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ORIGINAL ARTICLE

Expression of nucleoside transporters, deoxycitidine kinase, ribonucleotide reductase regulatory subunits, and gemcitabine catabolic enzymes in primary ovarian cancer

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Abstract
Purpose Gemcitabine (2′,2′-difluorodeoxycytidine) (GEM) is one of the most actively investigated drugs in ovarian cancer. Many molecular mechanisms have been proposed to be involved in GEM sensitivity/resistance including the equilibrative nucleoside transporter-1 (hENT1), the concentrative nucleoside transporter-1 (hCNT1), and deoxycytidine kinase (dCK). The expression of the ribonucleotide reductase regulatory subunits M1 (RRM1) and M2 (RRM2), and the catabolic enzymes 5′nucleotidase (5′NT), and cytidine deaminase (CDA) play also an important role. The aim of the study is to investigate the potential clinical role of hENT1, hCNT1, dCK, RRM1, RRM2, CDA, and 5′NT in a single institutional series of 25 primary ovarian carcinomas.
Methods The expression levels of the target genes were assayed by means of real-time quantitative PCR. The clinical role of the expression levels of each parameter was analyzed by Kaplan and Meier method and the Cox's proportional hazards model.
Results hENT1, hCNT1, dCK, CDA, 5′NT, RRM1, and RRM2 gene expression was documented in all samples; undifferentiated and clear cell carcinoma exhibited higher levels of hENT1, dCK, 5′NT, and RRM1 compared to serous ovarian tumors. As of May 2009, the median follow-up was 32 months (range 10–80), and death of disease were observed in 17 cases (68.0%). A statistically significant direct association of RRM2 expression levels and the relative risk of death was observed ($X^2 = 8.18$, $P = 0.0043$). Moreover, cases with high RRM2 expression had a shorter OS (median OS = 19 months) than cases with low RRM2 levels (median OS = 36 months) ($P = 0.0506$).
Conclusions We first reported that the most relevant genes involved in gemcitabine metabolism are expressed in ovarian carcinoma, and might be associated with more aggressive histotypes. The assessment of the expression levels of RRM2 as marker of clinical outcome deserves further investigation in a larger series of ovarian cancer patients.

Keywords Gemcitabine · Ovarian cancer · Ribonucleotide reductase subunit M2 · Prognosis

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Introduction

Despite the advances in surgical efforts, and the achievement of high response rates with platinum/paclitaxel front-line treatment, ovarian cancer remains the most lethal gynecological malignancy with a 5-year survival rate of 25–30% in advanced stage disease [1]. The major determinants of unfavorable prognosis are represented by the extent of residual tumor at primary surgery and resistance to platinum-based chemotherapy [2, 3]. In this context, efforts aimed at identifying molecular factors possibly predictive of clinical outcome are ongoing.

Gemcitabine (2′,2′ -difluorodeoxycytidine) (GEM), a synthetic nucleoside analogue, is one of the most actively investigated drugs in the salvage as well as upfront setting in ovarian cancer [4–6]. Many molecular mechanisms have been proposed to be involved in GEM sensitivity/resistance [7, 8]; in particular, much attention has been focused on the complex machinery associated with GEM transport, activation, and metabolism; indeed, the equilibrative nucleoside transporter-1 (hENT1) and the concentrative nucleoside transporter-1 (hCNT1) are required to carry the drug inside the cell, and deoxycytidine kinase (dCK) plays a crucial role in phosphorylating and activating the pro-drug [7]. Moreover, the expression of the ribonucleotide reductase regulatory subunits M1 (RRM1) and M2 (RRM2), and the levels of GEM catabolic enzymes 5′nucleotidase (5′NT), and cytidine deaminase (CDA) have been documented to be associated with GEM chemosensitivity [8, 9]. Although the major biological role attributed to most of these molecules is their involvement in determining GEM susceptibility [7–9], evidences have also been reported that some of them may play a critical role in other aspects of tumor biology; for instance, RRM1 has been associated with suppression of tumor proliferation and metastatic potential [10, 11], while RRM2 has been shown to cooperate with several oncogenes in cellular transformation [12], and to potentiate tumor cell growth and cellular invasiveness in vitro and in vivo [13, 14].

The expression and/or activity of the molecules involved in GEM metabolism have been mainly investigated in patients with pancreatic, bladder, and lung cancer, as well as in leukemias [15–21], but very few data are available about their expression in ovarian cancer; indeed, very early studies documented low levels of 5′NT in ovarian cancer homogenates compared to normal ovaries [22]. On the other hand, serum levels of 5′NT were higher in ovarian cancer patients than in control women, suggesting the existence of a shedding of this enzyme from tumor to systemic circulation; moreover, within ovarian cancer patients elevated levels of 5′NT were observed in 50% of cases with active disease compared to 11% of cases in clinical remission [22]. Recently, hENT1 and hCNT1 have been shown by immunohistochemistry in 91% and 67% of ovarian carcinomas, respectively, and found to be more frequently expressed in serous tumors [23].

We recently reported a case of recurrent ovarian cancer resistant to several cytotoxic drugs, but exhibiting high sensitivity to GEM not only at first administration, but also at re-challenge [24]. This case showed the highest levels of parameters involved in GEM transport and activation and, overall, the molecular profile most likely to show a high sensitivity to GEM [24]. More important, in our case, the association between gene expression profile in the primary tumor and GEM responsiveness was maintained over subsequent GEM treatments, thus leading to hypothesize that the transcription analysis of the panel of molecules affecting GEM activity could be reliably performed in primary tumor, and maintain its ability to predict GEM responsiveness in the recurrent setting [24]. No data are currently available about the potential prognostic role of the expression levels of these molecules in primary ovarian cancer.

The aim of the study is to investigate by means of real-time quantitative PCR, the presence, the expression levels, and the potential clinical role of the nucleoside transporters (hENT1, hCNT1), the pro-drug activating enzyme (dCK), the ribonucleotide reductase regulatory subunits (RRM1, RRM2), and GEM catabolic enzymes (CDA, 5′NT) in a single institutional series of primary ovarian carcinomas; the expression levels of these molecules have also been investigated in wild-type and gemcitabine resistant A2780 ovarian cancer cell lines.

Materials and methods

Cell cultures

A2780 wild-type ovarian cancer cells (A2780wt) were purchased from the European Collection of Cell Cultures (ECACC). Culture media was selected according to ECACC suggestions. A2780 gemcitabine resistant cells (A2780GEM) were generated in our laboratory via continuous exposure to stepwise increases of GEM (Ely Lilly, Sesto Fiorentino, Italy) concentrations (up to 10 mmol/l). For growth experiments, cells were seeded (20,000 cells/well) in 96-well flat bottom plates (Culture plates, Perkin-Elmer Life Science). After 24 h, cell cultures were washed and the medium was replaced with GEM containing medium. Three independent experiments were performed in quadruplicates. After 72 h of culture in the presence of the tested compounds, plates were harvested and the number of viable cells was estimated by dosing ATP using the ATPlitekit (Perkin-Elmer Life Science) and the automated luminometer Topcount (Perkin-Elmer Life Science). The kit was employed according to the manufacturer's suggestions. For each drug/cell line a dose–response curve was plotted and the IC_{50} values were then calculated by fitting the concentration–effect curve data obtained in the three experiments with the sigmoid-Emax model using nonlinear regression, weighted by the reciprocal of the square of the predicted effect [25].

Patients

The study included 25 ovarian cancer patients admitted to the Gynecologic Oncology Unit, Catholic University of

Rome, and Campobasso. Staging was performed according to FIGO classification. Within 2–3 weeks after surgery, all patients underwent 4–6 cycles of platinum/paclitaxel containing chemotherapy. Clinical and pathological features are summarized in Table 1: median age was 60 years (range 40–85). Eighteen cases (72.0%) were stage IIIC and 7 (28.0%) were stage IV disease. Optimal cytoreduction (residual tumor $\leq$ 1 cm) was achieved in 12 (48.0%) of cases, while suboptimal (residual tumor > 1 cm) cytoreduction was accomplished in 13 (52.0%) of cases. Fourteen patients (56.0%) received GEM as salvage treatment later on progression/recurrence of disease, while 11 patients (44.0%) were administered different cytotoxic agents as second-line treatment.

Tissue sampling and processing

Tumor tissue specimens obtained at time of surgery from ovarian carcinomas were first cut into two halves: one half was immediately frozen in liquid nitrogen, and then stored at −80°C until assay, while the other half was used for histopathological analysis, aimed at documenting the presence of sufficient tumor cells in the tumor specimen (at least 80%).

Quantitative PCR analysis

Total RNA was extracted from cells using the TRI Reagent LS. RNA was dissolved in RNase-free water containing 10 mmol/l DTT and 200 units/ml of RNase inhibitor and measured at λ_{abs} 260 nm. RNA (500 ng) was reverse transcribed as described previously [15]. After determining RNA quality by reverse transcription-PCR (RT-PCR) amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the resulting cDNA was amplified with the 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Forward and reverse primers and probes were designed as previously described [15, 17]. Therefore, data were expressed as GAPDH/target gene ratio. Specimens were amplified in triplicate with appropriate nontemplate controls, and the coefficient of variation was <2% for all replicates.

Table 1 Clinico-pathological characteristics of cases examined

Characteristics	Number of patients (%)
All cases	25
Age (years)	
<60	13 (52.0)
≥60	12 (48.0)
FIGO stage	
IIIC	18 (72.0)
IV	7 (28.0)
Histotype	
Serous	21 (84.0)
Undifferentiated	2 (8.0)
Clear cell	2 (8.0)
Extent of residual tumor (RT)	
RT < 1 cm	12 (48.0)
RT ≥ 1 cm	13 (52.0)
Grade	
G1–2	2 (8.0)
G3	21 (84.0)
n.a.	2 (8.0)

Statistical analysis

Gene expression levels in ovarian cancer cells were expressed as mean ± SE, and analyzed by Student's *t* test or ANOVA followed by the Tukey's multiple comparison. *T* test was also used to analyze the distribution of the expression levels of hENT1, hCNT1, dCK, CDA, 5′NT, RRM1, and RRM2 according to clinico-pathological features.

Overall survival (OS) was calculated from the date of diagnosis to the date of death of disease or date last seen. Medians and life tables were computed using the product-limit estimate by the Kaplan and Meier method [26] and the log-rank test was employed to assess the statistical significance [27]. The clinical role of the expression levels of each parameter analyzed as a continuous variable was assessed by Cox's proportional hazards model [28]. Statistical analysis was carried out using SOLO (BMDP Statistical Software, Los Angeles, CA).

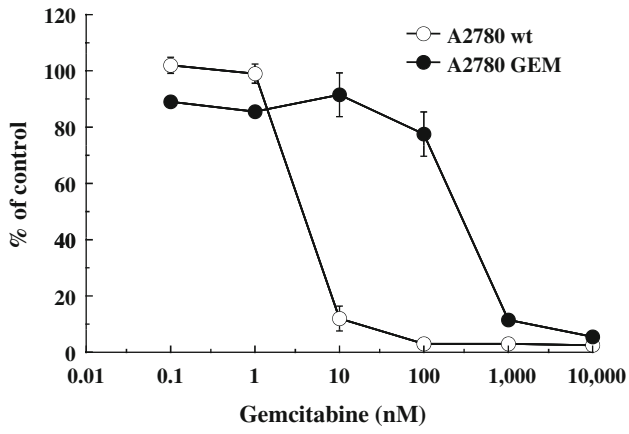

Fig. 1 Growth inhibitory effects of GEM in A2780 wild-type (A2780wt) and A2780 GEM resistant (A2780GEM) cells. Results are expressed as mean ± SD of three different experiments performed in triplicate

Table 2 Gene expression of hENT1, hCNT1, dCK, CDA, 5′NT, RRM1, and RRM2 in ovarian cancer cell lines

	Ovarian cancer cell lines[a]		*P*
	A2780 wild-type	A2780 GEM resistant	
hENT1	0.858 ± 0.009	0.837 ± 0.005	0.026
hCNT1	0.540 ± 0.002	0.557 ± 0.012	0.075
dCK	0.794 ± 0.009	0.737 ± 0.006	0.001
CDA	0.612 ± 0.008	0.630 ± 0.011	0.078
5′NT	0.832 ± 0.013	0.813 ± 0.010	0.117
RRM1	0.879 ± 0.005	0.858 ± 0.016	0.088
RRM2	0.811 ± 0.012	0.802 ± 0.006	0.304

[a] Values are expressed as mean ± SE of at least two experiments performed in triplicate

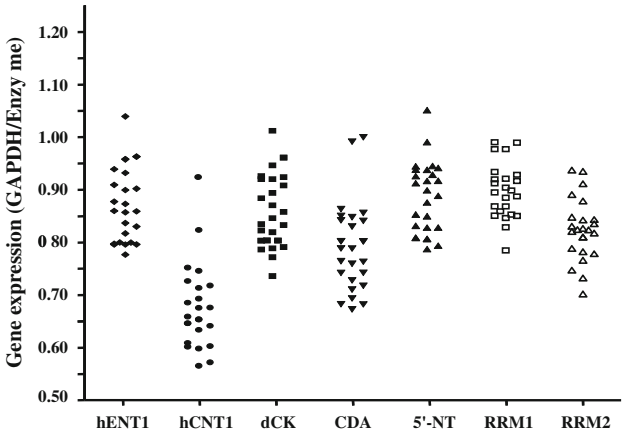

Fig. 2 Distribution of gene expression levels in primary ovarian cancer tissue. Results were calculated by the GAPDH/target gene ratio

Results

Figure 1 shows the growth inhibitory curves of GEM in A2780wt and A2780GEM cells: the IC50s were 4.7 ± 1.1 and 199.2 ± 17.1 nM for A2780wt and A2780GEM, respectively, with a resistance index = 42.38.

The relative levels of gene target expression in both cell lines are summarized in Table 2. The expression of dCK and hENT1 was significantly lower in A2780GEM compared to A2780wt cells (hENT1, 0.837 ± 0.005 vs. 0.858 ± 0.009 in A2780GEM vs. A2780wt, $P = 0.026$; dCK, 0.737 ± 0.006 vs. 0.794 ± 0.009, in A2780GEM vs. A2780wt, $P = 0.001$). On the other hand, there was no statistically significant difference in the expression levels of genes for the remaining molecules involved in GEM metabolism.

Figure 2 shows the distribution of hENT1, hCNT1, dCK, CDA,5′NT, RRM1, and RRM2 gene expression levels, as analyzed by quantitative RT-PCR: the genes were detected in all samples, with hCNT1 and CDA showing the most pronounced variability, and RRM1 showing the lowest level of variability. In the 14 patients administered GEM as salvage treatment, only 1 complete response was observed (7.1%); therefore, the analysis of the distribution of GEM-related molecules according to response to GEM treatment was not attempted.

As summarized in Table 3, the mean value of the levels of target genes in the overall series of ovarian cancer specimens was comparable to that observed in A2780 ovarian cancer cells. No apparent relationship between the expression levels of the genes examined and clinico-pathological parameters was documented with the exception of tumor histotype; indeed, undifferentiated and clear cell carcinoma exhibited higher levels of hENT1, dCK, 5′NT, and RRM1 compared to serous ovarian tumors. There was no difference in the distribution of target gene expression levels between patients who had or had not received GEM during their clinical outcome, with the exception of hCNT1, dCK, and CDA, whose levels were significantly higher in the group of patients who were administered GEM versus other agents in the salvage setting.

Follow-up data were available for all patients. As of May 2009, the median follow-up was 32 months (range 10–80). During the follow-up period, death of disease was observed in 17 cases (68.0%).

Given the large intertumor variability of the expression levels of our gene targets, the absence of a well-established scoring system, and the need to minimize any source of bias related to the use of a specific cut-off value, analysis of the survival data was first carried out using the values of each parameter as a continuous variable.

As summarized in Table 4, the presence of more advanced stage of disease and the values of RRM2 expression levels, as calculated by COX's proportional hazard regression model, were significantly associated with an unfavorable OS. In particular, Fig. 3a shows the plot of the estimates of the relative risk of death of disease as a prediction RRM2 expression levels: a statistically significant direct association of RRM2 expression levels and the relative risk of death was observed ($X^2 = 8.18$, $P = 0.0043$). We were then prompted at defining the cut-off value of RRM2 that more closely correlated with risk of death in Kaplan and Meier curve: the most significant association was observed at the cut-off value of 0.826 (corresponding to the median value): in the overall series, cases with high RRM2 expression showed a trend to a shorter OS (median OS = 19 months) than cases with low RRM2 levels (median OS = 36 months) ($P = 0.0506$) (Fig. 3b).

Table 3 Mean ± SD of hENT1, hCNT1, dCK, CDA, 5′NT, RRM1, and RRM2, gene expression levels in the whole series according to the clinico-pathological characteristics

Variable	hENT1 (n = 22)	hCNT1 (n = 22)	dCK (n = 25)	CDA (n = 25)	5′NT (n = 24)	RRM1 (n = 24)	RRM2 (n = 23)
All cases	0.859 ± 0.057	0.676 ± 0.078	0.857 ± 0.068	0.788 ± 0.085	0.893 ± 0.065	0.897 ± 0.052	0.854 ± 0.059
Age							
≤60	0.854 ± 0.058	0.663 ± 0.055	0.840 ± 0.053	0.761 ± 0.054	0.893 ± 0.049	0.877 ± 0.031	0.815 ± 0.042
>60	0.865 ± 0.048	0.694 ± 0.098	0.876 ± 0.080	0.818 ± 0.103	0.893 ± 0.082	0.918 ± 0.062	0.834 ± 0.073
P	0.75	0.81	0.15	0.14	0.82	**0.03**	0.52
Stage							
IIIc	0.847 ± 0.055	0.654 ± 0.067	0.854 ± 0.076	0.776 ± 0.083	0.887 ± 0.075	0.900 ± 0.058	0.828 ± 0.066
IV	0.886 ± 0.058	0.686 ± 0.056	0.865 ± 0.049	0.792 ± 0.052	0.905 ± 0.032	0.881 ± 0.031	0.815 ± 0.036
P	0.16	0.16	0.68	0.48	0.66	0.80	0.82
Histotype							
Serous	0.848 ± 0.055	0.660 ± 0.060	0.846 ± 0.068	0.774 ± 0.078	0.877 ± 0.054	0.888 ± 0.048	0.815 ± 0.055
Other	0.912 ± 0.033	0.776 ± 0.141	0.915 ± 0.030	0.865 ± 0.084	0.977 ± 0.057	0.944 ± 0.052	0.868 ± 0.065
P	**0.03**	0.09	**0.03**	0.07	**0.009**	**0.04**	0.18
Residual tumor							
≤1 cm	0.849 ± 0.051	0.670 ± 0.101	0.827 ± 0.059	0.769 ± 0.087	0.895 ± 0.078	0.891 ± 0.055	0.814 ± 0.066
>1 cm	0.868 ± 0.063	0.682 ± 0.071	0.881 ± 0.068	0.803 ± 0.083	0.892 ± 0.057	0.901 ± 0.052	0.833 ± 0.053
P	0.32	0.34	0.062	0.15	0.97	0.80	0.50
GEM							
Yes	0.880 ± 0.058	0.699 ± 0.062	0.893 ± 0.062	0.818 ± 0.077	0.901 ± 0.058	0.910 ± 0.049	0.834 ± 0.055
No	0.846 ± 0.069	0.638 ± 0.053	0.811 ± 0.055	0.743 ± 0.051	0.893 ± 0.076	0.880 ± 0.052	0.816 ± 0.063
P	0.48	**0.025**	**0.0054**	**0.007**	0.42	0.31	0.47

Statistically significant values are indicated as bold values

The direct association between RRM2 expression levels and the relative risk of death was documented both in the subgroup of cases who had received ($X^2 = 3.86$, $P = 0.049$) or not ($X^2 = 3.38$, $P = 0.061$) (data not shown).

Discussion

To our knowledge, this is the first investigation of gene expression levels of several parameters involved in GEM metabolism in primary ovarian carcinomas. Indeed, the complexity of the machinery involved in determining GEM efficacy requires to proceed to the simultaneous, multiparametric evaluation of several molecular targets.

In particular, hENT1, hCNT1, dCK, CDA, 5′NT, RRM1, and RRM2, specifically involved in intracellular transport, activation and metabolism of nucleosides have been analyzed by means of the highly sensitive quantitative RT-PCR, which represents a valuable tool to investigate any profile of gene expression. Moreover, a homogeneous series of advanced (stage IIIC/IV) ovarian carcinomas treated and followed in the same institution has been studied.

We showed that all genes were expressed in primary ovarian tumors at levels comparable to those documented in A2780 ovarian cancer cells. Moreover, the documentation of certain heterogeneity in the expression levels of each parameter in primary ovarian cancer strongly supports the existence of a biological variability, which would allow in principle, the stratification of patients according to the gene expression profile, in order to attempt a correlation with clinical outcome.

Several evidences have shown the association between the overexpression of nucleoside transporters and dCK enzyme to higher susceptibility to GEM; for instance, adenoviral-mediated overexpression of hENT1 enhances gemcitabine sensitivity in human pancreatic cancer in vivo [9], while nucleoside transport-deficient or not functional cell variants have been shown to exhibit a higher resistance to nucleoside analogues, including gemcitabine [29, 30]. We also documented lower levels of hENT1 and dCK in A2780 gemcitabine resistant versus the parental cell line, as also shown in in vitro models [21, 31, 32], thus suggesting that resistance to GEM could be, at least in human established cell lines, mainly sustained by a lower expression of genes encoding proteins involved in gemcitabine transport and activation. On the other hand, we found that the expression of hENT1, hCNT1 as well as dCK activating enzyme were not associated with clinical outcome, thus leading to hypothesize that a more complex, yet unknown, regulation of GEM-related biochemistry could be active in vivo.

Table 4 Univariate analysis of clinico-pathological parameters and biological markers as predictors of OS in the whole series

Variable	Univariate analysis		
	Association with poor survival	X^2	P^a
Age, years			
≤65			
>65	No difference	0.32	0.57
FIGO stage			
IIIC	−		
IV	+	**4.67**	**0.003**
Histotype			
Serous			
Other	No difference	0.41	0.53
GEM			
Yes			
No	No difference	0.11	0.74
hENT1			
Continuous value	No difference	3.30	0.07
hCNT1			
Continuous value	No difference	0.02	0.88
dCK			
Continuous value	No difference	0.94	0.33
CDA			
Continuous value	No difference	0.31	0.57
5′NT			
Continuous value	No difference	1.47	0.22
RRM1			
Continuous value	No difference	2.23	0.13
RRM2			
Continuous value	+	**10.41**	**0.0013**

[a] Positive (+) values indicate the variables directly associated with poor overall survival while negative (−) values indicate the variables inversely associated with poor overall survival

Conversely, with the limits inherent in the small sample size and limited number of events, we showed that patients whose tumors express high levels of RRM2 experience a shorter OS compared to cases with low RRM2 content. These results were obtained using RRM2 gene expression levels as a continuous value thus avoiding or at least minimizing the potential bias inherent in the use of an arbitrary cut-off point, and this further supports the association between increasing levels of RRM2 expression and increasing risk of death of disease. The role of RRM2 overexpression as marker of poor prognosis in ovarian cancer seems to be independent from clinico-pathological parameters; moreover, our results are unlikely related to tumor sensitivity to GEM in the salvage setting; indeed, with the limits inherent in the relatively small sample series, there was no difference in the clinical outcome according to type of treatment administered in the salvage setting. Moreover, the association between the expression levels of RRM2 and the relative risk of death was documented both in the group of cases who were treated with GEM as well as in patients receiving other salvage treatments. These observations suggest that in ovarian cancer, elevated levels of RRM2 might more likely indicate tumor intrinsic biological aggressiveness, rather than represent only a marker of GEM sensitivity. In this context, it is worth noting that in our series, more aggressive histotypes such as undifferentiated and clear cell carcinomas exhibited higher levels of hENT1 compared to serous carcinomas, contrary to previous data [23], and also higher levels of dCK, 5′NT, and RRM1.

Moreover, RRM2 overexpression has been found to be associated with the enhancement of tumor cell proliferation and induction of metastatic potential through NF-κB-mediated MMP9 activation [33, 34].

It is worth noting that ribonucleotide reductase inhibition has been recognized as an appealing anticancer approach

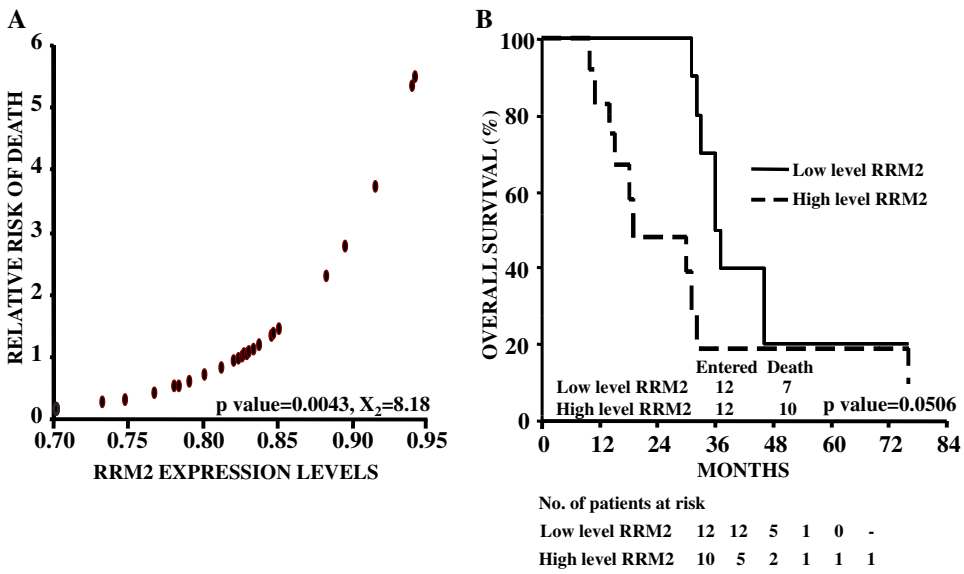

Fig. 3 Plot of the estimates of the relative risk of death of disease as a prediction of RRM2 as continuous variable (**a**) calculated by Cox's proportional hazard regression model, and Kaplan and Meier curve (**b**) of overall survival according to RRM2 status, as defined on the basis of the chosen cut-off value (median level of gene expression)

[34]; in particular, specific antisense molecules against RRM2 have been shown to significantly reduce the growth of human cancer cells (including ovarian cancer cells) in vitro and in vivo [35, 36]; moreover, small interfering RNA (siRNA) duplexes are able to inhibit cell proliferation [33] and to sensitize colon and pancreatic cancer cells to chemotherapeutic agents [37, 38].

In conclusion, we first reported that the most relevant genes involved in gemcitabine metabolism are expressed in ovarian carcinoma, and might be associated with more aggressive histotypes.

The potential clinical role of RRM2 expression remains to be further investigated in a larger series with a longer follow-up.

Conflict of interest statement The authors declare that there are no conflicts of interest.

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