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Chapter 14

MammaPrint for Individualized Recurrence Risk Assessment and Treatment Recommendations for Early-Stage Breast Cancer Patients

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14.1 Introduction

Breast cancer is the second most common cancer and the second leading cause of cancer-related deaths in women worldwide. In 2013 an estimated 232,340 women are expected to be diagnosed with new cases of invasive breast cancers in the United States, and 39,620 women are expected to die from the disease [1, 2]. The majority of these deaths are due to disease recurrence or distant metastasis after initial treatment. Adjuvant systemic therapy with either endocrine therapy and/or chemotherapy has been shown to reduce the risk of distant recurrence and death from invasive breast cancer after local treatment with surgery with or without radiation therapy. To save lives, existing guidelines, aimed at avoiding under use of adjuvant

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systemic therapy in early-stage breast cancer patients, recommend considering adjuvant therapy in all age of patients whose tumors are at least 1.0 cm [3, 4]. The decision to administer adjuvant therapy is based on several prognostic factors, including patient age, co-morbidities, tumor size, tumor grade, number of involved axillary lymph nodes, and possibly HER2 tumor status. These clinical and pathological features of breast cancer can be calculated using several Web-based tools such as Adjuvant! Online (www.adjuvantonline.com). Under this guideline, only 15–20% of node-negative breast cancer patients whose tumors are 1.0 cm or less are considered "low-risk," given that the 10-year recurrence risk is 10% and less. As only 30% of node-negative and 70% of node-positive breast cancer patients have recurrent disease, this recommendation has led to overtreatment with chemotherapy in many breast cancer patients.

The current clinical and pathological risk assessment tool also does not take into account the influence of tumor genetics or biology on disease recurrence for individual patients. With the advances in molecular technologies, molecular profiling has been increasingly used to subtype breast cancers and stratify patients into different prognostic and/or predictive subsets for risk assessment and individualized cancer therapy for both standard and novel targets [5]. While many of these molecular profiling assays are still in preclinical development, two diagnostic tests, Oncotype Dx and MammaPrint®, which use multi-gene expression signatures of breast tumors to assess the genetic risk of breast cancer recurrence in individual patients, have reached clinical testing. Like other new diagnostic biomarker assays, the development of these tests occurs in several phases before widespread clinical use, i.e., discovery, retrospective clinical validation, analytic validation, and prospective clinical validation. The utility of these tests in guiding clinical treatment decision are being evaluated in two large-scale, prospective randomized trials, the TAILORx for Oncotype Dx and the MINDACT for MammaPrint. In this chapter, we will summarize the development and clinical use of the MammaPrint assay. The Oncotype Dx test is reviewed in another chapter of this book.

14.2 Discovery of MammaPrint

The MammaPrint assay uses microarray technology to assess a 70-gene expression profile to assess a breast cancer patient's risk of

developing recurrence or distant metastases or death [4, 6–8]. The 70-gene expression profile was discovered by researchers at the Netherlands Cancer Institute, and is now performed and marketed by Agendia (www.agendia.com). This test was developed using an unbiased 3-step supervised biostatistical method to identify common gene expression patterns in 78 primary breast tumors from lymph-node negative patients [7]. First, gene expression microarray analysis using an Agilent Hu25K array, which contains approximately 25,000 human genes, identified 5,000 genes that were commonly regulated genes in at least 80% of samples. Next, 231 genes were selected based on significant association with disease outcome from patient data using the magnitude of the correlation coefficient. Finally, a leave-one-out method was used to optimize this gene list, resulting in a final list of 70 genes. The expression level of these 70 genes was used to calculate a recurrence score that is either low risk or high risk. "Low Risk" MammaPrint result means that a patient has a 10% chance that her cancer will recur within 10 years without any additional adjuvant treatment, either hormonal therapy or chemotherapy. A "High Risk" MammaPrint result means that a patient has a 29% chance that her cancer will recur within 10 years without any additional adjuvant treatment, either hormonal therapy or chemotherapy [9]. Patients at low risk might be safely spared adjuvant systemic chemotherapy, while patients at high risk might benefit from adjuvant systemic chemotherapy. The positive predictive value of tumor recurrence in high-risk patients at 5 and 10 years is 23% and 29%, respectively. The negative predictive value of tumor recurrence in low-risk patients at 5 and 10 years is 95% and 90%, respectively. Thus, compared to the current clinical-pathological risk assessment tools, the MammaPrint test more accurately identifies breast cancer patients with low-risk for recurrence than patients with high risk for recurrence.

14.3 Retrospective Clinical Validation

The MammaPrint test was first validated in a study of lymph-node negative breast cancer patients that were less than 56 years old with tumors less than 5 cm [7]. To date, the MammaPrint assay has been validated in various other retrospective studies in more than 5,600 patients [8–27]. These studies support that MammaPrint

could accurately identify those patients with low risk for distant recurrence and thus could avoid unnecessary adjuvant systemic chemotherapy. Table 14.1 summarizes the published validation tests of the MammaPrint assay in different populations of breast cancer patients, including various European (Dutch, German, Italian, Spanish), American, and Japanese cohorts.

Small tumors (T1 or ≤2 cm) are generally considered to have a low risk of recurrence after surgical resection. However, some stage 1 breast cancers do metastasize, and it has been a challenge to try and identify those small tumors with metastatic potential. MammaPrint evaluation of 964 patients with T1 tumors showed that 46% had a poor prognosis signature, suggesting that MammaPrint is a better tool for selecting early-stage breast cancer patients with smaller tumors for adjuvant therapy compared to current clinical-pathological risk assessment tools [15, 23]. The MammaPrint test was developed using fresh tumor specimens obtained at the time of surgery. However, more recently, Mayordomo and colleagues showed that sufficient RNA could be isolated from 14-gauge core biopsies to perform array analysis [22, 28]. This broadens the clinical applicability of MammaPrint as it eliminates the reliance on surgical specimens, which are often fixed immediately. Further optimization is required for smaller tumors since one in seven tumor specimens taken from tumors smaller than 2 cm had insufficient tumor material for analysis. Recently, studies were conducted to compare gene signatures obtained from MammaPrint using matched samples from fresh frozen tissue and samples from formalin-fixed, paraffin embedded tissue. Results showed that the gene signatures from both samples were highly similar, increasing the potential resources available for the MammaPrint test [29]. While most of the validation studies have retrospective analyses, one study by Bueno-de-Mesquita *et al.* showed that MammaPrint could be used prospectively for risk assessment and that MammaPrint is a feasible option for use in community hospitals [12].

MammaPrint has also been validated in many different clinical scenarios, such as in patients ranging from 35 to 70 years of age, in pre- or postmenopausal women, and in Stage T0–T4 breast cancers that are either node positive or node negative (Table 14.1). Multivariate analyses show that MammaPrint risk assessment is independent of ER, PR and HER2 status [10].

Table 14.1 Summary of published validation tests of MammaPrint assay

First author (year)	Patient population	Period of specimen collection	No. of patients	Age of patient	Histopathology	Median follow-up (years)	Distant metastasis-free survival (DMFS)			Overall survival (OS)		
							Good prognostic signature	Poor prognostic signature	Hazard ratio (HR)	Good prognostic signature	Poor prognostic signature	Hazard ratio (HR)
Van de Vijver (2002)	Dutch	1984–1995	295	<53	pTI-pT2, N0 or N+	6.7	*n* = 115	*n* = 180	N.A.			
							(5y) 94.7% ± 2.1%	(5y) 60.5% ± 3.8	<5y: HR 8.8; 95% CI 3.8–20; *P* < 0.001	(5y) 97.4 ± 1.5%	(5y) 74.1 ± 3.3%	HR 8.6; 95% CI 4–19; *P* < 0.001
							(10y) 85.2% ± 4.3%	(10y) 50.6 % ± 4.5	>5y: HR 1.8; 95% CI 0.69–4.5 *P* = 0.24	(10y) 94.5 ± 2.6	(10y) 54.6 ± 4.4%	N.A
Buyse (2006)	European	1980–1998	307	<61	T1-T2, N0, ER-	13.6	*n* = 113	*n* = 194	N.A.			
							(5y) 96%	(5y) 83%	N.A.			
							(10y) 88%	(10y) 71%	(10y) HR 1.5 95% CI 1.04–2.16 *P* < 0.001	(10y) 88%	(10y) 69%	(10y) HR 2.79: 95% CI 1.6–4.87; *P* = 0.032

(*Continued*)

Table 14.1 (*Continued*)

First author (year)	Patient population	Period of specimen collection	No. of patients	Age of patient	Histopathology	Median follow-up (years)	Distant metastasis-free survival (DMFS)			Overall survival (OS)		
							Good prognostic signature	Poor prognostic signature	Hazard ratio (HR)	Good prognostic signature	Poor prognostic signature	Hazard ratio (HR)
Bueno-de-Mesquita (2007)	Dutch	2004–2006	427	<61	T1-4, N0, M0	1.16 (14 months)	*n* = 219	*n* = 208	N.A.	N.A.	N.A.	N.A
Bueno-de-Mesquita (2008) [Update report of Van de Vijver (2002)]	Dutch	1984–1995	151	<53	N0	10.2	*n* = 60	*n* = 91	N.A.			
							(10y) 86 ± 5%	(10y) 50 ± 6%	HR 5.5; 95% CI 2.5–12 *P* < 0.01	(10y) 94 ± 3%	(10y) 51 ± 5%	HR 10.7; 95% CI 3.9–30; *P* < 0.01
Bueno-de-Mesquita (2008)	Dutch	1996–1999	123	<56 years	pT1-T2, N0	5.8	*n* = 64	*n* = 59				
							(5y) 98 ± 2%	(5y) 78 ± 6%	HR 5.7; 95% CI 1.6–20; *P* = 0.007	(5y) 97 ± 2%	(5y) 82 ± 5%	HR 3.4; 95% CI 1.2–9.6; *P* = 0.021

First author (year)	Patient population	Period of specimen collection	No. of patients	Age of patient	Histopathology	Median follow-up (years)	Distant metastasis-free survival (DMFS)			Overall survival (OS)		
							Good prognostic signature	Poor prognostic signature	Hazard ratio (HR)	Good prognostic signature	Poor prognostic signature	Hazard ratio (HR)
Mayordomo (2008)	Spain	2006–2008	35	all ages	T2-T3 (Core needle biopsy)	N.A.	*n* = 4	*n* = 31				
Wittner (2008)	US	1985–1997	100	median age 63	pT1-T3, pN0	11.3	*n* = 27	*n* = 73				
							(5y) 93%	(5y) 51%	N.A.	85%	52%	N.A
							(10y) 75%	(10y) 72%	N.A.	N.A.	N.A.	N.A
Mook (2008)	European	1994–2001	241	<71	T1-T3, N1-N3	7.8	*n* = 99	*n* = 142				
							(5y) 98 ± 2%	(5y) 80 ± 4%	N.A.	(5y) BCSS 96 ± 2%	(5y) BCSS 76 ± 4%	BCSS HR 5.7; 95% CI 2.01–16.23; *P* = 0.001
							(10y) 91 ± 4%	(10y) 76 ± 4%	HR 4.13; 95% CI 1.72–9.96; *P* = 0.002	(10y) BCSS 99 ± 1%	(10y) BCSS 88 ± 3%	(Overall) HR 5.4; 95% CI 2.11–13.8; *P* < 0.001
Mook (2008)	Dutch	1984–1996	148	<71	T1-T2, N0	12.5	*n* = 91	*n* = 57				

(*Continued*)

Table 14.1 (*Continued*)

First author (year)	Patient population	Period of specimen collection	No. of patients	Age of patient	Histopathology	Median follow-up (years)	Distant metastasis-free survival (DMFS)			Overall survival (OS)		
							Good prognostic signature	Poor prognostic signature	Hazard ratio (HR)	Good prognostic signature	Poor prognostic signature	Hazard ratio (HR)
							(5y) 93 ± 3%	(5y) 72 ± 6%	(5y) HR 4.6; 95% CI 1.8–12; P = 0.001	(5y) BCSS 99 ± 1%	(5y) BCSS 80 ± 5%	HR 19.1; 95% CI 2.5–148; P = 0.005
							(overall DMFS) 81%	(overall DMFS) 68%	(Overall) HR 1.8; 95% CI 0.9–3.5; P = 0.07	N.A.	N.A.	(Overall) HR 2.0; 95% CI 1.0–4.0; P = 0.04
Generali (2008)	Italy	N.A	13	N.A.	T2-T4, N0-N1, ER+, Postmenapausal	N.A.	n = 4	n = 9	N.A.	N.A.	N.A.	N.A
Kunz (2009)	German	2005–2008	140	N.A.	T1-T2	N.A.	n = 77	n = 59	N.A.	N.A.	N.A.	N.A
Saghatchian (2009)	European	N.A	153	N.A.	T1-T3, N = 4–6	N.A.	n = 65	n = 88				
							N.A.	N.A.	N.A.	(5y) 97 ± 2%	(5y) 79 ± 5%	HR 2.4; 95% CI 1.0–5.5; P = 0.04

First author (year)	Patient population	Period of specimen collection	No. of patients	Age of patient	Histopathology	Median follow-up (years)	Distant metastasis-free survival (DMFS)			Overall survival (OS)		
							Good prognostic signature	Poor prognostic signature	Hazard ratio (HR)	Good prognostic signature	Poor prognostic signature	Hazard ratio (HR)
							N.A.	N.A.	N.A.	(10y) 91 ± 4%	(10y) 74 ± 5%	N.A
Bender (2009)	European	N.A	1637	N.A.	T1-T2, N0 or N+	7.1	*n* = 772	*n* = 865				
							(10y) 90%	(10y) 30%	HR 3.88; 95% CI 1.99–7.58; *P* < 0.01	N.A.	N.A.	N.A
Knauer (2009) [Update report of Bender (2009)]	European	1984–2009	541	N.A.	pT1-T3, N0-N1, M0	7.1	*n* = 252	*n* = 289	N.A.	N.A.	N.A.	N.A
							95%	82%	HR 3.88; 95% CI 1.99–7.58; *P* < 0.01	(5y) BCSS 97%	(5y) BCSS 87%	HR 4.01; 95% CI 1.98–11.67; *P* = 0.05
Ishitobi (2010)	Japanese	1998–2001	102	<70	N0	7.1	*n* = 20	*n* = 82				

(*Continued*)

Table 14.1 (*Continued*)

First author (year)	Patient population	Period of specimen collection	No. of patients	Age of patient	Histopathology	Median follow-up (years)	Distant metastasis-free survival (DMFS)			Overall survival (OS)		
							Good prognostic signature	Poor prognostic signature	Hazard ratio (HR)	Good prognostic signature	Poor prognostic signature	Hazard ratio (HR)
							(5y) 100%	(5y) 94%	N.A.	N.A.	N.A.	N.A
Straver (2010)	Dutch	2000–2008	162	<69	T1-T2	1.04 (25 months)	*n* = 23	*n* = 144	N.A.	N.A.	N.A.	N.A
Kunz (2010)	German	2004–2008	44	<56	T1-3, N0-3	N.A.	*n* = 29	*n* = 15	N.A.	N.A.	N.A.	N.A
Somlo (2010)	US	2006–2009	68	<70	T2-T3 (Core needle biopsy)	N.A.	*n* = 15	*n* = 53	N.A.	N.A.	N.A.	N.A
Mook (2010)	Dutch	N.A	964	N.A.	N.A.	7.1	*n* = 525	*n* = 439				
							(5y) 95 ± 1%	(5y) 80 ± 2%	N.A.	(5y) BCSS 99 ± 1%	(5y) BCSS 88 ± 2%	N.A
							(10y) 87 ± 2%	(10y) 72 ± 3%	(10y) HR 2.7; 95% CI 1.88–3.88; $P < 0.001$	(10y) BCSS 91 ± 2%	(10y) BCSS 72 ± 2%	(10y) HR 4.22: 95% CI 2.7–6.6; $P < 0.001$

Abbreviations: BCSS, breast cancer specific survival; N.A., not available.

14.4 Analytic Development for MammaPrint as a Diagnostic Test

All MammaPrint tests are performed using a customized microarray in the Agendia's Clinical Laboratory Improvement Amendments (CLIA)-accredited service laboratories at the company's headquarters in Amsterdam, the Netherlands since 2004 and in Irvine, California since 2008. In February 2007, it became the first *in vitro* diagnostic multivariate index assay cleared by the US Food and Drug Administration (FDA) to assess the risk of tumor recurrence in lymph node negative breast cancer patients under 61 years of age with tumors or less than 5 cm, who were within 10 years of diagnosis. FDA clearance under the *in vitro* diagnostic multivariate index assay (IVDMIA) guidelines requires clinical and analytical validation and reporting systems to ensure patient safety issues are addressed. The FDA label indicates that as a diagnostic tool, MammaPrint has a 98.9% degree of accuracy in classifying patients as Low Risk or High Risk and technical reproducibility of 98.5%. It is recommended that MammaPrint® results are used by physicians as a prognostic marker only, along with other clinicopathological factors in planning treatment with adjuvant chemotherapy.

Since its initial FDA clearance, several modifications of the cleared device have been made such as changing the specimen type from fresh frozen tissue to fresh tissue stored in a specific RNA preservative solution (i.e., RNARetain room temperature tissue fixative) and XPrint software v1.33 to v1.40. Recently, the FDA cleared the MammaPrint test for breast cancer recurrence in all ages and all stage I and II breast cancer patients, including patients with negative and up to three positive lymph nodes. Agendia received its fifth FDA clearance for MammaPrint in early 2011 to allow the test to be performed using two additional Agilent Microarray scanners and two Agilent Bioanalyzers in CLIA- and College of American Pathologists (CAP)-accredited central laboratories in Amsterdam, the Netherlands. With this approval, Agendia could provide a consistent testing service in case one machine is down and could accommodate the increasing need for clinical testing.

It is currently covered by Centers for Medicare and Medicaid Services and most major insurance carriers in the US. In Europe, MammaPrint has been approved for all ages since 2004. The cost of the assay in the US is $4,200. In Europe, the test costs EUR 2675. Some

insurance companies will pay for the total cost of the MammaPrint test, while others may pay a portion of the cost. Agendia created the Agendia Cares Program to help with insurance and payment issues.

Table 14.2 summarizes the current approved indication for MammaPrint in the United States and Europe.

Table 14.2 Current approved indication for MammaPrint in the United States and Europe

Eligibility	United States	Europe and others
Staging and histology of breast cancer	stage I or stage II invasive hormone-receptor-positive AND hormone-receptor-negative smaller than 5 centimeters lymph node: 0–3	stage I or stage II invasive hormone-receptor-positive AND hormone-receptor-negative smaller than 5 centimeters lymph node: 0–3
Age	Women diagnosed with cancer must be 61 or younger All (since 2011)	All (since 2004)
Date of Approval	February 2007	2004
Cost	$4,200	EUR 2675
Coverage	Most of the insurance carriers	Depending on the country

14.5 Prospective Clinical Validation of MammaPrint

MINDACT (Microarray In Node-negative and 1 to 3 positive lymph node Disease may Avoid ChemoTherapy), as illustrated in Fig. 14.1, is a prospective randomized study comparing the 70-gene signature MammaPrint assay with the common clinical-pathological criteria in selecting patients for adjuvant chemotherapy in breast cancer with 0–3 positive nodes (EORTC Protocol 10041 -BIG 3–04; http://www.eortc.be/services/unit/mindact/MINDACT_websiteii.asp; ClinicalTrials.gov Identifier: NCT00433589) [30, 31]. The objective of MINDACT is to test whether patients with a low risk signature may

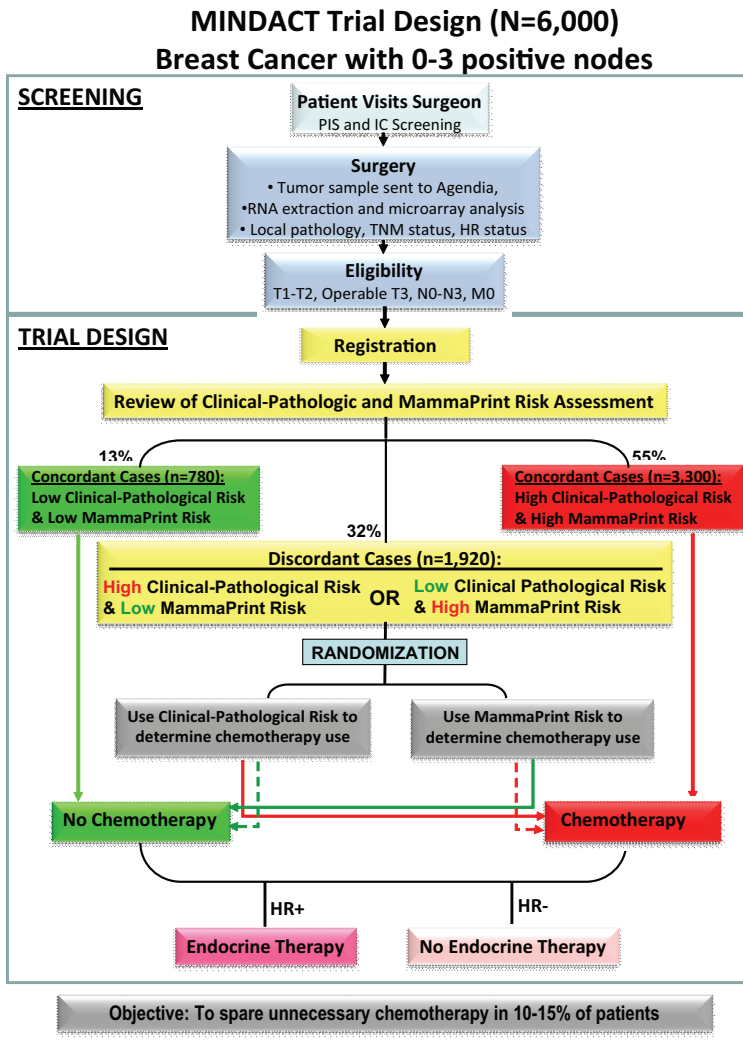

Figure 14.1 Schema of MINDACT study. *Abbreviations:* PIS, patient information sheet; IC, informed consent; T: tumor stage; N: nodes; M: metastasis; HR: hormone receptor.

safely be spared the toxicities of chemotherapy without affecting their survival. The study is complex in that it further tests which of two chemotherapy treatments offers better survival and which of two endocrine regimes is more effective. Each enrolled breast cancer patient will be assessed using both MammaPrint and conventional

prognostic tools. For most women, the results of the two tests are expected to be the same. Chemotherapy, either anthracycline- or docetaxel-based, will be offered to those who are categorized as high risk by both assays. It will not be offered if they are shown to be at low risk by both assays. Women for whom the conventional and MammaPrint tests do not agree will be randomized according to conventional or MammaPrint test results and allocated to either adjuvant chemotherapy or no adjuvant chemotherapy. Additionally, all women who are estrogen receptor positive will be offered one of two endocrine treatment regimens: 7 years of single agent letrozole or the sequential strategy of 2 years of tamoxifen followed by 5 years of letrozole. It is hoped that not only will 10% to 20% of patients be spared treatment but that there will be considerable savings for the national health services. The MINDACT was first activated in the Netherlands on March 22, 2007, and was amended to increase the sample size to 6600. By July 2011, the study reached the accrual goal with 6700 breast cancer patients have been enrolled from 119 participating institutions in 9 European Countries. From 119 participating institutions in 9 European Countries. Similarly to the TAILORx trial in the United States, the MINDACT study has progressed well in accruing patients, suggesting the general acceptance of using new molecular risk assessment tools in guiding clinical decisions for treatment planning. The results from these two studies are highly anticipated.

14.6 Biologic Implication of MammaPrint Results

14.6.1 Understanding of Tumor Biology

It is interesting that a prognostic signature could be developed using the transcriptional profile of primary breast tumors. This suggests that the metastatic potential is inherently expressed in the initial tumor rather than being acquired at a later stage. A recent report provides functional annotation of the 70 genes in the MammaPrint with the hallmarks of cancer [32]. Figure 14.2 illustrates the genes that are known to be functionally involved in recurrence, such as signal transduction and cell cycle, invasion, metastasis and angiogenesis genes that are found to be significantly upregulated

in the poor prognosis signature. Many of these candidate genes should be investigated to test their feasibility as new therapeutic targets. It is interesting to note that individual genes that have been previously correlated with disease outcome, such as cyclin D1, ERS1, HER2, c-myc, UPA and PAI-1 are not present in the 70-gene MammaPrint profile. This is most likely due to the overlap and redundancy inherent in biological processes as well as our own lack of knowledge. Indeed, genes regulated by ERα and HER2 are represented in the 70-gene profile. A network association map of the 70 genes that comprise MammaPrint shows that key players in cancer, such as p53, Rb, c-myc, Jun and CDKN2, are central regulators although their change in expression is not integral to the profile [32]. It is known that clinical factors, such as co-morbidities (e.g., diabetes, chronic inflammation, etc.) affect patient's risk of tumor recurrence and survival. Currently, it is not known how these clinical factors affect the results of 70-gene expression in MammaPrint test.

14.6.2 Revealing New Therapeutic Targets

Most of the genes in the MammaPrint profile are not targets of current drug discovery efforts; however, many can be categorized into one of the six "Hallmarks of Cancer," as defined by Hanahan and Weinberg [32, 33]. Eleven genes fall into the sustained proliferative signaling hallmark, 5 into the evading growth suppressors hallmark, 15 into the enabling replicative immortality hallmark, 5 into the resisting cell death hallmark, 8 into the activating invasion and metastasis hallmark and 12 into the inducing angiogenesis hallmark (Fig. 14.2). Often, the most druggable targets are the genes involved in signal transduction pathways, and several were found to be included in the MammaPrint profile, including FLT1, HRASLS, STK32B, TGFB3, RASSF7, MELK, IGFBP5, FGF18, and DCK. Four of these genes, each of which represents a different signaling pathway, stand out as pathway-specific drug targets for therapy. FLT1, a member of the VEGF pathway, is a receptor tyrosine kinase that binds to VEGFR-A, VEGFR-B and placental growth factor and plays an important role in angiogenesis and vasculogenesis [34]. TGFβ3 is part of the TGFβ pathway and is involved in cell differentiation [35]. IGFBP5 is a member of the insulin pathway and is linked to senescence and autophagy, which are often associated with apoptosis [36, 37]. FGF18 is a growth factor in the FGF signaling pathway and is thought to

promote cell survival under stress, through autocrine and paracrine signaling, and angiogenesis [38]. While these represent mechanisms to target individual pathways, targeted therapy could be successful since they may be involved in multiple hallmark functions. While these are individual pathways, targeted therapy could be successful since they can overlap in their hallmark function. For instance, targeting FLT1 could prevent tumor cells from evading apoptosis and initiating/maintaining angiogenesis, and antibodies against TGFβ3 could resensitize cells to growth inhibitory therapies.

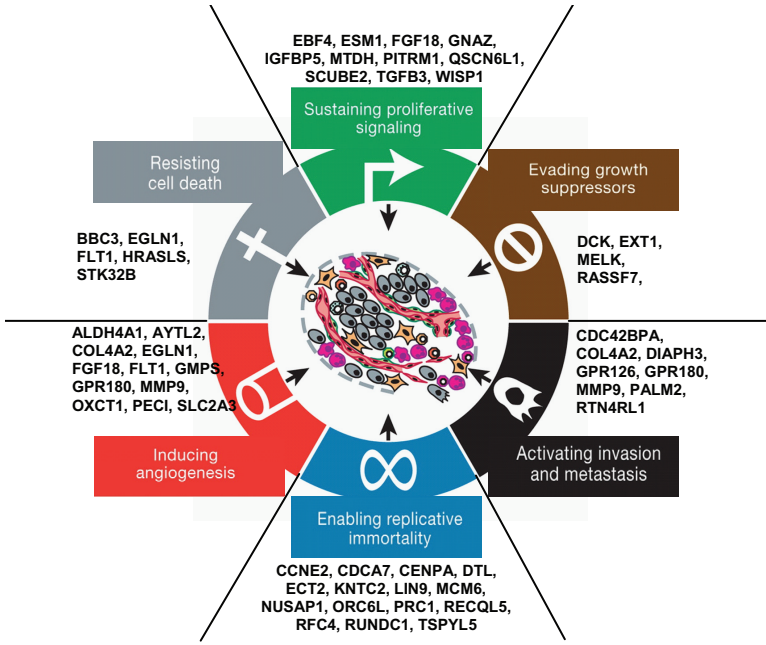

Figure 14.2 Biological function of MammaPrint® 70 genes and the hallmarks of cancer. The 70 genes are involved in at least eight well-defined hallmarks of cancer, in tumor progression and metastasis related biological processes, as well as epithelial-mesenchymal transition. Adapted from Biomark Insights. 2010, Tian *et al*., Biological Functions of the Genes in the MammaPrint Breast Cancer Profile Reflect the Hallmarks of Cancer, 5: 129–138. Copyright © 2010 the author(s), publisher and licensee Libertas Academica Ltd. Modified with permission.

In addition to genes in specific pathways, there were several genes that may play a role in multiple signaling pathways. HRASLS is

a RAS tumor suppressor [39], STK32B has been linked to the NFκB pathway [40], RASSF7 is a JNK inhibitor and involved in mitosis [41, 42], MELK inhibits apoptosis and has been associated with poor prognosis in breast cancer [43, 44], and DCK is involved in metabolism of nucleosides and is clinically important because of its relationship to drug resistance and sensitivity [45]. The advantage of targeting these genes is that signaling pathways often converge on downstream signaling molecules thereby targeting multiple pathways in a single agent.

Finally, several other genes are worth mentioning due to their importance in breast tumor biology. For instance, CCNE2 is a member of the cyclin family, is tightly controlled and mediates the G1/S transition during the cell cycle. It binds to CDK2, is regulated by estrogen, and has been shown to be upregulated in tumor cells [46, 47]. CENPA is a key player in mitosis and is required for recruitment of most proteins to the centromere [48]. COL4A2 is a type IV collagen and is a basement membrane protein that contributes to tumor structure, is degraded during tumor invasion and metastasis, and is overexpressed on ER-negative breast cancer patients [49]. MMP9 is a metalloproteinase extracellular matrix protein that is very important in metastasis. It is involved in the degradation of type IV and V collagens [50]. These are all potential drug targets. A recent update to the "Hallmarks of Cancer" added four new categories: inflammation, evading the immune system, unstable DNA, and deregulated metabolism [51], of which MammaPrint genes fit into the two latter categories (deregulated metabolism: ALDH4A1, AYTL2, DCK, GMPS, GSTM3, OXCT1, PECI, PITRM1, SLC2A3 and unstable DNA: CCNE2, CENPA, MCM6, NUSAP1, ORC6L, PRC1, RASSF7 and RFC4) as illustrated in Fig. 14.3. It is unclear yet how these new categories will aid in the development of more potent and beneficial therapy.

14.6.3 Prediction for Response or Resistance to Chemotherapy

MammaPrint has been established and validated as an accurate prognostic tool that categorizes patients according to their recurrence risk (low or high) for breast cancer and thus identifies patients for whom adjuvant therapy would be most beneficial. The next clinical challenge now becomes how to manage the high-risk

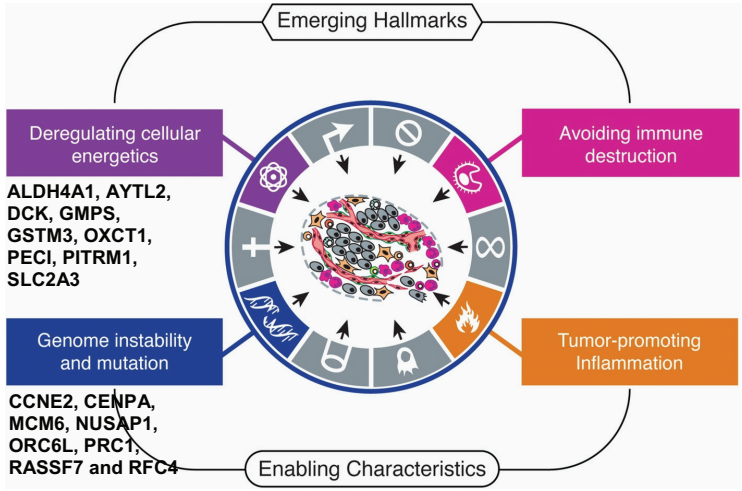

Figure 14.3 Biological function of MammaPrint® 70 genes and new hallmarks of cancer.

patients characterized by MammaPrint test. Breast cancer patients who were at high risk for recurrence had a 32% pathological complete response to chemotherapy in a recent neoadjuvant trial. In contrast, those patients who were low-risk had a 9% pathological complete response rate [26]. To determine the predictive value of MammaPrint for neoadjuvant chemotherapy, Knauer *et al.* reported on a meta-analysis of data gathered from seven large studies, comparing 541 patients that were given endocrine therapy alone or chemotherapy plus endocrine therapy [10, 17, 19]. MammaPrint analysis identified 252 of the 541 patients as low risk, with a 5-year distant disease-free survival (DDFS) of 95%, and 289 patients as high risk, with a 5-year DDFS of 82%. In the low-risk group, those who received chemotherapy plus endocrine therapy had only a slight increase in their 5-year DDFS (99% vs. 93%). In contrast, high-risk patients who received the combination therapy demonstrated a significantly higher 5-year DDFS compared to those who received endocrine therapy alone (88% vs. 76%). Multivariate analysis of clinical pathologic criteria that may have affected the results showed that there was a statistically significant correlation between high-risk signatures and tumor size, number of positive lymph nodes and positive PR status. Similar studies conducted with smaller numbers of patients also showed that patients with a low-risk gene signature

are less sensitive to chemotherapy compared to patients with a high-risk gene signature, further confirming that neoadjuvant therapy is the most beneficial to patients with poor prognosis profiles, and that MammaPrint may be useful for predicting patient response to chemotherapy. MammaPrint assays have been incorporated in the two ongoing, large-scale neoadjuvant studies I-SPY1 Trial (Investigation of Serial Studies to Predict Your Therapeutic Response with Imaging And moLecular Analysis or The I-SPY Trial) and I-SPY2 Trial (Neoadjuvant and Personalized Adaptive Novel Agents to Treat Breast Cancer; ClinicalTrials.gov Identifier: No. NCT01042379). While I-SPY I aims to evaluate biomarkers and imaging for predicting response to standard neoadjuvant chemotherapy, I-SPY II will use biomarkers to stratify patients based on their predicted likelihood of response to treatment with molecularly targeted drugs in combination with standard chemotherapy in the neoadjuvant setting. More insights will be highly anticipated from these studies.

14.6.4 Elucidation of Resistant Mechanisms to Chemotherapy

MammaPrint is a good prognostic tool for predicting the risk of recurrence and metastasis in early-stage breast cancer patients. However the question remains: How do tumors become resistant to chemotherapy? Since the probability of recurrence and metastasis can be determined from the primary tumor, the possible mechanisms of resistance may also be part of the MammaPrint gene signature. The first steps of metastasis require cells to undergo a series of biochemical changes including cytoskeletal rearrangement and loss of cell polarity, breaking down the extracellular matrix and invasion into surrounding tissue. During these processes the cells go from an epithelial phenotype to a mesenchymal phenotype, called the epithelial to mesenchymal transition (EMT), also known to be part of the developmental process. Genes that mediate EMT may not be targeted by current chemotherapy regiments. There are several EMT related genes that are expressed in the MammaPrint gene signature. TGFβ signaling is known to induce EMT, and TGFβ1 and TGFβ3 have been shown to play complementary roles in the process [35]. IGFBP-5 is a member of the insulin pathway, but has also been linked to tissue remodeling by inducing the TGFβ pathway [52]. In addition, IGFBP-5 has been found to be involved in different mechanisms mediating

resistances to tamoxifen in cell lines and mouse models [53]. MMP9, as mentioned above, is a matrix metalloproteinase that degrades extracellular matrix (ECM) proteins and may be upregulated by TGFβ signaling [54]. COL42A, another ECM protein mentioned in previous section, may also be involved in the metastasis process. While EMT is thought to mitigate the first steps of metastasis, the reverse process, mesenchymal to epithelial transition (MET) is required to establish the tumor cells in their distal locations. Re-depositing basement membrane protein at that time is consistent with re-establishing cell polarity, and the surrounding stromal environment. The WNT signaling pathway has also been implicated in EMT. WISP1, a downstream signaling molecule of WNT pathway, is expressed in fibroblasts and is thought to promote tumor cell migration as well as prevent apoptosis [55]. The role of EBF4 gene in EMT is unknown. However, it is a transcription factor involved in neuronal development, and therefore may modulate at least some part of the EMT process [56].

After tumor cells undergo EMT, they intravasate into blood vessels, extravasate to the metastatic site, initiate and sustain angiogenesis and through the process continue to survive through anti-apoptotic signals. Several proteins related to angiogenesis have already been described (see Fig. 14.2) including FLT1, FGF18, COL4A2, and MMP9. Other relevant proteins include: GPR180, a G protein-coupled receptor, which regulates vascular remodeling [57]; GPR126, a G protein-coupled receptor that is increased in human umbilical vein endothelial cells; ESM1, a protein secreted in endothelial cells [58], and SCUBE2, a developmental protein expressed in the vascular endothelium [59]. The role of these proteins in metastasis has yet to be elucidated.

14.7 Potential Advantages of MammaPrint as a Prognostic Test

Approximately 30% of node-negative patients will need chemotherapy to reduce the risk of recurrence. Currently, 47% of breast cancer patients in the US are identified as node-negative at the time of diagnosis; this could reach to 60–70% worldwide in regions with widespread breast cancer screening and disease awareness [60]. As the tumor biology is inherited in individual

tumors, the expected increase in the prevalence of breast cancer will need a reliable risk assessment tool for clinical management of these patients. A substantial decrease, from 63% in 2009 to 49% in 2010, in the use of chemotherapy for node-negative breast cancer patients, has seemingly been driven by clinician's use of the two prognostic genomic assays Oncotype Dx and MammaPrint. This corresponds to the determination that, at 10-year follow-up, low-risk patients with node positivity gained absolutely no benefit from chemotherapy with 5-fluorouracil, doxorubicin (Adriamycin), and cyclophosphamide (FAC) chemotherapy with or without tamoxifen [61].

Compared to conventional risk assessment tools, microarray data are much more quantitative and reproducible as they are subjected to less human variability. In support of this, Paik *et al.* compared the performance of the 21-gene recurrence score with the histologic grading performance of three pathologists [62]. They concluded that the 21-gene recurrence score was more robust in the multivariate analyses as substantial inter-observer variation (discordance 57%, kappa 0.23–0.36) existed between the three pathologists in that study. As long as inter-observer variation between pathologists is large [62, 63] there is a need for molecular prognostic tests such as the 70-gene prognosis signature. In patients who have intermediate-risk features (e.g., grade 2) in which inter-observer variation is most distinct, the prognosis signature could potentially add valuable prognostic information.

14.8 Challenges in Clinical Application of MammaPrint

The most limiting factor for widespread clinical use of MammaPrint is that the tumor sample needs to be fresh or fresh frozen tissue. Unlike the Oncotype DX assay, MammaPrint analyses cannot be obtained from tumor tissues that have been preserved in a formaldehyde solution and embedded in wax, which is a common way to preserve tissue samples in routine pathology labs. Therefore, the decision to have a MammaPrint test must be made before surgery. It is recommended that a tumor sample be taken from an unfixed tumor specimen using a 3-mm punch within an hour of surgery and immediately shipped to Agendia. This process, which allows a fresh sample to be obtained, is not the normal process whereby samples

are surgically removed and immediately fixed. Furthermore, the immediate degradation of the fresh samples has the potential to negatively affect downstream processes such and IHC, DNA and RNA isolation. There is a pressing need to establish a quality control measurement to ensure preanalytic variables for sample collection and processing could be tracked and controlled. The other issue is the time for initiating the test. Although Agendia has made every effort to expedite the sample collection and turnaround time of the test, the need to perform the test in a single company in the Netherlands has the potential to impede the ability to remain competitive, with regards to extra time and high cost, with other emerging tests that could be performed locally at CLIA-certified laboratories.

14.9 Summary and Perspectives

Tailoring therapy to an individual breast cancer patient by state-of-art technologies is a promising approach for selecting the most appropriate therapeutic regimen to maximize efficacy and minimize unwanted toxicity. The MammaPrint assay is the first FDA-approved, multi-gene molecular profiling test using fresh breast cancer tissue samples to qualitatively assess a patient's 5- to 10-year risk for distant metastasis. Over the past few years, it has been validated in several cohorts of early-stage breast cancer patients. Early-stage breast cancer patients with a low MammaPrint risk score are at low risk (≤10%) of developing distant metastases or death, and therefore might safely be spared chemotherapy after surgical treatment alone. Currently, the MammaPrint assay is performed at two Agendia laboratories in the Netherlands and approved by global regulatory agencies for all age of breast cancer patients with 0–3 lymph node metastasis. MammaPrint is currently being tested prospectively for the ability to predict benefit of chemotherapy in the MINDACT trial for its feasibility as a prognostic (versus predictive) test. As the prevalence of early-stage breast cancer with node-negative or 1–3 positive lymph nodes is expected to increase with screening and education, the MammaPrint assay is potentially an important tool to avoid over treatment with cytotoxic chemotherapy in breast cancer patients with good prognostic features. Further studies are needed to assess whether systemic therapy targeting the key genes in the 70-gene signature will reduce the risk of breast cancer recurrence

and how co-morbidities might affect the expression of 70-gene signature.

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