally select patients for treatment with this drug.

Immunohistochemical (IHC) staining has been approved as a screening method for HER2 protein activity, accepting a 3+ score as “positive” for treatment. However, there are some problems with IHC interpretation due to its subjective nature, so it is generally accepted that a 2+ score requires gene status confirmation by an amplification test. Further, there are indications that gene amplification status predicts response better than IHC [42]. The most widely used gene amplification test is fluorescence *in situ* hybridization (FISH). However, FISH is difficult, expensive, probes have a limited half life, and interpretation is not without its problems, so FISH is not very suitable for routine use. Chromagen ISH (CISH) overcomes some but definitely not all these drawbacks. Other more easy to perform tests are therefore urgently required. Recently, a test based on multiplex ligation dependent probe amplification [43] has become available that deserved to be validated.

II. GENETIC ASPECTS OF HEREDITARY BREAST CANCER

Approximately 5% of breast cancers show a familial pattern of occurrence [44]. This is often related to germline mutations in different (tumor suppressor) genes of which the proteins have a crucial function in the breast. Like in the classical tumor suppressor, in patients with a germline mutation, loss of expression of the other allele by point mutations or deletions will lead to a significant or complete loss of protein function. In familial breast cancer patients, germline mutations have been described in *BRCA1*, *BRCA2*, *PTEN*, *p53*, *ATM* and *CHEK2*. Together, these account for most but certainly not all hereditary cases, so the search for other hereditary breast cancer genes continuous. *BRCA1* and *BRCA2* will be dealt with extensively further on as they form the focus of this thesis, so here we mainly discuss the other proteins.

The *BRCA1* gene, located on chromosome 17q12-21, was cloned in 1994 [18]. *BRCA1* is involved in many transcriptional processes. It has been associated with more than 15 different proteins involved in transcription, either in transcriptional activation or transcriptional repression [45]. It also plays a role in apoptosis. As a tumour suppressor, *BRCA1* is a factor in maintaining genomic stability. It interacts with various proteins, and the complexes formed are involved in DNA recognition

and repair [46, 47]. The *BRCA2* gene is located on chromosome 13q12-13. The gene codes for proteins involved in DNA repair, cell cycle control and transcription [48], and may have a function in terminal differentiation of breast epithelial cells [49]. In sporadic breast cancer, mutational inactivation of *BRCA2* is rare as inactivation requires both gene copies to be mutated or totally lost [47, 48, 50]. Surprisingly, despite the inherited predisposition to cancer associated with *BRCA1* and *BRCA2* (see below) somatic disease-causing mutations in either of these genes are extremely rare in sporadic breast cancers [51, 52].

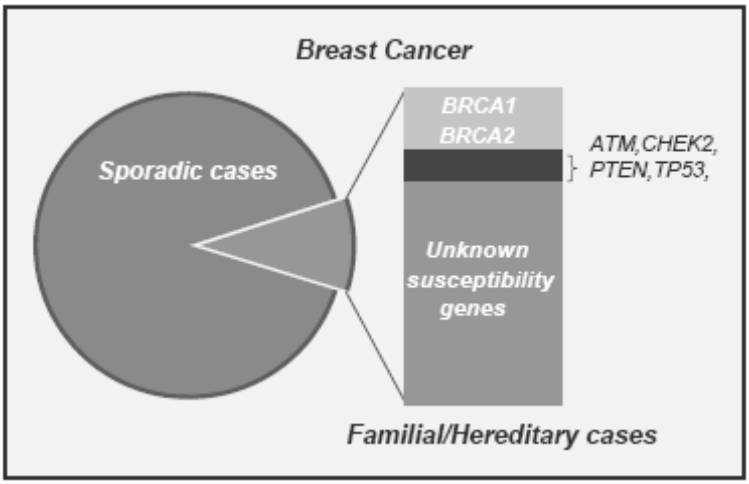

Figure 1. — Breast cancer susceptibility genes. Hereditary breast cancer (right) constitutes only approximately 5% to 10% of all breast cancer cases (left). Germline mutations in the two major susceptibility genes *BRCA1* and *BRCA2* account for less than 5% of all breast cancer cases, while mutations in genes such as ATM, *CHEK2*, *PTEN*, and T*P53* account for only about 1% of all breast cancer cases (adopted from Dapic et al, 2005 [53]).

TP53 is a tumor suppressor gene located on 17p13.1 encoding a nuclear phosphoprotein that acts as a transcription factor involved in the control of cell cycle progression, repair of DNA damage, genomic stability, and apoptosis [54]. In response to DNA damage, the *p53* protein arrests cells in the G1 phase of the cell cycle, allowing the DNA repair mechanism to proceed prior to DNA synthesis. Loss of *p53* function abolishes this growth arrest response to DNA damage. T*P53* is one of the most frequently mutated genes in sporadic human cancer [50]. Most mutations are point mutations leading to proteins defective for sequence-specific DNA binding and activation of *p53*-responsive genes [55-57]. In sporadic breast carcinomas the occurrence of T*P53* mutations is usually a late event. Interestingly, *TP53* mutations are frequently found in *BRCA1*-linked tumors and several studies have suggested that the status of *BRCA1/BRCA2* influences the type and distribution of *TP53* mutations in breast cancer [57-59]. Germline mutations in *TP53* (Li-Fraumeni syndrome) are very rare. Analysis of 475 tumors in 91 families with p53 germline mutations showed that breast carcinomas are most frequent (24.0%), followed by bone sarcomas (12.6%), brain tumors (12.0%), and soft tissue sarcomas (11.6%) [60]

PTEN (also known as *MMAC1*) on chromosome 10q23.3 was originally identified as a tumor suppressor gene defective in a variety of human cancers [61, 62]. Germline mutations in *PTEN*, causing Cowden disease, a rare autosomal dominant inherited cancer syndrome characterized by a high risk of breast, thyroid, and endometrial carcinomas and hamartomas (a common benign tumor as a result from an abnormal formation of basically normal tissue components). Hamartomas, while generally benign, can cause problems due to their location. They are particularly likely to cause major health issues when located in the hypothalamus, spleen or kidneys. [63-65]. Most cancer associated *PTEN* mutations are truncations that cause a 25% to 50% lifetime breast cancer risk among women affected with Cowden disease [66, 67]. *PTEN* mutations are rare in sporadic breast cancer and have been found in only 5% of the sporadic cases [68, 69]. However, 29% to 48% of sporadic breast cancer cases show loss of heterozygosity at the *PTEN* locus, while no alterations have been found in the remaining allele [70]. In addition, approximately 40% of breast cancers show a decrease or absence of *PTEN* protein levels.

The cell cycle checkpoint kinase *CHEK2* gene on chromosome 22q12.1 is a key mediator in DNA damage-response [71, 72]. In mammalian cells, *CHEK2* is phosphorylated by *ATM* in response to double strand breaks (DSB) [73]. Activated *CHEK2* phosphorylates a number of target proteins that in turn prevent cellular entry into mitosis and activate DNA repair pathways. In addition, *CHEK2* acts in the G1-S checkpoint by phosphorylating *p53* and mediating activation and stabilization of *p53* by *ATM* [74, 75]. In another important connection, *CHEK2* phosphorylates *Cdc25C* and *BRCA1* [76, 77]. Mutation screening of the *CHEK2* gene among Li-Fraumeni cases revealed the *CHEK2 1100delC* mutation which inactivates the kinase activity of the protein [78]. This allele has also been proposed to be a low-penetrance breast cancer susceptibility allele [79, 80]. Additional screening of *CHEK2* did not identify any other variant that occurs at significantly elevated frequency, indicating that *1100delC* may be the only *CHEK2* allele with a significant contribution to breast cancer susceptibility [81]. Interestingly, *CHEK2 1100delC* is associated with breast cancer only in non-carriers of *BRCA1* and *BRCA2* [79]. A recent search for new breast cancer susceptibility genes among families with no *BRCA1* and *BRCA2* mutation suggested a model in which *CHEK2 1100delC* interacts with an as yet unknown gene to increase breast cancer risk [82].

The ATM *(*ataxia telangiectasia-mutated) protein was identified as the product of the gene mutated in the rare human autosomal recessive disorder ataxia telangiectasia (AT) [83]. *ATM* plays a key role in monitoring genomic integrity and triggering appropriate cell-cycle checkpoints, DNA repair, or apoptotic pathways in response to DNA double-strand breaks. In response to ionizing radiation, a potent inducer of DNA double strand breaks, *ATM* associates with and phosphorylates a number of different substrates, including p53, MDM2, Nibrin, CtIP and BRCA1 [84-87]. Phosphorylation of p53 and MDM2 results in p53 stabilization and accumulation that activates the G1/S cell cycle checkpoint. *ATM* phosphorylation of CtIP in response to radiation exposure modulates the ability of *BRCA1* to induce expression of DNA-damage response-molecules such as GADD45 [87]. ATM also directly phosphorylates BRCA1, and this phosphorylation is required for normal cellular survival after exposure to ionizing radiation [86]. Therefore, there are direct functional links between *ATM* and *BRCA1*, both of which have been implicated in

breast carcinoma susceptibility. *ATM* heterozygotes have an approximately ninefold-increased risk of developing a type of breast cancer characterized by frequent bilateral occurrence, early age at onset and long-term survival [88].

2.1. *BRCA1* and *BRCA2*

In 1990, genetic studies provided initial evidence that the risk of breast cancer in some families is linked to chromosome 17q21 [89]. This 17q-associated syndrome was characterized by autosomal dominant inheritance with incomplete penetrance. In fact, loss of heterozygosity (LOH) at 17q was found in most familial breast and ovarian tumors, suggesting the involvement of tumor suppressor gene(s) [90, 91]. In 1994, the breast-cancer susceptibility gene, *BRCA1*, was identified by positional cloning; subsequently, this gene has been the subject of intensive research [18]. *BRCA1* is composed of 22 coding exons distributed over 100 kb of genomic DNA. This gene encodes 1863 amino acids, and more than 200 different germline mutations associated with cancer susceptibility have so far been identified (http://www.research.nhgri.nih.gov/projects/bic). Many disease-predisposing alleles of *BRCA1* concern insertions, deletions, frameshifts, base substitutions and inferred regulatory mutations [92], the majority resulting in premature truncation of the protein leading to loss of protein function. Germline mutations in *BRCA1* confer susceptibility to breast, ovarian and Fallopian tube cancer, as well as cancers of the corpus uteri, the cervix and the peritoneum.

Because only 45% of familial breast cancers showed evidence of linkage to *BRCA1*, the search for a second breast cancer susceptibility gene continued. In 1995, the *BRCA2* gene was identified at chromosome 13q12.3 [19, 93]. So far, around 3000 different germline mutations associated with cancer susceptibility have been identified (http://www.research.nhgri.nih.gov/projects/bic). Germline mutation carriers also have increased susceptibility to ovarian, Fallopian tube, pancreatic, prostate, and male breast cancers.

2.1.1. Structure of *BRCA1* and *BRCA2*

Although there is no sequence similarity between the two genes, many structural and functional features of *BRCA1* and *BRCA2* are similar. Both genes have complex genomic structures (*BRCA1* is composed of 24 exons and *BRCA2* of 27 exons), and they both encode very large proteins (*BRCA1* 1863 amino acids and *BRCA2* 3418 amino acids) [18, 19, 94]. In both, exon 1 is noncoding and exon 11 is unusually large, 3.4 kb in *BRCA1* and 5 kb in *BRCA2*. *BRCA1* and *BRCA2* are expressed in a wide range of tissues and show remarkably similar temporal and spatial patterns of expression.

Both *BRCA1* and *BRCA2* exhibit approximately 60% amino acid identity with their murine counterparts, although several short domains of both proteins are relatively well conserved compared to the remainder [95-97]. This level of evolutionary conservation is lower than that of other cancer-susceptibility genes, most of which exhibit more than 90% human/mouse sequence identity.

Three regions of *BRCA1* show sequence similarity to previously described proteins. A highly conserved zinc-binding RING finger domain is located close to the amino-terminus (residues 20–68). Although some classes of zinc finger act as transcriptional regulators by binding to specific DNA sequences in promoter/enhancer regions, there is little current evidence in favor of this function

for the RING class of zinc finger. In contrast, exist data indicating that RING fingers are involved in protein-protein interactions [98]. Indeed, a search for interacting proteins, using the BRCA1 RING finger domain as bait, uncovered a further RING-domain protein designated BARD1 (*BRCA1*-associated RING domain), which binds to BRCA1. The RING domains of both *BARD1* and *BRCA1* are necessary, but not sufficient, to mediate this interaction [99]. Toward the carboxyl terminus of *BRCA1* are two tandem copies of a motif (designated the BRCT domain), which are located at residues 1699–1736 and 1818–1855 [100]. Similar BRCT motifs have been found in 53BPI, a protein capable of binding *p53*, and several proteins involved in cell cycle regulation or DNA repair such as RAD9, XRCC1, RAD4, RAP1, Ect2, terminal deoxynucleotidyltransferases, and three DNA ligases [101]. The BRCT domains show strong sequence conservation in the murine protein and are found in a similar position in BARD1. Their function is unknown, but they are located within the region of *BRCA1* that is reported to activate transcription when fused to a DNA-binding domain [102].

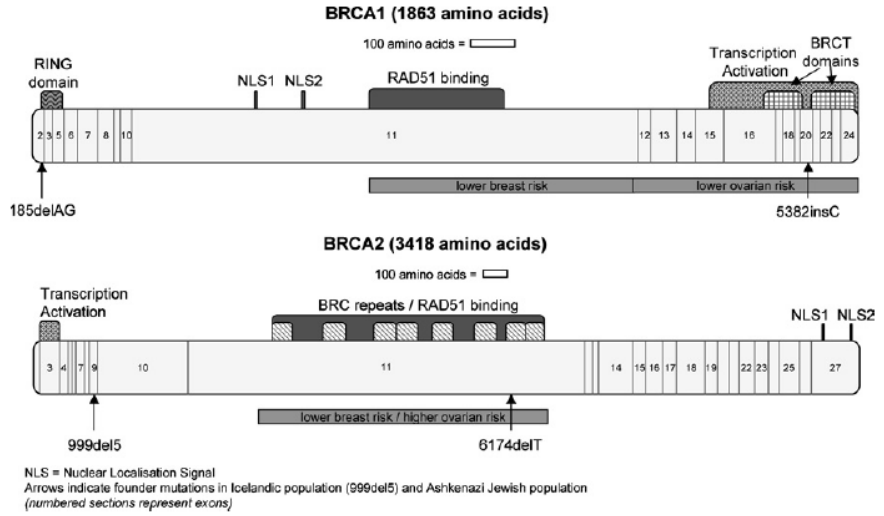

Figure 2. The *BRCA1* and *BRCA2* genes, showing some functional domains and founder mutations (adopted from Thompson and Easton, 2004 [103])

RING finger or BRCT domains are not present in *BRCA2*, nor does it bear substantial similarity to any other sequence presently registered in the databases. However, eight copies of a 20–30 amino acid repeat (termed BRC repeats) are located between residues 1000–2030. Currently, the only other protein known to contain a similar sequence is a predicted *C. elegans* protein of unknown function. Most of the BRC repeats in *BRCA2* show >80% amino acid sequence identity between human, mouse, and chicken, and are therefore better conserved than the protein overall [95].

2.1.2. Function of *BRCA1* and *BRCA2*

2.1.2.1. DNA repair

Subsequent studies demonstrated the involvement of *BRCA1* and *BRCA2* in complexes that activate the repair of doublestrand breaks (DSBs) and initiate homologous recombination (HR), linking the maintenance of genomic integrity to tumor suppression. *BRCA1* and *BRCA2* co-localize with *Rad51* to form complexes [104, 105]. Eukaryotic Rad51 proteins are homologues of bacterial RecA and are required for recombination during mitosis and meiosis, as well as for HR repair of DSBs [106]. Rad51 coats single-stranded DNA to form a nucleoprotein filament that invades and pairs with a homologous region in duplex DNA, and then activates strand exchange to generate a crossover between the juxtaposed DNA [107, 108]. Co-localization of BRCAs with Rad51 at sites of recombination and DNA damage-induced foci strongly suggests that *BRCA*s have a role in both the detection and the repair of DSBs [104]. In this regard, focus formation of Rad51 is reduced after treatment with DNA-damaging agents and is deficient during repair of DSBs by HR in *BRCA1*-deficient cells [109, 110]. However, accumulating evidence suggests that *BRCA1* might not directly regulate Rad51, since interactions between BRCA1 and Rad51 are indirect and stoichiometrically negligible [111].

The roles played by *BRCA1* and *BRCA2* in the repair of DSBs by HR appear to differ. Available evidence indicates a more direct role of *BRCA2*. *BRCA2*-deficient cells exhibit increased sensitivity to ionizing radiation, indicative of a defect in DSB repair, whereas the cell cycle checkpoint and apoptotic responses to DNA damage remain intact [112, 113]. In addition, *BRCA2*-deficient cells accumulate chromosomal breaks and aberrant mitotic exchanges during culture. Rad51-deficient cells show similar phenotypes, providing genetic evidence that interactions of *BRCA2* with Rad51 are fundamental for the maintenance of cell division and chromosome structure. Physiologically, interactions between *BRCA2* and Rad51 are mediated by the BRC repeat and an unrelated domain located at the C-terminus. Recent studies have shown that *BRCA2* regulates the intracellular localization and function of Rad51 [114]. In *BRCA2*-deficient cells, nuclear transport of Rad51 is impaired, suggesting that *BRCA2* moves Rad51 from the site of synthesis to the site of DNA damage processing [114].

Some unexpected and potentially informative insight into the role of *BRCA* genes in human DNA repair has come from recent studies of Fanconi anemia (FA) [115-117]. To date, twelve genetic FA subgroups have been identified based on the complementation analysis, and eleven of these have been cloned (FancA/B/C/D1/D2/E/F/G/J/L/M) [118, 119]. The proteins encoded by FA genes are intimately related to each other in molecular pathways involved in DNA repair, and *FANCA, FANCC, FANCE, FANCF, and FANCG* interact directly to form a multisubunit nuclear complex [120]. In response to DNA damage this complex is translocated to DNA repair foci containing *BRCA1* and *BRCA2* [121]. Howlett *et al.* [115] have provided evidence that *FANCD1* is identical with *BRCA2*. The cellular consequences of homozygosity for mutated *BRCA2*, including spontaneous chromosome instability and hypersensitivity to DNA crosslinking agents, are similar to those observed in cells derived from FA patients. Another FA-complementation-group protein, *FANCD2*, can interact and colocalize with *BRCA1* [121]. Moreover, both *FANC*D2 and *BRCA1* can be phosphorylated by *ATM* [122], which has also recently been implicated in susceptibility to breast cancer [123]. Hence, it appears

that the pathways involved in FA and breast-cancer susceptibility are closely linked on several levels.

2.1.2.2. Transcriptional response to DNA damage

BRCA1 has been implicated in the transcriptional regulation of several genes activated in response to DNA damage. The first line of evidence came from an observation that the C-terminus of *BRCA1* binds and activates the basal transcriptional machinery [102, 124]. A subsequent series of studies demonstrated that the C-terminus of human *BRCA1* (amino acids 1528–1863) complexes with RNA polymerase II through RNA helicase A [125]. This interaction appears to involve several proteins associated with the core polymerase complex. In fact, *BRCA1* protein is a component of the RNA polymerase II holoenzyme, and deletion of the C-terminal 11 amino acids of *BRCA1* attenuates the association with this holoenzyme.

Finding target genes regulated by *BRCA1* would shed considerable light on the transcriptional role of *BRCA1*. Studies using microarray technology have shown that *p53*-responsive cell cycle progression inhibitor and stress-response factors such as p21 and GADD45 are stimulated by *BRCA1* overexpression [126]. Subsequent investigations have revealed that *BRCA1* serves as a co-activator for *p53* [127]. Co-immunoprecipitation experiments have also demonstrated that *BRCA1* interacts with *p53*. Deletion of the N-terminus (amino acids 224–500) impairs *in vitro* interactions with *p53*. Furthermore, a truncated mutant of *BRCA1* that retains the *p53*-binding site exhibits a dominant negative effect in *p53*-mediated transcription, thereby substantiating a pivotal role for interactions of *BRCA1* and *p53 in vivo*. A recent study shows that *p53* is stabilized by overexpression of *BRCA1*, suggesting that *BRCA1* functions to stimulate *p53* pathways [126].

The possible function of *BRCA2* as a transcriptional regulator is far less certain. Available evidence suggests that the product of *BRCA2* exon 3 (amino acids 23–105) activates transcription and that a missense mutation (Tyr42Cys) of *BRCA2* reduces the transactivation potential. The basis for this mutation and its relevance to carcinogenesis remain to be defined. Other studies have shown that overexpression of *BRCA2* is associated with down-regulation of basal *p53* transcriptional activity. In contrast, *BRCA2* might activate transcription by modulating histone acetylation. *BRCA2* interacts with the transcriptional co-activator protein P/CAF (p300/CBP-associated factor) and its associate p300/CBP, both of which possess histone acetylase activity. *BRCA2* might recruit these histone modifiers to the transcription complex to induce transcriptional activity [128]

2.1.2.3. DNA damage-responsive cell cycle checkpoints

Cell cycle checkpoints play an essential role in cell survival by preventing the propagation of DNA damage through cell cycle progression before DNA repair. Recent studies using cells defective for different DNA damage-responsive proteins have demonstrated that both *ATM* and *BRCA1* are required for effective S-phase and G2/M-phase checkpoints. Expression of *BRCA1* variants defective for *ATM*-mediated phosphorylation is associated with a defect in G2/M arrest, suggesting that *BRCA1* phosphorylation by *ATM* is indispensable for G2/M checkpoints in the DNA damage response [86]. Other work has indicated that *BRCA1* regulates G2/M

DNA damage induced checkpoints through its ability to activate Chk1 kinase and thereby induce signaling cascades downstream of Chk1 [129]. In this context, the finding that *BRCA1*-deficient cells exhibit defective G2/M arrest in response to ionizing radiation further supports a role of *BRCA1* in the regulation of G2/M checkpoints.

As mentioned above, *BRCA1* functions as a co-activator of *p53*-mediated gene transcription. In *BRCA1*-deficient cells, the expression of 14-3-3σ, which is regulated by *p53*, is significantly diminished [130]. Since 14-3-3σ is a major G2/M checkpoint control gene, 14-3-3σ induction by *BRCA1* may also be involved in *BRCA1*-mediated G2/M checkpoints. Other studies have shown that overexpression of *BRCA1* results in the transcriptional activation of GADD45 in a *p53*-dependent manner [131, 132]. As GADD45 has been implicated in G2/M checkpoints, *BRCA1* may in part activate G2/M checkpoints by induction of GADD45 protein. Interestingly, another *p53* target gene, the G1 cyclin-dependent kinase inhibitor p21, is also transactivated by exogenous expression of *BRCA1* to block S phase entry in a *p53*-independent manner [133]. Importantly, cancer-associated mutant *BRCA1* failed to activate the p21 promoter. *BRCA1* has also been found to transactivate the cyclin-dependent kinase inhibitor $p27^{KIP1}$ [134]. The induction of G1 arrest by exogenous *BRCA1* expression is likely to be associated with activation of $p27^{KIP1}$.

There is evidence that *FANCD2*, like *BRCA1*, participates in the events that are triggered by DNA damage during S and G2 phases and that lead to cell-cycle arrest. As well as modifying *BRCA1*, the *ATM* protein kinase can phosphorylate *FANCD2* on several residues, including serine 222, following exposure to ionizing radiation [122]. Ser222 phosphorylation is required for the intra-S-phase arrest and is, in turn, dependent on the protein kinase NBS1, which is a component of the trimeric MRE11–RAD50–NBS1 complex [135]. *FANCD2* can also be phosphorylated in an *ATM*-independent manner by the ATR kinase [136], which signals an intra-S-phase arrest that is triggered by ultraviolet radiation or DNA-crosslinking agents [137]. Here, too, *FANCD2* phosphorylation depends on NBS1, which is required to fully enforce the S-phase checkpoint. The participation of *FANCD2* in ionizing radiation-activated checkpoint responses through phosphorylation on Ser222 seems to be independent [135] of its mono-ubiquitylation on Lys561, which promotes the translocation of *FANCD2* to damage-induced nuclear foci that contain *BRCA1* and RAD51, and that are presumed sites of DNA repair. Similarly, *FANC*D2 foci do not require ATR-dependent phosphorylation [136]. So, these findings distinguish functions of *FANCD2* in two aspects of DNA-damage responses during S and G2 phases through apparently discrete post-translational modifications — through Ser/Thr phosphorylation in checkpoint arrest and through Lys561 mono-ubiquitintation in DNA repair.

It remains unclear whether *BRCA2* participates directly in cell cycle regulation or checkpoint functions. Available evidence suggests that *BRCA2* mediates G2/M-phase control by interacting with a novel protein, *BRCA2*-associated factor 35 (BRAF35), which binds to branched DNA structures [138]. Nuclear staining has revealed a close association of BRAF35/*BRCA2* complex with condensed chromatin, coincident with histone H3 phosphorylation. Importantly, antibody microinjection experiments suggest a role of *BRCA2*/BRAF35 complex in modulation of metaphase progression [138]. However, it is premature to conclude

that *BRCA2* is directly involved in mitotic progression. Since *BRCA2* has a major role in DNA repair, its suppression is thought to induce unrepaired DNA lesions, which cause cell cycle arrest by activating checkpoint signaling, including mitotic progression.

2.1.3. Germline mutations in *BRCA1* and *BRCA2*

BRCA1 and *BRCA2* are the most important breast cancer susceptibility genes in high-risk families, and identification of mutations in these genes forms an important component of the management of high-risk women. The Breast Cancer Information Core (BIC) database had recorded (as of June 2006) 1536 distinct germline *BRCA1* mutations and 1885 *BRCA2* mutations. Of these, 878 (57%) and 1140 (60%) have been reported just once. Mutations appear to be reasonably evenly distributed across the coding sequences, with no obvious “mutation hot-spots.” Most mutations found in breast and/or ovarian cancer families are predicted to truncate the protein product. The most common types of mutation are small frameshift insertions or deletions, nonsense mutations, or mutations affecting splice sites resulting in deletion of complete or partial exons or insertion of intronic sequence. The Breast Cancer Linkage Consortium (BCLC) has estimated that approximately 70% of *BRCA1* mutations and 90% of *BRCA2* mutations in linked families are of this type [103]. Large-scale rearrangements, including insertions, deletions, or duplications of more than 500kb of DNA, have also been identified, but as these are not identifiable by exonic sequencing or other conventional screening techniques they are likely to be underreported. To date there have been reports of at least 19 distinct large genomic rearrangements in *BRCA1* and two in *BRCA2*, identified using protein truncation analyses, Southern blots.or relatively new method, multiplex ligation dependent probe amplification (MLPA) [139, 140].The majority are deletions of one or more exons [140]. The higher density of Alu repetitive sequences in the *BRCA1* gene (42% vs. 20%) [141] is thought to contribute to the larger number of large deletions and duplications observed in this gene. In addition to protein truncating mutations, large numbers of amino-acid substitutions have been identified in both *BRCA1* and *BRCA2*. A small number of these, principally involving cysteine residues in the *BRCA1* RING domain, have occurred consistently in high-risk families and are regarded as disease-associated missense mutations. Some do not cause amino acid changes and are thereby harmless polymorphisms, but the status of the majority (termed “unclassified variants”) is uncertain. Given their frequency and the fact that many occur in patients with another, deleterious, mutation, it is clear that the large majority of these variants cannot be strongly associated with disease. At present no reliable functional assay exists to determine whether such a variant is likely to be deleterious, and only the epidemiological evidence on the frequency of the variant in breast cancer cases and controls, or co-segregation of the variant with disease in families, can be regarded as definitive. Also association with the typical morphology of BRCA related cancers (see below) may help in this respect. Unfortunately this evidence is lacking for most variants. Only two variants outside known functional domains of *BRCA1* are classified as missense mutations by BIC, and for some of these the evidence that they are pathogenic is not totally convincing. No clearly deleterious missense *BRCA2* mutations have yet been defined.

It has been suggested that common polymorphisms in *BRCA1* and *BRCA2* may be associated with moderately increased risks of breast or ovarian cancer. This hypothesis has been tested by comparing polymorphism frequencies in cases and controls, but there is no consistent evidence that any of the *BRCA1* polymorphisms tested so far confers an increased risk of breast cancer [142, 143]. One common *BRCA2* variant, N372H, has been shown to be associated with a moderately increased risk of breast cancer [144, 145]. Intriguingly, among female controls including newborns, the frequency of homozygotes was significantly lower than that expected under Hardy-Weinberg equilibrium, whereas among newborn males a deficit of heterozygotes was identified, suggesting that *BRCA2* has different roles in the fetal development of males and females, leading to differential selection [144].

2.1.4. Ethnic differences in frequency of *BRCA1*/2 mutations

Whilst the majority of *BRCA1* and *BRCA2* mutations are infrequently observed, certain mutations in *BRCA1* and *BRCA2* have been observed to be common in specific populations. Such founder mutations in *BRCA1* and *BRCA2* have been described in French Canadians [146], Swedes [147], Icelandic [148], Norwegians [149], Finns [150], Dutch [151, 152], Russians [153], Japanese [154], African Americans [155] and Ashkenazi Jews [156-158]. The best characterized examples occur in The Icelandic and the Ashkenazi Jewish Population.

Three mutations are commonly found in the Ashkenazi Jewish population: 185delAG and 5382insC in *BRCA1* [146] and 6174delT in *BRCA2* [94]. Although the large majority of 185delAG carrier families are Ashkenazi, the mutation has also been reported in other Jewish groups, indicating an older origin [159]. The 6174delT mutation appears to be virtually restricted to the Ashkenazi Jews, and has only once been reported in anyone of proven non-Ashkenazi Jewish heritage [160]. The 5382insC mutation is, however, more widespread, being common in Poland, Russia, and other parts of Eastern Europe and occurring in most European populations. On the basis of a pooled analysis of five population studies, the frequencies of the 185delAG and 6174delT mutations in the Ashkenazi Jews have been estimated to be about 1 in 100, with the frequency of 5382insC being about 1 in 400 [161]. In this population, these mutations are present in approximately 30% of breast cancer cases diagnosed below age 40 years [158, 162-165] and in 40–60% of ovarian cancer cases [166, 167].

A single *BRCA2* mutation, 999del5, has been identified in the geographically isolated population of Iceland, and is present in the majority of multiple case breast cancer families in this population [148, 168]. About 1 in 200 Icelanders are thought to carry a 999del5 mutation, a much higher frequency than that of all mutations together in larger, genetically more heterogeneous populations [169, 170]. In this population, the 999del5 mutation is estimated to account for around 8% of ovarian cancers and female breast cancers, rising to 24% of female breast cancers diagnosed before age 40 years, and 38% of male breast cancer cases [148, 169].

2.1.5. Penetrance of *BRCA1* and *BRCA2* mutations

Penetrance estimates from a recent meta analysis of 22 population studies [171] showed that the cumulative risks of both breast and ovarian cancer are lower in *BRCA1* carriers than *BRCA2* carriers, but the difference is more marked for ovarian cancer (39% vs. 11% by age 70). The difference is also more marked for

breast cancer at younger ages. This is a consequence of the fact that *BRCA1* breast cancer incidence rates rise steeply to approximately 3–4% per annum in the 40–49 age group, and are roughly constant thereafter, whereas the *BRCA2* rates show a pattern similar to that in the general population (though approximately 10-fold higher), rising steeply up to age 50 and more slowly thereafter. Ovarian cancer risk in *BRCA1* carriers is very low below age 40, rising thereafter to 1–2% per annum, whereas it is very low below age 50 in *BRCA2* carriers but thereafter increases sharply.

2.1.6. Pathology of *BRCA1* and *BRCA2* related breast cancer

The morphology of breast tumours arising in *BRCA1* carriers is markedly different from those occurring in non-carriers. Several studies have demonstrated that *BRCA1*-associated tumours tend to be high grade (usually grade 3) and, more specifically, have high mitotic count [172, 173]. The majority of *BRCA1*-associated tumours are infiltrating ductal, but there is a significantly higher frequency of tumours classified as medullary or atypical medullary type than in non-carriers (21% vs. 2% in the BCLC study). Conversely, *BRCA1* tumours are less likely to be lobular, mucinous, cribriform or tubular, or to be associated with ductal or lobular carcinoma *in situ*. More detailed analysis has demonstrated that *BRCA1* tumours are more likely to exhibit continuous pushing margins and marked lymphocytic infiltration. Consistent with their higher grade, *BRCA1* tumours have been shown to more often be DNA aneuploid, with a higher average S-phase fraction [174, 175]. Other studies have suggested that *BRCA1* tumours are larger [176-178] and more often associated with axillary lymph node involvement [179], although the evidence for these associations is less convincing than for grade. Some studies have indicated that somatic *p53* mutations are more common in these tumours [180], although the evidence from immunohistochemical staining with T*P53* antibodies is less clear [181]. Several studies have shown that *BRCA1* tumours are likely to be estrogen (ER) and progesterone receptor (PR) negative; in the largest study, over 90% of *BRCA1* tumours exhibited no staining for ER [181]. This finding suggests that breast tumours arising in *BRCA1* carriers are less likely to be responsive to hormonal therapies such as tamoxifen, and moreover that tamoxifen might be unable to prevent breast cancer in *BRCA1* carriers, although this has not always been seen in practice (e.g., [182]). Tumours in *BRCA1* carriers are also often EGFR positive [183-186], but unlikely to be HER2 positive [175, 181]. More recently, microarray studies have suggested that *BRCA1* tumours fall into a category of “basal-like” tumours, recognized by staining for high molecular weight cytokeratin types 5,6 and 14 [187, 188].

Although much less is know about the pathological phenotype of breast precursor lesions, it is our experience that DCIS in carriers is also usually of high grade and shows associated lymphoplasmocytic infiltrate. The infiltrate already presents in high frequency in the morphologically normal breast in prophylactic mastectomies as T-cell lobulitis [189]. In prophylactic mastectomies, a high frequency has been found of ductal hyperplasia of usual type, atypical ductal/lobular hyperplasia, LCIS, DCIS, and invasive cancer [190], also when compared to age matched mammoplasties in non-carriers [189, 191], although this was not conformed in one study [192]. A patient with a family history of

breast/ovarian cancer has been described that presented with multiple fibroadenomas harbouring DCIS and LCIS [193].

Recently, gene expression profiling studies have included some hereditary breast cancers [194-198]. In one study, the molecular profiles of sporadic breast cancers were compared with those in *BRCA1* and *BRCA2* germline mutation carriers [199]. Almost 200 genes were found to be significantly differently expressed between tumours associated with *BRCA1* and *BRCA2* germline mutations [199]. Interestingly, one patient with a sporadic breast cancer was demonstrated to have a gene expression profile that was highly similar to that of *BRCA1* mutants, suggesting a mechanism of the inactivation of this gene. They found that the down-regulation of the expression of *BRCA1* in this tumor was associated with hypermethylation of the promoter region.

Wessels et al. [200] described a molecular classification of breast cancer based on somatic genetic profiles using comparative genomic hybridization (CGH). They developed a profile and classification rule with which tumors with a *BRCA1* mutation can be distinguished from control tumors with accuracy of 84% in regions on chromosomes 3p, 3q and 5q. Whereas van Beers et al., [201] report the chromosomal gains and losses as measured by CGH in 25 *BRCA2*-associated breast tumors and compared them with existing 36 *BRCA1* and 30 control profiles. All chromosomal regions were compared and the regions of differential gain or loss were determined between tumor classes and controls. *BRCA2* and control tumors have very similar genomic profiles. As a consequence, and in contrast to *BRCA1*-associated tumors, CGH profiles from *BRCA2*-associated tumors could not be distinguished from control tumors using the classification methodology developed previously by Wessels et al,. The largest number of significant differences existed between *BRCA1* and controls, followed by *BRCA1* compared with *BRCA2*, suggesting different tumor development pathways for *BRCA1* and *BRCA2* [201]

In summary, these features establish the following pathological phenotype of *BRCA1* germline mutation related breast cancer: morphologically ductal or medullary type, pushing margins, marked lymphoplasmocytic infiltration, high grade, high mitotic index, and immunophenotypically ER/PR/HER2 negative, and CK5/CK6/EGFR positive. This phenotype may provide a powerful basis for identifying likely *BRCA1* carriers amongst early onset breast cancer patients, and help to establish the deleterious nature of “unclassified variant” BRCA mutations.

The pathological characteristics of tumours in *BRCA2* carriers are less clear than for *BRCA1,* and overall their pathological phenotype appears to be between carriers and non carriers [184, 202]. The distribution of ER and PR is similar to that in non-carriers [203].

2.1.7. Clinical presentation of *BRCA1* and *BRCA2* related breast cancer

Patients with *BRCA1*/2 germline mutation related breast cancer present at younger age, have more often bilateral breast cancer and also more often develop multiple tumors in other organs than patients with sporadic breast cancer [ref]. The above described pathological characteristics of breast cancers in *BRCA1* germline mutation carriers would suggest that the prognosis in these patients is likely to be quite poor. Direct evidence of bad prognosis in *BRCA1* carriers is, however, still conflicting (e.g., [173, 174, 177, 204]). Although overall *BRCA1*/2 carriers may

perhaps have a slightly worse prognosis compared to sporadic cases, it is clear prognosis is not as poor as would be expected from the pathological phenotype.

2.2. Mutation detection methods

The increasing demand for mutation detection in disease genes, either known or presumed, can be solved by automated sequencing using fluorescent dyes [205]. However, only a few laboratories are equipped for the broad application of this costly and labour intensive strategy. As alternatives to sequencing methods, which determine the exact nature and location of each base along a DNA fragment, various mutation scanning procedures have been developed. These methods, which rely on the recognition of a sequence variation between mutant and wild-type DNA on the basis of an altered electrophoretic migration pattern, provide a simple means for determining whether a given DNA sample harbours a mutation in a particular gene. The most well established scanning procedures are single strand conformational polymorphism (SCCP) analysis, DGGE, chemical cleavage of mismatch, RNase cleavage, the protein truncation test (PTT), and heteroduplex analysis.

Among these methods, SCCP, DGGE, PTT, and heteroduplex analysis are the most widely used because of their accuracy, simplicity, lack of toxicity, and/or relative affordability. DGGE is believed to have the highest mutation detection rate (close to 100%) [206] compared with SCCP and heteroduplex analysis. Additional advantages of this methodology are the possibility of optimising the analysis by computer simulation and the non-radioactive approach. Double gradient DGGE is based on the combination of two linear gradients, a primary denaturing gradient (urea and formamide) and a collinear secondary porosity gradient (polyacrylamide) [207]. This secondary gradient suppresses band broadening during electrophoresis and thus improves the resolution of the DGGE banding pattern. Hayes et al compared double gradient DGGE gels with various porosity gradients to a standard 9% polyacrylamide gel, and showed that mutations with different melting profiles cannot be appropriately detected using a single DGGE condition [208]. Even though DGGE seems to be the most attractive technique to screen naïve populations such as the Indonesian population for *BRCA1*/2 mutations, standard DGGE techniques are not high throughput and mutation detection based on standard DGGE would be very laborious since *BRCA1* and *BRCA2* are very large genes. Therefore, DGGE needs to be high-throughputized first.

PTT is a widely applied screening technique [209] but is especially helpful for known mutations in particular populations, and less suitable as a primary screening approach for new mutations in naive populations. Furthermore, PTT only detects mutations that result in stop codons and lead to premature termination of translation, thereby producing truncated proteins. A possible advantage of PTT is that it conveniently misses harmless polymorphisms. PTT is usually only applied for detecting mutations in exon 11 of the *BRCA1* gene and exons 10 and 11 of the *BRCA2* gene, which account for more than 60% of the coding sequence. However, mutations are distributed throughout the entire coding sequence, with no apparent clustering or hot spots [207].

In spite of these methods being well established for mutation detection of *BRCA1* and *BRCA2*, they do not detect all mutations. With sequencing, especially

large genomic deletions can be missed. Recently, an MLPA technique has been developed that may especially detect such large genomic deletions [139].

In summary, to screen Indonesian breast cancer patients for *BRCA1*/2 mutations, a combination of high-throughput DGGE and MLPA seems to be the best combination of techniques.

2.3. Epigenetics of sporadic and hereditary breast cancer

Decades of research have led to a substantial understanding of the factors involved in the development of breast cancer. All these factors cause or are at least associated with development of breast cancer and lead to a `new type of tissue' (neoplasm) characterized by a variety of genetic events including gene amplifications, gene deletions, point mutations, chromosomal rearrangements, and chromosomal aneuploidy.

Besides the above mentioned well known genetic alterations, epigenetic alterations are among the most common molecular alterations in human neoplasia [20-22]. Epigenetic changes differ from genetic changes mainly in that they occur at a higher frequency than genetic changes, are reversible upon treatment with pharmacological agents and occur at defined regions in a gene, usually the promoters. Epigenetics can be understood as the mechanisms that initiate and maintain heritable patterns of gene expression and gene function in an inheritable manner without changing the sequence of the genome. Therefore, epigenetics provides the best explanation about how the same genotype can be translated to different phenotypes.

The epigenetics network has many layers of complexity that could be summarized in four: DNA methylation, histone modifications, chromatin remodeling and microRNAs. The latter 3 are beyond the scope of this thesis and will not be discussed here. DNA methylation is the most well known epigenetic-mechanism and it has become clear in recent years that there is a synergy between genetic and epigenetic changes and that Knudson's two-hit hypothesis has to be revised: instead of two possibilities (loss of heterozygosity or homozygous deletion), a third possibility - transcriptional silencing by DNA methylation of promoters - can disable tumor-suppressor genes [21].

2.3.1. DNA methylation

Cytosines are methylated in the human genome mostly when located 5' to a guanosine. These CpG nucleotides have been severely depleted in the vertebrate genome to about 20% of the predicted frequency and most CpG dinucleotides (over 70%) are methylated. However, in small stretches of DNA termed CpG islands, which are about 500 to 2000 bp in length [210, 211], the CpG dinucleotide occurs at near the expected frequency and these areas are frequently located in and around the transcription start sites of approximately half of human genes. It has been increasingly recognized over the past 4 to 5 years that the CpG islands of a large number of genes, which are mostly unmethylated in normal tissues, are methylated to varying degrees in human cancers, including breast cancer [212].

In human cancer, the observed DNA methylation aberrations can be considered as falling into one of two categories: transcriptional silencing of tumor suppressor genes by CpG island promoter hypermethylation [21, 213, 214], or genomic hypomethylation that takes place predominantly in DNA repetitive sequences and

has been linked to the generation of chromosomal instability [213, 215]. CpG islands become hypermethylated with the result that the expression of the contiguous gene is shut down. If this aberration affects a tumor suppressor gene, it confers a selective advantage on that cell and is selected generation after generation. A long list of hypermethylated genes in human neoplasias has been identified, and this epigenetic alteration is now considered to be a common hallmark of all human cancers affecting all cellular pathways [21, 213, 214]. Extremely important genes in cancer biology, such as the cell cycle inhibitor $p16^{INK4a}$, the *p53*-regulator $p14^{ARF}$, the DNA-repair genes hMLH1, *BRCA1* and MGMT the cell-adherence gene E-cadherin, or the estrogen and retinoid receptors undergo methylation-associated silencing in cancer cells [21, 213, 214]. The profiles of CpG island hypermethylation are known to depend on the tumor type [216, 217]. Each tumor subtype can now be assigned a DNA hypermethylome that almost completely defines that particular malignancy in a similar fashion as do genetic and cytogenetic markers. Establishing a DNA hypermethylome can be very useful for classifying these malignancies according to their aggressiveness or sensitivity to chemotherapy. Single-gene approaches can also be extremely useful, such as it was firstly demonstrated with the DNA repair gene MGMT [218].

2.3.2. CpG island methylator phenotype

Two important issues in the study of DNA hypermethylation pattern are the following: (*a*) whether gene-specific patterns of methylation can distinguish breast cancer phenotypes; and (*b*) whether there is a CpG island methylator phenotype for breast cancer. Both of these possibilities might be expected based on methylation patterns that have been observed in other types of human cancers. For example, unique profiles of methylation for 12 different genes have been found to distinguish 15 different types of human cancer [217], leading us to question whether subsets of breast cancer, which is biologically heterogeneous, could be distinguished in a similar manner.

In addition, a distinctively high frequency of methylation has been described for a subset of colorectal cancers [219, 220], leading us to consider the possibility of a similar CpG island methylator phenotype for breast cancer. There is some evidence in breast cancer that gene methylation might identify phenotypes with different histology or clinical properties. For example, a recent study using an array-based method found that poorly differentiated tumours exhibit more hypermethylated CpG islands than their moderately- or well-differentiated counterparts [221].

2.3.3. Hypermethylation of genes in breast cancer

A significant amount of data has established a list of genes hypermethylated in cancer and recently whole genome approaches have identified methylation signatures of breast cancer cells [222-224]. These methylation signatures, which are the unique combination of methylated CpG islands in a cancer cell, were correlated with breast cancer stage and have been proposed to be a diagnostic marker of breast cancer cells. In addition to their diagnostic value in breast cancer it is clear from the repertoire of methylated genes that silencing of these genes by DNA methylation plays a role in the transformation process. Amongst the methylated genes are tumor suppressor genes such as *p16* and *CCND2* whose

methylation is proposed to silence this gene and override cell growth regulatory signals [225, 226]. p16 methylation in DNA prepared from plasma of breast cancer patients was associated with nodal metastasis [227]. Another group of methylated genes in breast cancer is composed of damage response genes such as *BRCA1* and *GSTP1* [228], which is also mutated in familial breast cancer. Disruption of repair genes might increase sporadic mutations frequency, a hallmark of cancer cells. Steroid receptor genes family members such as the estrogen receptor [229] and retinoic acid beta 2 (RARb2) receptor are methylated and silenced in a fraction of breast cancers [230]. Interaction of RARb2 receptor with retinoic acid might have an antiproliferative effect and its silencing confers a selective advantage on advanced breast cancer cells. Cell adhesion and cell surface molecules such as E-cadherin, *CDH13* [231, 232] and inhibitors of proteases such as *TIMP-3* [233] whose silencing might promote metastases are also found to be methylated in breast cancer as well as two alternative forms of tumor suppressor in the Ras mediated signal transduction pathway: *RASSF1* [234], *PTEN* [235] and *APC* [236].

All this information has been gained on sporadic breast cancer (cells), and as yet no studies haven been performed on hereditary cases.

III. SUMMARY AND SCOPE OF THESIS

Little is known about the role of *BRCA1*/2 germline mutations in Indonesian breast cancer. Since breast cancer shows a high and increasing incidence in Indonesia, and many females develop breast cancer at young age, it is likely that such mutations do play an important role in the Indonesian population. We therefore set out to screen a series of Indonesian breast cancer patients and their family member for germline mutations in *BRCA1* and *BRCA2*. To this end, a fast and cheap method to screen for these mutations was needed. In ***chapter 2***, we describe a rapid and sensitive method to screen for *BRCA1*/2 mutations based on pooled DGGE and targeted sequencing. Using this method, and adding MLPA to detect genomic deletions, we analyzed a group of 116 early onset breast cancer patients and some of their family members for *BRCA1*/2 mutations in ***chapter 3***.

The phenotype and genotype of a group of early onset Indonesian breast cancer patients is described in ***chapter 4 and chapter 5*****,** as such phenotype can give clues for the “BRCA-ness” of a breast cancer. Absence of HER-2/*neu* amplification and overexpression is a feature of *BRCA1*/2 germline mutated breast cancer. In view of the fact that HER-2 status determines the type of adjuvant chemotherapy, we studied in ***chapter 4*** we evaluated for the first time the potential value of a new MLPA based PCR based technique to assess HER-2/*neu* amplification. In ***chapter 5***, we further investigated the histopathological and immuno-histochemical characteristics of early onset ($\leq$ 40 years) Indonesian breast cancer patients, as such features can be used as to distinguish between *BRCA* and non-*BRCA* carriers among these young women. This could help to limit expensive mutation screening to those patients at highest risk to harbour a germline *BRCA* mutation.

Breast cancer derives through accumulation of a wide variety of genetic and epigenetic events, also in patients predisposed to breast cancer due to a germline *BRCA1*/2 mutation. As promoter methylation of tumor suppressor genes and amplification of oncogenes are well known phenomena in sporadic breast cancer but have hardly been studied yet in hereditary breast cancer, we analyzed a group

of early onset Indonesian breast cancer for promoter methylation of tumor suppressor genes in ***chapter 6*** and for gene copy number in ***chapter 7***.

In ***chapter 8***, the different papers are discussed together to underline their coherence within the framework of early onset and hereditary breast cancer.

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Chapter 2:

A novel *BRCA2* mutation in an Indonesian family found with a new, rapid, and sensitive mutation detection method based on pooled denaturing gradient gel electrophoresis and targeted sequencing

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ABSTRACT

Background: Breast cancer is increasing in Indonesia and other developing countries. Germline mutations in the *BRCA1*/2 genes are most strongly associated with a high risk for breast cancer development. There have been no reports on *BRCA1*/2 gene mutations in the Indonesian population. Genetic research yielding insight into mutations affecting the Indonesian population can help in risk assessment of individual patients.

Aims: To screen the *BRCA1*/2 genes for mutations in early onset Indonesian breast cancer patients and their families with a new, simple, and sensitive *BRCA1*/2 mutation screening strategy based on denaturing gradient gel electrophoresis (DGGE) and targeted sequencing.

Methods: DNA was isolated from the blood of four Indonesian breast cancer patients from high risk families and seven family members, and the polymerase chain reaction was performed with specially designed primers throughout the *BRCA1*/2 coding sequences to produce fragments suitable for pooled DGGE analysis. The aberrantly migrating samples were reamplified and sequenced.

Results: Two mutations were found in exons 13 and 16 of *BRCA1* and two mutations in exons 2 and 14 of *BRCA2*, which turned out to be established polymorphisms according to the Breast Cancer Information Core. In addition, a novel 6 bp deletion in exon 11, leading to a premature stop, was found in *BRCA2*.

Conclusion: Pooled DGGE and targeted sequencing revealed four *BRCA1*/2 polymorphisms and one novel *BRCA2* mutation in a group of Indonesian patients at high risk of hereditary breast cancer. This illustrates that the proposed method is sensitive and particularly suited for screening unknown populations.

INTRODUCTION

Breast cancer is the most common malignancy in women in the Western world, accounting for 32% of all female cancers, and is responsible for 18% of cancer deaths in women. The hereditary form of breast cancer constitutes about 5% of breast cancer cases overall [1]. The first major gene responsible for hereditary breast cancer susceptibility, *BRCA1*, was mapped to 17q21 in 1990 [2] and was cloned in 1994 [3]. A second gene involved in the hereditary breast cancer syndrome, *BRCA2*, was identified several months later.4 Both genes have long coding sequences and complex genomic structures: *BRCA1* comprises 5592 bp, composed of 22 coding exons that encode 1863 amino acids [3], and *BRCA2* comprises 10443 bp, with 26 coding exons that encode 3418 amino acids [4]. In addition to breast cancer, ovarian cancer [5] fallopian tube cancer [6] and primary peritoneal cancer are part of the *BRCA1*/2 cancer spectrum in women.

''By identifying the endemic Indonesian mutations, we hope to offer better risk assessment for women in the Indonesian population who are susceptible to the *BRCA1*/ 2 related hereditary cancers''

Over 80% of families with two or more cases of premenopausal breast cancer and two or more cases of ovarian cancer are believed to carry a germline *BRCA1* or *BRCA2* mutation [7, 8]. Comprehensive evaluation of breast cancer susceptibility genes can be used to provide women with information concerning their risk of developing cancer, and guide the decision on the necessity of preventive surgical measures. It has been shown that ethnically different populations exhibit different germline mutation spectra in the *BRCA1* and *BRCA2* genes [9-11]. There are no previous publications on *BRCA1*/2 mutation detection in the Indonesian population. By identifying the endemic Indonesian mutations, we hope to offer better risk assessment for women in the Indonesian population who are susceptible to the *BRCA1*/2 related hereditary cancers.

There are no exact data on breast cancer incidence in Indonesia, but a study by Soeripto et al in 1982 (unpublished results) showed that the age standardized incidence rate in the Jogjakarta region was 6.17, ranking second after cervical cancer (7.69).

One of the central themes in cancer risk assessment is the efficiency and accuracy of genetic screening methods. Currently, the ability to conduct large scale, population based studies is constrained by the lack of an accurate and inexpensive method for mutation detection. The large size of the *BRCA1* and *BRCA2* genes, and the scattered distribution of mutations throughout the genes, complicate the task of mutation detection and make rapid screening for mutations a major technical challenge. A technique that scans stretches of DNA for unknown mutations should be optimised to detect as close to 100% of the sequence alteration as possible. Here, we describe the results of the application of a recently developed rapid and sensitive method for the detection of *BRCA1*/2 mutations [12] based on denaturing gradient gel electrophoresis (DGGE) [13] and targeted sequencing on an Indonesian group of high risk patients. This method appears to be particularly suited for the analysis of unknown populations.

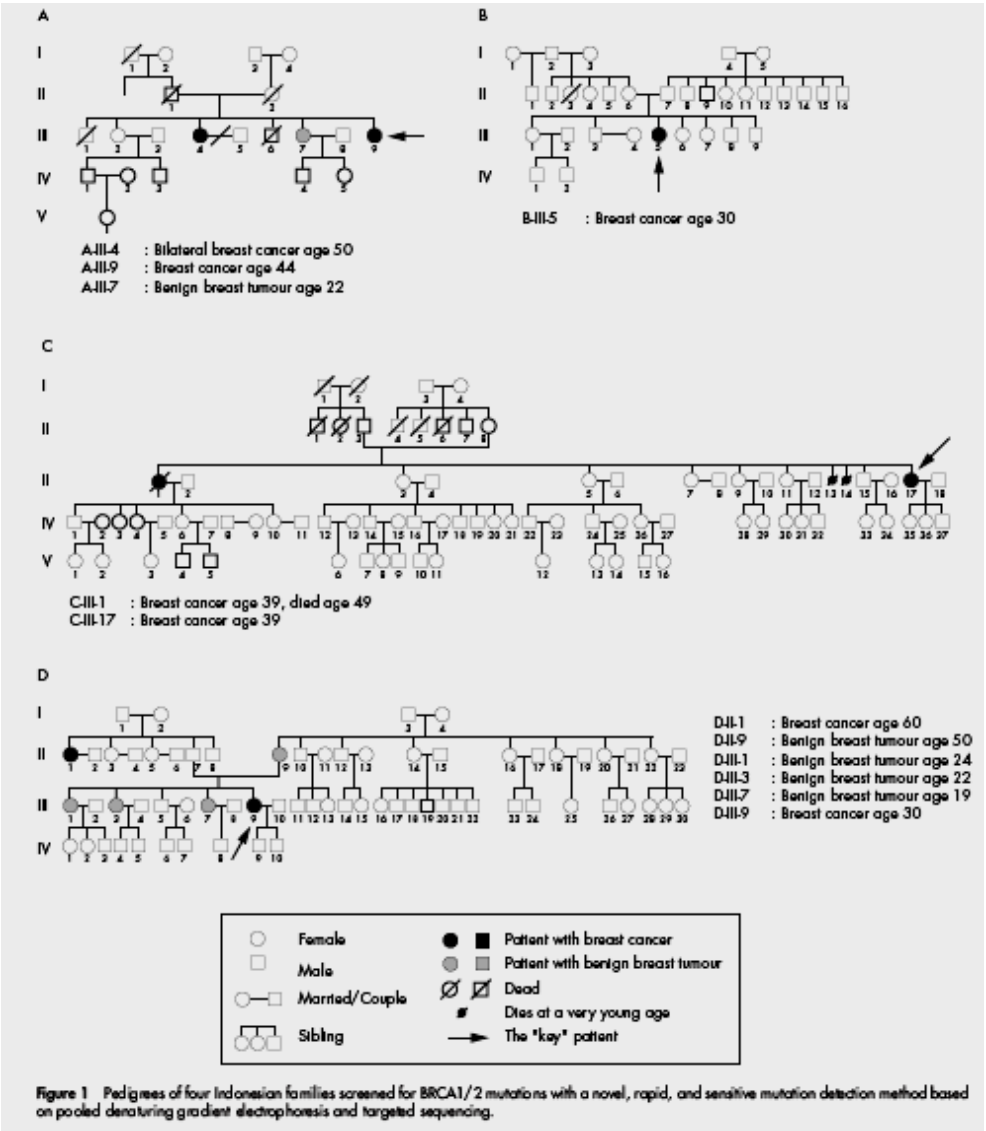

Figure 1 Pedigrees of four Indonesian families screened for BRCA1/2 mutations with a novel, rapid, and sensitive mutation detection method based on pooled denaturing gradient electrophoresis and targeted sequencing.

MATERIALS AND METHODS

Patients

From 1996 to 1998, four Indonesian families were selected from patient pools at three hospitals in Jogjakarta, Indonesia; three with hereditary breast cancer based on Moller's criteria [14], and one with one early onset patient without a family history. Figure 1 shows the pedigrees of these four families. Information regarding potential risk factors for breast cancer was obtained through a structured face to face interview. Information on family history (up to the date of diagnosis of the patient's breast cancer) was elicited by asking each patient to identify all first and

second degree female blood relatives. For each identified relative, the interviewer then asked the year of birth, vital status, year of death (if applicable), history, and type of cancer (if any). The patients were interviewed for family history using standardised questionnaires and blood was taken after informed consent.

DNA amplification

DNA was isolated from peripheral blood using simple and rapid genomic DNA extraction. Two types of buffer—cell membrane lysis buffer, containing sucrose and triton X-100, and nuclear membrane lysis buffer, containing guanidine thiocyanate, sodium N-lauroyl sarcocinate, and b mercaptoethanol—were used. By using this method, only a small amount of peripheral blood (0.5 ml) was needed. The DNA was stored at 4˚C. Polymerase chain reaction (PCR) fragments suitable for DGGE analysis were designed based on the theoretical melting profile of each exon, as described previously.12 A 40 mer GC clamp was attached to the short oligonucleotide to obtain optimal melting profiles. Tables 1 and 2 provide the PCR primer sequences for *BRCA1* and *BRCA2*, respectively. Genomic DNA was amplified using 100–200 ng of template DNA, 10 pmol of the mixture of 60 mer primers, 1mM MgCl, and 1 U Platinum Taq in 50 ml PCR reactions. The amplification conditions in a Robocyler (Stratagene, Canada) were five minutes at 94˚C (hot start), followed by five cycles of one minute at 94˚C, one minute at 52˚C, and one minute at 72˚C; five cycles of one minute at 94˚C, one minute at 50˚C, and one minute at 72˚C; and 30 cycles of one minute at 94˚C, one minute at 48˚C, and one minute at 72˚C; with five minutes extra at 72˚C. The final incubation was followed by five minutes at 94˚C and 15 minutes at 50˚C to enhance the formation of heteroduplex molecules.

Denaturing gradient gel electrophoresis

Primers for DGGE were obtained from Ingeny (Goes, the Netherlands). Aliquots (4–6 ml) of the PCR products were mixed with 2 ml of standard dye loading buffer and electrophoresed through a 20 cm 9% polyacrylamide gel (acrylamide/bisacrylamide, 37.5/1) containing a linear 20– 65% denaturing gradient (100% UF: 7M urea/40% deionised formamide). A 12% polyacrylamide stacking gel was poured to create solid slots for efficient loading of the PCR products, which prevented difficulties caused by the high urea concentration. Electrophoresis was performed in 0.5 Tris acetate EDTA buffer at 58˚C for 16 hours [15]. Gels were stained with ethidium bromide and photographed under an ultraviolet transilluminator.

To increase the throughput of the DGGE procedure, three to four different amplicons with relatively large melting temperature differences were pooled. The fragment pool was designed using a computer program that was based around melting profiles and sequences (tables 3 and 4). For each different exon, seven samples from four different families were loaded side by side to compare their band patterns. The aberrantly migrating samples were reamplified using sequencing primers and sequencing was performed using the Big Dye cycle sequencing kit according to the manufacturer's instructions. The reaction products were analysed using an ABI DNA sequencer 310 and sequence files were edited using the Sequence Navigator program.

Table 1 Primer sequences used to amplify BRCA1 exon by exon for denaturing gradient gel electrophoresis[12]

Fragment	Forward primer	Reverse primer	Size (bp)
Exon 2-1	ATGATAAAATGAAGTTGTC*	ACACTCTAAGATTTTCTGC	204
Exon 2-2	TTATCTGCTCTTCGCGTTG	CTTCCCTAGTATGTAAGGTC*	202
Exon 3	GCGCGTTGAGCCTCATTTATTTTC	ACAAAAGCTAATAATGGAGC*	185
Exon 5	GTATTCTTTCTACAAAAGG*	TCCAACCTAGCATGATTAC	208
Exon 6	GGTTGATAATCACTTGCTG*	CACTTGAGTTGCATTCTTG	223
Exon 7	ATACATAGGGTTTCTCTTG*	AGAAGAAAACAAATGGTTT	293
Exon 8	TTGCTTGACTGTTCTTTACC*	ACTTAAAAAACCTGAGACC	209
Exon 9	CCCTTTTAATTAAGAAAAC*	ACTAAATAGGAAAATACCAG	191
Exon 10	CATTTGACAGTTCTGCATAC*	TTCAGTGCCTGTTAAGTTG	217
Exon 11-1	ATGACAATTCAGTTTTGAG*	TATTACTGGGTTGATGATG	147
Exon 11-2	AGCTGCTTGTGAATTTTCTG*	ATAAACTGCTGTTCTCATGC	243
Exon 11-3	TTTTTACAAATACTCATGCCAGCTC*	TAGGATTCTCTGAGCATGGC	313
Exon 11-4	TTTTTTTTTTGTGTGAGAGAAAAGAATGG*	CATCTACCTCATTTAGAACG	272
Exon 11-5	GAATCAAATGCCAAAGTAGC*	GGACGCTCTTGTATTATCTG	329
Exon 11-6	ATTATAGGAGCATTTGTTAC	TTTTCGAGTGATTCTATTGG*	324
Exon 11-7	CAAAAGGTGATTCTATTCAG*	ATAAGGTGGGCTTAGATTTC	275
Exon 11-8	CAGGCATATTCATGCGCTTG*	GAAAGTATCGCTGTCATGTC	281
Exon 11-9	GAGTAACAAGCCAAATGAAC	GGGGTCTTCAGCATTATTAG*	242
Exon 11-10	TTGTCAATCCTAGCCTTCC*	ATTAGTCCCTTGGGGTTTTC	324
Exon 11-11	AATAAATGTGTGAGTCAGTG*	ACATTCCTCTTCTGCATTTC	300
Exon 11-12	ATTCAAGGTTTCAAAGCGCC	GTATATTAACTGTCTGTAC*	238
Exon 11-13a	AGGAAGAAAATCAAGGAAAG*	TAATGAGTCCAGTTTCGTTG	223
Exon 11-13b	GCCAAATGTAGTATCAAAGG	CAGGTGACATTGAATGTTCC*	248
Exon 11-13c	AAAATCTGCTAGAGGAAAAC	TCATCACTGGAACCTATTTC*	259
Exon 11-13d	TAAAGAAGCCAGCTCAAGC	CTGAAATCAGATATGGAGAG*	325
Exon 11-13e	GCAAGAATGTCAAGAAGTAG*	CCATCATCTAACAGGTCATC	181
Exon 11-13f	AGTCATGCATCTCAGGTTTG*	ATAAGTTCTCTTCTGAGGAC	280
Exon 11-14	CTTTCACCCATACACATTTG	TGCAGTCATTTAAGCTATTC*	277
Exon 11-15	GAGTGTCTGTCTAAGAACAC*	TATTTGCAGTCAAGTCTTCC	221
Exon 11-16	GTTTCTTCACAGTGCAGTG	AAATAGACTGGGGCAAACAC*	296
Exon 12	GTCTGCTTTTACATCTGAACC*	AATGCAAAGGACACCACACAC	221
Exon 13	GCGCGATTTCATTTTCTTGGTGCC	GGGAAGGAAAGAATTTTGC*	305
Exon 14	TCAGAACAAAGCAGTAAAG*	AAGATGTCAGATACCACAG	257
Exon 15-1	ATTGGTGGCGATGGTTTTC*	CTCCTCCACATCAACAACCT	204
Exon 15-2	ACTACCCATCTCAAGAGGAG	AAATCAAAGTGTTTGTTCC*	195
Exon 16-1	GACCAGAACTTTGTAATTC*	CCAGCAGTATCAGTAGTAT	299
Exon 16-2	AAAGTTGCAGAATCTGCCC	TAAGTCTTAGTCATTAGGG*	252
Exon 17	GTGCTAGAGGTAACTCATG*	CAGCAGATGCAAGGTATTC	213
Exon 18	ACAGCACTTCCTGATTTG	TCTGAGGTGTTAAAGGGAG*	222
Exon 19	TCTATCTCCGTGAAAAGAG	CTGGTTAGTTTGTAACATC*	176
Exon 20	TGCTCCACTTCCATTGAAG*	TTTGTCAACTTGAGGGAGG	220
Exon 21	CCTCTCTCCATTCCCCTG*	AAGGCTGGTGCTGGAACTC	182
Exon 22	GCCTGGGTTAAGTATGCAG*	ATTGTGTCCTCCCTCTCTG	210

*GC clamp sequence (CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCGCCCG) is attached to this site.

Table 3 Amplicon pools created for BRCA1 to increase the throughput of denaturing gradient gel electrophoresis

Gene	Pool	Exons in amplicon pool
	A	7,18, 10, 20
	B	9, 6, 8, 23
	C	5, 2B, 11-3, 16B
	D	14, 11-4, 22, 24
BRCA1	E	2A, 11-1, 16A
	F	19, 13, 21
	G	17, 11-14, 3
	H	11-15, 15A, 15B
	I	11-16, 11-2, 12
BRCA1 exon 11	A	11-5, 11-8, 11-10
	B	11-13b, 11-13e, 11-13f
	C	11-7, 11-12, 11-13c
	D	11-9, 11-11, 11-13a
	E	11-6, 11-13d

Each amplicon pool contained three to five amplicons with relatively large melting temperature differences.

Table 4 Amplicon pools created for BRCA2 to increase the throughput of denaturing gradient gel electrophoresis

Gene	Pool	Exons in amplicon pool
BRCA2	A	11-1, 27-1, 19
	B	23-1, 18-1, 10-4, 11-3
	C	8, 20, 27-3, 10-5, 21-1
	D	15-1, 23-2, 26, 10-6
	E	12, 10-7, 2, 24, 27-2
	F	5, 6, 11-17, 15-2
	G	11-16, 9, 27-4, 18-2
	H	10-1, 10-3, 10-10, 10-8
	I	25-1, 14-2, 22-1, 25-2
	J	14-3, 10-9, 22-2
	K	4, 3, 7
	L	16, 11-2, 21-2
	M	13, 17, 14-1, 10-2
BRCA2 exon 11	A	11-4, 11-8, 11-15h
	B	11-5,11-10,11-13, 11-15i
	C	11-6, 11-12, 11-15d
	D	11-7, 11-14, 11-15c
	E	11-9, 11-15g, 11-15m
	F	11-15e, 11-15l, 11-15n
	G	11-15a, 11-15f, 11-15j
	H	11-11, 11-15b, 11-15k

Each amplicon pool contained three to five amplicons with relatively large melting temperature differences.

Table 2 Primer sequences used to amplify BRCA2 exon by exon for denaturing gradient gel electrophoresis[12]

Fragment	Forward primer	Reverse primer	Size (bp)
Exon 2	TCCCTGTGTAAGTGCATT	CACTTTCTCGGTGTAATT*	229
Exon 3	ACTAAGGTGGGGATTTTTT*	CGCCCCCCCAGTCTACCATATTG	350
Exon 4	CACTGAATTATTGTACTG	ATATGTAGGAAAATGTTTC*	226
Exon 5	AAATAACCTAAGGGATTT	CATTTCTAGTATTCTAAG*	170
Exon 6	CTTAACAATTTCCCCCTT	GCTATTGTCAAATTCTCA*	169
Exon 7	GATCAGGGCATTTCTATA*	CGCCCCTCATCTGCTCTTTCTTG	248
Exon 8	GTTTTTGCATTCTAGTGAT*	GTTAGCAATTTCAACAGTCT	184
Exon 9	GAGAGTTTTTATACTAGTGA*	ACAGAGCAAGACTCCACCTC	278
Exon 10-0	ATGTGCTTCTGTTTTATACT*	CGCCCGCCAAACTATCTCTTCAGAGGT	305
Exon 10-1	TTTTTTTTTAGTATATGAAACAGTTGTAG*	CTTCTGATTTGCTACATTTG	291
Exon 10-2	CTCATTTGTATCTGAAGTGG*	CGCCCTTTTGGTCACATGAAGAAAT	255
Exon 10-3	GGAGCCCAGATGGCGAAAA*	CTGTTTCCTCATTTAATGGC	234
Exon 10-4	GCGCCACGTATTTCTAGCCTACC	TTAAAGTTTGGATCAGTCAT*	294
Exon 10-5	GAGAATCACCTAAAGAGACT*	GGGGGGCCAGCTTCCATTATCAAT	210
Exon 10-6	GTTTGCTCACAGAAGGAGGA*	CCTGCATTCTTCAAAGCTAC	256
Exon 10-7	AGCCACCACCACACAGAATT	CTTTGGGTATTTTTTTCCT*	171
Exon 10-8	GCTATACATGATGAAACATC*	GTACCTGAATCAGCATTTGC	177
Exon 10-9	GCTTTTGAAGCACCACTTAC	GGAATCGTCATCTATAAAAC*	138
Exon 11-1	CGCGTGAATGTGATTGATGGTAC	CTGTAGTTTTTCCTTATTAC*	244
Exon 11-2	ATCTTGATTATAAAGAAGCA*	TGAATGTTGTACTGGGTGAC	219
Exon 11-3	AAAAGAAGAGGTCTTGGCTG	GACTAGGTTTGACAGAACA*	214
Exon 11-4	AGCACTCTTATTTAACTCC	TTTTCAGGTGGCAACAGCTC*	255
Exon 11-5	AATGTCAGACAAGCTCAAAG*	TTGGATTACTCTTCGATTTG	245
Exon 11-6	TAGCATCACCTTCAAGAAAG*	CTGTGTCTCCATATAAAACC	316
Exon 11-7	TGACTTGTGTAAACGAACCC	GTCCTGCCCATTTGTTCATG*	286
Exon 11-8	GGACATCTCCTTGAATATAG*	TTCAACACAAGCTAAACTAG	265
Exon 11-9	GAGCAAAATGTTCTTCAAAG*	AATTTCTGCCTTTTGGCTAG	301
Exon 11-10	CCCCTCAGATGTTATTTTCC*	GATCAGCATCTCTGCATTCC	271
Exon 11-11	AAGTGCCTGAAAACCAGATG	ATGAGCAGAATATAAAGCCCC*	282
Exon 11-12	AGTGCTTCTGGTTATTTAAC	CTTACAGTTTTATCATTATG*	285
Exon 11-13	GCAGAGGTACATCCAATAAG*	TTACTTGAATCACTGCCATC	312
Exon 11-14	GAAGATAACAAAATATACTGC*	TTACCATGACATGCTTCTTG	288
Exon 11-15a	GAAGATTTGTCAGATTTAAC	TATGTCAGAATCTAATTCAG*	310
Exon 11-15B	ATCAGAAACCAGAAGAATTG*	TACCAACTGGGACACTTTCT	184
Exon 11-15C	CAGACATAGTTAAACACAAA	ATTTCACTAGTACCTTGCTC*	265
Exon 11-15D	AGGAATCTTTGGACAAAGTG	GTTTTGAGATTTTCAGTTTG*	325
Exon 11-15E	TTCTATTGAGACTGTGGTGC*	TGACCATCAAATATTCCTTC	327
Exon 11-15F	TGTGAGTCAGACTTCATTAC	GTTAGACATGCTACTGTTAC*	250
Exon 11-15G	GTACTATAGCTGAAAATGAC*	TCCTCAACGCAAATATCTTC	319
Exon 11-15H	AAAAGATGCAAATGCATACC	CCCTACCTCAAAATTATTAC*	179
Exon 11-15I	ATGCAGCCATTAAATTGTCC	TACTTTACTGAAACTGTCTG*	197
Exon 11-15J	CAGTGGTAAAATCGTTTGTG*	TCTTCACTCTGAATGTCAGC	283
Exon 11-15K	TTATGATGAATGTAGCACGC	CTTCCCTATACTACATTTAC*	306
Exon 11-15L	CACCTTGTGATGTTAGTTTGG	ACCACATTATATGAAAAGCC*	362
Exon 11-15M	GAAGAAAATACTGCTATACG*	ATCAAATTCCTCTAACACTC	211
Exon 11-15N	TACAGCAAGTGGAAAGCAAG	TTATTTTCTGAAGAACCACC*	301
Exon 11-16	CTTCCTCGTGTTGATAAGAG*	GGCATGACTTGGCAGTTTAG	472
Exon 11-17	GGAAGATGATGAACTGACAG	GTGATTGGCAACACGAAAGG*	214
Exon 12	GACTTTTGAGAAATAAAAACTG*	GATCCACCTGAGGTCAGAAT	278
Exon 13	CGCCCGTAATATAAAATAATGGTTTCC*	AAACGAGACTTTTCTCATAC	196
Exon 14-1	CGCCCGCCGTGTACTAGTCAATAAACTT	AGCAGAAACTTGATAAAATG*	244
Exon 14-2	ATCTTCAAGCAATTTAGCAG*	TACTATCATCAGAGCCATGT	277
Exon 14-3	AAAACAGACAAAAGCAAAAC*	CATCACACAATTGTCATAC	225
Exon 15-1	GGTGTGCTTTTTAAATTC*	CTTCTTAATTCGCATATCCTG	151
Exon 15-2	ATTACAAGTCTTCAGAATGCC*	GCGGGAAAAGCCATCAGTATTGTAG	254
Exon 16	GTGTGATACATGTTTACTT*	GGTAGAGGGAATACATAAA	358
Exon 17	CAGTATCATCCTATGTGGTT*	CTGCCGTATATGATTACGTA	303
Exon 18-1	CTCAGTTATTCAGTGACTTG*	GATCTAACTGGGCCTTAACA	354
Exon 18-2	TTGAACTTACAGATGGGTGG	CTGATTTTTACCAAGAGTGC*	248
Exon 19	CGCCCGCCATATTTATTAATTTGTCC	TATGGTAAGTTTCAAGAATAC*	252
Exon 20	TATGTGACTTTTTTGGTGTG	CTCTAAGACTTTGTTCTCAT*	281
Exon 21-1	GTGAATTAATAATCCTTTTG*	GCACGAACTTGCTGTCTTGT	127
Exon 21-2	CATATTTACCATCACGTGCA	GCCTCATTATATGTCCTCTT*	177
Exon 22-1	CTAGTTACAATAGATGGAAC*	TGTGACATCCCTTGATAAAC	254
Exon 22-2	TGGAATCTGCTGAACAAAAG	CTGATAAAAACAAAGCATTTAC*	169
Exon 23-1	GATAATCACTTCTCCATTTG	GTTAGCTCTTTCAGATTTAC*	205
Exon 23-2	GAAGGAAAGAGATACAGAAT*	CCCGCCCGTTCCATAAACTAACAAGCAC	219
Exon 24	GTGCTTGTTAGTTTATGGAA*	CGCCCAAAAATTAACTATATTGTGC	265
Exon 25-1	CTTGCATCTTAAAATTCATC*	GGTTGCTTGCAGCAATTAAC	221
Exon 25-2	CCCGCCCTGGCAATAAAGTTTTGGAT	CAAAATGTGTGGTGATGCTG*	283
Exon 26	GGAAATACTTTGGAAACAT*	TTCCTTGAGTTTACATTAAC	282
Exon 27-1	ATGATAGGCTACGTTTTCAT*	TTGCAGTTCTTTTGGTCATC	220
Exon 27-2	GTCTTGTAAAGGGGAGAAAG*	TGAGGAGAATTCAGTTCTT	256
Exon 27-3	CTGCACAGAAGGCATTTCAG*	CAGAGATGTAGTACAACGTC	333
Exon 27-4	ACCAGTTCAGAAGATTATCT	GTCAATAATTTATTGTCGCC*	256

*GC clamp sequence (CGCCCGCCCGCCGCCCCGCCCGGCCCGCCGCCCCGCCCG) is attached to this site.

RESULTS

In the 11 patients, aberrantly migrating bands (fig 2) were found in exons 13 and 16 of the *BRCA1* gene, and in exons 2, 11, and 14 of the *BRCA2* gene (table 5). We then sequenced the exons with aberrantly migrating bands (fig 3). The aberrantly migrating bands found in exons 13 and 16 of *BRCA1* and exons 2 and 14 of *BRCA2* appeared to be caused by single nucleotide substitutions producing the same amino acid. Consultation with the Breast Cancer Information Core (last accessed 1 February 2005; http://www.research.nhgri.nih.gov/projects/bic) revealed that these substitutions were indeed established polymorphisms.

We detected one deleterious *BRCA2* mutation in patient BIII-5 (fig 4). This mutation was a deletion of six nucleotides in exon 11 (c.2472delTAAATG) at codon 824. The mutation did not cause a shift of the reading frame, but affected three codons and resulted in a premature stop codon. There is no such mutation registered in the Breast Cancer Information Core (http://www.research.nhgri.nih.gov/projects/bic), so this is a novel mutation. This patient had early onset breast cancer and there was no family history of cancer. Table 5 summarises the genetic aberrations found, and table 6 shows the clinicopathological data of the patients with breast cancer.

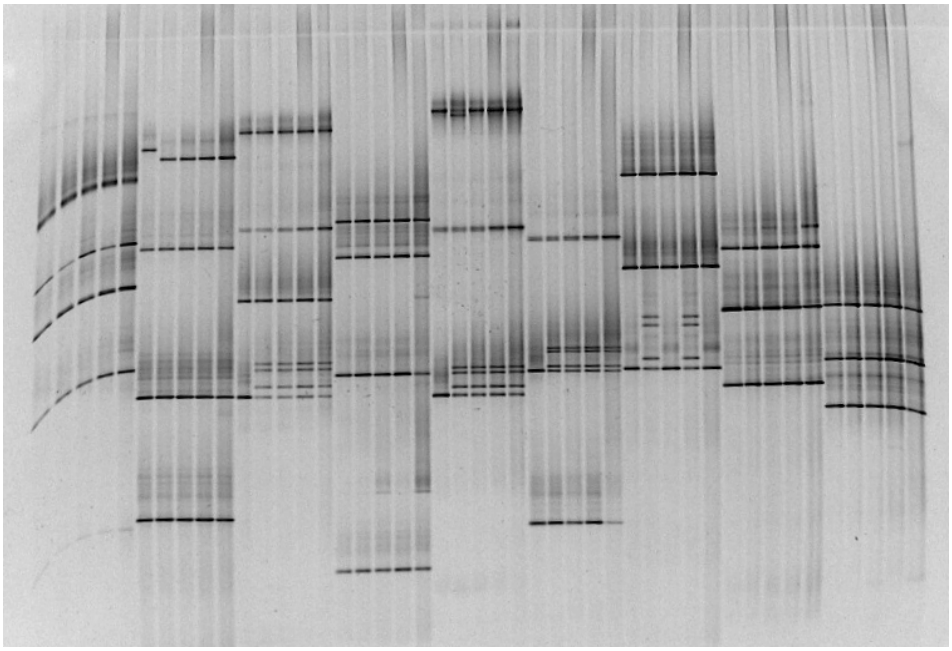

Figure 2. Pooling of five samples of 24 exons in *BRCA1* gene for DGGE. Each pool consist of three to four amplicons selected based on their melting behaviour. The fragment for each pool can be seen in Table 3. The aberrant bands appeared in fragment 9 (in Pool B), fragment 16B (in Pool C), fragment 2A and 16A (in Pool E), fragment 13 (in Pool F) and fragment 3 (in Pool G), and fragment 11-15 (in Pool H) were further analysed by sequencing.

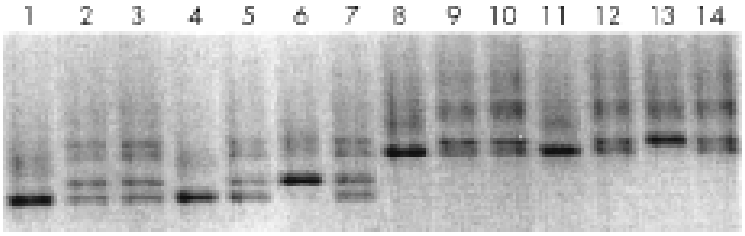

Figure 3. Results of DGGE analysis of *BRCA1* DGGE of Indonesian patients at high risk for hereditary breast cancer. Exon 16, Lanes 1, 4 and 6 display a darker single band as opposed to samples in lanes 2, 3, 5 and 7 which showed lighter double bands. The dark band sat in the same position as one of the double bands. Exon 13 was similar: lanes 8, 11 and 13 displayed a dark single band, whereas lanes 9, 10, 12 and 14 showed light double bands. These aberrant bands appeared to represent single nucleotide substitutions with no consequences for the amino acid sequence of the protein (polymorphisms).

DISCUSSION

The increasing demand for mutation detection in disease genes, either known or presumed, can be solved by automated sequencing using fluorescent dyes [16]. However, only a few laboratories are equipped for the broad application of this costly and labour intensive strategy. As alternatives to sequencing methods, which determine the exact nature and location of each base along a DNA fragment, various mutation scanning procedures have been developed. These methods, which rely on the recognition of a sequence variation between mutant and wild-type DNA on the basis of an altered electrophoretic migration pattern, provide a simple means for determining whether a given DNA sample harbours a mutation in a particular gene.

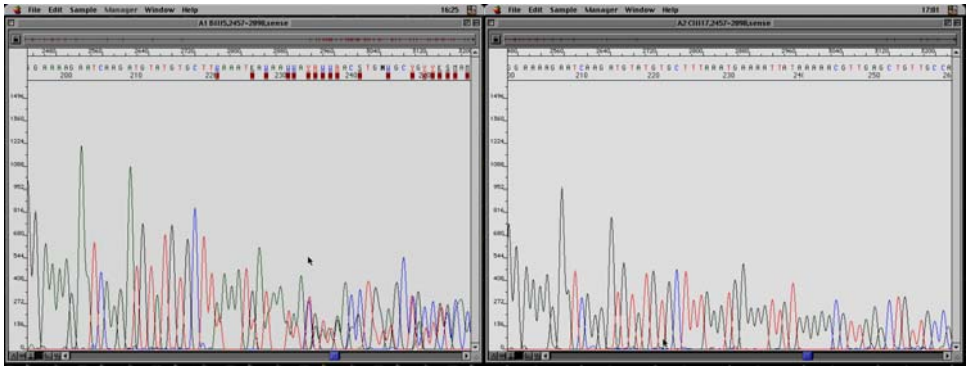

Figure 4. Sequencing graphs of exon 11 of *BRCA2* of a 30 years old early onset breast cancer patient showing the 2699delTAAATG mutation on the left with the normal control on the right.

The most well established scanning procedures are single strand conformational polymorphism (SCCP) analysis, DGGE, chemical cleavage of mismatch, RNase cleavage, the protein truncation test (PTT), and heteroduplex analysis. Among these methods, SCCP, DGGE, PTT, and heteroduplex analysis are the most widely used because of their accuracy, simplicity, lack of toxicity,

and/or relative affordability. We focused on DGGE because this method, when optimised, has the highest mutation detection rate (close to 100% [17]) compared with SCCP and heteroduplex analysis. Additional advantages of this methodology are the possibility of optimising the analysis by computer simulation and the non-radioactive approach. In DGGE, during electrophoresis, double stranded DNA amplified by PCR migrates through a gel containing an increasing concentration of denaturant (urea and formamide). As double stranded DNA is electrophoresed through the denaturing gradient, it will melt and change its conformation in such a way that the mobility of the molecule is dramatically reduced. To prevent complete strand dissociation and to facilitate the detection of mutations in the higher melting domains, a GC rich fragment (GC clamp) is introduced during fragment amplification. The GC clamp increases the percentage of single base changes detectable by DGGE, theoretically to 100% [18, 19]. In principle, four bands are detectable in a heterozygous state after denaturation and renaturation, corresponding to the two homodimers and two heterodimers. Although DGGE has been applied to BRCA mutation screening before [20-22] we used a new technique based on DGGE after exon by exon PCR amplification of the complete *BRCA1* and *BRCA2* sequences [12], and sequenced aberrantly moving bands. This yielded four polymorphisms consisting of single nucleotide substitutions, underlining the sensitivity of the method. Intelligent pooling of the amplicons before electrophoresis greatly improved the throughput of the method.

Table 5 Mutations and polymorphisms found in the BRCA1 and BRCA2 genes using the proposed method

Patient	Exon, gene	NT	Codon	Base change	AA change	Designation	Mutation type
B-II-5	11, BRCA2	2699	824	Del TAAATG	Leu to stop	2699 del TAAATG	Nonsense
A-II-9	13, BRCA1	4427	1436	T to C	Ser to Ser	4427T/C	Polymorphism
B-II-5							
D-II-9							
A-II-9	16, BRCA1	4956	1613	A to G	Ser to Gly	S1613G	Polymorphism
B-II-5							
D-II-9							
D-II-9	2, BRCA2	203	–	G to A	5'UTR	203 >A	Polymorphism
D-II-9	14, BRCA2	7470	2414	A to G	Ser to Ser	7470A/G	Polymorphism

AA, amino acid; NT, nucleotide; UTR, untranslated region.

Double gradient DGGE is based on the combination of two linear gradients, a primary denaturing gradient (urea and formamide) and a collinear secondary porosity gradient (polyacrylamide) [23]. This secondary gradient suppresses band broadening during electrophoresis and thus improves the resolution of the DGGE banding pattern. Hayes et al compared double gradient DGGE gels with various porosity gradients to a standard 9% polyacrylamide gel, and showed that mutations with different melting profiles cannot be appropriately detected using a single DGGE condition [15]. PTT is a widely applied screening technique [5], but is especially helpful for known mutations in particular populations, and less suitable as a primary screening approach for new mutations in unknown populations. Furthermore, PTT only detects mutations that result in stop codons and lead to premature termination of translation, thereby producing truncated proteins. A possible advantage of PTT is that it conveniently misses harmless polymorphisms. PTT is usually only applied for detecting mutations in exon 11 of the *BRCA1* gene and exons 10 and 11 of the *BRCA2* gene, which account for more than 60% of the

coding sequence. However, mutations are distributed throughout the entire coding sequence, with no apparent clustering or hot spots [24].

''Intelligent pooling of the amplicons before electrophoresis greatly improved the throughput of the method''

Table 6 Clinicopathological data of the patients with breast cancer showing BRAC1/2 mutations and/or polymorphisms

Patient	Age	Tumour type	Tumour size	TNM	Stage	lymph nodes positive	Pregnancies	Children	Contraceptives
A	44	Ductal	2	T2N0M0	II A	0/11	0	0	None
B	30	Ductal	1	T1N0M0	I	0/10	0	0	None
C	39	Ductal	3	T2N0M0	II A	0/10	5	3	IUD
D	30	Ductal	2.5	T2N0M0	II A	0/10	2	2	Oral contraceptives for 4 years, IUD

IUD, intrauterine device.

More than 1893 distinct germline *BRCA2* mutations have been found to date (http://www.research.nhgri.nih.gov/projects/bic), and the number is expected to increase further. The pattern of mutations is similar to that seen in *BRCA1* and in many other tumour suppressor genes. At present, all mutations that clearly cause disease result in premature termination of translation or absence of a transcript. Approximately 75% of truncating mutations are small deletions, 15% are small insertions, and 10% are base substitutions leading directly to termination codons (http://www.research.nhgri.nih.gov/projects/bic) [25-27]. In addition, the novel 2699delTAAATG mutation described in our present paper leads to a premature stop codon. The patient with this mutation had early onset breast cancer and no family history of cancer. It has been suggested that the probability of harbouring a *BRCA2* mutation among patients with early onset breast cancer is relatively independent of a positive family history for the disease [25].

Mutations in the *BRCA2* gene have been found mainly in families with a high incidence of female and male breast cancer [4, 26, 28, 29], whereas the risk of ovarian cancer is lower than in *BRCA1* families [4, 26, 30, 31]. Loss of heterozygosity has been found in 30–45% of sporadic breast tumours [32, 33], indicating a role for *BRCA2* also in sporadic breast tumours, although very few somatic mutations have been reported [24, 34, 35]. Other tumour types seen repeatedly in *BRCA2* families include those of the prostate, larynx, pancreas, and colon [36, 37].

In conclusion, we describe a novel *BRCA2* mutation and four polymorphisms in the *BRCA1*/2 genes found in the Indonesian population using a new high throughput and highly sensitive method to screen *BRCA1* and *BRCA2* for mutations based on exon by exon PCR amplification followed by pooled DGGE and sequencing of aberrant bands. This is an ideal approach for screening populations for unknown mutations, and has the ability to detect single base differences using non-toxic and relatively simple and inexpensive methods.

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Chapter 3

BRCA1 and *BRCA2* germline mutation analysis in the Indonesian population

Submitted for publication

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ABSTRACT

Specific mutations in *BRCA1* and *BRCA2* genes have been identified in specific populations and ethnic groups. However, little is known about the contribution of *BRCA1* and *BRCA2* mutations to breast cancers in the Indonesian population.

One hundred-twenty moderate to high risk breast cancer patients were tested using PCR-DGGE, and any aberrant band was sequenced. Multiplex ligation-dependent probe amplification (MLPA) was performed on all samples to detect large deletions in the two genes.

Twenty-three different mutations were detected in 30 individuals, ten were deleterious mutations and 20 were “unclassified variants” with uncertain clinical consequences. Three of seven (c.2784_2875insT, p.Leu1415X and del exon 13-15) and two of four (p.Glu2183X and p.Gln2894X) deleterious mutations that were found in *BRCA1* and *BRCA2* respectively, are novel.

Several novel, pathogenic *BRCA1* and *BRCA2* germline mutations are found in early onset Indonesian breast cancer patients, these may therefore be specific for the Indonesian population.

INTRODUCTION

Breast cancer is the most common cancer in women. In 5% to 10% of breast cancer cases, the disease results from a hereditary predisposition [1, 2], which can to a large extent be attributed to mutations in either of two tumour suppressor genes, *BRCA1* (MIM# 113705) and *BRCA2* (MIM# 600185) [3-5]. These genes are involved in DNA repair as well as transcriptional regulation [6, 7]. Women carrying pathogenic germline mutations in either of these genes tend to develop breast cancer at an early age [8, 9].

The *BRCA1* and *BRCA2* genes encode large proteins of 1863 and 3418 amino acids, respectively. Over 300 distinct mutations in *BRCA1* and *BRCA2* have been described [10, 11]. These mutations are widely scattered across both genes and most affect the structure and function of the gene. Nevertheless, a significant proportion (34% of *BRCA1* and 38% of *BRCA2* mutations) (http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic) are missense mutations that alter one amino acid, but do not truncate the protein and are rare sequence variants of unknown functional consequence. Moreover, a number of base substitutions do not alter the amino acid sequence or result in amino acid changes not associated with disease (polymorphisms) [12]. Hence the biggest challenge in interpreting the mutation analysis of *BRCA1* and *BRCA2* genes is to distinguish between harmless polymorphisms and deleterious mutations associated with increased cancer risk.

In addition, mutations specific for certain populations and ethnic groups have been identified in both genes. For example, specific *BRCA1* and *BRCA2* mutations were reported for Ashkenazi Jews [13]. Other common *BRCA1* mutations were especially found in Italian, Canadian, Belgian or Dutch breast cancer families [14-16]. In Indonesia, the contribution of the *BRCA1*/*BRCA2* mutations to the population incidence of early-onset breast cancer is largely unknown. In one pilot study, however, a new *BRCA2* mutation was identified [17] indicating that it was worthwhile to more extensively study the Indonesian population, which was the aim of this study. The accumulating knowledge about the prevalence and nature of *BRCA1* and *BRCA2* mutations in specific populations may facilitate the interpretation of genetic analysis with regard to breast cancer risk of individual patients.

MATERIALS AND METHODS

Patients

A total of 120 unrelated breast cancer patients and 16 of their family members from three Indonesian cities (Jakarta and Jogjakarta on the Java island, Denpasar on the Bali island) were analyzed. Breast cancer patients at moderate to high-risk of a hereditary predisposition were selected according to the following criteria: A. Breast cancer before the age of 41 (n=102); B. Two cases of breast cancer in the same family before the age of 60 (n=9); C. Three or more cases of breast cancer in the same family (n=2); D. Bilateral breast cancer (n=7). Subjects were asked to fill out questionnaires to evaluate their personal and family histories, and blood specimens were collected for determination of BRCA mutations. Informed consent was obtained from all the subjects in this study.

DNA extraction and PCR amplification

Genomic DNA was isolated by the saturated salt extraction procedure as described in [18]. All 22 coding exons of BRCA1 and 26 coding exons of BRCA2 were amplified using primer sequences developed by the University of Groningen, The Netherlands [19]. Primers for DGGE were obtained from Ingeny (Goes, The Netherlands). Genomic DNA was amplified using 100 – 200 ng of template DNA, 10 pmol of the mixture of 40-mer primers, 30 mM of MgCl, 3 mM dNTPs (Invitrogen) and 0.7 unit of Platinum Taq (Invitrogen) in 9 μl PCR reactions. In order to speed up the test, the PCR reaction was placed in 384 well plates using a pipetting robot (TECAN Miniprep 75). PCR conditions were performed as previously described [17].

Denaturing Gradient Gel Electrophoresis and DNA sequencing

A 4 – 6 μl aliquot of each PCR product with relatively large melting temperature differences were pooled as previously described [17] with some modifications for optimal results. The fragment pool was designed based on melting profiles and sequence. Electrophoresis was performed in 0.5 TAE buffer at 58°C, 120 V for 16 hours for BRCA1 gene, and 55°C, 100V for 18 hours for BRCA2. Gels were stained with ethidium bromide and photographed under a UV transilluminator. The aberrantly migrating samples were re-amplified using sequencing primers and sequencing was performed using Big Dye Cycle-sequencing kit according to the manufacturer's instructions. The reaction products were analyzed using an ABI 3100 DNA Sequencer (Applied Biosystems, Torrence, CA, USA) and sequence files were edited using the Bio Edit program. The classification of gene alterations was performed in accordance with the entries in the Breast Cancer Information Core (BIC, Bethesda, MD).

Multiplex ligation-dependent probe amplification (MLPA)

The principle of the MLPA technique has been described elsewhere [20]. The MLPA test for BRCA1 (P02) and BRCA2 (P45) mutations were obtained from MRC-Holland, Amsterdam, The Netherlands. The fragments were analyzed on an ABI model 310 capillary sequencer (Applied Biosystems, Torrence, CA, USA) using Genescan-TAMRA 500 size standards (Applied Biosystems). Fragment analysis was performed with Genescan software.

RESULTS AND DISCUSSIONS

We identified 120 incident Indonesian breast cancer cases diagnosed before the age of 41 years, or having family history of breast cancer, or harboring bilateral breast cancer during September 1999 – April 2005 (Jogjakarta) and during July 2004 – April 2005 (Jakarta and Denpasar). In addition, 16 of their family members were analyzed.

The entire coding regions and exon-intron junctions of *BRCA1* and *BRCA2* were screened in these 136 persons of breast cancer patients and their families using PCR-DGGE (Figure 1) followed by sequencing (Figure 2) for samples with aberrant migrating bands. To optimize the screening, MLPA, a relatively new technique, was also performed in all samples (Figure 3). Here, we report on 116/120 women (96.7%) for whom *BRCA1*/2 analysis were completed. The

remaining 4 patients (all from group A) had to be excluded due to the small amount of extracted DNA that did not allow complete screening of the *BRCA1* and *BRCA2* genes.

BRCA1 and BRCA2 pathogenic mutations

The analysis of 116 unrelated breast cancer patients with breast cancer revealed that 9 patients (7.8%) carried pathogenic germline mutations especially the early onset patients: 3 within *BRCA1* (2.6%) and 6 within *BRCA2* (5.2%) which is comparable to previous studies [21]. We only found *BRCA1* and *BRCA2* mutations in groups A ("early onset", n=7 out of 98, 7.1%) and B (two cases of breast cancer in the same family before the age of 60, n=2 out of 9 (22.2%)) (Table 1). There were twice as many BRCA2 mutations as BRCA1 mutations. Although the absolute numbers are low and no firm conclusions can therefore be drawn, this is comparable to other Asian regions [22-24] but seems to discern the Indonesian population from non-Asian ethnic groups where the reverse trend is seen.

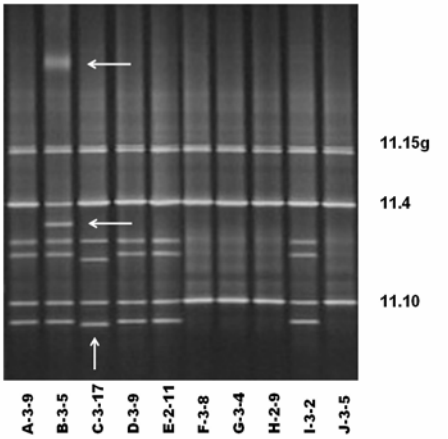

Figure 1. DGGE analysis of fragments 11.15g, 11.4 and 11.10 of the *BRCA2* gene in ten unrelated breast cancer patients. The arrows show altered band mobility compare to other patients.

Seven pathogenic mutations were found in nine probands: three in *BRCA1* (c.2784_2785insT, pL1415X (c.4361_4362insT), del exon 13-15) and four in *BRCA2* (c.3040_3043delGCAA, p.Glu2183X (c.6775G>T), p.Leu824X (c.2699_2704delTAAATG), p.Gln2894X (c.9008C>T)). All these mutations were classified as pathogenic as they are predicted to result in protein truncation. The three pathogenic mutations found in *BRCA1* were not previously reported in the BIC database as well as two novel nonsense mutations (p.Glu2183X and p.Gln2894X) identified in *BRCA2*. The p.Glu2183X mutation was found in 2 related patients that had breast cancer above the age of 60.

One of seven pathogenic mutations found in *BRCA1* and *BRCA2* showed a significant clinical impact on the patient (Table 2). Patient AE with a one nucleotide insertion (Thymine) between nucleotide 2784 and 2785 (c.2784_2785insT) in exon 11 of *BRCA1* suffered from bilateral breast cancer at a relatively early age (25 years). The insertion leads to frameshift and creates a premature stop codon in

exon 11. The mutation takes place in the sequence within *BRCA1* encoding for aminoacids 758-1064 which interact with RAD51 protein that is required for homologous recombination (HR) repair of double strand breaks (DSBs) [25], which is one of the most important functions of the *BRCA1* protein. This patient presented in a late stage (stage III for both breasts) and only survived for 9 weeks after treatment. Her mother did not carry this mutation. Although her father may be carrier, the mutation is probably *de novo* as there was no family history of breast or other cancers.

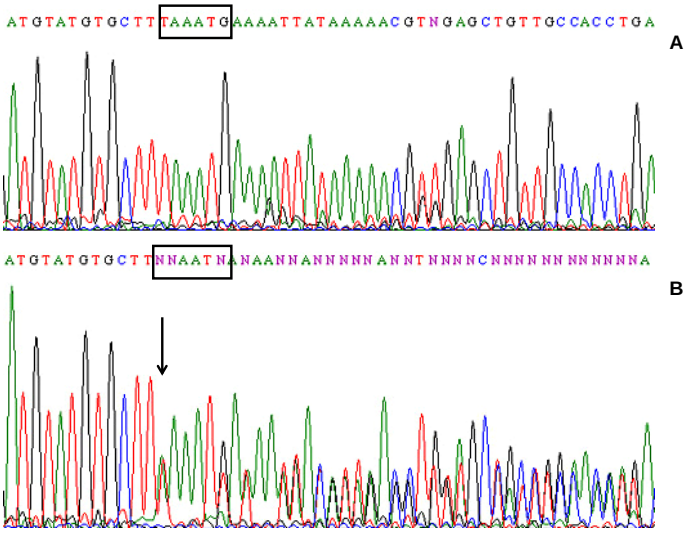

Figure 2. Sequence electropherogram of a normal individual showing (A) wild-type *BRCA2* exon 11 sequence and (B) of breast cancer patient (B-3-5) showing c.2699_2704delTAAATG mutation.

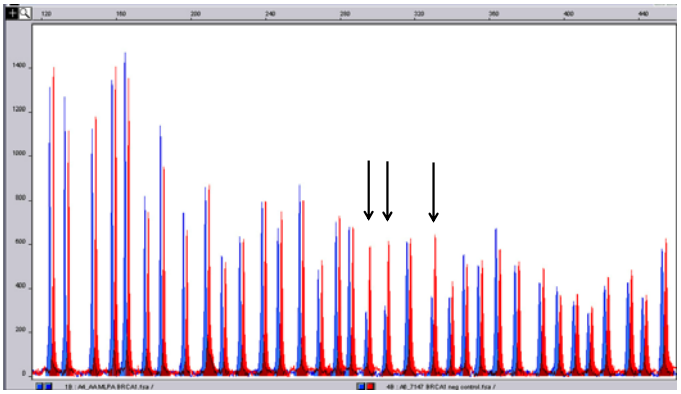

Figure 3. MLPA analysis of *BRCA1* gene of patient sample (blue) compare to the normal control (red). X and Y axis represent peak size and peak height respectively. There are reduced peaks in the patient sample compared to the normal control in exons 13, 14 and 15 (arrows) indicating deletions.

Table 1. *BRCA1* or *BRCA2* germline mutations in Indonesian women with early onset breast cancer.

Patient	Age[1]	gene	Exon	Mutation[2]	mutation type	Pathogenic mutation	BIC[3]
AE	25	*BRCA1*	11	c.2784_2785insT	frameshift	+	no
B10	31	*BRCA1*	13	p.Leu1415X	nonsense	+	no
AA	40	*BRCA1*	13-15	-[4]	large rearrangement	+	no
AB	34	*BRCA2*	11	c.3040_3043del4	frameshift	+	1
B5	66	*BRCA2*	11	p.Glu2183X	nonsense	+	no
B6	65	*BRCA2*	11	p.Glu2183X	nonsense	+	no
B-III-5	30	*BRCA2*	11	p.Leu824X	nonsense	+	no
AZ	40	*BRCA2*	11	p.Leu824X	nonsense	+	no
W-II	37	*BRCA2*	21	p.Gln2894X	nonsense	+	no
Q-II	40	*BRCA1*	2	c.101-10T>C	IVS	±	6
P-III-19	19	*BRCA1*	9	p.Val191Ile	Missense	±	6
J22	32	*BRCA1*	11	p.Leu1209Val	Missense	?	No
AZ	40	*BRCA1*	16	p.Met1652Ile	Missense	±	35
B1	24	*BRCA1*	20	c.5313-31A>G	IVS	?	No
B7	31	*BRCA1*	24	p.Arg1835Gln	Missense	?	No
216	33	*BRCA1*	24	p.Thr1852Ile	Missense	?	No
P-III-19	19	*BRCA2*	5	p.Gln147Arg	Missense	±	6
B3	24	*BRCA2*	10	p.Gln609Glu	Missense	?	no
C-II-7	39	*BRCA2*	11	p.Met1149Val	Missense	±	5
AO	28	*BRCA2*	11	p.Met1149Val	Missense	±	5
AQ	44	*BRCA2*	11	p.Met1149Val	Missense	±	5
BH	38	*BRCA2*	11	p.Met1149Val	Missense	±	5
172	36	*BRCA2*	11	p.Gln699Leu	Missense	?	no
J32	29	*BRCA2*	11	p.Arg2108Cys	Missense	±	16
J6	33	*BRCA2*	11	p.Val950Ile	Missense	?	No
206	37	*BRCA2*	25	c.9485-16T>C	IVS	±	4
BC	35	*BRCA2*	27	p.Ile3412Val	Missense	±	109
166	33	*BRCA2*	27	p.Ile3412Val	Missense	±	109
J24	35	*BRCA2*	27	p.Ile3412Val	Missense	±	109
206	37	*BRCA2*	27	p.Lys3326X	nonsense	±	289

[1] Age at time of diagnosis
[2] Gen Bank Accession number, *BRCA1*: U14680, *BRCA2*: U43746
[3] number of times reported in BIC
[4] not determined, detected by MLPA

The second pathogenic mutation with a significant clinical manifestation was a cytosine for thymine substitution on nucleotide 9008 of BRCA2 leading to a premature stop codon in position 2894, c9008C>T (p.Gln2894X). Patient W presented at age 37 in a late stage and survived for only 107 weeks after initial treatment. She had no family history of breast or other cancers. This mutation lies within exon 21 of *BRCA2* which is the proposed site for interaction with the DSS1 protein that seems to have a fundamental role in enabling the BRCA2-RAD51 complex to associate with sites of DNA damage [26].

Table 2. Clinicopathological features of Indonesian breast cancer patients with deleterious *BRCA1* or *BRCA2* germline mutations

Patient	Age[1]	Gene with germline mutation	Mutation[2]	stage	Diagnosis	Menopausal status	family history of cancer	Survival status
AE	25	*BRCA1*	c.2784_2785insT	IIIB/IIIA	IDC, bilateral	pre	No	DOD 9 w
B10	31	*BRCA1*	p.Leu1415X	I	IDC	pre	No	DOD 57 w
AA	40	*BRCA1*	- [3]	IIIB	IDC N+	pre	No	AWD
AB	34	*BRCA2*	c.3040_3043del4	IIIB	IDC N+	pre	Sister, Int	DOD 17 w
B5	63	*BRCA2*	p.Glu2183X	IV	Tubular	post	Sister, Br	AWD
B6	65	*BRCA2*	p.Glu2183X	III	IDC	post	Brother, Br	AWD
B-III-5	30	*BRCA2*	p.Leu824X	I	IDC	pre	No	AWD
AZ	40	*BRCA2*	p.Leu824X	IV	IDC	pre	Sister, Cv	DOD 46 w
W-II	37	*BRCA2*	p.Gln2894X	IIIA	IDC	pre	No	DOD 107 w

[1] Age at time of diagnosis
[2] Gen Bank Accession number, *BRCA1*: U14680, *BRCA2*: U43746
[3] not determined, detected by MLPA
IDC: invasive ductal carcinoma; DOD: dead of disease; bil : bilateral breast cancer; N+: with metastatic to lymph node; Int: intestinum cancer, Br: breast cancer; Cv: cervical cancer

The c.2699_2704delTAAATG (p.Leu824X) in *BRCA2* that has been reported previously by us in the Indonesian population [17], was found in one other patient in the present study (Table 1). This mutation lies in exon 11 *BRCA2*, within the BRC repeats domain. The truncating mutation causes loss of three quarters of the protein leading to lack of interaction with the RAD51 protein. Different from *BRCA1*, the repair of DSBs by HR is the most important function of the *BRCA2* protein [27]. Patient B-III-5 was diagnosed with early stage breast cancer at age 30 with no family history of breast or other cancers. Her sister carried the same mutation, but with no present clinical manifestation as yet. Patient AZ who was diagnosed at 40 years of age, presented in late stage, only survived 46 weeks after initial treatment. This patient also harbored a mutation in exon 16 of *BRCA1*, a G to A substitution in nucleotide 5075 (c.5075G>A), which leads to amino acid change from Methionine

to Isoleucine, (p.Met16521Ile) which has to date been reported 35 times in BIC as a UV mutation. As the c.2699_2704delTAAATG mutation was found in two unrelated patients, this mutation could be a good candidate as a founder mutation.

None of the families with more than 3 cases of breast cancer and families with bilateral breast cancer showed pathogenic mutations in the *BRCA1* and *BRCA2* genes. Family U had four first-degree relatives that were affected by breast cancer. Two of four members had bilateral breast cancer. In spite of this high familial breast cancer incidence, no *BRCA1*/2 mutations were found.

BRCA 1 and BRCA2 unclassified variants

Sixteen (7 *BRCA1* and 9 *BRCA2*) rare mutations of so far unknown significance ("unclassified variants", UVs) were detected in 18 patients: 13 missense changes and 3 intronic variants. Of these 16 UVs, 7 were novel, whereas the other UVs have been previously reported in the BIC database (Table 1). From the 18 patients which carried UV mutations, two patients were detected in families from group D; one patient in a group B family and the other fifteen patients in families from group A.

Seven UV were found in the *BRCA1* gene, two mutations occurring in the intronic region between exons 1 and 2 (c.101-10T>C) and between exons 19 and 20 (c.5313-31A>G), and five missense mutations identified: p.Val191Ile (c.690G>A), p.Leu1209Val (c.3744T>G), p.Met1652Ile (c.5075G>A), p.Arg1835Gln (c.5623G>A) and p.Thr1852Ile (c.5674C>T).

Four out of seven *BRCA1* missense mutations; p.Leu1209Val (c.3744T>G), c.5313-31A>G, p.Arg1835Gln (c.5623G>A) and p.Thr1852Ile (c.5674C>T) were have not been described previously in the BIC. The p.Leu1209Val may not be a significant change as both Leucine and Valine belong to the same group of non polar amino acids. However, p.Arg1835Gln is possibly an important alteration since a positively charged Arginine is replaced by an uncharged Glutamine, which may have an effect on the structure and/or function of the protein. Another potentially important alteration concerns p.Thr1852Ile, where the hydrophilic amino acid Threonine is replaced by a hydrophobic Isoleucine. The sites of mutation of both p.Arg1835Gln and p.Thr1852Ile also have to be considered as they lie within the site for the activation domain of the *BRCA1* protein [28]. The intronic UV c.5313-31A>G also deserves further investigation as it may theoretically have an effect on splicing. However, according to splice site finder (http://www.genet.sickkids.on.ca/~ali/splicesitefinder.html), the splicing sites in the wild type and mutant alleles are similar, so therefore we can suggest that the c.5313-31A>G has no effect on splicing.

Nine different UVs of the *BRCA2* gene were found in fourteen patients (Table1), and three of them were novel; p.Gln609Glu 9c.2053C>G), p.Gln699Leu (2324A>T) and p.Val950Ile (3076G>A). One truncating mutation near the C-terminal end of BRCA2, p.Lys3326X (c.10204A>T) is probably not pathogenic. Since the truncating mutation is at the very end of the protein, it is possible that protein functions are not affected. Most of the few entries in databanks describing nonsense mutations near the C terminus of *BRCA2* between codon 3308 and 3408 are described as UVs. Thus, the effect of this truncating mutation on cancer predisposition remains unclear.

The p.Val950Ile may not be a significant change as both Valine and Isoleucine belong to the same group of non polar, hydrophobic amino acids. However, pGln609Glu and p.Gln699Leu are potentially important alterations as for p.Gln609Glu, a non acidic, polar, hydrophilic Glutamine is replaced by a negatively charged Glutamic acid, whereas for p.Gln699Leu, an uncharged hydrophilic Glutamine is replaced by a hydrophobic Leucine. As it takes place within the BRC repeats of the *BRCA2* protein, the pGln699Leu alteration might affect protein structure and function.

To know more about the importance of amino acid substitutions for protein function, we compared the amino acid sequence of interest in seven other species, i.e. *Mus musculus, Rattus rattus, Bos taurus, Gallus gallus, Canis familiaris, Macaca mullata* and *Monodelphis domestica.* The missense mutation p.Leu1209Val lies in the conserved region of exon 11 of the BRCA1 gene as the sequence is maintained in seven other species, whereas p.Arg1835Gln and p.Thr1852Ile are only conserved in four and three other species (comparison of p.Arg1835Gln and p.Thr1852Ile with *Bos taurus* sequence is not possible because the *BRCA1* gene is shorter). Therefore, even tough the Leucine to Valine changes may not give any effect on amino acid charge, its conservation in evolution is suggestive of a functional role. Interestingly, p.Gln609Glu and p.Gln699Leu of BRCA2 that result in a quite dramatic amino acid subtitution that might lead to protein structure changes, are only conserved in four and five species respectively. As for the p.Val950Ile, the conservation in evolution is quite low. Although p.Gln609Glu is less conserved, we still believe that Glutamine to Glutamic acid substitution may have an effect on protein conformation as two adjacent acidic amino acids will be formed as the result of the substitution.

Table 3. The amino acid properties of novel unclassified mutations in *BRCA1* and *BRCA2* within an Indonesian breast cancer population

Gene	Amino acid Change	Change of Charge	Change of amino acid group	Similarity score*	# species with conserved sequence
BRCA1	Leu to Val	None	No	32	$7^{a,b,c,d,e,f,g}$
BRCA1	Arg to Gln	Pos to no charge	Yes	43	$4^{a,c,f,g}$
BRCA1	Thr to Ile	polar to non polar	Yes	89	$3^{a,c,g}$
BRCA2	Gln to Glu	No charge to neg	Yes	29	$4^{a,b,c,g}$
BRCA2	Gln to Leu	Polar to non polar	Yes	113	$5^{a,b,d,e,f}$
BRCA2	Val to Ile	None	No	29	$2^{f,g}$

* based on Grantham table [Grantham et al. 1974], a score above 100 indicates significance changes

a = *Macaca mullata*, b = *Bos taurus*, c = *Canis familiaris*, d = *Rattus rattus*, e = *Mus musculus*, f = *Gallus gallus*, h = *Monodelphis domestica*

Glycosylation moiety of an amino acid also plays a role in protein function. Amino acid substitutions involving Serine, Threonine and Asparagine, should also be checked for their O-GlcNac potential and threshold. Here we have a Threonine to Isoleucine substitution (p.Thr1852Ile) that after checking with YinOYang (http://www.cbs.dtu.dk/services/YinOYang) showed no significant threshold changes between the wildtype and the mutant allele.

The possible effect of amino acid changes in proteins can also be assessed using similarity scores (based on Grantham table [29]), in which a value above 100 for an amino acid substitution indicates a higher chance of impact on protein function. Among seven novel UVs in the BRCA1 and BRCA2 genes found in the present study, only p.Gln699Leu in BRCA2 has a similarity score above 100, whereas p.Gln609Glu and p.Val905Ile in BRCA2 have the lowest score (Table 3).

Overall, we propose that among the seven novel UVs, there are three mutations that are possibly pathogenic: p.Leu1209Val for its location in a conserved region, and p.Gln609Glu and the p.Gln699Leu because of two adjacent acidic amino acid being formed and a high similarity score, respectively.

When comparing the three different Indonesian regions, the percentages of breast cancer patients with pathogenic *BRCA1/2* mutations was significantly higher in Denpasar (Bali island) than in Jogjakarta and Jakarta (Java island) ((25% (3/12), 7.2% (6/83) and 0% (0/25) respectively (p=0.0255, chi-square test)). The percentages of breast cancer patients with UV mutations in Jakarta, Jogjakarta, and Denpasar were 16% (4/25), 12% (10/83), and 25% (3/12), respectively (n.s.). Although the number of patients is too small to draw firm conclusions, these data may point to geographic differences within Indonesia.

It was initially suggested that the *BRCA1* and *BRCA2* genes would be responsible for most cases of inherited breast cancer, but more recent studies suggest that they would account for a far smaller proportion, with considerable variation among different populations [30]. We found that the incidence of mutations in these genes varies, depending on the diagnostic group. In this sense, mutations were present in (22/102) 21.6% of early onset patients (group A), 28.7% (2/7) in patients with bilateral breast cancer (group D) and (2/9) 22.2% of patients with two cases of breast cancer before the age of 60 (group B). The proportion of families affected by *BRCA1*/2 mutations depends on the population analyzed and on the criteria used to select the patients. Family history of breast cancer was, however, absent or not suggestive of a hereditary predisposition in three-fourth of the deleterious mutations carriers and in more than 90% of UV carriers. This suggests that BRCA screening policies based on family history only would miss a considerable proportion of mutation carriers.

In conclusion, a relatively high percentage of early onset Indonesian breast cancer patients carry a germline mutation in either *BRCA1* or *BRCA2.* Several novel, pathogenic *BRCA1* and *BRCA2* germline mutations have been found, as well as a variety of novel “unclassified variant” mutations that may therefore be specific for the Indonesian population. It is likely that some of the “unclassified variant” mutations may have a functional role in breast cancer development, which deserves to be explored further.

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Chapter 4

Comparison of multiplex ligation dependent probe amplification to immunohistochemistry for assessing HER-2/*neu* amplification in invasive breast cancer

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ABSTRACT

The HER-2/*neu* transmembrane tyrosine kinase receptor is both a prognostic marker and a therapeutic target for breast cancer. Accurate determination of HER-2/*neu* status is a prerequisite for selecting breast tumors for HER-2/*neu* immunotherapy or for taxan based chemotherapy. Unfortunately, there is no consensus concerning how this determination should be reached. We compared assessment of HER-2/*neu* status using Multiplex ligation-dependent probe amplification (MLPA) and immunohistochemistry (IHC). The patient group comprised 60 Indonesian breast cancers patients. IHC was performed on paraffin sections using the CB11 antibody from Novocastra. Results were scored according to the Hercept test. For MLPA, DNA was extracted from frozen samples, PCR amplified with a probe set containing three hemi-primer sets for the HER-2 locus and another nine control probes spread over chromosome 17 and other chromosomes, and analyzed on a gene scanner. A ratio above two for at least two HER-2 locus probes compared to the control probes was regarded as amplification. IHC for HER-2/*neu* was negative in 36 cases, and 24 cases (40%) showed expression. Seven, eight and nine of the latter cases were 1+, 2+ and 3+ positive, respectively. Forty-seven cases showed no amplification by MLPA, and 13 cases (22%) were amplified. Comparison of IHC and MPLA showed that none of the 36 IHCnegative or seven IHC 1+ cases was amplified. Five of the eight (63%) 2+ cases were amplified, and eight of nine (89%) of the IHC 3+ tumors showed gene amplification by MLPA assay. For HER-2/*neu*, there is a good correlation between gene amplification detected by MLPA and overexpression by IHC in invasive breast cancer. It appears that MLPA can detect the HER-2 amplified cases in the IHC 2+ class. Because MLPA is quick and inexpensive, it is an attractive method for detecting HER-2/*neu* amplification in daily laboratory practice.

Key words: amplification, breast cancer, HER-2/*neu*, MLPA, polymerase chain reaction

INTRODUCTION

The HER-2/*neu* proto-oncogene located on human chromosome 17 encodes a 185 kD transmembrane tyrosine kinase growth factor receptor of the epidermal growth factor receptor family (Bargmann et al. 1986) that is involved in cell growth and development (Popescu et al. 1989). HER-2 is amplified in 20-30% of breast carcinomas. Amplification of HER-2/*neu* is now known to confer a poor prognosis (Slamon et al. 1987; Baak et al. 1991) and may also predict a poor response to hormonal therapy (Wright et al. 1992) and standard chemotherapy regimens (Konecny et al. 2004). HER-2/*neu* proto-oncogene amplification usually is accompanied by overexpression of its protein (Slamon et al. 1989) as determined by immunohistochemistry (IHC).

The recent development of trastuzumab (Herceptin), a humanized monoclonal antibody to the extracellular domain of HER-2/*neu*, offers a new therapeutic approach for women with HER-2/*neu* positive breast cancer. Further, HER-2/*neu* positive breast cancer patients respond better to taxane chemotherapy. The significant cost and toxicity of trastuzumab (Sparano 2001) and taxanes, however, have focused attention on accurate determination of HER-2/*neu* status. Currently, HER-2/*neu* status is determined using two methods: one that reveals gene amplification and one aimed at detecting the overexpressed HER-2/*neu* protein (Hanna 2001; Tubbs et al. 2001; Di Leo et al. 2002; Press et al. 2002). Some studies claim that gene amplification status predicts response to therapy better than protein overexpression (Mass et al. 2001).

IHC is the most commonly used method for assessing protein overexpression. It is a rather easy morphological method that has many advantages, but it may be hampered by technical problems and it requires strict quality control and standardization (Ginestier et al. 2004). Moreover, the different IHC technical steps are highly dependent on fixation conditions that significantly modify membrane staining (Penault-Llorca et al. 1994; Press et al. 2002; Rhodes et al. 2002; Rhodes et al. 2002). Consequently, significant variability of IHC results has been demonstrated in inter-laboratory quality control studies. For scoring IHC staining, the 0 to 3+ visual system developed for the Herceptest (Dako) is widely used. While there is little difficulty assigning the 0 and 3+ scores, interpretation is more problematic for the two intermediate levels. For cases scoring 2+ (10-15% of all breast cancers), the concordance with gene amplification by FISH is barely 25%, yet a proportion of these 2+ cases are true HER-2/*neu* amplified tumors. These cases, therefore, require a second amplification test.

Fluorescence hybridization (FISH) currently is the most popular method for HER-2/*neu* gene amplification testing. FISH is expensive, technically challenging and sensitive to differences in digestion methods, however, and the commercially available kits have a limited half-life. Therefore, FISH is not a practical primary screening tool (Jacobs et al. 1999), although it has been recognized for this purpose by the United States Food and Drug Administration (Birner et al. 2001). Its use, therefore, usually is limited to equivocal cases.

We evaluated the potential value of a new PCR based technique to assess HER-2/*neu* amplification called multiplex ligation-dependent probe amplification (MLPA). MLPA permits detection of a gain in copy number in samples containing only 20-100 ng of human DNA extracted from paraffin blocks. MLPA reactions are inexpensive, simple to perform, and the results are easy to interpret.

MATERIALS AND METHODS

Breast tumor samples

Sixty frozen and accompanying formaldehyde fixed, paraffin embedded breast cancer samples were obtained from women treated at Sardjito Hospital, Yogyakarta, Indonesia. The collection and storage of samples conformed to specific local guidelines and local ethical guidelines were followed for this study.

Immunohistochemistry

Immunohistochemical staining was performed using a two-step immunoperoxidase technique on 4 mm paraffin sections. Sections were mounted on silan coated glass slides and stored overnight at 60^0 C. Endogenous peroxidase was blocked prior to immunohistochemical staining. The primary antibody c-ErbB2 (CB11, Novocastra, Newcastle upon Tyne, UK) was used at a dilution of 1:100 in phosphate buffered saline (PBS) containing 1% bovine serum albumin and 0.2% sodium azide. After incubation for 1 h, the slides were incubated with a horse anti-mouse biotine-conjugated antibody (Vector laboratories, Burlingame, CA) followed by a streptavidin-peroxidase conjugate (Immunotech, Margency, France), both for 30 min. Visualization of the peroxidase label was accomplished with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO). The sections were counterstained with Mayer's hematoxylin. Scoring was performed as proposed for the Hercept test by one experienced pathologist (PvD) as negative, 1+, 2+ or 3+ (Fig. 1).

DNA extraction

DNA was extracted from the frozen samples using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

MLPA analysis

The principle of the MLPA technique has been described elsewhere (Schouten et al. 2002) and is summarized in Fig. 2. The MLPA test for HER-2/*neu* amplification was obtained from MRC-Holland (Amsterdam, The Netherlands). The P012 probe mix contains three sets of hemi-probes that recognize different sequences of the human erbB2/HER-2 gene. In addition to these HER-2/*neu* specific probes, nine control hemi-probe sets are included: one probe for TOP2A, a gene located on chromosome 17 a short distance from the HER-2 gene, and eight probes for single copy sequences on regions on other chromosomes that harbor infrequent copy number changes in breast cancers (Fig. 3) according to Comparative Genomic Hybridization analysis (Kallioniemi et al. 1992) experiments. Briefly, 50-500 ng of target DNA per 5 ml of 10 mM pH 8 Tris 0.1 mM EDTA was denatured for 5 min at 98^0 C after which 3 ml of the probe mix was added. The mixture was heated at 95^0 C for 1 min and incubated at 60^0 C overnight (16 h). Ligation was performed with the temperature stable Ligase-65 enzyme (MRC-Holland) for 15 min at 54^0 C. Next, the ligase was heat inactivated in the thermocycler for 5 min at 98^0 C. Ten microliters of the ligation mixture was premixed with 30 ml of PCR buffer and put in a PCR machine at 60^0 C. Subsequently, a 10 ml mix was added that contained deoxynucleoside triphosphate, Taq polymerase, and one unlabeled and one carboxyfluorescein labeled PCR primer that are complementary to the universal primer sequences.

PCR was carried out for 33 cycles (30 sec at 95^0 C, 30 sec at 60^0 C, and 1 min at 72^0 C). The fragments were analyzed with an ABI model 310 capillary sequencer (Applied Biosystems, Torrence, CA, USA) using Genescan-TAMRA 500 size standards (Applied Biosystems). Fragment analysis was performed using Genescan software. DNA from Centre d'Etude Polymorphisme duHumain (CEPH) was used as a control sample and analyzed simultaneously with breast cancer samples in each run.

To objectify interpretation of the fragment analysis, the relative quantity of the amplified probes in each sample was determined using an Excel template. For this purpose, the relative peak areas for each probe were calculated as fractions of the sum of peak areas in a given sample. Subsequently, the fraction of each peak was divided by the average peak fraction of the corresponding probe in control samples. Finally, the values were normalized using the values obtained for the autosomal control probes that served as a reference for the copy number of 2.0. Cases that showed a copy number greater than 2 for at least two of the probes on the HER-2/*neu* locus could be considered amplified.

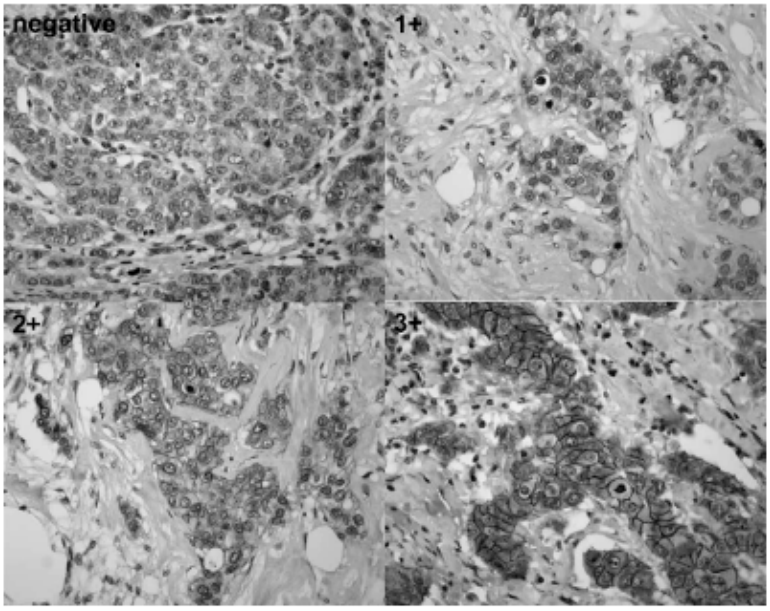

Fig. 1. Examples of scoring of HER-2/*neu* immunohistochemical staining. (Top left) Negative staining: no staining, only cytoplasmic staining or less than 10% cells with membrane staining. (Top right) 1+ staining: more than 10% cells with membrane staining that is incomplete. (Bottom left) 2+ staining: more than 10% cells with complete membrane staining that is not strong. (Bottom right) 3+ staining: more than 10% cells with complete, intense membrane staining.

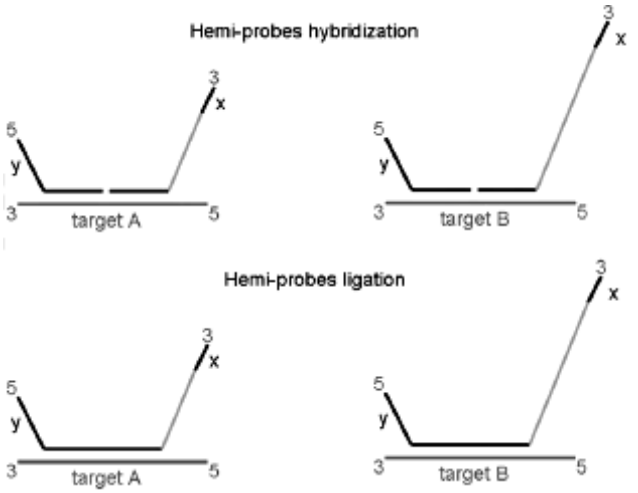

Fig. 2. MLPA uses a mixture of hemi-probe sets that consist of two oligonucleotides, both having PCR primer sequences (x/y) on the outer ends, while both on the inner ends have a sequence complementary to a part of the target sequence (A or B). One of the primers has a spacer (green) of variable length between the PCR primer sequence and the complementary target sequence. When the complementary target sequences of both hemi-probes hybridize adjacent to each other on the target sequence (top figure), they can be ligated to each other (bottom figure), and subsequently amplified using the PCR primer sequences. Because the PCR primers are the same for all hemi-probe sets, they can be amplified in a single PCR, which will provide amplicons of unique and defined lengths due to the specific spacer length within each probe set.

RESULTS

Figure 3 shows an example of a HER-2/*neu* MLPA test on a HER-2 amplified invasive breast cancer compared to a normal sample. Table 1 shows the correlation of IHC staining intensity with MLPA assay. Of the 60 cases, 36 were HER-2/*neu*-negative and seven, eight and nine cases showed 1+, 2+ and 3+ HER-2/*neu* overexpression, respectively. A total of 13/60 cases (22%) showed gene amplification by MLPA assay. Of these amplified cases, eight (62%) showed 3+ IHC, five (38%) 2+ IHC and no cases were 1+ or IHC negative. None of the 36 IHC negative and none of the seven 1+ cases were amplified. Five of the eight (63%) 2+ cases were amplified, and as many as eight of nine (89%) IHC 3+ tumors showed gene amplification by MLPA assay.

Table 1. Comparison of HER-2/*neu* IHC and MLPA to assess protein overexpression for detecting HER-2/*neu* gene amplification.

		MLPA	
IHC score		**normal**	**amplified**
0	36	36	0
1 +	7	7	0
2 +	8	3	5
3 +	9	1	8
Total	60	47	13

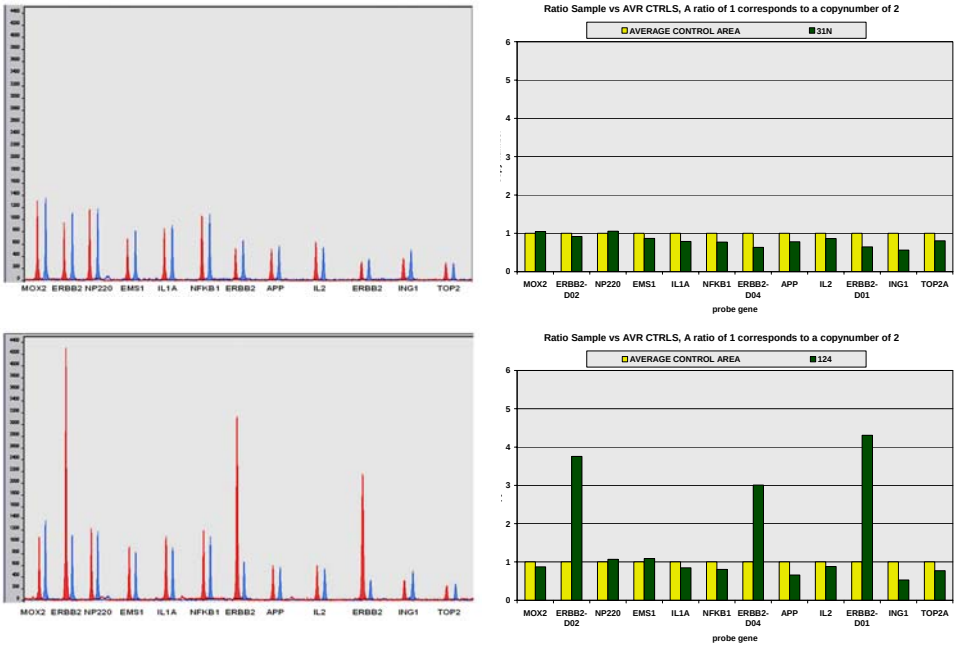

Fig 3. Example of a HER-2/*neu* MLPA test in two invasive breast cancers (31N and 124) and copy number calculations. Amplification products were separated by capillary electrophoresis and compared to normal control DNA (blue peaks). (A) Breast cancer without HER-2 amplification showing peaks comparable peaks for the all probes (left one of the peak pairs) and the control peaks (right one of the peak pairs), further demonstrated by copy number calculations (B) yielding a ratio of approximately 1 for all probes. (C) Invasive breast cancer with HER-2 amplification as demonstrated by three tumor HER-2 peaks that are clearly higher than the red autosomal control peaks for this sample and the normal control peaks indicating increased HER-2 gene copy number, further demonstrated by copy number calculations (D) yielding a ratio between 3 and 5 for the HER-2 probes compared to ratios of approximately 1 for the control probes. Note that the chromosome 17 located TOP2A gene (far right) shows a normal copy number, excluding chromosome 17 polysomy as an explanation for the HER-2 gene amplification.

DISCUSSION

Since the first reports by Slamon et al. (Slamon et al. 1987; Slamon et al. 1989) showing that HER-2/*neu* amplification in breast carcinoma correlates with poor prognosis, accurate detection of HER-2/*neu* gene alteration status has become increasingly important. Moreover, the selection of patients for trastuzumab and taxane chemotherapy relies on the presence of gene amplification or protein overexpression. Given the toxicity of trastuzumab (Sparano 2001) and taxane chemotherapy, accurate determination of HER-2/*neu* status is crucial.

Several methods are in use for detecting HER-2/*neu* gene amplification or protein overexpression including immunostaining of the protein, FISH, quantitative Southern blotting, and real time PCR. The most widely applied test for HER-2/*neu* is IHC. Depending on the antibody and scoring system used, however, HER-2/*neu* overexpression rates in the literature vary between 14 (van de Vijver et al. 1988) and 60% (Roche and Ingle 1999). The subjectivity of IHC tends to decrease with increasing positivity, so that inter-observer correlation is higher for strongly positive cases. Chromosome 17 polysomy has been postulated to have played a role in other studies showing discrepancies between protein expression and gene

amplification. Pauletti et al. (Pauletti et al. 2000) attributed 3+ positive, FISH-negative cases to chromosome 17 polysomy and also found this subset of patients to have clinical outcomes similar to those for patients without the HER-2/*neu* gene alteration. In the literature, concordance rates between IHC and FISH range from 79 to 100% for 3+ cases (Lebeau et al. 2001; Tubbs et al. 2001) and between 12% and 36% for 2+ cases (Ridolfi et al. 2000; Perez et al. 2002), which demonstrates the importance of a gene amplification test.

Recently, two new methods have been described for measuring gene copy number: multiplex amplifiable probe hybridization (Armour et al. 2000) and MLPA (Schouten et al. 2002). Both techniques rely on comparative quantitation of specifically bound probes that are amplified by PCR with universal primers. The introduction of universal primers has advantages because multiplexing numerous targets becomes much easier, and when fluorescence detection of products is used, only one fluorescent primer is required, thus reducing the cost compared to buying fluorescent probes for each target. Technically, FISH has disadvantages compared to MLPA for determining partial gene deletions and remains a relatively low throughput method compared to other molecular genetic techniques. The latter limitation also applies to Southern blotting, where only a few samples can be run per gel, a limited number of loci can be queried per blot, and the tests may take several days (Sellner and Taylor 2004). Real time PCR also can be used as a semiquantitative technique when an internal amplification control is incorporated, and it has the advantage of not requiring post-PCR analysis; however, the number of targets that can be interrogated in a reaction is limited by the number of fluorophores available and the detection capabilities of the instrument. In general, PCR-based techniques for gene dosage determination offer a less labor intensive alternative with higher throughput.

In the study reported here, we evaluated for the first time MLPA as a simple and rapid PCR based test for HER-2/*neu* gene amplification. In our group of 60 Indonesian breast cancer patients, HER-2/*neu* overexpression was found in 24 cases (40%). Forty-seven cases showed no amplification by MLPA, and 13 cases (22%) were amplified. Comparison between IHC and MPLA showed that none of the 36 IHC negative cases were amplified and as many as eight of nine (89%) IHC 3+ tumors showed gene amplification by MLPA assay. Therefore, there was complete concordance between IHC and MLPA in (36+8)/60 (73%) of cases. None of the IHC 1+ cases was MLPA amplified. FISH has been shown to detect amplification in 6- 7% of negatively staining tumors (Pauletti et al. 2000); MLPA also may prove to have some additional value in these cases in larger future studies. As many as five of eight (63%) 2+ cases, however, were amplified by MLPA, indicating that the additional value of MLPA lies especially in the IHC 2+ cases. Therefore, we propose MLPA as a quick and inexpensive method for detecting HER-2/*neu* amplification in daily laboratory practice. Although IHC still is useful as an initial screening tool, MLPA may be an attractive alternative to FISH for amplification testing for IHC 2+ cases. Because MLPA can be used readily on paraffin embedded tissue, the method is widely applicable.

The percentage of patients with tumors that overexpressed HER-2/*neu* in our study was similar to the 10-30% that has been described for Western women, as well as other studies of white, African- American and Hispanic women (Elledge et al. 1994). Another study of HER-2/*neu* expression in Asian women also reported a

positivity rate similar to that found in Western women (Horiguchi et al. 2000). A higher incidence of HER-2/*neu* positivity (approximately 50%) has been noted, however, in Japanese (Osaki et al. 1992; Yokota et al. 1999) and Korean women (Choi et al. 2003) who are ethnically related. Merchant et al. (Merchant et al. 1999) compared expression levels of HER-2/*neu* in an effort to explain the differences in outcome between British and Japanese women. Those authors found similar rates of expression in both populations. Overall, there are no strong indications that there are significant differences in HER-2/*neu* overexpression or amplification between Western and Asian women.

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Chapter 5

Histopathological features of early onset Indonesian breast cancer pointing to *BRCA1*/2 germline mutations

Manuscript

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ABSTRACT

Breast cancer under 40 years concerns a relatively small subgroup of cases that tend to display a more aggressive phenotype. Compatible with this, early age of onset has been known as one of clinical characteristic of hereditary breast cancers associated with germline *BRCA1* or *BRCA1* mutations. As early onset breast cancer is frequent in Indonesia, we investigated the histopathological and immunohistochemical characteristics of early onset ($\leq$ 40 years) Indonesian breast cancer patients, as such features can be used to distinguish between BRCA and non-BRCA germline mutation carriers among these young women.

Thirty-five formalin-fixed and paraffin-embedded tissue sections of young women (mean 36 years, range 22-40 years) who underwent surgical resection at the Department of Surgery of the Sardjito Hospital, Jogjakarta were examined for pathological features, estrogen and progesterone receptor status, proliferation as determined by Ki67 labeling, EGFR and CK5/6 and the presence of HER-2/*neu* and p53 protein. Additionally, mutation analysis for *BRCA1* and *BRCA2* was performed in 30 young women. The control group consisted of carcinomas from women above 50 years (mean 59.02, range 50-80 years).

Carcinomas occurring in women aged below 40 years were more often advanced stage and higher proliferating (p=0.006). Among the early onset breast cancer patients, ductal type, grade 3, ER and HER-2/*neu* negativity, high Ki67 index and CK5/6 and EGFR positivity were typical for *BRCA1* patients. Unfortunately, there were no typical phenotypical features for *BRCA2* carriers. However, grade I and lobular cases were never BRCA1/2 germline mutated.

In conclusion, early onset Indonesian breast cancer shows increased proliferation compared to late onset patients. Within the early onset group, the strongest features pointing to a sporadic cancer seem to be grade I and lobular differentiation. Features increasing the chance of a germline *BRCA1*/2 mutation are CK5/6 and EGFR expression, p53 accumulation and high proliferation as measured by Ki67 labeling. This is potentially useful to optimize selection of early onset breast cancer patients for *BRCA1/2* mutation testing.

INTRODUCTION

Breast cancer in patient under 40 years old is uncommon. The National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program reveals that 75% of breast tumors occur in women age >50 years, only 6.5% in women age <40 years, and a mere 0.6% in women age <30 years [1, 2]. Carcinomas from younger patients tend to be more aggressive with a higher incidence of poorly differentiated (grade III) carcinomas and more hormone insensitive (ER/PR negative) tumors. Additionally, the tumors are often rapidly proliferating and express abnormal p53 [2-4].

Of the this relatively small fraction of young patients, a disproportionally large fraction has hereditary breast cancer. It has been known that over 80% of families with two or more cases of premenopausal breast cancer and two or more cases of ovarian cancer carry a germline *BRCA1* or *BRCA2* mutation [5, 6]. Therefore, a diagnosis of breast carcinoma at young age greatly impacts these patients and their families.

Clinically, a hereditary basis of breast cancer is, apart from early onset and family history of (early onset) breast cancer, recognized by contralateral breast cancer, male breast cancer, and ovarian/Fallopian tube cancer [7, 8]. Sometimes, however, family history is incomplete, even in developed countries. In Indonesia, incompleteness of family history is common as a significant number of patients do not seek medical treatment, families tend to be bigger, and patients are thereby often poorly informed on the exact medical problems in their families. So, young age at presentation is in developing countries even more important to indicate a potential hereditary condition.

Genetic testing for *BRCA1* and *BRCA2* is complex and time-consuming due to the large size of both genes, and the presence of mutations throughout their entire coding regions. Therefore, it is important to find additional clinical or pathological factors that could suggest the presence of *BRCA1* or *BRCA2* mutations in a patient.

Several studies have compared the characteristics of breast cancers in *BRCA1* carriers and sporadic controls. Distinct features of *BRCA1*-associated tumor have been proposed, such as high tumor grade, estrogen (ER) and progesterone receptor (PgR) negativity [9, 10], accumulation of p53 [9, 10], expression of the epidermal growth factor receptor (EGFR) [11-13], and absence of amplification and overexpression of HER-2/*neu* [14, 15]. Additionally, hereditary breast cancers are preferentially of the ductal and medullary carcinoma types [16, 17]. cDNA expression analyses and expression of cytokeratins 5/6 have suggested a basal epithelial phenotype for *BRCA1* related cancers [18]. However, the phenotype of *BRCA2* related cancers is much less outspoken, and seems to be in between that of *BRCA1* related and sporadic cancers [19]. Whereas in Western countries most hereditary breast cancers are *BRCA1* related, the reverse trend is seen in Indonesia where most hereditary breast cancers seem to be *BRCA2* related [20]. In the current study, we therefore investigated the histopathological and immunohistochemical characteristics of early onset ($\leq$ 40 years) Indonesian breast cancer patients, as such features can be used as to identify the *BRCA1/2* germline mutation carriers among these young women. This could help to limit expensive

mutation screening to those patients at highest risk to harbor a germline *BRCA* mutation.

MATERIALS AND METHODS

Tumor specimens

Formalin-fixed and paraffin-embedded tissue sections were obtained from 35 young women (mean age 36.11 years, range 22–40 years) who underwent surgical resection of primary breast cancer between 2002-2004 at the Department of Surgery, Sardjito Hospital, Yogyakarta, Indonesia. Tumors were staged according to the 5th edition of the American Joint Committee on Cancer TNM classification system [21]. Fourteen tumors were early stage (I/II) and nineteen tumors presented at late stage (III/IV). Specimens included 33 invasive ductal carcinomas (IDCs), and 2 invasive lobular carcinomas (ILCs) according to the WHO [22]. Three tumors were classified as Grade I, 15 as Grade II and 17 as Grade III according to the modified Elston and Ellis grading [23]. Concerning family history, 3/35 patients had a history of breast or ovarian cancers in first-degree female relatives and 3/35 patients had a family history positive for other cancer types. Germline mutation analyses of the entire coding region of *BRCA1/BRCA2* was done in 30 of these patients by pooled denaturing gradient gel electrophoresis with direct sequencing of aberrantly moving bands as described before [20], complemented by multiplex ligation dependent probe amplification to detect genomic deletions [24]. There were two mutations in *BRCA1* (deletion exon 13-15 and exon 16 p.Met165Ile) and five mutations in *BRCA2* (c.3040_3043del 4, p.Met1149Ile, p.Glu699Leu, p.Leu824X all in exon 11 and c.9485-16T>C in exon 25). One of these latter patients (AZ) had in addition to a pathogenic c.2699_2704 delTAAATG mutation in exon 11 of *BRCA2* an unclassified variant mutation in exon 16 of *BRCA1* (p.Met165Ile).

Fifty-four late onset breast cancer (>50 years) from the same hospital were included as a control group (mean 59.02, range 50 – 80 years). On this series, data concerning family history, *BRCA* mutation status, lymph node status, and EGFR and CK5/6 status were lacking.

Immunohistochemistry

Immunohistochemistry was performed on 4 µm thick sections. After deparaffination and rehydration, endogenous peroxidase activity was blocked for 30 minutes in a methanol solution containing 0.3 % hydrogen peroxide. After antigen retrieval in citrate buffer (autoclave except for ER where the microwave was used, a cooling off period of 30 minutes preceded the incubation (overnight at 4°C) with the primary antibodies (p53: DO7, Dako, 1:500; EGFR: Novocastra, 1:10; ER: Dako, 1:50; PR: Novocastra, 1:50; HER-2/*neu*: Novocastra 1:100; Ki67: Dako, 1:40). The primary antibodies were detected using a biotinylated rabbit anti-mouse antibody (DAKO). The signal was amplified by avidin-biotin complex formation and developed with diaminobenzidine followed by counterstaining with haematoxylin, dehydrated in alcohol and xylene and mounted. For Ki67, p53, ER, and PR, only nuclear staining was considered and diffuse cytoplasmic staining was ignored, leading to an estimated percentage of positively stained nuclei. HER-2/*neu* was scored according to the HERCEPT system as negative, 1+, 2+ or 3+, and EGFR staining was scored positive when a clear membrane staining pattern was seen.

Scoring was done blinded to *BRCA1*/2 mutation status by a single experienced pathologist (PvD).

HER-2/neu amplification testing

In addition to immunohistochemistry, HER-2/*neu* was tested for amplification by multiplex ligation dependent probe amplification (MLPA) as described before [25]. In short, 50- 500 ng of target DNA was denatured and the probe mix was added afterward. The mixture was heated and incubated overnight (16 h). Ligation was performed with the temperature stable Ligase-65 enzyme (MRC-Holland) then was inactivated in the thermocycler. Ligated mixture was amplified with one unlabeled and one carboxyfluorescein labeled PCR primers that are complementary to the universal primer sequences. PCR was carried out for 33 cycles. The fragments were analyzed with an ABI model 310 capillary sequencer (Applied Biosystems, Torrence, CA, USA) using Genescan-TAMRA 500 size standards (Applied Biosystems). Fragment analysis was performed using Genescan software. DNA from Centre d'Etude Polymorphisme duHumain (CEPH) was used as a control sample and analyzed simultaneously with breast cancer samples in each run. To objectify interpretation of the fragment analysis, the relative quantity of the amplified probes in each sample was determined using an Excel template. For this purpose, the relative peak areas for each probe were calculated as fractions of the sum of peak areas in a given sample. Subsequently, the fraction of each peak was divided by the average peak fraction of the corresponding probe in control samples. Finally, the values were normalized using the values obtained for the autosomal control probes that served as a reference for the copy number of 2.0. Cases that showed a copy number greater than 2 for at least two of the probes on the HER-2/*neu* locus could be considered amplified. HER-2/*neu* was considered to be "positive" when it was either 3+ overexpressed or amplified by MLPA.

Statistics

Statistical analysis was carried out by SPSS software version 11 for Windows. Continuous variables were tested for differences between the non-mutated and mutated cases using the Mann-Whitney test, and discrete variables with the chi-square test using logical classes. P-values <0.05 were considered significant.

RESULTS

Table 1 shows the main clinicopathological features of our early onset Indonesian breast cancer study group compared to the control group and data from other relevant published studies. There was high prevalence of advanced stage, tumors with poor differentiation, and tumors with lymph node metastases. Most of the cancers were of the ductal type, and special type cancers were lacking. With regard to the immunophenotype, about half the cases were ER and PgR positive, 30% were EGFR positive, 23% were HER-2/*neu* positive, and 23% were CK5/6 positive. The mean percentages of p53 and MIB-1 positive cells were both 30%.

Table 1. Clinicopathological features of a group of early onset (≤ 40 years) Indonesian breast cancers compare to control group (>50 years) and relevant studies.

		Present study		Sidoni et al		Rodrigues et al	
		early	late	early	late	early	late
Feature		**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**
Stage	I/II	13 (41%)	32 (60%)	nd	Nd	Nd	Nd
	III/IV	19 (59%)	21 (40%)	nd	nd	Nd	Nd
		p=0.105					
Type	Lobular	2 (6%)	2 (4%)	2 (4%)	1 (2%)	Nd	Nd
	Ductal	33 (94%)	45 (83%)	44(88%)	45 (90%)	Nd	Nd
		p=0.165					
Grade	I	3 (9%)	2 (4%)	2 (4%)	4 (8%)	3 (15%)	7 (21%)
	II	15 (43%)	19 (36%)	27 (58%)	36 (75%)	9 (45%)	14 (41%)
	III	17 (48%)	32 (60%)	19 (38%)	8 (17%)	8 (40%)	13 (38%)
		p=0.438		P=0.04		P=0.88	
nodal			nd	22(47%)	29 (58%)	Nd	Nd
status	Negative	11 (37%)					
	Positive	19 (63%)	nd	25(53%)	21 (42%)	Nd	Nd
family	Negative		nd	26 (76%)	Nd	11 (55%)	22 (65%)
history		25 (86%)					
	Breast/ovarian		nd	8/34	5/30	8 (40%)	9 (26%)
	cancer	1 (3%)		(24%)	(17%)		
	Other cancers	3 (10%)	nd	nd	Nd	1 (5%)	Nd
ER	Negative	19 (58%)	23 (43%)	23 (46%)	11 (20%)	2 (10%)	7 (21%)
	Positive	14 (42%)	31 (57%)	27 (54%)	39 (80%)	18 (90%)	27 (79%)
		p=0.175		P=0.01		P=0.31	
PgR	Negative	19 (58%)	25 (46%)	25 (50%)	16 (30%)	4 (20%)	10 (30%)
	Positive	14 (42%)	29 (54%)	25 (50%)	34 (70%)	16 (80%)	23 (70%)
		p=0.307		P=0.067		P=0.41	
HER-2/*neu*	not amplified	27 (77%)	43 (80%)	26 (52%)	37 (74%)	7 (35%)	21 (62%)
	Amplified	8 (23%)	11 (20%)	24 (48%)	13 (26%)	13 (65%)	13 (38%)
		p=0.78		P=0.03		P=0.06	
EGFR	Negative	23 (70%)	nd	nd	Nd	Nd	Nd
	Membranous	10 (30%)	nd	nd	Nd	Nd	Nd
CK5/6	Negative	27 (77%)	nd	nd	Nd	Nd	Nd
	Positive	8 (23%)	nd	nd	Nd	Nd	Nd
p53	Negative	21 (62%)	39 (72%)	35 (70%)	46 (92%)	16 (84%)	28 (82%)
	Positive	13 (38%)	15 (28%)	15 (30%)	4 (8%)	3 (16%)	6 (18%)
		p=0.305		P=0.01		P=0.86	
Ki67	low	9 (26%)	30 (56%)	nd	Nd	12 (60%)	25 (74%)
	high	26 (74%)	24 (44%)	nd	nd	8 (40%)	9 (26%)
		p= 0.006				P=0.30	

Comparison with control group of late onset (>50 years) breast cancer for several clinicopathologic features (Table 1) showed that high Ki67 (>10%) was more frequent in early onset patients (74% to 44%, p= 0.006). Tumor stage, tumor type and ER staining only showed tendency for significance (p=0.105, p=0.165 and p=0.175 respectively) while the other features showed no significance at all.

Comparison of continuous features between non-*BRCA* and *BRCA* mutation carriers did not reveal statistically significant differences (Table 2). Further comparison between non-*BRCA* and *BRCA* carriers among early onset breast cancer with regard to the discrete histopathological features did also not show significant differences although BRCA germline mutation carriers had more advanced stage and had never lobular or grade I carcinomas (Table 3), except for

family history (p=0.025) where twenty-two non-*BRCA* cases all had negative family history for breast/ovarian cancer or other tumors.

Table 2. Means of continuous clinicopathological features of early onset Indonesian breast cancers (=≤ 40 years) according to *BRCA1/2* mutation status. None of the values were statistically significant between *BRCA* mutation carriers and patients without mutations.

	N	no mutation		*BRCA* mutation		*BRCA1* mutation		*BRCA2* mutation	
		mean	N	Mean	N	mean	n	Mean	N
Age	30	35.96	23	36.14	7	40	2	34.6	5
Tumor size	29	3.09	23	3.33	6	4	2	3	4
ER	28	29.81	21	41.43	7	0	2	58	5
PgR	28	24.95	21	24.43	7	25	2	24.2	5
Ki67	30	25.96	23	32.14	7	20	2	37	5
p53	29	24.23	22	53.14	7	90	2	38.4	5

DISCUSSION

It is widely believed that breast cancer in young women is characterized by relatively unfavorable prognostic pathologic features. Published studies on this issue are however not easily compared because of differences in the age ranges, and the prognostic features considered. The majority of epidemiological studies have documented an adverse outcome of breast cancer in young patients [26, 27] independent from other factors [27].

With these considerations in mind the present study was undertaken to analyze some well-defined pathological prognostic factors in a series of 35 invasive breast cancer patients below 40 years of age and compared them with a control group of postmenopausal aged over 50 years.

The results of our study show that breast carcinoma in Indonesian women below 40 years of age differ especially in proliferation as measured by Ki67 positivity. As rate of proliferation is of overriding prognostic importance and the most important component of grade [28], this is consistent with those studies showing higher prevalence of grade 3 cancers [29, 30] and worse prognosis in young women [3, 26, 31], although Rodrigues et al [30] found no significance for Ki67 in their study. Grade itself was not significant in our study, and the results on grade in the literature and conflicting [29, 30]. ER, PR, p53 and HER-2/*neu* were not significant in our study as well, and also for these features other studies are conflicting [29, 30].

Early onset breast cancer is often hereditary [32]. In recent years, the histopathological features of *BRCA1* germline mutation related tumors have been well characterized. They are most frequently of ductal and medullary histological type [6], more frequently poorly differentiated [6, 17, 33-36], usually ER [14, 17, 34-38] , PR [14, 34-36, 39] and HER-2/*neu* [40, 41] negative, and show a high degree of positivity for CK5/6 [40], EGFR [11-13] and p53 accumulation [39, 42, 43]. Fully consistent with this, our few *BRCA1* related cases were advanced stage, ductal type, grade 3, ER and HER-2/*neu* negative, and EGFR, CK5/6 and p53 positive.

Table 3. Frequencies of discrete clinicopathological features of early onset Indonesian breast cancers (=< 40 years) according to *BRCA1/2* mutation status.

Features	n	no mutation		BRCA mutation		BRCA1 mutation		BRCA2 mutation	
		freq	(%)	freq	(%)	freq	(%)	freq	(%)
stage	30								
I/II	12	9	39	3	25	0	0	3	60
III/IV	18	14	61	4	22	2	100	2	40
type	30								
lob	2	2	9	0	0	0	0	0	0
duc	28	21	91	7	100	2	100	5	100
grade	29								
well	3	3	14	0	0	0	0	0	0
mod	11	7	32	4	57	0	0	4	80
poor	15	12	55	3	43	2	100	1	20
nodal status	29								
negative	10	8	36	2	29	1	50	1	20
positive	19	14	64	5	71	1	50	4	80
fam history	29								
no	25	22	100	3	43	1	50	2	40
yes	1	0	0	1	14	1	50	0	0
other	3	0	0	3	43	0	0	3	60
		p= 0.025							
ER	28								
negative	15	11	52	4	57	2	100	2	40
positive	13	10	48	3	43	0	0	3	60
PgR	28								
negative	14	11	52	3	43	1	50	2	40
positive	14	10	48	4	57	1	50	3	60
her2	30								
not ampl	23	17	74	6	86	2	100	4	80
amplified	7	6	26	1	14	0	0	1	20
EGFR	28								
negative	19	16	76	3	43	0	0	3	60
positive	9	5	24	4	57	2	100	2	40
CK5/6	30								
negative	24	20	87	4	57	0	0	4	80
positive	6	3	13	3	43	2	100	1	20
p53	29								
negative	18	15	68	3	43	0	0	3	60
positive	11	7	32	4	57	2	100	2	40
Ki67	30								
negative	8	7	30	1	14	1	50	0	0
positive	22	16	70	6	86	1	50	5	100

The phenotype and genotype of breast cancers in *BRCA2*-mutation carriers seems to be less outspoken and lies between that of *BRCA1* mutation carriers and sporadic controls [19]. Better defining the phenotype of *BRCA2* related breast cancers is especially important in Asian populations like ours, as the prevalence of *BRCA2* related cancers exceeds that of *BRCA1* related cancers [24]. *BRCA2* tumors are more frequently of ductal type [44] as in the present study where all

BRCA2 related tumors were ductal. They are also more frequently grade 2 and 3 than sporadic controls [6, 34, 35]. In line with these previous studies, *BRCA2* carriers had in the present study moderately or poorly differentiated tumors and none of them were well differentiated. The frequency of ER and PR expression in *BRCA2* tumors has been reported as similar to that in sporadic breast tumors most studies [14, 35, 36, 39], in line with the present study. As to p53, some studies have found p53 accumulation in around 20-50% of *BRCA2* related carcinomas [14], In the present study, 60% of the *BRCA2* associated tumors showed p53 accumulation. Data on HER-2/*neu* expression in *BRCA2*-associated tumors vary from series to series, probably as a consequence of differences in the techniques employed. For example, Armes et al. [39] and Eerola et al. [36] found no differences in the expression of HER-2/*neu* in *BRCA2* and sporadic breast tumors. However, other studies revealed low frequencies between 0 and 3% in HER-2/*neu* overexpression in *BRCA2* tumors [14, 17, 35]. Combining immunohistochemistry with a new amplification test, we found a 20% frequency of HER-2/*neu* positivity in *BRCA2* associated tumors.

Concerning cytokeratin 5/6, 20% of our *BRCA2* cases were positive, compared to 15.4% (N=48) in the literature [13]. There are few data on *EGFR* and Ki67 index in breast cancers of *BRCA2* mutated cases. One study reported a 100% EGFR positivity [19] in *BRCA2* related breast cancer (N=5), another one 8% (N=48) [13] compared to 40% in the present study. As to Ki67, one study reported a median Ki67 of 20% in *BRCA2* cancers, a little lower than the 37% mean in our study. These studies, like ours, concern however just a few cases, so these results will not be significantly different.

The results of the present study add to the concept that breast cancer arising in *BRCA1* and *BRCA2* mutation carriers of mutation in the genes differ from sporadic breast cancer of age matched controls. This is especially clear for *BRCA1* related cancers. Unfortunately, also our study has not firmly established a clear phenotype for *BRCA2* related breast cancers. One of the most important applications of this information would be its use as a guide for genetic testing. Although we have developed a fast and relatively cheap method for BRCA1/2 mutation detection [20], such testing is still difficult to afford for most Indonesian patients. Currently, young age and positive family history are the best predictors of a high likelihood of carrying a *BRCA1/2* mutation. However, to optimize screening, it would for early onset Indonesian patients that are often oblivious to their family history be quite useful if histopathological features, in conjunction with clinical data, could be used to raise the a priori chance of a germline mutation (table 3). To this end, the strongest features pointing to a sporadic cancer seem to be grade I and lobular differentiation. Features increasing the chance of a germline *BRCA1*/2 mutation are CK5/6 and EGFR expression, p53 accumulation and high proliferation as measured by Ki67 labeling.

In conclusion, early onset Indonesian breast cancer is characterized by increased proliferation. Within the early onset group, the strongest features pointing to a sporadic cancer seem to be the absence of family history of breast and or ovarian cancer, grade I and lobular differentiation. Features increasing the chance of a germline *BRCA1*/2 mutation are family history of breast and or ovarian cancer, CK5/6 and EGFR expression, p53 accumulation and high proliferation as

measured by Ki67 labeling. This may be potentially useful to optimize selection of early onset breast cancer patients for *BRCA1/2* mutation testing.

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Chapter 6

High throughput analysis of promoter hypermethylation status of 22 tumor suppressor genes in invasive breast cancer

Manuscript

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ABSTRACT

A number of different tumor suppressor genes are known to be inactivated by aberrant hypermethylation in breast cancer, but it is still unknown to what extent these epigenetic alterations differ according to specific breast cancer phenotypes. This is largely caused by the fact that most studies concern only a few genes due to the low throughput of traditional techniques. We therefore investigated hypermethylation of 22 tumor suppressor genes in 168 breast cancers by methylation-specific multiplex ligation-probe dependent amplification (MS-MLPA), a new high throughput technique, to assess whether hypermethylation identifies breast cancers with distinctive clinicopathological features.

MS-MLPA showed methylation frequencies ranging from 0% for *CDKN2A* (*p14*^{ARK}), *CDKN1B* (*p27*^{KIP1}), *ATM*, *PTEN*, *BRCA2* and *VHL* to 26% for CDH13, 33% for GSTP1, 40% for APC and 61% for *RASSF1*. Tumors with frequent methylation (4-8 genes) were more often poorly differentiated (P=0.007) and HER-2/*neu* amplified (P=0.041) compared to those with infrequent methylation (0-2 genes).

In conclusion, this comprehensive analysis of tumor suppressor gene promoter methylation status in invasive breast cancer by high throughput MS-MLPA reveals remarkable differences in methylation frequency for various genes. Promoter methylation of multiple genes seems to be correlated to poor differentiation and HER-2/*neu* amplification. A more comprehensive hypermethylation profile as assessed by MS-MLPA could therefore potentially be useful for breast cancer detection and classification, and understanding the biology of this disease.

INTRODUCTION

Breast cancer is the most common malignancy in women and represents 18% of all female cancers. The incidence of breast cancer increases with age, and in Western countries the disease is the single most common cause of death among women. The natural history of breast cancer is characterized by a long duration and marked heterogeneity between patients. Tumors with similar histopathological appearance can follow significantly different clinical courses and show different responses to therapy. The presence of hormone or growth factor receptors identify subsets of patients responsive to targeted or endocrine therapy, but even in these groups the clinical outcome can be heterogeneous [1].

Cancer is a disease in which cells suffer from multiple genomic changes resulting in the aberrant expression of many genes which in turn form tumor specific phenotypes [2-5]. These genomic changes may comprise genetic changes such as gene copy number changes or point mutations, but also epigenetic gene silencing due to promoter methylation can contribute to the tumor specific profile of gene expression. In the mammalian genome, methylation takes place only at cytosine bases that are located 5′ to a guanosine in a CpG dinucleotide which is significantly under-represented [6]. Most cytosines within CpG dinucleotides are methylated in the human genome, but some remain unmethylated in short regions of 0.5–4 kb in length that are rich in CpG content, known as CpG islands [6, 7]. Over 50% of the protein coding genes have at least one CpG island within or near their promoters, and the expression of these genes is sensitive to the methylation status of such CpG islands [5], being generally unmethylated in normal cells. In cancer, however, hypermethylation of these promoter regions within a background of global hypomethylation is now the most well-categorized epigenetic change to occur, found in virtually every type of human neoplasm [5, 8]. Hypermethylation of the CpG islands is associated with delayed replication, condensed chromatin, and inhibition of transcription initiation. Therefore, there must be a gene-specific mechanism that determines the methylation profile of the promoter CpG islands both in normal and tumor cells.

Many recent studies demonstrated that methylation can contribute to inactivation of tumor suppressor genes, a key event in tumorgenesis of a wide spectrum of human tumors [5, 9-12], including breast cancer [13, 14]. However, the majority of such studies have dealt with only one or just a few genes [15, 16], applying techniques based on the conversion of unmethylated cytosine residues into uracil after sodium bisulphite treatment [17], which are converted to thymidine during subsequent PCR. Thus, after bisulphate treatment, alleles that were originally methylated have different DNA sequences compared to their corresponding unmethylated alleles. These differences can be exploited by several techniques, such as methylation specific PCR (MSP), restriction-digestion (COBRA), methyl-light, direct sequencing, denaturing high-performance liquid chromatography (DHPLC), nucleotide extension assays (MS-SnuPE), methylation-specific oligonucleotide (MSO) microarray and HeavyMethyl [17-24]. However, most of these methods are labor intensive and/or allow the study of methylation status of only one gene at the time. Here, we performed for the first time a high throughput easy method based on methylation specific Multiplex Ligation dependent Probe Amplification (MS-MLPA) [25] to assess the methylation status of 22 selected tumor suppressor genes in a large group of breast cancer patients.

MATERIALS AND METHODS

Tissue samples

Tumor tissues were obtained during surgery from 168 breast cancer patient at the Sardjito Hospital Jogjakarta, Indonesia in the years 2002-2004. All samples were freshly obtained and put into a -80º C freezer for storage. The histological type and grade of the tumors were classified according to the World Health Organization criteria. Patient age at diagnosis ranged from 19 to 80 years (mean 49). Genomic DNA was extracted from frozen tissues using Qiamp DNA mini kit (Qiagen, Germany) as described in the manufacturer's protocol. HER-2/*neu* gene amplification status was assessed by MLPA as described before [26].

For comparison, 46 paraffin embedded tissue of Dutch invasive breast cancer samples from the archives of the Department of Pathology of the VU University Medical Center were used.

Methylation specific – MLPA (MS-MLPA)

The principle of MLPA has been described elsewhere [27]. MLPA probes to assess methylation are similar to ordinary MLPA probes except that the sequence detected by the MS-MLPA probe contains a recognition sequence for the methylation sensitive restriction enzyme *Hha*I. Upon digestion of the sample DNA/probe hybrids with *Hha*I, probes of which the recognition sequence is unmethylated in the sample analyzed, will not generate a signal. In contrast, a normal probe signal will be detected if the site is methylated. We used the ME001 MS-MLPA probe mix (MRC Holland, Amsterdam, The Netherlands) which contains 24 sequences that correspond to the promoters of a set of tumor suppressor genes that are frequently silenced by methylation in different tumors (including breast cancer), but are unmethylated in blood derived DNA of healthy individuals: *hMLH1, BRCA2, VHL, RABβ, TIMP3, DAPK1, CDKN2B (p15^{INK4b}), CDKN2A (p14^{ARF}), APC, PTEN, CDKN1B (p27^{KIP1}), ATM, HIC1, CASP8, CD44, ESR1, RASSF1, TP73, FHIT, IGSF4, CDH13, and GSTP1* (there are two probes for both *RASSF1* and *MLH1*). In addition, 15 different control probes are present that are not influenced by the *Hha*I digestion.

The initial hybridization step of the probemix to the DNA is similar to conventional MLPA [27]. However, after 16 hrs when hybridization of the probes to the sample DNA is completed, the sample reaction is divided into two parts. The first part is processed as an ordinary MLPA reaction and provides information on copy number changes. The second part of the MLPA hybridization reaction is digested with *Hha*I while the hybridized probes are ligated. Hybrids of (unmethylated) probe DNA and unmethylated sample DNA are digested by the *Hha*I enzyme and thus can not be exponentially amplified by PCR and will not generate a signal when analyzed by capillary or gel electrophoresis. However, if the sample DNA is methylated, the hemi-methylated probe-sample DNA hybrids cannot be digested by *Hha*I and the ligated probes will generate a MS-MLPA probe signal after PCR.

Thereby, each MS-MLPA reaction will yield two samples to be analyzed; one undigested sample that is only ligated and one sample that is simultaneously digested and ligated. Quantification of the methylation status can be done by

comparing the relative peak signal of these two samples for the 22 different genes. Aberrant methylation was scored when the digested:undigested peak ratio was >= 0.1. Any peak ratios below this level were regarded as background.

In order to validate the MS-MLPA results, we also applied MS-PCR on 32 samples for *ESR1* and *hMLH1* because these genes represent high and low methylation frequencies in breast cancer, respectively [14, 28].

Statistical Analysis

The χ2-test was used to determine associations between the methylation status of individual genes on the one hand and phenotypic and molecular features of breast cancer on the other. The early onset patients (=< 40 years) were analyzed separately as this subgroup is relatively large in Indonesia. Fisher's exact test was used when individual cell numbers were less than 5. All P values were derived from two-tailed statistical tests and significance was assumed at <0.05. The Mann-Whitney U test was used to compare stage, histological type and histological grade between tumors with and without methylation. All analyses were performed using the SPSS 11.0 for Windows statistical software package.

RESULTS

We assessed aberrant promoter methylation of CpG islands in a total of 22 gene promoter regions in a single experiment in 168 breast tumor samples applying for the first time MS-MLPA, a new high throughput method, to invasive breast cancer. Six genes displayed no promoter methylation in any of 168 tumor samples: *CDKN2A (p14^{ARK})*, *CDKN1B (p27 ^{KIP1}))* *ATM*, *PTEN*, *BRCA2* and *VHL*. Methylation frequencies for the other genes ranged from 1% for *TP73* and *FHIT* to 61% for *RASSF1* (Table 1).

Of the 168 tumor samples, 33 (20%) showed no methylation at any promoter site. 30 cases (18%) were methylated at the promoter site of only 1 gene, 35 (21%) at 2 sites, 32 (19%) at 3 sites, 21 (13%) at 4 sites, 11 (7%) at 5 sites, 2 (1%) at 6 sites, 3 (2%) at 7 sites and 1 (1%) at 8 sites. No tumor was methylated at all 22 sites.

Figure 1 shows an example of a breast cancer showing methylation on *APC* (148 bp), *RASSF1* (328 bp), *RASSF1* (382 bp) and *GSTP1*(454 bp) (Figure 1c). Figure 1b and 1d show capillary electrophoresis-pattern from the same sample but without *HhaI* treatment, showing the undigested peak heights that were used for quantification of the methylation levels. For *APC*, *RASSF1* (382 bp) and *GSTP1*, 100% methylation occurred, indicating that methylation is present in both alleles, whereas the *RASSF1* (328 bp) probe showed a 50% reduction of the MS-MLPA signal, corresponding to the presence of only one methylated allele.

When comparing methylation status between early onset patients (≤ 40 years) with the remaining patients, there were no significant associations between methylation of each of the gene with the age of patients.

Table 1 also shows associations between the methylation status of each gene and various clinicopathological features. Most associations were weak, with significant associations between *CDH13* methylation and poor differentiation and amplification of HER-2/*neu*, *RASSF1* methylation with ductal histological type, poor differentiation and positive HER-2/*neu*, *TIMP3* methylation with lobular histological

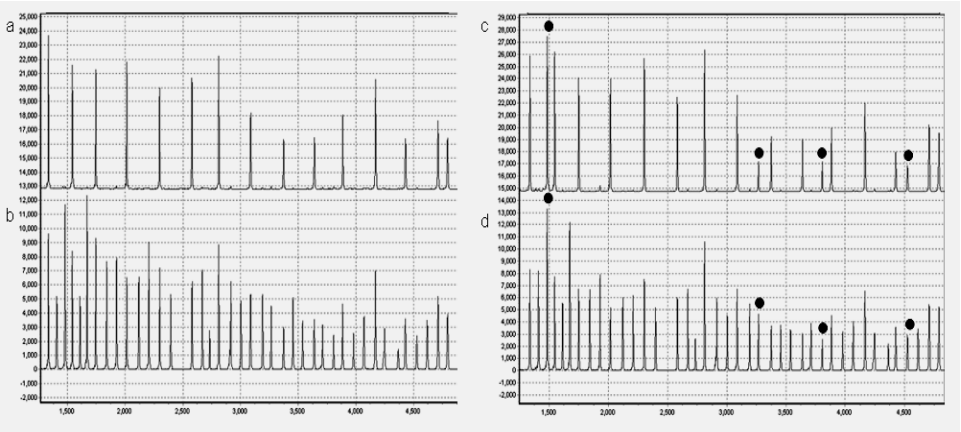

Figure 1. Detection of aberrant methylation pattern in breast cancer samples by MS-MLPA using the ME001 probe mixture. (a). Capillary electrophoresis (CE) pattern from a control sample, showing no methylation in any probe. Total absence of all MS-MLPA probes indicates 100% efficiency in the digestion reaction. (b) CE-pattern from the same sample but without *HhaI* treatment, showing undigested peak heights that were used for quantification of the mehylation levels. (c) CE-pattern from a breast cancer sample showing promoter methylation of *APC* (148 bp), *RASSF1* (328 bp), *RASSF1* (382 bp) and *GSTP1* (454 bp) (black dots). (d) CE-pattern from the same sample [as shown in c] but without *HhaI* treatment

type, *APC* methylation with HER-2/*neu* positivity, *RARb* methylation with poor differentiation, and *GSTP1* methylation with positive lymph node status and HER-2/*neu* amplification. Most associations were thus seen with HER-2/*neu* status, where four genes showed a higher methylation frequency in tumors with amplification of HER-2/*neu*. Tumors with infrequent methylation (0–2 genes) were more often negative for HER-2/*neu* compared to those with frequent methylation (3-8 genes; 63% vs 37%, P=0.041). Tumors with infrequent methylation were more often well differentiated than those with frequent methylation (63% vs 38%, P=0.007). No significant associations were seen between nodal involvement or stage or menopausal status and methylation of any gene.

Methylation of *APC* and *RASSF1*, *APC* and *GSTP1*, *RASSF1* and *GSTP1, RARb* and *CD44* genes showed strong positive associations, while methylation of *RARb* showed trends for association with methylation of *DAPK1*, *RASSF1* and *GSTP1*, as well as *CDKN2B* and *TIMP3*, *HIC1* and *IGSF4*, *GSTP1* and *CD44* (Table 2).

In the 46 Dutch invasive breast cancer samples, two of the six genes that displayed no methylation in Indonesian samples (*ATM* and *VHL*) also did not show any methylation, whereas the other four genes ((*CDKN2A (p14*^{ARK}), *CDKN1B (p27*^{KIP1}), *PTEN*, and *BRCA2)* showed low methylation frequencies of 4% (2/46), 2% (1/46), 4% (2/46) and 4% (2/46), respectively. Methylation of *RASSF1* (32/46, 70%) as well as *GSTP1* (26/46, 57%) and *RARB* (4/46, 9%) were in line with Indonesian samples, whereas methylation frequencies were higher for *ESR1* (13/46, 28%, p<0.0001) and *TIMP3* (9/46, 20%, p=0.002).

Table 1. Association between gene promoter methylation status assessed by MS-MLPA and clinicopathological features of 168 breast cancer cases. Six genes (*CDKN2A (p14^{ARK})*, *CDKN1B (p27 ^{KIP1})*) *ATM*, *PTEN*, *BRCA2* and *VHL*) that did not show methylation in any of the cases are not listed.

Feature (n)		TIMP3		APC		RARβ		CDKN2B		HIC1		CASP8		CD44		DAPK1		ESR1		RASFF1		TP37		FHIT		IGSF4		CDH13		GSTP1		MLH1	
		N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Total	168	9	5	68	40	15	9	14	8	3	2	13	8	5	3	14	8	4	2	103	61	1	1	1	1	2	1	44	26	56	33	3	2
Age	167																																
<=40	44	2	5	17	39	4	9	1	2	0	0	5	11	0	0	4	9	1	2	22	50	0	0	0	0	0	0	10	23	16	36	0	2
>40	123	7	6	50	41	11	9	13	11	3	2	7	6	5	4	10	8	3	2	80	65	1	1	1	1	2	2	34	28	40	33	3	2
Menopausal status	168																																
pre-menopause	92	3	3	33	36	7	8	5	5	0	0	6	7	2	2	7	8	3	3	51	55	0	0	0	0	0	0	24	26	31	34	1	1
post-menopause	76	6	8	35	46	8	11	9	12	3	4	7	9	3	4	7	9	1	1	52	68	1	1	1	1	2	3	20	26	25	33	2	3
Stages	164																																
I/II	57	2	4	21	37	4	7	6	11	0	0	4	7	3	5	2	4	2	4	32	57	1	2	0	0	0	0	13	23	17	30	2	4
III/IV	107	7	7	45	42	10	9	7	7	2	2	8	7	1	0.9	12	11	2	2	68	64	0	0	1	1	1	1	31	29	37	35	1	1
Histological type	167																																
Ductal	154	6	4	66	43	15	10	11	7	3	2	13	8	5	3	14	9	3	2	100	65	1	1	1	1	2	1	43	28	53	34	3	2
Lobular	5	2	40	1	20	0	0	2	40	0	0	0	0	0	0	0	0	0	0	1	20	0	0	0	0	0	0	1	20	2	40	0	0
Other	8	1	13	1	13	0	0	1	13	0	0	0	0	0	0	0	0	1	13	2	25	0	0	0	0	0	0	0	0	1	13	0	0
		P=0.004																		*P=0.003*													
Histological grade	161																																
I	8	0	0	3	38	0	0	1	13	0	0	0	0	0	0	1	13	1	13	4	50	0	0	0	0	0	0	2	25	0	0	0	0
II	76	3	4	32	42	5	7	3	4	1	1	5	7	3	4	6	8	1	1	43	57	1	1	0	0	1	1	12	16	27	36	0	0
III	68	3	4	51	75	10	15	7	10	2	3	8	12	2	3	6	9	1	1	50	74	0	0	0	0	1	1	27	40	25	37	3	4
						P=0.03														*P=0.002*								*P=0.002*					
Nodal involvement	151																																
Negative	39	3	8	13	33	1	3	3	8	0	0	1	3	1	3	2	5	3	8	23	59	0	0	0	0	0	0	8	21	8	21	2	5
Positive	112	6	5	47	42	2	2	9	8	3	3	11	10	3	3	11	10	1	1	70	63	0	0	1	1	2	2	13	12	43	38	1	1
																														P=0.042			
HER-2/*neu*	168																																
Not amplified	122	7	6	42	34	8	7	11	9	3	2	11	9	3	2	12	10	3	2	65	53	1	1	0	0	2	2	22	18	34	28	3	2
Amplified	46	2	4	25	54	7	15	3	7	0	0	2	4	2	4	2	4	1	2	38	83	0	0	1	2	0	0	22	48	22	48	0	0
				P=0.024																*P=0.001*								*P=0.000*		*P=0.014*			

Table 2. Associations between promoter methylation of different genes assessed by MS-MLPA in 168 breast cancer cases. Six genes (*CDKN2A (p14^{ARK})*, *CDKN1B (p27 ^{KIP1})*) *ATM*, *PTEN*, *BRCA2* and *VHL*) that did not show methylation in any of the cases are not listed.

	APC	***RARβ***	***CDKN2B***	***HIC1***	***CASP8***	***CD44***	***DAPK1***	***ESR1***	***RASFF1***	***TP37***	***FHIT***	***IGSF4***	***CDH13***	***GSTP1***	***MLH1***
TIMP3	NS	NS	0.029	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.007	NS
APC		0.007	NS	NS	NS	NS	NS	NS	0.000	NS	NS	NS	NS	0.000	NS
RARβ			NS	NS	NS	0.000	0.024	NS	0.035	NS	NS	NS	NS	0.022	NS
CDKN2B				NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
HIC1					NS	NS	NS	NS	NS	NS	NS	0.036	NS	NS	NS
CASP8						NS	0.014	NS	NS	NS	NS	NS	NS	NS	NS
CD44							NS	NS	NS	0.03	NS	NS	NS	0.043	NS
DAPK1								NS	NS	NS	NS	NS	NS	0.016	NS
ESR1									NS	NS	NS	NS	NS	NS	NS
RASFF1										NS	NS	NS	0.004	0.000	NS
TP37											NS	NS	NS	NS	NS
FHIT												NS	NS	NS	NS
IGSF4													NS	NS	NS
CDH13														0.018	NS
GSTP1															NS

When comparing MS-MLPA with MS-PCR, methylation status of *hMLH1* by MS-MLPA and MS-PCR was concordant. The six *ESR1* MS-MLPA methylated samples showed also methylation by MS-PCR, as well as 19/26 of the non-methylated MS-MLPA samples.

DISCUSSION

In an attempt to better understand the epigenetic events involved in breast cancer development and progression, we examined the promoter methylation status of multiple tumor suppressor genes in invasive breast cancers in a single experiment using a new high throughput method: MS-MLPA. We included cell cycle regulators operating in the $p16^{INK4a}$ pathway ($p15^{INK4b}$) or in the p53 pathway ($p14^{ARK}$), as well as $p27^{KIP1}$, a cyclin-dependent kinase inhibitor. The remaining genes were the p53 analogue p73, the alternative form of a tumor suppressor in the Ras mediated signal transduction pathway (*RASSF1*), as well as *VHL*, *APC* and *PTEN*. Also included were *CDH13, a* gene encoding a cell membrane protein active in intercellular interactions, *hMLH1* and *BRCA2* that act in DNA repair and a gene acting in apoptosis, *DAPK1*.

The MS-MLPA technique described here shows to be a robust method for high throughput analysis of DNA methylation. In MS-MLPA, the ligation of the probes while hybridized to their target sequence is combined with simultaneous digestion of these complexes with methylation sensitive restriction endonucleases such as *Hha*I. Complete digestion was also apparent by the disappearance of all MS-MLPA probes in a MS-MLPA reaction (figure 1a), whereas incomplete digestion would result in general background peak signals of all MS-MLPA probes. The different probes have been designed to differ enough in length so that, after multiplex amplification in one reaction, the PCR products can well be separated on a gene scanner and quantitatively analyzed. In order to avoid false negative results, MS-MLPA probes were included that only harbor *Hha*I recognition sites within the hybridizing sequences. Sometimes (figure 1), the results for different probes for the same gene were not fully concordant. In general, a CpG island is either completely methylated or not, but in certain genes there seems to be a critical region for methylation. In addition, several genes have parts that are always methylated. Contamination by non-tumor DNA could be another reason that leads to a reduced signal in the MS-MLPA for genes that show tissue specific expression.

We also compared MS-MLPA with MS-PCR for *ESR1* and *hMLH1* in order to validate our results, because these two genes respectively represent high and low methylation frequencies in breast cancer in the literature [14, 28], whereas in our study both genes had low methylation frequencies. For *hMLH1,* MS-MLPA and MS-PCR results were completely consistent. MS-PCR on *ESR1*, however, showed a high frequency of methylation (25/32, 78%). The six *ESR1* MS-MLPA-methylated samples also showed methylation using the MS-PCR, as well as 19/26 of the MS-MLPA non-methylated samples. By checking the primer and probe sequence, we think that the different CpG recognition site between MS-MLPA and MS-PCR is the source of the difference. Not all CpGs within a promoter region are analyzed by MS-MLPA, but only those CpGs that block digestion of methylation-sensitive endonucleases [25]. In the MS-MLPA probes, only one methylation-sensitive restriction site should be present within the recognition sequence, because not all

CpG sites in a CpG island need to be methylated to silence the transcription of a particular gene [29, 30]. Thus, if a signal is generated from one MS-MLPA probe but not from a second probe located elsewhere in the same promoter, this indicates that the particular gene is methylated and additional tests should be performed. The MS-MLPA is more quantitative than other methods, but is restricted to the specific sites chosen in each promoter region.

CDKN2A (p14^{ARK}), *CDKN1B (p27 ^{KIP1})*) *ATM*, *PTEN*, *BRCA2* and *VHL* displayed no promoter methylation in any of our 168 Indonesian breast cancer samples. For *p14^{ARF}*, *VHL* and *BRCA2*, this is in line with a previous study [31]. However, other studies using methylation-specific PCR did show methylation for *PTEN* (15/44 cases, 34%) [32] and *ATM* (18/23 cases, 78%) [33]. Hypermethylation of the Von Hippel-Lindau (*VHL*) gene is mostly found in renal call carcinoma [34], while the reason behind non-hypermethylation of *BRCA2* remains unsolved [35].

Methylation frequencies of individual genes reported in the literature for breast cancer vary widely and are likely to depend on the sensitivity of the procedure, quality of DNA, the number of PCR cycles and the PCR conditions used, and (ethnic) differences between study populations. Frequencies of *ESR1* and *TIMP3* in the present study were much lower (2.4% for *ESR1* and 5.3% for *TIMP3*) than previous studies where their methylation ranged from 27% [13, 36] to 84% [14] and 20% [37] to 27% [28], respectively. On the other hand, the frequency of methylation of *APC* was at the higher end of the range compared to previous studies [13, 28], whereas the frequencies of *GSTP1* and *DAPK* methylation were consistent with the previous findings [28]. We therefore think that the sensitivity of the new method for methylation detection that we applied for the first time to breast cancer in this study, MS-MLPA, is at least as good as conventional MS-PCR. MS-MLPA has the big advantage that it enables to detect promoter methylation in a series of genes in one reaction.

For comparison, 46 Dutch invasive breast cancer samples were analyzed. Two of the six genes (*ATM* and *VHL*) that displayed no methylation in Indonesian samples also showed no methylation in Dutch samples, and methylation of the other four genes (*CDKN2A (p14^{ARK})*, *CDKN1B (p27^{KIP1})*, *PTEN*, and *BRCA2)* was infrequent in Dutch samples without significant statistical differences. Methylation of *RASSF1* as well as *GSTP1* and *RARB* were in line with Indonesian samples, but *ESR1* and *TIMP3* methylation was significantly more frequent in Dutch samples, consistent with previous studies [13, 36, 37]. The result of comparison of MS-MLPA between Dutch and Indonesian breast cancers thereby strongly suggest that variation of methylation frequencies in Indonesian patients are due to ethnic differences rather than technical limitations of MS-MLPA.

Epigenetic inactivation of *RASSF1A* is known to be widespread in carcinomas of lung, ovary, bladder, kidney and breast [38-42]. The function of *RASSF1A* is thought to involve regulation of Ras-like GTPases, due to interactions at the Ras-Rab association domain, a region homologous with the Ral GDS superfamily [43, 44]. In the present study, the *RASSF1A* gene promoter was hypermethylated with the highest frequency (61%, 103/168). Although *RASSF1A* hypermethylation may be highly frequent in carcinomas, Lehmann *et al.* [45] recently reported epigenetic inactivation of *RASSF1A* expression in benign diseases like usual ductal hyperplasia and papillomas, although inactive normal breast epithelium and proliferating lactating epithelium were unmethylated. Dammann *et al.,*[46] also

found *RASSF1A* hypermethylation in 7.5% of normal breast specimens. Hence, as Lehmann *et al.* have stated, low levels of *RASSF1A* hypermethylation may reflect non-physiologic proliferation in breast cells and/or lesions that however not morphologically qualify as carcinoma. Whether such proliferations are, in fact, of higher clinical risk than is suggested by their morphology remains to be determined. *RASSF1A* methylation may be correlated with bad prognosis as it showed strong association with ductal histological type, poor differentiation and amplification of HER-2/*neu*. *RASSF1A* methylation also strongly correlated with other genes that are often found methylated in breast cancer; *APC* (P=0.000), *RARβ* (P=0.035), *CDH13* (P=0.004) and *GSTP1*. Significant correlations of *RASSF1A* with *APC* and *RARβ* which are both involved in signal transduction, suggest that epigenetic inactivation of signal transduction genes plays a role in breast carcinogenesis, as well as silencing of genes for cell-cell interaction and detoxification.

The most consistent association observed in the present study of specific gene methylation was with amplification of HER-2/*neu* where 4 genes (*APC*, *RASSF1A*, *CDH13* and *GSTP1*) reached statistical significance. The subgroup of tumors with frequent methylation (4-8 genes) were often poorly differentiated and HER-2/*neu* amplified compared to those with infrequent methylation (0-2 genes). This finding is important because it links DNA hypermethylation with the well established pathological prognostic and predictive features of breast cancer. Previous studies have also reported associations between CpG island methylation and poor histological differentiation of breast tumors [47].

When comparing the early onset with the remaining patients, no significant differences were found. We analyzed this subgroup as in Indonesia it relatively large with many patient haboring BRCA1/2 mutations [48]. Although previous studies have shown genetic differences between early onset and older breast cancer patients [49, 50] such differences are not apparent with regard to promoter methylation of the tumor suppressor genes analyzed.

Finally, our study allowed us to compare different genes to each other for patterns of methylation across a spectrum of breast cancers. A consistent pattern of coordinated methylation of two or more genes could suggest that these genes have complementary rather than overlapping functions in breast cancer biology. Although the functional significance of concordant gene methylation remains unclear, such findings have practical significance related to the use of methylation markers for the detection of cancer in ductal lavage fluids or other biological specimens [51]. In particular, multiple genes that show parallel profiles of methylation are likely to be redundant as markers for cancer detection, whereas non-redundant combinations of genes could provide a more useful set of markers for breast cancer detection in biological specimens.

In conclusion, this comprehensive analysis of tumor suppressor promoter methylation status in invasive breast cancer by high throughput MS-MLPA reveals remarkable differences in methylation frequency for various genes. Promoter methylation of multiple genes seems to be correlated to poor differentiation and HER-2/*neu* amplification. A comprehensive hypermethylation profile as assessed by MS-MLPA could therefore potentially be useful for breast cancer detection and classification, and understanding the biology of this disease.

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Chapter 7

High throughput analysis of gene amplification of 27 genes in invasive breast cancer by MLPA

Manuscript

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ABSTRACT

Genomic instability is a hallmark of cancer, and specific copy number changes are thought to play a driving role in the transformation of normal cells to malignant clones. Gene copy number alteration has mostly been studied using conventional or array CGH or single gene analyses. Here we performed Multiplex Ligation dependent Probe Amplification (MLPA) to detect copy number changes of 27 genes that often have an increased copy number in one or more types of human cancer on 191 Indonesian breast cancer samples. These genes span the human genome, from *MYCL1* that is located on 1p34.2 to *BCL2L13* on 22q11.

No cases were amplified for *BIRC4, PDGFRA, PDGFRB* or *HMGA*. Amplification frequencies by MLPA ranged from 1% for *MYBL2, BIRC2, hTERT, BCL2A1, BCL2L1, BCL2L13* and *MYCN* to 26% for HER-2*/neu*, consistent with previous studies on single genes and CGH. Tumors with frequent amplification (>2 genes) were more often poorly differentiated (p=0.021) and presented at advanced stage (p=0.025) compared to those with infrequent amplification (0-2 genes).

In conclusion, we present a novel high throughput MLPA method that allows a reliable, quick and cheap comprehensive analysis of copy number of a large set of genes that play a role in carcinogenesis in breast and other cancers. This method thereby allows efficient analysis of gene copy number status for better understanding the biology and possibly clinical behavior of breast and other cancers.

INTRODUCTION

Breast cancer is the most prevalent cancer worldwide and is the second leading cause of cancer-related deaths in women [1, 2]. It is a complex disease in which multiple genetic factors combine to drive pathogenesis [3-5]. Genomic instability is a hallmark of cancer, and specific subchromosomal copy number changes are thought to play a driving role in the transformation of normal cells to malignant clones. These genomic copy number changes may result in deletion of one or both alleles of tumor suppressor genes, overexpression of oncogenes and rearrangements that may alter transcription of target and downstream genes [6]. Several recent studies suggest that specific genetic abnormalities in human cancers may be highly predictive of response to targeted therapeutics. For example, HER-2/*neu* amplification may be more predictive of response to trastuzumab than protein overexpression with normal gene copy number. Changes in copy numbers of genes such as HER-2/*neu* and c-*MYC* have been extensively documented in breast cancer [7, 8].

Because of the stability of DNA, gene amplification is easier to measure than RNA or protein overexpression. Determination of gene amplifications would therefore be optimally suited for diagnostic applications. Indeed, gene amplification analysis is now increasingly being used for diagnostic analysis of the *HER2* status instead of immunohistochemistry in clinical breast cancer samples [9].

Presence of gene amplification may not only be important because of the consecutive overexpression of the respective oncogene. Gene amplification may also serve as a surrogate parameter for increased genetic instability of a cancer and, as such, represent an indicator of poor prognosis. This may especially apply to tumors that have multiple amplifications. Indeed, a trend toward a worse prognosis in tumors with multiple amplifications was described recently in a study investigating 640 breast cancers by Southern blot for amplification of eight different oncogenes [10].

Conventional karyotyping, fluorescence *in situ* hybridization (FISH), spectral karyotyping, multicolor FISH, chromosomal comparative genomic hybridization (CGH) and array CGH have found a wide application in studying chromosomal alterations and detection of specific genes with copy number changes in different female cancers like breast cancer [11-13], ovarian [14] and Fallopian tube [15] cancer. Although the resolution of CGH is increasing, the loci found amplified or lost still harbour multiple genes, and the other techniques targeting single genes often suffer from low throughput. Here we performed a high throughput easy method based on Multiplex Ligation-dependent Probe Amplification (MLPA) to assess the amplification of 25 genes in a large group of breast cancer patients.

MATERIALS AND METHODS

Tissue samples

Tumor tissues were obtained during surgery from 191 breast cancer patient at the Sardjito Hospital Jogjakarta, Indonesia in the years 2002-2004. All samples were freshly obtained and put into a -80º C freezer for storage. The histological type and grade of the tumors were classified according to the World Health Organization criteria. Patient age at diagnosis ranged from 19 to 80 years (mean 49). Genomic DNA was extracted from frozen tissues using Qiamp DNA mini kit (Qiagen, Germany) as described in the manufacturer's protocol.

MLPA analysis

The principle of the MLPA technique has been described elsewhere [9, 16]. The Salsa MLPA kit P171 (MRC-Holland, Amsterdam, The Netherlands) was used to detect copy number changes of several human genes that often have an increased copy number in different tumors including breast cancer. The P171 probemix contains 42 different probes, with amplification products between 112 and 472 bp, for the following 27 genes: *BCAS1, BCL2A1, BCL2L1, BCL2L13, BIRC2, BIRC4, CENPF, EGFR, ERBB2, ERBB4, ESR1, FGFR1, GNAS, GSTP1, HMGA1, MET, MYBL1, MYBL2, MYC, MYCL1, MYCN, PDGFRA, PDGFRB, PTK2, PTP4A3, SERPINB9,* and *hTERT*. Several genes have more than one probe; *ERBB2, MYC, MET, MYCN* and *PDGFRB* has three probes, *ESR1*, *EGFR, PTK2, PTP4A3* and *BCL2L1* has two probes. In addition, one synthetic control probe is present.

Briefly, 50_ 500 ng of target DNA per 5 ml of 10 mM pH 8 Tris _0.1 mM EDTA was denatured for 5 min at 98 C after which 3 ml of the probe mix was added. The mixture was heated at 95°C for 1 min and incubated at 60ºC overnight (16 h). Ligation was performed with the temperature stable Ligase-65 enzyme (MRC-Holland) for 15 min at 54ºC. Next, the ligase was heat inactivated in the thermocycler for 5 min at 98ºC. Ten microliters of the ligation mixture was premixed with 30 microliters of PCR buffer and put in a PCR machine at 60ºC. Subsequently, a 10 microliters mix was added that contained deoxynucleoside triphosphate, Taq polymerase, and one unlabeled and one carboxyfluorescein labeled PCR primer that are complementary to the universal primer sequences. PCR was carried out for 33 cycles (30 sec at 95ºC, 30 sec at 60ºC, and 1 min at 72ºC). The fragments were analyzed with an ABI model 310 capillary sequencer (Applied Biosystems, Torrence, CA, USA) using Genescan-TAMRA 500 size standards (Applied Biosystems). Fragment analysis was performed using Genescan software. DNA from Centre d'Etude Polymorphisme duHumain (CEPH) was used as a control sample and analyzed simultaneously with breast cancer samples in each run.

To objectify interpretation of the fragment analysis, the relative quantity of the amplified probes in each sample was determined using Gene Marker software version 1.2. For this purpose, the relative peak areas for each probe were calculated as fractions of the sum of peak areas in a given sample. Subsequently, the fraction of each peak was divided by the average peak fraction of the corresponding probe in control samples. Followed the default software settings, cases that showed ratios greater than 1.5 were considered amplified. For genes with multiple probes, at least two of the probes needed to fulfill this criterion to be amplified.

Statistical Analysis

The $\chi 2$-test was used to determine associations between the amplification status of individual genes on the one hand and phenotypic and molecular features of breast cancer on the other. The early onset patients (=< 40 years) were analyzed separately as this subgroup is relatively large in Indonesia. Fisher's exact test was used when individual cell numbers were less than 5. All P values were derived from two-tailed statistical tests and significance was assumed at <0.05. The Mann-Whitney U test was used to compare tumor size, histological type and

histological grade between tumors with and without amplification. All analyses were performed using the SPSS 11.0 for Windows statistical software package.

RESULT

DNA copy number changes (table 1) were detected in 110 of 191 (58%) of cases; 52 cases (27%) were amplified at only 1 gene, 24 (13%) at 2 genes, 17 (9%) at 3 genes, 9 (5%) at 4 genes, 5 (3%) at 5 genes and 4 (2%) at 6 genes. Four genes displayed no amplification in any of 191 tumor samples: *BIRC4, PDGFRA, PDGFRB* and *HMGA*. Amplification frequencies for the other genes ranged from 1% for *MYBL2, BIRC2, hTERT, BCL2A1, BCL2L1, BCL2L13* and *MYCN* to 26% for HER-2/*neu*. The relative quantitative measurement of copy number showed that *EGFR* (amplified in 2% of the cases) had the highest copy number change with a mean ratio of 8.05 (range 1.50-13.10), corresponding to a 16 times amplification. *HER2* (amplified in 26%), *FGFR1* (amplified in 6%) and *ESR1* (amplified in 2%) were on average amplified eight (range 1.60-8.78) and six times range (2.01-6.50) and (range 1.52-5.93), respectively, whereas, *MYBL2*, *BCL2L13*, *ERBB4, BIRC2* and *MET* were amplified three times. The other genes were amplified between four times.

Associations between amplification of each gene and various clinicopathological features are given in Table 2. Most association were weak, with significant associations between *MYC* amplification and poor differentiation, between *CENPF, BCAS* and *BCL2A1* amplifications on the one hand and tumor size on the other, and between *BCAS* amplification and stage. All these were positive associations except for amplification of *BCL2A1* that correlated with smaller tumor size. No significant associations were seen between menopausal status, nodal status or histological type and amplification of any gene.

Tumors with frequent amplification (>2 genes) presented more often at advanced stage (p=0.025) and were more often poorly differentiated (p=0.021) compared to those with infrequent amplification (0-2 genes). When comparing gene amplification between early onset patients ($\leq$ 40 years) with the remaining patients, there were no significant differences in frequency of gene amplification.

MYC, GNAS and *PTK2* were most often coamplified with other genes (Table 3). Amplification of *MYC* was significantly associated with amplification of *PTK2* (p=0.000), *PTP4A3* (p=0.000), *BCL2L1* (p=0.032), *MYBL1* (p=0.000) and *FGFR1* (p=0.046), while amplification of *GNAS* was significantly associated with HER-2/*neu* (p=0.047), *ESR1* (p=0.021), *PTK2* (p=0.006), *BCL2L1* (p=0.007), and *BCAS* (p=0.007) amplification, and amplification of *PTK2* was significantly associated with *PTP4A3* (p=0.000), *MYBL1* (p=0.000), *MYCL1* (p=0.004), *GNAS* (p=0.006) and *MYC* (p=0.000) amplification.

DISCUSSION

Multiplex ligation-dependent probe amplification (MLPA) is a high throughput method for detecting copy number variations in genomic sequences [16]. Available evidence suggests that MLPA is a robust assay, which offers several advantages over existing techniques [17]. Multiplex ligation-dependent probe amplification has rapidly gained acceptance in genetic diagnostic laboratories due to its simplicity, relatively low cost, capacity for reasonably high throughput and robustness [18]. Here we performed MLPA to detect copy number changes of 27 human genes

spanning the human genome that often have an increased copy number in one or more types of tumors including breast cancer.

From the chromosomal region point of view, this study observed three amplified regions consistent with current breast cancer literature [19-21], by showing 38% gain of region 8q (attributed to *MYC, PTK2* and *PTP4A3*), 26% of 17q (attributed to *HER2*) and 17% of 20q (attributed to *BCAS1* and *GNAS*).

Table 1. Copy number changes of 25 oncogenes as assessed by multiplex ligation dependent probe amplification of 191 breast cancer cases (n.a. = not applicable). In addition, amplification ratios (ratio=1 means 2 gene copies) are given

gene	# amplified cases (%)	amplification ratio (range)
BCAS1	16 (8%)	1.91 (1.52-3.43)
BCL2A1	1 (1%)	1.71
BCL2L1	2 (1%)	1.99 (1.93-2.05)
BCL2L13	2 (1%)	1.61 (1.53-1.69)
BIRC2	1 (1%)	1.62
BIRC4	0	n.a.
CENPF	16 (8%)	1.75 (1.51-2.12)
EGFR	3 (2%)	8.05 (1.5-13.1)
ERBB2	50 (26%)	4.04 (1.60-8.78)
ERBB4	4 (2%)	1.57 (1.52-1.66)
ESR1	3 (2%)	3.30 (1.52-5.93)
FGFR1	12 (6%)	3.30 (2.01-6.5)
GNAS	17 (9%)	1.98 (1.52-3.70)
GSTP1	4 (2%)	2.14 (1.59-2.57)
HMGA1	0	n.a.
MET	3 (2%)	1.59 (1.53-1.67)
MYBL1	15 (8%)	2.08 (1.51-3.00)
MYBL2	1 (1%)	1.52
MYC	35 (18%)	2.21 (1.55-4.82)
MYCL1	7 (4%)	2.01 (1.59-2.52)
MYCN	1 (1%)	3.13
PDGFRA	0	n.a.
PDGFRB	0	n.a.
PTK2	21 (11%)	2.05 (1.50-2.72)
PTP4A3	18 (9%)	1.98 (1.52-2.62)
SERPINB9	4 (2%)	1.76 (1.55-1.99)
hTERT	1 (1%)	1.97

Table 2. Associations between gene amplification status assessed by MLPA and clinicopathological features of 191 breast cancer cases. Four genes (*PDGFRA*, *PDGFRB*, *BIRC4* and *HMGA*) that did not show amplification in any of these cases are not listed.

Feature	n	HER-2		MYC		MET		MYCN		ESR1		EGFR		PTK2		PTP4A3		BCL2L1		MYLB1		MYLB2		CENPF	
		N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Total	**191**	50	26	35	18	3	2	1	1	3	2	3	2	21	11	18	9	2	1	15	8	1	1	16	8
Age	191																								
<= 40	49	10	20	11	22	2	4	1	2	1	2	0	0	9	18	8	16	1	2	5	10	0	0	3	6
> 40	142	40	28	24	17	2	1	0	0	2	1	3	2	13	9	10	7	1	1	10	7	1	1	13	9
Menopausal status	191																								
pre-menopause	109	23	21	23	21	3	3	1	1	1	1	1	1	16	15	12	11	2	2	9	8	0	0	5	5
post-menopause	82	27	33	12	15	1	1	0	0	2	2	2	2	6	7	6	7	0	0	6	7	1	1	11	13
Tumor size	185																								
T1	8	2	25	0	0	0	0	0	0	0	0	0	0	1	13	0	0	0	0	0	0	0	0	0	0
T2	46	9	20	8	17	0	0	0	0	1	2	2	4	4	9	3	7	0	0	3	7	0	0	2	4
T3	36	16	44	7	19	1	3	0	0	0	0	0	0	5	14	3	8	0	0	2	6	0	0	1	3
T4	95	21	22	17	18	3	3	1	1	1	1	1	1	10	11	11	12	2	2	8	8	1	1	13	14
																								p=0.019	
Stage	190																								
I/II	65	18	28	12	18	0	0	0	0	1	2	2	3	6	9	4	6	0	0	4	6	0	0	2	3
III/IV	125	32	26	22	18	4	3	1	1	2	2	1	1	15	12	14	11	2	2	10	8	1	1	14	11
Histological grade	184																								
0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	10	0	0	0	0	0	0	1	10
1	6	2	33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	90	25	28	12	13	1	1	0	0	2	2	1	1	10	11	9	10	1	1	5	6	1	1	8	9
3	78	20	26	20	26	3	4	1	1	1	1	2	3	12	15	7	9	1	1	9	12	0	0	7	9
				p=0.005																					
Histological type	191																								
ductal	174	46	26	32	18	4	2	1	1	3	2	3	2	21	12	16	9	2	1	13	7	1	1	15	9
lobular	4	1	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
others	13	2	15	2	15	0	0	0	0	0	0	0	0	1	8	1	8	0	0	1	8	0	0	1	8
Nodal status	170																								
negative	46	9	20	9	20	2	4	1	2	0	0	1	2	8	17	2	4	0	0	4	9	1	2	5	11
positive	124	34	27	20	16	2	2	0	0	2	2	2	2	14	11	15	12	1	1	9	7	0	0	11	9

Table 2 (continued). Associations between gene amplification status assessed by MLPA and clinicopathological features of 191 breast cancer cases. Four genes (*PDGFRA*, *PDGFRB*, *BIRC4* and *HMGA*) that did not show amplification in any of these cases are not listed.

Feature	n	BCL2L13		BCAS		ERBB4		BIRC2		GSTP1		TERT		FGFR1		BCL2A1		MYCL1		GNAS		SERP	
		N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Total	**191**	2	1	16	8	4	2	1	1	4	2	1	1	12	6	1	1	7	4	17	9	4	2
Age	181																						
<= 40	49	0	0	5	10	2	4	0	0	0	0	0	0	5	10	0	0	0	0	4	8	0	0
> 40	142	2	1	11	8	2	1	1	1	4	3	1	1	7	5	1	1	7	5	13	9	4	3
Menopausal status	191																						
pre-menopause	109	0	0	8	7	3	3	0	0	1	1	1	1	10	9	1	1	4	4	9	8	2	2
post-menopause	82	2	2	8	10	1	1	1	1	3	4	0	0	2	2	0	0	3	4	8	10	2	2
Tumor size	185																						
T1	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	13	0	0	0	0	1	13
T2	46	0	0	0	0	1	2	1	2	0	0	0	0	2	4	0	0	3	7	4	9	0	0
T3	36	0	0	3	8	1	3	0	0	0	0	0	0	3	8	0	0	2	6	4	11	0	0
T4	95	2	2	12	13	2	2	0	0	4	4	1	1	6	6	0	0	2	2	8	8	3	3
				p=0.009												**p=0.043**							
Stage	190																						
I/II	65	0	0	0	0	1	2	1	2	0	0	0	0	2	3	1	1.54	4	6	5	8	1	2
III/IV	125	2	2	15	12	3	2	0	0	4	3	1	1	10	8	0	0	3	2	12	10	3	2
				p=0.004																			
Histological grade	184																						
0	10	0	0	0	0	1	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	6	0	0	1	17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	17	0	0
2	90	0	0	4	4	2	2	0	0	1	1	1	1	5	6	1	1.11	5	6	10	11	2	2
3	78	2	3	10	13	1	1	1	1	3	4	0	0	6	8	0	0	2	3	6	8	2	3
Histological type	191																						
ductal	174	2	1	15	9	3	2	1	1	4	2	1	1	12	7	1	0.57	6	3	17	10	4	2
lobular	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
others	13	0	0	1	8	1	8	0	0	0	0	0	0	0	0	0	0	1	8	0	0	0	0
Nodal status	170																						
negative	46	1	2	5	11	2	4	0	0	2	4	0	0	2	4	1	2.17	3	7	4	9	2	4
positive	124	1	1	9	7	2	2	1	1	2	2	0	0	8	6	0	0	4	3	12	10	2	2

Table 3. Associations between gene amplification of different genes assessed by MLPA in 192 breast cancer cases. Four genes (*PDGFRA*, *PDGFRB*, *BIRC4* and *HMGA*) that did not show amplification in any of these cases are not listed.

	MYC	MET	MYCN	ESR1	EGFR	PTK2	PTP4A3	BCL2L1	MYLB1	MYLB2	CENPF	BCL13	BCAS	ERBB4	BIRC2	GSTP1	TERT	FGFR1	BCL2A1	MYCL1	GNAS	SERP
HER-2											0.014							0.038			0.047	
MYC						0.000	0.000	0.032	0.000									0.046				
MET																						
MYCN																						
ESR1																					0.021	
EGFR												0.031										
PTK2							0.000		0.000											0.004	0.006	
PTP4A3									0.000		0.048											
BCL2L1																	0.01				0.007	
MYLB1																						
MYLB2																0.021						
CENPF																						
BCL13																						0.041
BCAS																					0.007	0.036
ERBB4																						
BIRC2																						
GSTP1																						
TERT																						
FGFR1																						
BCL2A1																						0.021
MYCL1																						
GNAS																						0.04

Consistent with previous studies on Indonesian breast cancer [9] and other studies on HER-2/*neu* amplification [22-24], we found 26% of the samples to show HER-2/*neu* amplification. A recent meta-analysis summarizing data from 81 studies with 27,171 patients, revealed that in the great majority (90%) of studies either HER-2/*neu* amplification or protein overexpression correlated with poor outcome of the patients [25]. Studies using CGH have also found gain of 17q12-q21.1 where HER-2/*neu* is located [20].

MYC amplification was found in 18% of the samples. This result is in line with previous studies that found 8 to 37% *MYC* amplification in breast cancer [26, 27]. A significant association between *MYC* amplification and poor differentiation was found in this study, consistent with a previous study that found a highly significant association between 8q24 copy number gains and high tumor grade [28]. By CGH, gains of 8q23-q24 were more often observed in grade III tumors [20].

Another gene that showed amplification frequencies above 10% was *PTK2* (21/191; 11%). *PTK2* (protein tyrosine kinase 2) gene is located in 8q24-ter, encodes a cytoplasmic protein tyrosine kinase which is found concentrated in the focal adhesions that form between cells growing in the presence of extracellular matrix constituents [29, 30]. The encoded protein is a member of the FAK subfamily of protein tyrosine kinases but lacks significant sequence similarity to kinases from other subfamilies. Activation of this gene may be an important early step in cell growth and intracellular signal transduction pathways triggered in response to certain neural peptides or to cell interactions with the extracellular matrix [31-33]. *PTK2* was one of the amplified genes that was only recently found amplified in breast cancer, mostly together with *ERBB2 (*HER-2*/neu)*, *EGFR* and *MYC* [19]. *PTK2* amplification has also been found in hepatocellular carcinoma (HCC) together with amplification of *MYC, MOS* and *EXT1* [34], and elevated expression of *PTK2* was associated with a large tumor size in HCC [34]. Additionally, over expression of *PTK2* is seen in a number of different tumor types, including carcinoma of colon [35], prostate [36], ovary [37] and mesenchymal tissues [38].

Several studies [11, 19, 20, 39] found 8q24 as the most frequently gained region in breast cancer samples. Additionally, gains at chromosome arm 8q were found to be correlated with the mean nuclear area (MNA), mitotic activity index (MAI) and occurred in the 'poor prognostic features' group [11], and Isola *et al*. found gain of 8q to be associated with recurrence [40]. There are three potentially important genes located in this region: *MYC* (8q24.12), *PTK2* (8q24-ter) and *PTP4A3* (8q24.3).

The protein product of oncogene c-*myc* acts as a transcription factor involved in cell differentiation and apoptosis. Deregulation of this gene may lead to impaired differentiation and apoptosis pathways and thus to growth advantage [11]. The amplification of *MYC* in primary breast tumors and tumor cell lines is associated with the formation of a distinct type of chromosome changes referred as double minute chromosomes and abnormally banded or homogeneously stained regions (HSR). Both abnormalities reflect DNA amplification and like other structural alterations are associated with genomic instability and contribute to the process of carcinogenesis [41-44]. Their occurrence leads to loss of control of copy gene number that may generate subpopulations of tumor cells with increased growth

potential, invasiveness and refractory to chemotherapeutic agents [42-44]. These alterations are also viewed as important in cancer development because they frequently involve proto-oncogenes which can alter proliferation of cells [41].

PTK2 encodes focal adhesion kinase (FAK), a non-receptor protein tyrosine kinase that is a major mediator of integrin-stimulated signal transduction pathways. There is evidence that FAK is essential for integrin stimulated cell migration [31, 45], cell spreading [45], and proliferation [31-33]. Upon activation by integrins, FAK induces cell migration through activation of small GTPases of the Rho and Ras families, as well as activation of MAPK and JNK cascades. Furthermore, it induces cell invasion through expression of motility-and invasion-associated proteins such as matrix metalloproteinases [46, 47]. Some investigators have suggested that over expression of *PTK2* is a late event in carcinogenesis, contributing to invasiveness and metastasis [48, 49].

Expression of *PTP4A3* or *PRL3* was found clearly elevated in tumor endothelial cells relative to normal endothelial cells, bulk tissue samples, and breast tumor cell lines. Therefore it was an additional support to a preferential expression of *PRL3* in breast tumor vasculature relative to epithelial cells [50]. *PRL3* is a proposed tyrosine phospatase with a COOH-terminal prenylation motif that allows its association with the plasma membrane. This gene was reported recently to have role in colorectal cancer metastasis [51].

High level 20q copy number gain was shown to contribute to the cancer phenotype, especially aspects of immortalization, genome instability, apoptosis and increased proliferation [52]. Amplification at 20q13 is particularly interesting because this aberration occurs in a variety of tumor types and is associated with aggressive tumor behavior [53]. The initial CGH study showed increased copy number involving 20q13 in 40% of breast cancer cell lines and 18% of primary breast tumor [54]. Positional cloning of cancer-associated 20q13.2 amplicon identified two genes that display high mRNA levels in breast tumors. One of them was designated Novel Amplified in Breast Cancer-1 (*NABC1*) or *BCAS1*. *BCAS1* was found to be highly expressed in three amplified breast cancer cell lines and in one breast tumor without amplification at 20q13.2. However, Beardsley et al [55] in their study to characterize the *BCAS1* gene product, revealed that *BCAS1* overexpression alone does not promote cellular transformation. Concerning that cancer is a multistep process generally involving altered function of several protein, this protein can not be excluded as a determinant in breast tumorigenesis. as overexpression of *ZNF217*, a gene that is very closely located to *BCAS1,* has been shown to lead to immortalization of breast cancer cell lines [56]. Thus, it is possible that *BCAS1* may have a similar effect when overexpressed in this cell type as well, or *BCAS1* expression works cooperatively or synergistically with *ZNF217* to promote malignant growth. Although not consistently overexpressed in tumors [53] , *BCAS1* is a candidate oncogene. In the present study, we found amplification of *BCAS1* in 8% of breast cancers. Despite the low frequency of amplification, a significant association of *BCAS1* amplification with advance tumor stage (p=0.004) and bigger tumor size (p=0.009) was found. This result strengthens the previous studies of association of *BCAS1* as gene attributed to increased copy number of 20q with more aggressive tumor phenotype.

CENPF amplification associated with bigger tumor size. *CENPF*, centromere protein F, 350/400ka (mitosin) is located on 1q32-q41 and encodes a protein that

associates with the centromere-kinetochore complex. High copy number of 1q32 region has been found esophageal squamous cell carcinoma as well as glioblastoma and breast cancer [57].

BCL2A1 located on 15q24.3 was isolated from human fetal liver, is a member of the *BCL-2* gene family, inhibits p-53 induced apoptosis and exhibits a potent cooperative transforming activity [58, 59]. *BCL2A1* or *BFL-1* was also shown to be induced by inflammatory cytokines TNF-α or IL-1β, suggesting that it may play a protective role during inflammation [60]. Yoon et al [61] observed that *BCL2A1* gene was related to more advanced breast cancer and to factors that have favorable prognosis. In the present study, *BCL2A1* amplification was associated with smaller tumor size which is not easy to understand.

Tumors with frequent amplification (>2 genes) were more often poorly differentiated and at advanced stage compared to those with infrequent amplification (0-2 genes). This is well understandable as increasing genomic instability as also reflected in more amplified genes will accompany tumor progression. This finding is important because it links gene amplification with the well established pathological prognostic and predictive features of breast cancer.

When comparing gene amplification between early-onset patients (≤ 40 years) with the remaining patients, there were no significant associations. Apparently, there are no clear differences between early and late onset Indonesian breast cancer patients with regard to amplification of the genes studied.

In conclusion, we present a high throughput MLPA method that allows a reliable, quick and cheap comprehensive analysis of copy number of a large set of genes that play a role in carcinogenesis in breast and other cancers. This method thereby allows efficient analysis of gene copy number status for better understanding the biology and possibly clinical behavior of breast and other cancers.

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Chapter 8:

General Discussion

Breast cancer shows a high and increasing incidence in Indonesia, and many females develop breast cancer at young age. It is therefore likely that *BRCA1* and *BRCA2* mutations play an important role in breast carcinogenesis in the Indonesian population. However, little is known about the role of *BRCA1*/2 germline mutations in Indonesian breast cancer. We therefore set out to screen a series of Indonesian breast cancer patients and their family member for germline mutations in *BRCA1* and *BRCA2*. To this end, a fast and cheap method to screen for these mutations was needed.

BRCA1 and *BRCA2* germline mutations

Over 80% of families with two or more cases of pre-menopausal breast cancer and two or more cases of ovarian cancer are believed to carry a germline *BRCA1* or *BRCA2* mutation [1, 2]. It has been shown that ethnically different populations exhibit different germline mutation spectra in the *BRCA1* and *BRCA2* genes [3-5]. There are no previous publications on *BRCA1*/2 mutation detection in the Indonesian population. By identifying the endemic Indonesian mutations, we hope to offer better risk assessment for women in the Indonesian population who are susceptible to the *BRCA1*/2 related hereditary cancers. However, the large size of the *BRCA1* and *BRCA2* genes, and the scattered distribution of mutations throughout the genes, complicate the task of mutation detection and make rapid screening for mutations a major technical challenge. A technique that scans stretches of DNA for unknown mutations should be optimized to detect as close to 100% of the sequence alteration as possible. In ***Chapter 2***, we describe the results of the application of a recently developed rapid and sensitive method for the detection of *BRCA1*/2 mutations [6] based on pooled denaturing gradient gel electrophoresis (DGGE) [7] and targeted sequencing to an Indonesian group of high risk patients. Theoretically, this method should be particularly suited for the analysis of naive populations.

As alternatives to sequencing methods, which determine the exact nature and location of each base along a DNA fragment, various mutation scanning procedures have been developed. These methods, which rely on the recognition of a sequence variation between mutant and wild-type DNA on the basis of an altered electrophoretic migration pattern, provide a simple means for determining whether a given DNA sample harbors a mutation in a particular gene. The most well established scanning procedures are single strand conformational polymorphism (SCCP) analysis, DGGE, chemical cleavage of mismatch, RNase cleavage, the protein truncation test (PTT), and heteroduplex analysis. Among these methods, SCCP, DGGE, PTT, and heteroduplex analysis are the most widely used because of their accuracy, simplicity, lack of toxicity, and/or relative affordability. We focused on DGGE because this method, when optimized, has the highest mutation detection rate (close to 100% [8]) compared with SCCP and heteroduplex analysis. Although DGGE has been applied to BRCA mutation screening before [9-11] we used a new technique based on DGGE after exon by exon PCR amplification of the complete *BRCA1* and *BRCA2* sequences [12] in an Indonesian breast cancer population, and sequenced aberrantly moving bands. This yielded four polymorphisms consisting of single nucleotide substitutions, underlining the sensitivity of the method. Intelligent pooling of the amplicons before electrophoresis greatly improved the throughput of the method. In addition, a novel *BRCA2*

mutation (2699delTAAATG) was found. In conclusion, a new high throughput and highly sensitive method to screen *BRCA1* and *BRCA2* for mutations based on exon by exon PCR amplification followed by pooled DGGE and sequencing of aberrant bands seems to be the ideal approach for screening naïve populations for mutations, and has the ability to detect single base differences using non-toxic and relatively simple and inexpensive methods.

Using this method, and adding by Multiplex ligation dependent probe amplification (MLPA) technique to detect genomic deletions in BRCA1/2, we analyzed a group of 116 early onset breast cancer patients and 16 of their family members from three Indonesian cities (Jakarta and Jogjakarta on the Java island, and Denpasar on the Bali island) for *BRCA1*/2 mutations in ***chapter 3***. Mutations specific for certain populations and ethnic groups have been identified in both genes. For example, specific *BRCA1* and *BRCA2* mutations were reported for Ashkenazi Jews [13]. Other common *BRCA1* mutations were especially found in Italian, Canadian, Belgian or Dutch breast cancer families [14-16]. In Indonesia, the contribution of the *BRCA1*/*BRCA2* mutations to the population incidence of early-onset breast cancer was largely unknown before. The analysis of 116 unrelated breast cancer patients with breast cancer revealed that 9 patients (7.8%) carried pathogenic germline mutations especially the early onset patients: 3 within *BRCA1* (2.6%) (c.2784_2785insT, pL1415X (c.4361_4362insT), del exon 13-15) and 6 within *BRCA2* (5.2%) (c.3040_3043delGCAA, p.Glu2183X (c.6775G>T), p.Leu824X (c.2699_2704delTAAATG), p.Gln2894X (c.9008C>T)) which is comparable to previous studies [17]. All these mutations were classified as pathogenic as they are predicted to result in protein truncation. The three pathogenic mutations found in *BRCA1* were not previously reported in the BIC database as well as two novel nonsense mutations (p.Glu2183X and p.Gln2894X) identified in *BRCA2*. There were twice as many *BRCA2* mutations as *BRCA1* mutations. Although the absolute numbers are low and no firm conclusions can therefore be drawn, this is comparable to other Asian regions [3, 18, 19] but seems to discern the Indonesian population from non-Asian ethnic groups where the reverse trend is seen. The c.2699_2704delTAAATG (p.Leu824X) in *BRCA2* that has been reported previously by us in the Indonesian population [20], was found in one other patient in the present study. This mutation lies in exon 11 *BRCA2*, within the BRC repeats domain. The truncating mutation causes loss of three quarters of the protein leading to lack of interaction with the RAD51 protein. Different from *BRCA1*, the repair of DSBs by HR is the most important function of the *BRCA2* protein [21]. As the c.2699_2704delTAAATG mutation was found in two unrelated patients, this mutation could be a good candidate as a founder mutation.

Sixteen (7 *BRCA1* and 9 *BRCA2*) rare mutations of so far unknown significance (“unclassified variants”, UVs) were detected in 18 patients: 13 missense changes and 3 intronic variants. Of these 16 UVs, 7 were novel, whereas the other UVs have been previously reported in the BIC database. Seven UV were found in the *BRCA1* gene, two mutations occurring in the intronic region between exons 1 and 2 (c.101-10T>C) and between exons 19 and 20 (c.5313-31A>G), and five missense mutations identified: p.Val191Ile (c.690G>A), p.Leu1209Val (c.3744T>G), p.Met1652Ile (c.5075G>A), p.Arg1835Gln (c.5623G>A) and p.Thr1852Ile (c.5674C>T). Four out of seven *BRCA1* missense mutations; p.Leu1209Val (c.3744T>G), c.5313-31A>G, p.Arg1835Gln (c.5623G>A) and p.Thr1852Ile

(c.5674C>T) were have not been described previously in the BIC. Nine different UVs of the *BRCA2* gene were found in fourteen patients, and three of them were novel; p.Gln609Glu 9c.2053C>G), p.Gln699Leu (2324A>T) and p.Val950Ile (3076G>A). When comparing the three different Indonesian regions, the percentages of breast cancer patients with pathogenic *BRCA1/2* mutations were significantly higher in Denpasar on the Bali island than in Jogjakarta and Jakarta on the Java island. Although the number of patients is too small to draw firm conclusions, these data may point to geographic differences within Indonesia.

In conclusion, a relatively high percentage of early onset Indonesian breast cancer patients carry a germline mutation in either *BRCA1* or *BRCA2.* Several novel, pathogenic *BRCA1* and *BRCA2* germline mutations have been found, as well as a variety of novel "unclassified variant" mutations that may therefore be specific for the Indonesian population. The finding of all these novel mutations illustrates the power of the optimized DGGE/MLPA technique to detect BRCA1/2 mutations in naïve populations like the Indonesian one. It is likely that some of the "unclassified variant" mutations may have a functional role in breast cancer development, which deserves to be explored further.

The phenotype and genotype of a group of early onset Indonesian breast cancer patients is described in ***chapter 4 and chapter 5*****,** as such phenotype can give clues for the "BRCA-ness" of a breast cancer. Distinct features of *BRCA1*-associated tumor have been proposed, such as high tumor grade, estrogen (ER) and progesterone receptor (PgR) negativity [22, 23], accumulation of p53 [22, 23], expression of the epidermal growth factor receptor (EGFR) [24-26], and absence of amplification and overexpression of HER-2/*neu* [27, 28]. Additionally, hereditary breast cancers are preferentially of the ductal and medullary carcinoma types [29, 30]. cDNA expression analyses and expression of cytokeratins 5/6 have suggested a basal epithelial phenotype for *BRCA1* related cancers [31]. However, the phenotype of *BRCA2* related cancers is much less outspoken, and seems to be in between that of *BRCA1* related and sporadic cancers [32]. Whereas in Western countries most hereditary breast cancers are *BRCA1* related, the reverse trend is seen in Indonesia where most hereditary breast cancers seem to be *BRCA2* related [20]. Better defining the phenotype of *BRCA2* related breast cancers is especially important in Asian populations like ours, as the prevalence of *BRCA2* related cancers exceeds that of *BRCA1* related cancers [3, 33].

Currently, HER-2/*neu* status is determined using two approaches: one that reveals gene amplification and one aimed at detecting the overexpressed HER-2/*neu* protein [34-37]. In ***chapter 4*** we evaluated for the first time the potential value of a new MLPA based PCR based technique to assess HER-2/*neu* amplification. MLPA relies on comparative quantitation of specifically bound probes that are amplified by PCR with universal primers [38]. The introduction of universal primers has advantages because multiplexing numerous targets becomes much easier, and when fluorescence detection of products is used, only one fluorescent primer is required, thus reducing the cost compared to buying fluorescent probes for each target. Technically, FISH has disadvantages compared to MLPA for determining partial gene deletions and remains a relatively low throughput method compared to other molecular genetic techniques. Comparison between IHC and MPLA showed that none of the 36 IHC negative cases were amplified and as many

as eight of nine (89%) IHC 3+ tumors showed gene amplification by MLPA assay. Therefore, there was complete concordance between IHC and MLPA in (36+8)/60 (73%) of cases. None of the IHC 1+ cases was MLPA amplified. FISH has been shown to detect amplification in 6-7% of negatively staining tumors [39]; MLPA also may prove to have some additional value in these cases in larger future studies. As many as five of eight (63%) 2+ cases, however, were amplified by MLPA, indicating that the additional value of MLPA lies especially in the IHC 2+ cases. Therefore, we propose MLPA as a quick and inexpensive method for detecting HER-2/*neu* amplification in daily laboratory practice. Although IHC still is useful as an initial screening tool, MLPA may be an attractive alternative to FISH for amplification testing for IHC 2+ cases. Because MLPA is relatively cheap and can be used readily on paraffin embedded tissue, the method is widely applicable, also in a developing country like Indonesia. This method was further used in the following chapter.

In ***chapter 5***, we further investigated the histopathological and immunohistochemical characteristics of early onset ($\leq$ 40 years) Indonesian breast cancer patients, as such features can be used as to distinguish between *BRCA* and non-*BRCA* carriers among these young women. This could help to limit expensive mutation screening to those patients at highest risk to harbour a germline *BRCA* mutation. Fully consistent with previous studies, our few *BRCA1* related cases were advanced stage, ductal type, grade 3, ER and HER-2/*neu* negative, and EGFR, CK5/6 and p53 positive. *BRCA2* tumors are more frequently of ductal type [40] as in the present study where all *BRCA2* related tumors were ductal. They are also more frequently grade 2 and 3 than sporadic controls [2, 41, 42]. In line with these previous studies, in the present study *BRCA2* carriers had moderately or poorly differentiated tumors and none of them were well differentiated. The frequency of ER and PR expression in *BRCA2* tumors has been reported as similar to that in sporadic breast tumors most studies [27, 42-44], in line with the present study. As to p53, some studies have found p53 accumulation in around 20-50% of *BRCA2* related carcinomas [27], In the present study, 60% of the *BRCA2* associated tumors showed p53 accumulation. Data on HER-2/*neu* expression in *BRCA2*-associated tumors vary from series to series, probably as a consequence of differences in the techniques employed. For example, Armes et al. [44] and Eerola et al. [43] found no differences in the expression of HER-2/*neu* in *BRCA2* and sporadic breast tumors. However, other studies revealed low frequencies between 0 and 3% in HER-2/*neu* overexpression in *BRCA2* tumors [27, 30, 42]. Combining immunohistochemistry with a new amplification test, we found a 20% frequency of HER-2/*neu* positivity in *BRCA2* associated tumors. While immunohistochemistry result for *EGFR*, *CK5/6* and *Ki67* for *BRCA2* associated tumors showed 40%, 20% and 100% positivity, respectively. The results of the present study add to the concept that breast cancer arising in *BRCA1* and *BRCA2* mutation carriers of mutation in the genes differ from sporadic breast cancer of age matched controls. This is especially clear for *BRCA1* related cancers. Unfortunately, also our study has not firmly established a clear phenotype for *BRCA2* related breast cancers. In conclusion, early onset Indonesian breast cancer is characterized by increased proliferation. Within the early onset group, the strongest features pointing to a sporadic cancer seem to be the absence of family history of breast and or ovarian cancer, grade I and lobular differentiation. Features

increasing the chance of a germline *BRCA1*/2 mutation are family history of breast and or ovarian cancer, CK5/6 and EGFR expression, p53 accumulation and high proliferation as measured by Ki67 labeling. This is potentially quite useful to optimize selection of early onset breast cancer patients for *BRCA1/2* mutation testing.

Breast cancer, like other cancers, derives through accumulation of a wide variety of genetic and epigenetic events [45-51], also in patients predisposed to breast cancer due to a germline *BRCA1*/2 mutation. As promoter methylation of tumor suppressor genes and amplification of oncogenes are well known phenomena in sporadic breast cancer but have hardly been studied yet in hereditary breast cancer, we analyzed a group of early onset Indonesian breast cancer for promoter methylation of tumor suppressor genes in ***chapter 6*** and for gene copy number in ***chapter 7***.

The majority of previous studies on methylation have dealt with only one or just a few genes [52, 53], applying techniques based on the conversion of unmethylated cytosine residues into uracil after sodium bisulphite treatment [54], which are converted to thymidine during subsequent PCR. Here, we performed for the first time a high throughput easy method based on methylation specific Multiplex Ligation dependent Probe Amplification (MS-MLPA) [55] to assess the methylation status of 22 selected tumor suppressor genes in a large group of breast cancer patients. We used in ***chapter 6*** the ME001 MS-MLPA probe mix (MRC Holland, Amsterdam, The Netherlands) which contains 24 sequences that correspond to the promoters of a set of tumor suppressor genes that are frequently silenced by methylation in different tumors (including breast cancer), but are unmethylated in blood derived DNA of healthy individuals. Six genes displayed no promoter methylation in any of 168 tumor samples: *CDKN2A (p14^{ARK}*), *CDKN1B (p27 ^{KIP1}*)) *ATM*, *PTEN*, *BRCA2* and *VHL*. Methylation frequencies for the other genes ranged from 1% for *TP73* and *FHIT* to 61% for *RASSF1.* For *p14^{ARF}*, *VHL* and *BRCA2*, this is in line with a previous study [56]*.* However, other studies using methylation-specific PCR did show methylation for *PTEN* (15/44 cases, 34%) [57] and *ATM* (18/23 cases, 78%) [58]. Hypermethylation of the Von Hippel-Lindau (*VHL*) gene is mostly found in renal call carcinoma [59] while a fundamental matter still remains completely unresolved regarding the reason behind non-hypermethylation of *BRCA2* [60]. Of the 168 tumor samples, 33 (20%) showed no methylation at any promoter site. 30 cases (18%) were methylated at the promoter site of only 1 gene, 35 (21%) at 2 sites, 32 (19%) at 3 sites, 21 (13%) at 4 sites, 11 (7%) at 5 sites, 2 (1%) at 6 sites, 3 (2%) at 7 sites and 1 (1%) at 8 sites. No tumor was methylated at all 22 sites. The most consistent association observed in the present study of specific gene methylation was with amplification of HER-2/*neu* where 4 genes (*APC*, *RASSF1A*, *CDH13* and *GSTP1*) reached statistical significance. Tumors with infrequent methylation (0–2 genes) were more often negative for HER-2/*neu* compared to those with frequent methylation (3-8 genes; 63% vs 37%, P=0.041). Tumors with infrequent methylation were more often well differentiated than those with frequent methylation (63% vs 38%, P=0.007). This finding is important because it links DNA hypermethylation with the well established pathological prognostic and predictive features of breast cancer. Previous studies have also reported associations between CpG island methylation and poor

histological differentiation of breast tumors [61]. In conclusion, this comprehensive analysis of tumor suppressor promoter methylation status in invasive breast cancer by high throughput MS-MLPA reveals remarkable differences in methylation frequency for various genes. Promoter methylation of multiple genes seems to be correlated to poor differentiation and HER-2/*neu* amplification. A comprehensive hypermethylation profile as assessed by MS-MLPA could therefore potentially be useful for breast cancer detection and classification, and understanding the biology of this disease.

Genomic instability is a hallmark of cancer, and specific subchromosomal copy number changes are thought to play a driving role in the transformation of normal cells to malignant clones. These genomic copy number changes may result in deletion of one or both alleles of tumor suppressor genes, overexpression of oncogenes and rearrangements that may alter transcription of target and downstream genes [62]. In ***chapter 7*** we applied a high throughput easy method based on Multiplex Ligation-dependent Probe Amplification (MLPA) to assess the amplification of 25 genes in a large group of breast cancer patients. Four genes displayed no amplification in any of 191 tumor samples: *BIRC4, PDGFRA, PDGFRB* and *HMGA*. Amplification frequencies for the other genes ranged from 1% for *MYBL2, BIRC2, hTERT, BCL2A1, BCL2L1, BCL2L13* and *MYCN* to 26% for HER-2/*neu*. Of 191 tumor samples, 52 cases (27%) were amplified at only 1 gene, 24 (13%) at 2 genes, 17 (9%) at 3 genes, 9 (5%) at 4 genes, 5 (3%) at 5 genes and 4 (2%) at 6 genes. From the chromosomal region point of view, this study observed three regions that consistent with other breast cancer literatures [63-65], by showing 38% gaining in region 8q (attributed to *MYC, PTK2* and *PTP4A3*), 26% in 17q (attributed to *HER2*) and 17% in 20q (attributed to *BCAS1* and *GNAS*). *MYC* amplification was found in 18% of the samples. This result is in line with previous studies that found 8 to 37% *MYC* amplification in breast cancer [66, 67]. A significant association between *MYC* amplification and poor differentiation was found in this study, consistent with a previous study that found a highly significant association between 8q24 copy number gains and high tumor grade [68]. By CGH, gains of 8q23-q24 were more often observed in grade III tumors [64]. Consistent with our previous study on Indonesian breast cancer [69] and other studies on HER-2/*neu* amplification [70-72], we found 26% of the samples to show HER-2/*neu* amplification. A recent meta-analysis summarizing data from 81 studies with 27,171 patients, revealed that in the great majority (90%) of studies either HER-2/*neu* amplification or protein overexpression correlated with poor outcome of the patients [73]. Studies using CGH have also found gain of 17q12-q21.1 where HER-2/*neu* is located [64]. The subgroup of tumors with frequent amplification (>2 genes) were more often poorly differentiated and at advanced stage compared to those with infrequent amplification (0-2 genes). This is well understandable as increasing genomic instability as also reflected in more amplified genes will accompany tumor progression. This finding is important because it links gene amplification with the well established pathological prognostic and predictive features of breast cancer. In conclusion, we present a novel high throughput MLPA method that allows a reliable, quick and cheap comprehensive analysis of copy number of a large set of genes that play a role in carcinogenesis in breast and other cancers. This method thereby allows efficient analysis of gene copy

number status for better understanding the biology and possibly clinical behavior of breast and other cancers.

When comparing methylation and gene amplification status between early onset patients (≤ 40 years) with the remaining patients, there were no significant differences. We analyzed this subgroup as in Indonesia it relatively large with many patient haboring BRCA1/2 mutations [33]. Although previous studies have shown genetic differences between early onset and older breast cancer patients [74, 75], such differences are not apparent with regard to promoter methylation of the tumor suppressor genes or amplification of the oncogenes analyzed.

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Chapter 9:

Samenvatting in het Nederlands
Summary in English
Ringkasan dalam bahasa Indonesia

SAMENVATTING

Borstkanker is een belangrijk volksgezondheidsprobleem in Indonesië, met name onder jonge vrouwen. Presentatie op jonge leeftijd is een bekende factor die wijst op een mogelijke kiembaan mutatie in *BRCA1/2*. Gezien het feit er etnische verschillen zijn in het soort *BRCA1*/2 mutatie en er nog geen publicaties zijn verschenen over *BRCA1*/2 mutatie detectie in de Indonesische populatie, is het identificeren van Indonesië specifieke mutaties belangrijk voor betere risk assessment van Indonesische vrouwen die een predispositie hebben voor het krijgen van *BRCA1*/2 gerelateerde erfelijke kankers. De grootte van de *BRCA1* en *BRCA2* genen en de verspreide ligging van mutaties binnen de genen maken mutatie detectie echter moeilijk, en snelle mutatie screening is daarmee een grote technische uitdaging. In ***hoofdstuk 2*** wordt een nieuwe slimme methode voor *BRCA1/2* mutatie detectie beschreven die is gebaseerd op gepoolde DGGE. Deze methode lijkt ideaal voor het screenen van naïeve populaties op mutaties en bleek gevoelig genoeg te zijn voor het detecteren van veranderingen in een enkel base paar op een niet-toxische, relatief simpele en goedkope manier.

Met deze methode werd een grotere groep van jonge borstkanker patiënten en enkele familie leden uit 3 Indonesische steden gescreend op *BRCA1*/2 mutaties in ***hoofdstuk 3.*** Een relatief hoog percentage van deze jonge Indonesische patiënten bleek een kiembaan mutatie te hebben in *BRCA1* of *BRCA2,* waaronder verschillende nieuwe pathogene mutaties en nieuwe “unclassified variant” mutaties die specifiek zouden kunnen zijn voor de Indonesische populatie. Er leken ook wat geografische verschillen te zijn tussen de mutaties in de 3 Indonesische regio’s.

In ***hoofdstuk 5*** wordt de waarde van histopathologische en immuunhistochemische markers voor het onderscheiden van BRCA mutatie draagsters en sporadische jonge patiënten (≤ 40 jaar) met borstkanker onderzocht. Positieve familie anamnese, CK5/6 en EGFR expressie, p53 accumulatie en hoge proliferatie maakten een kiembaan *BRCA1*/2 mutatie waarschijnlijker. Deze bevindingen zijn mogelijk waardevol voor het optimaliseren van de selectie van Indonesische patiënten voor *BRCA1/2* mutatie detectie.

De Multiplex Ligation dependent Probe Amplification (MLPA) PCR techniek werd in ***hoofdstuk 4*** gevalideerd als een attractief alternatief voor FISH als amplificatie test voor HER-2/*neu*, en voor het tegelijk testen op amplificatie van een set van 27 andere oncogenen in **hoofdstuk 7**. Tumoren met veel amplificaties (>2 genen) waren vaker slecht gedifferentieerd en van hoger stadium dan tumoren met weinig amplificaties (0-2 genen). Deze bevinding linkt gen amplificatie met bekende pathologische prognostische en predictieve borstkanker markers. In ***hoofdstuk 6*** is de methylering-specifieke MS-MLPA techniek gebruikt om een uitgebreide analyse te doen van de promotor methyleringsstatus van een set van 22 tumor suppressor genen in een grote groep borstkanker patiënten. Er waren opmerkelijke verschillen in methylerings frequenties voor de geteste genen. Als meerdere genen promotor methylering toonden was er vaak sprake van slechte differentiatie graad en HER-2/*neu* amplificatie.

Alhoewel eerdere studies genetische verschillen vonden tussen jonge en oudere borstkanker patiënten, waren er in onze Indonesische borstkanker populatie geen duidelijke verschillen in promotor methylering van tumor suppressor genen of amplificatie van oncogenen.

SUMMARY

Breast cancer is a major health problem in Indonesia, especially among young women. Early onset breast cancer has been known as one of the indicators harboring germline *BRCA1/2* mutation. Giving the fact that different *BRCA1*/2 mutations were found in different ethnic populations and no publications on *BRCA1*/2 mutation detection in the Indonesian population are available, identifying the Indonesian mutations is important for better risk assessment of Indonesian women who are susceptible to the *BRCA1*/2 related hereditary cancers. However, the large size of the *BRCA1* and *BRCA2*, and the scattered distribution of mutations complicate the task of mutation detection and make rapid screening for mutations a major technical challenge. In ***chapter 2***, we describe an intelligent pooled DGGE method to screen for *BRCA1/2* mutations. This method seems to be the ideal approach for screening naïve populations for mutations and was able to detect single base differences using non-toxic and relatively simple and inexpensive procedures.

A larger group of early onset breast cancer patients and their family members from three Indonesian cities were screened for *BRCA1*/2 mutations in ***chapter 3.*** A relatively high percentage of early onset Indonesian breast cancer patients were observed to carry a germline mutation in either *BRCA1* or *BRCA2,* which comprises of several novel pathogenic and a variety of novel "unclassified variant" mutations that could be specific for the Indonesian population. Comparison of the three different Indonesian regions for *BRCA1/2* mutations pointed to geographic differences.

To distinguish between *BRCA* and non-*BRCA* carriers among these young women, we investigated in ***chapter 5*** their histopathological and immuno-histochemical characteristics. Within the early onset group (≤ 40 years), family history of breast and or ovarian cancer, CK5/6 and EGFR expression, p53 accumulation and high proliferation features increased the chance of a germline *BRCA1*/2 mutation. This finding is potentially useful to optimize selection of early onset breast cancer patients for *BRCA1/2* mutation testing.

The Multiplex Ligation dependent Probe Amplification (MLPA) PCR technique was validated as an attractive alternative to FISH for amplification testing of HER-2/*neu* (***chapter 4***) as well as for multiplex testing of amplification of twenty-seven oncogenes (**chapter 7**). Tumors with frequent amplification (>2 genes) were more often poorly differentiated and at advanced stage compared to those with infrequent amplification (0-2 genes). This finding is important because it links gene amplification with well established pathological prognostic and predictive features of breast cancer. In ***chapter 6***, we used the methylation specific MS-MLPA technique to do a comprehensive analysis of promoter methylation status of 22 tumor suppressor genes in invasive breast cancer, which revealed remarkable differences in methylation frequency for various genes. Promoter methylation of multiple genes was correlated to poor differentiation and HER-2/*neu* amplification.

Although earlier studies have shown genetic differences between early and late onset breast cancer patients, such differences were not apparent with regard to promoter methylation of the tumor suppressor genes or amplification of the oncogenes analyzed in our Indonesian breast cancer population.

RINGKASAN

Kanker payudara merupakan salah satu penyakit yang banyak ditemukan pada wanita muda di Indonesia. Kejadian kanker payudara pada usia muda telah diketahui karena adanya mutasi pada gen *BRCA1* dan *BRCA2.* Karena terdapat perbedaan jenis mutasi *BRCA1* dan *BRCA2* pada berbagai etnis populasi, serta tidak ada data mengenai deteksi mutasi *BRCA1* dan *BRCA2* sebelumnya di Indonesia, maka identifikasi variasi mutasi di Indonesia sangat penting untuk skrining terhadap wanita yang mempunyai risiko kanker payudara turunan terkait *BRCA1* dan atau *BRCA2*. Namun demikian mengingat besarnya ukuran gen *BRCA1* dan *BRCA2* serta acaknya penyebaran mutasi, maka dibutuhkan teknik skrining mutasi secara tepat dan cepat. Metode yang digunakan untuk skrining mutasi *BRCA1* dan *BRCA2* adalah metode pengelompokan *DGGE*, yang dijabarkan pada ***bab 2***. Metode ini tidak hanya merupakan metode yang ideal untuk mendeteksi mutasi pada populasi baru namun juga aman, sederhana dan ekonomis.

Pada ***bab 3***, dijabarkan tentang deteksi mutasi *BRCA1/2* pada sejumlah besar penderita kanker payudara usia muda dari tiga kota besar di Indonesia. Sebagian besar penderita tersebut diketahui mempunyai mutasi turunan pada *BRCA1* atau *BRCA2* yang bersifat patogenik maupun jenis mutasi yang spesifik untuk populasi di Indonesia yang belum diketahui dampak klinisnya. Sedangkan perbandingan mutasi yang ditemukan diantara 3 kota besar tersebut menunjukkan adanya perbedaan.

Untuk membedakan karier *BRCA* dengan non-*BRCA* pada wanita muda, dilakukan karakterisasi berdasarkan histopatologis dan imunohistokimianya seperti dijabarkan dalam ***bab 5***. Pada kelompok kanker payudara usia muda ($\leq$ 40 tahun), riwayat kanker payudara dan atau kanker rahim pada keluarga, ekspresi CK5/6 dan EFGR, akumulasi p53 dan tingkat pertumbuhan yang tinggi meningkatkan kemungkinan mutasi *BRCA1/2*. Temuan ini bermanfaat untuk mengoptimalkan seleksi pada penderita kanker payudara usia muda yang akan dilakukan uji mutasi gen *BRCA1/2*.

Teknik *Multiplex Ligation dependent Probe Ampification* (MLPA) terbukti sebagai metode alternatif terbaik pengganti *FISH* untuk mengalisis amplifikasi *HER2-2/neu* ***(bab 4),*** seperti juga dalam menganalisis amplifikasi dua puluh tujuh onkogen ***(bab 7).*** Tumor yang mengalami amplifikasi pada lebih dari 2 onkogen mempunyai kecenderungan berdiferensiasi buruk dan berada pada stadium lanjut. Penemuan ini berhasil menyimpulkan adanya hubungan antara amplifikasi gen dengan gambaran patologis yang digunakan untuk menentukan prognosis kanker payudara. Pada **bab 6**, dibicarakan mengenai analisis komprehensif status metilasi 22 promotor gen tumor supresor dengan teknik *MS-MLPA* pada kanker payudara invasif. Studi ini menemukan adanya perbedaan yang signifikan pada frekuensi metilasi berbagai gen. Metilasi promoter pada berbagai gen ditengarai mempunyai korelasi pada diferensiasi yang buruk serta amplifikasi *HER-2/neu.*

Meskipun penelitian yang telah dilakukan sebelumnya menunjukkan perbedaan genetik antara penderita kanker payudara usia muda dan usia yang lebih tua, perbedaan ini tidak dijumpai pada analisis metilasi promoter gen tumor supresor dan amplifikasi onkogen yang dilakukan pada studi ini.

A word of gratitude
Curriculum Vitae
List of Publications

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CURRICULUM VITAE

Dewajani Purnomosari was born in Surabaya, East Java, Indonesia, on December 15, 1969. She received her degree in Biology in 1993 at Gadjah Mada University, Yogyakarta, Indonesia, and pursued her master program in Medical Biotechnology in 1994 at the same University.

After graduating from her master study, she joined the department of Histology and Cell Biology at the Faculty of Medicine of the Gadjah Mada University in 1997, and was appointed to help in the Tumor Biology Laboratory in 1998 as a researcher. In 1999 she had the opportunity to do research training in the Department of Pathology of the VU University Medical Centre (VUmc) of Amsterdam, The Netherlands, and the Fräuen Klinik, Heidelberg, Germany, for 3 months. In 2000, she worked in the field of molecular genetics of breast cancer in the Department of Clinical Genetics of the VUmc for 3 months.

From 2003-2006 she conducted a sandwich research program at Molecular Biology Laboratory, Medical Faculty, Gadjah Mada University and at the Molecular Diagnostics Laboratory, Department of Clinical Genetics, VUmc for 3 months each year, within the framework of a Dutch Cancer Society development project. During this period she improve her knowledge and skills as a researcher in the Laboratory Facilities of the Department of Health, Hawaii State, Hawaii, USA for 1 month in 2005 and represented Indonesia in the Novartis International Biotechnology Leadership Camp in Taiwan in the same year. Her work in The Netherlands was supervised by Prof. dr Paul J van Diest (Department of Pathology, Utrecht Medical Center (formerly at the VUmc)) and dr. Gerard Pals, (Molecular Diagnostics laboratory, Department of Clinical Genetics, VUmc), and resulted in this PhD thesis.

LIST OF PUBLICATIONS

Purnomosari D, Aryandono T, Setiaji K, Nugraha SB, Pals G, van Diest PJ. *Comparison of multiplex ligation dependent probe amplification to immunohistochemistry for assessing HER-2/neu amplification in invasive breast cancer*, Biotechnic & Histochemistry 2006, 81(2-3); 79-85

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