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Research Paper

Lack of HIN-1 Methylation Defines Specific Breast Tumor Subtypes Including Medullary Carcinoma of the Breast and BRCA1-Linked Tumors

Pascaline Tisserand[1]
Coralie Fouquet[1]
Michel Barrois[2]
Catherine Gallou[1]
Rémi Dendale[3]
Dominique Stoppa-Lyonnet[4]
Xavier Sastre-Garau[5]
Alain Fourquet[3]
Thierry Soussi[1]

[1]EA 3493 Laboratoire de Génotoxicologie des Tumeurs; Paris, France

[2]The Institut Gustave Roussy; Villejuif, France

[3]The Département de radiothérapie; [4]The service de génétique oncologique; [5]The Département de pathologie; Institut Curie; Paris, France

*Correspondence to: Thierry Soussi; EA3493; laboratoire de pneumologie; Hôpital Tenon; 4 rue de la Chine; 75970 Paris, France; Tel.: +33.1.56.01.65.15; Email: thierrysoussi@yahoo.fr

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KEY WORDS

medullary carcinoma of the breast, HIN-1, methylation, BRCA1, p53

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ABSTRACT

Medullary carcinoma is a poorly differentiated breast cancer with a high histologic grade and a paradoxically good prognosis. It accounts for only 3% of all breast cancers except in BRCA-1 families, in which it can account for as many as 13% of cancers. To date, only histologic criteria have been used to define this tumor type. In an attempt to more clearly define the genetic pathway leading to this subtype of cancer, we recently demonstrated that nearly 100% of these carcinomas display *p53* mutations. In the present study, we extended our analysis to include *HIN-1*, a candidate tumor suppressor that has been shown to be silenced by methylation in the majority of breast tumors. In striking contrast to unselected sporadic invasive ductal carcinoma, we show that medullary carcinomas do not display a high frequency of *HIN-1* methylation ($p<0.001$). This feature is also found in BRCA-1 associated tumors that shared several histologic characteristics with medullary carcinomas of the breast. Medullary carcinoma of the breast should therefore be considered to be a unique entity defined by specific histologic and molecular traits.

INTRODUCTION

Human breast tumors can be classified into various histopathologic subtypes with distinct biological and prognostic characteristics.[1] One such subtype, medullary carcinoma (MC) of the breast, represents a minor morphologically and biologically distinct group (3-5% of all breast cancers).[2] Initially identified half a century ago as a favorable prognostic form, MC was classified by Ridolfi in 1977 on the basis of 5 essential features:[3,4] seemingly well-circumscribed histologic limits, syncytial architecture in at least 75% of the areas examined, inflammation of the stroma containing round cell infiltrate, moderate or marked anisonucleosis and absence of tubular differentiation and/or an intraductal component. Despite the fact that these tumors are histologic grade III, the outcome is significantly more favorable than that of usual invasive breast carcinoma.[5] This finding has been linked to the round cell infiltrate that could stimulate the host immune response.[6] The genetic pathway leading to MC has not been fully elucidated due to the low frequency of this tumor. Nevertheless, recent studies have suggested that MC display certain genetic features that are distinct from those of other breast tumors. Although *p53* mutations are found in 20% of breast tumors and are associated with poor prognosis, nearly 100% of MC display p53 alterations detected by either immunohistochemical or molecular analysis.[7,8] This feature is specific for typical medullary carcinoma. Analysis of the expression of secreted Frizzled-related protein 1 (SCFRP1), a member of a protein family that contains a cysteine-rich domain similar to the WNT-binding site of Frizzled receptors and regulating the WNT pathway, is deleted in more than 80% of invasive breast cancers, except for MC.[9] These findings suggest that MC is a genetically distinct entity from DC.

A higher frequency of MC has been observed among tumors arising in patients with *BRCA1* germline mutation (13% versus 2% in the control group).[10,11] A high frequency of BRCA1 methylation has also been observed in sporadic MC.[12] Like MC, *BRCA1*-associated tumors are mostly grade 3 and negative estrogen- and progesterone- receptors. The prognosis of *BRCA1*-associated tumors is still controversial.[13-15] In an attempt to define the genetic pathway leading to MC, we conducted a comparison of the most common molecular alteration found in MC versus ductal carcinoma. Among the new genes that have been recently characterized in breast tumors, *HIN-1*, a putative tumor suppressor gene of unknown function, has been shown to be silenced by methylation in the majority of sporadic breast carcinomas.[16] In the present study, we show that *HIN-1* methylation is found at a very low frequency in either MC or other breast tumors from patients with *BRCA1* germline mutation, suggesting that the natural history of these tumors is different from that of ductal breast tumors.

MATERIAL AND METHODS

Tissue Samples. Twenty-two samples of breast cancer initially entered in the tissue bank as medullary carcinomas, were obtained from the Institut Curie database (between 1989 and 1995). They were all selected on the basis of the availability of blocks and frozen material. According to the criteria defined by Rapin et al, tumors presenting syncytial architecture in at least 75% of the tumor, complete histological circumscription of the tumor, a diffuse mononuclear infiltrate, nuclear polymorphism and the absence of a glandular structure and in situ component were classified as typical medullary carcinomas (TMC, 16/22).[2] Other cases were classified as atypical medullary carcinomas (AMC, 6/22) when they presented a syncytial architecture and 2 or 3 of the criteria listed above.

Apart from patient M10 who presented a *BRCA1* germline mutation, none of these patients had a family history of breast cancer. Tumor samples from 14 patients with *BRCA1* germline mutations and ductal carcinoma and 20 patients with ductal carcinoma with no family history of cancer were obtained from the Institut Curie.

DNA and RNA Extraction. DNA and RNA extraction was performed simultaneously using the DNA/RNA minikit (QIAGEN 14123) and resuspended in either TE (10 mM Tris, pH 8.0, 1 mM EDTA) (DNA) or water (RNA) in a final volume of 20 and 25 microliters, respectively.

***p53* and *BRCA1* Mutations.** The *p53* and *BRCA1* status of these patients has been previously described. The patient nomenclature has not been changed since the previous reports.[8,17]

Methylation Specific PCR. Briefly, 1 µg of genomic DNA was denatured by NaOH and modified by sodium bisulfite and hydroquinone, which converts all unmethylated cytosines into uracils, whereas methylated cytosines remain unchanged. The modified DNA was purified using the Wizard DNA clean up system (Promega). Primers for the analysis of *HIN-1* or *BRCA1* analysis have been previously described.[12,16] Initial denaturation at 95°C for 10 min was followed by 40 cycles of denaturation at 94°C for 30 s, an annealing step at 69°C for BRCA1 (63°C for HIN-1) for 60s and an extension step at 72°C for 1 min, and a final extension step of 72°C for 5 min was added. The products were separated by electrophoresis on 2% NuSieve-agarose gel. Control experiments were performed using normal DNA methylated in vitro using the CpG methylase SssI (New England Biolabs).

Quantitative PCR Analysis of BRCA1 Expression. The BRCA1 transcription level was quantified by real-time quantitative PCR using the ABI PRISM 7700 sequence detection system (PE Biosystems). The PCR reaction mixture was prepared in accordance with the manufacturer's recommendations and contained 5 mmol/L MgCl2. The following primers were used to determine the BRCA1 transcript level: forward primer for cDNA 5'ATTTTAACCACTCAGCAGAGGGAT3', reverse primer 5' CTCAAGGGCAGAAGAGTCACTTATG3', and fluorescent probe FAM 5'AACAGCATGGGAGCCAGCCTTCTAACAG3' TAMRA. After analysis, Ct values (defined as the fractional cycle number at which the fluorescence signal generated by cleavage of the probe crosses a fixed threshold) were obtained. Normalization of samples was performed with endogenous controls (18S rRNA). The relative quantity of BRCA1 transcripts was obtained using cDNA of MCF7 as calibrator (21 mRNA copies of BRCA1 per cell or 250 x 10^3 mRNA copies/0.3 µg of total RNA.18)

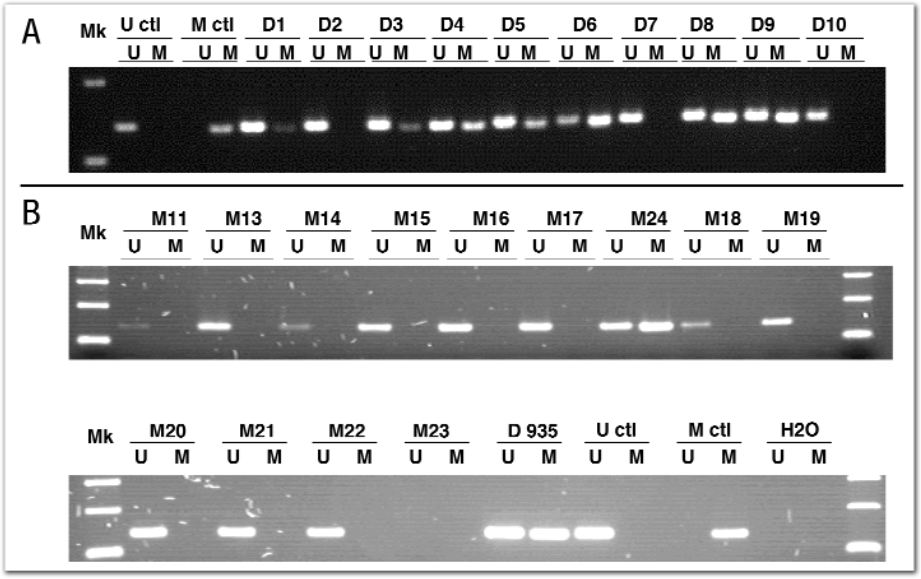

Figure 1. Analysis of CpG island promoter methylation of HIN-1 gene by MSP. MSP analysis was performed with either DC (A) or MC (B). U and M indicates amplification using primers specific for unmethylated or methylated DNA, respectively. U Ctl: amplification performed with normal DNA (lymphocyte from an healthy individual); M Ctl: amplification performed with normal DNA methylated in vitro; H2O: Control with no DNA; MK: marker.

RESULTS AND DISCUSSION

***HIN-1* Methylation in Medullary Breast Cancer.** We used MSP to analyze the methylation status of the *HIN-1* promoter in ductal breast tumors and MC. A control experiment with ductal carcinoma showed a high frequency of *HIN-1* methylation (15/20), as previously described by Krop et al (Fig. 1).[16] In contrast, only two out of 22 cases of MC were methylated (Fig. 1B and Table 1). This difference was statistically significant despite the small number of cases ($p<0.001$). As medullary breast tumors are more frequent in *BRCA1* families, *HIN-1* status was also analyzed in breast and ovarian tumors from patients with *BRCA1* germline mutation. Only 1 out of 10 breast tumors had a methylated hIN-1 promoter (Fig. 2 and Table 2), a statistical difference compared to ductal carcinoma ($p=0.001$). In ovarian tumors from BRCA1 families, only one out of 4 tumors displayed hypermethylation of *HIN-1*. Our findings are supported by the results recently reported by Krop et al. showing that *HIN-1* is not methylated in *BRCA1*-associated tumors and in sporadic *BRCA-1*-like basaloid tumors.[19]

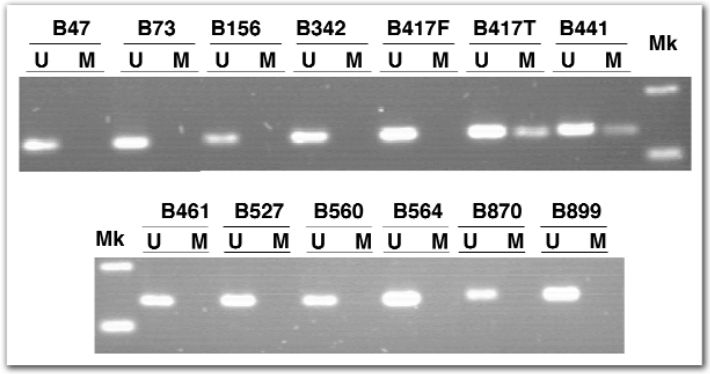

Figure 2. Analysis of CpG island promoter methylation of HIN-1 by MSP. MSP analysis was performed with BRCA1-associated tumors for HIN-1 promoter. U and M indicates amplification using primers specific for unmethylated or methylated DNA, respectively. MK: marker.

Table 1. Histologic and molecular status of medullary carcinoma of the breast

Patient	Histologic Classification[a]	BRCA1[b]	BRCA1 Exp[c]	HIN-1[b]	p53 Mutation[d]		
M1	TMC	U	ND	U	248	CGG>CAG	arg>gln
M2	TMC	U	ND	U	213	CGA>TGA	arg>stop
M3	TMC	U	ND	U	339	GAG>TAG	glu>stop
M4	TMC	U	ND	U	220	TAT>TGT	tyr>cys
M5	AMC	M	ND	U	NF	-	-
M7	TMC	U	ND	U	252	del 252 CTC	leu>-
M8	TMC	U	ND	M	236	TAC>TGC	tyr>cys
M9	TMC	M	low	U	175	CGC>CAC	arg>his
M10	AMC	gl	low	U	99	delC 99	ser>-
M11	AMC	U	ND	U	NF	-	-
M13	TMC	U	inter	U	195	ATC>ACC	ile>thr
M14	AMC	U	ND	U	-	-	-
M15	TMC	U	ND	U	125	ins CACG	-
M16	TMC	U	Inter	U	194	CTT>CGT	leu>arg
M17	TMC	U	ND	U	175	CGC>CAC	arg>his
M18	TMC	U	inter	U	175	CGC>CAC	arg>his
M19	TMC	U	ND	U	127	TCC>CCC	ser>pro
M20	TMC	M	low	U	159	GCC>GTC	ala>val
M21	AMC	M	low	U	205	TAT>TGT	tyr>cys
M22	TMC	U	ND	U	220	TAT>TGT	tyr>cys
M23	AMC	U	ND	ND	NF	-	-
M24	TMC	U	high	M	ND		

aTMC, Typical MC; AMC, Atypical MC; bU, Unmethylated; M, Methylated; gl, germline BRCA1 mutation; cBRCA expression; dp53 mutations were previously described.[8]

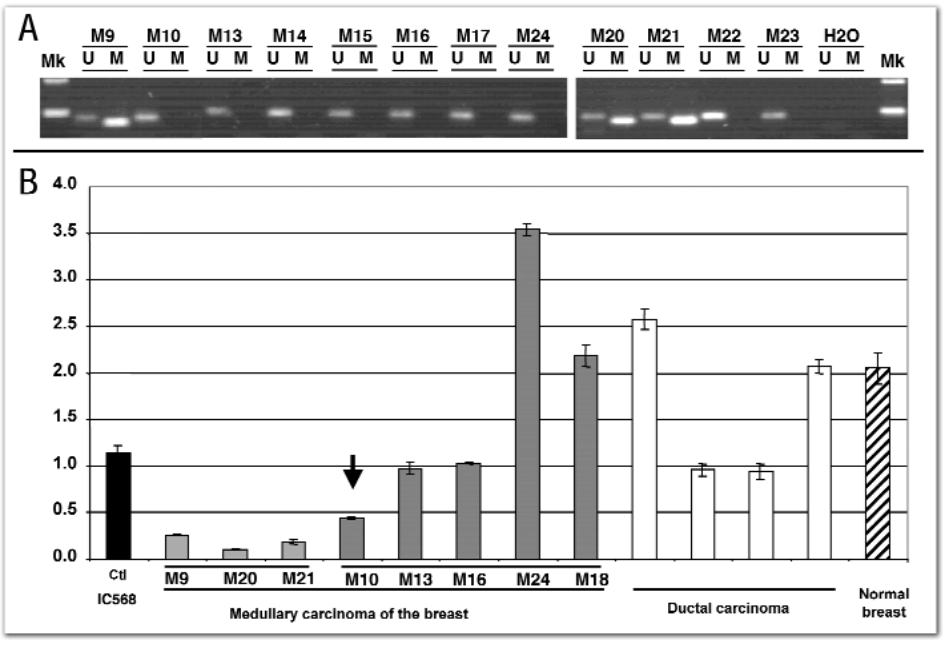

Figure 3: Analysis of CpG island promoter methylation of BRCA1 by MSP and quantification of BRCA1 expression. (A) MSP analysis was performed with MC for BRCA1 promoter. U and M indicates amplification using primers specific for unmethylated or methylated DNA, respectively. MK: marker. (B) Quantification of BRCA1 expression by quantitative PCR. Ductal carcinoma from four patients with methylation of the Hin-1 promoter. Arrow: Tumor M10 with BRCA1 germline mutation.

As a high frequency of BRCA1 methylation has been described in MC, lack of *HIN-1* methylation may be correlated with BRCA1 inactivation. MSP analysis of MC showed that only 4 MC tumors had methylated inactivation of the BRCA1 promoter and one had a *BRCA1* germline mutation (Fig. 3A and Table 1). Quantitative PCR analysis for BRCA1 expression was performed for tumors for which RNA was available. For 4 tumors with *BRCA1* methylation or germline mutation, BRCA1 expression was low compared to tumors with unmethylated BRCA1 or normal breast tissue (Fig. 3B and Table 1). This result differs from those reported by Esteller et al., showing hypermethylation of BRCA1 in 70% of MC, but suggests that the absence of *HIN-1* methylation is not linked to BRCA1 status.[12]

The large diversity of the various histologic groups of breast cancer is supported by a heterogeneous series of alterations that target various genetic pathways.[20] In sporadic ductal carcinoma, tumor progression occurs from normal cells to DCIS, and then to invasive and metastatic carcinoma.[21] This progression is accompanied by specific somatic genetic and epigenetic alterations that are the hallmark of this subgroup of cancers. p53 alterations in breast cancer are a good example of the heterogeneity of these pathways. In sporadic ductal breast cancer, *p53* mutations constitute an early event, as they are

Table 2. Analysis of BRCA1-associated tumors

		BRCA1 Status[a]					p53 Status[a]	
Patient Families	HIN-1[d]	Exon	Codon	Nucleotide	Effect	Tumor[b]	p53 Mutation	IHC Analysis
B417T[c]	M	20	1756	5382insC	stop1829	Right breast	ND	ND
B417F[c]	U	20	1756	5382insC	stop1829	left breast	281 GAC>CAC	70% (4)
B564	U	11	270	926ins10	stop289	left breast	175 CGC>CAC	100%
B560	U	11	1281	3962delG	stop1307	right breast	196 CGA>TGA	negative
B156	U	IVS5	-	+3A/G	stop64	right breast	NF	80%
B635	U	11	825	2594delC	stop845	left breast	NF	90%
B899	U	18	1708	C5242A	Ala1708Glu	left breast	NF	100%
B527	U	5	47	G259A	Cys47Tyr	left breast	NF	negative
B870	U	11	1301	4020delAG	stop1301	right breast	NF	negative
B461	U	20	1756	5382insC	stop1829	left breast	NF	negative
B441	M	13	1404	T4330GTG->GCG	Leu1404Arg	ovary	ND	ND
B342	U	20	1756	5382insC	stop1829	ovary	NF	50-60%
B73	U	11	855	2683ins5	stop894	ovary	NF	negative
B47	U	13	1416	4366del2	stop1427	ovary	NF	ND

[a]BRCA1 and p53 status have been previously described[17,26]; [b]Breast tumors were either grade II or III; [c]Two different members of the same family; [d]U, Unmethylated; M, Methylated; IHC; immunohistochemical analysis: ND; Not done: NF; Not found

detected in pure DCIS.[22] The frequency of these mutations ranges from 20% to 30% with a similar pattern of mutational event to that of other types of cancers. *p53* mutations are usually associated with a poorer prognosis and poor response to treatment.[23] The frequency of p53 alterations in tumors arising in *BRCA1* patients is higher (50-60%) with an unusual pattern of mutations that could be induced by the defect of DNA repair due to the lack of BRCA1.[24] Despite the good prognosis of MC, the frequency of *p53* mutations is nearly 100% and the pattern of these mutations is not different from those observed in sporadic breast cancer.[8]

The very low frequency of *HIN-1* methylation in MC and *BRCA1*-associated tumors compared to ductal carcinoma, demonstrated in this study, constitutes an additional novel feature in the particular genetic pathway leading to these tumors. *HIN-1* is a putative cytokine that is highly expressed in terminally differentiated cells, such as airway epithelial cells or luminal mammary epithelial cells.[25] One of the common characteristics of MC and *BRCA1*-associated tumors is their high grade and poor differentiation, as *HIN-1* methylation has been associated with DCIS.

The present finding, combined with previous observations, indicates that the genetic pathway leading to MC is different from that of ductal carcinoma, with a high frequency of *p53* mutations[8] and a low frequency of SCFRP[19] and *HIN-1* methylation. As MC is often associated with a good prognosis, it would be of interest to check whether this specific signature is also associated with a good prognosis in ductal breast cancer.

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