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Promyelocytic leukemia (PML) gene expression is a prognostic factor in ampullary cancer patients

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Background: Promyelocytic leukemia (PML) tumor suppressor gene plays a key role in acute PML pathogenesis but its involvement in pathogenesis and prognosis of solid cancers has not been defined yet.
Patients and methods: In all, 62 ampullary adenocarcinoma patients who underwent curative surgery between 1996 and 2005 were included. Expression analysis of PML was carried out by immunohistochemical staining and correlated with disease-free survival (DFS) and overall survival (OS).
Results: In 24 tumor specimens (38.7%), PML was classified as absent, in 16 (25.8%) as focally expressed and in 22 (35.5%) as diffusely expressed. By univariate analysis, DFS was significantly influenced by pathological T stage ($P = 0.03$), lymph nodal involvement ($P = 0.002$), and PML expression ($P = 0.001$). DFS in patients without PML expression was 28.0 months versus 45.1 and 75.5 for patients with focal and diffuse expression, respectively. OS in the group of patients without PML expression, with focal expression, and with diffuse expression was 40, 48, and 77 months, respectively ($P = 0.002$). By a multivariate analysis, PML expression was the strongest prognostic factor for DFS ($P = 0.003$) and the only statically significant prognostic factor for OS ($P = 0.009$).
Conclusions: Our preliminary data suggest PML as a novel prognostic tool for ampullary cancer patients.
Key words: ampullary cancer, PML, survival

introduction

Although carcinoma of the ampulla of Vater is an uncommon entity, it accounts for 20%–40% of the resected cases of all periampullary neoplasms [1, 2]. Many studies have examined the outcomes of radically resected ampullary cancer, but most studies had insufficiently large sample sizes to assess prognostic factors, and in larger series there remains substantial differences in the main independent prognostic variables. At present, the most reliable prognostic variable in resected ampullary cancer is the presence and number of lymph nodes with metastatic deposits, and no molecular prognostic factors have been definitively validated in this disease [3, 4]. Similarly, the adjuvant therapy for resected ampullary carcinoma is poorly studied due, in part, to the rarity of the cancer. Case series of adjuvant chemoradiotherapy, generally infusional fluorouracil with concurrent radiotherapy [5, 6], achieved median survivals on the order of 3 years. Furthermore, there is no generally accepted chemotherapy for those with recurrent or distant metastatic disease.

Literatures still show few data on the role of clinical, histopathologic, and molecular factors in predicting length of survival. More specifically, there are only some interesting data that report on the role of allelic loss (loss of heterozygosity) of chromosomes 5q, 17p, and 18q as molecular significant prognostic markers for ampullary cancer patients [7–9]. Achille et al. [7] reported that chromosome 5 allelic losses signify early events in tumors of the papilla of Vater. Moreover, Scarpa et al. [8] evaluated the prognostic role of loss of heterozygosity on chromosomes 17p and 18q in a cohort of 53 ampullary cancers [9].

More recently, Santini et al. [10] reported a positive statistically significant correlation between survival and Cox-2 in ampullary cancer patients These data suggest that, in ampullary carcinoma, Cox-2 may play a relevant role in determining the biological phenotype and the aggressiveness of tumor.

A novel molecular marker candidate for human cancer pathogenesis and progression, promyelocytic leukemia (PML), is a tumor suppressor gene implicated in the pathogenesis of leukemia and human cancers [11]. PML belongs to a large

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family of proteins harboring a distinct zinc finger domain designated the RING finger [12].

The PML protein is typically concentrated in subnuclear structures, known as PML nuclear bodies. In the vast majority of acute promyelocytic leukemia patients, PML is fused to the *retinoic acid receptor* α (*RARα*) gene as a consequence of chromosomal translocation [13]. The PML-RARα protein inhibits RARα transcriptional function, and physically associates with PML, leading to its delocalization from nuclear bodies and consequently deregulation of the functions of PML and nuclear bodies. Recent studies suggest that PML and PML nuclear bodies play a role in the regulation of apoptosis, growth and DNA repair, in addition to tumor suppression and transcription [13].

PML colocalizes with >30 different proteins, including p53, pRb, Daxx, Sp100, cAMP-responsive element-binding protein, and small ubiquitin-related modifier protein-1 [14–16].

PML protein expression is reduced or abolished in various human malignancies, including carcinomas of the prostate, colon, breast, and lung, as well as lymphomas, CNS tumors, and germ-cell tumors [17].

Furthermore, loss of PML expression is associated with tumor progression in prostate, breast, gastric, and CNS cancers [17, 18], even if inconclusive data have been reported about the impact on survival. However, the clinicopathological significance of PML expression in ampullary cancer is not yet established.

In order to investigate the potential role of PML in this disease, we retrospectively analyzed the immunohistochemical expression of PML in a very homogenous cohort of patients treated with radical surgery.

patients and methods

clinical data and tumor specimen acquisition

This retrospective study was restricted to patients with ampullary carcinoma consecutively treated at the Catholic University School of Medicine of Rome and at the University Campus Bio-Medico of Rome from 1986 to 2006. To be eligible for this analysis, each subject underwent surgical resection for tumors of ampullary origin with curative intent, and only patients without known residual disease were analyzed. All patients were staged before surgery by clinical examination, computed tomography of the thorax, abdomen, and pelvis, and, when indicated, intraoperative ultrasound of the liver, excluding the presence of overt distant metastases. Data on clinical variables, including sex, age, preoperative assessment of disease state, and type of operative procedure, were gathered retrospectively from patient records. All specimens underwent gross anatomical examination according to the procedure described by Rosai [19] including evaluation of all anatomic structures (pancreatic duct, ampulla of Vater, common bile duct, and pancreatic head). All tumors included in the study were limited to the ampulla or were primarily located in the ampulla and secondarily spreading into the neighboring structures. Pathological findings (tumor size and spread, and lymph node status) were obtained from the pathologists' original reports. In addition to the original pathological reports, tumor–node–metastasis status classification was reassessed as International Union Against Cancer [20]. Overall survival (OS) was determined from the date of initial surgery to the date of death or the last contact. Disease-free survival (DFS) was defined as the interval between the initial surgery and the documented disease representation. Follow-up data were available for all included patients. The study was carried out with approval of the relevant local institutional research boards.

immunohistochemistry

Representative tumor blocks were sectioned at 3-μm thickness for immunohistochemical studies. Immunohistochemistry was carried out by the streptoavidin–biotin method. Endogenous peroxidase in the section was blocked by incubating them in 3% hydrogen peroxide. The used antibody was a rabbit polyclonal antibody against PML protein (clone PG-M3, Santa Cruz Biotechnology, Inc.) at 1:50 dilution. This antibody has been used and validated previously by others [17, 21]. Sections were incubated with LSAB2 (Dakocytomation). 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 clinicopathologic data.

Immunostaining was considered positive if appropriate brown staining was seen in tumor cell. PML expression was established calculating the percentage of nuclear immunoreactive cells in a total of 1000 neoplastic cells.

scoring and quantification of the immunoreactivity

In terms of PML immunohistochemical staining results, all cases were divided into complete loss (nuclear immunoreactivity in <10% of tumor cells), focal positivity (in ≥10% or more but <50%), and diffuse positivity (in ≥50%), as previously reported by Lee et al. [18].

statistical analysis

Standard descriptive analysis was used to describe patients' features. The χ^2 test or Fisher's exact test (two sided) was used for searching possible correlations between PML expression status in the gastric carcinomas and clinicopathological parameters.

Univariate survival analysis for each prognostic variable on OS was estimated according to the Kaplan–Meier method [22]. The terminal event was death attributable to cancer or noncancer causes. The statistical significance of the differences in survival distribution among the prognostic groups was evaluated by the log-rank test [23]. The Cox proportional hazards model was applied to the multivariate survival analysis [24].

P value <0.05 was regarded as statistical significant in two-tailed tests. SPSS software (version 14.00, SPSS, Chicago, IL) was used for statistical analysis.

results

patient characteristics

The main clinicopathological features are summarized in Table 1. The cohort consisted of 62 patients with pathological diagnosis of radically resected cancer of the ampulla (34 men and 28 women). The median age at diagnosis was 59 years (range 38–82). Twelve patients (19.3%) were classified as T1, 24 (38.7%) as T2, 22 (35.5%) as T3, and only four (6.5%) as T4. Twenty-one (63.13%) patients had locoregional lymph node metastasis. Adjuvant radiotherapy and/or chemotherapy for ampullary cancer were not routinely offered in the hospitals involved in the study. The median duration of follow-up after surgery was 66 months (range 6–118 months). The median OS was 64 months (range 6–98). The 1-, 3-, and 5-year OSs were 85.5%, 51.6%, and 33.8%, respectively.

PML staining in normal and cancer tissues

PML immunostaining showed an intense nuclear immunoreactivity of the normal ampullary epithelia in all 62

Table 1. Patient's features

Total no. of patients	62
Median age (range)	59 (range 38–82) years
Gender	
Male versus female	34 versus 28 (54.8% versus 45.2%)
Pathological T stage	
1	12 (19.3%)
2	24 (38.7%)
3	22 (35.5%)
4	4 (6.5%)
Pathological lymph nodal involvement	
Assent (N0)	41 (66.13%)
Present (N+)	21 (33.9%)
Grading	
G1	18 (29.0%)
G2	28 (45.2%)
G3	6 (9.7%)
Promyelocytic leukemia expression	
Absent	24 (38.7%)
Focal	16 (25.8%)
Diffuse	22 (35.5%)
One-year OS rate	53 (85.5%)
Three-year OS rate	32 (51.6%)
Five-year OS rate	21 (33.8%)
Median OS (median, range)	64 (6–98) months

OS, overall survival.

tissues examined (Figure 1A). PML displayed a nuclear speckled staining pattern compatible with its normal localization in the PML nuclear body. In some instances, a concomitant diffuse nucleoplasmic immunostaining was found. By contrast, in tumor specimens, PML staining was frequently focally or completely lost. In particular, of the 62 tissue samples, in 24 (38.7%) ampullary adenocarcinomas PML was classified as absent (1B), in 16 (25.8%) as focally expressed (1C), and in the other 22 (35.5%) as diffusely expressed (1D). Endothelial cells and tumor-infiltrating lymphocytes were strongly positive for PML expression in both normal and tumor tissues, thus serving as internal positive controls.

PML expression and clinicopathological variables

PML immunostaining was correlated with all clinicopathological parameters. The only parameters that resulted to be negatively correlated with PML were the pathological ones. In particular, PML downregulation was correlated with T-stage increase with a P value of 0.004, with lymph nodal involvement with a P value of 0.001 and with tumor grading increase with a P value of 0.03. In addition, no differences of PML expression were recorded between patients of different ages and gender.

univariate analysis of survival

To determine the prognostic impact of PML downregulation, all the cases were classified as absent expression, focal expression, and diffuse expression of PML as previously reported.

By univariate analysis, DFS in our cohort of patients was influenced significantly by pathological T stage, lymph nodal involvement, and PML expression. In particular, DFS in patients without PML expression was 28.0 [95% confidence interval (CI) 24.13–31.86] months versus 45.1 (95% CI 24.77–65.22) and 75.5 (95% CI 62.75–91.24) for patients with focal and diffuse expression, respectively ($P = 0.0001$). The median DFS in patients with T1–2 tumors was 59.40 versus 34.00 in patients with T3–4 tumors ($P = 0.03$). Moreover, median DFS was longer in patients with lymph nodal involvement than DFS recorded in patients without (61.00 versus 31.50, $P = 0.002$). All the data regarding the univariate analysis of DFS are summarized in Table 2 and Kaplan–Meier curves according to PML expression plotted in Figure 2.

Concerning OS, PML expression represents in our population a statistically significant prognostic factor together with lymph nodal involvement. OS in the group of patients without PML expression, with focal expression and with diffuse expression was 40.00 (95% CI 28.95–49.04), 48.00 (95% CI 31.80–68.19), and 77.00 (95% CI 55.97–88.02) months, respectively ($P = 0.002$). In addition, median OS was shorter in patients with lymph nodal involvement than this documented in patients without (74.25 versus 42.00) ($P = 0.007$). The data about OS are presented in Table 3 and Kaplan–Meier curves according to PML expression plotted in Figure 3.

multivariate analysis of survival

By a multivariate Cox regression analysis, PML expression was the strongest prognostic factor for DFS ($P = 0.003$) and the only statically significant prognostic factor for OS ($P = 0.009$).

The calculated relative risk of disease progression in ampullary cancer patients with focal PML expression was 0.464 (95% CI 0.333–0.668) and this in patients with diffuse PML expression was 0.217 (95% CI 0.178–0.477). The other pathological parameter that continued to be a statistically significant prognostic factor for DFS was the lymph nodal involvement ($P = 0.004$).

In addition, PML expression was the only parameter that represented a prognostic significant factor also in multivariate analysis ($P = 0.009$). The relative risk of death in ampullary cancer patients with focal PML expression was 0.593 (95% CI 0.401–0.863) and this in patients with diffuse PML expression was 0.359 (95% CI 0.259–0.562).

The results of the multivariate analysis for DFS and OS are summarized in Tables 4 and 5.

discussion

There is a need for a better understanding of the biology of ampullary adenocarcinoma, the identification of potential prognostic factors, and clinically relevant molecular targets for therapy.

There are few substantial data reporting significant prognostic markers for ampullary cancer patients. The influence of pathological (tumor size, lymph node involvement, status of resection margins, and perineural invasion) [3], surgical (aggressive surgical approach versus limited resection) [25], and biological (p53, c-erbB2, bax-2, mapsin, and apoptotic index) factors is not clear [26, 27].

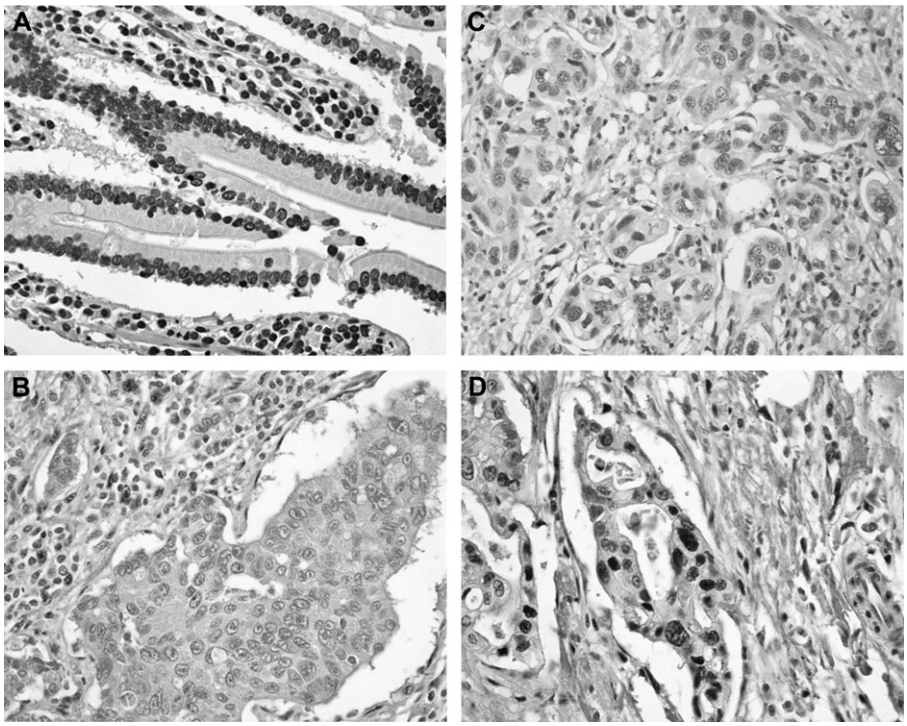

Figure 1. Promyelocytic leukemia (PML) immunostaining. (A) PML immunostaining in peritumoral normal epithelia cells; (B) absent PML immunostaining in ampullary adenocarcinoma cells; (C) focally expressed PML immunostaining in ampullary adenocarcinoma cells; and (D) diffusely expressed PML immunostaining in ampullary adenocarcinoma cells.

An increasing interest is focused on new molecular markers to select patients with better prognosis and, therefore, in need of more aggressive treatments.

Recently, Santini et al. [10] reported that Cox-2 expression is a poor prognostic factor for patients with cancer of the ampulla of Vater and may represent a possible and appropriate target for novel targeted therapies. However, in medical literature, there are controversial data about the prognostic role of Cox-2 in ampullary carcinoma patients. Kim et al. [28] have investigated the prognostic role of Cox-2 overexpression in ampullary cancer but failed to show any correlation with survival and other clinicopathologic factors. On the basis of these counteracting results, no further studies were carried out to elucidate the potential role con Cox-2 as a target for therapy in ampullary cancer patients.

Some other interesting data about molecular aspects of ampullary cancer have been published by Santini et al. [29]. The authors clearly showed that high immunohistochemical staining for the human equilibrative nucleoside transporter 1 (hENT1) is significantly correlated with poor prognosis in patients with resected cancer of the ampulla of Vater. Given the positive association between hENT1 protein and benefit from gemcitabine in pancreatic cancer, this population might particularly derive benefit from gemcitabine-based therapy.

As already stated, PML seems to have an important role in solid cancer, both in tumorigenesis and progression. Gurrieri et al. [17] demonstrated that although PML was more frequently completely lost in advanced cancers, it was also lost in early stages of tumorigenesis. This observation raises the

Table 2. Univariate analysis of DFS in radically resected ampullary cancer patients

	Median DFS (months)	95% confidence interval	*P* value
Gender			
Male	44.90	35.90–78.93	0.976
Female	51.20	39.21–67.90	
Age			
<65 years old	49.00	34.72–65.91	0.656
>65 years old	55.60	39.4–69.56	
Pathological T stage			
T1–2	59.40	37.11–79.44	**0.03**
T3–4	34.00	24.38–49.36	
Pathological lymph nodal involvement			
N0	61.00	35.56–74.70	**0002**
N+	31.50	23.56–44.93	
Grading			
G1	54.30	41.94–62.11	0.160
G2	45.70	35.22–61.02	
G3	42.20	28.56–59.30	
Promyelocytic leukemia expression			
Absent	28.00	24.13–31.86	**0.001**
Focal	45.10	24.77–65.22	
Diffuse	75.50	62.75–91.24	

DFS, disease-free survival.
Bold indicates statistically significative differences.

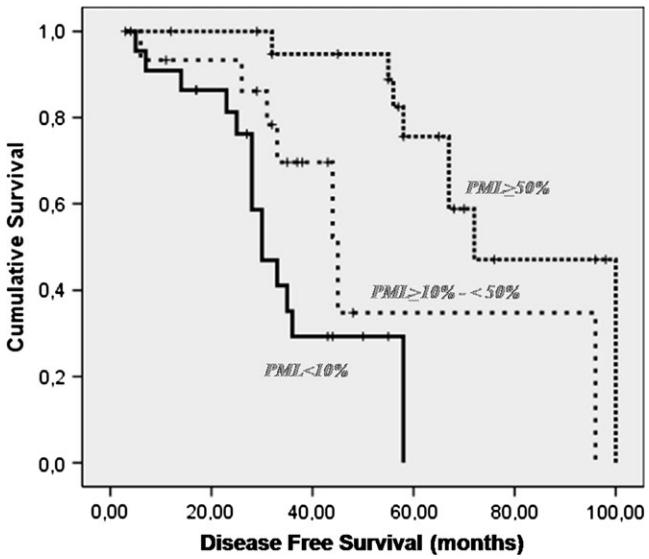

Figure 2. Kaplan–Meier survival plot for disease-free survival in radically resected ampullary cancer patients according to promyelocytic leukemia expression.

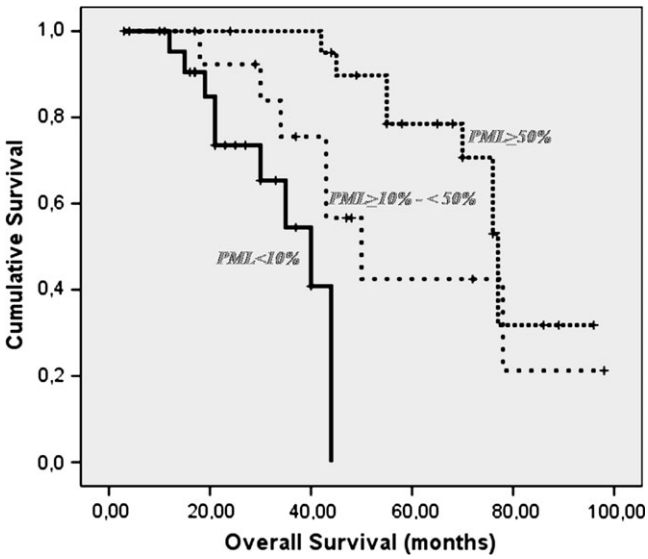

Figure 3. Kaplan–Meier survival plot for overall survival in radically resected ampullary cancer patients according to promyelocytic leukemia expression.

Table 3. Univariate analysis of overall survival in radically resected ampullary cancer patients

	Median disease-free survival (months)	95% confidence interval	*P* value
Gender			
Male	59.90	38.21–67.90	0.451
Female	70.20	25.90–78.93	
Age			
<65 years old	61.02	31.25–71.93	0.737
>65 years old	66.90	39.89–77.89	
Pathological T stage			
T1–2	67.32	43.56–78.23	0.231
T3–4	53.50	31.00–64.45	
Pathological lymph nodal involvement			
N0	74.25	45.65–85.56	**0.007**
N+	42.00	24.76–48.76	
Grading			
G1	67.32	39.09–83.28	0.104
G2	59.77	40.45–76.36	
G3	51.23	24.67–59.93	
Promyelocytic leukemia expression			
Absent	40.00	28.95–49.04	**0.002**
Focal	48.00	31.80–68.19	
Diffuse	77.00	55.97–88.02	

Bold indicates statistically significative differences.

Table 4. Multivariate analysis of disease-free survival in radically resected ampullary cancer patients

	Relative risk of progression	95% confidence interval	*P*
Pathological T stage			
T1–2	1	–	0.356
T3–4	0.815	0.467–1.961	
Pathological lymph nodal involvement			
N0	1	–	**0.040**
N+	0.371	0.390–0.976	
Promyelocytic leukemia expression			
Absent	1	–	**0.003**
Focal	0.464	0.333–0.668	
Diffuse	0.217	0.178–0.477	

Bold indicates statistically significative differences.

question of whether PML loss is an important event in tumor initiation and/or progression. The ability of PML to control proliferation following oncogenic stimulation and apoptosis in cells experiencing DNA damage or other proapoptotic stimuli provides a straightforward explanation for how loss of PML protein would favor tumor initiation. This hypothesis has been demonstrated for lymphoma and other hemopoietic malignancies, in which the survival advantage conferred to cancer cells by PML loss could be a critical determinant in oncogenesis. However, PML loss could also favor tumor initiation in non-hemopoietic malignancies.

On this basis, we design this study to investigate for the first time in literature the role of PML suppression in ampullary carcinoma patients. Our findings suggest, for the first time in literature, that PML downregulation may play an important role in pathogenesis and progression of ampullary carcinoma as already demonstrated for other solid cancer histotype. Moreover, in our population, PML seems to play also a central role in the determination of prognosis both in terms of DFS and OS. As a consequence, PML could represent an ideal target for anticancer therapy for patients affected by this disease and

Table 5. Multivariate analysis of overall survival in radically resected ampullary cancer patients

	Relative risk of death	95% confidence interval	*P*
Pathological lymph nodal involvement			
N0	1	–	0.380
N+	0.710	0.548–1.289	
Promyelocytic leukemia expression			
Absent	–	–	**0.009**
Focal	0.593	0.401–0.863	
Diffuse	0.359	0.259–0.562	

Bold indicates statistically significative differences.

that actually receive only very modest benefit from the available treatments.

In addition, Lee et al. [18], in order to investigate the mechanism of PML protein loss, analyzed PML messenger RNA (mRNA) levels by RT-PCR and found that PML mRNA levels were similar in all gastric carcinoma cell lines regardless of PML protein level. These findings prompted the authors to state that a posttranslational proteasomal degradation mechanism underlies the loss of PML protein. Moreover, a proteasome-dependent pathway has been previously proposed for the loss of PML protein [30] and the results of our proteasome inhibitor experiment suggest that a proteasome-dependent pathway is responsible for PML protein loss in the gastric carcinoma cell lines. On this basis, the present data could represent the rationale to investigate if a proteasome-dependent pathway is responsible of PML degradation also in ampullary cancer and if confirmed this hypothesis, a proteasome inhibitor, could represent an attractive therapeutic option to test in this patients' population.

In conclusion, our data clearly define PML as a prognostic factor for ampullary cancer patients and this could represent a parameter to indicate which patients need to receive a more aggressive therapeutic approach after surgery.

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