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ORIGINAL ARTICLE

PLK-1 Expression is Associated with Histopathological Response to Neoadjuvant Therapy of Hepatic Metastasis of Colorectal Carcinoma

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Abstract Polo-like kinase 1 (PLK1) is a serine/threonine-protein kinase expressed during mitosis and overexpressed in multiple human cancers, including leukemia and also many solid tumors. PLK1 knockdown has been shown to block proliferation of leukemic cell lines and the clonogenic potential of tumor cells grown from patients with cancer. PLK1 inhibition is a promising strategy for the treatment of some tumors. We aim to analyze expression of PLK1 in metastatic colorectal carcinoma. Retrospective analysis of colorectal carcinomas with hepatic metastasis during follow-up receiving neoadjuvant chemotherapy (NAC), based on oxaliplatin. Immunohistochemistry for PLK-1 in paraffin-embedded tissue from the primary and also from the metastasis. 50 patients. 32 % showed good histopathological response. 43 % of the primaries were positive for PLK1, as opposed to 23.5 % of the metastasis. Expression of PLK1 was significantly reduced in metastasis compared with the primaries ($p = 0.05$), what could be due to therapy or to a phenotypic change of the metastatic nodule. Analysis of the prognostic influence of PLK1 expression showed significant association between PLK1 expression in metastasis and lower overall survival ($p = 0.000$). We have also found a significant association between PLK1 expression and histopathological response ($p = 0.02$). All the tumors with high expression of PLK1 showed minor response (11/11). This study shows the association between survival and poor histopathological response to therapy and high expression of PLK1 in metastasis. Our results could open a new therapeutic approach through the inhibition of PLK1.

Keywords Hepatic metastasis · Colorectal carcinoma · Neoadjuvant therapy · PLK1 · Histopathological response · Targeted drugs

Introduction

Colorectal carcinoma is one of the most frequent human malignancies, mainly in developed countries [1]. Despite recent advances in therapy, mainly after the introduction of targeted drugs against EGFR and VEGFR [2], mortality remains high for advanced stages, especially for metastatic disease. The best therapeutic option for metastasis amenable to resection is surgery [3], but neoadjuvant chemotherapy (NAC) is being increasingly used as a therapeutic alternative for patients with metastatic disease before resection [4]. Several strategies have been developed to grade histopathological response of the metastatic tumor to NAC [5] and this factor is very important to predict the outcome of the patients [6]. Besides, there is a growing interest in predicting response to therapy to avoid delays in efficient management in cases of NAC failure. It is also essential to find factors that can guide the need of adjuvant therapy after metastasis resection surgery. Targeted drugs could have a crucial importance in this setting.

As it is known, cells protect their genome integrity by a conserved DNA damage response pathway (DDR) that coordinates DNA repair with control of the cell cycle progression. Many molecules are involved in these repair mechanisms. PLK1 is a serine/threonine-protein kinase expressed during

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mitosis; PLK1 plays an essential role in the control of mitotic spindle formation and cytokinesis. In addition, it is required for recovery from the G2 checkpoint. Some recent reports have proposed that PLK1 participates in the phosphorilation of 53BP1 and this phosphorilation leads to an altered recognition and repair of the DNA damaged sites [7]. PLK1 is overexpressed in many human cancers, including most cases of acute myelogenous leukaemia (AML). PLK1 knockdown by small interfering RNA blocks proliferation of leukemic cell lines and the clonogenic potential of primary cells from patients. PLK1 inhibition is a promising strategy for the treatment of AML. Depletion or inhibition of kinase activity of PLK1 is sufficient to induce cell-cycle arrest and apoptosis in cancer cell lines and in xenograft tumor models. Recent studies have also shown that PLK1 expression is a prognostic marker for urothelial [8], renal cell [9], and triple negative breast carcinoma [10], among other solid tumors [11–13]. The chance to selectively target this molecule could open new therapeutic approaches in these tumors. We have found few references to the prognostic and pathogenic significance of PLK1 in colorectal carcinoma [13], but no references to the possible prognostic influence of this kinase in gastrointestinal metastatic malignant tumors. The aim of the present study is to analyze PLK1 expression in metastatic colorectal carcinoma treated with NAC.

Material and Methods

Patient and Tissue Samples

We have retrospectively reviewed the electronic records of the patients with colorectal carcinoma treated at the Fundación Jiménez Díaz Hospital in Madrid (Spain). From these we have included in the study those with initially resectable hepatic metastasis that received NAC and were subsequently operated with disease free margins. After surgery they received standard adjuvant therapy.

We have collected general demographic and clinical data and also data from the primary tumor (location, differentiation grade, vascular invasion, inflammatory response, TNM staging).

Evaluation of Histological Response to NAC

We have reviewed the stained slides of the metastatic hepatic nodules to describe the morphological response to therapy according to the criteria established by Blazer et al. [5] which was employed as one of the outcome variables in the present study. In short we considered cases with complete response as the total absence of tumor in the resection specimen; major response as the persistence of less than 50 % of tumor cells in the specimen; and minor response as the persistence of more than 50 % of tumor cells. Prognosis of the patients was measured by time to recurrence (disease free survival; DFS) and by the time to death due to the tumor (overall survival; OS), both expressed in months.

Immunohistochemistry

We selected formalin-fixed paraffin-embedded (FFPE) tissue samples from 50 primary tumors and from 50 hepatic metastasis and used them for Tissue Microarray (TMA) construction. Representative tumor regions from biopsies were identified by a pathologist on hematoxylin and eosin-stained tissue sections. After pathologist review, TMAs were assembled from triplicate 0.6 mm cores of FFPE biopsy tumor samples using a TMA workstation MTA-1 (Beecher Instruments). All the immunohistochemical techniques were performed in the Surgical Pathology Department at Fundación Jiménez Díaz. Antigen retrieval was performed in PT-Link (Dako) for 20 min at 95 °C in high pH buffered solution (Dako). Endogenous peroxidase was blocked, by immersing the sections in 0.03 % hydrogen peroxide for 5 min. Slides were washed for 5 min with Tris buffered saline solution containing Tween 20 at pH 7.6 and incubated with the primary antibodies anti-PLK1 (Abcam) for 20 min at room temperature, followed by incubation with the appropriate anti-Ig horseradish peroxidase-conjugated polymer (EnVision, Dako) to detect antigen-antibody. Sections were then visualized with 3, 3'-diaminobenzidine as a chromogen for 5 min and counterstained with hematoxylin. All immunohistochemical stainings were performed in a Dako Autostainer. FFPE tissue samples from healthy testis were stained as positive controls for PLK1.

Evaluation of Immunohistochemistry

PLK1 expression was scored positive when >10 % of tumor cells showed immunoreactivity (Fig. 1). The cut-off point for PLK was established with an operator-receiver characteristics (COR) curve. As all our cases showed an intense immunohistochemical reaction, we employed the percentage of positive cells to classify cases instead of the H-score previously employed by other authors [7].

Statistical Analysis

Data was analyzed with SPSS for Windows 20.0 statistical package (IBM corporation). Association between PLK1 expression and clinicopathological and outcome variables were evaluated by chi-squared (or Fisher's exact test) or Student's T test for mean comparison, as indicated. For survival analysis we compared the Kaplan-Meier curves with the log-rank test. The level of statistical significance was defined as a P value less than 0.05.

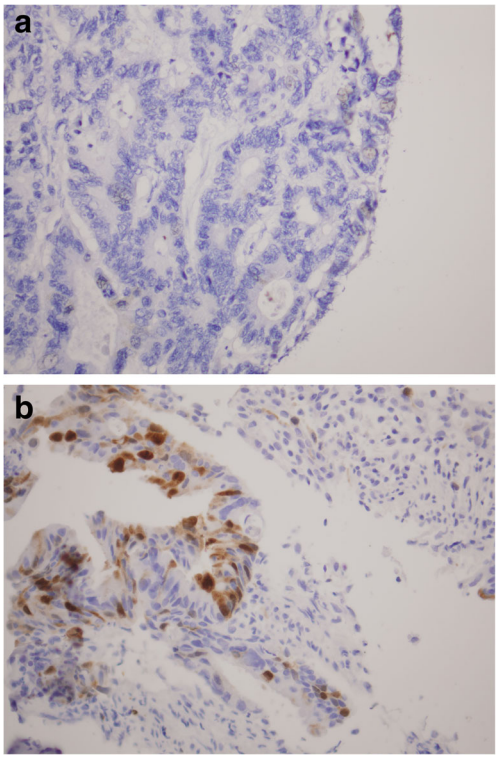

Fig. 1 **a** and **b** PLK1 immunohistochemical expression. On the right there is a negative case (**a**) and on the left a positive case (**b**) (immunohistochemistry for PLK1, ×200)

Permission for this study was obtained by the Ethical Committee on Scientific Investigation of our hospital (given in written form in April 2014). This study is in accordance to national regulations regarding personal data protection and also to the Declaration of Helsinki.

Results

Fifty patients fulfilled inclusion criteria for the present study. Table 1 summarizes the general characteristics of the series. Table 2 summarizes the histopathological features of the colon primaries, according to the histopathological response of the hepatic nodule to NAC, following Blazer's criteria (data of the analysis of the prognostic influence of these histopathological features are not shown). All our patients received a NAC regimen based on oxaliplatin and fluoropyrimidines following the local guidelines for this disease and targeted drugs were only added in 18 % of the patients (mainly anti-VEGF drugs).

Immunohistochemistry for PLK1 of the primary tumor revealed that 43 % of the tumors were positive, as opposed to 23.5 % of the metastasis ($p = 0.05$). PLK1 was significantly more frequently expressed in colonic tumors (64 %) than in rectal-sigmoid ones (32 %) ($p = 0.02$) and PLK1 expression in metastasis was found in 35 % of colonic primaries as opposed to 16 % of rectal tumors (although with a p value 0.16, this difference did not reach statistical significance). We found no significant association between PLK1 expression and any of the demographic characteristics of the patients. We only found a statistically significant association between the kind of growth at the leading edge of the primary and PLK1 expression. Of the tumors with a pushing leading edge, 72 % showed expression of PLK1 as opposed to 33 % of tumors, which showed an infiltrative edge ($p = 0.03$ for the Fisher's exact test).

We analyzed the possible association between outcome and PLK1 expression both in the primary tumor and the metastasis. We found that PLK1 expression in the tissue from the primary tumor was not significantly associated to DFS or OS, but we found a statistically significant difference in OS between metastasis expressing PLK1 and those that lost expression ($p = 0.000$; 95 % CI: 16.1–48.9 mo) (Table 3). This prognostic significance was also shown through the

Table 1 General characteristics of the 50 patients fulfilling inclusion criteria

	Percentage
Gender	Male: 54 %
	Female: 46 %
Age	62.3 (11.3)
Comorbidities	No: 70 %
	Yes: 30 %
Family history of cancer	No: 74 %
	Yes: 26 %
Personal history of cancer	No: 82 %
	Yes: 18 %
Location of primary tumor	Rectosigmoid: 66 %
	Other: 44 %
T stage of primary	T1: 2 %
	T2: 10 %
	T3: 80 %
	T4: 8 %
N stage of primary	N0: 50 %
	N1: 30 %
	N2: 20 %
Histopathological response to therapy	Complete: 6 %
	Major: 20 %
	Minor: 68 %
Recurrence	No: 12 %
	Yes: 88 %
DFS	20.9 (14.3)
Death	No: 36 %
	Yes: 62 %
	Lost: 2 %
OS	52.5 (36.5)

Data are expressed either as percentages or mean (SD), as indicated

Table 2 Histopathological features of the primary tumors

	Complete response (n = 3)[a]	Major response (n = 10)	Minor response (n = 34)[a]
Differentiation	Low grade 1 (50 %)	Low grade 3 (30 %)	Low grade 27 (84.5 %)
	High grade 1 (50 %)	High grade 7 (70 %)	High grade 5 (15.5 %)
Location	Colon 0	Colon 3 (30 %)	Colon 13 (38 %)
	Sigmoid-rectum 3 (100 %)	Sigmoid-rectum 7 (70 %)	Sigmoid-rectum 21 (62 %)
Vessel invasion	Present 0	Present 1 (10 %)	Present 8 (25 %)
	Absent 2 (100 %)	Absent 9 (90 %)	Absent 24 (75 %)
Lymphohistiocytic Inflammatory reaction	Absent 1 (50 %)	Absent 5 (50 %)	Absent 17 (53 %)
	Scarce 1 (50 %)	Scarce 3 (30 %)	Scarce 6 (19 %)
	Intense 0	Intense 2 (20 %)	Intense 9 (28 %)
Leading front	Pushing 0	Pushing 3 (30 %)	Pushing 9 (28 %)
	Infiltrative 2 (100 %)	Infiltrative 7 (70 %)	Infiltrative 23 (72 %)
Desmoplasia	Absent 2 (100 %)	Absent 7 (70 %)	Absent 28 (87.5 %)
	Present 0	Present 3 (30 %)	Present 4 (12.5 %)
Mucin production	Absent 2 (100 %)	Absent 10 (10 %)	Absent 27 (84.4 %)
	Present 0	Present 0	Present 5 (15.6 %)
pT stage	T1 0	T1 1 (10 %)	T1 0
	T2 1 (33 %)	T2 1 (10 %)	T2 2 (6 %)
	T3 2 (64 %)	T3 7 (70 %)	T3 29 (85 %)
	T4 0	T4 1 (10 %)	T4 3 (9 %)
pN stage	N0 3 (100 %)	N0 4 (40 %)	N0 15 (44 %)
	N1 0	N1 2 (20 %)	N1 13 (38 %)
	N2 0	N2 4 (40 %)	N2 6 (8 %)

[a] Histopathological data of the primaries were missing in one patient from the complete response group and two from the minor response group

comparison of the survival curves between groups with the log-rank test (Fig. 2). In our study we did not confirm the prognostic significance of histopathological response in terms of OS and DFS, as shown by other studies (Table 4).

As for histopathological response, we found a significant association between PLK1 positivity in the metastatic nodule and a minor histopathological response. In our series none of the 11 patients with high PLK1 expression in the metastasis showed major response to therapy (Table 5).

Discussion

It is well known that almost 50 % of the patients with colon carcinoma will develop metastasis during follow-up and to improve survival it is essential to design aggressive therapeutic schemes that try to reduce the burden of disease and enhance response to adjuvant chemotherapy and also to targeted drugs [2]. Surgery of metastasis is the best therapeutic alternative [3], but sometimes it is difficult to achieve complete resection or surgery is technically complex, and some groups advocate the use of NAC [14]. NAC is usually based on oxaliplatin or irinotecan associated to fluoropyrimidines. Some schemes also employ targeted drugs (namely, anti-EGF and anti-VEGF drugs) in this setting. The response to therapy is usually followed with serial CT scans during NAC, for progression of disease could even make surgical resection impossible or recommend NAC cessation. However, it is rather clear than the RECIST criteria, which rely on size reduction, are not well correlated to the histopathological response [15] and also that they do not precisely predict prognosis after resection. Acknowledging this, radiologists have developed the so called morphological criteria [16, 17], which seem to correlate better with histopathological response.

On the other hand, selective targeting of small molecules of the tyrosine kinase family is becoming an ever-more used strategy to manage cancer [13]. PLK1 is a serine-threonin kinase that plays an essential role in control of mitotic spindle formation and cytokinesis [7]. In addition, PLK1 is required for recovery from the G_2 checkpoint. PLK1 has been reported

Table 3 Association between outcome measures and PLK1 expression both in the primary tumor and metastasis

	DFS	p value	OS	p value
PLK1 expression primary tumor	Negative: 19.9 Positive: 18.5	0.77	Negative: 51.7 Positive: 45	0.26
PLK1 expression metastasis	Negative: 21.7 Positive: 16.8	0.55	Negative: 59.7 Positive: 27.1	0.00

Mean comparison for DFS and OS values in months

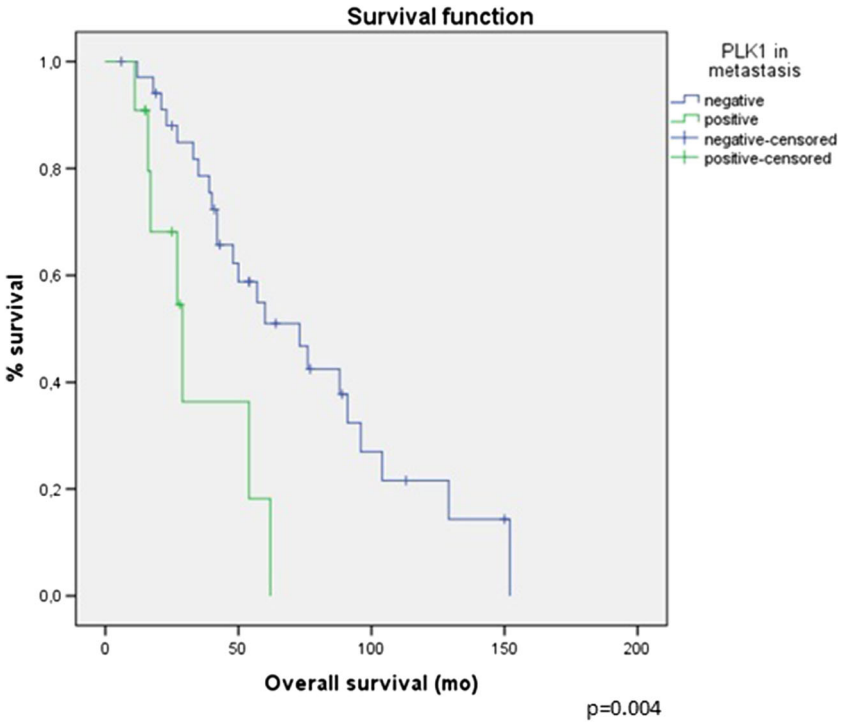

Fig. 2 Kaplan-Meier curves for overall survival in the PLK1 positive and negative metastatic nodules

to phosphorylate Chk2 in the FHA domain and to prevent its activation in mitosis [18]. Several reports show that expression of PLK1 is significantly lower in non-transformed cells and this makes this molecule an especially interesting target which might be antagonized selectively in neoplastic cells. Depletion or inhibition of kinase activity of PLK1 is sufficient to induce cell-cycle arrest and apoptosis in cancer cell lines and in xenograft tumor models [19]. There are some reports with anti-PLK1 drugs both for leukaemia [20] and for solid tumors [21] and there have been phase I clinical trials using intravenous [22] or oral [23] PLK1 inhibitors, with promising results. In conclusion, PLK1 seems to be a promising target for future development of targeted drugs against both leukaemia and many kinds of solid tumors.

However, in our literature review we have found no reference to the possible prognostic influence of PLK1 expression in metastatic colorectal cancer. Therapy of metastasis has greatly evolved in recent years and aggressive management with chemotherapy and surgical resection is now the standard of care for these patients, with an important improvement in prognosis. Nevertheless, these patients tend to recur and many eventually die of disease. In our study we first compared the immunohistochemical expression of PLK1 between the primary tumor and the metastasis, showing a significant reduction of expression in the metastatic nodules. Also, our study had 50 patients treated with conventional NAC associated or not to targeted drugs against EGF and VEGF, and successfully showed a significant association between PLK1 expression in the metastasis and overall survival. If in the near future experimental studies on human tumor cell lines and clinical trials confirmed the efficacy of some of the selective antagonists for PLK1 to control growth and induce apoptosis in colorectal carcinoma (as has been shown in leukaemia both clinically and experimentally) [24], these drugs could become an option for the adjuvant therapy in selected cases of metastatic colorectal carcinoma. In the present study PLK1 expression has been shown to predict a minor response to NAC and a worse prognosis of disease and the chance to control the activity of this molecule could become a useful therapeutic tool in patients with metastatic colonic carcinoma after conventional therapy. Interestingly, this effect has been shown to be

Table 4 Prognostic value of the histopathological response to NAC for DFS and OS

	DFS	*p* value	OS	*p* value
Response to NAC	Minor: 20.22 Major: 23	0.55	Negative: 47.2 Positive: 54.4	0.58

Table 5 Association between PLK1 expression in metastasis and response to therapy

		Negative	Positive	Total
Response	Minor response	23	11	34
	Major response	13	0	13
Total		36	11	47

p value for the xi squared test: 0.02

independent of the use of anti-EGF or anti-VEGF drugs in a stratified subgroup analysis.

The present study has several drawbacks, which deserve to be mentioned. The most important drawback is the small number of cases. Despite the series being small, the patients are rather homogeneous and we consider them representative of standard disease management in developed countries. Despite the small number of cases, which could compromise the power of the study, we have found significant differences, which should be confirmed by larger studies. Also, the retrospective nature of the study is another limitation to the design. It is also necessary to design better powered studies that take into account other well-known factors that can influence both prognosis and response to therapy in these patients, including lymph node involvement at diagnosis, CEA levels, among others, that could not be analysed in the present study due to the small sample size. The forementioned size limitation also makes it difficult to draw sound conclusions regarding the possible influence of tumor location (rectal as opposed to colonic) or therapeutic differences due to targeted drugs. Prospective better powered studies should be undertaken to confirm the preliminary results found by our group.

Conclusion

We herein report the first series of cases in which PLK1 expression has been shown to predict lack of response to neoadjuvant therapy of metastatic colorectal disease and also bad prognosis in terms of overall survival. The existence of small drugs selectively antagonizing PLK1 could open a new way for the management of this complex group of patients in the near future, if our results are confirmed in larger prospective studies or clinical trials.

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Specific Contributions to the Study MJFA and LE: Study design, pathologic review, IHC, writing
DCG, CPI and AC: Patient selection, files review
AC, TGP, JMU and JGF: Files review, statistical analysis

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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