POLR2F, ATP6V0A1 and PRNP Expression in Colorectal Cancer: New Molecules with Prognostic Significance?

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Abstract. *Background: DNA-directed RNA polymerase II subunit F (POLR2F), a subunit of the V0 domain of the vacuolar ATPase (ATP6V0A1) and the prion protein (PRNP) are molecules of potential importance in carcinogenesis and targeted cancer therapy. However, their expression has not been studied in colorectal carcinomas. Patients and Methods: Expression microarray data were analyzed using a novel computational tool to reveal elevated levels of POLR2F, ATP6V0A1 and PRNP in relapsed colorectal carcinoma patients. The mRNA levels of POLR2F, ATP6V0A1 and PRNP were evaluated by quantitative RT-PCR in 70 colorectal carcinomas and 17 normal tissue specimens and were correlated with clinicopathological parameters. Results: POLR2F and PRNP were up-regulated in colorectal carcinomas. Moreover, a significant difference in the expression levels of all three molecules between the right colon and the rectum was observed. High expression levels of POLR2F and ATP6V0A1 correlated with improved 3-year survival. Moreover, PRNP expression constituted an independent prognostic factor of the 3-year survival in multivariate analysis. Conclusion: POLR2F and PRNP exhibited elevated levels in carcinomas compared to normal tissue samples suggesting a possible role for these molecules in colorectal cancer. The association of the three molecules with survival or disease prognosis warrants further investigation.*

Colorectal cancer is the second leading cause of cancer death in the western world. Colorectal carcinogenesis is a gradual process during which alterations in the expression of particular genes constitute important steps. The control of gene expression can occur in various steps, but the majority of regulatory events occur at the transcriptional level. An enzyme with an important role in transcription is DNA dependent RNA polymerase II (POLRII). POLRII is responsible for synthesizing mRNA from protein-encoding genes and it is composed of 10-14 subunits. The sixth largest of these, is the subunit F (POLR2F). POLR2F is shared by the other two DNA-directed RNA polymerases (I and III). Its basic function is to catalyze the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates (1).

POLRII is phosphorylated by the transcriptional cyclins (cyclin-dependent kinases 7 and 9) and hence, it facilitates the efficient transcription initiation and elongation of genes encoding antiapoptotic proteins. It has been reported that the inhibition of these cyclins reduces the accumulation of transcripts with short half lives including those encoding anti-apoptotic proteins and cell cycle regulators (2). Indeed, diminution of POLRII levels leads to decreased expression of the anti-apoptotic proteins myeloid cell leukemia sequence 1 (MCL-1) and X-linked inhibitor of apoptosis (XIAP) in non-small cell lung cancer and osteosarcoma cells (3). Moreover, the inhibition of POLRII phosphorylation by the nucleoside analogue 4-amino-6-hydrazino-7-beta-D-ribofuranosyl-7H-pyrrolo(2,3-d)-pyrimidine-5-carboxamide induced potent apoptosis in cancer cell lines and reduced angiogenic activity (4). Therefore, the up-regulation of POLRII expression and/or phosphorylation may result in an increase of anti-apoptotic factors.

The prion protein (*PRNP*) gene encodes the prion glycoprotein PrP, which has been implicated in various types of transmissible neurodegenerative spongiform encephalopathies (5). Although the function of normal PrP remains under investigation, it has been suggested that is possesses a neuroprotective function, possibly by mediating the activation of several signal transduction pathways including phosphatidylinositol 3-kinase/v-akt murine thymoma viral oncogene (PI3K/Akt) known to promote survival (6). There is limited information regarding its role in cancer. It has been

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implicated in the resistance to tumor necrosis factor α-induced death of human breast carcinoma cell lines (7). Moreover, it is suggested that it prevents apoptosis in gastric cancer cell lines, through B-cell CLL/lymphoma 2 (Bcl-2)-dependent pathways (8). The expression of *PRNP* has not as yet been evaluated in colorectal cancer.

ATP6V0A1 is an isoform of one of the 5 subunits of the V0 domain of the vacuolar ATPase (V-ATPase). V-ATPase is an ATP-dependent proton pump that moves protons across the apical plasma membrane of osteoclasts and certain epithelial cells and acidifies intracellular compartments (9). As a result, a variety of intracellular processes, such as zymogen activation, receptor-mediated endocytosis and protein degradation can take place (10,11). ATP6V0A1 binds to phosphofruktokinase 1, the enzyme, which catalyses the rate-limiting step in glycolysis (9). This suggests a direct link between proton transport and glycolysis, the preferred energy producing pathway in cancer cells (12). Moreover, specific inhibition of V-ATPase induces epidermal growth factor receptor (EGFR) dependent apoptosis in EGFR overexpressing cancer cells (13).

Publicly available microarray data for stage B colorectal carcinomas have been analysed with a novel computation tool (14) and the results suggested that *POLR2F, PRNP* and *ATP6V0A1* overexpression was associated with early disease relapse. The purpose of the current study was to investigate the role of *POLR2F, PRNP* and *ATP6V0A1* in colorectal cancer by evaluating their mRNA expression in both malignant and normal colonic tissue from patients with stage B and C colorectal carcinomas with and without disease relapse.

Patients and Methods

Colorectal samples. This study comprised 70 surgical specimens of primary colorectal carcinomas and 17 specimens of normal colorectal tissue, from patients with colorectal cancer who had undergone curative resections at the University Hospital of Patras, Greece, between 1995 and 2005. The patients were randomly selected so that approximately half had disease relapse. None of the patients had received any preoperative neoadjuvant chemotherapy or radiotherapy.

Out of the 87 patients, 52 were men and 35 women ranging from 30 to 90 years of age (median age 66 years). Of the 70 patients with carcinomas, 37 (52.9%) had stage B disease and 33 (47.1%) stage C. Twenty eight (40.0%) tumors were located in the rectum, 17 (24.3%) in the right colon, and 25 (35.7%) in the left colon and 15 (21.4%) were grade I (well differentiated), 52 (74.3%) grade II (moderately differentiated) and 3 (4.3%) grade III (poorly differentiated). The patients were followed for a period of 17 to 144 months with a median of 56 months. During the period of follow-up, 31 (44.3%) patients relapsed, while 21 (30 %) died of the disease.

Before post-operative chemotherapy was initiated, all the patients were assessed by physical examination, routine hematological and biochemical analysis, chest X-ray, and abdominal CT scans. The patients with colon cancer were treated with chemotherapy consisting of a six-week course of leucovorin, 200 mg/m^2 as a 2 hour intravenous infusion, and an intravenous bolus of 5-fluorouracil, 500 mg/m^2 weekly, followed by a two-week rest period. The chemotherapy was continued for four cycles. The patients with rectal cancer were treated with a four-week course of the same chemotherapy followed by pelvic radiotherapy and 5-fluorouracil as a rapid intravenous administration (30 minutes before radiotherapy) at a dose of 400 mg/m^2 on the first three and the last three days of radiotherapy. Chemotherapy started again 2-4 weeks after completion of radiotherapy and five more cycles were administered, each of which consisted of a four-week course of leucovorin and 5-fluorouracil, followed by a two-week rest period.

Microarray data analysis. The gene expression profile data of 16 patients with stage B colorectal cancer with or without recurrence within a 5 year follow-up period that were evaluated using Human 19K Oligo Array slides (Center for Applied Genomics, University of Medicine of New Jersey, USA) was obtained from the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database (Accession Number: GDS1263, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE2630). The data values were edited in order to perform the analysis. The intensity value associated with each probe was the result of subtracting a gaussian function of the noise from the foreground values (15). After this background subtraction, base 2 logarithms of all the data were calculated and the genes with more than two missing values were excluded from the analysis. The remaining missing values were replaced by using the K nearest neighbor imputation method (16). The normalization method of Bolstad and colleagues was then used (17). In order to analyze the microarray data from the 16 samples, the MicroArray Processing Software (MAPS) tool was used (14). More specifically, in order to identify gene markers that could best discriminate between relapsed and non-relapsed patients, a supervised class prediction method was followed. From the 19,200 total genes all 16 patients were divided into a training set of 10 samples and a test set of 6 samples (Table I). The training set was used to select gene markers so as to build a prognostic signature. The test set contained the remaining samples and was used for independent validation. It should be noted that the arbitrary selection of these sets did not affect the performance of the classifier and the results remained the same for every possible sample selection.

Two methods were used to find the differentially expressed genes between relapsed and non-relapsed patients. The first test was the simple permutation *t*-test for comparison of two means and the second was a novel method that is implemented in the MAPS computation tool (14, 18). This method is entitled Mean Value Trust (MVT, see supplementary material).

RNA preparation. Total RNA was extracted from the formalin-fixed paraffin embedded primary colorectal carcinoma and normal tissue specimens as previously described (19). The DNA-free total RNA was quantified using Ribogreen (Molecular Probes, Leiden, The Netherlands) and the MX3000p (Stratagene, La Jolla, USA) according to the manufacturer's instructions.

cDNA synthesis. The first strand cDNA was synthesized from a constant quantity (1.6 μg) of total RNA from each sample using

Table I. *The training set (Tr) containing 6 samples of non-relapsed patients and 4 relapsed patients. The remaining 4 disease free samples and 2 relapsed patients' samples formed the testing set (Te). CS: colon sigma, LC: left colon, RC right colon.*

Samples	Gender	Age at diagnosis	TNM	Location	Recurrence	Set
1GSM50474	M	66	T3N0M0	CS	NO	Tr
1GSM50496	F	45	T3N0M0	LC	NO	Tr
1GSM50504	M	78	T3N0M0	RC	NO	Tr
1GSM50505	F	69	T3N0M0	RC	NO	Tr
1GSM50506	M	67	T2N0M0	CS	NO	Tr
1GSM50507	M	67	T2N0M0	CS	NO	Tr
2GSM50473	M	42	T3N0M0	CS	YES	Tr
2GSM50475	M	72	T3N0M0	CS	YES	Tr
2GSM50510	M	72	T3N0M0	CS	YES	Tr
2GSM50513	M	72	T2N0M0	CS	YES	Tr
1GSM50508	F	66	T3N0M0	CS	NO	Te
1GSM50509	M	81	T3N0M0	CS	NO	Te
1GSM50511	M	67	T2N0M0	RC	NO	Te
1GSM50512	F	48	T2N0M0	CS	NO	Te
2GSM50514	M	60	T2N0M0	RC	YES	Te
2GSM50515	F	58	T3N0M0	LC	YES	Te

random nonamers (ITE, Crete, Greece) and 50U Stratascript reverse transcriptase (Stratagene). In addition, Human Reference RNA (Stratagene) was used as a calibrator sample to allow adjustment for run-to-run variation. A no enzyme control was also included to assure lack of DNA contamination.

Primer design. The primers for *POLR2F, PRNP* and *ATP6V0A1* were designed using mRNA sequences from NCBI (http://www.ncbi.nlm.nih.gov/) and Primer3 (20) and then subjected to a Basic Local Alignment Tool (BLAST, NCBI) analysis to ensure gene specificity. The sequences are presented in Table II. In addition, primers for the Alu-sq expressed repeat sequence were designed by Vandesompele J *et al.*, (manuscript in preparation). The primers were synthesized by the Foundation for Research and Technology-Hellas (Crete, Greece).

Real-time PCR. Quantitative RT-PCR was used due to its ability to detect subtle changes in the expression levels. *POLR2F, PRNP* and *ATP6V0A1* mRNAs were quantified using SYBR Green I intercalation dye in Brilliant Sybr Green QPCR Master Mix (Stratagene, La Jolla, USA). The reactions contained 5-carboxy-x-rhodamine (ROX) as a passive reference dye and cDNA equivalent to 100 ng of total RNA. The PCR reactions were performed in triplicate in the MX3000p (Stratagene) under the following conditions: 10 min at 95˚C followed by 45 cycles of 30 sec at 95˚C, 40 sec at 57˚C and 40 sec at 72˚C. Additionally, a melting curve analysis was included. The relative expression levels of each gene were derived using the standard curve method, after calibration to the cycle number obtained for the calibrator sample. Furthermore, mRNA levels were normalised to the Alu-Sq levels. Alu-Sq was validated to be a stably expressed repeat sequence in normal and tumor tissue of different grades and stages.

Table II. *Primer sequences. F: forward primer, R: reverse primer.*

Gene/NCBI accession number		Primer Sequence (5'-3')	Amplicon size
POLR2F	F	ACAGATCCTCTGCTCATTGC	83 bp
NM_021974	R	CTCCCATCTGGCAGGTAAC	
PRNP	F	TCAGAGGACGCAGACGA	82 bp
NM_000311	R	GGCAGTACTCGATGGTGTG	
ATP6V0A1	F	TGTCACAACACTGAACCTCTG	73 pb
NM_005177	R	CAAGTCCCAGAAGCCTTTC	

Statistical analysis. Intergroup comparisons, regarding the correlation of clinicopathological parameters with the expression levels of *POLR2F, PRNP* and *ATP6V0A1* were performed using the Kruskal Wallis and Mann-Whitney nonparametric tests for continuous or ordinal variables and the Chi-square test for nominal variables. Because of multiple comparisons, these tests were followed by a *post hoc* Bonferroni test. The Spearman rank correlation analysis was used to detect any potential relationships between the expression levels of the three genes. The Kaplan-Meier analysis was used to compare the survival curves. The latter included the 3-year survival rates and the time to disease progression rates. Finally, the Cox hazard regression model assessed the prognostic value of the mRNA levels in conjunction with the clinicopathological parameters. The data were analyzed using the SPSS statistical package (SPSS, Release 14, Chicago, IL, USA). The level of significance was set at p-value <0.05.

Results

The union of the genes identified by both analysis methods of the microarray data yielded a group of 139 differentially expressed genes between the relapsed and non-relapsed groups. This 139 gene signature was then evaluated in the 6 independent patients (test set) to test its predictive performance in stage B colon cancer patients. Two out of 2 relapsed patients and 4 out of 4 disease-free patients were predicted correctly (Figure 1). Six genes were up-regulated in the relapsed patient carcinomas (Table III).

POLR2F, PRNP and *ATP6V0A1*, which were found to be overexpressed by both methods of microarray data analysis in the carcinomas from the patients who relapsed, were arbitrarily chosen for further analysis to assess any possible concordance between the computationally obtained results and the biological data.

POLR2F, PRNP and *ATP6V0A1* genes were expressed in all the normal tissues with median values of 0.0545 (0.0138-0.3867) for *POLR2F*, 0.1266 (0.0781-0.3025) for *PRNP* and

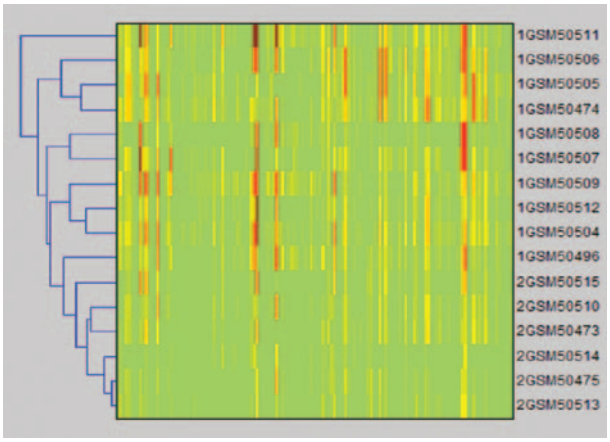

Figure 1. *Hierarchical clustering for the 139 gene list. All the relapse-free samples (prefix in sample name is 1) are in the same clusters. The same is valid for the non-relapsed samples (prefix is 2).*

Table III. *Six overexpressed genes in the recurrence samples. The results of the MVT method and the t-test method were almost identical except for the C21orf86 gene, which was not overexpressed according to the t-test.*

Probe	Gene	MVT	Certainty	*t*-test
s13976	POLR2F	0.96	1	0.01
s11555	NULL	0.87	1	0.04
s11571	ATP6V0A1	0.83	1	0.02
s11530	RORA	0.82	1	0.01
s13073	C21orf86	0.8	0	0.06
s11507	PRNP	0.76	1	0.04

0.031753 (0.0799-0.2244) for *ATP6V0A1*. Expression of POLR2F was detected in 68 (97.14%), *ATP6V0A1* in 67 (95.71%) and *PRNP* in all of the carcinomas. The corresponding median levels of *POLR2F, PRNP* and *ATP6V0A1* expression in the carcinomas were 0.1015 (0.0184-9.6910), 0.1874 (0.1061-0.7996) and 0.0443 (0.0092-0.3591), respectively. The expression levels of *POLR2F* and *PRNP* were higher in the carcinomas than in the normal tissue ($p=0.034$ and $p<0.001$, respectively). In the carcinomas, the *POLR2F* levels strongly correlated with *PRNP* and *ATP6V0A1* levels ($r=0.625$, $p<0.001$; $r=0.835$, $p<0.001$, respectively). Moreover, the *PRNP* levels strongly correlated with the *ATP6V0A1* levels ($r=0.689$, $p<0.001$).

Additionally, using the median of expression as a cut-off, the carcinomas were divided into two categories, overexpressing (above median) and underexpressing (below median), and different combinations of over/underexpression of the three genes were examined. POLR2F, PRNP and ATP6V0A1 levels were simultaneously overexpressed in 27 out of 67 (40.29%) specimens and underexpressed in 21 out of 67 specimens (31.34%) expressing all three genes. Moreover, elevated levels of PRNP with concomitant low levels of POLR2F and ATP6V0A1 were noted in 5 out of 67 cases (7.46%). Only these three combinations were used for statistical analysis as less than three cases were observed for the other combinations.

No significant difference was observed in the mRNA levels of *POLR2F, PRNP* and *ATP6V0A1* between stage B and stage C carcinomas. Moreover, the expression was not related to tumor differentiation, age or gender. However, the lowest expression levels of all three genes were found in carcinomas of the left colon and the highest in carcinomas of the rectum, although the difference was statistically significant only between carcinomas of the rectum and the right colon (Table IV).

Kaplan-Meier analysis using the 3-year survival rate as an end-point, revealed that high expression levels of *POLR2F* or *ATP6V0A1* conferred a survival benefit compared to lower levels ($p=0.002$, $p=0.002$, respectively) (Figures 2 and 3). Univariate Cox regression analysis revealed that *POLR2F* as well as *ATP6V0A1* levels constituted prognostic factors of the 3-year survival (95% CI: 1.587-99.120; $p=0.016$ and 95% CI: 0.010-0.649, $p=0.018$, respectively). Moreover, elevated levels of *PRNP* with concomitant low levels of *POLR2F* and *ATP6V0A1* correlated with shorter survival ($p=0.019$) (Figure 4) and were found to be significant predictors of survival (95% CI: 0.036-0.905; $p=0.037$).

In multivariate Cox regression analysis, in which gender, age, stage, grade, primary site, *POLR2F, ATP6V0A1* and *PRNP* expression levels were considered, *POLR2F* and *ATP6V0A1* were not found to be independent prognostic factors. In contrast, primary site and up- or down-regulation of PRNP proved to have prognostic value regarding the 3-year survival (95% CI: 0.098-0.926, $p=0.036$; 95% CI: 0.002-0.385, $p=0.007$; respectively).

Discussion

The *POLR2F, PRNP* and *ATP6V0A1* expression levels in publicly available microarray data were found to differ significantly between recurrent and non-recurrent colorectal adenocarcinomas. In the current study, elevated levels of *POLR2F* and *PRNP* were exhibited in carcinoma specimens compared to normal tissue samples. Although the differences in mRNA levels are relatively small, they may reflect functional significance. The increased expression of *POLR2F* may reflect a higher transcriptional activity in tumor cells. Although data regarding *POLR2F* expression in cancer are scarce, Orian-Rousseau and colleagues reported elevated levels of *POLR2F* in the metastatic and undifferentiated colon cancer cell line HT29 compared to its differentiated and non-metastatic derivative, HT29MTX. However, this finding was not

Table IV. *Gene expression levels in relation to primary tumor site. p-value is shown only when <0.05.*

		Expression levels					
		POLR2F		*PRNP*		*ATP6V0A1*	
Primary site	n	Median (Range)	*p*	Median (Range)	*p*	Median (Range)	*p*
Left Colon	25	0.0446 (0.0382-4.5553)		0.16 (0.0215-0.2609)		0.0191 (0.0227-0.0917)	
Right Colon	17	0.0951 (0.0211-9.6910)	0.001	0.1874 (0.1254-0.712)	0.007	0.0469 (0.0124-0.295)	<0.001
Rectum	28	0.519 (0.0255-9.3564)		0.207 (0.1195-0.800)		0.070 (0.0091-0.3590)	

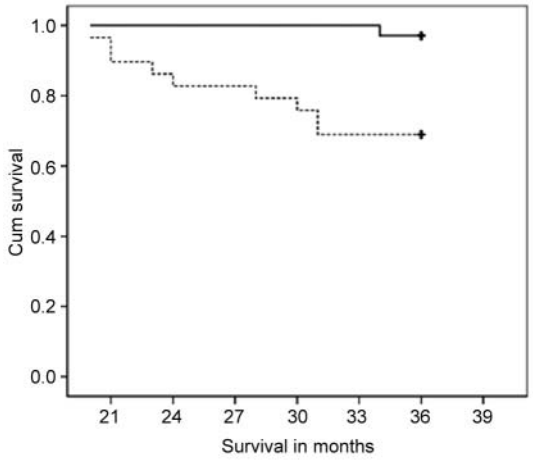

Figure 2. *Kaplan-Meier curves demonstrating longer survival at the 3 year end-point in cases with high expression levels of POLR2F. The continuous and dotted lines indicate high and low levels, respectively.*

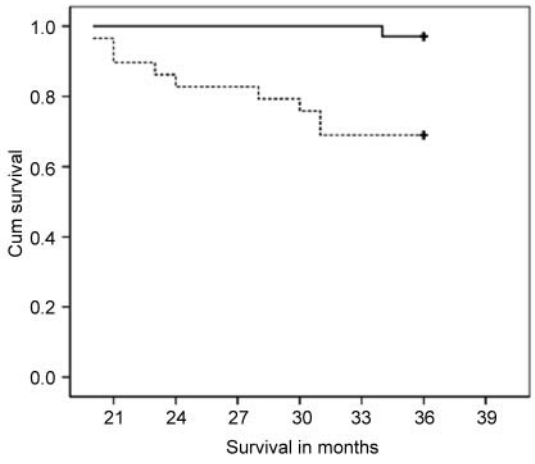

Figure 3. *Kaplan-Meier curves demonstrating longer survival at the 3 year end-point in cases with high expression levels of ATP6V0A1. The continuous and dotted lines indicate high and low levels, respectively.*

evaluated in tissue samples (21). With regard to *PRNP*, elevated levels in the carcinomas are in agreement with the recently reported up-regulated PRNP levels in gastric carcinomas and gliomas (22,23) as well as with the antiapoptotic function of this molecule. Increased levels of *ATP6V0A1* were expected in the carcinomas as they would reflect the effort of the cancer cell to achieve intracellular pH homeostasis in the hypoxic conditions inside the tumor. This is in agreement with the findings of Sennoune and colleagues, who have reported that V-ATPases not only participate in pH homeostasis but their increased levels are also correlated with higher metastatic potential of breast cancer cells (24). However, no such association was noted in the current study in colorectal carcinomas.

The expression levels of *POLR2F, PRNP* and *ATP6V0A1* were not related to age, gender, grade or stage of the disease. Relevant information has been reported only for PrP by Liang and colleagues (23), who evaluated protein

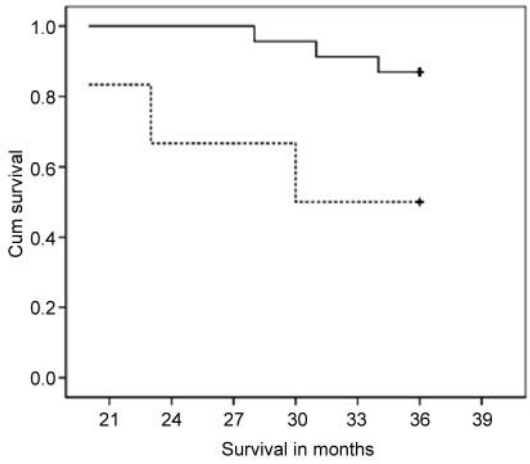

Figure 4. *Kaplan-Meier curves demonstrating shorter survival at the 3 year end-point in cases with high expression levels of PRNP and concomitant low levels of POLR2F and ATP6V0A1. The dotted line indicates high PRNP and low POLR2F and ATP6V0A1 levels. The continuous line corresponds to all other combinations.*

levels in a large cohort of gastric carcinomas and found that PrP levels correlated with tumor grade and stage. This discrepancy may be attributed to differences between mRNA and protein levels or between the populations studied.

The expression levels of the three genes were not associated with any other clinicopathological parameters with the exception of the primary tumor site. In particular, the expression levels differed significantly between the right colon and the rectum. This finding is in agreement with the different epidemiology and distinct gene expression profiles displayed by tumors located in these two anatomical sites (25). Furthermore, none of the molecules correlated with disease recurrence or disease free survival. This finding contradicts the microarray data used for the selection of the studied genes. This may be attributed to the different tissue specimens used for the microarrays and the RT-qPCR validation.

The *POLR2F* and *ATP6V0A1* levels were related to survival and constituted prognostic factors in univariate analysis. Relevant information regarding colorectal cancer or other malignancies remains elusive. Although POLR2F levels were found to be elevated in parental HT29 cells (15), it is not known whether this was due to an involvement of POLR2F in metastasis or differentiation. Therefore, as in the case of estrogen receptor alpha in breast cancer, POLR2F could serve as a tumor-promoting factor, but at the same time high levels of expression may render patients more responsive to therapy and thus result in improved 3-year survival. In the multivariate analysis, the primary tumor site had prognostic value. This is in agreement with previous reports (26). Furthermore, *PRNP* expression appeared to be an independent prognostic factor, when *POLR2F* and *ATP6V0A1* expression levels were also taken into account. In particular, high PRNP levels with concomitant low *POLR2F* and *ATP6V0A1* levels offered no advantage in survival. This may suggest that the tumor driving activity of *PRNP* may not be impaired by low levels of *POLR2F* and *ATP6V0A1*.

To conclude, a prognostic value for PRNP was shown. Moreover, the elevated expression levels of PRNP and POLR2F observed in carcinomas compared to normal tissue suggest a role for these molecules in colorectal cancer. However, further studies with a larger number of samples are necessary to confirm our results and elucidate such a role.

Supplementary Material

The computation formula of the MVT method is the following:

IF (means_ln(i)-means_ln(i)) > ln(threshold) AND min(genesAln(i:)) > max(genesBln(i:)), then gene(i) is overexpressed in condition A, otherwise in B

çhere, A is the group of the non-relapsed patients and B is the group of the relapsed patients. MeansAln(i) is the mean value of the natural logarithm of gene(i) expression for samples belonging to group A. Min(genesAln(i:)) is the minimum value of gene(i) in group A samples. ln(threshold) is a constant value that the user can define according to the desired accuracy. The higher the desired accuracy the lower the value must be set, typically smaller than 2.

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