PPM1D gene amplification and overexpression in breast cancer: a qRT-PCR and chromogenic *in situ* hybridization study

Maryou B Lambros^{1,5}, Rachael Natrajan^{1,5}, Felipe C Geyer¹, Maria A Lopez-Garcia^{1,2}, Konstantin J Dedes¹, Kay Savage¹, Magali Lacroix-Triki^{1,3}, Robin L Jones⁴, Christopher J Lord¹, Spiros Linardopoulos¹, Alan Ashworth¹ and Jorge S Reis-Filho¹

¹The Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, London, UK; ²Hospital Universitario Virgen del Rocío, Seville, Spain; ³Institut Claudius Regaud, Toulouse, France and ⁴Breast Unit, The Royal Marsden Hospital, London, UK

PPM1D (protein phosphatase magnesium-dependent 1δ) maps to the 17q23.2 amplicon and is amplified in ~8% of breast cancers. The *PPM1D* gene encodes a serine threonine phosphatase, which is involved in the regulation of several tumour suppressor pathways, including the p53 pathway. Along with others, we have recently shown that *PPM1D* is one of the drivers of the 17q23.2 amplicon and a promising therapeutic target. Here we investigate whether *PPM1D* is overexpressed when amplified in breast cancers and the correlations between *PPM1D* overexpression and amplification with clinicopathological features and survival of breast cancer patients from a cohort of 245 patients with invasive breast cancer treated with therapeutic surgery followed by adjuvant anthracycline-based chemotherapy. mRNA was extracted from representative sections of tumours containing >50% of tumour cells and subjected to TaqMan quantitative real-time PCR using primers for *PPM1D* and for two housekeeping genes. *PPM1D* overexpression was defined as the top quartile of expression levels. Chromogenic *in situ* hybridization with in-house-generated probes for *PPM1D* was performed. Amplification was defined as >50% of cancer cells with >5 signals per nucleus/large gene clusters. *PPM1D* overexpression and amplification were found in 25 and 6% of breast cancers, respectively. All cases harbouring *PPM1D* amplification displayed *PPM1D* overexpression. *PPM1D* overexpression was inversely correlated with expression of *TOP2A*, *EGFR* and cytokeratins 5/6 and 17. *PPM1D* amplification was significantly associated with *HER2* overexpression, and *HER2*, *TOP2A* and *CCND1* amplification. No association between *PPM1D* gene amplification and *PPM1D* mRNA overexpression with survival was observed. In conclusion, *PPM1D* is consistently overexpressed when amplified; however, *PPM1D* overexpression is more pervasive than gene amplification. *PPM1D* overexpression and amplification are associated with tumours displaying luminal or *HER2* phenotypes. Co-amplification of *PPM1D* and *HER2/TOP2A* and *CCND1* are not random events and may suggest the presence of a ‘firestorm’ genetic profile.

Modern Pathology (2010) 23, 1334–1345; doi:10.1038/modpathol.2010.121; published online 11 June 2010

Keywords: breast cancer; chromogenic *in situ* hybridization; p53; prognosis; real time reverse transcriptase PCR; therapeutic target

PPM1D (protein phosphatase magnesium-dependent 1δ), also known as WIP1 (wild-type p53-induced phosphatase 1), is a member of the nuclear

type 2C protein phosphatase family.¹ The *PPM1D* gene maps to 17q23.2, a genomic region recurrently amplified in several types of tumours including medulloblastomas, neuroblastomas, pancreatic adenocarcinomas, ovarian clear cell carcinomas and breast cancers.^{2–10} We have recently shown in a series of 95 grade III invasive breast cancers that *PPM1D* is amplified in 7% of luminal and 20% of *HER2* amplified cancers and that *PPM1D* is consistently overexpressed at the mRNA level in cases harbouring *PPM1D* gene amplification.⁹

Correspondence: Dr JS Reis-Filho, MD, PhD, FRCPath, The Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, London SW3 6JB, UK.

E-mail: Jorge.Reis-Filho@icr.ac.uk

⁵These authors contributed equally to this work.

Received 6 April 2010; revised and accepted 10 May 2010; published online 11 June 2010

There are several lines of evidence suggesting that *PPM1D* may constitute a driver of the 17q23.2 amplicon^{8–10} and that it may have oncogenic properties when amplified.¹¹ *PPM1D* is induced by p53 upon DNA damage and has a pivotal role in the negative regulation of p53 itself and also a series of additional proteins involved in the response to cellular stress such as ATM, Chk2, p38 mitogen-activated protein kinase, INK4, ARF, MDM2 and H2AX.^{12–17} In particular, *PPM1D* provides a negative feedback mechanism in the p38/p53 signalling cascade by inhibiting irradiation-induced activation of p38, thus eliciting suppression of p53 activation.^{3,18} In addition to its role in the regulation of the p53 pathway, *PPM1D* is also involved in the negative regulation of G1-S and G2/M checkpoints in response to DNA damage,¹⁹ DNA repair mechanisms,^{11,20} progesterone receptor signalling²¹ and apoptosis inhibition.¹¹ *PPM1D* also seems to be involved in the regulation of the nuclear factor- κ B (NF- κ B) pathway, as there is evidence to suggest that *PPM1D* acts as a negative regulator of NF- κ B signalling;^{22,23} however, NF- κ B is a transcriptional enhancer of *PPM1D* expression.²⁴

In previous studies, we have shown that *PPM1D* expression and phosphatase activity are required for the survival of cancer cells derived from breast^{9,25} and ovarian clear cell carcinomas⁸ harbouring amplification of 17q23.2. Our previous results provide direct evidence that *PPM1D* may be one of the drivers of this amplicon^{8–10} and that *PPM1D* may constitute a therapeutic target for a subgroup of breast and ovarian cancers harbouring *PPM1D* gene amplification.

The aims of this study were to investigate whether *PPM1D* is significantly overexpressed when amplified and to define the correlations between *PPM1D* gene amplification and mRNA overexpression with clinicopathological characteristics of primary breast cancers. As a secondary end point, we sought to define whether *PPM1D* gene amplification or mRNA overexpression are associated with the outcome of breast cancer patients uniformly treated with therapeutic surgery followed by anthracycline-based chemotherapy alone or with endocrine therapy. As part of this study, we also assess the potential of using chromogenic *in situ* hybridization as a method to assess *PPM1D* gene status (ie, amplification).

Materials and methods

Tissue Microarrays

A cohort of 245 patients with invasive breast cancer (185 invasive ductal carcinomas, 27 invasive lobular carcinomas, 25 invasive mixed carcinomas and 8 invasive breast carcinomas of other special types) treated with therapeutic surgery followed by adjuvant anthracycline-based chemotherapy were included in this study. All patients were diagnosed and managed at the Royal Marsden Hospital,

London, UK, between 1994 and 2000. All patients were primarily treated with surgery (69 mastectomy and 156 wide local excision) followed by anthracycline-based chemotherapy. Adjuvant endocrine therapy was prescribed for patients with oestrogen receptor-positive tumours (tamoxifen alone in 96% of the patients for the available follow-up period). Complete follow-up was available for 244 patients, ranging from 0.5 to 125 months (median 67 months, mean 67 months). Tumours were graded according to a modified Bloom–Richardson scoring system²⁶ and size was categorized according to the TNM staging.²⁷ The study was approved by the Royal Marsden Hospital ethics committee.

Chromogenic *In Situ* Hybridization

Chromogenic *in situ* hybridization for *PPM1D* gene amplification was performed on 2- μ m-thick tissue microarray sections mounted on polylysine-coated slides, using an in-house-generated probe as previously described.²⁸ This probe comprises three bacterial artificial chromosome contigs (RP11-15E18, CTD-2327L2 and RP11-67D12), which map to the region 58.54–58.83 Mb on chromosome 17q23.2 (<http://www.ensembl.org>) and encompass the *PPM1D* gene. Heat pretreatment of deparaffinized sections consisted of incubation for 15 min at 98°C in CISH pretreatment buffer (SPOT-Light tissue pretreatment kit; Invitrogen) and digested with pepsin for 5.5 min at room temperature according to the manufacturer's instructions. Slides were hybridized and developed as previously described.²⁸ Appropriate *PPM1D* gene-amplified breast cancers ($n=2$), as defined by microarray-based comparative genomic hybridization analysis, were used as controls to determine the sensitivity of the probe and included in the slide run. Negative controls comprised cases defined as lacking *PPM1D* gene amplification by microarray-based comparative genomic hybridization analysis ($n=2$). Chromogenic *in situ* hybridization experiments were analysed by three of the authors (FCG, MAL-G and ML-T) on a multi-headed microscope. Only unequivocal signals were counted. Signals were evaluated at $\times 400$ and $\times 630$ magnification and 30 morphologically unequivocal neoplastic cells in each core were assessed for the presence of the *PPM1D* gene signals. Amplification was defined as $>50\%$ of the neoplastic cells harbouring either >5 copies of the gene or large gene clusters. Chromogenic *in situ* hybridization analysis was performed with observers blinded to clinicopathological parameters, patients' survival and results of the quantitative real-time reverse-transcriptase PCR (qRT-PCR) analysis. In addition to probes for *PPM1D* gene, the tissue microarray was hybridized with SPOT-Light probes (Invitrogen) for *CCND1*, *MYC*, *HER2*, *TOP2A*, and chromosome 8 centromere. Results not in relation to *PPM1D* amplification and/or expression were reported elsewhere.^{29,30}

Quantitative Real-Time Reverse-Transcriptase PCR

Briefly, all cases were reviewed by two pathologists (FCG and ML-T). Representative 8- μ m-thick sections were cut from archival blocks and microdissected to ensure at least 50% of tumour cell content. RNA was extracted from microdissected sections using the RNeasy FFPE RNA Isolation Kit (Qiagen) followed by an additional DNase treatment as previously described.²⁹ RNA quantification was performed using the RiboGreen Quant-iT reagent (Invitrogen) and reverse transcription was performed with Superscript III (Invitrogen) using 400 ng of RNA per reaction with triplicate reactions performed for each sample. Quantitative real-time PCR was performed using TaqMan chemistry on the ABI Prism 7900HT (Applied Biosystems), using the standard curve method.²⁹ PPM1D probe was purchased from Applied Biosystems (PPM1D ID: Hs00186230_m1-PPM1D). In addition, two reference genes (TFRC ID: Hs00174609_m1-TFRC and MRPL19 ID: Hs00608522_g1-MRPL19) were used, having been previously selected as effectively normalizing for degradation of RNA.²⁹ Target gene expression levels were normalized to the geometric mean of the two reference genes and normalized to a calibrator (pool of tumour cDNAs from the same series). PPM1D overexpression was defined as the top quartile of expression levels.

Immunohistochemistry

The details of the immunohistochemical methods and scoring systems for oestrogen receptor, progesterone receptor, HER2, epidermal growth factor receptor (EGFR), cytokeratin (Ck) 14, Ck5/6 and Ck17, Ki-67, p53, topoisomerase II α (TOP2A), caveolin-1 (CAV1), caveolin-2 (CAV2), FOXA1, E-cadherin, CD44, Bcl2, MDM2, MDM4, nestin and cyclin D1 are described elsewhere^{31–35} and summarized in Supplementary Table 1. On the basis of the expression of HER2, oestrogen receptor, Ck 5/6 and EGFR, tumours were classified into basal, HER2 and luminal according to the immunohistochemical panel proposed by Nielsen *et al.*³⁶

Statistical Analysis

The SPSS statistical software package version 11.5 (SPSS Inc., IBM, Chicago, IL, USA) was used for all statistical analysis. Correlations between categorical variables were performed using the χ^2 -test and Fisher's exact test. Disease-free survival was expressed as the number of months from diagnosis to the occurrence of distant, local relapse or death (disease-related death). Metastasis-free survival was expressed as the number of months from diagnosis to the occurrence of distant relapse. Overall survival was expressed as the number of months from diagnosis to the occurrence of breast cancer-related

death. Cumulative survival probabilities were calculated using the Kaplan–Meier method. Differences between survival rates were tested with the log-rank test. A *P*-value of 0.05 was considered as statistically significant.

Results

PPM1D Gene Amplification

PPM1D was found to be amplified in 10 out of 181 interpretable cases (6%, Figures 1–4). Table 1 summarizes the correlations between PPM1D amplification and clinicopathological features, immunohistochemical and chromogenic *in situ* hybridization findings in invasive breast carcinomas. PPM1D gene amplification was significantly associated with HER2 overexpression and HER2, TOP2A and CCND1 amplification. All but one case harbouring PPM1D amplification displayed oestrogen receptor expression (five were oestrogen receptor+/HER2–, four were oestrogen receptor+/HER2+ and one was oestrogen receptor–/HER2+). As expected, no grade I breast cancer displayed PPM1D gene amplification. PPM1D gene amplification was observed in 4% of luminal cancers, 19% of HER2 tumours and none of basal-like and triple negative breast cancers. A statistically significant association between PPM1D gene amplification and HER2 phenotype as defined by Nielsen *et al.*³⁶ (*P*=0.04) was found. No inverse association between p53 nuclear expression, a surrogate marker for TP53 mutations and PPM1D gene amplification was identified.

It should be noted that although an association between PPM1D gene amplification and amplification of HER2 and TOP2A was observed, PPM1D was not necessarily co-amplified with these genes. In fact, out of the 10 cases harbouring PPM1D gene amplification, only 5 and 3 displayed HER2 and TOP2A gene amplification, respectively (Table 1).

PPM1D Gene Expression

PPM1D overexpression was more pervasive than amplification as it was found in 45 (25%) out of 181 assessable cases that yielded sufficient amounts of optimal quality mRNA for qRT-PCR assay. PPM1D overexpression was inversely correlated with the expression of 'basal' markers, including EGFR, Ck5/6, Ck14 and Ck17, and with TOP2A expression. There was no significant association between HER2 expression and PPM1D expression; however, one-third of the cases that showed HER2 amplification or overexpression also showed overexpression of PPM1D. There was no association between the overexpression of PPM1D with TOP2A, HER2 or CCND1 gene amplification. The correlations

Figure 1 Representative micrographs of a grade III invasive ductal carcinoma of no special type (a) of HER2 phenotype as defined by Nielsen *et al*³⁶ criteria, displaying *PPM1D* gene amplification (b), oestrogen receptor expression (c), focal progesterone receptor expression (d) and *HER2* gene amplification (e), and lacking *TOP2A* gene amplification (f).

between *PPM1D* expression and clinicopathological features and immunohistochemical findings in breast carcinomas are summarized in Table 2.

Results for both status of *PPM1D* amplification and mRNA expression were available in 142 cases. *PPM1D* amplification was found to be strongly

Figure 2 Representative micrographs of a grade III invasive ductal carcinoma of no special type (a) of HER2 phenotype as defined by Nielsen *et al*³⁶ criteria, displaying *PPM1D* gene amplification (b), oestrogen receptor expression (c), progesterone receptor expression (d), and *HER2* (e) and *TOP2A* (f) gene co-amplification.

associated with *PPM1D* mRNA expression levels (Mann–Whitney *U*-test, $P < 0.00001$, Figure 5). In fact, all cases harbouring *PPM1D* gene amplification

displayed *PPM1D* mRNA overexpression; however, *PPM1D* mRNA overexpression was more pervasive than gene amplification.

Figure 3 Representative micrographs of a grade III invasive ductal carcinoma of no special type (a) of luminal phenotype as defined by Nielsen *et al*³⁶ criteria, displaying *PPM1D* gene amplification (b), oestrogen receptor expression (c) and focal progesterone receptor expression (d), and lacking *HER2* (e) and *TOP2A* (f) gene amplification.

Figure 4 Representative micrographs of a grade III invasive ductal carcinoma of no special type (a) of basal-like phenotype as defined by Nielsen *et al*³⁶ criteria, lacking *PPM1D* gene amplification (b), oestrogen receptor expression (c), progesterone receptor expression (d), and *HER2* (e) and *TOP2A* (f) gene amplification.

Table 1 Correlations between *PPM1D* gene amplification as defined by chromogenic *in situ* hybridization and clinicopathological parameters and immunohistochemical markers in 181 invasive breast carcinomas

Parameter	N	<i>PPM1D</i> not amplified	<i>PPM1D</i> amplified	P-value
<i>Size, TNM</i>	180			> 0.1
T1		85	8	
T2		73	1	
T3		12	1	
<i>Grade</i>	176			> 0.1
I		17	0	
II		49	2	
III		100	8	
<i>Type</i>	179			> 0.1
IDC		123	7	
ILC		27	1	
Mixed		13	1	
Other		7	0	
<i>LVI</i>	179			> 0.1
Negative		58	2	
Positive		111	8	
<i>LN mets</i>	175			> 0.1
Negative		63	3	
Positive		103	6	
<i>ER</i>	181			> 0.1
Negative		31	1	
Positive		140	9	
<i>PR</i>	181			> 0.1
Negative		44	3	
Positive		127	7	
<i>HER2</i>	181			< 0.005
Negative		151	5	
Positive		20	5	
<i>HER2 CISH</i>	173			0.007
Not amp		143	5	
Amp		20	5	
<i>EGFR</i>	181			> 0.1
Negative		154	9	
Positive		17	1	
<i>Ck14</i>	179			> 0.1
Negative		156	10	
Positive		13	0	
<i>Ck5/6</i>	172			> 0.1
Negative		146	9	
Positive		17	0	
<i>Ck17</i>	178			> 0.1
Negative		148	10	
Positive		20	0	
<i>Basal Cks</i>	179			> 0.1
Negative		144	10	
Positive		25	0	
<i>Basal Cks or EGFR</i>	179			> 0.1
Negative		140	9	
Positive		29	1	
<i>Nielsen groups</i>	178			0.004
Basal		22	0	
Luminal		125	5	
HER2		21	5	

Table 1 Continued

Parameter	N	<i>PPM1D</i> not amplified	<i>PPM1D</i> amplified	P-value
<i>Triple negative</i>	179			> 0.1
No		149	10	
Yes		20	0	
<i>P53</i>	172			> 0.1
Negative		118	8	
Positive		45	1	
<i>MIB-1</i>	171			> 0.1
< 10%		70	4	
10–30%		69	4	
> 30%		23	1	
<i>TOP2A</i>	166			> 0.1
Low		75	4	
High		82	5	
<i>TOP2A CISH</i>	173			0.036
Not amp		152	7	
Amp		11	3	
<i>Cyclin D1</i>	173			> 0.1
Low		19	0	
Intermediate		34	1	
High		111	8	
<i>CCND1 CISH</i>	181			0.006
Not amp		150	5	
Amp		21	5	
<i>MYC CISH</i>	161			> 0.1
Not amp		138	9	
Amp		14	0	
<i>Caveolin 1</i>	181			> 0.1
Negative		155	10	
Positive		16	0	
<i>FOXA1</i>	152			> 0.1
Negative		34	0	
Positive		108	10	
<i>E-cadherin</i>	169			> 0.1
Negative		49	3	
Reduced		12	1	
Normal		99	5	
<i>Nestin</i>	159			> 0.1
Negative		135	7	
Positive		17	0	
<i>BCL2</i>	155			> 0.1
Negative		57	2	
Positive		89	7	
<i>MDM2</i>	155			> 0.1
Negative		92	8	
Positive		54	1	
<i>MDM4</i>	155			> 0.1
Negative		121	7	
Positive		25	2	
<i>p21</i>	154			> 0.1
Negative		96	6	
Positive		49	3	

AMP: amplification; ER: oestrogen receptor; CISH: chromogenic *in situ* hybridization; Ck: cytokeratin; IDC: invasive ductal carcinoma of no special type; ILC: invasive lobular carcinoma; LN mets: lymph node metastasis; LVI: lymphovascular invasion; Nielsen groups³⁶: molecular subgroups defined based upon the immunophenotypic expression of ER, HER2, Ck5/6 and EGFR; PR: progesterone receptor.

Table 2 Correlations between PPM1D mRNA overexpression as defined by qRT-PCR and clinicopathological parameters and immunohistochemical markers in 181 invasive breast carcinomas

Parameter	N	PPM1D not overexpressed	PPM1D overexpressed	P-value
<i>Size, TNM</i>	178			<0.1
T1		69	23	
T2		52	22	
T3		12	0	
<i>Grade</i>	178			>0.1
I		11	6	
II		43	10	
III		79	29	
<i>Type</i>	177			>0.1
IDC		102	34	
ILC		15	6	
Mixed		10	4	
Other		5	1	
<i>LVI</i>	178			>0.1
Negative		39	16	
Positive		94	29	
<i>LN mets</i>	173			>0.1
Negative		47	13	
Positive		84	29	
<i>ER</i>	178			<0.1
Negative		29	4	
Positive		104	41	
<i>PR</i>	178			>0.1
Negative		37	9	
Positive		96	36	
<i>HER2</i>	178			>0.1
Negative		118	38	
Positive		15	7	
<i>HER2 CISH</i>	172			>0.1
Not amp		114	37	
Amp		14	7	
<i>EGFR</i>	178			0.007
Negative		116	45	
Positive		17	0	
<i>Ck14</i>	178			<0.1
Negative		118	44	
Positive		15	1	
<i>Ck5/6</i>	171			0.028
Negative		109	43	
Positive		18	1	
<i>Ck17</i>	178			0.028
Negative		114	44	
Positive		19	1	
<i>Basal Cks</i>	178			0.027
Negative		109	43	
Positive		24	2	
<i>Basal Cks or EGFR</i>	178			0.010
Negative		106	43	
Positive		27	2	
<i>Nielsen groups</i>	173			<0.1
Basal		21	1	
Luminal		93	35	
HER2		16	7	

Table 2 Continued

Parameter	N	PPM1D not overexpressed	PPM1D overexpressed	P-value
<i>Triple negative</i>	174			>0.1
No		110	41	
Yes		20	3	
<i>P53</i>	167			>0.1
Negative		82	32	
Positive		44	9	
<i>MIB-1</i>	169			>0.1
<10%		48	17	
10–30%		57	21	
>30%		23	3	
<i>TOP2A</i>	159			0.026
Low		51	25	
High		69	14	
<i>TOP2A CISH</i>	170			>0.1
Not amp		118	37	
Amp		10	5	
<i>Cyclin D1</i>	168			>0.1
Low		20	2	
Intermediate		25	8	
High		82	31	
<i>CCND1 CISH</i>	177			>0.1
Not amp		115	37	
Amp		17	8	
<i>MYC CISH</i>	154			>0.1
Not amp		103	33	
Amp		15	3	
<i>Caveolin 1</i>	178			<0.1
Negative		117	44	
Positive		16	1	
<i>FOXA1</i>	141			>0.1
Negative		24	9	
Positive		81	27	
<i>E-cadherin</i>	165			>0.1
Negative		34	11	
Reduced		10	2	
Normal		81	27	
<i>Nestin</i>	140			>0.1
Negative		93	32	
Positive		12	3	
<i>BCL2</i>	137			>0.1
Negative		35	15	
Positive		68	19	
<i>MDM2</i>	137			>0.1
Negative		68	24	
Positive		35	10	
<i>MDM4</i>	137			>0.1
Negative		83	29	
Positive		20	5	
<i>p21</i>	136			>0.1
Negative		69	26	
Positive		34	7	

AMP: amplification; ER: oestrogen receptor; CISH: chromogenic *in situ* hybridization; Ck: cytokeratin; IDC: invasive ductal carcinoma of no special type; ILC: invasive lobular carcinoma; LN mets: lymph node metastasis; LVI: lymphovascular invasion; Nielsen groups³⁶: molecular subgroups defined based upon the immunophenotypic expression of ER, HER2, Ck5/6 and EGFR; PR: progesterone receptor.

Figure 5 Box and Whisker plot showing distribution of PPM1D mRNA levels assessed by qRT-PCR in breast cancers according to PPM1D gene amplification status. Cases with amplification ($n=8$) are labelled as 1, whereas cases without amplification ($n=134$) are labelled as 0. Extremes and outliers are identified by 'o' and '*', respectively.

Survival Analysis

Univariate survival analysis failed to show any association between PPM1D gene amplification and PPM1D overexpression, and disease-free, metastasis-free or overall survival. Exploratory subgroup analysis of the impact of PPM1D gene amplification and mRNA overexpression on the disease-free, metastasis-free and overall survival of patients with ER-positive, ER-negative, HER2-positive, HER2-negative, lymph node-positive and lymph node-negative breast cancer failed to identify any statistically significant associations (data not shown).

Discussion

Here we show that PPM1D gene amplification is present in 6% of cases in a consecutive cohort of 181 breast cancers. PPM1D amplification was significantly more prevalent in tumours of HER2 phenotype (19%) than in luminal cancers (4%, Fisher's exact test P -value = 0.007), whereas it was absent in tumours of basal-like and triple-negative phenotype. Furthermore, we confirmed by chromogenic *in situ* hybridization and qRT-PCR that PPM1D is significantly overexpressed when amplified expanding to previous observations derived from microarray-based comparative genomic hybridization and expression array analysis.⁹

Although PPM1D has been shown to have a pivotal role in the regulation of p53 signalling by dephosphorylating p38 and suppressing activation of downstream targets of p53, PPM1D amplification and overexpression were not mutually exclusive with p53 nuclear expression. Presence of p53

nuclear expression, albeit not a perfect surrogate for TP53 mutations, is strongly associated with TP53 mutation.³⁷ This observation suggests that PPM1D amplification may confer advantages to tumour cells above and beyond p53 pathway inactivation. In fact, PPM1D has been shown to be involved in a series of interactions that could account for non-P53-related functions, including the regulation of progesterone receptor expression, specific DNA repair mechanisms and control of the NF- κ B pathway.²⁴ In fact, our previous studies have shown that PPM1D silencing and inhibition of its phosphatase activity are selectively lethal for breast and ovarian clear cell cancer cell lines in a manner that does not completely correlate with TP53 gene status.^{8,9}

In agreement with our previous observations, we show here that PPM1D overexpression is more prevalent than gene amplification. This is not surprising, as a substantial number of oncogenes, such as MYC, EGFR and CCND1, are more frequently overexpressed than amplified;^{30,38,39} however, in a way akin to these *bona fide* oncogenes, PPM1D is consistently and significantly overexpressed when amplified in support of the contention that PPM1D may constitute one of the drivers of the 17q23.2 amplicon.

At variance with TOP2A amplifications, which are almost exclusively found in association with HER2 gene amplification (Arriola *et al*²⁹, Slamon and Press⁴⁰ and references therein), PPM1D is not necessarily co-amplified with HER2. This is not surprising, as PPM1D maps 20 Mb distal to the smallest region of amplification of the HER2 amplicon on chromosome 17q.²⁹

It should be noted that PPM1D gene amplification was associated with amplification of other loci on chromosome 17 (eg, HER2 (17q12) and TOP2A (17q21.2)) and on other chromosomes (eg, CCND1 on 11q13). This observation suggests that cases harbouring PPM1D gene amplification may display a 'firestorm' pattern (ie, tumours whose genome is characterized by multiple, clustered high-level amplifications).^{9,41,42} Re-analysis of the data from Natrajan *et al*⁹ revealed that all cases harbouring PPM1D gene amplification ($n=8$) displayed a 'firestorm' profile (Supplementary Figure 1).⁹ Taken together, it is plausible that amplification of 17q23.2 may stem from a global pattern of genetic instability that favours the acquisition of multiple, high-level gene amplifications throughout the genome.

Although we could not find an association between PPM1D gene amplification and PPM1D mRNA expression with the outcome of breast cancer patients, our findings should be interpreted with caution, as this is a retrospective, single-institution study. Furthermore, the prevalence of cases displaying PPM1D gene amplification is relatively low (6%); therefore, it is plausible that in larger cohorts with longer follow-up, associations with survival may be identified. Further studies testing the

prognostic impact of *PPM1D* gene amplification in larger cohorts of breast cancer patients, in particular in tumours of HER2 and luminal phenotype, are warranted.

Given the increasingly more coherent data to suggest that *PPM1D* may constitute a therapeutic target in tumours harbouring 17q23.2 amplification and the availability of preclinical compounds that selectively inhibit *PPM1D*, it is probable that novel drugs inhibiting *PPM1D* will be developed and tested *in vivo* in the near future.^{25,43} Hence, the availability of molecular diagnostics to identify the group of patients that may benefit from *PPM1D* inhibitors would facilitate the development of biology-driven clinical trials to test *PPM1D* as a therapeutic target. Here, we described reagents that can be used for the identification of *PPM1D* amplification and overexpression in breast cancer archival samples, including a chromogenic *in situ* hybridization probe for *PPM1D* that can be readily applied to formalin-fixed paraffin-embedded tissue samples. Given the lack of validated anti-*PPM1D* antibodies applicable for immunohistochemistry, the TaqMan-based qRT-PCR we described in this paper may constitute an alternative to assess *PPM1D* expression levels in routinely processed pathological samples, provided that the samples have an excess of 50% of tumour cells.

Acknowledgement

This study was funded in part by Breakthrough Breast Cancer. KJD is the recipient of a Swiss National Science Foundation (SNF) fellowship.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (<http://www.nature.com/modpathol>)