

PML as a Potential Predictive Factor of Oxaliplatin/Fluoropyrimidine-Based First Line Chemotherapy Efficacy in Colorectal Cancer Patients

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PML regulates a wide range of pathways involved in tumorigenesis, such as apoptosis, which is also one of the main mechanisms through which oxaliplatin and fluoropyrimidine exert their antineoplastic activity. The present study aims to investigate PML expression as a predictive factor of oxaliplatin/fluoropyrimidine therapy efficacy. Seventy-four metastatic colorectal cancer patients who received oxaliplatin/fluoropyrimidine-based first line therapy have been included in this retrospective study. PML expression was assessed by immunohistochemistry. PML down-regulation was detected in 39 (52.7%) patients (14 complete and 25 partial PML loss). RR was significantly lower (25.6%) in patients with PML down-regulation than in patients with preserved PML expression (60%) ($P = 0.006$). Median TTP was 5.5 months when PML was down-regulated versus 11.9 months in case of preserved PML expression ($P < 0.0001$). A statistical significant difference was also detected in OS (15.6 and 24.5 months, respectively, $P = 0.003$). The impact of PML down-regulation on TTP and OS was statistically significant also in a multivariate model. This study represents the first evidence of a possible correlation between PML protein expression and outcome of metastatic colorectal cancer patients treated with oxaliplatin/fluoropyrimidine-based first line therapy.

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The promyelocytic leukemia (PML) gene, located at 15q22, consists of 9 exons which encode for alternatively spliced isoforms with both cytoplasmatic and nuclear localization. PML nuclear isoforms (PML I, II, III, IV, V, VI) are characterized by a nuclear location signal (NLS), coded by exon 6, which is absent in the cytoplasmatic isoforms. The NLS allows PML localization in the nucleoplasm or into complex protein structures known as PML nuclear bodies (PML-NBs).

PML-NBs appear as ring-like structures composed by highly ordered PML multimers which, together with S100 proteins, represent the only constitutive elements of nuclear bodies. This basic structure works as a platform on which many other proteins are transiently recruited (Jensen et al., 2001; Shen et al., 2006; Weidtkamp-Peters et al., 2008). The importance of PML as tumor suppressor gene was postulated since its identification at the breakpoint of t(15;17) translocation, which brings to the fusion protein PML/RAR α that characterizes

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Contributions section: AMR, AOM carried out the molecular genetic studies, participated in the sequence alignment and drafted the article. GP, CR carried out the immunoassays. BV, DS, FL, FG and AMF participated in the design of the study and performed the statistical analysis. GT, AR, SG, AZ, AF and SR conceived the study, and participated in its design and coordination and helped to draft the article. All authors read and approved the final article.

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promyelocytic acute leukemia. Thanks to the interaction with many different partners in the context of PML-NBs, PML is able to regulate key pathways of tumorigenesis and to control basic cellular functions such as senescence (Chan et al., 1997; Ferbeyre et al., 2000; Pearson et al., 2000; Mallette et al., 2004), apoptosis (Wang et al., 1998b; Zhong et al., 2000; Salomoni et al., 2005; Halazonetis et al., 2008), and protein synthesis (Culjkovic et al., 2007). Furthermore, a recent work by Bernardi et al. shows how PML regulates HIF α levels through the interaction with mTOR. In fact, HIF α levels seem to be higher in PML $-/-$ mice, with consequently increased neoangiogenesis and tumor vascularization (Bernardi et al., 2006).

Despite all the previous reported evidences, a few studies up to date evaluated the expression of PML gene in different neoplasms. In 2004, Gurrieri et al. assessed PML expression in human cancers of multiple histologic origins through microarrays. They detected a partial reduction or complete loss of PML expression in prostatic adenocarcinoma, colon adenocarcinoma, breast cancer, lung cancer, lymphoma, CNS tumors, and germ cells tumors (Gurrieri et al., 2004). Further studies have shown a decreased PML expression in breast cancer, gastric cancer, NSCLC, and invasive epithelial tumors (Koken et al., 1995; Gambacorta et al., 1996; Zhang et al., 2000; Lee et al., 2007).

Oxaliplatin is one of the most widely used and effective agents in the treatment of colorectal cancer. It is a third generation platinum drug whose two-steps activation leads to the production of reactive compounds (Jerremalm et al., 2003) that bound to DNA, constituting cross-links between two adjacent guanosine residues (stable adducts). Oxaliplatin ability of inducing DNA structural changes confers to the drug a cytotoxic and antineoplastic action. In the treatment of metastatic colorectal cancer (mCRC), oxaliplatin (85 mg/m² every 2 weeks) is given in association with 5-fluorouracil (FU) and folinic acid (FOLFOX) or with capecitabine (XELOX). FOLFOX regimen has been proven to be associated with a longer progression free survival (PFS) (9 vs. 6.2 months) and a better response rate (RR) (50.7% vs. 22.3%) when compared with LV5FU2 regimen in patients affected by mCRC. Although the difference in terms of overall survival (OS) does not reach a statistical significance (16.2 vs. 14.7 months), FOLFOX resulted to be significantly superior to IFL in terms of 5-years OS (9.8% vs. 3.7%; $P = 0.04$) (de Gramont et al., 2000). Moreover, IFL regimen was previously shown to be associated with longer survival when compared with LV5FU2 alone (Saltz et al., 2000). Thus, there are currently strong evidences that FOLFOX improves survival when compared to fluoropyrimidines alone.

A study presented by Chan et al. (1997) analyzed PML expression by immunofluorescence staining in HeLa cells treated with ionizing radiation (20 Gy) and cisplatin (6 μ g/ml), showing a 5–10-fold increase in PML expression. These data underline the key role played by PML in the cellular response to DNA damage. However, at the moment there are no preclinical or clinical studies demonstrating a direct link between PML and sensitivity/resistance to oxaliplatin.

Given the possible relationship between oxaliplatin mechanism of action and PML involvement in apoptosis, the aim of the present study was to assess PML immunohistochemical (IHC) expression in colorectal cancer and evaluate the value of PML down-regulation as a predictive factor of resistance to oxaliplatin-based therapy in mCRC patients.

Patients and Methods

The present study is retrospective, thus it was not designed in "a priori" model. Seventy-four consecutive (40 males and 34 females, median age 61 years) mCRC patients were included from January 2005 to December 2006. This cohort represents

all the patients that started a first line treatment for mCRC in the Department of Medical Oncology at University Campus Bio-Medico of Rome, the Unit of Medical Oncology at Hospital of Pesaro, and the Unit of Medical Oncology 2, Azienda-Ospedaliero Universitaria Pisana, Istituto Toscano between January 2005 and December 2005. Inclusion criteria were only the availability of representative tumor blocks for IHC analysis.

All tumor samples were collected at the time of surgical resection with written informed consent. All patients received an oxaliplatin/fluoropyrimidine-based first line regimen for metastatic disease (FOLFOX IV and XELOX regimen in 22 and 52 patients, respectively) (Table I). The decision to treat each patient according to one of the two regimens was made according to the physician's personal decision. Treatment was continued until the occurrence of radiologic progression as defined by RECIST criteria or the occurrence of either unacceptable toxicity or death. There was no difference in terms of dose intensity between patients treated with FOLFOX IV and those treated with XELOX regimen (data not shown). Tumor response was evaluated every 8 weeks by appropriate imaging techniques (computed tomography or magnetic resonance imaging). Assessment was performed by the investigators on the basis of the response evaluation criteria in solid tumors (RECIST). All the radiological tumor responses were confirmed by an independent radiological review. The cut-off time for survival analysis was May 2009 and the median follow-up was 28 months.

PML immunohistochemical analysis

PML expression was evaluated through immunohistochemistry by three independent observers. Representative tumor blocks were sectioned at 3 μ m thickness for IHC studies. Immunohistochemistry was carried out by the streptavidin-biotin method. A mouse monoclonal antibody against PML protein (clone PG-M3, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at 1:50 dilution. This antibody has been previously used and validated by our group and by others (Falini

TABLE I. Baseline characteristics of the patients

Patient's characteristics	Number of patients (%)
Total number	74 (100%)
Male/female	40/34 (54/46%)
Age (years)	
Median	63 years
Range	29–78 years
Performance status (ECOG)	
Median	1
Range	1–2
Primary tumor site	
Colon	48 (64.8%)
Rectum	26 (35.1%)
Tumor grade	
G2	23 (31.0%)
G3	44 (59.5%)
G4	7 (9.5%)
No. of metastatic sites	
1	31 (41.9%)
2	23 (31.1%)
≥ 3	20 (27.0%)
Sites of metastases	
Liver	42 (56.7%)
Lung	25 (33.8%)
Lymph nodes	27 (36.5%)
Local	15 (20.3%)
Other	26 (35.1%)
Prior adjuvant therapy	
None	25 (33.8%)
FU/LV (Mayo Clinic or De Gramont schedules)	49 (66.2%)
First line regimen	
XELOX	52 (70.3%)
FOLFOX	22 (29.7%)

et al., 1997; Mallette et al., 2004; Vincenzi et al., 2009). DakoCytomation Labelled Streptavidin-Biotin2, Horseradish Peroxidase (LSAB2 System, HRP, Milan, Italy) system was utilized as staining kit. 3-3-Diaminobenzidine was used for color development and hematoxylin was used for counterstaining. Negative control slides processed without primary antibody were included for each staining. Slides were examined without knowledge of the corresponding clinico-pathological data. Immunostaining was considered positive if appropriate brown staining was seen in tumor cells. PML expression was defined by calculating the percentage of nuclear immunoreactive cells in a total of 1 000 neoplastic cells.

Scoring and quantification of the immunoreactivity

PML protein expression patterns were divided into complete loss (nuclear immunoreactivity in <10% of tumor cells), focal positivity (in $\geq 10\%$ but <50%), and diffuse positivity (in $\geq 50\%$), as previously reported by Lee et al. (2007). Agreement in IHC evaluation among the three observers was >90% (Kappa value: 0.931). In cases of disagreement, a final score was determined by consensus after re-examination. We compared RR and TTP between patients with and without PML down-regulation.

MSI status determination

DNA extracted from each tumor was amplified by standard polymerase chain reaction using microsatellite markers defined during the 1998 National Cancer Institute Workshop on Microsatellite Instability: BAT25, BAT26, D17S250, D5S346, ACTC, D18S55, BAT40, D10S197, BAT34c4, and MycL. In most cases, normal control tissue was obtained from a separate, non-tumor tissue block. When this was not possible, non-cancer control tissue was obtained by microdissection. Whenever necessary, microdissection was performed to enrich tumor specimens for cancer cells, ensuring a minimum of 60% tumor within the sample. Tumors were considered MSI-high (MSI-H) if instability was identified at $\geq 50\%$ of the loci screened, MSI-low (MSI-L) if at least one but $\leq 50\%$ of the loci showed instability, and microsatellite stable (MSS) if all loci were stable. For analysis, MSI-L and MSS cases were combined (MSI-L/S). A minimum of five successfully amplified loci were required for classification.

Statistical analysis

Time to progression (TTP) was calculated as the period from the date of starting treatment to the first observation of disease progression or death from any cause. The duration of response was defined as the period of time from initiation of treatment (in a patient responding to therapy) until radiological or symptomatic disease progression. OS was calculated as the period from the date of starting treatment until death from any cause or until the date of the last follow-up, at which point data were censored. TTP and OS were determined by Kaplan–Meier product-limit method (Kaplan and Meier, 1958). The other prognostic variables tested were: tumor grading, liver involvement, previous adjuvant chemotherapy, albumin, alkaline phosphatase, gamma-GT, lactate dehydrogenase (LDH), and basal carcinoembryonic antigen (CEA) levels.

Stratified permutation tests were carried out to explore the association between tumor response and PML expression. Moreover, the differences in terms of TTP and OS according to PML expression were evaluated by log-rank test. Finally, Cox proportional hazards model was applied to the multivariate survival analysis. Chi square test was used to test the association between PML expression and tumor RR. SPSS software (version 17.00, SPSS, Chicago, IL) was used for statistical analysis. A *P* value of less than 0.05 was considered to indicate statistical significance.

Results

PML expression

In normal colon epithelium, PML displayed a diffuse nuclear staining pattern (Fig. 1A). Moreover, endothelial cells and tumor-infiltrating lymphocytes were also strongly positive for PML expression in both normal and tumor tissues, thus serving as internal positive controls. In contrast, PML staining was frequently focally or completely lost in tumor specimens.

PML down-regulation (defined as either complete loss or focal expression) was detected in 39 (52.7%) patients. In the remaining 35 patients PML tumor expression was preserved.

In particular, 14 (18.9%) of the total 74 colon adenocarcinoma samples were classified as PML complete loss (Fig. 1B), and 25 (33.7%) samples as focal expression (Fig. 1C). In the remaining 35 (47.2%) tissue samples PML was diffusely expressed (Fig. 1D).

Correlation between PML expression and treatment efficacy

Patients with PML down-regulation showed a RR of 25.6% versus 60.0% in patients with preserved PML expression (*P* = 0.006). Moreover, the tumor control rate, defined as the percentage of patients who had a best-response rating of complete response, resulted significantly lower in those patients with PML down-regulation (43.6% vs. 82.8%, *P* = 0.001) (Table 2).

No difference was identified between patients with complete loss or partial PML down-regulation in terms of RR and tumor control rate.

The median TTP was 5.5 (95%CI: 2.78–7.43) months in patients with PML down-regulation and significantly longer in patients without PML reduced protein expression (11.00 months, 95%CI: 6.56–14.90; *P* < 0.0001). Moreover, also in terms of duration of response, PML protein expression showed a statistically significant impact in our cohort. OS was significantly different between the two groups of patients (15.60 and 24.50 months, respectively, *P* = 0.003). All these data are summarized in Table 3 and Kaplan–Meier plots are presented in Figure 2.

Notably, no differences in terms of efficacy measurements were detected between the group of patients treated with FOLFOX and XELOX regimen and no difference in terms of oxaliplatin dose-intensity was recorded according to PML down-regulation (data not shown).

Finally, we performed survival analysis using a multivariate model both for TTP and OS, by introducing all the clinical variables that showed a statistical significant impact on prognosis in univariate analysis.

As reported in Table 4, PML maintained a statistically significant impact on TTP (*P* = 0.008) and OS (*P* = 0.011) in multivariate analysis.

MSI status determination

MSI-H and MSI-L were observed in 19 (25.7%) and 15 (20.3%) of the 74 primary tumors studied, respectively. For analysis, MSI-L and MSS cases were combined and compared with the remaining patients (with MSI). In our cohort MSI did not correlate with efficacy (RR) (*P* = 0.450) and did neither predicted TTP nor OS (*P* = 0.951 and 0.121, respectively).

Discussion

Although there are still some enigmatic aspects in PML biology, research in this field has moved forward in the last few years and produced evidences suggesting a PML tumor suppressive function in non-hematopoietic neoplasms (Trotman et al., 2006; Ferguson et al., 2007).

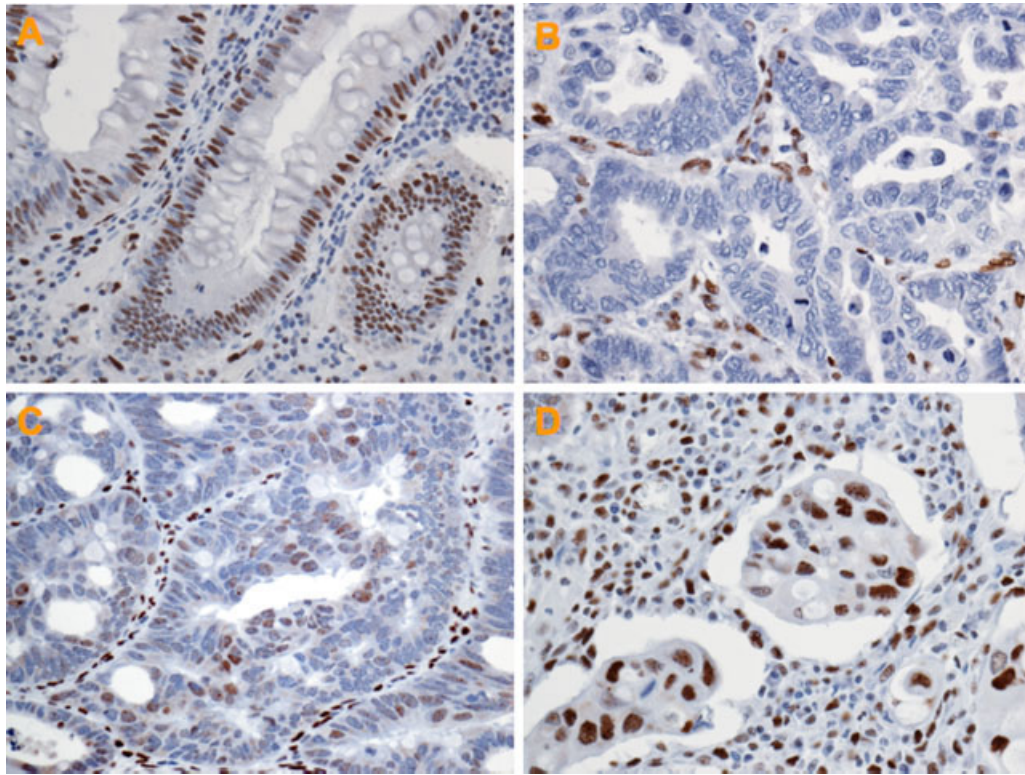


Fig. 1. Promyelocytic leukemia (PML) protein detection by immunohistochemistry. (A) Positive PML immunostaining in peritumoral normal epithelial cells; (B) loss of PML protein expression in colon adenocarcinoma cells (nuclear immunoreactivity in <10% of tumor cells); (C) focally expressed PML protein in colon adenocarcinoma cells (nuclear immunoreactivity in $\geq 10\%$ but <50%); and (D) diffuse expression of PML protein in colon adenocarcinoma cells (nuclear immunoreactivity in $\geq 50\%$). In all images, nuclear PML staining in endothelial cells and tissue-associated lymphocytes represents positive control. Original magnification 400 \times . [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/jcp>]

Loss of PML expression is associated with transformation, progression or high tumor grade in some tumors and PML has been proven to be lost or down-regulated in many cancers of multiple histologic origins. For example, Gurrieri et al. found that PML gene is partially or completely lost in 31% and 17% of colon adenocarcinomas, respectively (Gurrieri et al., 2004). In our cohort (74 tissue samples), we observed preserved PML expression in 47.2% of cases and partial or complete PML loss in 52.7% of patients (18.9% and 33.7%, respectively).

PML has growth suppressive and pro-apoptotic properties and displays an altered expression pattern during human oncogenesis (Koken et al., 1995). Its increased expression suppresses the growth and tumorigenicity in cancer cells in vivo and PML deficient cells are resistant to apoptosis induced by multiple stimuli (He et al., 1997; Quignon et al., 1998; Wang et al., 1998a; Pearson et al., 2000; Wu et al., 2003). Apoptosis is also one of the main mechanisms through which platinum compounds, such as oxaliplatin, exert their antineoplastic activity.

Oxaliplatin is a third-generation platinum-containing drug, and oxaliplatin/fluoropyrimidine therapy has significantly improved efficacy compared with cisplatin-based regimens in the treatment of colorectal cancer. The combination of capecitabine and oxaliplatin is particularly attractive because of its favorable tolerability profile and efficacy. Furthermore, it has been shown that oral fluoropyrimidines are preferred by patients, obviating the drawbacks of prolonged intravenous infusion. Phase III trials comparing capecitabine/oxaliplatin with infusional regimens of 5-FU +/- LV and oxaliplatin in mCRC clearly point out how efficacy, in terms of median PFS and OS, and tolerability profile are similar between the two regimens as first and second line treatment (Cassidy et al., 2004; Cassidy et al., 2008; Rothenberg et al., 2008).

Different studies underline that oxaliplatin dose intensity is related both to efficacy and tolerability profile. Oxaliplatin dose intensification probably improves RR and PFS in pre-treated metastatic patients (dose below 85 mg/m² may be associated with a worse outcome) with a slightly increased toxicity, mainly

TABLE 2. Radiological tumor response to oxaliplatin/fluoropyrimidine anticancer regimen according to PML protein expression

	CR (%)	PR (%)	SD (%)	PD (%)
Down-regulated PML (39 pts)	—	10 (25.6%)	7 (17.9%)	22 (56.5%)
Normally expressed PML (35 pts)	1 (2.9%)	20 (57.1%)	8 (22.9%)	6 (17.1%)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

TABLE 3. Influence PML down-regulation on TTP, duration of response and OS

	Median TTP (95% C.I.) in months	P value
TTP		
Down-regulated PML	5.50 (95%CI: 2.78–7.43)	<0.0001
Normally expressed PML	11.00 (95%CI: 6.56–14.90)	
Duration of response		
Down-regulated PML	2.8 (95%CI: 1.50–6.90)	0.006
Normally expressed PML	6.5 (95%CI: 4.40–9–20)	
OS		
Down-regulated PML	15.60 (95%CI: 11.30–19.50)	0.003
Normally expressed PML	24.50 (95%CI: 21.00–28.60)	

neuropathy (Maindrault-Goebel et al., 2000, 2001). In the OPTIMOX1 study (Tournigand et al., 2006), the first study evaluating the stop–start treatment strategy, previously untreated patients were randomly assigned to receive FOLFOX4 until progression or FOLFOX7 (oxaliplatin 130 mg/mq) for six cycles, then maintenance without oxaliplatin for 12 cycles, and reintroduction of FOLFOX7. RR was 58.5% in arm A and 59.2% in arm B. Grade 3 sensory neuropathy was observed in 17.9% of the patients in arm A versus 13.3% of

patients in arm B. On the contrary, in the OPTIMOX2 study (Andre et al., 2007) oxaliplatin was administered at 100 mg/mq with significantly lower incidence of grade 3 sensory neuropathy (9.5%). It is noteworthy that oxaliplatin dose intensity was not different between the groups of patients treated with FOLFOX IV and XELOX regimen.

The identification of predictive factors of response to standard chemotherapy and immunotherapy in patients affected by colorectal cancer is today a matter of strong clinical interest. Recently, several studies pointed out how the presence of K-Ras mutations in mCRC abrogates the efficacy of anti-EGFR therapy (Lievre et al., 2006; De Roock et al., 2008), leading the European Medicine Agency to restrict its use to patients with wild-type K-Ras tumors. BRAF status, EGFR amplification and cytoplasmic expression of PTEN seem to be also associated with outcome in K-Ras wild-type patients treated with a cetuximab-based regimen and they are currently under evaluation as possible additional predictive factors (Laurent-Puig et al., 2009). On the contrary, there is no evidence that patients with K-Ras/BRAF mutated tumors are less likely to benefit from standard chemotherapy agents such as irinotecan and oxaliplatin (Richman et al., 2009). Recently, a possible link between up-regulation of the nucleotide excision

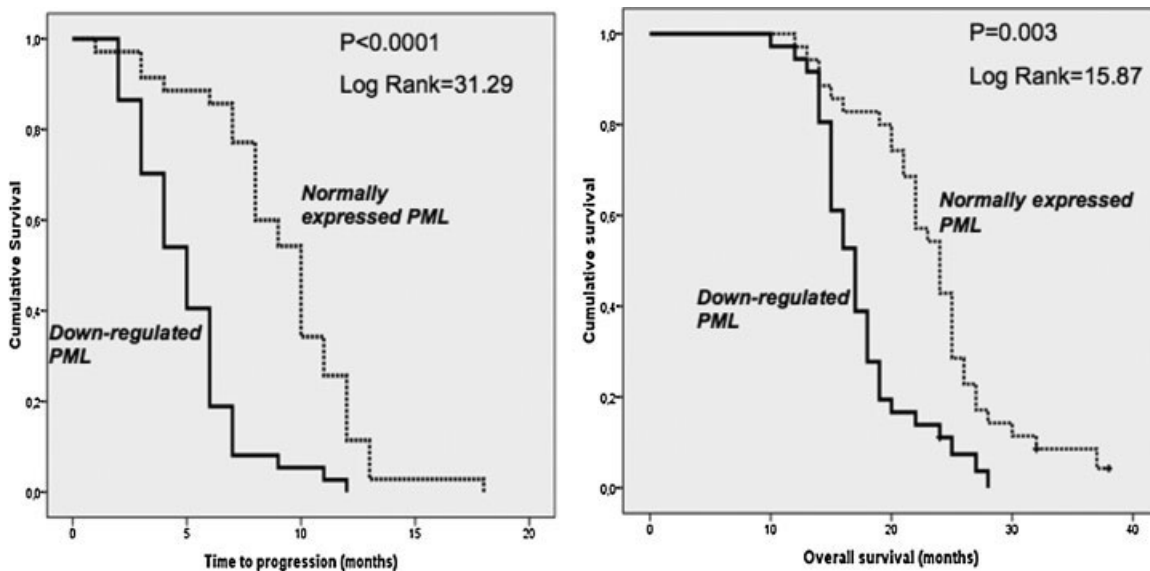


Fig. 2. Kaplan–Meier survival plots for PFS and OS in patients with normally expressed and down-regulated pml metastatic colorectal cancer treated with oxaliplatin/fluoropyrimidine-based first line chemotherapy.

TABLE 4. Multivariate analysis for PFS and OS

Factors	Univariate			Multivariate		
	RR	95% CI	P	RR	95% CI	P
Progression free survival						
PS (ECOG) (70–80 vs. 90–100)	1.56	1.23–1.85	0.004	1.23	0.75–1.53	0.120
Number of organs involved (1 vs. more)	0.77	0.37–0.95	0.015	0.86	0.54–0.99	0.048
PML down-regulation	0.45	0.31–0.78	<0.0001	0.67	0.34–0.81	0.008
Overall survival						
PS (ECOG) (70–80 vs. 90–100)	1.98	1.23–2.07	0.002	1.56	1.18–1.99	0.009
Carcinoembryonic antigen ^a	1.63	1.11–1.76	0.001	1.02	0.89–1.13	0.138
Number of organs involved (1 vs. more)	0.57	0.40–0.81	0.002	0.72	0.66–0.89	0.030
PML down-regulation	0.63	0.34–0.88	0.003	0.69	0.44–0.93	0.011

^aContinuous variable for every 50-point CEA increase.

repair pathway and response to platinum-based therapy has been supposed. In colorectal cancer, single nucleotide polymorphism (SNP) at codon 188 of the ERCC1 resulted associated with a reduced median survival, and it has been suggested as a possible predictor of clinical outcome in mCRC patients treated with platinum-based chemotherapy (Pare et al., 2008). Furthermore, gene polymorphisms of EGF (Spindler et al., 2011), EGFR and its downstream effectors (such as IL-8) have been proposed to be associated with platinum compounds sensitivity in colorectal cancer. However, further studies are necessary to confirm these initial findings (Zhang et al., 2005).

The present study provides, for the first time, evidences suggesting a possible correlation between PML protein expression and outcome of mCRC patients treated with oxaliplatin/fluoropyrimidine-based first line therapy. The link between PML expression and efficacy of oxaliplatin/fluoropyrimidine therapy has been evaluated through several clinical parameters such as TTP, duration of response and OS. A lower RR characterized patients with PML down-regulation (partial or complete loss) compared to those with preserved PML expression (25.6% vs. 60.0%, respectively), without any significant difference between patients with complete loss or partial PML down-regulation. Patients with PML down-regulation showed a significantly shorter median TTP than patients without reduced PML protein expression (5.5 vs. 11 months). The difference in terms of OS rate appears extremely relevant, revealing a decreased OS time for patients with PML down-regulated expression. All the data regarding the impact of PML down-regulation on survival (TTP and OS) were confirmed also in multivariate analysis. In conclusion, this study seems to support the role played by PML in cellular response to DNA damage and the value of its down-expression as a factor predicting resistance to oxaliplatin/fluoropyrimidine therapy in mCRC.

Nevertheless, the significance of our study is limited by a small sample size, absence of a validation cohort, retrospective nature of the study itself and inability to determine prognostic versus predictive role of PML down-regulation. In order to confirm the value of our assumptions, further larger prospective studies appear mandatory. The identification of a predictive factor of resistance to oxaliplatin/fluoropyrimidine therapy, together with reliable markers of efficacy, would enable clinicians to quickly select patients most suitable for management by this strategy.

Finally, the evaluation of PML possible involvement in the pathogenesis of colorectal cancer (for example by assessing its expression in normal tissue, precancerous lesion and tumor samples) and the possible prognostic value of its down-regulation would be of great interest.

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