Genomic evolution from primary breast carcinoma to distant metastasis: Few copy number changes of breast cancer related genes

Cathy B. Moelans [a,*,1], Petra van der Groep [a,g,1], Laurien D.C. Hoefnagel [a], Marc J. van de Vijver [b], Pieter Wesseling [c,d,e], Jelle Wesseling [f], Elsken van der Wall [g], Paul J. van Diest [a]

[a] Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands
[b] Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands
[c] Department of Pathology, Radboud University Medical Centre, Nijmegen, The Netherlands
[d] Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands
[e] Department of Pathology, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands
[f] Department of Pathology, The Netherlands Cancer Institute, Amsterdam, The Netherlands
[g] Division of Internal Medicine and Dermatology, University Medical Center, Utrecht, The Netherlands

ARTICLE INFO

ABSTRACT

Cancer initiation and progression is characterized by (epi)genetic aberrations. However, little is known about the changes that occur during breast cancer metastasis. In the present study, multiplex ligation-dependent probe amplification was used to compare copy numbers of 21 established oncogenes and tumor suppressor genes between 55 primary breast cancer samples and corresponding distant metastases. Distant breast cancer metastases generally showed similar gene copy number aberrations compared to their corresponding primary tumors. The few genes that showed differences between primary tumor and metastasis (*PRDM*14, *MED*1, *CCNE*1, *TRAF*4, *MTDH*, *CDH*1) have been implicated in the development of therapy resistance.

© 2013 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Despite advances in early diagnosis and treatment of breast cancer patients, still about 25% eventually die from distant metastases [1]. Little is known about the timing of genomic and other changes responsible for developing distant metastasis. Based on *in vitro* tumor cell cultures subsequently transplanted in mice [2–5], a model of metastasis was proposed showing that the metastatic capacity is acquired late in tumorigenesis. Moreover, the metastases were thought to originate from particular subclones with a distinct "metastatic" profile. Others, however, have shown that metastases develop through stochastic events from primary tumor cells with an equal metastatic potential [6,7]. This was confirmed by gene expression profiling where human primary breast tumors were strikingly similar to the distant metastasis of the same patient. These findings suggest that metastatic capability in breast cancer may be an inherent feature and is not based on clonal selection [8]. More evidence for this theory was provided by comparative genomic hybridization (CGH) studies showing, in a small number of cases, that metastases have similar aberration patterns to those found in the primary tumor [9,10]. Furthermore, a complex 54-gene breast cancer set that marks and mediates breast cancer metastasis to the lungs [11] and a functionally diverse gene set that, when overexpressed, cooperatively promotes the metastasis of breast cancer cells to bone [12] also confirms that the metastatic potential exists already in the primary tumor. Other whole genome approaches have identified gene sets in primary tumors that can predict the occurrence of distant metastases but these genes were not analyzed in the tissue of the distant metastases as well [13,14].

Only few high resolution studies have sequenced the complete genome of primary breast cancers as well as their distant metastases. Massive parallel DNA sequencing of a basal-like primary breast cancer and a brain metastasis of the same patient showed that the metastasis contained two *de novo* mutations and a large deletion not present in the primary tumor and was significantly enriched for 20 shared mutations. The differential mutation frequencies and structural variation patterns in the metastasis compared with the primary tumor indicate that secondary tumors may arise from a minority of cells within the primary tumor [15]. Similar findings were observed in another study, 19 of the 32 somatic non-synonymous coding mutations present in a metastasis were not detected

in the primary lobular breast cancer, suggesting that mutational heterogeneity can be a property of low or intermediate grade primary breast cancers and that significant evolution can occur with disease progression.

So far, studies investigating metastatic profiles have been performed *in vitro*, *in vivo* and in primary tumors alone. Only few studies have compared primary tumors with distant metastases of the same patient and those are often limited by the availability of tissue, especially fresh frozen tissue.

Gene copy number and/or expression studies allow the discovery of markers that can help identify patients who are most likely to develop metastatic disease, and would therefore benefit from adjuvant chemotherapy. In addition, such studies might identify markers that are new drug targets or that can predict the site of metastasis. This might enable oncologists to start tailoring treatment for individual patients. Therefore, in order to understand the molecular background of the metastatic process and to find new clues for prevention and therapy, we investigated gene copy number changes in 21 established breast cancer genes between 55 primary breast cancers and their corresponding distant metastases by multiplex ligation-dependent probe amplification (MLPA), an inexpensive and reliable high-throughput technique that allows analysis of small amounts of DNA derived from more widely available paraffin embedded material [16–18].

2. Materials and methods

2.1. Tissue selection and DNA isolation

Fifty-five formalin fixed embedded primary breast cancer specimens and corresponding first biopsied distant metastases from different sites (11 brain, 12 lung, 10 liver and 22 skin) were obtained from the Departments of Pathology of the University Medical Center Utrecht (UMCU), Isala klinieken Zwolle, Erasmus Medical Center Rotterdam, the Academic Medical Center Amsterdam, the Radboud University Nijmegen Medical Center, Gelre Hospital Apeldoorn, Laboratory Sazinon Hoogeveen, and the Laboratory for Pathology Oost Nederland, all in The Netherlands. Table 1 shows basic clinicopathological characteristics of the primary tumors studied.

Use of anonymous or coded 'left over' material for scientific purposes does not require informed consent according to our institutional medical ethical review board and according to Dutch legislation [Medical Research Involving Human Subjects Act, http://www.ccmo-online.nl/main.asp?pid=10&sid=30&ssid=51] [19].

Haematoxylin-eosin stained slides were reviewed by an experienced pathologist (PvD) to confirm the presence of malignancy in tumor samples. Only samples with a tumor percentage of at least 80% were included in this study. After deparaffinization, tumor tissue was scraped off (using a clean scalpel blade) from the marked tumor area on two 8 µm thick unstained sections, and incubated for 1 h in proteinase K (10 mg/ml; Roche, Almere, The Netherlands) at 56 °C followed by boiling for 10 min. Areas with lymphocytic infiltrate or ductal carcinoma *in situ* were avoided.

2.2. Multiplex ligation-dependent probe amplification

Five µl of this DNA solution was, after centrifugation, used in the MLPA analysis according to the manufacturers' instructions, using the P078-B1 breast kit (MRC Holland, Amsterdam, The Netherlands), as before [20]. Table 2 shows the contents of this kit and includes chromosomal locations of all probes. The kit also contains a probe to the *AURKA* gene, but as the results for this gene were unstable, further analysis was omitted. All tests were performed in duplicate on an ABI 9700 PCR machine (Applied Biosystems, Foster City, CA, USA). PCR products were analyzed on an ABI310 capillary sequencer (Applied Biosystems). Gene copy numbers were analyzed using Genescan (Applied Biosystems) and Coffalyser (version 7.0) software (MRC-Holland). Six negative reference samples (two blood and four formalin-fixed paraffin embedded normal breast tissue specimens) were taken along in each MLPA run to normalize MLPA ratios. For genes with more than one probe present in the kit, the arithmetic mean of all the probe peaks of this gene in duplicate was calculated. A mean probe ratio value below 0.7 was defined as loss, a value between 0.7 and 1.3 was defined as normal, 1.3–2.0 as gain/low-level amplification, and values >2.0 were defined as high-level amplification, as established previously [21].

2.3. Intrinsic subtypes

Immunohistochemical staining had been previously used to classify the tumors into five different subtypes: luminal type A (ER+ and/or PR+, HER2- and mitotic activity index <10), Luminal type B (ER+ and/or PR+, and HER2+ and/or mitotic

Table 1
Baseline clinicopathological data.

		N	%
Site	Brain	11	20
	Skin	23	42
	Liver	10	18
	Lung	11	20
Histology	Ductal	45	82
	Lobular	5	9
	Other	5	9
ER	Positive	33	60
	Negative	22	40
PR	Positive	32	58
	Negative	23	42
HER2	0/1+	47	85
	2+	1	2
	3+	7	13
LN	Positive	26	47
	Negative	23	42
Grade	1	1	2
	2	14	25
	3	40	73
MAI	⩾13	39	71
	<13	16	29
Age	⩾50	25	45
	<50	30	55
	Mean (median)	53 (51)	
	Range	27–88	
Tumor	⩾2 cm	18	33
	<2 cm	31	56
	Mean (median)	1.99 (1.5)	
	Range	0.2–9.5	
Time P-M	Mean (median)	46 (29)	
	Range	1–181	

LN status = lymph node status; MAI = mitotic activity index; time P–M = time in months between primary tumor and first distant metastasis. LN status and tumor size were available for 49/55 patients (89%).

activity index ⩾10), HER2 driven (HER2+ and ER-/PR-), basal-like (ER-/PR-/HER2-, and CK5/6+ and/or CK14+ and/or EGFR+), and unclassifiable triple negative (negative for all six markers).

2.4. Statistical analysis

The mean copy number ratio including all 21 genes in all 55 patients was compared between primary tumor and metastases by Mann–Whitney test. We compared the mean copy number ratio of each individual gene between the pooled primary tumors on the one hand and the metastases on the other by paired *T*-test (normally distributed variables, tested by Kolmogorov–Smirnov) or Wilcoxon test. Next, MLPA data were dichotomized as non-loss vs. loss (cut-off 0.7) and as non-amplified vs. gain (cut-off 1.3) or high-level amplified (cut-off 2.0). These gene dosage categories were compared between primary tumor and metastases by McNemar's test. Finally, the number of alterations (total gains, high-level amplifications and losses) between primary tumor and the different distant sites were compared by ANOVA. Copy number differences according to site were calculated by ANOVA. To investigate parameters influencing copy number differences between primary tumor and metastasis, we substracted the absolute copy number ratio in the metastasis from that in the primary tumor and used these copy number differences to analyze their relationship to the timing between primary and distant metastasis, intervening chemotherapy (CT) and hormonal therapy (HT), tumor grade, mitotic activity index and tumor size.

All statistical analyses were conducted with SPSS 15.0 statistical software, regarding two-sided *p*-values below 0.05 as significant. Correction for multiple comparisons was performed by resetting the 0.05 threshold according to the Bonferroni–Holm approach.

3. Results

3.1. Comparison between primary tumors and metastases

Supplementary Table S1 shows raw MLPA copy number data and clinico-pathological variables from each of the 55 paired