ORIGINAL ARTICLE

Methylated *PLK2* is a prognostic biomarker in taxane-treated breast cancers

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Received: February 29, 2020 **Accepted:** September 18, 2020 **Online Published:** October 22, 2020
DOI: 10.5430/dcc.v7n2p7 **URL:** https://doi.org/10.5430/dcc.v7n2p7

ABSTRACT

Background: In search of potential biomarkers of drug responsiveness the tumour suppressor *PLK2* has been identified as a mediator of taxane sensitivity in some tumour types, based on its role of G2M checkpoint regulation.
Objective: The current study set out to evaluate *PLK2* methylation in breast cancer treated with neo-adjuvant chemotherapy including a taxane, as a biomarker of disease progression.
Methods: Silencing of *PLK2* in MCF-7 cells followed by taxane treatment was assessed using apoptotic readout for proof of principle. DNA was extracted from 64 cases of diagnostic surgical FFPE sections. Using pyrosequencing, levels of *PLK2* promoter methylation were measured.
Results: Silencing of *PLK2* resulted in a significantly reduced apoptotic response following paclitaxel treatment (compared with scramble transfected controls). An association with higher levels of CpG island promoter methylation was seen for those cases with a progression-free survival (PFS) of less than 12 months. Kaplan-Meier analysis showed there was an association between overall survival (OS) and level of methylation ($p = .06$).
Conclusions: Thus, based on data obtained from this pilot study, further larger studies evaluating the utility of *PLK2* methylation as a potential predictive biomarker in breast cancer are warranted.

Key Words: Breast cancer, PLK2, DNA methylation, Drug resistance, Taxanes

1. INTRODUCTION

Polo-like kinases (*PLKs*) are critically involved in development, cell cycle regulation and DNA damage responses.[1] *PLK2* has been shown to induce apoptosis in Burkitt's lymphoma and appears to be under transcriptional control of wild type *p53*.[2] In addition, PLK2 protein is required for centriole duplication near the G1-S transition.[3] Taken together, these properties are consistent with a tumour suppressor role for *PLK2*. However, *PLK2* expression levels indicate that for breast cancer (BC) the levels are relatively high, in comparison with other cancers, with levels of being at a similar level as normal breast tissue for a large proportion of cases (`www.proteinatlas.org`).

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We have previously described how the functional inactivation of *PLK2* via methylation of promoter may be used as a biomarker of chemoresponse in patients with epithelial ovarian cancers (EOC).[4,5] In support of this, De Viron et al.[6] described how *PLK2* mRNA induction was shown to correlate with chemosensitivity of B-cell chronic lymphocytic leukaemia treated with purine analogues. In that paper the authors described a correlation between drug resistance and lack of *PLK2* mRNA induction in leukaemia cells, assessed *ex vivo* for response to chlorodeoxy-adenosine and fludarabine, using the MTT assay. Hence, high *PLK2* expression was consistently associated with high levels of drug sensitivity, as assessed *in vitro*.[6]

In spite of BC currently being treated with a variety of targeted agents, chemotherapy using agents such as anthracyclines and taxanes is still regarded as the main approach to treatment, particularly in the case of triple negative disease.[7] An approach to assess the response to chemotherapy using clinical samples from BC patients is to evaluate the clinical outcome to neo-adjuvant therapy. Previously, high expression of beta-III tubulin has been shown to correlate with resistance to taxanes[8] and, subsequently, low estrogen receptor expression with low microtubule tau protein expression has been associated with taxane sensitivity in BC.[9] A number of studies used the approach of GWAS in assessing predictive response to chemotherapy[10] and predictive gene lists have been derived. In particular, it was noticed that genes associated with taxane-associated therapies were enriched for cell cycle control processes. Such studies can then be followed up by focused studies that consider immunohistochemical (IHC) analysis of tissue sections for the gene protein product. We have used a different approach in using a discovery platform of novel drug sensitive and resistant cell line models to achieve the same goal. We now describe the evaluation of methylation of the *PLK2* promoter by pyrosequencing of target regions as potential high throughput method for predictive marker for BC patients.

2. METHODS

2.1 Patient demographics

Sixty-four patients were treated in the neo-adjuvant setting with gemcitabine, epirubicin and paclitaxel (GET) (see Table 1).

Table 1. BC patients' demographics

PARAMETER		
Number of cases	64	
Age at diagnosis (y)	mean	52.3
	range	35-73
Receptor status	ER/PR pos	26 (41%)
	HER2 pos	6 (9%)
	ER/PR, HER2 pos	23 (36%)
	Triple neg	8 (13%)
Disease stage at diagnosis	II	7 (11%)
	III	35 (55%)
	IV	13 (20%)
	NA	9 (14%)
Progression free survival (mo)	mean	45.9
	range	0-161
Overall survival (mo)	mean	63.2
	range	3-208

Note. y: years; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; pos: positive; neg: negative; NA: not available; mo: months.

2.2 Ethical approval for use of patient tumour samples

An informed consent for collection and research use of biological material, approved by the Ethical Committee of S. Croce & Carle Teaching Hospital in Cuneo, Italy, was obtained from alive patients enrolled in the study, which was conducted in accordance with the Helsinki Declaration.

2.3 Chemical and reagents

AllPrep formalin-fixed paraffin-embedded (FFPE) DNA/RNA mini kit (#80234), EpiTect Bisulfite kit (#59104), HotStar Taq Plus DNA Polymerase (#203605) and the reagents for pyrosequencing were obtained from Qiagen (UK). EZ DNA Methylation gold kit (#D5005) was procured from Cambridge Biosciences, UK.

2.4 Sample processing

Sixty-four FFPE samples were obtained at diagnosis from patients at the Pathology and Oncology Departments at S. Croce & Carle Teaching Hospital in Cuneo, Italy. AllPrep FFPE DNA/RNA isolation mini kit (Qiagen) was used for the simultaneous isolation of the DNA/RNA. Depending on the visual estimation of the sample size, different volumes were used for elution of DNA from the column, varying from 40-55 μl.

2.5 Bisulfite conversion

Bisulfite conversion was carried out using either EpiTect Bisulfite conversion kit (Qiagen) or EZ DNA methylation Gold kit (Cambridge Biosciences). Both the kits were used before in our lab for other samples with equally successful results. 250-500 μg of DNA with A260/A280 absorbance ratio > 1.7 was used for bisulfite conversion.

2.6 Identification of the target region and primer designing

Information available in public domain on UCSC Genome Browser was used as the starting material. CpG island CGI165 which covered the promoter region of the *PLK2* was targeted for the purpose. The target regions for amplification were determined based on our previous report.[4] We also took into account the available information on methylation on the UCSC Genome Browser showing methylation in some of the reported cancer cell lines (e.g., on Illumina 450K Beadchip and RRBS assay) and included some of these sites in the targeted sequence.

The primers were designed using Pyromark Assay Design v2.0.0 software (Qiagen, UK). The final primer sets were selected based on the GC content, possible mis-priming and the final overall scores of the primers.

Primer set CpG165_1025: PLK2_F1 TTTGGTGGTAGGGTAGTG;
PLK2_R1: CCTTAACACAAAAAACCCC-5' biot; fragment length: 107 bp
Primer set CpG165_926: PLK2_F2 GTGTTAAGGTATTAGGGTG;
PLK2_R2: CTACCCCAACTAAACACC- 5'biot; fragment length: 139 bp

One primer set (CpG165_1025) had a score of 92 and the other 80 (CpG165_926). Any primer sets beyond the score of 80 was discarded. The annealing temperatures for these primers were determined by temperature gradient PCR using already tested bisulfite-converted samples. A non-template control sample was used with all the PCR experiments for avoidance of false-positives.

2.7 *PLK2* silencing of BC cells and the impact on taxane-sensitivity

MCF-7 human BC cell line (obtained from ATCC) were cultured in 6-well tissue culture plates and subjected to transfection of SiRNA (On Target plus SmartPool product code J-003325-18, Dharmacon), according to manufacturer's instructions. This included the use of SiRNA directed to non-coding DNA sequences, constituting the "scramble" control. Following 72 h post-transfection, cells were plated into 6-well plates and subjected to treatment with paclitaxel at 20 nM and 50 nM for 48 h. This was followed by estimation of the apoptotic population of cells using annexin V conjugated to FITC with PI using flow cytometry. The knockdown of the *PLK2* gene was confirmed with western immunoblotting using SDS-PAGE gels (Novex, Invitrogen) using the antibody to PLK2/snk (H-90), rabbit polyclonal antibody (code sc-25421), obtained from Santa Cruz Technology (obtained from Insight Biotechnology, Wembley, UK).

2.8 Statistical analysis

Levels of statistical significance were evaluated using SPSS (version 21, IBM), with one way ANOVA analysis with post-hoc Bonferroni correction. Clinical data and levels of *PLK2* methylation were correlated using Kaplan Meier analysis. Overall Survival (OS) analysis was based on the time from diagnosis to death or last contact in which the survivors were censored. Progression Free Survival (PFS) analysis was based on the time from diagnosis to first event (loco-regional recurrence or distant metastasis); patients without an event were censored at their last follow-up. Patients that died from toxic death were considered as not progressed. Survival curves were plotted by Kaplan-Meier method and compared by with log-rank test for statistical significance.

3. RESULTS

3.1 Silencing *PLK2* in human breast cancer cells result in taxane resistance

As shown in Figure 1, the levels of apoptosis in MCF-7 cells subjected to silencing of the *PLK2* gene were significantly reduced, compared with the scramble (non-coding) control culture following treatment with paclitaxel. For cells treated at the 50 nM dose level, the difference in the level of surviving non-apoptotic cells fraction (as shown in the bottom left hand side of the quadrant) was statistically significant ($p <$ .002).

3.2 Levels of *PLK2* methylation from extracted DNA from breast cancer tissues shows higher levels are associated with reduced overall survival

As shown in Figure 2, the levels of methylation along 5 Variable Methylation Positions (VMP) of the CpG island were frequently higher in patients with a PFS of less than 12 months (see Figure 2A), compared with the other patient cohorts (i.e., 13-24, 25-60, > 60 month PFS). Levels of *PLK2* methylation for patient cohorts with > 13 months PFS were very similar and not shown to be statistically different from each other. Thus, we were able to distinguish patients with PFS of less than 12 months from those who progressed between 13 and 48 months (p = .035) comparing methylation levels.

Using Kaplan-Meier analysis for OS, the level of statistical significance for patients with a median *PLK2* methylation (calculated on the average of all VMPs) value above versus below the median level of methylation gave a value of p = .0614, which was close to statistical significance (see Figure 3).

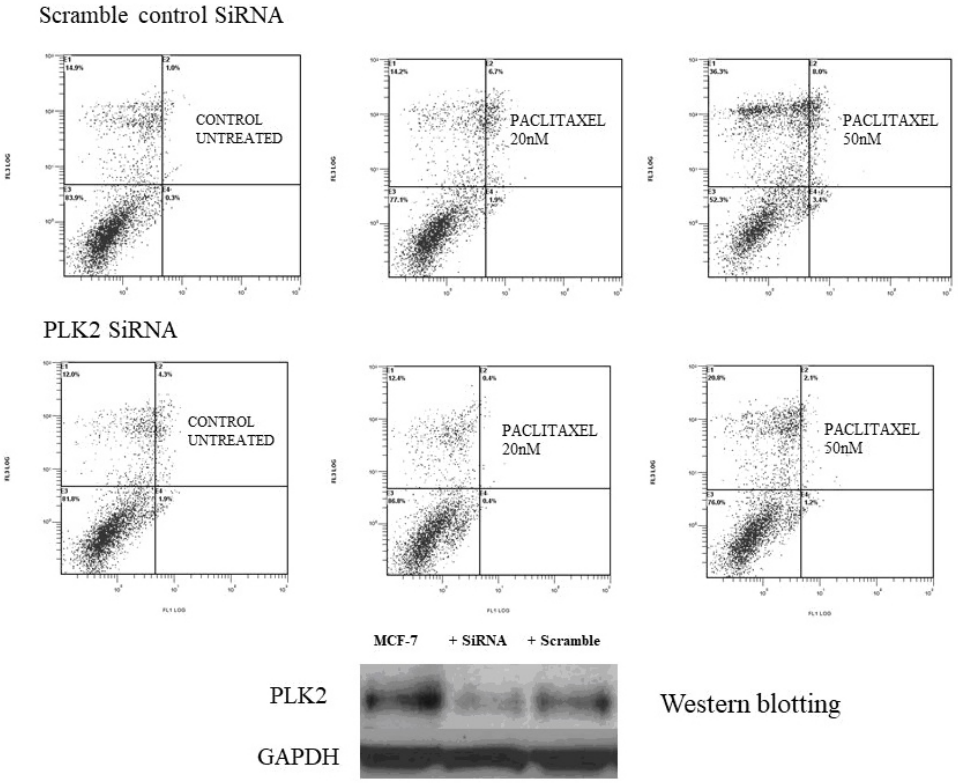

Figure 1. Flow cytometric data obtained using the annexin assay to examine levels of apoptosis in taxane treated MCF-7 cells, comparing *PLK2* silenced and scramble control samples. Data shown are typical of repeat analyses (n = 3). Western immunoblotting (bottom panel) shows knockdown of *PLK2* following transfection with SiRNA, compared with untreated cultured cells and scramble transfected cells.

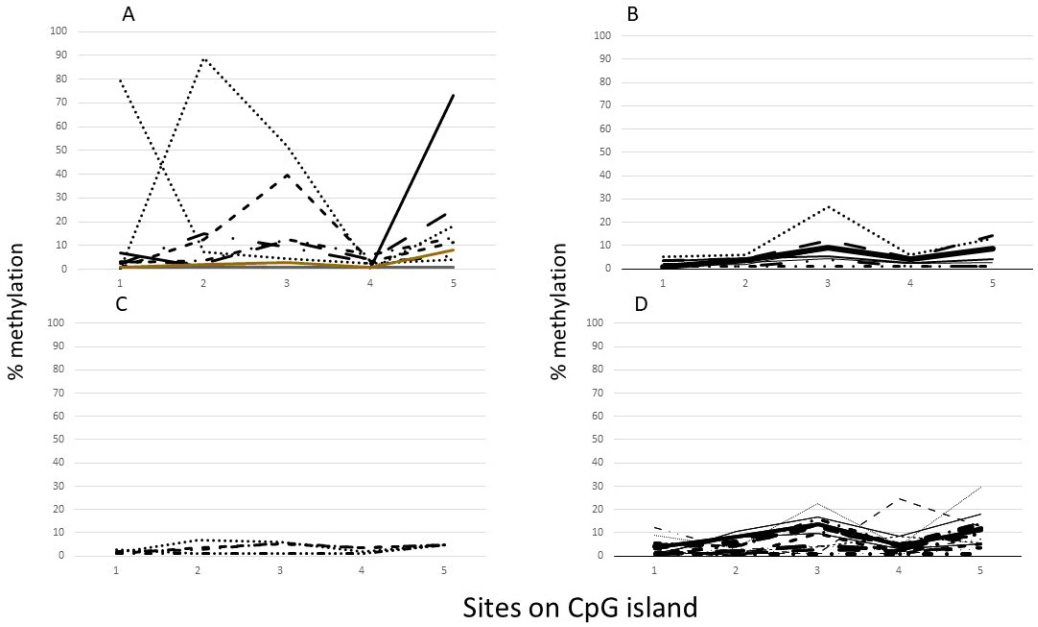

Figure 2. Graphs showing % levels of *PLK2* promoter methylation for CpG islands 4-8, according to progression free survival (PFS)

A : patients with PFS of less than 12 months; B: patients with PFS 12.1-24 months; C: patients with PFS 24.1-60 months; D: patients with PFS > 60 months.

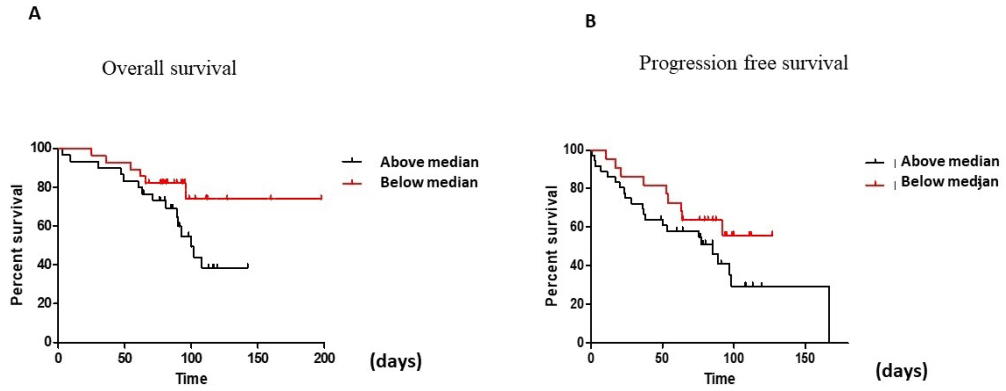

Figure 3. Kaplan-Meier curves plotted for levels for methylation below and above the cohort median level, with time
A: overall survival (OS), p value for differences in the 2 curves was .0614; B: progression free survival (PFS), p value for differences was .154, using the log rank (Mantel Cox) test.

4. Discussion

Personalised medicine in the management of breast cancer is now well established and has been facilitated in part by the advent of the Oncotype Dx™ 21 gene panel assay.[11] This RT-PCR based approach includes genes associated with proliferation, invasion, *HER2* status, estrogen status and currently 3 others: *CD68, BAG1* and *GSTM1*. Oncotype Dx is associated with a robust predictive power for chemotherapy benefit as well as showing a statistical link with metastatic disease.[11,12] There are several other genes that warrant evaluation for their potential in disease monitoring for BC e.g., *claudin*,[13] *ribonucleotide reductase*.[14] Other tests, such as the EndoPredict signature, comprising 11 genes, has predictive power for response to neoadjuvant chemotherapy, defining patients as either low-risk or high risk.[15] A recent study by Akashi-Tanaka et al.[16] described "*BRCAness*" as a strong correlate with progression of triple negative BCs treated with taxanes. The implementation of gene-expression profiling for risk stratification in BC is yet to be used widely and poses the problem of RNA instability. Further approaches include the use of complex focal chromosomal rearrangements which relate to genomic instability and make use of copy number analysis.[17]

The data we show herein make the case for *PLK2* methylation as a candidate biomarker for monitoring BC. Methylated *PLK2* was shown to be predictive of OS, with cases with a PFS of less than 12 months showing the highest levels compared with the remainder of the cohort. Our data showing the relationship between *PLK2* drug induced apoptosis are supported by Liu et al.[18] who showed that *PLK2* silenced gastric carcinoma cells were resistant to apoptosis, showing reduced levels of caspase-3 protein. The authors also provided evidence for *PLK2* as a tumour suppressor, since gastric carcinoma cells showed a significantly enhanced growth rate when transfected with *PLK2* SiRNA. Hu et al.[19] gave further evidence for *PLK2* acting as an oncogene by phosphorylating TAp73 in a human osteosarcoma model. In this study, PLK2 inhibition and combined use of cisplatin resulted in significant reduction of cell proliferation and increased apoptosis.[19]

A meta-analysis study by Xu et al.[20] considered the role of tumour suppressor genes and taxane resistance in cancer. The authors discovered alteration/perturbation of 22 tumour suppressor genes; several among them were involved in cell cycle and DNA repair, including *PLK2*. The authors concluded that the genes could have direct and indirect effects on each other and data indicated protein-protein interaction networks. In line with other studies, for *PLK2* this was shown to be chiefly interaction with p53 in a network involving *CDKN1A, BRCA1, PTEN* and *CDKN2A*.[20]

Based on the pilot study data presented herein, we propose *PLK2* methylation as a potential new predictive marker for the evaluation of taxane-treated BC. The method of plate pyrosequencing could readily lend itself to high throughput screening. Our analysis of samples used in this pilot study has identified a subgroup of BC patients with the poorest outcome (PFS of less than 12 months) by the observation of increased levels of *PLK2* methylation consistent with inhibition of tumour suppressor function. Further studies are necessary using larger sample numbers to increase statistical power and to further assess the utility of the *PLK2* as a useful marker in the management of BC patients.

Acknowledgements

We would like to acknowledge funding from Breast Cancer Campaign UK for a grant to support this work (HMC).

Conflicts of Interest Disclosure

The authors declare they have no conflicts of interest.

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